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Volume 1

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Volume 38B BASIC, CLINICAL, AND THERAPEUTIC ASPECTS OF ALZHEIMER'S AND PARKINSON'S DISEASES
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BASIC, CLINICAL, AND THERAPEUTIC ASPECTS OF ALZHEIMER'S AND PARKINSON'S DISEASES
Volume 1

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PREFACE

This two-volume work contains the full text of the oral and poster presentations and the general discussion at the round table discussion of the Second International Conference on Alzheimer's and Parkinson's Diseases: Basic and Therapeutic Strategies, held at the Kyoto Park Hotel in Kyoto, Japan, on November 6-10, 1989.

The First Conference was held at the Aviya Sonesta Hotel in Eilat, Israel, on March 24-27, 1985. The record of this First Conference was published by Plenum Press in 1986 as Volume 29 in Advances in Behavioral Biology, under the title "Alzheimer's and Parkinson's Diseases: Strategies for Research and Development." We are happy that the comprehensive texts of the oral and poster presentations of the Second Conference could again be published within the framework of this series.

Since the First Conference in 1985, rapid progress has been made in both basic and therapeutic aspects of these diseases. About 700 scientists from all over the world participated in the Second Conference, and 300 papers were presented in oral and poster sessions.

Many people and organizations have helped to organize this multidisciplinary international conference and hence have contributed to the scientific quality of these two volumes. We thank the members of the organizing committee, the organizations that provided financial support, and the contributing scientists for their enthusiastic participation.

These two volumes follow the same publishing philosophy as the volume derived from the First Conference. They span a broad spectrum of topics and bridge preclinical and clinical concepts related to these diseases. They review the old literature and emphasize new findings. They pose important questions, which have yet to be answered, as summarized in the round table discussion in Volume 2.

Alzheimer's and Parkinson's diseases are the most frequent age-related neurodegenerative diseases. The idea of bridging basic and therapeutic research and of comparing Alzheimer's and Parkinson's diseases is fascinating. The two diseases have similarities in etiology, in clinical and pathological features, and in drug development strategies like the neurotransmitter-supplementation therapy.

Research on these two most common age-related diseases will present important clues for the elucidation of the mechanism and therapy of brain aging in general.

We hope that the two books will mark another milestone on the road to effective treatment of Alzheimer's and Parkinson's diseases, and that they will be a valuable addition to the libraries of many students and scientists interested in these two devastating neuropsychiatric disorders, in neuroscience in general, and in brain aging in particular.

Toshiharu Nagatsu
Abraham Fisher
Mitsuo Yoshida

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CONTENTS

PLENARY LECTURES

AMYLOID

THE NEURONAL BREAKDOWN-1 (ALZHEIMER)

NEURONAL BREAKDOWN-2 (PARKINSON)

PATHOLOGY AND HISTOLOGY

TRANSMITTER

RECEPTOR

NGF AND PHYSIOLOGY

MODEL

GRAFT

Functional Principles Implied in Neurological Symptoms

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Characteristic symptoms of a neurological disease should reflect a specific mechanism with which brain tissues normally operate. One may hope that a rigorous analysis of such symptoms will easily reveal mechanisms of the central nervous system. However, looking at the history of neurology and neurophysiology, it is clear that this is not an easy task. There are two obvious reasons for this difficulty. First, to trace from results back to a cause is in general much harder than to deduce results from a cause. Once a central mechanism is known, it will be relatively easy to understand symptoms derived from its disturbance, but without knowing the central mechanism, symptoms would just be puzzling. Second, central mechanisms causal to neurological symptoms are in fact highly complex and in most cases, of unknown nature to any field of science.

Control Principles Represented by Cerebellar Symptoms

Dysmetria and incoordination are characteristic symptoms of cerebellar diseases. Apparently, the cerebellum is normally responsible for orthometria and coordination. However, how do the cerebellar tissues perform such a task, and what is the mechanism for it ? These have been puzzling questions, but a helpful analogy is now available in machine systems. Dysmetria is analogous to predictive control in which precise control is performed based on preceding experiences even without ongoing feedback. A normal person can thus accurately touch his nose with a finger with his eye closed, i.e., without visual feedback. Coordination signifies that a motor system effectively handles more than one input and output at the same time, a situation equivalent to multivariable control. These modern forms of control are all computer-aided. Without a computer, these could not be achieved. Thus, in analogy with a machine control

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

system, the cerebellum may be viewed as a computer helping neural control mechanisms (Ito, 1979).

Entity of the Cerebellum as a Neural Computer

To say that the brain is a computer may mean little, but characterization of the cerebellum as a sort of computer has a more concrete basis. The architecture of the cerebellar machinery has so far been dissected to an extent where we can grasp its operational principles as a computer (Ito, 1984).

let us assume a major signal flow pathway from the brain stem through a cerebellar or vestibular nucleus back to the brain stem. A microzone of the cerebellar cortex is connected to a small cell group in a vestibular or cerebellar nucleus, and these conjointly form a cerebellar corticonuclear microcomplex. Signals flowing from the nuclear cell group are also fed to the cortical microzone via mossy fibers, and after information processing in the cortical network, Purkinje cells send out inhibitory signals which modulate the signal flow through the nuclear cells. Thus, mossy fiber signals represent excitatory input signals driving a nuclear cell group, and at the same time, these generate inhibitory signals of Purkinje cells which modulate the signal flow through the nuclear neurons. By contrast, climbing fibers encode errors produced during the performance of an entire system executed under the control by a cerebellar corticonuclear microcomplex. Climbing fiber signals induce long-term depression, a specific type of synaptic plasticity in Purkinje cell synapses receiving mossy fiber signals via parallel fibers (Ito, 1989). Thus, the relationship between mossy fiber inputs and Purkinje cell outputs will be modified so that the dynamic characteristics of the entire system will be improved toward minimization of the error signals.

Concerning operational principles of the cerebellar cortical network, there are two excellent models so far proposed: three-layered neuronal network models for processing spatial information (Marr, 1969; Albus, 1971) and temporal information (Fujita, 1982). The cerebellum is thus viewed as a perceptron-like or adaptive filter-like neural computer, which endows various control systems of our body with error-correcting adaptability.

Cerebellum as an Adaptive Feedforward Controller

The actual control function of a cerebellar corticonuclear microcomplex has been studied in connection with the vestibulo-ocular reflex (Ito, 1982). The VOR drives eyes in a direction opposite to head movement so that retinal images of the external world will be stably maintained. Performance of the VOR requires

precision because eyes should be driven by the appropriate amount to just compensate for head movement. Whenever the VOR performs inadequately, retinal error signals conveyed by climbing fibers will act to modify signal transfer characteristics of the flocculus and consequently to improve dynamic characteristics of the VOR.

In order to interpret the floccular role in VOR, it is important to recognize the difference between two modes of control, feedback and feedforward. A feedforward system lacks feedback, as typically represented by the VOR. Such a system by itself would only perfom poorly, because its performance is not corrected by feedback. However, when attached with an adaptive mechanism for self-correction, as the flocculus is attached to the VOR, it can perform a precision control. An important conclusion of cerebellar physiology is that a cerebellar corticonuclear microcomplex constitutes such an adaptive feedforward control system.

Cerebellar aid of cerebral function

Learning by practice is a characteristic feature of our voluntary motor control. This feature can be represented by combination of a feedback control and adaptive feedforward control, both acting on the skeletomuscular system as a common control object (Ito 1990). Initially, movement is performed in a feedback mode, but during exercise, the feedforward system would complete adaptation and take over the feedback control. When this scheme is applied to brain structures, the feedback system may be located in the cerebral cortex, and the adaptive feedforward system in the cerebellum. In a learned state, a voluntary movement would be performed through the cerebellar adaptive system in a feedforward manner, so that feedforward control such as the finger-to-nose test reveals a cerebellar disorder.

Probably, the same scheme applies to a mental control, such as silent arithmetic counting, where one area of the cerebral cortex would act as a controller and another as a a control object. Here again, the cerebellum is viewed as an adaptive feedforward controller. A control which is initially performed by a cerebral cortical area by referring to feedback will gradually be taken over by the cerebellar feedforward controller adapted through repeated practice. Contribution of the cerebellum to silent counting has recently been suggested by a positron emission tomography study (Ingvar, 1989).

Control Principles of Basal ganglia

The basal ganglia act not only upon the brain stem, but also upon the cerebral

cortex as one of the so-called subcortical trio (cerebellum, basal ganaglia and thalamus). While the cerebellum helps the cerebral cortex by providing an adaptive feedforward control mechanisms, what role is played specifically by the basal ganglia ? Do the characteristic symptoms of basal ganglia disorders imply such a role ?

When akinesia and chorea are taken as characteristic symptoms of basal ganglia disorders, some stabilizing action of the basal ganglia upon operation of other brain tissues is suggested. Akinesia may be viewed as an overstabilized state, while chorea is apparently a less stabilized state. This stabilization concept may find a counterpart in the active control technology for highly complex vehicles such as aeroplane or space rocket (Ito, 1986). These complex systems require augmentation of control, and at the same time augmentation of stabilization. These two purposes cannot be achieved by a single device, and there must be independent devices dedicated to each purpose. While the cerebellum serves for augmentation of control, could the basal ganglia be an organ specifically evolved for augmentation of stabilization of highly complex systems of our body ? Augmentation of stabilization could be achieved with intensification of negative feedback. Can one speculate that the basal ganglia are something like an optimal regulator, as typically represented by Calman filter, utilized for feedback of an internal state of a complex system ? There is still a large gap in our current knowledge of the basal ganglia at cellular levels, but such modelistic consideration on the function of basal ganglia should be more encouraged.

Limbic System and Dementia

A characteristic feature of dementia is that emotional behavior involving food and sex becomes abnormal. This reminds us of a conspicuous symptom of destruction of the limbic system, especially the amygdala. An animal with lesioned amygdala becomes indifferent to fearful stimuli. He also become hyperactive in sexual behavior and approaches food as if it is a sexual partner. Biological values of stimuli appear to be confused in such an animal. Thus, the symptoms of amygdala lesions would suggest that the amygdala is a specific organ evolved for biological evaluation of external stimuli. The limbic system also includes the hippocampus, which apparently plays a key role in cognitive memory and learning. A recent report that the amygdala and hippocampal regions are atrophied in dementia patients (Matsuzawa, 1989) is in accordance with these postulated limbic system functions.

Comment

While diverse symptoms are all products of the elaborate neuronal machinery of

our brain, many of them emerge from mechanisms of unknown scientific nature. Neurological and psychiatric symptomatology will continue to provide insight into the profound mechanisms of the brain.

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THE NEUROBIOLOGY OF ALZHEIMER'S DISEASE

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INTRODUCTION

It is not possible in the space permitted to provide anything like a complete overview of the neurobiology of Alzheimer's Disease. I will thus simply attempt to identify the key questions raised by recent advances in this field. A much more comprehensive review of recent developments in selected areas of this research was recently published in Neurobiology of Aging (1).

AMYLOID

Deposition of amyloid is one part of the formation of neuritic or senile plaques in Alzheimer's Disease. Other elements comprising the plaque are degenerating neuronal processes, microglial cells and reactive astrocytes (2). There is now some understanding of the nature of the amyloid deposit, following the publication of a partial peptide sequence (3), and the successful cloning of cDNA's that encode the sequence (4,5). Alternate splicing appears to result in the synthesis of at least three different mRNA's, and hence three possible precursors of the deposited peptide (6,7,8). It appears that all three precursors are at least transiently inserted in the cell membrane, and that proteolytic cleavage may result in the release or secretion of truncated proteins. Many cell types can produce the potential amyloid precursors, although the shorter form (called APP695) appears to be present in higher levels in neurons than in other tissues. An important question for future work is the cellular source of the material which is deposited in plaques, and the identification of the precursor (s) that is cleaved or processed to yield the insoluble deposit.

Recent work showing that two forms of the presumed amyloid precursor include a protease inhibitor domain (6,7,8) have now been complemented by the suggestion that these are in fact a previously identified molecule described as protease nexin II (9). It is obvious that over the next few years we will see much published work on the possible role of proteases in the generation of amyloid. The function of nexin II in the normal brain must now be an important priority, and special attention to the possible role of this protein in interactions with growth factors (9) should be informative.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Perhaps even more important will be studies of the effects of amyloid deposition on neurons.

It is now clear from immunopathologic studies that amyloid deposition is much more common and widespread that was previously apparent. I believe we will have to revise our definitions of what constitutes a plaque. Certain histologic techniques do not allow distinction between amyloid deposits without detectable neuronal degeneration, and more classically defined neuritic plaques, the letter usually containing both amyloid and dystrophic neurites (as well as the glial elements mentioned above). It will be important for future studies to define plaques on the basis of the elements present: sensitive immunocytochemical techniques now exist to allow visualization of amyloid (10,11), the neuritic components (eg, Alz-50 (12)), as well as glial cells (eg, GFAP, (11)). These will be especially important given that the presence of amyloid deposits does no appear to correlate well with cognitive dysfunction (10), and older histologic studies demonstrated that several individuals could be found free of obvious dementia but with numerous senile plaques (13,14). As these studies did not classify plaques according to their composition, it is unclear what precisely was being included in the total plaque count. As both amyloid deposition and Alzheimer's Disease both increase in frequency with age, it is likely that elderly patients will have amyloid plaques, neuritic plaques, and in some cases, probably both. The association of these lesions with specific aspects of cognitive function will need to be evaluated with immunopathologic techniques. Such studies are in progress at our institution (eg, 15).

Although recent work suggests that a high proportion of the elderly develop amyloid plaques, a considerably smaller percentage develop the neuronal abnormalities and severe cognitive dysfunction characteristic of Alzheimer's Disease. There are two ways to interpret this observation. First, amyloid deposition may be the first detectable sign that Alzheimer's Disease has begun. After some time period which is probably lengthy, the amount or location of amyloid would begin to lead to degenerative changes in neurons, precipitating the numerous other abnormalities of the Alzheimer brain. In this scenario, the deposition of amyloid would be the prime target for therapeutic strategies: stopping this process would prevent the neuronal involvement. However, it appears possible that in Alzheimer patients we are seeing the results of two independent processes; the age related deposition of amyloid and the deposition as a result of a neuronal degeneration.

To propose these two different views of the possible role of amyloid in Alzheimer's Disease without a means to distinguish which is more likely would be armchair science. Although the development of testable hypotheses in this area is difficult, there are a number of different strategies which might be employed. Rapid and extensive amyloid deposition might be achieved through the use of transgenic mice using various constructs of the precursor cDNA's or genomic fragments from the amyloid precursor gene. A potential problem might lie in the limited life span of the mouse: we do not yet know how long amyloid would have to be present to induce the presumed neuronal pathology. Because we do not know the cellular source of amyloid, it is unclear which cells should be induced to over-express the transgene. Neurons of the cerebral cortex and hippocampus would probably

be the cells of first choice, but not all workers would agree with that statement (eg, 16). Although little has been published to date, I am aware of numerous groups attempting to employ transgenic techniques to address questions in this area.

Another way to address this question might be more careful evaluation of the types of lesions present in patients with Alzheimer's Disease of widely different ages. If amyloid deposition occurs first in all cases, then it should be possible to show that the relative number of amyloid plaques (without a neuritic or glial component) remained stable as the age of the patients increased. This would imply that all patients experienced the same basic process: amyloid deposition followed by neuronal involvement, regardless of the age of onset. On the other hand, if age and Alzheimer's disease lead to amyloid deposition by different mechanisms, one would predict that younger patients would have fewer amyloid plaques, and the younger the patient, the greater the percentage of neuritic plaques. In at least some situations, neuritic plaques can form with much evidence for amyloid deposition (17). It would be essential to use the best available antibody techniques for the above studies, since histologic techniques rarely allow precise and sensitive identification of the number or type of lesion present (see 2).

To summarize this section, the most compelling questions at present concern the nature and function of the potential amyloid precursor in normal brain, the processing events responsible for the formation of amyloid deposits in both aging and Alzheimer's Disease, and the significance of amyloid deposition for the development of neuronal abnormalities and cognitive dysfunction. We now have many of the tools with which to pursue these fundamental questions.

NEUROFIBRILLARY TANGLES

This author regards the presence of a neurofibrillary tangle in a neuron as the penultimate stage in the process leading to neuronal loss in Alzheimer's Disease. This is best appreciated in the entorhinal cortex, where numerous neuronal profiles appear to have tangles, and yet no cytoplasm or nucleus remains (18). Clearly, these "tombstones" are the result of the demise of the cell in response to increasing levels of pathologic changes. Numerous protein abnormalities can be visualized both in neurons with neurofibrillary tangles, and in some cases in neurons and neuronal processes in which no tangle is detectable. These abnormalities are prominent in proteins of the cytoskeleton, such as neurofilaments, microtubule associated proteins, tubulin and actin (reviewed in 19). It is also apparent that levels of several neurotransmitters and their associated enzymes are also markedly abnormal in affected regions of the Alzheimer brain (20). What is not yet clear is the temporal order of these numerous abnormalities. As with the formation of the neuritic plaque, there is a cascade of changes leading to the production of the lesion, but a great deal of uncertainty about the sequence in which these occur, and very little information about mechanisms of lesion formation.

An apparently early event in the development of neuronal pathology in Alzheimer's Disease is the appearance of immunoreactivity with the monoclonal antibody Alz-50.

Originally produced using ventral forebrain tissue from cases of Alzheimer's Disease as the immunogen (12), Alz-50 stains many neuronal elements in the Alzheimer brain, but demonstrates little reactivity with the normal adult brain. Although there may be low levels of reactivity with tissues from cases of other neurologic diseases (12,18,21), the presence of extensive immunoreactivity is, to date, unique to Alzheimer's Disease.

Alz-50 immunoreactivity is found in neuronal perikarya containing neurofibrillary tangles, in neurites involved in neuritic plaques, and in neurons and neuropil elements that do not appear abnormal by other histologic or immunocytochemical methods (12,18,21). There is no reactivity with amyloid deposits, nor with tombstone tangles. Light microscopy suggests that the antigen is present in the cytoplasm of neurons, throughout both axons, cell bodies and dendrites, as well as in the dystrophic processes of neuritic plaques. This interpretation is supported by studies demonstrating the perforant pathway in the Alzheimer brain, through studies of the cell bodies and terminals making up this pathway (24). The immunoreactivity appears to be due to the presence of a protein of apparent molecular weight 68,000 daltons, which we have designated A68. To date, A68 has not been detected in the normal human brain (12). A68 or a similar protein may be present transiently during human brain development (22).

The appearance of A68 in neurons that are not abnormal by other histologic criteria suggests that it appears before many of the other abnormalities of neurons in Alzheimer's Disease. We are currently attempting to test the validity of this hypothesis through studies of Down's Syndrome cases of various ages. A68 is expressed in older (aged 50 and above) Down's individuals (20,22), but we do not yet know at what age it first appears. These studies have taught us the valu of using immunocytochemical techniques: both amyloid and neuritic elements are detected with much greater sensitivity than with histologic techniques (Mattiace et al, unpublished data).

We are currently attempting to determine the nature and possible role of A68. Although Alz-50 shows some cross-reactivity with tau (23), A68 has very distinct biochemical properties. A68 has been purified to near homogeneity (Greenberg et al, in preparation), and sequencing studies, while hampered by an apparently blocked N-terminus, are ongoing. Our goal is to attempt to determine the relationship between the appearance of A68 and the development of the other protein abnormalities of Alzheimer's Disease. Perhaps this early marker is another consequence of disordered neuronal metabolism, or perhaps it is a participant in the sequence of degenerative events. At the least, it allows identification of neurons that have not progressed far down the path of changes that ultimately lead to cell death.

In summary, we now know of several specific abnormalities in neurons of the Alzheimer brain. Many of these have been discovered in just the last few years. The challenge now is to determine the temporal sequence of these changes, and to find either a common mechanism by which they occur, or at least identify how these various abnormalities interact to produce neurofibrillary tangles, and ultimately result in neuronal loss. It seems likely that understanding how and why neurons become so disordered will be necessary for the development of rational therapies for this condition.

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ROLE OF BRAIN MONOAMINES AND OTHER NEUROTRANSMITTERS IN NORMAL AND PATHOLOGICAL AGING

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INTRODUCTION

Although the life span of living organisms is ultimately determined by their genes, environmental factors are also important. The well-known "squaring off" of human survival curves illustrates this interaction. Also the general health of aged people appears to improve continuously, and this includes the mental health (Svanborg et al. 1986). In fact, the picture of senile dementia, i.e. "second childishness, mere oblivion" (quoted from "As you like it"), was familiar to William Shakespeare, even though very few people in his time reached the age when this condition starts to become prevalent in our time. This is encouraging, because it strengthens the hope that further environmental improvement as well as therapeutic/prophylactic measures will prove successful.

When trying to better understand what is going on in the aging brain, the neurotransmitter strategy has perhaps proven especially fruitful so far. It may help to identify the mechanisms immediately responsible for functional deficits and the underlying, long-term processes, and may thus be used for formulating strategies for treatment as well as prevention. The successful research on Parkinson's disease is often referred to as a model in this context.

MECHANISM OF THE AGE-RELATED CONFUSION LIABILITY

The liability to confusion or delirium in response to stress is generally recognized as a characteristic sign of the reduced vitality of the aging brain, which may otherwise function normally. In dementia it takes even less of a stressful impact to induce confusion. The mechanism underlying this increased vulnerability is thus of considerable interest. A starting-point is provided by the fact that confusional/delirious states in old age are efficiently alleviated by neuroleptics, i.e. predominantly antidopaminergic agents. Some neuroleptics have, in addition, alpha-adrenergic blocking properties (see Carlsson 1978). Although this does not seem essential for their therapeutic efficacy, it may contribute. In favor of this assumption is the likely involvement of locus ceruleus and the central noradrenergic system in stress reactions (see Elam 1985).

The efficacy of the neuroleptics indicates an imbalance involving a predominance of the dopaminergic system somewhere in the brains of deli-

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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rious people. A hyperdopaminergic state in absolute terms is unlikely, as the level of dopamine goes down with age. In fact, dopaminergic neurons appear to belong to the most age-sensitive neurons of the human brain (see Carlsson 1981). The mechanisms underlying this phenomenon will be discussed in a later section. The level of dopamine goes down with age in all brain regions examined post mortem, and so does tyrosine hydroxylase and the cell count of dopaminergic neurons. Recent PET data confirm the marked age dependence of the dopaminergic system (Tedroff et al. 1988).

The loss of dopaminergic neurons seems to be compensated by an increase in the physiological activity of the remaining neurons, as indicated by an increased HVA/DA ratio (see Carlsson 1988a).

A moderate, age-related involution of the dopaminergic system may, in fact, be favorable by reducing the risk of confusion or psychosis. This may be true also of the noradrenergic system, which is also age dependent.

If we are dealing with a transmitter imbalance due to a deterioration of a dopamine-antagonistic system, which overrides the dopamine deficit, the question arises which this one could be. Presumably the cholinergic system can be ruled out because it seems to be more resistant to normal aging than dopamine (see Carlsson 1981). (However, in Alzheimer's disease the reduction of the cholinergic system, compared to age-matched controls, is more severely affected than the dopaminergic system.) Another system to consider is the serotonergic system. In support of this are some recent findings of Gottfries and his colleagues with serotonin-uptake inhibitors. They observed that citalopram is capable of alleviating certain symptoms, including confusion, in demented patients (Nyth et al. 1987, 1988). Their observation is particularly interesting in view of the well-documented, marked reduction of the serotonergic system in dementia of Alzheimer type (see Carlsson 1988 a, Arai et al. 1984, Nagatsu and Iizuka 1989). Thus the increased confusion liability, at least in senile dementia, may be at least partly due to a serotonergic deficiency. However, recent neuroanatomical data suggest that other systems should also be considered.

STRIATUM: AN INHIBITORY STRUCTURE CONTROLLED BY THE CEREBRAL CORTEX

The profound action of neuroleptics on cortical functions, as indicated by their antidelirious and antipsychotic effects, is not necessarily due to a primary effect of these agents on the cerebral cortex. Both postmortem and more recent PET data indicate that the level of dopamine and the density of dopamine D-2 receptors, i.e. the subtype acted upon by most neuroleptics, are extremely low in the human cerebral cortex (for references, see Carlsson 1988b). Thus, not only the extrapyramidal but also the mental actions of neuroleptic drugs may depend largely on binding to receptor sites in the striatum. "Striatum" is used here in a wide sense and will thus comprise both the dorsal and the ventral, "limbic" part of this structure (see Nauta, 1989).

There is an increasing awareness of the role of the striatum as an inhibitory structure, acting on a variety of both motor and mental functions. The main targets for the dorsal and ventral striatal complexes, which besides the striatum include their "relay stations", the dorsal and ventral pallidum, respectively, appear to be the thalamus and the mesencephalic reticular formation. We have proposed that the inhibitory function of the striatum is at least partly brought about by restricting the flow of sensory information relayed through the thalamus on its way to the cortex and by counteracting the arousal induced by the mesencephalic reticular formation as well as by certain thalamic structures with similar function.

The striatum is controlled by several inputs. One is the mesostriatal

dopamine system, which appears to be inhibitory on the striatum and will thus open up the flow of sensory information to the cortex via the thalamus and increase the level of arousal. The noradrenergic and serotonergic systems, which originate in the mesencephalic reticular formation or in its close vicinity, may be part of this arousal-controlling system.

Another apparently very powerful input to the striatum is the corticostriatal glutamatergic pathway. (Whenever "glutamatergic" is used here, it will include other endogenous excitatory amino acids and related molecules, such as aspartic acid and quinolinic acid.) This pathway is excitatory and will thus enhance the inhibitory function of the striatum and counteract the dopaminergic influence. Apparently we are dealing here with a negative feedback loop, arising in the cerebral cortex and returning to the cortex, and, in fact largely to the same cortical area, via the striatum and the thalamus/mesencephalic reticular formation. By means of this feedback loop the cortex should be able to control the flow of sensory information as well the level of arousal. Moreover, the control is likely to be selective, that is, less relevant inputs are filtered off in favor of more relevant and novel information (for recent references on the neuroanatomy of the striatum and its afferent and efferent connections, see Heimer et al. 1985, Selemon and Goldman-Rakic 1985, Goldman-Rakic and Selemon 1986, Björklund and Lindvall 1986, Alexander et al. 1986, Penney and Young 1986, Nauta 1989, Albin et al. 1989).

Our hypothesis is supported by the fact that Parkinsonian symptoms can be alleviated by neurosurgical lesions in the pallidum or in certain thalamic nuclei. Especially impressive are the results reported by Narabayashi (1988), demonstrating marked improvement of Parkinsonian rigidity and tremors, respectively, following discrete lesions in two adjacent thalamic structures.

Some recent animal data emphasize the importance of glutamatergic mechanisms, and more specifically, those mediated via NMDA receptors, for psychomotor activity (M. Carlsson and A. Carlsson 1989 a and b, M. Carlsson and Svensson 1990). Blockade of these receptors in mice and rats by the specific, noncompetitive NMDA antagonist MK-801, exerts a pronounced stimulatory action on psychomotor activity, and that this effect, contrary to earlier belief, is largely independent of catecholaminergic mediation. Moreover, we find that already a partial blockade of NMDA receptors induces a marked increase in the responsiveness to the stimulating actions of the dopaminergic agonist apomorphine, the alpha-2-adrenergic agonist clonidine, and the muscarinic receptor antagonist atropine. We feel that the corticostriatal glutamatergic pathway is a good candidate for the antagonist to dopamine that we were looking for. Thus, we propose that the age-related increase in the liability to confusional/delirious states may be due to an insufficient capacity of the corticostriatal glutamatergic system to counterbalance the dopaminergic system and other arousal-inducing mechanisms. Among the latter, the noradrenergic system is interesting in view of the well documented responsiveness of the locus ceruleus to stressful stimuli. This will lead to hyperarousal and overflow of sensory information to an extent overthrowing the integrative capacity of the cerebral cortex, that is to a delirious state. Of course the effect of atropine in this model is also of considerable interest, given the well-known sensitivity of elderly people to the confusion-inducing action of antimuscarinic drugs.

Our own experiments are so far limited to systemic drug injections, but our interpretations concerning the role of the corticostriatal glutamatergic system are supported by data, showing that stimulation and blockade of NMDA receptors in the striatum by locally applied specific agonists and antagonists will inhibit and stimulate psychomotor activity, respectively (Schmidt and Bury 1988, Raffa et al. 1989).

LONG-TERM ASPECTS OF THE AGING PROCESS

The last part of this paper will be devoted to the area of oxygen toxicity. Even if we are dealing here with a rather special case of oxygen toxicity, it may have some general implications and may, in fact, serve as a model. The study of free oxygen radicals and how they are controlled by the complex antioxidant system present in tissues and body fluids, is somewhat elusive, indicating a need for additional experimental models.

The autoxidation of dopamine may be harmful in two respects. Firstly superoxide anions, that is free radicals, are formed, and secondly dopamine is converted to semiquinones and quinones which are also highly reactive and toxic species (see e.g. Moldéus et al. 1983). Further processing of the quinones is generally believed to result in neuromelanin, the normal occurrence of which is thus considered to support the existence of this autoxidation process, albeit at a low rate. It has been proposed that the autoxidation process may be responsible for the age-related loss of dopaminergic neurons as well as for Parkinson's disease. Possibly autoxidation-induced cell damage can be enhanced by a failure of the protective antioxidant system or of the scavenger system detoxifying the quinones.

To get a handle on the autoxidation mechanism we have made use of the well-known fact that quinones react very promptly with thiol groups, and thus with glutathione and cysteine. We have identified the product 5-S-cysteinyl-dopamine and some related metabolites, using HPLC (Fornstedt et al. 1986). Animal data show that 5-S-cysteinyl-dopamine accumulates when dopamine is released intraneuronally by reserpine (Fornstedt and Carlsson 1989), but not when it is released into the extraneuronal space by amphetamine. The ratio 5-S-cysteinyl-dopamine/dopamine is higher in the human brain than in other species investigated. It is also higher in the substantia nigra than in the terminal regions, suggesting at least a partial correlation to the occurrence of neuromelanin. In human brain analyzed postmortem the ratio of 5-S-cysteinyl-dopamine to dopamine is higher in the brains of individuals with a significant loss of dopaminergic neurons, as indicated by depigmentation of the substantia nigra (Fornstedt et al. 1989). To examine this phenomenon more closely we calculated the ratio 5-S-cysteinyl-dopamine/DOPAC. The rationale for doing so is that both the formation of 5-S-cysteinyl-dopamine and DOPAC occur largely, if not exclusively, intraneuronally in the cytoplasm of dopaminergic neurons. Thus a change in this ratio would indicate a switch from one metabolic process to another. There appears, in fact to be such a switch. There is a considerable individual variation in the dopamine level in the substantia nigra of these individuals, who may be considered to have been essentially normal for the age (72-91 years), with respect to the brain, except for one individual who had Parkinson's disease, and a few patients who had mild dementia, possibly to be considered normal for the age. Low dopamine levels, and thus a low number of surviving dopamine neurons, were correlated to a high 5-S-cysteinyl-dopamine/DOPAC ratio and thus apparently to a high rate of dopamine autoxidation. The highest ratio was found in the Parkinson case.

It is of course impossible to draw any conclusions about causal relations from these data, but it is tempting to suggest that the severe loss of dopamine neurons in some patients was due to a relatively high rate of dopamine autoxidation, leading to the formation of toxic oxygen species and quinonoid products.

CONCLUDING REMARKS

In the search for the mechanisms underlying the aging process and for approaches to alleviate the resulting functional losses, the neurotransmitter strategy appears to offer great promise. This strategy is based on the

hypothesis that the biological properties and vulnerabilities of nerve cells depend largely on the types of neurotransmitters they produce or are exposed to by innervation from other nerve cells. It permits an insight into the complex interactions and imbalances between neuronal systems that lead to age related functional losses. The aging process may not only cause transmitter deficiencies. Owing to failure of regulatory mechanisms neurotransmitters may prove harmful to the cells producing them as well as to cells exposed to them by innervation. The former mechanism is illustrated by the toxic potential of catecholamine autoxidation, the latter by excitotoxins.

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MOLECULAR GENETICS OF ALZHEIMER'S DISEASE

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INTRODUCTION

St. George Hyslop and colleagues (1987) reported that familial Alzheimer's disease was caused by a locus on the long arm of chromosome 21. In a cohort of families with early onset of disorder (<60 years), we have confirmed this observation (Goate et al., 1989). However, two groups (Schellenberg et al., 1988, Pericak-Vance et al., 1988) have failed to find evidence of linkage to chromosome 21 in families they have studied. The purpose of this article is to discuss possible reasons for this discrepancy.

GENETIC LINKAGE REPORTS

Persons with Down's syndrome (trisomy 21), apparently inevitably, devlop the pathological features (beta-amyloid containing plaques and neurofibrillary tangles) of Alzheimer's disease (Olsson and Shaw 1969). This observation made chromosome 21 a candidate chromosome for Alzheimer's disease. St. George Hyslop and colleagues used linkage analysis to examine the segregation of genetic markers from this chromosome in four extended pedigrees with early onset (<60 years), autosomal dominant Alzheimer's disease. They reported that the disease was genetically linked to two loci on this chromosome (D21S1/S11 and D21S16). We (Owen et al., 1990) and others (Van Broeckhoven et al., 1988) have mapped the locus D21S16 as the most proximal polymorphic marker on the long arm of the chromosome. Genetic and physical mapping of this section of the chromosome has led to the mapping of several genetic markers in the interval between D21S1/S11 and D21S16 (Tanzi et al., 1988, Warren et al., 1989, Van Broeckhoven et al., 1988, Owen et al., 1990). Using some of these markers (S1/S11, S52, S13 and S16), we were able to demonstrate linkage to the Alzheimer's disease locus in six families, selected at random from our cohort of families with an early onset of disorder (seletion criteria; 3 or more family members affected with a mean onset of >60 years) (Goate et al., 1989).

Diagram of showing order of genetic loci on proximal long arm

Centromere-S16-S13-S59-S52-S4-S1/S11-Beta-amyloid- - Telomere

Our genetic analysis confirmed that of St. George Hyslop and

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colleagues (1987), but suggested that the disease locus was more likely to be centromeric of S1/S11 than telomeric of this locus. Genetic analysis of two other pedigrees with a very early onset of disorder (ca. 35 years) by Van Broeckhoven and colleagues (1989 and unpublished) has suggested that the disease locus may be centromeric of D21S16. If this localisation of the disease locus relative to the genetic map of the chromosome is correct (and by the nature of the analysis, this localisation can only be considered provisional), then the marker S1/S11 is some distance from the disease locus.

Schellenberg and colleagues (1988) and Pericak-Vance and colleagues (1988) have both used many families with a late onset of illness in their genetic analyses. In addition, Schellenberg and colleagues used many families from a cultural isolate, the Volga Germans, who have a particularly high incidence of Alzheimer's disease, probably due to a founder effect. In both reports, the data derived from outbred families with an early onset of illness, are consistent with the positive reports described above. However, niether the families with a late onset of illness, nor the Volga Germans showed evidence for linkage to chromosome 21. We too have not been able to find evidence for linkage between Alzheimer's disease and chromosome 21 markers, if we restricted our analysis to families with a late onset (greater than 65 years).

Table 1. Linkage analysis of Chromosome 21 markers and late onset familial Alzheimer's disease.

Genetic Distance	0.00	0.05	0.10	0.20	0.03	0.04
D21S1/S11	-6.00	-2.90	-1.93	-0.88	-0.34	-0.09
D21S13/S16	-0.10	0.01	0.06	0.08	0.03	0.04

This analysis was performed on 6 families with Alzheimer's disease with an onset of greater than 65 years, using a gene frequency for Alzheimer's disease of 0.01. If a gene frequency of 0.05 were used, or a high phenocopy rate, or both, the effect was a flatten out of the curve. A value of -2.00 is taken as evidence against linkage. On this basis, the analysis above suggests that the Alzheimer's disease locus cannot be with a recombination fraction of 10% of D21S1/S11. There is no evidence, either for or against linkage to the D21S13/S16 locus. The TaqI polymorphisms of S11 and S13, the BamHI polymorphism for S1 and the XbaI polymorphism for S16 were used in the analysis.

Possible reasons for failure to observe linkage to Chromosome 21

There are four possible reasons why linkage between Alzheimer's disease and markers on chromosome 21 may not be observed:

(i) reports of linkage are incorrect
(ii) non-allelic genetic heterogeneity
(iii) technical reasons (disease locus on chromosome 21 but linkage not observed)
(iv) aetiological heterogeneity.

(i) Linkage reports are incorrect.
This would seem unlikely, as the original report has been confirmed (Goate et al., 1989) and others have positive data (Van Broeckhoven et al., 1988 and in preparation: Heston et al., unpublished).

(ii) Non-allelic genetic heterogeneity
This hypothesis is that cases of familial Alzheimer's disease can be

caused by genetic loci on other chromosomes. This hypothesis would be proved by the observation of such linkage in pedigrees which have not shown linkage to chromosome 21. However, the fact that four groups have independently observed linkage (above references), suggests that a large proportion of other families with a similar phenotype (most cases in the positive reports have an early onset of illness) must be caused at the chromosome 21 locus.

(iii) Technical reasons

Much of the data suggesting that familila Alzheimer's disease may not be caused on chromosome 21 have been derived using D21S1/S11. However, as described above, this marker may be some distance from the disease gene. This may have led to linkage not being observed. The development of more genetic markers around the previously uninformative D21S16 locus (Van Broeckhoven et al., 1990, Walker et al., 1990) will allow genetic linkage to this section of the chromosome to be tested more rigorously.

A particular problem in the application of linkage stratagies to inbred populations, such as the Volage Germans, is the possible and unrecognised occcurence of disease homozygotes. If this has occurred, this too might have led to linkage not being observed, even though the disease gene was on chromosome 21.

(iv) Aetiological heterogeneity

In our genetic analyses, we assume that the disease is genetic in aetiology. This is a safe assumption in large pedigrees where the pattern of autosomal dominance is well established. It becomes less safe in small pedigrees with few affected members. Here, we assume autosomal dominant inheritance only by analogy. Furthermore, as the disease is very common in the elderly, it is possible, even likely, that if the non-genetic disorder (phenopies) is common, then familial aggregatin will also be common.

We have analysed how frequently the disease would appear as an autosomal dominant in a small nuclear pedigree if it was never genetic in aetiology. We have postulated a population of families with four children ascertained by affected proband, and determined how frequently that both, one of the parents and one further sib would be affected by disease. Our modelling suggests that the disease would cluster in families with a mean onset of less than 60 years only once in 50,000 probands; however, in families with a mean onset of 85 years, it would cluster once in every 16 probands (Hardy et al., 1989). This simple modelling suggests that Alzheimer's disease in the elderly will frequently be familial even if it is not genetic. Thus, use of pedigrees with an onset in the range 65-90 years for linkage analysis makes the implicit assumption that most or all cases of the disease are genetic in aetiology. While some groups maintain this to be so (e.g. Breitner et al., 1988), most workers do not share this view. Further epidemiological investigations of the familial clustering of Alzheimer's disease in the old and very old are required to determine the proportion of genetic and non-genetic cases. Of course, it is also likely that many cases of the disease are caused by interactions between the genome and the environment: if this is ture, then it too will complicate linkage analysis.

These four explanations are not mutually exclusive.

DIRECTIONS FOR FUTURE WORK

Before substantive progress can be made toward isolating the genetic locus causing Alzheimer's disease, the issues relating to apparent

heterogeneity will have to be resolved. Fine genetic mapping requires the interpretation of individual recombinant events. If familial Alzheimer's disease is heterogenous in a non-predictable way, mapping the causative locus will be very difficult except through the use of the few large families because in small pedigrees, one will not know whether the disease is caused by the chromosome 21 locus. The issue of heterogeneity is likely to be resolved by the development of new polymorphic markers on the proximal long arm of chromosome 21, by the testing for genetic linkage elsewhere in the genome and by the joint analysis of all the available linkage data. Work in these directions is currently in progress.

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PROTEINS AND PROTEOLYSIS IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

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INTRODUCTION

Investigations concerning the pathogenesis of Alzheimer's disease initially focused on neurotransmitters, enzymes involved in their synthesis and their neuronal receptors. It eventually became apparent that abnormalities in neurotransmitter systems were probably not the cause of Alzheimer's disease, but rather the result of it, i.e. dead or dying neurons failed to synthesize the neurotransmitters and receptors found to be depleted in this disease. Since the major pathological findings in Alzheimer's disease are neuronal loss, neurofibrillary tangles, senile plaques and cerebrovascular amyloidosis, a major emphasis on delineating the nature of these lesions appeared to offer an approach to the understanding of the nature of this disease. These studies employing protein chemistry have resulted in new pathologic concepts and the introduction of molecular biology in the deciphering of the pathogenesis of Alzheimer's disease.

Proteins and Proteolysis

Earlier studies on amyloidosis had revealed that one of the mechanisms of formation of the ß-pleated sheet fibrils[1] characteristic of amyloid deposits was by proteolysis of the protein precursor of the fibril[2]. Those amyloid deposits for example composed of the N-terminal variable region of immunoglobulin light chains in such disorders as multiple myeloma are created by proteolytic cleavage of the intact light chain to produce ß-pleated sheet (amyloid) fibrils[3]. The presence of cerebrovascular amyloidosis in over 90% of Alzheimer's disease cases[4] prompted us to isolate the leptomeningeal vessels from such cases and purify from them the amyloid fibril protein. After extraction in 6 M guanidine, chromatography on a G-100 Sephadex column and HPLC reverse phase chromatography, a 4.2 kilodalton protein,

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ß protein, was isolated and its amino acid sequence was found to be unique and previously unreported[5].

Since adult Down's syndrome individuals are known to have the same lesions as Alzheimer's disease in 100% of cases, the amyloid protein from the leptomeningeal vessels of such cases was also purified[6]. This protein was essentially identical to the ß protein isolated from Alzheimer's disease patients. It was therefore concluded that ß protein was encoded by a gene on chromosome 21[6].

Four independent groups have isolated cDNA clones coding for the amyloid ß protein precursor (ßPP) by the use of oligonucleotide probes based on the amino acid sequence of the ß protein. Through the use of somatic cell hybrids the gene coding for the ßPP was localized to chromosome 21[7]. An analysis of an apparently full-length clone showed the gene contains an open reading frame coding for 695 amino acids. A small segment of this putative ßPP near the carboxyl terminus is identical with that of the ß protein. It was suggested that the 695 residue ßPP is a glycosylated cell-surface receptor of approximately 79 kDa[8]. The ß protein segment includes part of the membrane-spanning region and part of the adjacent extracellular domain. Two variants of ßPP having 751 and 770 amino acid residues contain an insert corresponding to a Kunitz-type inhibitor[9]. A familial Alzheimer's disease (FAD) gene marker was also reported on chromosome 21[10] .

ß Protein Antisera Localization

Antisera raised to the ß protein were reactive with intracortical amyloid-laden vessels and with senile plaques[11]. These results strongly suggested that the amyloid fibrils constituting the vascular deposits throughout the cerebrum as well as the amyloid core of senile plaques were composed of a protein sharing antigenic determinants with ß protein. In view of the fact that the vascular amyloid deposits resided in identical morphologic sites as that seen in systemic amyloidosis such as that of the AL and AA type in which dissemination of the amyloid fibril precursor is via the blood stream (3,4), it was suggested that here too vascular deposition of the ß protein was via the ßPP in the serum. Recent immunochemical evidence of a protein in the serum reactive to ßPP antibodies lends further support for this mechanism[12]. The reason for the localization of the amyloid deposits solely to the cerebrum in Alzheimer's disease and Down's syndrome can be attributed to the difference in the proteolytic enzyme complement between cerebral and peripheral endothelia[13].

Further application of antibodies to the ß protein has revealed diffuse granular (Type 4) and non-neuritic, non-amyloidotic plaque-like deposits (Type 3) as one of earliest lesions in the cerebral tissue in both Alzheimer's disease[14] and Down's syndrome individuals[15]. These lesions do not shown fibrillar deposits but rather consist of election

dense sheaves and rods[16] and apparently represent a preamyloid stage in plaque formation. It now appears that there exists a temporal sequence from the ß protein immunoreactive granular deposits to ill-defined non-neuritic and non-amyloid plaques to diffuse plaques containing amyloid fibrils and finally plaques with a compact amyloid core. These results and the recent immunohistochemical evidence that an antiserum to the N-terminal portion of ßPP, which excludes the ß protein residues, reacts with diffuse plaques[17] suggests that the ßPP enters the neuropil through an incompetent blood-brain barrier predominantly at the level of compromised capillaries[18] and is proteolytically cleaved in situ into amyloid fibers[17].

The ßP Gene and the Familial Alzheimer's Disease Gene Marker

The possibility that an increase in the gene dosage of the ß protein gene may lead to amyloid fibril formation has been presented[19], but not confirmed[20]. Originally it was believed that the amyloid ß protein gene and the FAD gene marker on chromosome 21[10], were linked; but this has not been corroborated[21], suggesting that the gene abnormality associated with the familial form of the disease is distinct from that of the ß protein locus.

The Pathogenesis of Alzheimer's Disease

From the present state of our knowledge, a pathogenic sequence of events leading to cerebrovascular amyloidosis, plaques and tangles, can be devised. The possibility is that the amyloid ß protein gene encodes a normal ßPP which is abnormally glycosylated (or otherwise abnormally processed post-translationally) by an abnormal enzyme encoded by the FAD marker gene. Assuming the primary source of cerebral amyloid deposits is in peripheral sites[22], the abnormal ß protein is disseminated via the blood stream where it is acted upon preferentially by cerebral endothelial cells, the proteolytic enzyme complement of which cleaves the ßPP to amyloid fibers. Accumulation of these fibers disrupts the blood-brain barrier. This permits egress of ßPP into the neuropil where it is acted upon by the proteolytic complement of microglia to form senile plaques. ßPP acts as a protein or peptide ligand[22] to block receptors on the surface of neurons in the neocortex to perturb their environment and induce the formation of paired helical filaments. The plaques are destructive to traversing nerve fibers while the paired helical filaments prevent neuronal axonal transport and destroy those cells in which they are deposited.

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AMYLOID β PROTEIN PRECURSORS HAVING PROTEINASE INHIBITOR REGIONS ARE HIGHLY EXPRESSED IN ALZHEIMER'S DISEASE BRAINS

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the aged and is characterized by cerebral deposits of amyloid β-protein (β -AP) comprising about 40 amino acids as senile plaque core and vascular amyloid.[1,2] Since there is a correlation between the number of plaques and the degree of dementia,[3] it has been suggested that the formation of senile plaque is one of the pathogenetic features of AD. A complementary DNA (cDNA) clone of a β-AP precursor (APP) has been proved to encode a 695-amino acid precursor (APP695) having structural features characteristic of cell surface glycoproteins.[4]

THREE TYPES OF mRNA OF β-AP PRECURSORS

From a cDNA library of a human glioblastoma cell line, we found two other types of APP mRNA's encoding a 751-amino acid molecule, APP751 (with 168-bp insert) and a 770-amino acid one, APP770 (with 225-bp insert) (Fig.1).[5] APP751[5-7] and APP770[5] bear an identical 56-amino acid sequence that is highly homologous with the Kunitz-type basic trypsin inhibitors, and the extract of COS-1 cells transfected with APP770 cDNA exhibits inhibitory activity against trypsin.[5] Cloning of genomic DNA revealed that the 225 bp insert in APP770 mRNA is derived from two exons, 168 bp and 57 bp long, and that these three types of APP mRNA are produced by alternative splicing of the premature APP gene transcript[5]. These two exons are the 7th and 8th of the entire 18 exons of APP770.[8]

EXPRESSION OF THE THREE TYPES OF APP mRNA IN HUMAN BRAIN

Conflicting findings have been reported on the relative occurrence of the three types of APP mRNA. Palmert et al. have found that the

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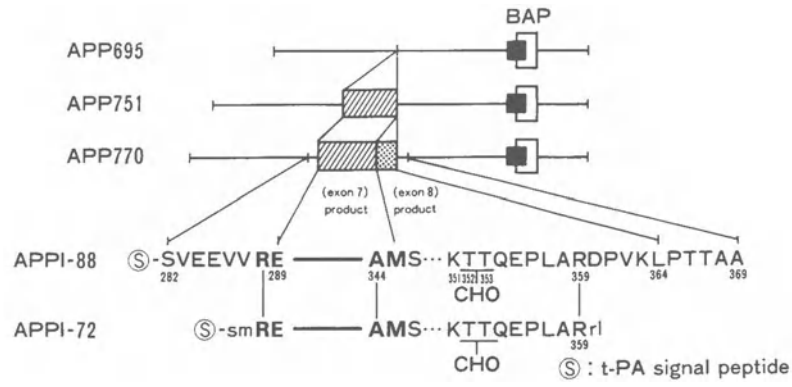


Fig. 1 Schematic structures of three types of APP's and two APPI's used as antigens. Open box:transmembrane region. Closed box:β -AP. Small letters represent amino acids encoded by the DNA linkers.

expression of APP695 mRNA is twice as strong in the specific region of AD brain (neurons of locus ceruleus and nucleus basalis) as in normal controls by in situ hybridization.[9] On the contrary, by RNA blot analysis using a probe specific for APP695 mRNA and a probe recognizing both APP751 and 770 mRNA, Johnson et al. have found a twofold increase in the ratio of [APP751 plus 770 mRNA] / [APP695 mRNA] in AD brain.[10,11] Further, utilizing oligonucleotide probes specific for each type of APP mRNA, we undertook RNA blot analysis of APP mRNA's obtained from the frontal cortices of 3 AD patients and 4 controls. Analyzing the density of APP bands normalized with the β -actin band revealed APP695 and APP751 mRNA's of AD patients to be substantially the same as in age-matched controls, but APP770 mRNA to be about doubly elevated.[12] We also observed a twofold elevation of APP770 mRNA and a 1.1-1.3 fold increase in APP751 mRNA in AD brain by an RNase protection assay.[13]

Based on Johnson's and our findings, the proteinase inhibitor regions of APP (APPI) are likely to foster plaque formation by inhibiting the APP-catabolizing proteinases.

INHIBITION SPECTRUM OF APPI

It has been suggested that the sequence of APPI is similar in some respects to that of protease nexin I, a serine proteinase inhibitor possessing neurite outgrowth activity.[14] From histological study, it is assumed that the senile plaque has trophic factors that promote aberrant growth of neurite into the plaque. Therefore, APPI is postulated to possess an inhibitory spectrum similar to that of protease nexin I and to be a potential promoter of neurite outgrowth.

As one step toward identification of the target enzyme(s) of APPI in the brain, which might also be the APP-catabolizing proteinase in vivo, and thus a step toward elucidation of the physiological role of APPI, we

studied the production of APPI in relatively pure and short form and investigated its in vitro inhibitory activity toward a number of enzymes, especially those sensitive to protease nexin I.[15]

A TaqI-AvaI fragment of APP770 cDNA that encodes APPI-72 (Fig.1) was inserted into an expression vector for mammalian cells downstream of the human tissue plasminogen activator (t-PA) signal sequence for secretion of APPI-72 into culture medium. The conditioned medium of monkey kidney COS-1 cells transfected with this expression plasmid was purified by a sequential acetone-precipitation, followed by affinity chromatography using immobilized trypsin, to give APPI-72 showing single band in SDS-PAGE.

The inhibitory activity of APPI-72 against various serine proteinases was measured using fluorogenic synthetic substrates, and a fairly broad spectrum of inhibition was revealed.[15] BPTI, one of the Kunitz-type basic trypsin inhibitors strongly homologous with APPI, was used as a control to show the reliability of our measurement. Equilibrium dissociation constants (Ki) were determined for all of the enzymes highly or moderately sensitive to APPI-72 (Table 1). A Green and Work plot of trypsin inhibition by APPI-72 suggests that APPI-72 forms a 1 : 1 complex with trypsin. The Ki value of APPI-72 for trypsin indicates an extremely

Table 1 Equilibrium dissociation constants (Ki) of APPI-72 and BPTI against serine proteinases[15] in comparison with association rate constants ($k_{assoc.}$) of protease nexin I[16]

Enzymes	Ki (M)		$k_{assoc.}$ (M^{-1} S^{-1})
	APPI-72	BPTI	Protease Nexin I
trypsin	(P)1.1×10^{-10}	(P)1.3×10^{-11}	(B)4.2×10^{6}
(B)chymotrypsin	5.8×10^{-9}	9.2×10^{-9}	no inhibition
factor Xa	(B)1.2×10^{-6}	(B)1.5×10^{-3}	(H)7.3×10^{3}
(H)kallikrein (urine)	4.7×10^{-7}	1.7×10^{-6}	n.d.
(H)kallikrein (plasma)	1.9×10^{-7}	5.7×10^{-7}	n.d.
(H)plasmin	4.6×10^{-8}	8.9×10^{-11}	1.3×10^{5}
(H)elastase (leukocyte)	7.9×10^{-7}	3.7×10^{-6}	no inhibition
(H)thrombin	no inhibition	no inhibition	6.0×10^{5}
(H)urokinase	no inhibition	no inhibition	1.5×10^{5}

P, Porcine; B, bovine; H, human. n.d., Not done.

strong inhibitory activity against this enzyme; that for chymotrypsin, though some 50 times higher, also indicates very strong inhibition. Those for other enzymes were some 10^2 to 10^4 times higher: however, they were still fairly small (10^{-7} M). Table 1 also indicates that APPI inhibits trypsin and plasmin less strongly than BPTI (APPI-72 Ki values some 10 and 10^3 times larger), and inhibits factor Xa more strongly than does BPTI (APPI-72 Ki value 10^3 times smaller).

The spectrum of APPI-inhibition observed in the present study was quite different from that of protease nexin I. Although the association rate constant $k_{assoc.}$ cannot be compared directly with the equilibrium constant Ki, relative inhibitory activity described in each parameter may be comparable (Table 1). Protease nexin I is reported to inhibit thrombin and urokinase as well as trypsin, plasmin and factor Xa, but not to inhibit chymotrypsin or leukocyte elastase.[16] However, APPI did not inhibit thrombin or urokinase and clearly inhibited chymotrypsin and leukocyte elastase. This pattern suggests that physiological roles of these two inhibitors are quite different. Actually, APPI-72 showed no effect on survival or neurite outgrowth of neonatal rat cerebral cortical neurons (Yoko Uchida, Masanori Tomonaga, et al., personal communication). Our results are consistent with the recent findings that APP having a proteinase inhibitor domain is protease nexin II.[17,18]

DETECTION OF APPI IN CEREBROSPINAL FLUID (CSF)

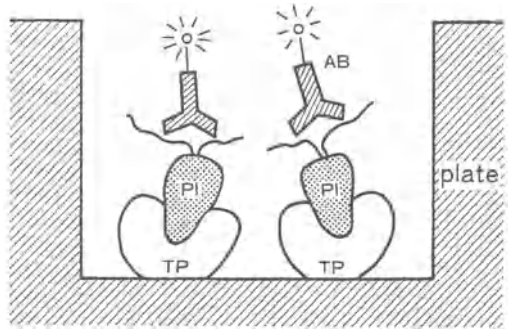
Based on the finding of the expression of APP mRNA's in the brain, APP proteins having APPI would be expected to increase in CSF of AD patients. There have been several reports on detection of APPI in CSF by immunoblot analysis. By using a monoclonal antibody against denatured APP-695, Weidemann and his colleagues have found that both soluble forms of APP, with APPI (112 KDa) and without APPI (91 KDa), are more abundant in CSF of AD than in that of controls.[19] Palmert et al. also has found that 125-KDa APP exists both in CSF and in soluble fractions of brain homogenate, and 58-KDa APP fragments are exists in CSF.[20]

For the diagnostic purpose, the concentration of APPI should be determined by a quantitative method that can assay many samples at a time such as the enzyme linked immunosorbent assay (ELISA), rather than immunoblot analysis. We developed a novel immunochemical method for detection of proteinase inhibitors.

As Palmert has reported[20], shorter molecular species of APPI may be present in CSF or in the brain, so we attempted to detect even the minimum size of APPI in APP770. The usual sandwich ELISA using an antibody against the exon 7 product (hatched box in Fig.1) and an antibody against the exon 8 product (dotted box in Fig.1) was found to be unsuccessful because we could not obtain a satisfactory antibody to recognize the exon 7 product. The weak immunogenisity of the exon 7 product may be caused by its high homology with the counterparts of other mammals. The mouse counterpart of the exon 7 product is different from human APPI at only one amino acid at its C-terminal,[21] and the rat counterpart differs in two residue.[22]

On the other hand, APPI has a very small Ki value against trypsin and other serine proteinases (Table 1). This strong binding ability of trypsin to APPI is comparable or superior to that of antibodies. The three-dimensional structure of the exon 7 product was calculated to show a tight wedge-like form with three disulfide bonds similar to other Kunitz type basic trypsin inhibitors.[22] The BPTI-trypsin complex is analyzed by X-ray crystallography to reveal that trypsin interacts with only several amino acids around the reactive site of BPTI. Therefore, the tip (reactive site Arg^{301} of APP 770) of the wedge-like APPI may come in contact with trypsin, and the exon 8 product may be situated opposite the "tip".

Based on this conception, we applied trypsin as the first antigen in the sandwich ELISA.[24] The schema of the new ELISA procedure with a trypsin-bound plate, trypsin-plate ELISA, is shown in Fig.2. APPI-72 and APPI-88 (Fig. 1) were used as antigens with or without denaturation.



TP : Trypsin PI : APPI
AB : anti APP(I) antibody

Fig. 2 Schema of trypsin-antibody sandwich ELISA (trypsin-plate ELISA)

In a preliminary trial to investigate CSF samples by this trypsin-plate ELISA, we found the APPI concentration in CSF of AD to be elevated compared with that of multi-infarct dementia (MID) and non-demented patients.[24] These results are consistent with the previous finding that mRNA's of APP with APPI increase in AD brain compared with their levels in controls.

Further investigation should be done with more samples of AD at various stages, MID, other dementias, depression, non-demented patients and healthy controls to evaluate the usefulness of this method for diagnosis of AD.

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DIFFERENTIAL EXPRESSION OF THREE TYPES OF AMYLOID PROTEIN PRECURSOR mRNA IN ALZHEIMER'S DISEASE BRAIN

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INTRODUCTION

Deposition of amyloid β-protein in senile plaque cores and cerebral vessels is one of the characteristic findings in Alzheimer's disease brain. Complementary DNA (cDNA) encoding the precursor of amyloid β-protein (APP695) was cloned and sequenced[1]. The precursor has structural features characteristic of cell surface receptors. We cloned APP cDNAs from a cDNA library of a human glioblastoma cell line, and found two

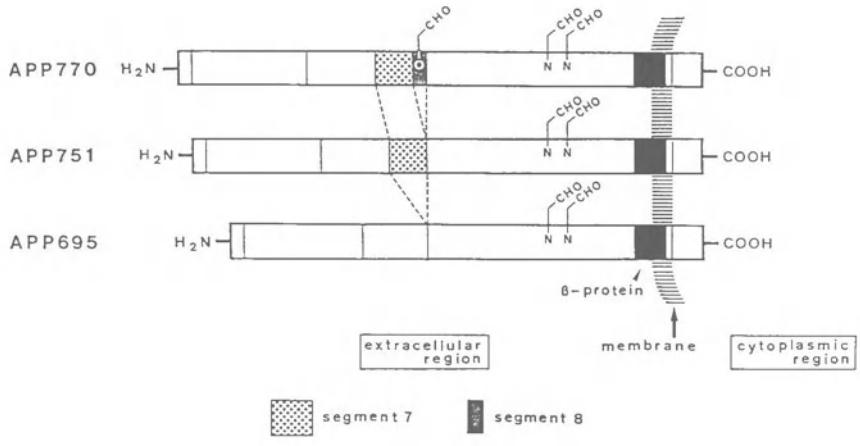

Fig. 1. Proposed structures of three types of APP mRNA[1,2]. The precursors have structural features characteristic of cell surface receptors; a large extracellular domain, a hydrophobic transmembrane domain, and a small cytoplasmic domain. β-protein is shown in the solid box, marked by arrowhead.

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more cDNAs encoding APPs (APP751 and APP770) together with APP695 cDNA previously reported[2]. APP751 has a 56 amino-acid insert, and APP770 has an additional 19 amino-acid insert (Fig. 1). These two inserts are encoded by exons, 7 and 8 [2,3], respectively. APP gene exists as a single copy, indicating that the three types of APP mRNA are produced by alternative splicing from a common transcript. The amino-acid sequence encoded by exon 7 is highly homologous to the basic trypsin inhibitor family. The fact that this APP fragment has actually a protease inhibitor activity was shown by an *in vitro* expression experiment[2].

The existance of protease inhibitor domain in the precursor has suggested a possibility that this inhibitor might interfere with the metabolism of APP and lead to amyloid deposition. In order to examine this possibility, we investigated the expression of various types of APP mRNA in the brain of AD.

MATERIALS AND METHODS

Postmortem brains were obtained from histologically confirmed AD patients and non-demented controls. The brains were removed within 3-10 hours after death, and kept frozen at -70°C until use. Total cellular RNA was prepared from frontal cortex (Brodmann areas 9 and 10) of each brain by the guanidinium/CsCl method, and poly(A)$^+$RNA was isolated by the oligo(dT)-cellulose chromatography.

Northern blot analysis was performed using four synthetic oligonucleotide probes designed to hybridize with one (or two) specific type(s) of APP mRNA[4]. For a quantitative comparison of APP mRNAs expression, the autoradiograms were analysed by densitometry.

Ribonuclease protection assay was carried out using anti-sense RNA probe after Gilman[5]. The RNA probe was synthesized using pSP64 plasmid (Promega). This probe contained the sequence complementary to inserts encoded by exons 7 and 8, and could differentiate three types of APP mRNAs. The hybrid of sample RNA and probe RNA was treated by ribonucleases A and T1. Double-stranded portions that were protected from digestion were denatured, elctrophoresed, and analysed by autoradiography.

RESULTS

The results of the quantitative analysis of Northern blotting are shown in Fig. 2. The ratios of the mean value for AD group to that for control group were 1.12 for APP695 mRNA, 1.11 for APP751 mRNA, and 2.04 for APP770 mRNA. The difference in APP770 mRNA was statistically significant ($p<0.05$, Student t-test); there was no significant change in APP695 and APP751 mRNAs.

Ribonuclease protection assay clearly revealed that APP695 mRNA was a major component of APP mRNA in human adult brain, followed by APP751 mRNA (Fig. 3). On the other hand, APP770 was a minor component, occupying less than 10%. In the brain of AD patients, the proportions of APP770 and APP751 mRNAs increased, while APP695 mRNA decreased. The ratios of AD to control were 0.81 for APP695 mRNA, 1.31 for APP751 mRNA, and 2.37 for APP770 mRNA. All these differences between AD and control were statistically significant ($p<0.05$, Student t-test).

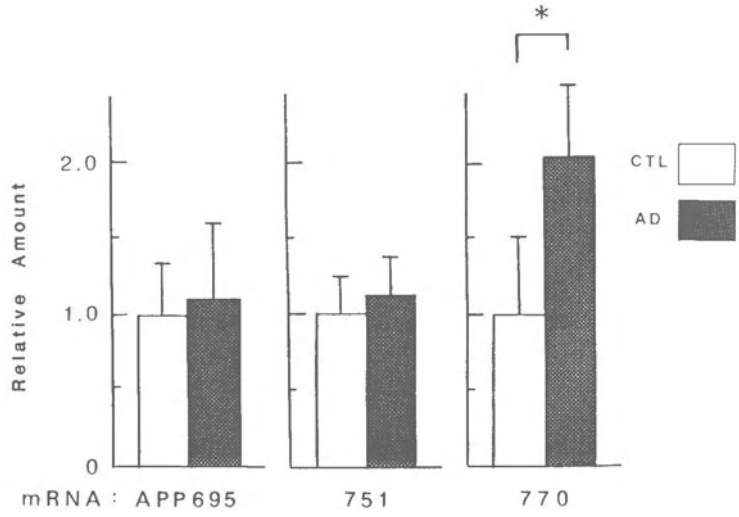

Fig. 2. Relative amounts of each type of APP mRNA, calculated from Northern blot analysis[4]. The mean value of the control group was taken as unity.
(*p<0.05, student t-test)
CTL: control, AD: Alzheimer's disease.

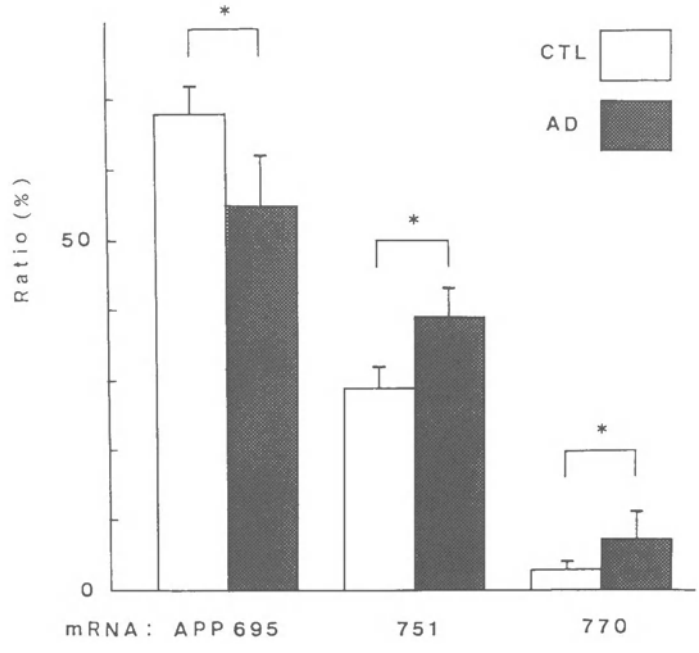

Fig. 3. Proportions of three types of APP mRNA, calculated from ribonuclease protection assay. Error bars denote standard deviation.
(*p<0.05, student t-test)

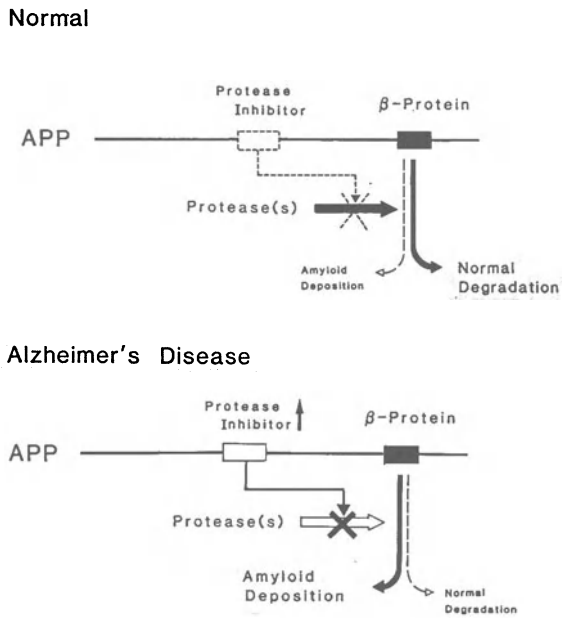

Fig. 4. A possible mechanism of amyloid deposition in the brain of AD.

DISCUSSION

Based on above results, we propose a working hypothesis, as shown in Fig. 4. Under normal conditions, APP is metabolized efficiently enough by proteases to match its biosynthesis, whereupon the role of protease inhibitor is minute. In the case of AD, by contrast, an increased production of protease inhibitor built in APP(s) may suppress protease(s) and interfere with the balance of APP biosynthesis and degradation, leading to eventually accumulation of incomplete APP metabolite(s) or amyloid. There might be brain-specific protease(s) engaged in this APP metabolism and regulated by the APP-derived protease inhibitor. Metabolic processes of APPs and proteases involved will be the next and more crucial problem in understanding amyloidogenesis of AD.

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PROTEASE INHIBITOR AND CHOLINERGIC SYSTEM IN ALZHEIMER'S DISEASE

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INTRODUCTION

The level of mRNA for an amyloid precursor (APP770) containing a Kunitz-type trypsin inhibitor (PI) is elevated in the autopsied Alzheimer brain.[1] However, the physiological significance of the augumented APP770 production has not been clarified thus far.

Alzheimer's disease affects the cholinergic system of the brain, depleting the cholinergic marker enzymes including choline acetyltransferase and acetylcholinesterase (AChE).[2-4] Moreover, histochemical study on AChE distribution in the cerbral cortex has revealed a difference between control subjects and Alzheimer patients.[5] However, neurochemical studies are required to determine if the AChE depletion is due to some difference in subcellular distribution or to an effect of various substances, including AChE inhibitor.

In order to investigate the effect of the protease inhibitor on the cholinergic system, we studied the abnormal subcellular distribution of acetylcholinesterase and its mechanism.

MATERIALS AND METHODS

Chemicals

Kunitz-type protease inhibitor (PI), containing 72 amino acids covering most part of the inserTion peptides of amyloid precursor protein, was prepared from the supernatant solution of cultured COS-1 cells.[6] Protease-free collagenase, (^{14}C)acetylcholine, and α-1-antichymotrypsin (AAC) were obtained from Advance Biofacture, ICN Biochemicals, and Calbiochem, respectively.

Brain samples

Autopsied human brains were obtained from 6 patients with non-neurological diseases and 8 patients with Alzheimer's disease. There was

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no significant difference in autopsy delay between the 2 groups. Brains were cut into 2 hemispheres at autopsy. One hemisphere was fixed in 10% formalin for pathological examination, and the other was stored at -80°C. Frozen brain was thawed and the frontal cortex was cut out for the biochemical study. The diagnosis of patients was made by clinical and radiological findings, and pathological examinations.

Preparation of subcellular fractions from control brains

The cerebral cortex was minced and gently homogenized in 0.32 M sucrose-10 mM Tris-acetate (pH 7.3) in the cold. Fractions P1, P2, P3, S3, A, B, and C were prepared by the method of Gray et al.[7]

Preparation of senile plaque-rich fractions

The fraction enriched in senile plaque was prepared from the frontal cortex of autopsied Alzheimer brain according to the method described by Candy et al.,[8] with modifications.[9] A block (5 g) cut out from the frozen autopsied brain was homogenized in 25 ml of 0.1 M potassium phosphate buffer (pH 7.0) for 30 sec in a Polytron homogenizer and sonicated for 1 min with a sonic oscillator. The homogenate was centrifuged at 20,000 g for 15 min, and the supernatant solution (S'1) was separated from the pellet (P'1). P'1 was suspended in 15 ml of 2% sodium laurylsulfate and homogenized again for 30 sec. The suspension was centrifuged at 35,000 g for 45 min and the supernatant solution (S'2) was separated from its pellet (P'2). The P'2 fraction was suspended in 6 ml of 20% sucrose, and a portion (2 ml) was layered over a discontinuous sucrose density gradient containing 2 ml of 45% sucrose and 2.5 ml of 30% sucrose. The gradient was centrifuged at 6,000 g for 15 min. Three fractions were obtained after centrifugation: fraction A' in the 20% sucrose, fraction B' at the boundary between 30% and 45% sucrose, and fraction C' at the bottom of the tube. Each fraction was suspended in 10 ml of 0.01 M potassium phosphate buffer (pH 7.0) and washed. Fraction B' showed a histological property associated with senile plaque, a green birefringence with congo-red staining.

Extraction of AChE from fraction B' with salt

We applied a differential extraction method[10] to the isolated senile-plaque rich fraction. The first extraction was carried out with a low ionic strength buffer (10 mM sodium phosphate buffer, pH 7.0, containing 1% Triton X-100, 5mM N-ethylmaleimide, 2mM benzamidine, and 10 mM EGTA). Fraction B' was homogenized with 10 volumes of low ionic strength buffer (H1). The centrifugation of H1 at 28,000 g for 30 min yielded supernatant solution E1 and pellet H2. A second extraction was also carried out with the same buffer, yielding supernatant E2 and precipitate H3. Fractions E1 and E2 have been reported to contain globular forms of AChE.[10] The homogenization of H3 with a high ionic strength buffer (low ionic strength buffer supplemented with 1.0 M NaCl) yielded supernatant solution E3 and pellet H4. The same procedure using H4 produced supernatant solution E4 and the final precipitate H5, which contained non-extractable AChE. E3 and E4 contained asymmetric forms of AChE as found in the skeletal muscle.[10]

Digestion with collagenase or proteases

Fraction B', E4, or H5 was incubated with collagenase or protease (0.2 mg/ml) for 10 min at 37°C. The incubated mixture (0.05 ml) was diluted with 1.0 ml of cold 0.01 M potassium phosphate buffer, pH 7.0, and centrifuged at 100,000 g for 60 min to yield supernatant solution S4 and pellet P4.

Determinations

We determined the sedimentation coefficient by centrifugation on a 5 - 20% linear sucrose density gradient of 4.5 ml on a 40% sucrose cushion, both prepared in the extraction buffer. The gradient was centrifuged at 100,000 g for 17.5 hours. The internal marker enzymes used were alcohol dehydrogenase (4.8 S), catalase (11.7 S), and beta-galactosidase (16.0 S). The sedimentation coefficient was calculated by the method of Martin et al.[11] AChE activity was measured spectrophotometrically by the thiocholine method.[12] The AChE assay was also performed radiochemically with (^{14}C)acetylcholine as the substrate.[13] Protein was determined by the method of Lowry et al.[14]

RESULTS

Subcellular distribution and extraction with Triton-NaCl of AChE

The highest specific activity of AChE was observed in the fraction B' enriched in senile plaque (Fig. 1). We extracted AChE from the control brain and fraction B' sequentially with Triton and salt. Large amounts of AChE were recovered in E1 and E2 (low ionic strength buffer). However, a small amount of AChE was extracted from the fraction B' into E1 and E2. The activity of AChE was high in fractions E3, E4, and H5 prepared from the senile plaque-rich fraction.

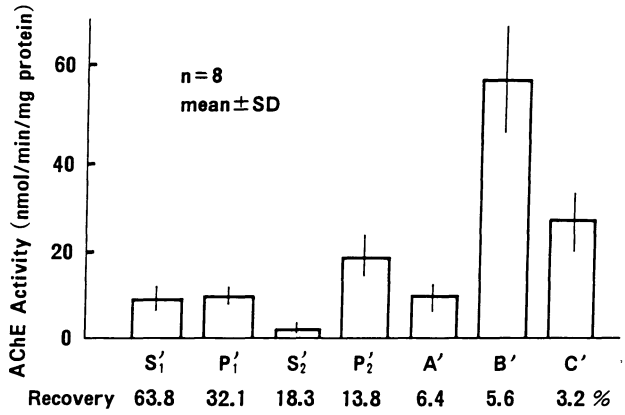

Fig. 1. Activity of AChE in the fraction enriched in senile plaque.

Digestion with collagenase or protease

More than 50% of the AChE activity in fraction B' appeared in the supernatant solution (S4) after incubation with either collagenase or protease for 10 min (Fig. 2). A prolonged incubation with collagenase or protease (40 min) resulted a complete solubilization of AChE, although the recovery of AChE activity was less than 60%. Congo-red positive structures were observed in the pellet (P4) after the incubation for 40 min.

The digestion was also performed in the presence of PI, AAC, or soybean trypsin inhibitor (STI). The solubilization of AChE with collagenase was inhibited by PI or AAC, but not by STI (Fig. 3). PI or STI decreased the solubilization with trypsin, but AAC did not.

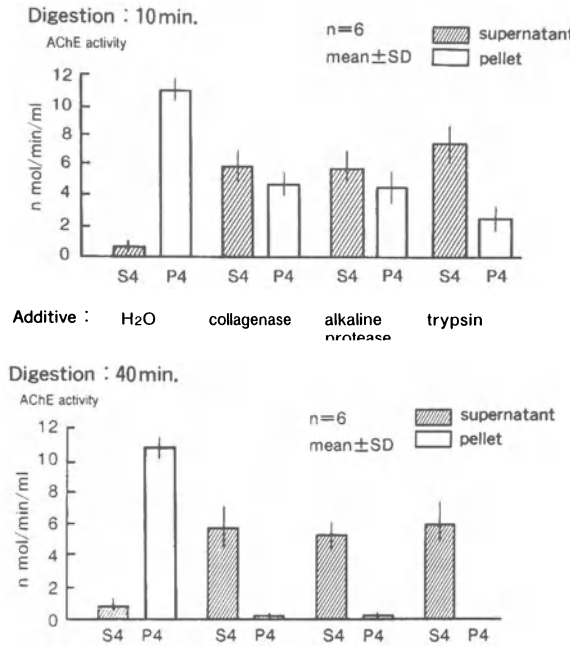

Fig 2. Digestion of Fraction B' with collagenase or proteases.

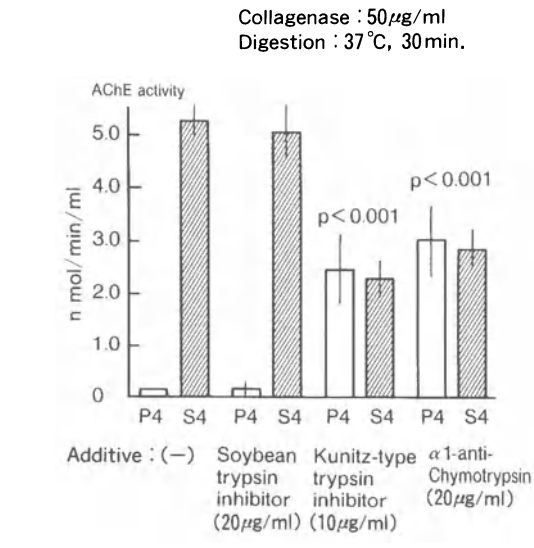

Fig. 3. Effect of protease inhibitors on solubilization of AChE.

Sucrose density gradient centrifugation

We determined by centrifugation on a linear sucrose density gradient the sedimentation coefficient of AChE in S4 obtained by digestion of fraction B' with collagenase for 40 min. Most of the AChE activity was detected in fractions calculated as 10S. Similar results were obtained with the preparations solubilized from fractions E3 or H5 by the collagenase digestion.

Inhibition of AChE with AChE inhibitors

Physostigmine was added to fractions S3 and B obtained from control brain and to fractions S'1 and B'. AChE activity in fractions S3, B, and S'1 was inhibited at a low concentration (ID50=80 nM), but a higher concentration (ID50>0.2 mM) was necessary to inhibit AChE in the fraction B'. Other AChE inhibitors including tetrahydroaminoacridine showed a similar pattern of inhibition.

DISCUSSION

The histochemical distribution of AChE has already been investigated in brains of patients with Alzheimer's disease.[5] The location of the enzyme was largely shifted to the senile plaques or neurofibrillary tan-gles. In the present neurochemical study, a considerable amount of AChE activity in Alzheimer brain (13.8%) was recovered in the pellet after solubilization, whereas most of the activity in control brain (>98%) was detected in the supernatant solution by the same procedure. The partic-ulate fraction (B') enriched in senile plaque showed the highest specific activity, which coincided with the histochemical observation.[5]

AChE was solubilized from isolated fraction B' after incubation with either protease or collagenase, while amyloid protein was left in the pellet, as earlier reported.[8] Solubilized AChE mainly showed a sedimentation coefficient of 10S, which corresponds with that of the G4 isozyme. Solubilization with protease-free collagenase suggests that AChE in the senile plaque, probably in the amyloid core, would be the A form possessing a collagen-like tail.[9] The globular G4 isozyme might be detached from the senile plaque by digestion with collagenase.

AChE is produced as an asymmetric form containing a collagen-like tail. Under normal conditions, this tail is cleaved by proteases, especially collagenase, and AChE is converted into the globular G4 form. The G4 form is transported to the nerve ending by axonal flow and exerts its function related to the cholinergic neurotransmission. In addition to its well-known esterase activity, AChE has been reported to show also a trypsin-like activity and the collagen-like tail is supposedly cleaved by autolysis.[16]

Recently, Kitaguchi et al.[6] reported a gene coding for a protease inhibitor near the locus of amyloid protein in chromosome 21. The presence of α-1-antichymotrypsin has also been demonstrated in the Alzheimer brain.[17] The increase in protease inhibitors such as Kunitz-type trypsin inhibitor or α-1-antichymotrypsin might prevent the conversion of AChE from an asymmetric form to a globular one as well as the breakdown of amyloid protein. The collagen-like tail of AChE would have an affinity for amyloid protein, thus causing precipitation of the enzyme in the senile plaque. The accumulation of AChE in the senile plaque and the decreased transportable G4 form would lead to a lack of AChE at the nerve terminals.

The effect of AChE inhibitor was less remarkable in the isolated senile plaque-rich fraction than in the soluble or normal particulate fraction. The present results might be ascribed either to a conformational change of AChE or to the inaccessibility of the drug. Further neurochemical studies will evaluate the utility or disutility of AChE inhibitor for the therapy of Alzheimer's disease.

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STRUCTURE AND EXPRESSION OF mRNA FOR THE MOUSE HOMOLOG OF ALZHEIMER AMYLOID BETA PROTEIN PRECURSOR

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INTRODUCTION

In the brain of patients with Alzheimer's disease (AD), fibrillar amyloid is deposited as senile plaque core and cerebrovascular amyloid[1,2]. The beta protein or A4 protein is a major constituent of this amyloid and is now known to be the cleavage product of a larger precursor protein (BPP) which has features characteristic of glycosylated cell surface receptors[3]. In human, at least three species of mRNA coding for the BPP of 695, 751 and 770-amino acid residues (hBPP695, hBPP751 and hBPP770) were found and the latter two were shown to encode a protease inhibitor domain[4-6]. The protease inhibitory activity could be related to aberrant BPP catabolism and eventually to amyloid fibril formation in AD.

Thus to understand the process of AD amyloid formation, it is important to know the biological functions as well as catabolism of these three species of BPP in detail. Because of limited availability of human tissue samples for experiments, it may be valuable to develop a suitable animal model. For this reason, we chose mouse as a model system. We have cloned and characterized the cDNA for the mouse homolog of hBPP (mBPP) and investigated the expression of the mBPP gene.

Our results indicate that BPP is highly conserved in mammalian evolution and that transcripts of the BPP gene are alternatively-spliced in a tissue specific manner in mouse as in human. Thus the mouse may be a good model system for understanding the expression, function and degradation of BPP in human.

MATERIALS AND METHODS

Cloning and DNA sequencing

For the isolation of cDNA clones, we screened mouse brain and kidney cDNA libraries purchased from Clontech (Palo Alto, USA). We also screened a mouse brain cDNA library constructed in our laboratory. The hBPP695 and hBPP751 cDNAs cloned in our laboratory were used as probes. A mouse genome DNA library constructed in our laboratory were screened to isolate the mBPP gene. Insert DNAs were subcloned into pUC plasmids and sequenced by the dideoxy chain-termination method.

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Northern blot analysis

Total RNA was extracted from various tissues of an adult mouse and brains of mice aged 0 day, 2, 6 and 22 months by the method of Chirgwin et al. Glyoxal-denatured RNAs were electrophoresed through 1% agarose gel and transferred to Zeta-probe membrane (Bio-Rad, Richmond, USA). To detect each species of mBPP mRNA specifically, four 40mer oligonucleotides complementary to the junctional sequences arising from four possible exon combinations were used (Fig.2A, HK, IK, IJ, HJ). Northern blots were stripped and rehybridized with a human beta actin gene (Wako, Osaka, Japan) to ensure the integrity and equal quantity of the samples. The ratio of intensity of the BPP band versus the beta actin band was calculated by transmission densitometry of autoradiograms.

RESULTS AND DISCUSSION

Isolation of the mBPP cDNA clones

After screening of the two mouse brain cDNA libraries, we obtained the two overlapping clones, which covered the whole protein-coding region of mBPP695[7]. We next screened the mouse kidney cDNA library. Two other species of cDNA clones containing 225-bp and 168-bp inserts in addition to the mBPP695 cDNA were obtained. These corresponded to the cDNAs for hBPP770 and 751, respectively[8].

Comparison of the nucleotide and amino acid sequences between mBPP and hBPP

When the nucleotide sequences of the protein-coding region of BPP695 and the 168-bp insert were compared between mouse and human, they shared high degree of homologies (89.1% and 93.5%, respectively). Comparison of the deduced amino acid sequences also revealed high degree of homologies (97.0% and 94.6% for BPP695 and the 168-bp insert, respectively). Among the amino acid residues of hBPP695, only twenty-one were replaced in mBPP695. Most of these substitutions appeared not to affect the secondary and tertiary structures. Three amino acid substitutions, R to G, Y to F, and H to R, were found within the amyloid beta protein region (Fig. 1). There were also three amino acid substitutions, A to V, A to V, and M to S, in the protease inhibitor domain (Fig. 1). None of the amino acid substitutions seem to affect the structural similarity of this region to the Kunitz family of serine protease inhibitors. These results strongly suggest the functional similarity of BPP in human and mouse. It should be, however, noted that five amino acid substitutions were found among 19 amino acid residues encoded by the 3' fifty-seven bases of the 225-bp insert. This region might be evolutionary variable.

beta protein region

```
mouse      DAEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
human      ....R....Y..H............................
```

inhibitor domain encoded by the 168-bp insert

```
mouse      VCSEQAETGPCRAMISRWYFDVTEGKCVPFFYGGCGGNRNNFDTEEYCMAVCGSVS
human      ..........................A..........................AM
```

region encoded by the 57-bp insert

```
mouse      TQSLLKTTSEPLPQDPDKL
human      S.......Q...AR..V..
```

Fig. 1 Amino acid sequences of the domains of mouse and human BPP770. Only those of hBPP770 replaced in mBPP770 are shown.

Tissue distribution of the mBPP mRNA

In human, the 225-bp insert is encoded by two exons of 168-bp and 57-bp long and three mRNA species (hBPP695, 751 and 770) are generated by alternative splicing[6]. Distribution of each mRNA species in mouse tissues was analyzed by Northern blot hybridization using 4 oligonucleotide probes HK, IJ, HJ and IK which were complementary to the junctional sequences of four possible combination of the exons (Fig. 2A). The probes HK, IK and IJ specifically detected the cDNAs for mBPP695, 751 and 770, respectively. The mBPP695 mRNA was present in the brain, kidney and intestine but most abundantly in the brain. The mBPP751 and 770 mRNAs were expressed in all the tissues examined but the signal was strongest in the kidney (Fig. 2B). No signal was, however, detected by the probe HJ in all the tissues examined (data not shown). Thus, the tissue distributions of individual mRNA species were markedly different each other. The mRNA for BPP with inhibitor domain is detected in all the tissues examined and the mRNA for BPP without that domain in some specific tissues. Thus tissue distribution of each species of BPP mRNA seems to be similar in mouse and human[4]. Although the biological significance of the alternative splicing of the BPP mRNA is still unknown, each species of BPP may play different functional roles in mammalian tissues.

Age-related change of the mBPP expression in the brain

It may be interesting to know whether the pattern of the mBPP gene expression in the brain is influenced by age. RNAs from the brains of mice of various ages were analyzed by Northern blot hybridization using HK and IK probes. The amount of the mBPP695 mRNA detected by the HK probe appeared low at 0 day, increased during the first 2 months, and remained almost steady thereafter (Fig. 2C). The relative amounts of the mBPP695 mRNA normalized by the amount of the beta actin mRNA were 0.068 (0 day), 0.583 (2 months), 0.603 (6 months) and 0.463 (22 months). The mBPP751 mRNA level, however, was so low that we could not obtain enough signal to asses age-related change by the IK probe (data not shown). Since immunocytochemical localization of BPP suggested a role for this protein in cell contact[9], the expression of BPP in the brain might be related with the maturation of the neuron network.

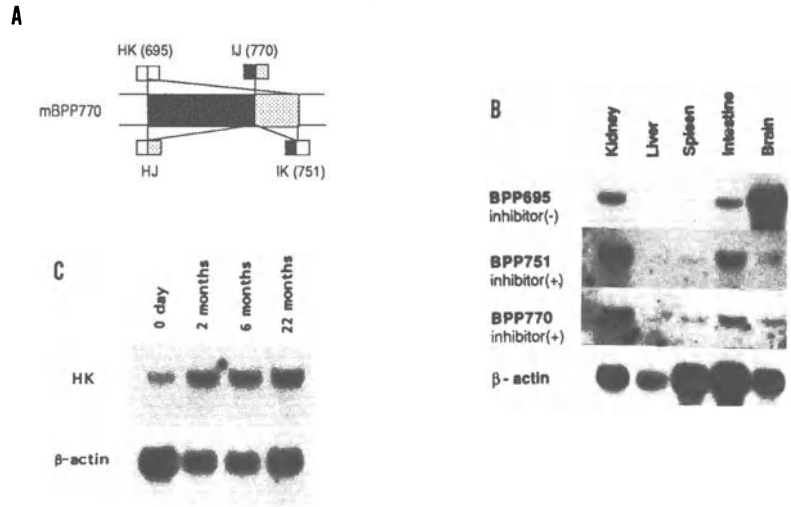

Fig. 2 Expression pattern of the mBPP mRNA. A. Schematic representation of the synthetic oligonucleotide probes. HK, IJ, HJ, IK: probes (40 mer). B. Northern blot analysis of RNA from various tissues of an adult mouse. C. Age-related change of the mBPP695 mRNA expression in the brain.

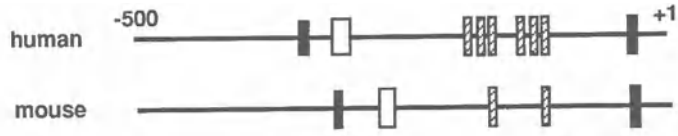

Fig. 3 Schematic representation of the mouse and human BPP promoter regions. ■, AP-1 binding site; □, heat shock element; ▨, 9-bp-long GC rich element; +1, common transcription start site.

Cloning of the mBPP gene containing the promoter region

In human, the promoter of the BPP gene resembles those of housekeeping genes and contains several possible regulatory elements[10]. Comparison of the promoter sequences between mouse and human may help to understanding of biological significances of these regulatory elements. We screened the mouse genome DNA library using the mBPP cDNA and the promoter of the hBPP gene as probes. Four clones containing the promoter region of the mBPP gene were isolated. Sequence analysis revealed that the mBPP promoter region lacked a typical TATA box and had a high GC content. It also contained sequences similar to several possible regulatory sequences in the human promoter, that is, two AP-1 binding sites, a heat shock control element and two GC rich elements (Fig. 3). Therefore, the mBPP gene might be regulated in the same manner as the hBPP one.

In conclusion, we have determined the structure and expression pattern of mouse BPP mRNAs and characterized the mBPP gene promoter. Since the structure and expression of the BPP gene are extremely similar between mouse and human, the mouse can offer a unique model system to investigate the biological function of BPP and its relation to AD pathogenesis.

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MOLECULAR CLONING AND STRUCTURAL ANALYSIS OF THE HUMAN AMYLOID β PROTEIN PRECURSOR GENE

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INTRODUCTION

Amyloid β protein in Alzheimer's brain is a cleavage product of the precursor protein (BPP). The sequence analysis of its BPP cDNA showed that BPP resembles a cell-surface receptor (Kang *et al.*, 1987) and that there are three types of BPP mRNA generated by alternative splicing, two of which encode a serine-protease inhibitor (serpin) domain (Kitaguchi *et al.*, 1988, Ponte *et al.*, 1988, Tanzi *et al*, 1988).

BPP is produced ubiquitously in human tissues but most abundant in the brain and kidney. It is reported that the amount, localization and type of the BPP mRNA are different between normal and Alzheimer's brains. These results probably implicate that the BPP gene regulation has some roles in the pathological process of Alzheimer's disease.

To analyze the regulatory mechanisms of the BPP gene transcription and alternative splicing, we isolated clones covering all the exons of the BPP gene and analyzed their structures. In this paper, we discuss the possible roles of the splicing acceptor sequences in alternative splicing and show that the BPP mRNA is induced by TPA in HeLa cells.

MATERIALS AND METHODS

Construction and screening of human genomic libraries

Several human genomic libraries were screened by plaque hybridization using a 2.8-kb cDNA fragment covering all the 5'-noncoding region and the entire coding region as a probe. The insert DNA fragments containing the exons were subcloned and their nucleotide sequences were determined.

Northern blot analysis

When HeLa cells grew to 70% confluence at 37°C, they were stimulated by addition of TPA to a final concentration of 60ng/ml for 12 hours, or incubated at 43°C for heat shock. Total RNAs were extracted, and 20mg/lane of them were electrophoresed. Hybridization was done with a mixture of probes of a 2.8-kb BPP cDNA fragment and β-actin cDNA. The ratio of the intensity of the BPP band versus β-actin band was determined by a transmission densitometer.

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RESULTS AND DISCUSSION

Overall structure of the human BPP gene

We obtained a total of 36 positive clones. These clones were organized by endonuclease mapping. The BPP gene consists of 18 exons and spans more than 170kb (Yoshikai *et al.*, 1989). The serpin domain is encoded by the 7th exon and additional 18 amino acids by the 8th exon. The amyloid β protein is encoded by the 16th and 17th exons (Lemire *et al.*, 1989).

Sequence analysis of the intron-exon organization

The BPP gene directs three types of mRNA products generated by alternative splicing. To speculate the mechanism of the alternative splicing, we analyzed the sequences of all the exon-intron boundaries (Yoshikai *et al.*, 1989). Figure 1 shows the sequences of the splicing acceptor site of the 6th, 7th and 8th introns. The consensus sequence of the branchpoints determined in vetrebrates is Py(13/16)NPy(16/16)U(14/16)Pu(13/16)A(16/16)Py(15/16) (Krainer and Maniatis, 1988), where the branchpoint is underlined. The branchpoints characterized thus far were located between -37 and -18 (nucleotides upstream of the acceptor site) (Krainer and Maniatis, 1988). In the 6th intron, TTTTTCAT or TGCTAAA (6/7 identity to the consensus) might be the branchpoint. On the other hand, the putative branchpoint sequence is less similar to the consensus in the 7th intron (TAGTTAT, 5/7 identity) and the region around this sequence is very T-rich. The lesser similarity to the consensus sequence of the putative branchpoint within the 7th intron may explain why BPP mRNA containing the 8th exon sequence is less abundant in all the human tissues. In the 8th intron, TATTAAA (6/7 identity) in -69 to -63 was similar to the consensus but distant from the exon-intron junction. We could not speculate the reason why BPP mRNA containing the 7th exon is less abundant in the brain, however, these sequences found within the 6th, 7th and 8th introns may be related to the alternative splicing. We also analyzed the possible secondary structures formed by the sequences within -163, -157, -116 bases of the 6th, 7th and 8th introns, respectively, by using an application computer program, but no stable secondary structure was found.

```
Intron6
5'-AAGAAGTAAACGTGTATACATGAACAGAGAGACAGTGCCT (Exon7. serpin)
   TTTCATGCTAAATGTGGTTCCCCACATCTCCTCTGATTAG  AGGTGT-3'

Intron7
5'-GTCAGTGGACTCGTGCATTTCACCATCATTCCCATGTTTC (Exon8. 18A.A.)
   TCTTTTTGTTTTTAGTTATGTTCTCTTATTTTTTCCATAG  TGTCCC-3'

Intron8
5'-ATACGGCTTTCTATTAAACGAGTGGATTATTCTGTTGTTG (Exon9)
   TTGGCTTTTTTCTCAAACCTCCTTCTCTTCTACTTTATAG  TTCCTA-3'
```

Figure 1. Sequences of the splicing acceptor sites of the 7th, 8th and 9th intron. Putative branchpoint sequences are underlined. A.A.,amino acids.

Regulation of the BPP gene transcription

We also cloned the promoter region of the BPP gene, which is identical to the data published by Salbaum *et al.* (1988). The promoter is very GC-rich and has multiple transcription start sites which is the feature of a housekeeping gene, and has two possible AP-1 binding sites (from -45 to -39, from -350 to -344, nucleotides upstream of the major transcription start site) and a sequence resembling the heat shock control element (-317 to -304). We asked whether the mRNA level is related to the altered methylation status in human tissues but the gross methylation patterns are very similar among tissues and

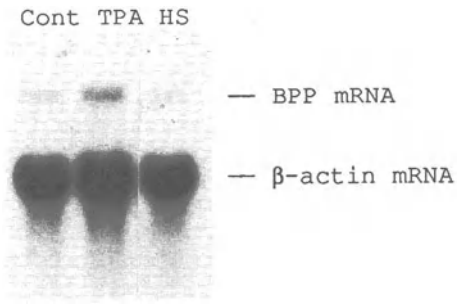

Figure 2. Northern blot analysis of BPP mRNA. Cont, control RNA; TPA, RNA induced by TPA; HS, RNA induced by heat shock.

between normal and Alzheimer's disease (Yoshikai *et al.*, 1989). We also asked whether the BPP gene transcription is induced by TPA or heat shock. Our results showed that total BPP mRNA increased 3.7-fold upon TPA stimulation (Figure 2). This suggests that the BPP gene transcription could be induced by AP-1,v-JUN, or c-FOS and BPP might be involved in the proliferation or the differentiation of cells. In the case of heat shock induction, the increase in the BPP mRNA synthesis was too small (1.2-fold) to access its relation to the stress condition.

In conclusion, we analyzed the sequences of the splicing acceptor sites and induction of the BPP gene transcription. The results should contribute to the studies on the regulation of the BPP gene and on the amyloidogenesis in Alzheimer's disease.

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PROTEASE NEXIN 1 IMMUNOREACTIVITY IN SENILE PLAQUES IN ALZHEIMER DISEASE AND AGED BRAIN

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Amyloid β-protein is deposited in senile plaques of Alzheimer and Down syndrome patients[1]. This β-protein is now known to be derived from a larger precursor protein (APP)[2]. However, the mechanism whereby the amyloid proteins are deposited is thus far not clarified. The recent finding that the APP contains a new domain with structural similarity to the Kunitz family of protease inhibitor[3-5] has raised the possibility that an imbalance of protease-protease inhibitor interaction is involved in the aberrant degradation of APP, which may lead to formation of amyloid β-protein. In fact, this domain does have protease inhibitor activity[3], and the amount of two larger versions of APP with a protease inhibitor domain appears to be elevated in Alzheimer brain. Recently, another completely unrelated protease inhibitor, α1-antichymotrypsin, was demonstrated to be closely associated with amyloid fibrils[6]. This fact also supports the possibility that the imbalance between protease and protease inhibitor brings about the anomalous degradation of the APP[7].

In the past few years, we have been interested in the astrocyte-derived factors that have biological activities such as neurite outgrowth-promoting activity. We have speculated that these factors may contribute to the abnormal thread formation and amyloid formation that are two of the neuropathological features seen in Alzheimer and aged brain. Since cultured astrocytes produce a neurite-promoting factor that has been found to be identical in amino acid sequence to nexin[8-10] and since this nexin 1 is a member of the Kunitz family of protease inhibitors, we focused our attention this protein. Here we report that immunoreactivity of antibodies against synthetic polypeptides corresponding to different portions of protease nexin 1 (PN1) is present in senile plaques and reactive astrocytes in Alzheimer and aged brain. This finding raises the possibility that protease nexin 1, which has two major activities protease inhibitoractivity and neurite-promoting activity[11], contributes to some of the neuropathology of dementia.

The synthetic polypeptides Asp-Gly-Thr-Lys-Ala-Ser-Ala-Thr-Thr-Thr-Ala-Ile-Leu-Ala-Arg-Ser-Ser-Pro-Pro (peptide T1) and Glu-Leu-Gly-Ser-Thr-Gry-Ile-Gln-Val-Phe (peptide T2) were of identical sequences to the residues 330-349 and 10-20, respectively, of PN1[10]. The peptides were coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits. Specificity of the antisera were checked by use of corresponding peptides and the unrelated synthetic peptides. Autopsy brains from two Alzheimer cases and three aged individuals without neurological disorders were used in the persent study. The samples were snap frozen inisopentane cooled by dry ice and kept at -80℃. Sections were cut on a cryostatand fixed in cooled acetone for 15 min. After fixation, the sections were incubated with antiserum against T1 or T2 (diluted 1:100) at 4℃ overnight. They were then reacted with biotinylated antirabbit IgG goat serum (Vector Laboratories; diluted 1:200) at

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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37℃ for 1 hr, followed by peroxidase-conjugated streptoavidin (BioGenex Laboratories; diluted 1:1000) at 37℃ for 1hr. The reaction product was visualized with 4-chloro-1-naphtol or diaminobenzidine.

In all the brain sections that were stained with anti-T1 antibody, immunoreactivity was associated with possible senile plaques (Fig.1a). To confirm the association of PN1 with the plaques, we carried out a succesive immunostaining of the sections with antibody against the amyloid β-protein after destaining of T1 immunoreactivity. The T1-positive structures were stained with β-antibody (Fig.1b). We estimated that more than two thirds of the β-protein-positive plaques were stained with antibody to T1. Another finding of the present study was that astrocytes, an abundant cell type in Alzheimer brain, were positively stained with anti-T1 antibody (Fig.2). Many astrocytes, especially fibrous and protoplasmic ones, were strongly stained. Neurons, oligodendrocytes, and capillary endothlial cells lacked immunoreactivity. The antibody against the T2 peptide of PN1 gave a similar staining pattern, although its intensity was weaker.

Our study thus demonstrated that the immumoreactivity for nexin 1 is localized in senile plaques and reactive astrocytes. In senile plaques, it appears that nexin 1 immunoreactivity is associated with amyloid. These results permit us to consider several points as to the role of nexin 1 in the pathological product formation in Alzheimer dementia and aged brain. Firstly, nexin 1 may participate in the processes of amyloidgenesis as a serine protease inhibitor. This notionis consistent with the protease-protease inhibitor imbalance hypothesis. It is possible that nexin 1 is associated with amyloid, because this protein has heparin and dextran sulfate binding activity[12-14] and amyloid contains heparin-like proteoglycan. Secondly, nexin 1 might contribute to the formation of abnormal neurites that are not only present in the vicinity of senile plaques but also seen as a massive neuropil in the brains of patients with Alzheimer dementia. So far, neurite outgrowth-promoting activity of nexin 1 has been demonstrated only in cultured neural cells of rodent peripheral neurons[11,15]. Therefore, it is necessary to see whether nexin 1 has such an activity for human central nervous system neurons. If so, nexin 1 may take part in this abnormal neurite formation. Thirdly, it is of considerable interest to note that nexin 1 binds to γ-NGF and inactivates its function to produce β-NGE[16]. An increase in the amount of nexin 1 in the brain might lead to the depletion of β-NGF, which may cause neuronal cell death.

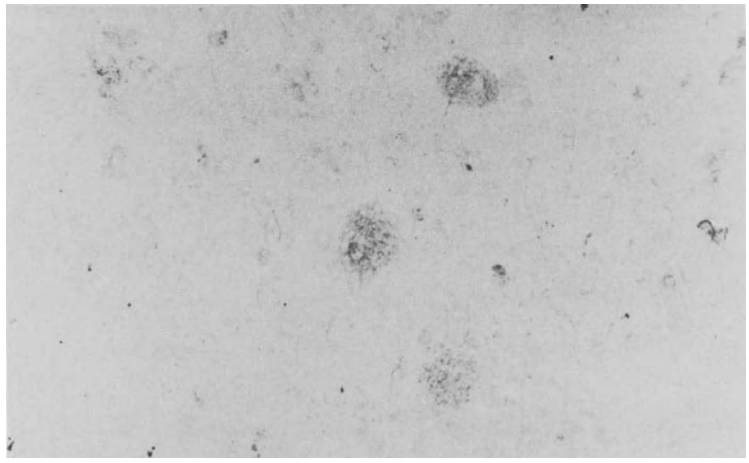

Fig. 1a Immunohistochemistry of the senile plaques.
PN1 immunoreactivity in the senile plaques visualized with 4-chloro-1-nephthol.

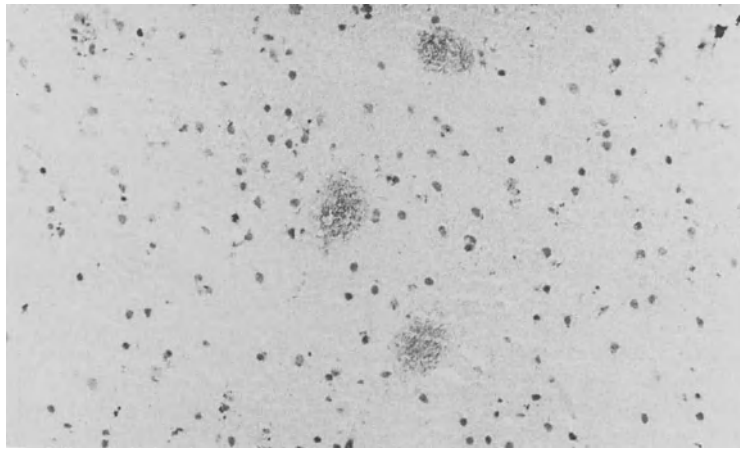

Fig. 1b Immunohistochemistry of the senile plaques.
β immunoreactivity in the senile plaques in the same field visualized with diaminobenzidine.

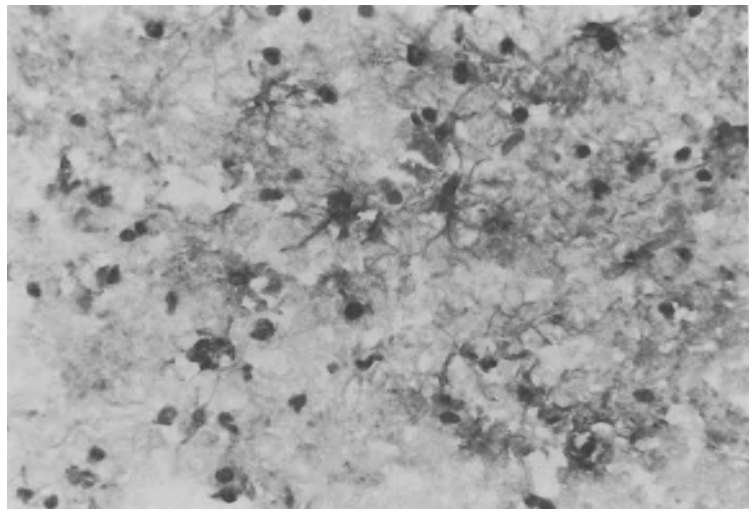

Fig. 2 PN1 immunoreactivity of Alzheimer brain.
Fibrillary astrocytes were stained.

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MONOCLONAL ANTIBODIES AGAINST SENILE PLAQUE AMYLOID IN ALZHEIMER'S DISEASE

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INTRODUCTION

Amyloid deposits occur in the forms of senile plaques (SP's), amyloid angiopathy(AA), and plaque-like vascular changes in the brains of Alzheimer's disease (Alz) patients. These pathological changes are important since the occurrence of these structures is correlated with the severity of the dementia. A protein consisting of 42-43 amino acids and with a molecular weight of about 4000 kd, designated as amyloid A4[1] or β-protein,[2] has been isolated as a component of amyloid protein (Am) from AA in the Alz brain. The same A4 protein as in AA is also found commonly in various types of SP's in Alz and Down's syndrome.

Recently, the gene encoding the amyloid precursor proteins (APP's) has been cloned and it has been elucidated that three alternative splicings produce three different APP's composed of 695, 751, and 770 amino acid residues.[3-5] But thus far all the components of amyloid are not known and the precise mechanism of amyloid deposition in the brain is not fully understood. In an effort to determine the antigenic character of Am, we established various monoclonal antibodies (mcAb's) against epitopes of native Am.

MATERIALS AND METHODS

1.Preparation of immunogen

Detergent-insoluble Am was prepared from pathologicaly confirmed Alzheimer's brain by modification of the methods of Masters et al.[1] and Kitamoto et al.[6] Native amyloid was prepared from Alzheimer's brain according to Yen et al.[7]

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

2.Establishment of monoclonal antibodies

McAb's against Am were established by the conventional method. Spleen cells from Balb/c mice, which were immunized with detergent-insoluble Am or native Am, were fused with myeloma cells, either P3-NS1-1-Ag4-1 or P3-X63-Ag8-6.5.3, by polyethyleneglycol. Hybridomas were selected by culturing in medium containing HAT. The antibody activities were checked by immunofluorecsence(IF) with cryostat sections of Alz brain, and the limiting dilution were performed by two times. Each positive clone was injected into the peritoneal cavity of Balb/c mice. McAb's were purified from ascites fluid by ion-exchange chromatography.

3. Characterization of mcAb's

After establishment of the mcAb's, immunoglobulin class and subclass were determined by Ouchterlony's method, and antibody titer were determined by IF. To characterize the epitopes detected by these mcAb's, we performed immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and Western-blot analysis as follows:

1) Immunohistochemical study. Cryostat sections were stained by IF. Paraffinized sections were also stained by modified ABC method(Streptoavidin-biotin complex peroxidase,BioGenex). Paraffinized sections were pretreated with formic acid solution for various short periods (3-5min). Brain sections were obtained from 5 cases of Alz, 3 cases of Parkinsonism dementia complex of Guam, 3 cases of non-demented aged, and 3 cases of non-demented youth.

2)ELISA. ELISA was carried out to identify the epitope seen by each mcAb. Antigens used for ELISA were 3 synthetic peptides homologous with amyloid precursor protein, β-28 (28 amino acid residues of N-terminus of the β-peptide), AI-23 (23 amino acid residues of 301-323 of APP 751, inhibitor domain), AC-24 (24 amino acid residues of C-terminus of APP), complements, (C1q, C1s, C3, C3b, C4, C4b, C4 binding protein, C5, factor B), Kunitz-type protease inhibitors, α1-antichymotrypsin (Calbiochemistry, α1-ACT), inter-α-trypsin inhibitor (ITI), and serum amyloid P component (SAP, which was kindly provided by Dr. I. Okubo, Nagoya City University). Each mcAb was diluted to 1-10 μg/ml. Alkaline phosphatase-conjugated anti mouse IgG, or IgM (1:3,000-10,000, TAGO) was used as the secondary antibody.

3) Western-blot analysis. Brain homogenate from Alz was prepared as antigen according to Selkoe et al.[8] Secondary antibody, the same as used in ELISA, was diluted (1:5,000) in T-TBS.

RESULTS

Ten mcAb's were established that reacted with SP. Eight of them were IgG(k); and 2, IgM(k). Their titer in ascites were $5x10^3$-$1x10^4$ by IF.

1) Immunohistochemical study

All 10 mcAb's stained SP (both classical primitive types), amyloid angiopathy, and plaque-like vascular change. The diffuse type of amyloid deposit was stained in gray matter, and granular deposits were noted in white matter, of Alzheimer's brain. But there were some differences in staining property of these mcAb's. Al 304/2 and Al 121/6 stained various forms of amyloid deposition most strongly. The Al 304/2-positive plaques were not necessarily stained with Al 121/6. Az 520/3 and Az 172/4 seemed to stain SP's more strongly than amyloid angiopathy and plaque-like vascular changes. None of our mcAb's stained neurofibrillary tangles in the brains of Alz, Parkinsonism dementia complex of Guam, or the non-demented aged.

2)ELISA (Table 1)

Four out of the 10 mcAb's reacted with the panel of antigens tested by ELISA. Al 304/2 recognized β-28, and Al 272/6 reacted with AI-23 weakly. Al 121/6 recognized C4, C4b, C4bp; and Am 519/8, C3, C3b. None of our mcAb's reacted with AC-24, α1-ACT, ITI, SAP, and other complements (C1q, C1s, C5, Factor B). The epitopes reacted with 6 of the mcAb's remained undetermined by ELISA.

3)Western blot analysis

Al 304/2 recognized several bands between 50 kd and 30 kd. Al 67/1 recognized 40kd band, and Az 172/4 recognized 2-3 bands of about 50 kd.

DISCUSSION

Only limited numbers of mcAb's raised against native Am have been reported.[9] In our attempt, 10 mcAb's were successfully established, and they stained various types Am in SP, AA, and plaque-like vascular changes. Differences in staining pattern of the mcAb's suggest that these mcAbs recognize different epitopes in Am.

Table 1. Results obtained by ELISA

	APP			ACT	ITI	SAP	Complement	
mcAbs	β-28	AI-23	AC-24				C3	C4
Al 67/1	-	-	-	-	-	-	-	-
Al 121/6	-	-	-	-	-	-	-	+
Al 272/6	-	+/-	-	-	-	-	-	-
Al 304/2	++	-	-	-	-	-	-	-
Am 519/8	-	-	-	-	-	-	++	-
Am 531/1	-	-	-	-	-	-	-	-
Am 567/1	-	-	-	-	-	-	-	-
Am 679/6	-	-	-	-	-	-	-	-
Az 172/4	-	-	-	-	-	-	-	-
Az 520/3	-	-	-	-	-	-	-	-

++:strongly positive +/-:weakly positive
+: positive -:negative

The epitopes of Am reactive with 4 mcAb's were determined by ELISA. Al 121/6 and Am 519/8 recognized C4 and C3, respectively. It was reported earlier that complement components are involved in amyloid plaques in Alz (Ishii et al.[10] , Eikelenboom et al.[11]) Complement C1q, C3, C4 and activated complement products C3c and C3d were found in amyloid deposits by an immunohistochemical technique. Eikelenboom et al. proposed that amyloid fibril formation triggers complement activation. Our present data added another piece of evidence that the complement system may play an important role in amyloidogenesis in Alz.

In the Western blotting experiment Al 304/2 reacted with β-28, recognizing several bands with molecular weights of 30-50 kd. Also using Western blot analysis, Selkoe et al.[8] reported that antisera raised against synthetic peptides of APP recognized amyloid precursors of 110-135 kd. Al 304/2 may recognize proteolytic products of APP.

The epitopes reactive with the other 6 mcAb's remain undetermined. Among these, 2 mcAb's recognized 40-50 kd bands in the Western blot analysis. These data suggest that there is unknown epitope in amyloid in Alz other than β-protein, Kunitz-type protease inhibitors, SAP, and complement component.

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IDENTIFICATION OF PUTATIVE AMYLOID A4-SPLITTING ENZYMES WITH TWO ENDOPEPTIDASES WIDELY DISTRIBUTED IN MAMMALIAN CELLS

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INTRODUCTION

Alzheimer's disease (AD) is characterized by the deposition of amyloid in the brain, especially in senile plaques and neurofibrillary tangles. Amyloid is thought to arise by abnormal cleavage of various proteins into self-aggregating fragments. The major component of AD amyloid is a 4.2-kD polypeptide referred to as the A4 or β-protein, and it corresponds to a membrane-spanning domain of a putative amyloid precursor protein (APP). A4 at positions 597 to 638 of the initially identified APP_{695} is hydrophobic and the C-terminal half of the A4 is buried in the membrane. The major peptide species in the amyloid plaque core in AD are peptide A4', which corresponds to Phe_{600} to Ala_{638}, and an A4 peptide.

On the basis of this structure, it has been suggested that the N-terminal portion of the A4 peptide is first cleaved off from the APP protein, and then the C-terminal end of A4 is cut off by another proteinase at the membrane to release the A4 or A4' peptide. To determine the initial N-terminal-cleaving enzyme for A4 production and the second C-terminal-splitting proteinase, we synthesized fluorogenic peptide substrates with cleavage points. Purification from rat tissues revealed that the N-terminal-splitting enzyme of amyloid A4 peptide is a multicatalytic proteinase, and that the C-terminal peptide is efficiently cleaved by a prolyl endopeptidase.

MATERIALS AND METHODS

The peptide substrates Suc-Ala-Glu-MCA (SAE-MCA) and Suc-Ile-Ala-MCA (SIA-MCA) were synthesized by Peptide Res. Inst., Osaka. Z-Val-Lys-Met-MCA was kindly provided by Dr. Hisashi Ito, Aoyama Gakuin University.

Standard assay mixtures comprised 50 mM Tris-HCl buffer, pH 7.0, containing 0.1 mM substrate and proteinase, in a total volume of 0.1 ml. Incubations were performed for 30 min at 37°C. The cleavage product, aminomethylcoumarin (AMC), was analyzed, after the reaction was stopped with 5% SDS, with a Hitachi F-3000 fluorescence spectrophotometer.

The multicatalytic proteinase ingensin was purified from rat liver according to the method of Ishiura et al. (1985,1986,1989a,1989b). Antibodies against the enzyme were raised in rabbits.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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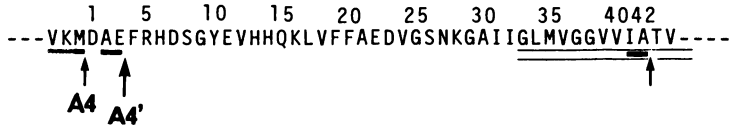

Fig. 1. Amino acid sequence of the amyloid A4 peptide.
The amino acid sequence of the A4 polypeptide, which is composed of 42 amino acids, is denoted by arrows. The amino-terminal Asp (D) of the A4 is probable membrane-spanning sequence of the A4 polypeptide. The most abundant species, which we tentatively named A4', begins at Phe_{600} (F) of APP. The model peptide sequence used in the search for the A4-splitting enzyme is indicated by a heavy underline.

RESULTS AND DISCUSSION

The sequence of the synthetic peptide-MCA is given in Fig. 1. The N-terminal peptide consists of N-blocked Val-Lys-Met-MCA. The tripeptide is adjacent to the N-terminal Asp of the A4 peptide. Since the most abundant amyloid A4 peptide in neurofibrillary tangles is composed of 39 amino acids (A4'), which is devoid of the three N-terminal amino acids Asp-Ala-Glu (Masters et al., 1985) of the A4 peptide, we synthesized Suc-Ala-Glu-MCA to identify the proteinase that splits the A4'peptide. We also synthesized a model peptide, SIA-MCA, homologous to the C-terminal portion of the A4 peptide.

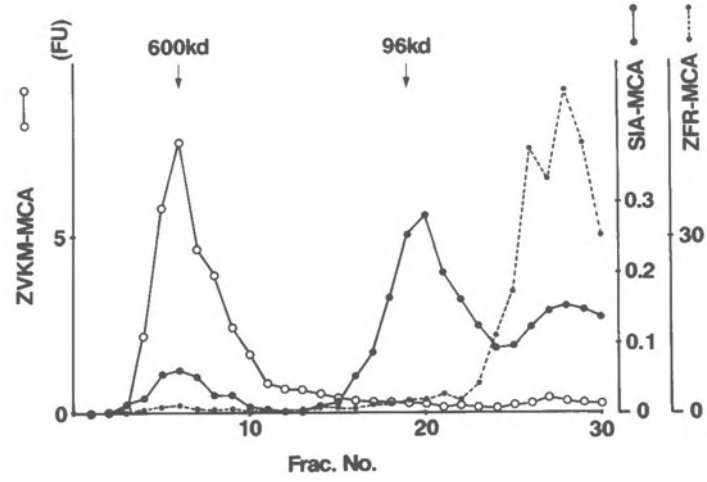

Fig. 2. Gel filtration chromatography of rat brain extract.
The crude supernatant of rat brain was applied to an HPLC gel filtration column (TSK G3000 SW; 0.8 x 60 cm) equilibrated with 0.1 M phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, at the flow rate of 1.0 ml/min. Proteolytic activities were assayed as described under MATERIALS AND METHODS. The molecular markers were rat brain multicatalytic proteinase (600-kD) and aminopeptidase M (96-kD). (Tsukahara et al., 1988, 1989).

The crude rat brain extract was subjected to HPLC gel filtration. ZVKM-MCA-degrading activity was eluted from a G3000 column at a position corresponding to a molecular weight of 600-kD (Fig. 2). SAE-MCA-degrading activity coincided with that of ZVKM-MCA (data not shown).

The proteinase was purified from rat liver and brain (Ishiura et al. 1989b). Western blot analysis revealed that antibody against rat liver multicatalytic proteinase crossreacted with the ZVKM-MCA-degrading or the SAE-MCA-degrading enzyme. The findings indicate that cytosolic multicatalytic proteinase is a candidate for the amyloid A4-splitting proteinase.

The material showing SIA-MCA-degrading activity, on the other hand, was separated into three entities (Fig. 2). The first, a minor 600-kD enzyme, coincided with the multicatalytic proteinase. This suggests that the multicatalytic proteinase has the ability to hydrolyze the C-terminal substrate. The major second peak material had a molecular weight of 80-kD. Subsequent chromatographies showed that a prolyl endopeptidase hydrolyzed the C-terminal portion of the A4 peptide (Ishiura et al., 1989 c). The last minor peak of SIA-MCA hydrolysis activity seemed to correspond to a cysteine-dependent, Z-Phe-Arg-MCA-degrading cathepsin with a molecular weight of 25-50 kD.

To investigate the specificity of the purified 80-kD SIA-MCA-degrading proteinase, various proteinase inhibitors were examined (Table 1). The activity of the purified sample was strongly inhibited on the addition of the prolyl endopeptidase-specific inhibitor Z-thiopro-thiazolidine (Tsuru et al., 1988).

Kunitz-type bovine pancreas trypsin inhibitor and aprotinin inhibited the rat brain prolyl endopeptidase. An *in situ* hydridization experiment reported by Palmert et al. (1988) demonstrated an increased transcription of APP mRNA lacking the Kunitz-type protease inhibitor

Table 1. Effects of proteinase inhibitor on the purified C-terminal-splitting enzyme of amyloid A4 peptide

Proteinase inhibitor	Concn.		Relative activity (%)	
			SGPLGP-MCA[a]	SIA-MCA[b]
none			100	100
Z-thiopro-thiazolidine	0.1	nM	100	99
	1	nM	92	90
	10	nM	59	61
	100	nM	24	17
Kunitz BPTI[c]	10	µg/ml	74	94
	100	µg/ml	25	30
Soybean TI[d]	10	µg/ml	100	100
	100	µg/ml	100	100
Aprotinin	10	µg/ml	91	40
	100	µg/ml	78	38
Bovine serum albumin	10	µg/ml	107	100
	100	µg/ml	91	100

[a]The purified enzyme most efficiently cleaved Suc-Gly-Pro-Leu-Gly-Pro-MCA (SGPLGP-MCA) in the presence of 2-mercaptoethanol. The rate of hydrolysis was 400-fold higher than that of SIA-MCA.
[b]The SIA-MCA-degrading activity was also activated by the addition of 2-mercaptoethanol.
[c]Bovine pancreas trypsin inhibitor
[d]Trypsin inhibitor

(KPI) domain in nucleus basalis and locus ceruleus neurons. If this KPI domain physiologically inhibits the A4-generating prolyl endopeptidase, an increased level of KPI-free APP should increase the rate of A4 production.

The SIA-MCA-degrading proteinase is abundant in the hippocampus, where large numbers of senile plaques and neurofibrillary tangles are observed in AD. Since the cleavage of the C-terminal portion of the A4 peptide has been thought to be the final step for the generation of the free A4 peptide, the enzyme responsible for this cleavage is the most important proteinase for the deposition of the A4 peptide. Interestingly, the distribution of the SIA-MCA-degrading enzyme is entirely different from that of the multicatalytic proteinase. These results suggest that the relative quantities of the proteinases and APP are important for A4 production and that an imbalance between them may induce nonfibrillar pre-amyloid deposition in AD. Further in vitro processing experiments on APP using these proteinases are required to determine if these proteinases promote the progression of A4 peptide deposition or not.

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PROTEOLYTIC PROCESSING OF β-PROTEIN PRECURSOR-RELATED SYNTHETIC PEPTIDES

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INTRODUCTION

In Alzheimer's disease, Down's syndrome and to a lesser extent in normal aging, abnormal proteinaceous deposits precipitate in the brain. The extracellular deposits, termed amyloid, are found in the center of senile plaques and in the blood vessel walls of the lepto-meninges and the brain. In 1984, Glenner and Wong purified the amyloid fibrils from the meninges of Alzheimer's disease (AD) and Down's syndrome (DS) and sequenced the first 28 amino acids of the 4Kd peptide they named the β-protein (Glenner and Wong, 1984). A similar, but not identical, peptide was also purified from the amyloid cores of senile plaques (Masters et al., 1985; Selkoe et al., 1986). The β-protein is a 39-42 amino acid fragment derived from a larger, 110-135 Kd precursor protein (β-PP) whose gene has been cloned and sequenced (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987a). The finding of multiple transcripts indicates alternative splicing. In addition to the β-protein, the brain amyloid contains a tightly associated serine protease inhibitor, α_1-antichymotrypsin (ACT) (Abraham et al., 1988).

Interestingly, two of the β-PP transcripts were shown to contain a domain homologous to the Kunitz-type of protease inhibitors (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). Today we know that the β-PP is identical to the previously described inhibitor—protease nexin 2 (PN2) (Van Nostrand et al., 1989; Oltersdorf et al., 1989). Other molecules that have been detected immunochemically or histologically in the brain amyloid are activated complement components (Eikelenboom et al., 1989) and heparan sulfate proteoglycans (Snow et al., 1987). Several serine protease inhibitors of the Kunitz type have binding sites for heparan sulfate proteoglycans (Guy Salvesen, personal communication).

Studying the enzymes involved in the pathway of proteolytic processing of the β-PP is fundamental to understanding the formation of amyloid deposits, which in turn are believed to be trophic/toxic to their surroundings (Whitson et al., 1988; Yanker et al., 1989). We searched for a brain protease which could make the N-terminal of the two cleavages necessary to generate the β-protein from its precursor. Such a cleavage of β-PP, between a methionine and an aspartic acid, will release a soluble extracellular protein which lacks the transmembrane and cytoplasmic domains (Figure 1). Indeed, a protein of 105-125 Kd was detected in the cerebral

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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spinal fluid with antibodies to a synthetic peptide from the N-terminus of the β-PP and was also previously described (then as PN 2) as being secreted into the medium by cultured fibroblasts (Palmert et al., 1989; Van Nostrand and Cunningham, 1987). Also, a 10 Kd peptide is seen on Western blots of membrane fractions with antibodies to the C-terminus of the β-PP (Selkoe et al., 1988). This 10Kd peptide is believed to be the transmembrane plus intracellular domain and recent evidence indicates that it can be toxic to neurons (Yanker et al., 1989).

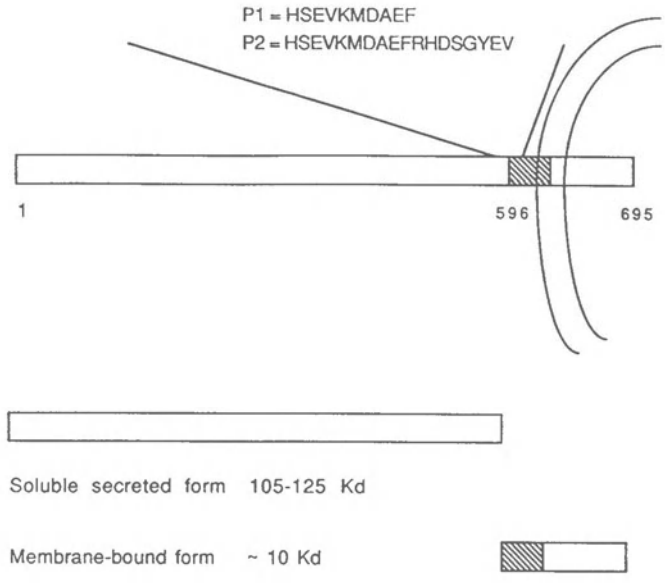

Figure 1

In order to find a protease which cleaves in the vicinity of the N-terminus of the β-protein, we synthesized two peptides according to the β-PP sequence (Figure 1). One peptide is ten amino acid long, peptide 1 (P1), and the second is eighteen amino acid long (P2). Both start at the same position in the β-PP, and extend differing distances across the putative cleavage site into the β-protein itself. The histidine was added at the N-terminus of the peptides for the purpose of radio-iodination. Iodinated peptides were used to follow the protease in two ways. First, the labelled peptide was incubated with the various brain fractions and then the resulting cleaved peptides separated on thin layer chromatography (TLC). The TLCs were exposed to film overnight. Second, a novel technique was applied to follow the specific cleavage enzymes. Brain fractions were incubated with the iodinated peptide and then treated with disuccinimidyl suberate (DSS), an agent which can cross-link amino groups that are 11.4 Angstrons apart. Only proteins that were in intimate contact with the peptide, i.e., a protease-substrate complex, will be cross-linked by DSS and thus radioactively labelled. This method has been successfully used to bind labelled ligands to receptors (Tsudo et al., 1987), and is here shown to be useful for identifying and following the enzymes through purification procedures.

RESULTS

Homogenates of Alzheimer's brain were spun at 10,000g and the supernatant subjected to ammonium sulfate precipitation, DEAE and CMC columns and finally a gel filtration column. At each step the ability of the protease to cleave the ^{125}I-P1 was checked by autoradiography after separating the cleaved products on TLC. The brain fractions were also reacted with both

iodinated peptides (P1 and P2), cross-linked with DSS, subjected to SDS-PAGE, and the gel dried and exposed to X-ray film. Both peptides bound to a single band in the fractions tested, suggesting a single peptide-binding protein.

Specific inhibitors were used to characterize the type of protease that we have purified: EGTA, a specific inhibitor of calcium-activated proteases, DFP, a specific inhibitor of serine proteases, and two serine protease inhibitory proteins potentially involved in the proteolytic degradation of the β-PP: α_1-antichymotrypsin (a gift from Jim Travis) and the purified Protease Nexin 2 from human brain (PN2 or β-PP) which includes two secreted forms, one with and one without the Kunitz inhibitory domain (a gift from William Van Nostrand and Dennis Cunningham). All above inhibitors prevented the cleavage of the ^{125}I-P1, indicating that the fraction is enriched in a calcium-activated, serine protease. Protease nexin 1 (a gift from Steve Wagner) and albumin did not influence the enzymatic activity.

Finally, since we suspected a chymotrypsin-like or a cathepsin G-like enzyme should cleave between the methionine and the aspartic acid at the N-terminus of the β-protein (corresponding to the middle of P1) we compared the sequence of the cleaved products generated by cathepsin G to the ones generated by our protease fraction. Both cathepsin G and our protease fraction cleaved before and after the methionine, and are thus capable, in principle, of generating the first cleavage required to release the β-protein from its precursor.

DISCUSSION

Thus far, two protease inhibitors have been described to be involved in the amyloid deposits of the β-protein type: β-PP (PN2), the β-protein precursor, and, intimately associated with the amyloid, ACT. In the brain, the two forms of the β-PP, with (751/770 amino acids) and without (695 amino acids) the protease inhibitory domain, are found in approximately equal amounts, in contrast to the β-PP in other organs where the inhibitor form prevails. The different ratios of the two forms may explain the almost unique accumulation of the β-protein type amyloid in the brain, although recently it was shown that β-protein antibodies label skin and intestine sections (Joachim et al., 1989). The 695/751 ratio was also measured in various parts of the AD brain and the results suggest that in affected areas the 695/751 ratio is higher than in unaffected areas (Palmert et al., 1988).

We described a protease inhibitor actually in the AD brain amyloid, i.e., ACT (Abraham et al., 1988). ACT was also detected in the amyloid of aged humans and monkeys (Abraham et al., 1989a) and in all brain amyloidoses that have the β-protein as their major component (Abraham et al., 1989b). It is not known whether ACT, which is a serum protein, gets into the amyloid from the circulation or from the local synthesizing cells—the astrocytes (Pasternak et al., 1989). There seems to be a very strong association between the β-protein and ACT in the amyloid filaments since harsh SDS/β-ME extraction cannot separate these two molecules (Abraham et al., 1988). We hypothesize that ACT interacts with a region in the β-protein which resembles the active site of a serine protease (Potter and Abraham, in preparation).

There are many indications that an abnormal post-translational processing of the β-PP results in the β-protein fragment which can adopt a β-pleated sheet conformation and precipitate as amyloid. First, unlike a mutated prealbumin protein which can form amyloid deposits in Familial Amyloidotic Polyneuropathy or a mutated cystatin C which precipitates in Hereditary Cerebral Hemorrhage of Icelandic type, the β-protein precursor is not mutated (Tanzi et al., 1987c). Nor is its DNA microduplicated as was suggested as a possible explanation for the excess amyloid deposits in the brains of AD and DS (Podlisny et al., 1987; Tanzi et al., 1987b; St. George Hyslop et al., 1987). Nor are the amounts of β-PP mRNA or protein significally

different between normal and diseased brain. In summary, it seems that the β-protein is formed as a result of an abnormal proteolytic degradation of a normal protein. The fact that two of the amyloid or amyloid-generating components, i.e., the β-PP and the ACT are protease inhibitors could potentially explain their resistance to degradation. Thus, our finding of a serine protease that normally degrades β-PP, but is inhibited by both β-PP (PN2) and ACT may shed light on the abnormal proteolytic processing of the β-PP. It is noteworthy that ACT is an acute phase protein, strongly induced in the liver by IL-1 (Baumann et al., 1987) and synthesized in reactive astrocytes (Pasternak et al., 1989). In cell culture β-PP can also be induced by IL-1 (Goldgaber et al., 1989) and recently it was shown that after neuronal injury, astrocytes in the injured area express high levels of β-PP (Siman et al., 1989). The amounts of IL-1 in AD brain are also elevated, as judged by immunohistochemistry (Griffin et al., 1989). The levels of ACT mRNA and protein in AD brain are highly increased (Abraham et al., 1988). When the amounts of the β-PP are compared between AD and controls they do not seem significantly different, but high levels of abnormal degradative forms of β-PP are found in AD neurons and neurites and on Western blots using β-PP antibodies (Cole et al., 1989). All of the evidence taken together suggests that an aberrant proteolytic degradation of the β-PP can contribute to all of the various aspects of AD pathology (see also Abraham, 1989).

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DEGENERATION OF NEURONAL PROCESSES IN RATS INDUCED BY THE PROTEASE INHIBITOR LEUPEPTIN

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INTRODUCTION

Recent findings have shown that there is a close relationship between protease inhibitors and amyloid deposits or amyloid precursor protein. Experimentally, an accumulation of neurofilaments in axon terminals, suggesting a connection between protease inhibitor and neurofibrillar pathology, and a prominent accumulation of lipofuscin in neuronal cytoplasma have been induced by the cysteine protease inhibitor leupeptin. These findings indicate that some derangement of protease activity plays a role in the pathological aging process of the CNS.

We investigated neuronal changes following continuous administration of leupeptin into the rat lateral ventricle and found a remarkable degeneration of neuronal processes and presynaptic terminals in addition to an accumulation of lipofuscin-like dense bodies in the perikaryon. The present findings show that the leupeptin-induced changes in the neuropil, which consist of aggregates of degenerated neuronal processes and axon terminals, closely resemble the fine structure of senile plaques in Alzheimer's disease except for the absence of amyloid deposition, and suggest that the degenerated neurites and synapses, the important consitituents of senile plaque, are caused by amyloid protein acting as a protease inhibitor.

MATERIALS AND METHODS

As we described the details of materials and methods elsewhere,[1] only an outline is given here. Sixteen Wistar rats, each weighing about 200g, were implanted with osmotic minipumps (Alzet 2002) containing leupeptin dissolved in phosphate buffer solution at 32.5mg/ml; and leupeptin was continuously infused into the lateral ventricles for two weeks at the daily dose of 0.47mg. Brains and spinal cords were processed for light and electron microscopic study according to the usual methods.

RESULTS

Leupeptin-treated rats showed weight loss and decreased motor activity beginning several days after the start of the experiment. No remarkable neurological deficit was observed during the initial ten days, but during the last five days, the rats gradually became inert and ataxic.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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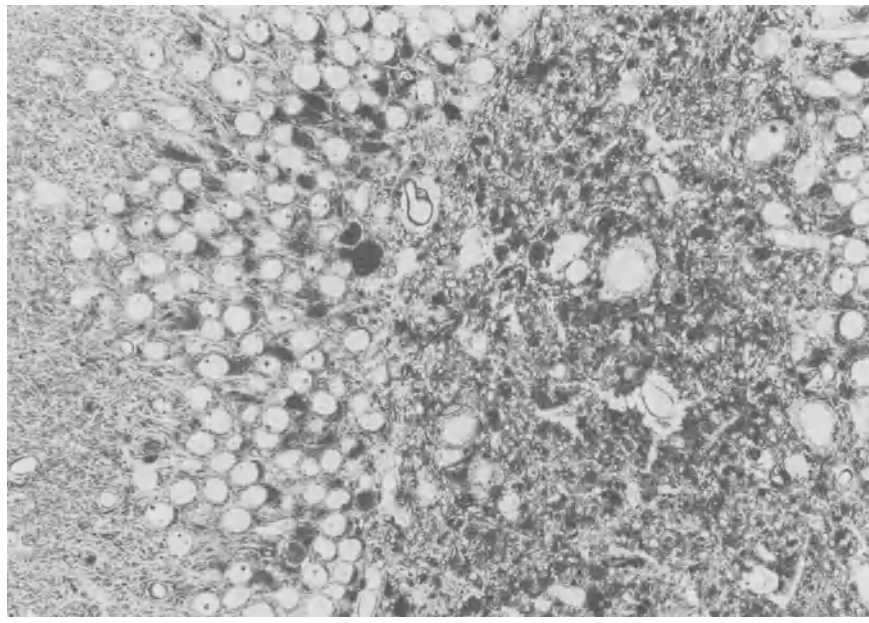


Fig.1. Most neurons in the gyrus dentatus contain abundant, dense granules; and darkly stained, swollen axons of various sizes are dispersed beneath the granule cell layer. 1-µm Epon section, toluidine blue staining.

Light Microscopic Findings

In H&E-stained sections, numerous eosinophilic spherical structures were seen in the hippocampus, corpus callosum, and zona incerta. A small number of the same structures existed in other parts of the cerebral cortex as well as in the basal ganglia. Cell bodies of cortical neurons were much more eosinophilic than they normally appear, and a marked predominance of these neurons was found in the hippocampus. The architecture of the cerebral cortex was preserved and no neuronal loss was detectable. In the cerebellar cortex, however, a decreased population of Purkinje cells showed severe degeneration and were presumably responsible for the ataxia. In semi-thin sections stained with toluidine blue (Fig.1), dark, spherical structures corresponding to spheroids were observed with the same distribution as the eosinophilic, spherical structures, and were especially abundant in the hilus of the dentate gyrus.

Electron Microscopic Findings

Perikaryon and dendrites. Most neurons of the dentate gyrus, and a large part of the cerebral cortex, contained numerous electron-dense granules resembling lipofuscin, whose existence in neurons is rare for 8-week-old rats. In spite of the accumulation of dense granules, the other cytoplasmic organellae were intact, and active protein synthesis was indicated by well-developed rough ER, free ribosomes, and an indented nucleus with distinct nucleolus. The dense granules also appeared in dendrites, and were distributed not only in the vicinity of the perikaryon, but also in the distal portion of dendritic processes where they formed small islets consisting of several granules. Accumulation of granules in dendrites caused no change in the caliber of the processes.

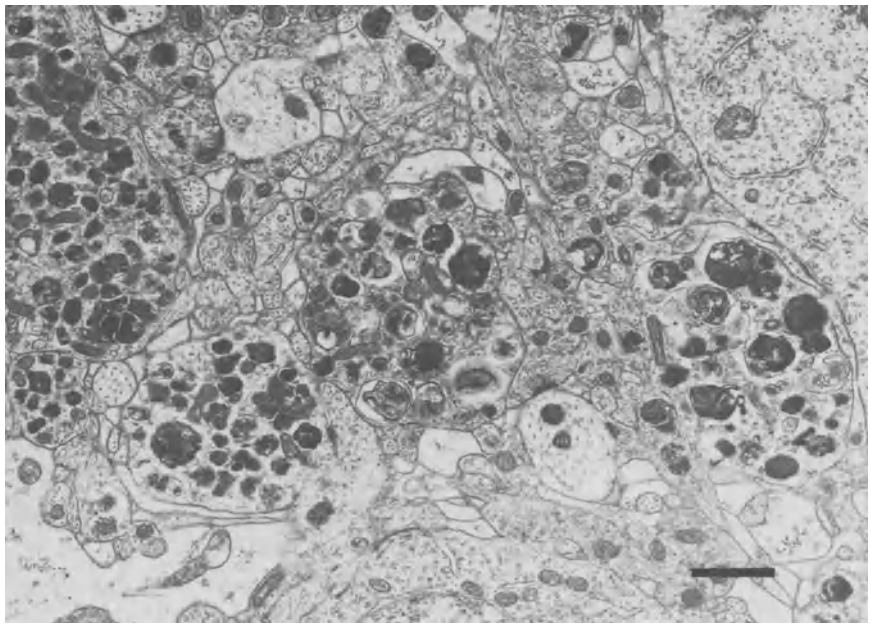


Fig. 2. A small cluster of abnomal neurites mingled with glial processes and filled with degenerated mitochondria and dense bodies resembles a primitive plaque in Alzheimer's disease. Bar, 1μm.

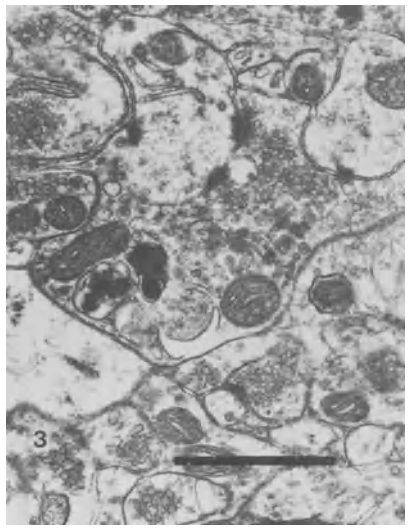
Axons. In the regions where axonal swelling had been detected by light microscopy, not only in myelinated axons but also in non myelinated ones, even the smallest axons were variously enlarged and contained numerous mitochondria and membranous, dense bodies. No microtubules were detected in the swollen axons, although, rarely, lamellated structures that are known to appear in dystrophic axons were observed. Dense bodies were identical to those in the perikaryon, showing various inner structures seeming to correspond to the stage of degradation of abnormal products.

Neuropil. The neuropil of the cerebral cortex, especially in the hippocampus, of leupeptin-treated rats presented an aspect very different from the normal appearance; namely, numerous degenerated neuronal processes containing dense bodies, dispersed randomly, were mingled with glial cells and their processes. Occasionally, we noted small aggregates of degenerated neurites closely resembling the aggregation of degenerated neurites appearing in neuritic plaques of Alzheimer's disease (Fig. 2). Presynaptic terminals also showed degenerative changes (Figs. 3 & 4). No amyloid fibrils nor degenerated neurites containing paired helical filaments were detected in the brains of leupeptin-treated rats.

DISCUSSION

It is generally accepted that senile plaques are composed of masses of amyloid fibrils, degenerated neurites and synapses, glial cells, and their processes.[2] The aggregation of degenerated neuronal processes and axon terminals in the neuropil induced by leupeptin points out a close morphological resemblance to the senile plaque of Alzheimer's disease.

Recent studies have demonstrated that the serine protease inhibitor α1-antichymotrypsin is one of the components of Alzheimer's amyloid depos-



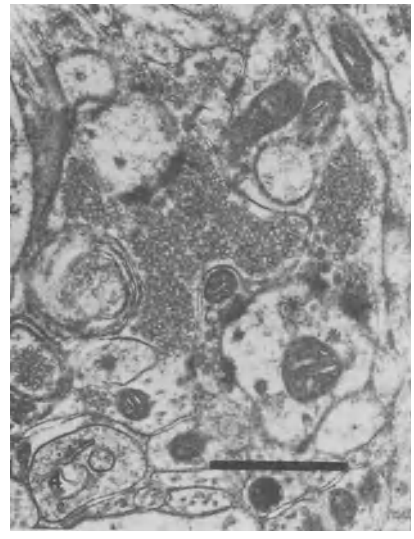


Fig. 3. A degenerated axon terminal observed in the hippocampus of a leupeptin-treated rat. The axon terminal is enlarged and contains dense bodies and vacuoles. Bar, 1μm.
Fig. 4. Compactly agglomerated synaptic vesicles and membranous whorls are seen in an irregularly distended axon terminal. Bar, 1μm.

its.[3] Furthermore, amyloid precursor protein has a domain containing a protease inhibitor sequence.[4] These findings suggest that protease inhibitors play roles in the mechanism of amyloid deposition by inhibiting normal degradation of the precursor protein.

Experimental findings concerning the effects of protease inhibitors on the CNS have been accumulating for several years. Especially, the changes caused by leupeptin have similarities to the main characteristics of the aging process in the CNS.[5] Taking these facts into consideration, the present findings are very suggestive that protease inhibitors are involved in the mechanism of senile plaque formation, and they also support the proposed inhibitor model of aging.[5] Although the role of protease inhibitors in senile plaque formation has not been clarified as yet, it is possible that protease inhibitors not only take part in the mechanism of amyloid deposition, but also cause changes in the neurites and synapses surrounding the amyloid core.

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α1-ANTICHYMOTRYPSIN INFLUENCES THE SURVIVAL OF NEURONS

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INTRODUCTION

Alzheimer's diseases(AD) is a degenerative disorder characterized by neuronal loss and brain lesions such as senile plaques and neurofibrillary tangles. In the AD brain, abnormal neuronal sprouting responses occur[1]. Many proteases and protease inhibitors have been shown to influence the extent of neurite outgrowth from several neuronal cell types in vitro [2,3,4]. α1-Antichymotrypsin (ACT), a serine protease inhibitor, is produced in the liver and its serum levels are elevated in patients with cancer or inflammatory diseases. ACT is associated with senile plaque amyloids[5,6]. Matsubara reported that its serum level was elevated in AD patients[7]. The presence of a functional Kunitz protease inhibitor domain in the amyloid protein precursor(APP) molecule carries profound implications for the process of amyloidogenesis in AD[8]. Thus we studied the effect of protease inhibitors on cultured neurons.

MATERIALS AND METHODS

Culture of Hippocampal Neuron

Cerebrum was taken from rat embryos on the 18th gestational day and the hippocampus was isolated, with the hippocampal fissure being regarded as a border (thus, the hippocampus as used here contained Ammon's horn but not the dentate nucleus and the subiculum). After incubation with 0.125% trypsin and DNase I (200 Kunitz units / 6 ml) at 37℃ for 15 min, the cells were dissociated by pipetting in the chemically defined medium (50% Dulbecco's modified Eagle medium, 50% Ham's F12 medium, 5μg/ml insulin, 100μg/ml transferrin, 20nM progesterone, 100μM putrescine, 30nM selenite). The dissociated neurons were plated on 24-well culture plates pre-coated with collagen. Various concentrations of protease inhibitors dissolved in the medium were added. The cultures were maintained for 48 hours in a humidified atmosphere consisting of 95%air-5%CO_2 at 37℃.

Culture of Dorsal Root Ganglion Cells

Dorsal root ganglions, taken from 8-week-old male mice, were digested by 2mg/ml collagenase for 1hour and pipetting in the chemically defined medium mentioned above. Cultures were started by seeding in collagen coated 96-well culture plate and maintained in the same condition as hippocampal cell culture.

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Quantification of the Number of Neuron with MAP 2 EIA

The cultured cells were fixed with 4% paraformaldehyde at room temperature for 45 min and washed with PBS (phosphate buffered saline). They were permeabilized with 0.1% Triton X-100 in PBS for 5 min, then followed by incubation with monoclonal anti-microtuble associated protein (MAP) 2 antibody (Amersham, 1:1000) for 1 hour. The cultures were incubated with biotinylated anti-mouse sheep IgG (1:200) for 1 hour, then with streptavidin-biotinylated horseradish peroxidase complex (1:300) for 30min, and finally incubated with ABTS (Kirkegaard and Perry Laboratories) for 30min. Color-development was stopped by the addition of 1.5mM sodium azide, absorbance at 405nm was measured.

We examined the MAP2 level and the number of MAP2-positive cells, and found the level of anti-MAP2 binding to the culture, quantified by enzyme immunoassay (EIA) to be correlated with the number of MAP2-positive cells. Thus, the level of anti-MAP2 binding, primarily reflecting neuronal survival in culture, can be an arbitrary index of neurotrophic activity (Fig 1).

RESULTS

Fig. 2. shows the cultured hippocampal neurons. As shown in Fig.3, ACT had neurotrophic activity on hippocampal cells. But ACT for dorsal root ganglion cells and aprotinin (another protease inhibitor) for hippocampal cells did not show neurotrophic activity (data not shown), and neurite outgrowth.

DISCUSSION

Neurite outgrowth is a complex and a most important cellular event leading to differentiation of the neuronal cell and maintaining the features as a neuron and the cell's survival. Many substances which increase neural sprouting and neurite elongation are becoming clear recently.

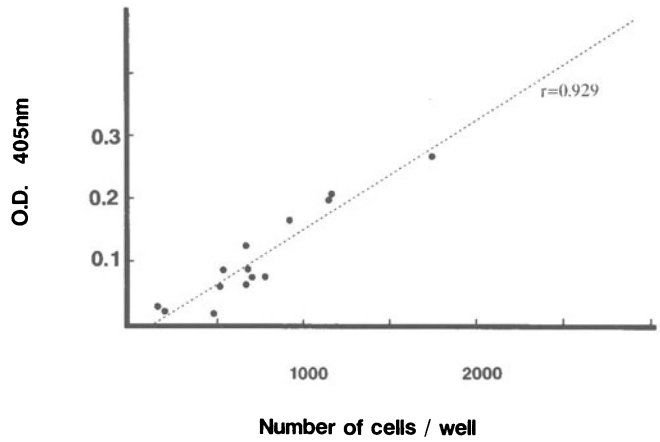

Fig. 1. Correlation between MAP2 level and the number of cells. Hippocampal neurons were seeded and maintained 48 hours, then fixed and labeled with a monoclonal antibody to MAP2. The number of MAP2-positive cells on plate was counted under a microscope. The level of anti-MAP2 was quantified by using EIA and expressed as absorbance at 405nm.

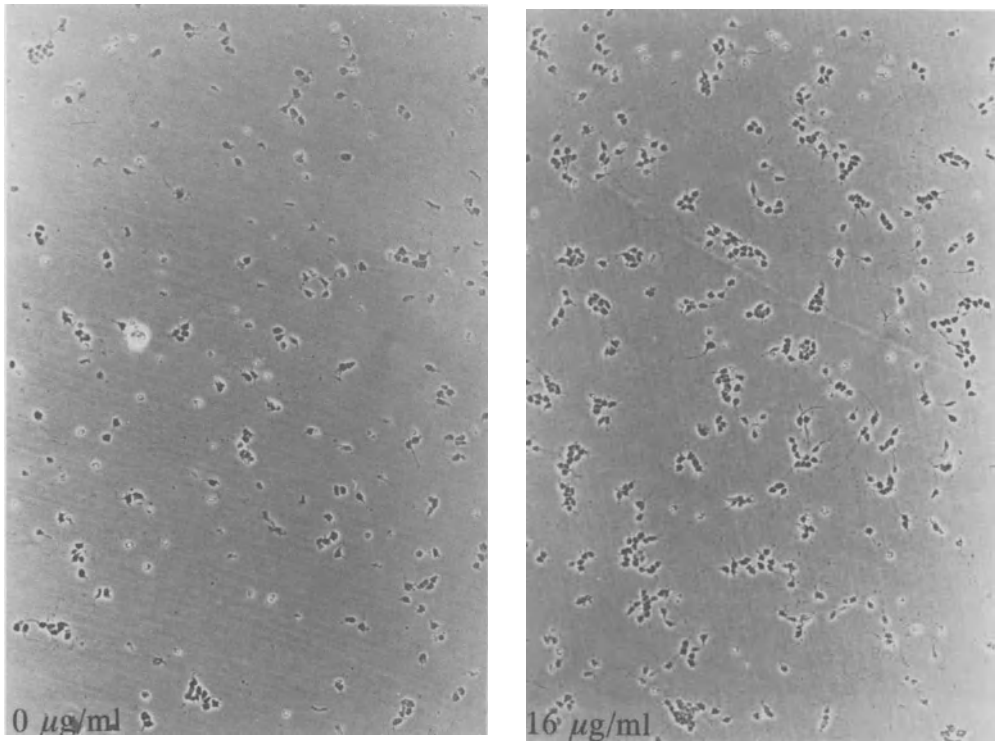

Fig. 2. Morphological effect of ACT on hippocampal neurons. They were cultured for 48 hours in the serum-free defined medium. (Left)Control culture, maintained without ACT to the medium. (Right) 16μg/ml of ACT was added to the medium.

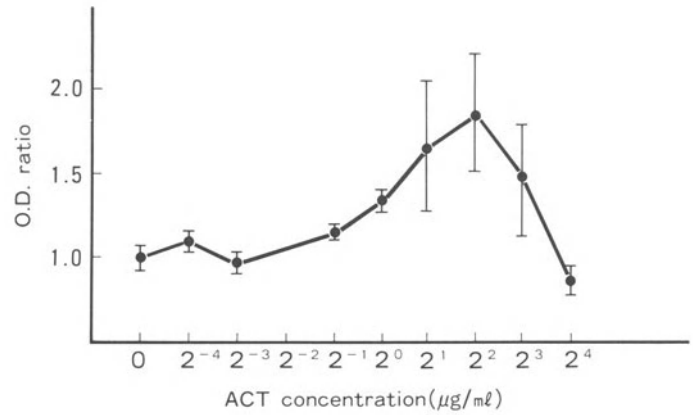

Fig. 3. Dose-response curve for neurotrophic activity in ACT. Hippocampal cells were cultured for 48 hours in serum-free chemically defined medium containing ACT at various concentrations. The binding of anti-MAP2 antibody to the cultures, quantified by EIA was expressed as O.D. at 405nm. O.D. ratio to control cultures was calculated.

APP has a Kunitz protease inhibitor domain and the deduced amino-terminal sequence of APP is identical to the sequence of a cell-secreted protease inhibitor, protease nexin-II[9]. The role of protease and protease inhibitors to regulate neuronal elongation has become clear.

Atterwill et al.[10] showed that although both normal and AD brain had neurotrophic factor the AD brain had more. Uchida et al.[11] reported that the AD brain extract had neurotrophic activity, because it lacked the inhibitory factors, and resulted in a relative increase in neurotrophic activity. Whitson et al.[12] reported amyloid β protein enhanced survival of hippocampal neurons. Yankner et al. opposed to this[13].

We examined the relationship between trophic effects on neurons and protease inhibitors. Of the protease inhibitors we tested, only ACT had neurotrophic activity for cerebral neuron. In addition to being an associated protein of senile plaque amyloids, ACT may act as a trophic factor in the AD brain.

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EVIDENCE FOR A NEURONAL ORIGIN OF SENILE PLAQUES IN DOWN'S SYNDROME BRAINS

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SUMMARY

A newly developed methenamine silver (MS)/Nissl stain was used to study the relationship of pre-plaques in Down's syndrome (DS) brains with glial nuclei, capillaries and neuronal perikarya. The larger pre-plaques often encompassed all of these tissue elements, but the smaller ones were almost always found immediately adjacent to, or around the cell bodies of neurons. Thus we consider an early stage of senile plaque (SP) formation to be the deposition of amyloid substance adjacent to the cell body of a morphologically normal neuron. We suggest that the amyloid progressively accumulates around the cell body until the enclosed neuron degenerates. Finally, the necrotic cell body is replaced by amyloid, resulting in a stellate formation of amyloid with degenerating neurites in the periphery. Larger SP may be formed from a cluster of several neurons. Our observations support the idea of a neuronal origin for SP and for SP amyloid.

INTRODUCTION

The origin of SP has remained an enigma ever since they were first described, although much progress has been made in the molecular pathology of SP amyloid. The SP amyloid protein (β-protein or A4) has been isolated (1) and cDNA clones encoding a larger membrane glycoprotein precursor (APP) have been identified (2). Recently, attention has been drawn to a type of SP that is not detected by Congo red or conventional silver stains (3-6). These "pre-plaques" (5) show little or no evidence of degenerating neurites and can be seen as an area of diffuse immunoreactivity after an immunohistochemical reaction for β-protein (3-6). Numerous pre-plaques in the absence of mature SP are found in many cases of DS aged 30-40 years, suggesting that these lesions are indeed the 'forerunners' of mature SP (5-7). We have observed that pre-plaques in these patients show a particularly close spatial relationship with the cell bodies of neurons.

MATERIALS AND METHODS

Paraffin-embedded, formalin-fixed sections from the superior temporal gyrus of 4 cases with DS (table 1) were stained by routine neurohistological methods, by immunohistochemistry using antibodies to residues

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table 1. Assessment of senile plaques in Down's syndrome brains

Case	Age (sex)	Estimation of plaque number: Congo red or silver[1]	Immuno-stain	MS	Reconstruction of pre-plaques (≤50μm) from serial sections: Number counted	Number with ≥1 neuronal cell body	Number with capillary
1	31(M)	-	+	++	30	28	15
2	37(F)	+	+++	+++	NC		
3	38(F)	-	+++	+++	70	65	24
4	42(F)	+	+++	+++	NC		
				Totals	100	93	39

[1]Bielshowsky or Bodian; NC = Not counted; (-) No lesions; (++) Moderate; (+++) Severe.

8-17 (8) or 1-24 (9) of β-protein, and by a sensitive MS method with cresyl violet Nissl counterstain. This MS method detects amyloid fibrils, and possibly "pre-amyloid" substance, with high sensitivity (10). Full details of the methods will be published elsewhere (11).

RESULTS AND DISCUSSION

In cases 1 and 3 the vast majority of SP were pre-plaques, which were particularly numerous in case 3; cases 2 and 4 showed many pre-plaques together with significant numbers of mature SP (table 1).

The larger pre-plaques (50-160 μm diameter) usually encompassed many tissue elements (neuronal perikarya, glial nuclei and capillaries) but a striking observation was the presence within many of the smaller pre-plaques of a single, morphologically intact neuronal cell body, often accompanied by 1 or 2 satellite oligodendrocytes. These smaller pre-plaques showed no obvious, consistent, close spatial relationship with any other tissue element visualised by the MS/Nissl stain. Many of the very small (presumably earliest) pre-plaques were found immediately adjacent to a neuronal cell body, without completely surrounding it. The deposits of MS stain resembled many fine, interlacing fibres scattered closely around, or adjacent to, the cell body, seemingly restricted to the proximal dendritic field of the closely associated neuron. In a few instances, the pattern of MS-positive fibres resembled the classical shape of the dendritic field of a cortical pyramidal neuron. Our observations suggest that the amyloid may originate from the dendrites (and possibly the cell body). A neuronal origin of SP amyloid has been proposed by others (1), and this is supported by the high levels of APP mRNA in some cortical neurons (12) and the immunohistochemical reaction of APP antibodies with the plasma membranes of nerve cell bodies and dendrites (13) and with the abnormal neurites around SP (9).

The 3-dimensional appearance of 100 smaller (≤50 μm) pre-plaques was reconstructed from serial sections of the 2 cases (1 and 3) with only pre-plaques (table 1). At least one neuronal cell body was found within, or immediately adjacent to, 93 of the 100 pre-plaques (with 2 or more in 39). A capillary vessel passed through the area occupied by 39 of the 100

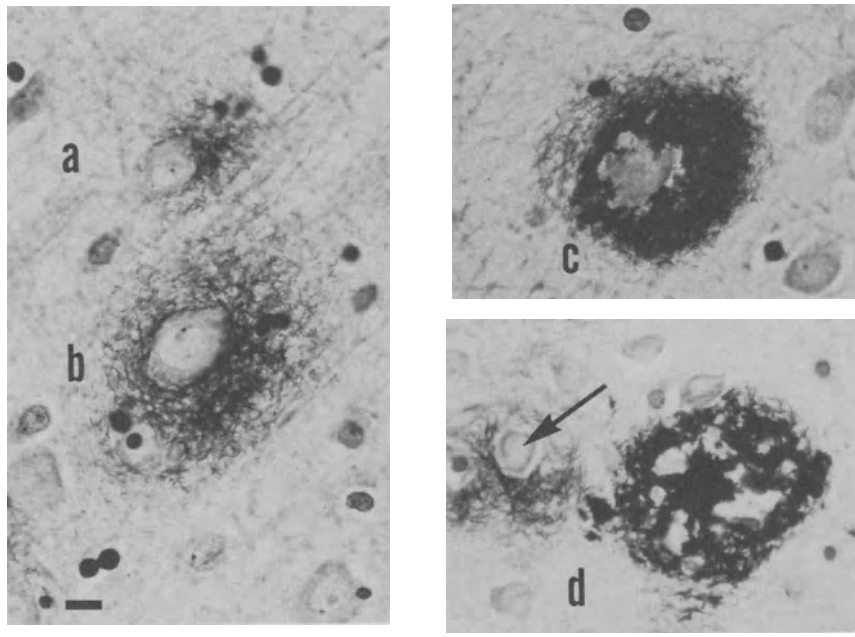

Figure 1. Possible stages (a,b,c,d) in the morphogenesis of a typical SP MS/Nissl stain. Note early plaque in (d) indicated by arrow. (a-c) case 3, (d) case 2. Bar = 10μm.

pre-plaques. This is significantly less than the proportion of pre-plaques with a neuronal cell body ($\chi^2 = 62.5$; $p < 0.001$).

From 'cross sectional' observations of pre-plaques and more mature SP within these 4 patients a series of transitional forms was selected demonstrating a possible progression of stages in the pathomorphogenesis of the typical SP (figure 1). The earliest stage consists of a few MS-positive fibrils (pre-amyloid ?) adjacent to a neuronal cell body (a), and the amyloid substance continues to accumulate until it surrounds the cell body (b), sometimes appearing to occupy the entire dendritic field. As the density of the amyloid increases, the enclosed neuron begins to degenerate (c), possibly due to problems in the exchange of metabolites. Finally, the amyloid "core" accumulates at the site of the cell body, which eventually degenerates and disappears (d). We imagine that the stellate form of the amyloid in a typical SP may represent the last vestige of the encased neuronal cell body and dendrites.

The spatial relationship between SP and neurons was not as simple as figure 1 might suggest. Many SP appear to fuse together, or engulf adjacent neuronal cell bodies as they expand outwards; some larger SP appear to originate from aggregates of several neurons. The latter might develop into the large "amorphous" kind of SP that is particularly common in DS (14).

The derivation of SP from neurons provides a simple and logical explanation for the morphology of SP in various stages, for the correlation between plaque number and degree of dementia, and for the limited size of SP. The restricted size may be determined by the neuronal dendritic field.

We conclude that each SP in DS (and by implication in Alzheimer's disease) may represent the "tombstone" of one or more neurons.

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SERUM AMYLOID P IMMUNOREACTIVITY IN CORTICAL TANGLES, PLAQUES AND VESSELS IN ALZHEIMER'S DISEASE: IMPLICATIONS FOR DYSFUNCTION OF THE BLOOD-BRAIN BARRIER?

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INTRODUCTION

Amyloid P component (AP) is an α_1-glycoprotein found in almost all types of amyloid deposits, representing 10-20% of the weight of the amyloid fibrils[1]. AP is a normal constituent of serum (SAP) synthesized by the liver, the only tissue that appears to exhibit its mRNA[2]. Investigators have previously attempted to demonstrate the immunocytochemical localization of SAP in brain amyloid deposits of subjects with Alzheimer's disease (AD). In earlier studies[3-5], positive SAP antigenicity was evident only in cerebral vessels of AD subjects and those of hereditary cerebral hemorrhage with amyloidosis of the Icelandic type[3,6]. More recently such immunoreactivity has been demonstrated in senile plaques of AD[5,6], as well as cerebral vessels, and in other related disorders exhibiting cerebral amyloid angiopathy[6] (CAA). However, with the exception of a recent preliminary report[7], SAP immunoreactivity has not been, so far, described in neurofibrillary tangles.

To explain the positive SAP immunoreactivity and that of other serum proteins[8,9], previous investigators[6,7] have implicated impairment of the blood-brain barrier (BBB) in AD[9]. However, recent immunohistochemical studies did not only show evidence for BBB impairment in AD but some aging controls were equally affected[8,10]. Despite these findings there appear to be abnormalities in some BBB proteins specific to AD, particularly in cases with CAA[11,12]. To address these issues on BBB permeability and to control for possible failure of antigen detection by conventional fixation and embedding methods applied in previous studies[3-6], we attempted the immunocytochemical localization of SAP in lightly fixed frozen tissue sections of neocortex and hippocampus from subjects with AD, Parkinson's disease (PD) and normal controls.

MATERIALS AND METHODS

Fresh samples of hippocampus including the parahippocampal gyrus, frontal and occipital cortex were obtained at autopsy. Tissue blocks of about 0.5 cm cube were fixed by immersion in

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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3-4% formalin in PBS, for 12-24 h at 4°C, and then transferred to 30% sucrose solution for an equivalent period, followed by freezing in dry-ice cooled n-hexane. Thirty to 40 μm coronal sections were cut in a cryostat, incubated free-floating with primary antiserum (SAP1; 1:500; Dako) containing 0.3 % Triton X-100 and processed according to the PAP method of Sternberger with intensification using nickel ammonium sulphate. Sections were counterstained with H & E. To ensure specificity, serial sections were immunostained with antiserum to serum amyloid A (SAA; 1:500), preimmune serum (1:500) and another SAP antiserum (SAP2; 1:500, Calbiochem) and stained with thioflavin S.

RESULTS AND DISCUSSION

Unlike in previous studies, strong staining was evident in hippocampal pyramidal neurons of Sommer's sector (CA1) and the subiculum with tangles and in neurites and fibrils remaining from degenerated neurons (Figure A and B). The distribution pattern of the reaction product obtained with the antisera to SAP was strikingly similar to the pattern of thioflavin S staining. Both the SAP antisera, SAP1 and SAP2 gave similar staining although as found by immunoblotting (unpublished results) the Dako antiserum, SAP1 was much superior. Heavy deposition of reaction product with antisera to SAP was also evident in tangles and plaques of the neocortex (Figure C). As previously described staining was also seen in vessels exhibiting CAA (Figure D). There was no evidence for staining in either plaques or tangles in any of the cases by rabbit preimmune serum or antiserum to SAA which is primarily present in secondary amyloid deposits[1]. The lack of SAP reaction product in sections from cases ascribed as controls remarkably paralleled the general lack of thioflavin S staining in the same cases.

These observations indicate the presence of SAP immunoreactivity consistently in all the three types of amyloid deposits found in AD. The localization of immunoreactivity was similar to the widely described distinct hippocampal and neocortical pathology. This supports the contention that cerebral amyloid deposits in AD are antigenically related[9]. Failure by previous investigators[3-6] to detect SAP in all three types of brain amyloid deposits, particularly tangles, suggests that conventional tissue fixation in 10% formalin followed by paraffin embedding are not appropriate for such immunocytochemistry. Here, we used brief fixation periods in a relatively low concentration of formaldehyde. However, it is less likely that there are antigenic differences in the antisera. The specificity of our observations is verified by use of two antisera to the same protein obtained from different sources and use of antiserum to SAA and preimmune serum. These observations are in agreement with a recent preliminary report[7] describing SAP immunostaining in both tangles and plaques in cortex of AD subjects. Studies at the ultrastructural level will enable us to assess exact localization of SAP in brain amyloid deposits.

Given that SAP is only synthesized in the liver[2], these observations provide evidence for extravasation of a human serum protein across the BBB in brains of subjects with AD and PD. However, expression of the protein by cellular elements

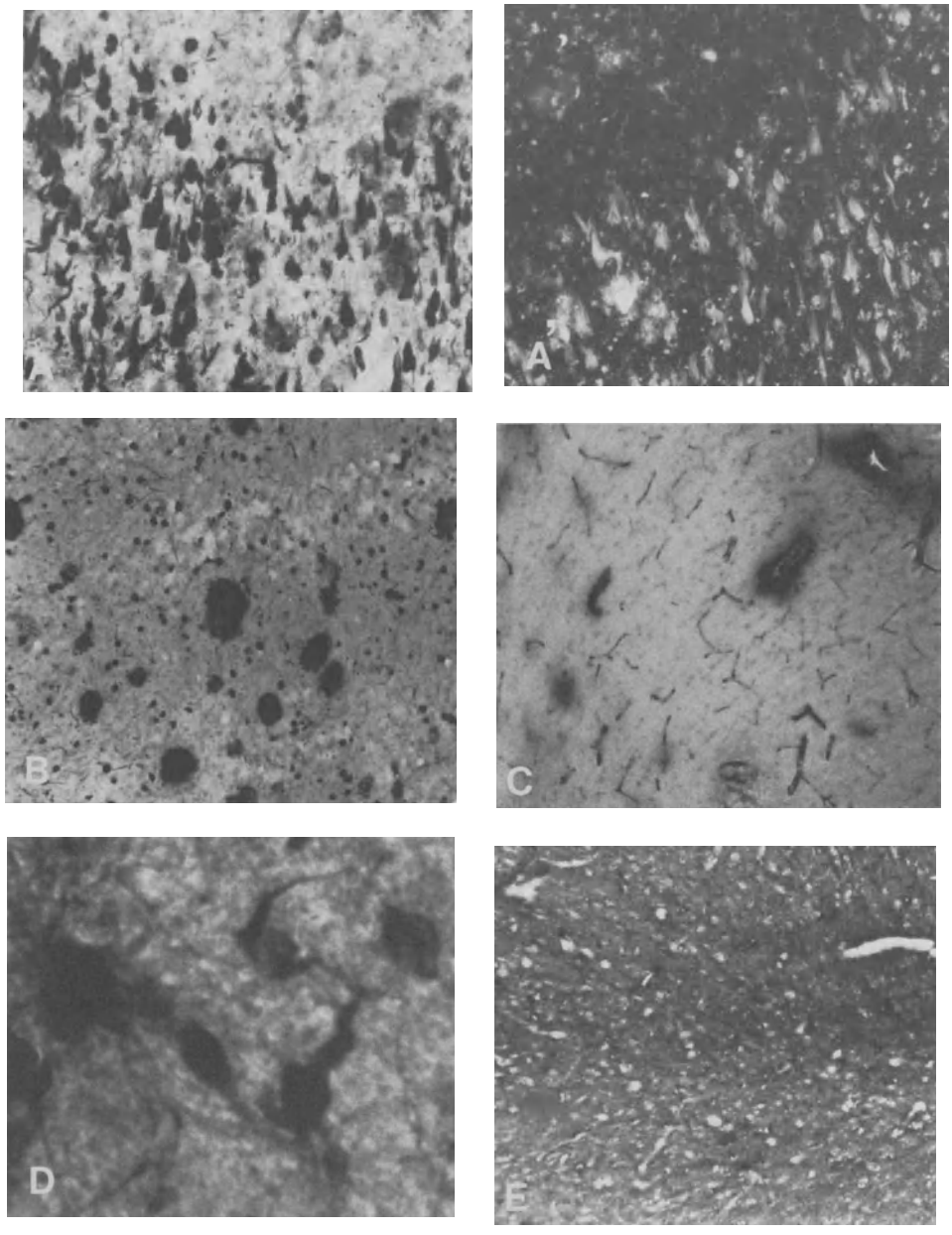

Figure 1. A, SAP immunostaining of neurofibrillary tangles (NFT) in Sommer's sector (CA1) in hippocampus from a 79 year old man with AD. A' demonstrates thioflavin S staining in the same field (as A) of an adjacent section. B shows staining in plaques in endplate of Ammon's horn (same subject as in A) and C, patchy staining in cortical vessels of an 80 year old AD subject. D, shows staining of NFT in frontal cortex from a 72 year old woman with PD. E, demonstrates lack of staining with preimmune serum (same field as in A). SAP2 (Calbiochem) antiserum also stained NFT's and SP's but SAA was negative. Magnification: A-C and E x50; D x125 (before reduction).

such as macrophages[13] or other immune system cells entering the brain through the barrier (unpublished results) cannot be ruled out. Another possibility is that SAP could be expressed by neurons predisposed to amyloid formation.

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MONOAMINE OXIDASE B ACTIVITY IN SENILE PLAQUE

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INTRODUCTION

Two types of monoamine oxidase (MAO; EC 1.4.3.4), MAO-A and MAO-B, has been demonstrated on the basis of their inhibitor specificities and substrate preferences (Johnston, 1968; Knoll and Magyar, 1972; Glover et al., 1977; Fowler et al., 1980; Garrick and Murphy, 1980). The age related increment of MAO-B activity has been described in the various regions of human brain, including the neocortex, hippocampus, basal ganglia and amygdala (Adolfsson et al., 1980; Oreland and Gottfries, 1986). Increased MAO-B activity has also been shown in the neocortex and hippocampus of dementia of Alzheimer type (DAT) (Adolfsson et al., 1980; Oreland and Gottfries, 1986; Reinikainen et al., 1988).

We studied the extracranially perfused postmortem human brains with MAO histochemical procedure and demonstrate the expression of MAO-B enzyme activity in astrocytes in association with senile plaques.

MATERIALS AND METHODS

Four DAT cases and three non-demented controls were studied in the present study. The DAT cases were clinically diagnosed as DAT and confirmed pathologically. Neuropathological examinations revealed none or only few senile plaques in the neocortex of controls. Postmortem human brains were perfused extracranially within 7 h after death (Beach et al., 1987). Tissue blocks, including either the insular cortex, basal ganglia, hippocampus, amygdala and brainstem, were dissected out from the perfusion-fixed brains. Specimens from three brains (one DAT and two controls) were fixed with 4% paraformaldehyde in 0.1 M PB for 24-48 h and soaked in the cryoprotectant containing 15% sucrose in 0.1 M PB for 24 h at 4 C. Tissue blocks from three brains (three DAT cases and a control) were placed immediately after perfusion in the cryoprotectant for 24 h at 4 C. Fifty to 80 um thick sections were cut on a freezing microtome.

The MAO enzyme histochemistry was performed according to the method by Arai et al. (Arai et al., 1986). To examine the relationship between MAO activity and astrocytes, glial fibrillary acidic protein (GFAP) immunohistochemistry was performed. GFAP immunohistochemistry was done as follows; MAO-stained sections were washed with 0.1 M PBS and incubated in

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0.3% H_2O_2 in 0.1 M PBS for 30 min at room temperature to block endogenous peroxidase, and incubated in anti-bovine GFAP serum (DAKO, Denmark) diluted (1:4000) in 0.1 M PBS containing 0.3 % Triton X-100 (PBST) for 24 h at 4 C. After washing with PBST, sections were processed for an avidin/biotin peroxidase system (Vector Labs., California) according to standard protocols, in which nickel ammonium sulfate was not used in the DAB solution to give brown colored reaction products. Under the present double staining procedure, MAO activity was shown as dark blue reaction products and GFAP immunoreactivity as brown. Some of the MAO stained sections were counterstained with either thioflavin S, the Bielshowsky's method, or Congo red to demonstrate senile plaques or amyloid deposits.

As a control, sections were incubated in the reaction medium with the omission of a substrate. The enzyme inhibition experiment was performed as follows. Sections were incubated in 0.1 M PBS containing 10^{-3} to 10^{-10} M L-Deprenyl or 10^{-4} to 10^{-10} M clorgyline for 1 h at room temperature. Sections were thoroughly washed and stained for MAO histochemistry in the same way as described above. Since the post-fixation apparently depressed the staining intensity as described below, the inhibition experiment was done in sections from tissue blocks without post-fixation.

RESULTS

In control sections, no reaction product was observed. The most intense staining was observed in sections without post-fixation. A positive reaction was also observed in sections from blocks of 24-48 h post-fixation, although only in the sections from the deep part of tissue blocks. In this case, it was assumed that the enzymatic activity of the deep part of the tissue blocks was not fully destroyed by the immersion of the post-fixative for up to 48 h.

In the neocortex, no MAO positive neuronal somata was observed. However, many glial cells were stained in the subcortical white matter (Fig. 1). The glial staining in the subcortical white matter appeared somewhat more intense around the vessels. The characteristic finding in brains from four DAT cases was the presence of round or oval shaped MAO-positive mass (Fig. 2). These masses were 50-200 um in diameter, which appeared to consist of small cells. The cell clusters were observed in the insular cortex, amygdala, hippocampus, putamen and brainstem, although the numerical density of cell clusters were clearly different among the brain regions examined. These clusters were most frequently observed in the amygdala, hippocampus and insular cortex. As shown in Fig. 1, no MAO positive mass was observed in controls. Careful examination of GFAP immunohistochemically counterstained sections showed that MAO positive cells were also stained immunoreactive for GFAP (Fig. 3). As seen in this figure, astrocytes negative for MAO were also observed. The double staining with Bielshowsky's method revealed that astrocytic clusters were in or around senile plaques (Fig. 4). Observation of sections counterstained with either Congo red or thioflavin S also revealed the close association between the MAO positive mass and senile plaques (data not shown).

Astrocytic MAO staining in the senile plaque was almost completely inhibited by pretreatment with L-Deprenyl at a concentration higher than 10^{-5} M (Fig. 5 and 6). At lower concentrations, the inhibition was either limited or never observed. Preincubation with clorgyline at a concentration lower than 10^{-5} M did not affect MAO activity in the senile plaque, although partial inhibition was observed by pretreatment with clorgyline at 10^{-4} M.

DISCUSSION

Ninety to 100% inhibition of type B MAO has been observed with preincubation by L-Deprenyl at concentrations of 10^{-4} M in the human cortex

(Garrick and Murphy, 1980). In the present study, glial MAO activity was almost completely inhibited with L-Deprenyl at lower concentrations. Since, in addition, high concentration of chlorgyline such as 10^{-4} M inhibits MAO-B activity as well (Knoll and Magyar, 1972; Glover et al., 1977; Fowler et al., 1980; Garrick and Murphy, 1980), partial MAO inhibition with 10^{-4} M clorgyline in the present study might be caused by MAO-B inhibition with clorgyline. Double staining with GFAP immunohistochemistry and MAO histochemistry has revealed that the MAO positive mass in DAT brain consisted of astrocytes. Thus, the present results clearly indicate that the histochemical reaction in glial cells in the present study represents MAO activity and fibrillary astrocytes in association with senile plaques contain type B. The recent immunohistochemical study demonstrated that glial cells in the non human primate and human brain exhibited MAO-like immunoreactivity of both types (Westlund et al., 1985; Westlund et al., 1988). Although the reason for this discrepancy between our results and others has yet to be determined, the immunohistochemical study also showed that MAO-B staining was much more consistent than MAO-A staining in the glial cell of the human brain (Westlund et al., 1988).

The increased activity of MAO-B without accompaying MAO-A change has been shown in Alzheimer's brain (Adolfsson et al., 1980; Oreland and

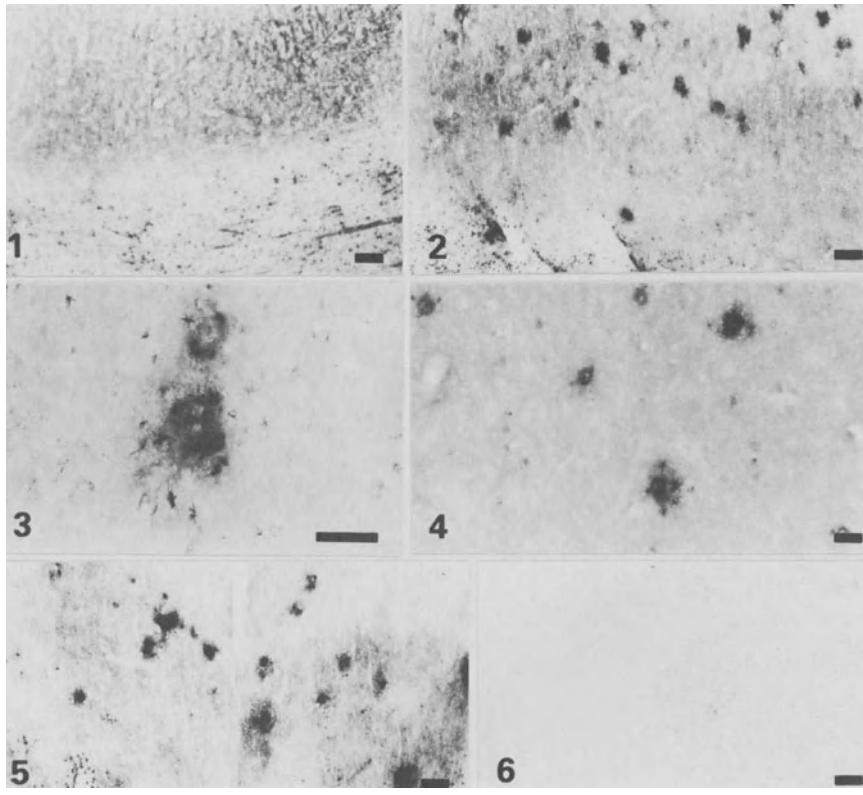

Figure 1 (1) MAO staining of the insular cortex of a control. (2) MAO staining of the insular cortex of a DAT case. (3) Double staining of MAO and GFAP. (4) Double staining of MAO and the Bielshowsky's method. (5) MAO staining of the insular cortex of a DAT case. (6) MAO staining of the neighboring section of (5) after pretreatment with 10^{-5} M L-Deprenyl. bar=100um

Gottfries, 1986; Reinikainen et al., 1988). It has been assumed that increased MAO-B activity in Alzheimer's brain results from the glial outgrowth (Oreland and Gottfries, 1986; Reinikainen et al., 1988). The present study indicates that reported MAO-B increment in various regions of Alzheimer's brain is at least partly due to the occurrence of MAO-B activity in astrocytes in senile plaques.

The pathophysiological role of MAO-B activity in astrocytes in senile plaques are yet to be determined. Although dopamine has been reported to be a substrate for MAO-B in the human brain (Glover et al., 1977), it seems unlikely that astrocytes in or around senile plaques exhibit MAO-B activity in order to metabolize dopamine, since the contents of dopamine and its metabolites have been described to be unchanged or even slightly decreased in Alzheimer's cortex compared with controls (Yates et al., 1979; Cross et al., 1983; Reinikainen et al., 1988). It has been understood that MAO-B deaminates oxidatively the exogenous amines (Fowler, 1982). MAO-B expressed in senile plaques might be involved in metabolizing extraneous amines within senile plaques. Further studies are needed to address this assumption.

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LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS OF DIFFUSE PLAQUE AND ITS RELATED CONDITIONS

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INTRODUCTION

Many ultrastructural studies appear to have confirmed the fine structures of senile plaques(SPs). SPs have been usually classified into three types:1)typical plaques with amyloid cores surrounded by degenerate neurites, 2)primitive plaques composed of many degenerate neurites and a few amyloid wisps,and 3)compact plaques consisting of a mass of amyloid without pathological neurites.Recent advances in staining methods such as β-protein immuno-histochemistry and the modified periodic-acid methenamine silver (PAM) method[1] have offered important information concerning amyloid substance. In the present study, diffuse plaques(DPs)[2],a type of SP,were investigated light and electron microscopically using various staining methods including modified-PAM electron microscopy[3].Ultrastructural findings characteristically seen in DPs were similar to those obtained in the superficial layer of the cerebral cortex and occasionally in peripheral coronas of typical SPs. Based on these observations we discuss the morphological origin of SP amyloid.

MATERIALS AND METHODS

The brains used for this study were obtained at autopsy from two(44 and 79 years old) male patients with Alzheimer-type dementia(ATD).Formol-fixed, paraffin-embedded 5-μm serial sections of the cerebral cortex were stained with the methenamine-Bodian, modified-PAM, and β-protein immunostaining methods.For electron microscopy,small pieces were cut from the fresh cerebral cortex of the two ATD cases and immersed in 2% glutaraldehyde solution. Some of the tissue was refixed in 1% OsO_4 solution and embedded in Araldite for routine electron microscopic examination. Using the remaining parts of the tissue,we cut 100-μm-thick sections using a vibratome and appropriated them for the modified-PAM electron microscopic examination, which shows the specific deposition of silver particles on amyloid substance.[3]

RESULTS

Diffuse plaques

DPs were,though fainter than SPs, clearly stained by the methenamine-Bodian method, but were never recognized with the original Bodian method, which has been widely used to detect SPs. DPs were less argyrophilic than

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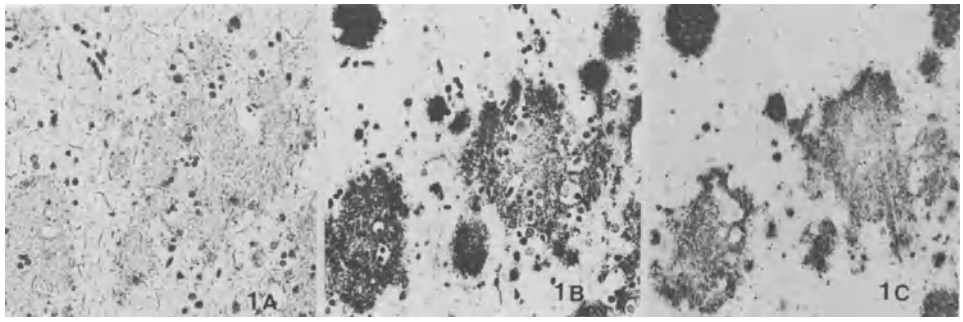


Fig.1. Serial sections of diffuse plaques stained by methenamine-Bodian(A), PAM(B) and anti-β-protein(C) methods.

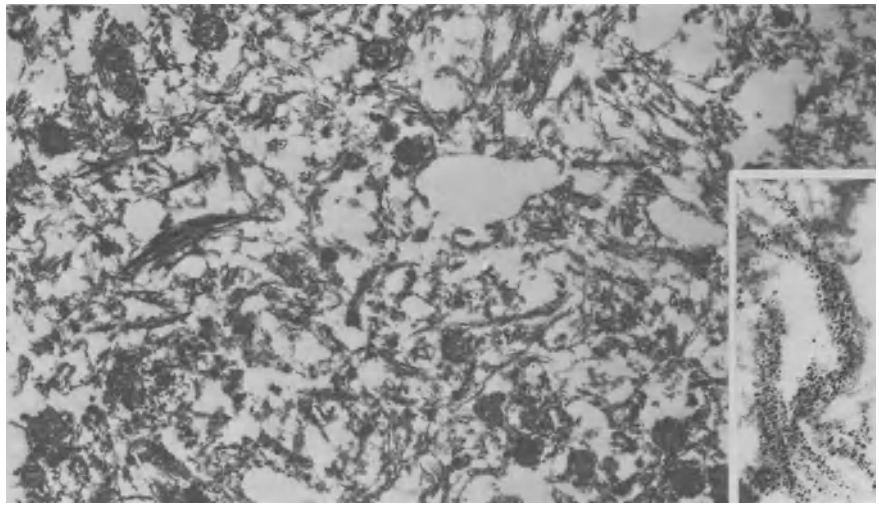


Fig.2. DPs revealed by PAM electron microscopy. Note the scattered wisps with silver granules. x17,500. Inset, x70,000

other types of SPs, and were seen as diffuse and uniform deposits of fine silver granules.They were irregular in form and variable in size. Large ones often contained a few neurons(Fig.1-A).No apparent glial reaction was seen within or around DPs. The plaques were clearly stained with β-protein immunostaining(Fig.1-C), as well as with the modified-PAM method(Fig.1-B), which was proved to stain SP amyloid specifically.[1,3] The predilection sites of DPs were similar to those of other types of SPs in the cerebrum. By the modified-PAM electron microscopic examination DPs were revealed as sparse aggregates of fusiform or bundle-like amyloid-like wisps accompanied by the deposition of silver granules (Fig.2). At higher magnification, no obvious amyloid fibrils were confirmed, but weakly electron-dense amorphous substances were recognized, on which silver granules had been deposited(Fig.2, inset). Upon routine electron microscopy,DPs were also shown as aggregates of scattered fusiform or bundle-like wisps (Fig.3) consisting of weakly electron-dense amorphous and partially fibrous substances (Fig.3, inset). A few small degenerate neurites and astrocytic processes were occasionally observed. The ground substance of DPs seemed to be somewhat rough in comparison with the surrounding tissue. Although the amorphous and partially fibrous amyloid-like wisps seemed to be free in the tissue and it was

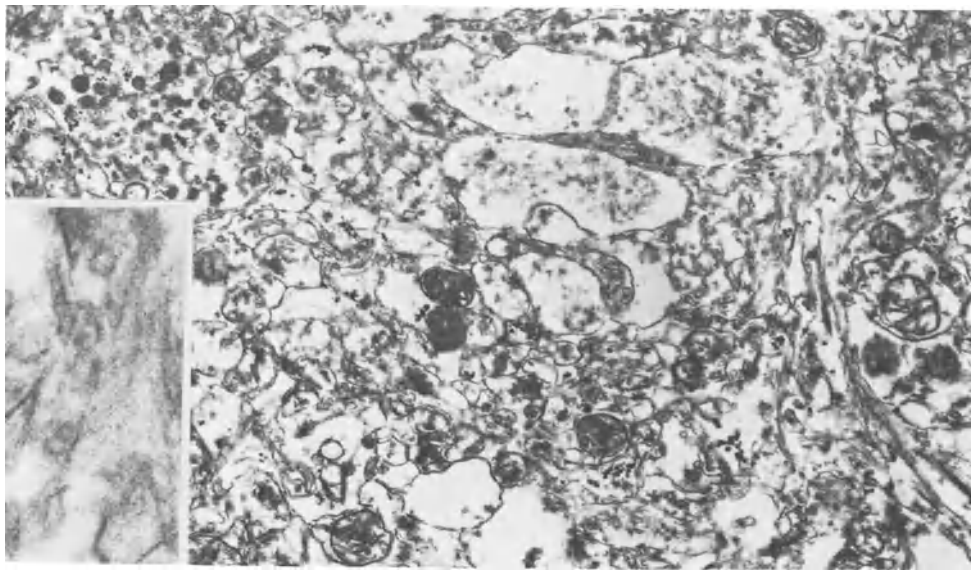
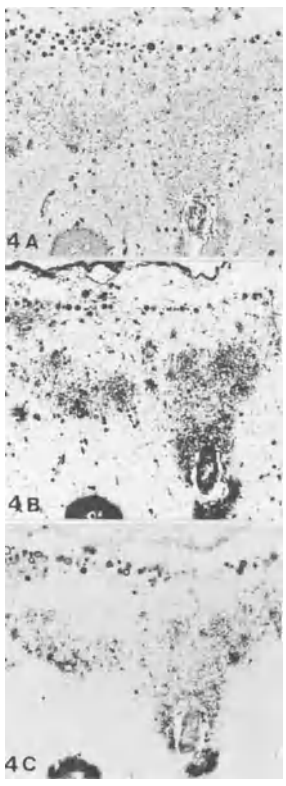


Fig.3. DP:many scattered amorphous but partially fibrous amyloid-like wisps with only a few degenerate neurites. x17,500. Inset, x87,000.



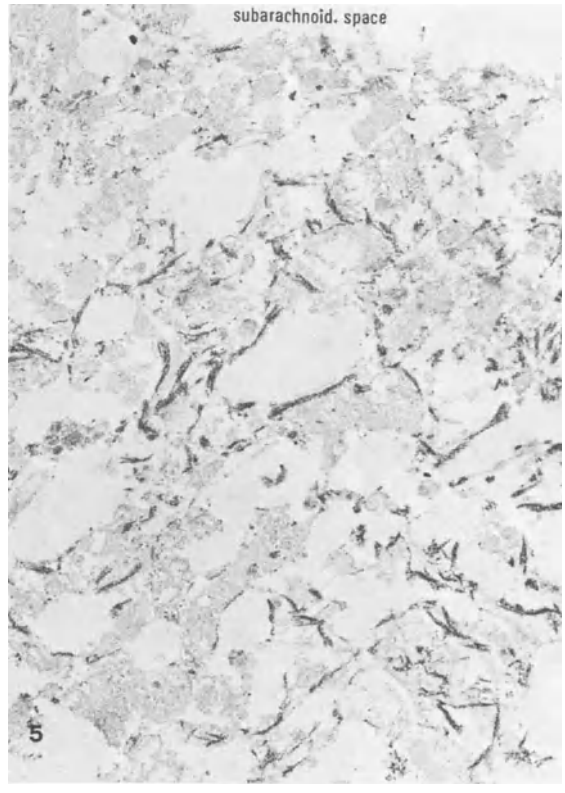


Fig.4. Subpial amyloid deposits are shown by methenamine-Bodian(A),PAM(B), and anti-β-protein(C) methods.
Fig.5. Same type of deposits visualized by PAM electron microscopy. x10,000.

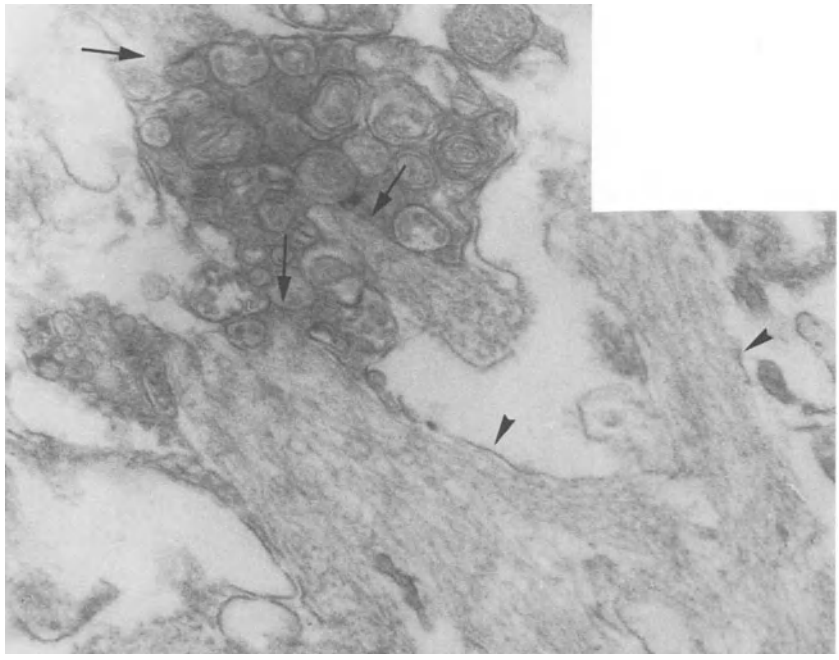


Fig.6.Peripheral coronal part of typical SP:In contrast to partially amorphous amyloid-like wisps(arrows) having close contact with degenerate neurites, amyloid bundle in the vicinity have an obvious fibrous structure. x57,000. Inset, β-protein immunostaining.

difficult to know the morphological relationship between these wisps and tissue elements,some of amyloid-like wisps were found in close proximity to postsynaptic endings as well as to unidentifiable cell processes.

Related conditions

Subpial amyloid-like deposits:In the superficial region of the cerebral cortex and just beneath the pial membrane, band-shaped weakly argyrophilic deposits were sometimes found in the methenamine-Bodian, modified PAM, and β-protein immunostain preparations (Fig.4,A-C). They were similar to DPs in staining pattern. These deposits were often continuous with perivascular amyloid deposits. The corresponding finding shown by the modified-PAM electron microscopy was diffusely scattered small fusiform wisps with fine silver granular deposits(Fig.5). Though no apparent fibrillar structure was confirmed,they resembled the amyloid-like wisps of DPs.

Peripheral amyloid-like deposits of typical SPs: By β-protein immunohistochemistry,besides the central amyloid core, β-immunoreactive substance was observed in the peripheral coronas of typical SPs(Fig.6, inset), where many degenerate neurites are usually distributed.Examination of the peripheral coronas by modified-PAM electron microscopy revealed an intermingling of scattered degenerate neurites and bundle- or fleck-formed, PAM-positive wisps.Such wisps were never seen within degenerate neurites,but were found in close proximity to or free from them. In contrast to obvious fibrous structures in the central core amyloid and freely present peripheral amyloid (Fig.6, arrowhead), some of the amyloid-like wisps closely associated with degenerate neurites were not always fibrous(Fig.6, arrows) when examined by both PAM and routine electron microscopy. Such an amorphous nature of the peripheral amyloid-like wisps was like that of DPs.

DISCUSSION

The purpose of this study was to present detailed ultrastructural findings of DPs[2,3] and similar structures, and to discuss the origin of SP amyloid. Hitherto, ultrastructural studies of SP amyloid have been focused upon fibrillar amyloid structures.Here we closely examined non-fibrillar pre-amyloid structures. A common ultrastructural characteristic of amyloid-like wisps in DPs,subpial deposits, and peripheral coronas of typical SPs was the presence of amorphous structures.The amorphous structures might be in a pre-stage of amyloid fibril formation and,therefore,be important in relation to recent biochemical reports of amyloid-precursor protein.[4,5] Some biochemical and immunohistochemical studies[6,7] suggest a neuronal origin of SP amyloid. Our observations support this hypothesis from a morphological aspect in the following points. Some amyloid-like wisps were closely associated with post-synaptic endings and unidentifiable processes. In peripheral coronas of typical SPs amorphous wisps were always in close proximity to degenerate neurites, while amyloid wisps found just nearby had an apparently fibrous structures. Subpial amyloid-like deposits were mostly restricted to the Ist layer of the cerebral cortex, where terminal dendrites of neurons are distributed. At the same time,however, astrocytic processes were also closely related to these structures. Although we lean toward a neuronal origin of SP amyloid,the possibility of astrocytic origin can still not be denied.Further investigations on this problem are necessary.

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β-AMYLOID PLAQUES AND DEMENTIA: AN IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT: Senile plaques (SPs) and NFTs were immunohistochemically studied in the temporal lobe of 246 autopsy brains from non-demented and demented old people, using antibodies to synthetic polypeptide (1-24) of β-protein of cerebrovascular amyloid and to human tau protein. Dementia was present in 64% of 28 cases with many NFTs, while it was present only 33% of 43 cases in which there were many SPs but a few NFTs. The rate of prevalence of β-immunoreactive SPs increased with age in both demented and non-demented subjects. It was not significantly different between non-demented group (40% of 161 cases), and senile (47% of 19 cases) or vascular (30% of 27 cases) dementia groups. These findings suggest that β-immunoreactive SPs may be related to aging but may not be closely linked to dementia.

INTRODUCTION

In the previous communication [Kuzuhara et al,1989], we have reported that tau-immunoreactive neurofibrillary tangles (NFTs) increase with age in the hippocampus and entorhinal cortex, but not in the temporal lobe neocortex in non-demented subjects; whereas many NFTs appear in the neocortex also, in senile dementia of Alzheimer type (SDAT). In the present study, we report the incidence and distribution of β-amyloid plaques (BAPs) in non-demented and demented old people.

SUBJECTS AND METHODS

Table1. Subjects

Age	non-demented	demented	total
50s	2	1	3
60s	23	6	29
70s	61	13	74
80s	73	33	106
90s	10	20	30
100s	2	2	4
total	171	75	246

Brains from 246 autopsy cases aged between 51-102 years, including 171 non-demented and 75 demented cases were submitted for the study (Table). They were fixed in 10% formalin. Coronally-cut slices of the brains were embedded in paraffin, and cut into 8 μm-thick sections. Sections of the temporal lobe through the lateral geniculate body, including the hippocampus, parahippocampal gyrus, and superior, middle and inferior temporal gyri were submitted for the immunohistochemical study.

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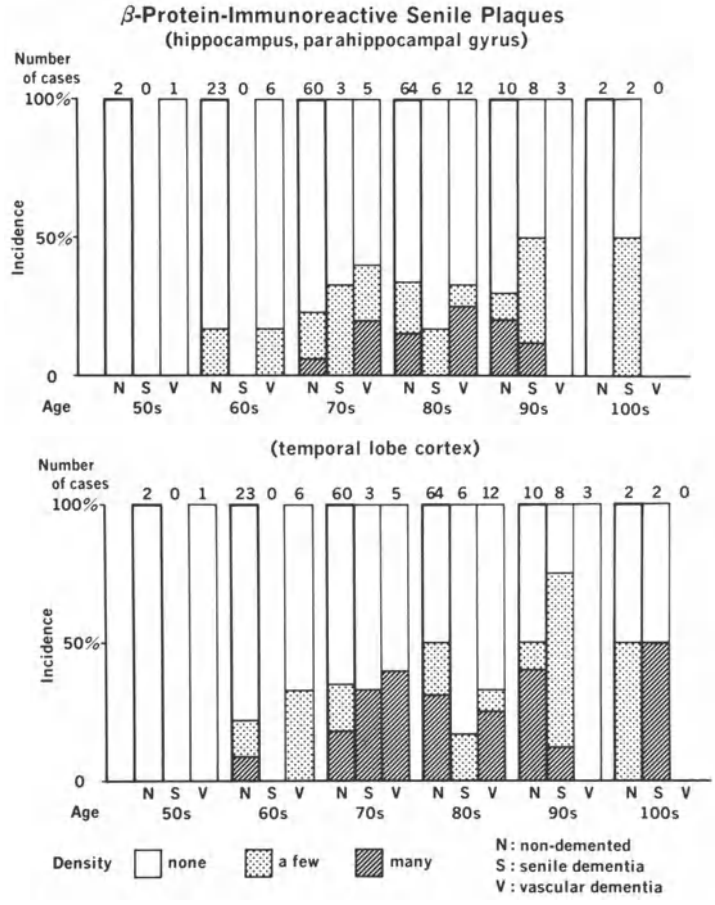

Fig.1. Rate of prevalence and density of β-protein-immunoreactive senile plaques

Primary antibodies used for the present study were antiserum to human phosphorylated tau (anti-tau)[Nukina et al, 1986] and antiserum to synthetic polypeptide (1-24) of β-protein of cerebrovascular amyloid (anti-β)[Glenner et al,1984]. The deparaffinized sections were incubated with diluted primary antibodies (anti-tau; 1:500, anti-β; 1:200) at room temperature overnight. For enhancing the immunohistochemical reaction of β-protein, the sections were treated with 98% formic acid for 10 minutes prior to incubation [Kitamoto et al,1987]. The immunoreaction products were visualized with avidin-biotin-peroxidase complex (ABC) technique (Vectastain, Vector, USA) as reported previously [Kuzuhara et al,1988]. The immunostained sections were faintly counterstained with hematoxylin.

RESULTS

1. Immunostained structures. With β-protein immunohistochemistry, immunostained were senile plaques (SPs) of classic, primitive and diffuse types, amyloid angiopathy and subpial deposits as shown by previous studies [Ogomori et al,1988; Wisniewski et al,1989a; Yamaguchi et al,1988]. With tau immunohistochemistry, immunostained were NFTs, degenerating neurites of SPs and curly fibers [Braak et al, 1986; Ihara,1988], a meshwork of degenerating neurites in the cerebral cortex.

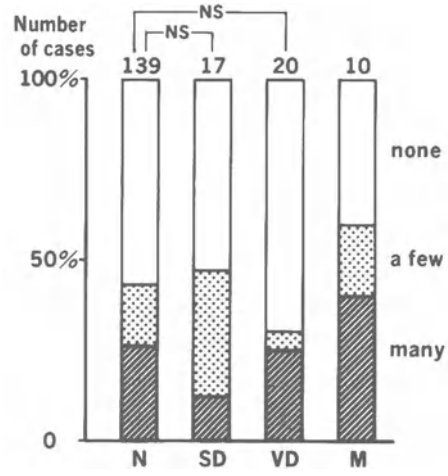

Fig. 2. Rate of prevalence and density of β-protein-immunoreactive senile plaques in nondemented and demented subjects.

2. NFTs and dementia. With tau immunohistochemistry, 28 cases showed many NFTs in the hippocampus, parahippocampul gyrus and temporal lobe neocortex. Eighteen of them (64.3%) had dementia.

3. Plaques and dementia. In order to study the relationship between BAPs and dementia, 28 cases with many NFTs and one case with unclear medical history were excluded. The remaining 217 cases with a few NFTs, or with considerable NFTs in the hippocampus and parahippocampal gyrus but few or none in the neocortex were classified into non-demented, SDAT and vascular dementia groups, according to their medical history, and BAPs of the hippocampus, parahippocampal gyrus and temporal lobe neocortex were semiquantitatively studied. As shown in Fig.1, the rate of incidence of cases showing BAPs increased with age in both demented and non-demented subjects. The rate of prevalence and density of BAPs were higher in the temporal lobe neocortex than in the hippocampus and parahippocampal gyrus. As to the subjects aged between 70 and 99 years, there was not significant difference in the rate of prevalence between non-demented cases and SDAT or vascular dementia cases (Fig.2). Out of the 43 cases which showed many BAPs but a few NFTs, only 14 cases (32.6%) had dementia.

DISCUSSION

SPs together with NFTs have been regarded as one of the most important pathological hallmarks of dementia of Alzheimer type. SPs are, however, seen in the brains of non-demented old people also although they are less dense. In recent years, immunohistochemical techniques using specific antibodies to β-protein of the cerebrovascular amyloid, paired helical filaments (PHF) of NFTs [Ihara et al,1983], or tau protein were introduced in the neuropathological studies of the demented or aged brains, and they have made NFTs and SPs visible far more sensitively and specifically than the conventional histological staining techniques [Duyckarts et al,1987; Wisniewski et al,1989b]. With pretreatment with formic acid, many SPs can be vesible in the brains of not only demented but also non-demented old people. Consequently have occurred arguments whether or not BAPs of various

forms can be regarded as a diagnostic criteria of dementia of Alzheimer type. Many studies have demonstrated that there is a strong correlation between the premortem diagnosis of dementia and the presence of numerous BAPs. The converse, however, does not hold: several studies have reported that many patients with abundant BAPs did not have premortem dementia [Davies et al,1988; Delaère et al, 1989]. Furthermore, some studies [Delaère et al,1989; Dickson et al,1988] have demonstrated that morphological changes closely linked to premortem dementia of Alzheimer type are NFTs shown by anti-tau.

In the present study, we excluded the cases with many NFTs in the hippocampus, parahippocampal gyrus and the temporal lobe neocortex and investgated the density and distribution of BAPs in the remaining cases, since our previous study demonstrated close relationship between dementia and NFTs. The number of cases with BAPs increased with age in both demented and non-demented subjects, and there was not significant difference of rates of prevalence between them. Only one third of 43 cases with abundant BAPs had premortem dementia. These findings suggest that BAPs may be related to aging but may not be closely linked to premortem dementia although further studies on the morphology, density and distribution of cerebral amyloid deposots as well as premortem prostective studies on the diagnosis of dementia seem necessary to confirm it.

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DIAGNOSIS OF CEREBRAL AMYLOID ANGIOPATHY WITH CEREBRAL HEMORRHAGE BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) OF CYSTATIN C IN CEREBROSPINAL FLUID

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SUMMARY

A lower level of cystatin C in cerebrospinal fluid (CSF) is one of the useful diagnostic markers of hereditary cerebral hemorrhage with amyloidosis in Iceland. Therefore we set up an assay to determine the level of cystatin C in CSF for diagnosis of cerebral amyloid angiopathy (CAA) associated with cerebral hemorrhage and cystatin C. We carried out an sandwich enzyme immunosorbent assay using monoclonal mouse anti-cystatin C and polyclonal rabbit anti-cystatin C antibodies. We examined CSF from nine cases of cerebral hemorrhage and fifty reference cases of other neurological diseases. Four patients with cerebral hemorrhage showed a low level of cystatin C and clinical manifestations suggestive of CAA. Our study showed that ELISA is useful for the diagnosis of CAA associated with cerebral hemorrhage and cystatin C.

INTRODUCTION

In 1972, Gudmundsson reported families of hereditary cerebral hemorrhage with amyloidosis in Iceland. The patients frequently develop cerebral hemorrhage (HCHWA) in their youth and they have marked amyloid deposition in the cerebral vessels.[1] Ghiso clearly demonstrated[2] that the amyloid protein is a variant of cystatin C (gamma-trace), which is one of the cysteine proteinase inhibitors. In 1984, Grubb reported[3] that concentrations of cystatin C in CSF of patients with HCHWA were lower than those of normal subjects. We also reported[4] the first case in Japan of CAA with the deposition of cystatin C. In addition, we reported a non-familial group of such patients. Maruyama reported[5] that cystatin C immunoreactive substance is important for pathogenesis of CAA with cerebral hemorrhage. However, a diagnosis of CAA during the patient's lifetime is difficult to make. At present, the immunohistological method following brain biopsy or autopsy is the only diagnostic tool available. In the present study, we determined concentrations of CSF cystatin C by ELISA in patients with cerebral hemorrhage including subcortical hemorrhage and in patients with other neurological disorders as the control. As a result developed a simple and easy diagnostic test for CAA associated with cerebral hemorrhage and cystatin C.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

MATERIALS

1. Standard antigen

We used cystatin C from patients with chronic renal failure. The protein was refined at the Otsuka Pharmaceutical company.

2. Antibodies

We used mouse anti-cystatin C monoclonal antibody and rabbit anti-cystatin C polyclonal antibody both of which were provided by Dr. Grubb, University of Lund, Malmö, Sweden.

3. Patients

(1) Cerebral hemorrhage group: Nine patients (multiple cerebral hemorrhage,1; lobular-type hemorrhage,2; thalamic hemorrhage,3; putaminal hemorrhage,2; and corona radiate hemorrhage,1).

(2) Control group: 50 patients with other than cerebral hemorrhage.

We added preservative solution containing benzamidium to CSF specimens from all of the patients and froze the fluid immediately.

METHODS

ELISA:We employed double antibody sandwich assay as follows:

(1) Coating of mouse anti-cystatin C monoclonal antibody.
(2) Blocking of immuno plate with 0.5% egg albumin.
(3) Addition of various levels of cystatin C (0 - 10μg) or the test CSF.
(4) Addition of rabbit anti-cystatin C polyclonal antibody.
(5) Addition of goat peroxidase-labeled anti-rabbit IgG antibody.
(6) Addition of ABTS solution. After color development had occurred we determined the absorbance (452nm) by microplate spectrometry. Based on the standard curve, we assayed CSF cystatin C levels.

CONTROL STUDIES

1. We examined the cross reaction of anti-cystatin C antibody with ALκ, AA amyloid protein, or trypsin inhibitor by the same manner as for cystatin C.

2. To investigate the effect of the part of CSF on this assay system, we carried out a recovery test of cystatin C by adding 50μl of each concentration of cystatin C (0 - 1000ng/ml) to a fixed amount (50μl) of control CSF.

3. To confirm the enzyme activity of cystatin C used as the standard antigen, we determined the cystatin C activity in an inhibition activation test of papain, one of the cysteine proteinases. In brief, we added papain solution, cystatin C (0-1000ng), and buffer (phosphate buffer with cysteine) and then preincubated the solution at 40°C for 5min. Then the substrate solution (BANA) was added and the mixture incubated at 40°C for 30min. Finally we added the color reagent (Fast Garnet GBC) and determined the absorbance by spectrophotometry.

RESULTS

As Fig. 1 illustrates, the relation between absorbance and cystatin C was linear over the concentration range of 10 ng/ml to 10μg/ml. In this assay system, anti-cystatin C antibody did not show any cross reaction with ALκ, AA or the trypsin inhibitor, thus demonstrating the specificity of the antibody for cystatin C. When cystatin C was added to CSF for the recovery test, the recovery rate was nearly 100%. In the enzyme activity test, the papain inhibition curve was linear, allowing between 100 ng and 750 ng of cystatin C. It allowed us to confirm the inhibitory activity of the cystatin C used as the standard antigen for ELISA.

The concentrations of cystatin C in the test CSF are shown in Fig. 2. In the cerebral hemorrhage group, four patients fell in the low-level group, below 50 ng/ml, and five in the high-level group. The former patients had subcortical hemorrhage or multiple relapsing cerebral hemorrhage without hypertension. We highly suspected that these patients had CAA. Grubb reported that cystatin C concentrations in CSF of patients with HCHWA were lower than those of normal subjects. [3] Maruyama reported that cystatin C immunoreactive substance is important for pathogenesis of CAA with cerebral hemorrhage.[5] In this respect, suspecting CAA with cerebral hemorrhage associated with cystatin C, we clinically examined the four cases in the low-level group.

Case 1 was a 63-year-old male patient who had left subcortical hemorrhage without hypertension. We highly suspected CAA. Case 2 was an 81-year-old female patient who had large cerebral hemorrhage in the left frontal lobe. Since, this was also a lobular-type cerebral hemorrhage patient without any previous history of hypertension, we highly suspected CAA. Case 3 was a 75-year-old female patient who had a total of four cerebral hemorrhages. MRI revealed a large hematoma in the right putamen by IR and a high-intensity area in the right cerebral hemisphere and multiple high intensity areas in the left hemisphere by long SE. There is a high probability of CAA manifested in a case of relapsing multiple cerebral hemorrhage without hypertension. Case 4 was an 84-year-old female patient who experienced hemorrhage of the right thalamus without any previous history of hypertension.

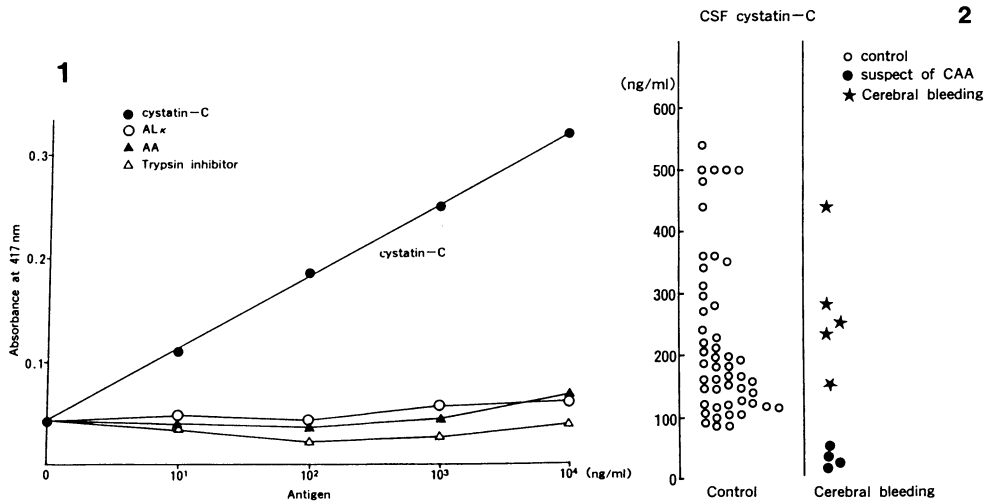

Fig. 1. Standard curve for cystatin C, showing linear line over the concentration range of 10 ng/ml to 10μg/ml. In this assay system, anti-cystatin C antibodies did not show any cross reaction with ALκ, AA or trypsin inhibitor.
Fig. 2. Cystatin C levels in the test CSF. In the control group, most values came between around 100 ng/ml and 550 ng/ml. In the cerebral hemorrhage group, four patients of nine fell in the low-level group, below 50 ng/ml.

DISCUSSION

Ghiso reported[2] that the amyloid of HCHWA was a variant of cystatin C, and Grubb suggested[3] that this amyloid was useful for the diagnosis of HCHWA because of the lower level of cystatin C in CSF of HCHWA patients

than in that of the controls. Löfberg formulated[6] the following hypothesis for the mechanism of action of cystatin C: Cysteine proteinases are released from the wall of small cerebral vessels of HCHWA patients. Consequently, cystatin C, one of the cysteine proteinase inhibitors, is consumed, thus accounting for the drop in the CSF level of the inhibitor. The inhibitor deposited in the form of amyloid is then having the structure of a cystatin C variant.

In the present study, we established an assay system for cystatin C in CSF based on ELISA. We confirmed the specificity of the antibody for cystatin C and the lack of interference by other substances in the CSF. also we confirmed the inhibitory activity of cystatin C, the standard antigen. In addition ,ELISA employed in this study is simpler and easier than RIA despite the reports by Löfberg et al. Using ELISA, we determined the CSF cystatin C in nine cases with cerebral hemorrhage and in 50 cases as the control. As a result, the four cerebral hemorrhage cases showed low CSF cystatin C levels. They included one case of multiple cerebral hemorrhage, two of lobular-type cerebral hemorrhage, and one of thalamic hemorrhage without hypertension. In consideration of the clinical findings, we strongly suspected CAA associated with cerebral hemorrhage and cystatin C. This assay for the cystatin C level in CSF can possibly be useful in the diagnosis of CAA involving cystatin C. Today, in the actual situation of CAA, no decisive diagnosis can be made during the patient's lifetime aside from that based on risky brain biopsy, and one must depend on post-mortem histological diagnosis. The present study findings offer the possibility that the CSF cystatin C level as assay by ELISA, may prove to be effective tool for diagnosis of CAA.

ACKNOWLEDGMENT

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CYSTATIN C (γ-TRACE) AND β-PROTEIN COEXIST IN THE CEREBRAL AMYLOID ANGIOPATHY CAUSING CEREBRAL HEMORRHAGE AND CONSEQUENTIAL VASCULAR DEMENTIA

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INTRODUCTION

Cerebral amyloid angiopathy (CAA) is one of the important pathological changes of Alzheimer's disease.[1] CAA also causes unique cerebral hemorrhages. In typical cases, recurrent, multiple and lobar hemorrhages occur in normotensive elderly people and cause consequential dementia of the vascular type.[2] As for the protein nature of the amyloid of CAA, there are at least two types of amyloid. One is β-protein (BP) and the other is variant cystatin C (CC). The former is found in CAA and in senile plaques of Alzheimer's Disease(AD).[3,4] CAA amyloid of normal elderly is also BP.[5] On the other hand, variant CC is the main component of CAA amyloid of hereditary cerebral hemorrhage with amyloidosis (HCHWA) found in Iceland.[6] Recently, we reported Japanese cases of CAA causing cerebral hemorrhage, of which amyloid shows the antigenicity of CC.[7,8] Two of them had a family history of possible CAA and the others were sporadic.[8] To characterize the Japanese cases of CAA with the deposition of CC and to compare them with Icelandic cases, we used the immunoperoxidase method in combination with anti-BP, conducted biochemical analyses, and studied the DNA of the CC gene.

MATERIALS AND METHODS

Examined Cases

Clinical and autopsy records of eleven cases of CAA associated with cerebral hemorrhage were collected from several hospitals in Japan. The cases comprised five males (average age : 75.5 years old) and six females (average age : 74.0 years old). We examined conventionally formalin-fixed and paraffin-embedded tissues.

Immunoperoxidase Method

We applied enzyme immunohistochemistry (avidin: biotinylated enzyme method) for the detection of CC.[7,8,9] Antisera against CC obtained

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Table 1. Results of immunohistochemistry

CASES						IMMUNOHISTOCHEMISTRY	
NO.	AGE	SEX	DEMENT	H.T.	F.H.	cystatin C	β-protein
1	70	F	VD	-	+	+	+
2	62	F	VD	-	+?	+	+
3	68	M	VD	-		+	+
4	78	M	+	-		+	+
5	73	F	+	-		+	+
6	73	M	VD	+		+	+
7	80	F	VD	-		+	+
8	77	M				+	+
9	81	M	+	+		-	+
10	82	F	+	+		-	+
11	77	F	VD	+		-	+
HCHWA			VD	-		+	-
G.A.	70	M	+	-		-	+
A.D.	55	F	AD			-	+

H.T.:hypertension, F.H.:family history
DEMENTIA (VD:vascular dementia, AD:Alzheimer type)
HCHWA :hereditary cerebral hemorrhage with amyloidosis of Iceland
G.A.:granulomatous angitis of central nervous system
A.D.:Alzheimer's disease

courtesy of Dr. Grubb[10] was used, as well as monoclonal antibody raised against synthetic peptide consisting of residues 8 to 17 of amyloid BP of AD, which was donated by Dr. Glenner.[11,12] Control amyloid tissues were those of HCHWA in Iceland[13], CAA associated with AD, and CAA associated with granulomatous angitis.[14]

Biochemical Analysis

Crude amyloid fibrils were extracted from the meninx of the frozen brain tissue of the patient reported previously[7] by a slight modification of Glenner's method.[3] Antigenicity of the fibrils was examined by Western blot.[15]

DNA Study

We searched for DNA of variant CC by the Southern blot hybridization method. DNA was extracted from frozen brain tissue of case 1 by SDS-proteinase digestion followed by phenol/chloroform extraction. The DNA was digested with *Alu* I and hybridized with the full-length CC cDNA probe after blotting.[16]

RESULTS

Immunoperoxidase Method

Results are summarized and shown in Table 1.

Each antisera reacted specifically with the correspondent control amyloid tissues. In eight of the eleven cases of CAA, amyloid showed a positive reaction with anti-CC. And in all eleven cases amyloid reacted positively with anti-BP, which antibody did not react with the amyloid of

HCHWA. Not all the BP-positive areas showed a positive reaction for CC.

In the CC-positive cases, the amyloid cores of the senile plaques (SP) also showed a positive reaction to anti-CC as well as to anti-BP. CAA amyloid and that of the SP of AD reacted with anti-BP but negatively with anti-CC.

Although two of the eight CC-positive cases had a family history of possible CAA, the remaining positive six were sporadic cases. Ten of the eleven CAA patients had a history of dementia of cerebrovascular origin. Seven of the CC-positive cases did not have a history of hypertension, whereas the three negative cases were hypertensive.

Biochemical Analysis

By immunoblotting, crude extracts of cerebrovascular amyloid from the patient of CAA with cerebral hemorrhage revealed a band with the antigenicity of CC at the migration position corresponding to a molecular weight of about 12,500 daltons.

DNA Study

DNA extracted from our patient (No.1) showed only the 600-bp *Alu* I fragment, while DNA of the HCHWA patients have two *Alu* I fragments, one 630 bp and one 600 bp (Fig. 1).

DISCUSSION

The results suggest the presence of not only familial but also sporadic cases of CAA with the deposition of CC associated with cerebral hemorrhage causing vascular dementia in the normotensive elderly.

CAA of the aged without demonstrable Alzheimer-type dementia is related to BP.[5] The average age of our cases was 75 years old. By immunohistochemistry not all parts of the BP-positive amyloid showed the antigenicity of CC, and not all the BP-positive cases were positive for CC. Thus, age-related BP may underlie or precede the deposition of CC which is related to CAA associated with cerebral hemorrhage.[17] Patients with HCHWA in Iceland may develop CAA with BP if they survive till old age.

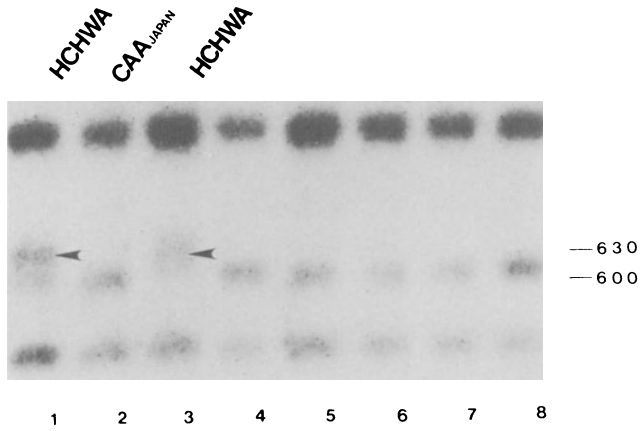

Fig. 1. Southern blot of cystatin C cDNA from patient No.1 (lane 2), patients with HCHWA (lane 1,3), and from normal controls(4-8). Our patient had 600-bp *Alu* I fragment only, while HCHWA patients also had 630-bp fragment (arrowhead), representing a mutation.

CAA and SP of HCHWA from the Netherlands are related to BP, and CAA of them showed only dubious staining for CC immunohistochemically.[18] In contrast, CAA of our cases showed clear antigenicity of CC as well as of BP. Immunoblotting showed that crude amyloid fibrils from one of our cases contain CC antigens. We speculate that abnormal metabolism of CC as a cysteine proteinase inhibitor may play some role in the cause of cerebral hemorrhage in CAA patients.[17]

A cDNA probe was used to identify a restriction fragment length polymorphism (RFLP) which detects a loss of an *Alu* I restriction site because of a mutation in the codon for the position 68 in the CC molecule.[16] This gene analysis did not show the same variant of CC gene of Japanese case as found in the Icelandic HCHWA cases. Further biochemical analysis of the amyloid fibril protein is necessary to clarify if the Japanese cases have the different variation of CC or normal CC.

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THE REGULATION OF CYTOSKELETAL ELEMENTS IN DIFFERENTIATING HUMAN NEUROBLASTOMA AND RAT PHEOCHROMOCYTOMA PC-12 CELLS

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INTRODUCTION

The appearance of neurofibrillary tangles (NFT) is one of the major structural changes that occur in neurons during Alzheimer's disease. They are composed almost entirely of paired helical filaments (PHF), intermixed with some straight filaments. Immunocytochemical studies of NFT have revealed that they have antigenic determinants in common with noncytoskeletal elements, neurofilaments (Perry et al., 1984) and microtubule associated tau proteins (Brion et al., 1984; Wood et al., 1986; Kosik et al., 1986, 1988a,b; Nukina and Ihara, 1986; Goedert et al., 1988), while the presence of microtubule-associated protein 2 (MAP2) in NFT has yet to be established (Rosemblatt et al., 1989). Both MAP2 and tau proteins have been shown to bind to peptides derived from the carboxyl-terminal region of β-tubulin. In addition, tau proteins but not MAP2 display a strong interaction with a peptide derived from the amino-terminal domain of α-tubulin (Littauer et al., 1986). Recently, it was found that tau proteins contain three 18 amino acid repeated sequences which appear to be involved in the binding to tubulin (Lee et al., 1988; Goedert et al., 1988, 1989; Lee et al., 1989; Kosik, 1989). It was also observed that MAP2 shares the tau repeated regions (Lewis et al., 1988). However, this is not the case for a recently cloned tubulin binding protein designated neuraxin, which does not contain the tau and MAP2 repeated sequences. Neuraxin was found to be immunologically related to MAP5 and is perhaps identical to this high molecular weight MAP (Rienitz et al., 1989).

On the cellular level, MAP2 and tau proteins were found to separate into dendrites and axons, respectively (cf. Matus, 1988). In Alzheimer's disease, this topographical segregation is violated. Tau-immunoreactive NFT occur generally in the pyramidal cell soma and the apical dendrite, regions which are normally devoid of tau proteins but are rich in MAP2 (Kosik et al., 1986; Wood et al., 1986; Kowall and Kosik, 1987). In this report, we show that the induction of differentiation in human neuroblastoma and rat pheochromocytoma PC-12 cells can serve as a model system to study neuronal maturation and examine the expression and reorganization of the relevant cytoskeletal elements.

RESULTS

Effect of Various Components of the Extracellular Matrix on the Differentiation of Human Neuroblastoma Cells

The adrenergic human neuroblastoma cell line LA-N1 (Seeger et al., 1979) can be induced to differentiate to some extent by the addition of retinoic acid, Bt_2cAMP or NGF to the

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culture medium. Various proteins of the extracellular matrix such as laminin, fibronectin or mixed extracellular matrix will contribute significantly to this process. The induction of differentiation in human neuroblastoma cells requires priming with a humoral factor such as NGF for 5 to 10 days prior to the stimulation of cellular receptors for ECM components. Primed LA-N1 cells extended long neurites on laminin (Kirsch et al., 1988; Ginzburg et al., 1989), fibronectin and mixed extracellular matrix, whereas collagens I and IV did not exert a synergistic effect on neurite outgrowth (Fig. 1). The time course of neurite extension varied among the various components with laminin and fibronectin acting faster than the mixed ECM, where neurite extension started only 2 to 3 days after replating. In the presence of laminin and fibronectin substrates, the extension of neurites was triggered within 12 hours after replating and proceeded for approximately 3 days.

If the priming phase is omitted and the cells are directly grown on any of the neurite stimulating substrates, only small extensions were observed which retracted after 48 hours, even in the presence of an inducing agent. Thus, even in the presence of both ECM components and the inducing agent, the neurites are unstable and eventually retract, unless the cells were subjected to a previous priming period.

LA-N1 cells primed with NGF displayed a different morphology than the Bt_2cAMP or retinoic acid primed cells. The NGF induced cells extend only one or two neurites which are mostly straight and unbranched, whereas Bt_2cAMP induced multiple curved and branched neurites with varicosities along the axis. It was also noted that the various cell extensions featured growth cone-like structures at their distal end.

Organization of Cytoskeletal Elements in Differentiating Human Neuroblastoma Cells

The expression and organization of cytoskeletal elements, and the growth associated protein 43 (GAP43) in differentiating human LA-N1 cells were investigated by immunocytochemical methods and Western blot analysis. The results are summarized in Table 1. GAP43, which is distributed uniformly throughout the cell body in undifferentiated cells, is down-regulated upon induction of differentiation. On the other hand, the high and low molecular weight neurofilament subunits are induced in NGF primed cells. There is a significant change in the organization of the neurofilament related protein peripherin and vimentin. These cytoskeletal elements are arranged as perinuclear whorls in uninduced cells, and are redistributed to the growth cone-like structures upon induced differentiation.

The microtubules (MTs) and their associated proteins are also subject to redistribution in differentiating neuroblastoma cells. In undifferentiated cells, the MT cytoskeleton consists mainly of individual filaments. Upon induction of differentiation, MT bundles are formed which span the entire length of the neurites. On the other hand, MAP2 and tau proteins are sparse, distributed diffusely, and their expression is not significantly increased by NGF, Bt_2cAMP, or retinoic acid. However, upon induced differentiation, there is a change in their distribution. In these cells, MAP2 and tau are also found in the proximal part of the neurites. In addition, MAP2 was also observed in neurite branching points. The distribution of MAP5 also changed during the differentiation period. In the undifferentiated state, its distribution was similar to that of MAP2 and tau. However, unlike these MAPs, in differentiating cells MAP5 was found throughout the entire length of the neurites.

The molecular events involving MT genes that occur during neurite outgrowth, were studied in PC-12 cells. Exposure to NGF initiates a cascade of events, one of which is neurite outgrowth. The formation of the processes involves initially an increase in tubulin and MAP mRNA levels and de novo protein synthesis. We have shown that NGF and ECM components can act synergistically in PC-12 cells yielding vigorous neurite outgrowth (Ginzburg et al., 1989).

The involvement of individual tubulin isoforms in the initial stage of neurite outgrowth was studied employing antisense oligodeoxynucleotides (Teichman-Weinberg et al., 1988). Inhibition of neurite outgrowth was observed using α-tubulin specific reagents which allowed

Table 1. Expression and Organization of Cytoskeletal elements in Human Neuroblastoma Cells

Cytomatrix	Undifferentiated Cells	Induced Neurites
Microtubules		
Distribution	Single Filaments	Dense Bundles
MAP5 (MAP1B)	Diffuse Cytoplasmic	Throughout
MAP2 (70kDa,250kDa,270kDa)	Diffuse Cytoplasmic	Proximal Part
Tau	Sparse Punctuated	Sparse, Proximal Part
Intermediate Filaments		
Neurofilaments	Sparse	68kDa, 200kDA, NGF Induced
Vimentin	Perinuclear Whorl	Only Growth Cone (Heavy)
Peripherin	Perinuclear Whorl	Only Growth Cone (Heavy)
GAP43		
Distribution	Diffuse Cytoplasmic	Down-regulated

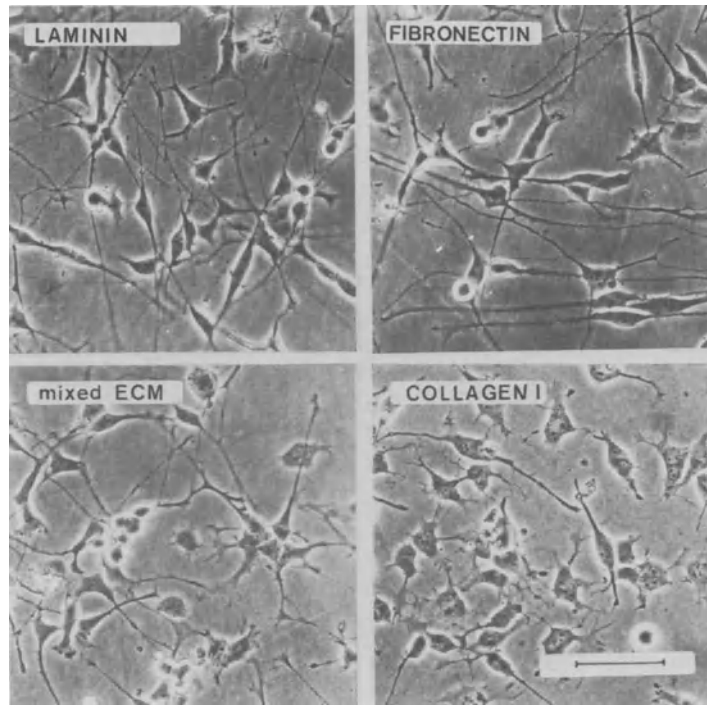

Fig. 1. The effect of various ECM components on the differentiation of human neuroblastoma cells. LA-N1 cells were primed for 10 days in the presence of 100 ng/ml of NGF in RPMI medium containing 10% fetal calf serum. The cells were then replated on the various ECM components for 3 days and then viewed with a phase contrast microscope. Bar represents 100 μm.

us to conclude that both isoforms are a prerequisite for the differentiation to proceed. This approach enables us to further study in vivo the involvement and function of endogenous genes, i.e., MT genes, during the process of MT reorganization and differentiation.

DISCUSSION

The expression and organization of cytoskeletal elements is controlled by a variety of factors. Humoral factors such as NGF and components of the extracellular matrix, influence this complex process synergistically, as we could demonstrate for human neuroblastoma cells.

It has been shown (Greene et al., 1982) that neurite outgrowth in rat pheochromocytoma PC-12 cells can be divided into two distinct phases. In this model, the NGF dependent priming phase leads to the synthesis of specific mRNA species. On the other hand, the actual event of neurite extension does not require RNA transcription and will proceed with little or no lag period, provided the PC-12 cells are plated on a suitable substrate. Unlike the PC-12 cells, little or no neurite outgrowth was observed when human neuroblastoma cells were stimulated simultaneously with NGF or other inducing agents and various components of the ECM. In contrast, the priming phase had to proceed the triggering of neurite outgrowth, probably involving the stimulation of cellular receptors for the ECM components. LA-N1 cells, in particular, responded well to the stimulation with laminin or fibronectin, but were neutral on collagen I and IV substrates. Likewise, treatment with either inducing agent or ECM alone resulted in small and unstable neurites. It is also interesting to note that cell shape and neurite fine morphology were found to depend on the inducing agent, but not on the ECM component.

These observations suggest that neurite extension can be divided into two distinct phases. The first phase involves the priming of the cells with humoral factors such as NGF, and may include the synthesis or modifications of proteins which are involved in neurite extension and their stabilization. In the second phase, the actual neurite outgrowth is triggered by the stimulation of ECM receptors which are likely to be linked to a signaling cascade. It can, therefore, proceed with little or no lag period. The separation between the two phases is very pronounced in human neuroblastoma cells, whereas NGF and ECM in PC-12 cells appear to act simultaneously.

Cytoskeletal elements, namely neurofilaments, and the MT associated tau proteins have been implicated in the generation of NFT in Alzheimer's disease. It was therefore of interest to investigate, whether the expression of these proteins and their cellular organization is linked during neuronal differentiation processes. Differentiating human neuroblastoma cells express a variety of cytoskeletal elements and appear to be a suitable model for this approach. The dramatic changes in cell morphology during differentiation corresponded with the reorganization of the MT cytoskeleton. In particular, the formation of thick MT bundles was observed spanning the entire length of the neurite and a redistribution of MAP2 and tau proteins. These polypeptides, although sparse, were found in the proximal part of the neurites, where they may be involved in MT stabilization and cross-linking to other cytoskeletal elements. The distal part of the extensions was devoid of these MAPs. By contrast, MAP5 was uniformly distributed along the entire neurite. In view of its close similarity (or identity) with neuraxin (Rienitz et al., 1989), MAP5 may link the microtubules to membrane associated proteins. Interestingly, immunostaining for these MAPs is diffuse, which may indicate that not all of them are associated with MTs.

Vimentin and the neurofilament related peripherin displayed a perinuclear whorl in undifferentiated cells. Upon NGF induction, the filamentous system was reorganized. The filaments in the perinuclear space could not be observed; instead, the distal part of the newly formed neurites and the growth cone-like structures were heavily decorated by the respective antibodies. Thus, the results would indicate that these cytoskeletal elements may play a significant functional role in neurite extension.

As in the case of the Alzheimer's diseased cortex, there was no segregation between MAP2 and tau proteins into different cell compartments. Both proteins were observed in the proximal part of the cell extensions. Other cytoskeletal elements showed a different distribution. Thus, the distal part of the neurites contained mainly intermediate type filaments, while microtubules containing MAP5 were distributed along the entire length of the neurites. It would be of interest, therefore, to follow closely the expression and distribution of all these cytoskeletal elements in the NFT during the pathogenesis of Alzheimer's disease.

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SOME FINDINGS ON INTERMEDIATE FILAMENTS IN ALZHEIMER'S DISEASE

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INTRODUCTION

The aberration of cytoskeletal systems is speculated in the pathogenesis of Alzheimer's disease (Selkoe,1989). In this present report, we communicate, 1) the presence of higher titers of IgG antibody against glial fibrillary acidic protein (GFAP) in Alzheimer sera than in control sera and further characterization of the lympocytes from Alzheimer patients, 2) aberration of vimentin fiber arrangement in Alzheimer fibroblasts, and 3) characterization of neurofilament fibers observed in aluminum encephalopathy in rabbit brain. From these independent findings about intermediate filaments in the central nervous system, we also the author will discuss the possible involvement of intermediate filament aberration in Alzheimer pathogenesis.

METHODS

Assay of Anti-GFAP Antibody. Sera of patients with early-onset Alzheimer's disease (n=13),late-onset Alzheimer's disease (n=26), cerebrovascular dementia (n=39), and those of healthy control subjects (n=219) were assayed for anti-GFAP IgG by ELISA (Tanaka,1988). For in vitro experiments, lymphocytes from Alzheimer patients were obtained by differential centrifugation in Ficoll-Paque. The Alzheimer lymphocytes were incubated for 7 days in 20% FCS-containing RPMI-1640 medium containing 10 ng/ml pokeweed mitogen, and the anti-GFAP IgG in the medium was assayed by ELISA. For the transformation experiment with Epstein-Barr(EB) virus, lymphocytes were incubated with cyclosporin A (2 ug/ml) and the filtrate of the medium conditioned by EB-infected B95-8 cells (Kingsley, 1988).The anti-GFAP IgG and anti-neurofilament 200K protein (NF200P) IgG were assayed in the culture medium 14 days after the transformation.

Alzheimer Fibroblast Culture. Cutaneous tissue from 2 familial Alzheimer patients (males, 54 and 69 years old) were obtained by biopsy and cultured in Dulbecco's minimum essential medium with 10% FCS at 37 C. Cells at 8-12 passage cells were grown on polylysine-coated cover glasses, fixed with 4% paraformaldehyde, and immunostained with the first antibody and FITC-labeled second antibody.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Production of Experimental Neurofibrillary Change. Adult rabbits were injected with 0.25ml of Holt's adjuvant solution intracerebrally (Klatzo,1965). Ten days after the injection, the animals were decapitated and the brain tissue was fixed in 10% formaldehyde. Tissue sections were prepared and silver-stained with Bodian's and immunostained with anti-NF200P or anti-neurofilament 160K protein(NF160P).

RESULTS

Anti-GFAP IgG Production of Alzheimer Lymphocyte. The anti-GFAP IgG titer was assayed in sera obtained from patients with early- and late-onset Alzheimer's disease, cerebrovascular dementia, as well as in samples from a large number of healthy subjects. The mean O.D. value of anti-GFAP IgG by ELISA was 0.909+0.363 for early-onset Alzheimer's disease, 0.816+0.332 for late-onset Alzheimer's disease, 0.559+0.302 for cerebrovascular dementia, and 0.533+0.260 for the healthy control. The Alzheimer serum showed the significantly higher titer of anti-GFAP IgG (Fig.1). When the O.D. value of the mean plus two standard deviations of the control was tentatively taken as the value demarcating positive cases, the ratio of the positive cases was 53.8% with early-onset Alzheimer's disease, 30.8% with late-onset Alzheimer's disease, 10.8% with cerebrovascular dementia, and 5.5% with the control subjects (Tab. 1).

To characterize the lymphocytes in Alzheimer blood, the lymphocytes were fractionated and studied in vitro. Alzheimer lymphocytes were chemically stimulated with pokeweed mitogen, and the production of anti-GFAP IgG was assayed by ELISA. The result showed the higher production of anti-GFAP IgG with Alzheimer lymphocytes than with control cells (Fig.2). Alzheimer lymphocytes were also transformed by EB virus, and the production of anti-GFAP IgG and anti-NF200P IgG was assayed on the 14th day after the transformation. The results showed that Alzheimer lymphocytes produce more anti-GFAP IgG than anti-NF200P IgG, and more of both of these than the control lymphocytes (Table 1).

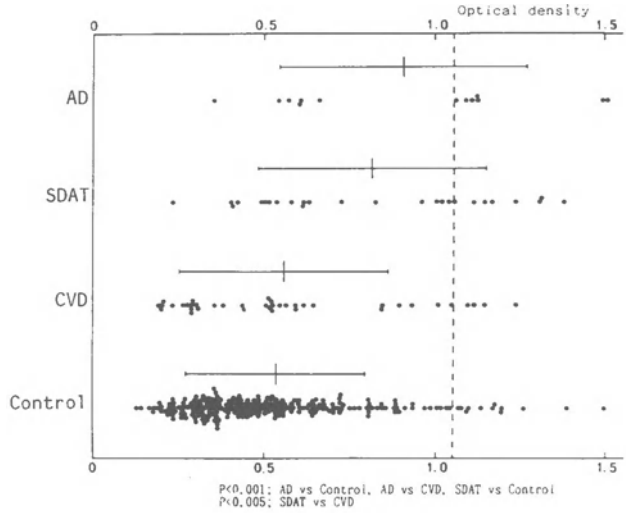

Fig.1. Anti-GFAP IgG antibody titer by ELISA in sera from early-onset Alzheimer(AD), late-onset Alzheimer(SDAT), cerebrovascular dementia(CVD), and healthy control (Control) subjects.

Table 1 The frequency of wells producing IgG against GFAP and NF200P after EB virus transformation

	GFAP	NF200P
ALZHEIMER	95%	82%
CONTROL	48%	35%

Aberration of Vimentin Array in Alzheimer Fibroblasts

Alzheimer fibroblasts were incubated in serum-free medium for 10 days, and the distribution of cytoskeletal proteins was then observed. The cells were fluroescently-stained with antibodies against cytoskeletal proteins, such as vimentin, actin, and phodrin. When the fibroblasts were stained with anti-actin antibody, the Alzheimer (Fig.3A) and control fibroblasts showed similar distribution of microfilaments. Likewise anti-phodrin antibody staining showed the same distibution pattern of phodrin between the Alzheimer (Fig.3B) and the control cells. As shown in Figure 3C, the control fibroblasts showed an even smooth distribution of vimentin fibers. The Alzheimer cells, however, showed a unique derrangement of the fibers (Fig.3D). To study the protein change in the Alzheimer fibroblast, we fractioned the crude cytoskeletal protein and immunostained with anti-vimentin and anti-phodrin. The crude cytoskeletal protein fraction from the Alzheimer cells showed no difference from that of the control cells (Fig.4A). The immunostaining revealed the same quantity and size of vimentin molecule from Alzheimer and control cellsb but the phodrin from the Alzheimer cells showed a higher molecular weight than that of the control cells.

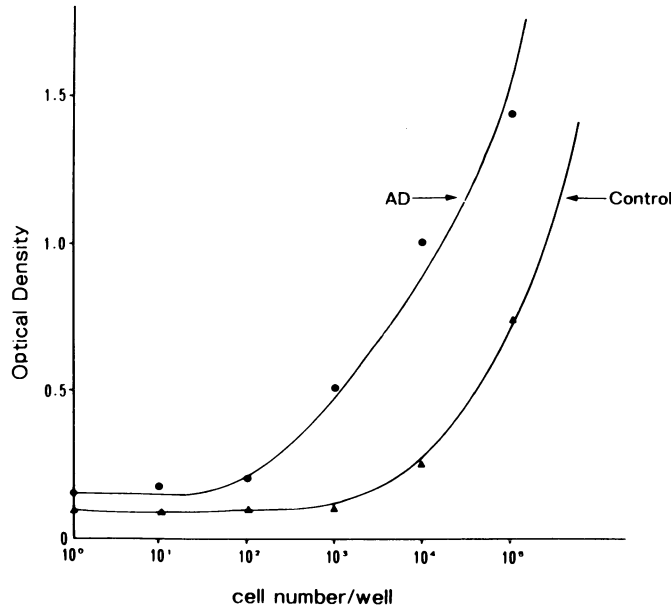

Fig.2 Anti-GFAP IgG production by lymphocytes after pokeweed mitogen stimulation.

Characterization of Experimental Neurofibrillary Change. The aluminium-injected rabbit brain showed the proliferation of 10-nm neurofilament-like fibers in neurons of the cervical spinal cord and pons. The crude cytoskeletal fraction purified from aluminium-injected rabbits showed increased intensity of 68 K, 160 K, and 200 K protein bands, which bands correspond to subunits of neurofilaments. The tissue obtained from the aluminum-injected rabbit brain was stained with Bodian's to show the production of experimental neurofibrillary changes (Fig.4A). Tissue sections were also immunostained with antibodies against 160 K and 200 K neurofilament subunit proteins. The immunostaining with anti-NF200P as well as with anti-NF160P was positive in the neurons containing the experimental neurofibrillary changes (Fig.4B,C).

DISCUSSION

The IgG antibody against GFAP was elevated in the serum of Alzheimer's disease patients. Since the anti-GFAP IgG titer of the healthy control subjects was unchanged by age and sex, this antibody could be a useful biological marker of Alzheimer's disease. Further, the anti-GFAP IgG titer could be used for differential diagnosis of Alzheimer and cerebrovascular type of dementia because the serum from cerebrovascular dementia patients did not show any increase in anti-GFAP IgG. The in vitro characterization of Alzheimer lymphocytes indicates the possibility that GFAP antigenicity is stronger than that of neurofilament subunit protein in Alzheimer's disease. The finding that fibroblasts obtained from familial Alzheimer's disease patients showed an aberrant distribution of vimentin fibers may correspond to the observed decreased attaching ability of Alzheimer fibroblasts (data not shown) and/or change in the phodrin molecules. Regarding the experimental neurofibrillary changes produced in rabbit brain by aluminium intoxication, the accumulation of neurofilament filaments in degenerating neurons was quite evident.

The experimental findings reported above are indicative of changes in glial, vimentin, and neurofilaments in the pathogenetic process of Alzheimer's disease. All of these filaments belong to the class of intermediate filaments in the central nervous system. Ubiquitin is a protein composing paired helical filaments (PHF) (Mori,1987) and senile plaque neurites (Perry,1987) of Alzheimer's disease. Recently ubiquitin was reported to be incorporated into intermediate filament inclusion bodies of diverse pathological conditions, such as Lewy bodies of Parkinson's disease, Pick bodies of Pick's disease, Mallory bodies of alcoholic liver disease, cytoplasmic bodies of a specific myopathy, and Rosenthal fibers in astrocytes (Lowe,1989). These findings support the hypothesis that there is a common pathological process mediated by ubiquitin conjugation, which results in the formation of intracellular accumulation of intermediate filaments. We wish to stress the importance of the dysfunction of the mechanisms governing the function and the distribution of intermediate filaments in the pathogenesis of Alzheimer's disease.

ACKNOWLEDGEMENTS

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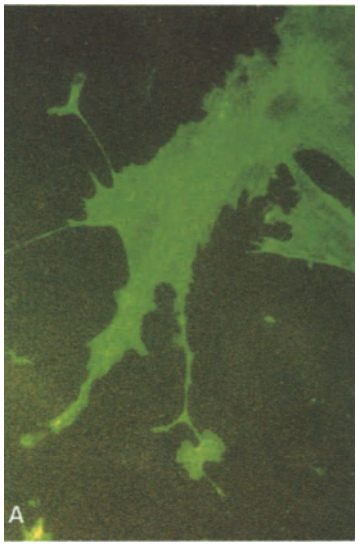


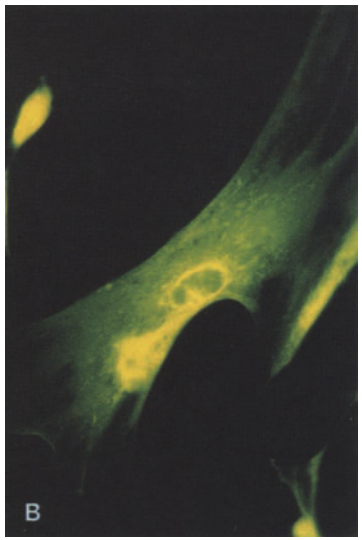
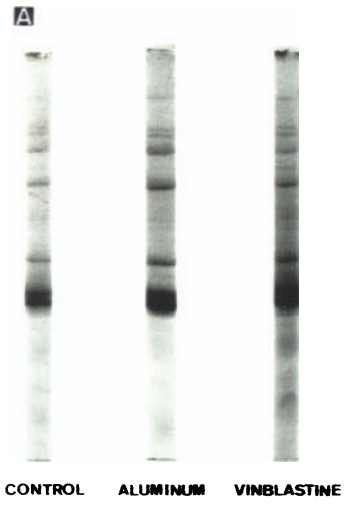
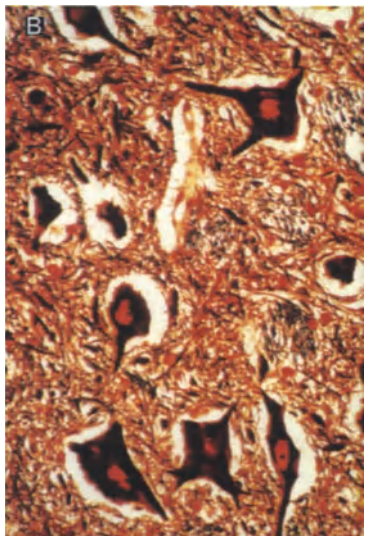
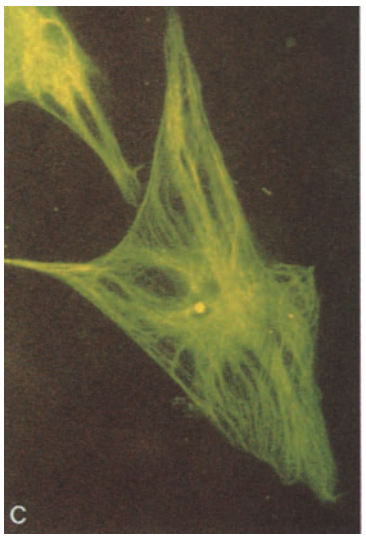


Fig. 3
Immunostaining of cytoskeletal proteins of Alzheimer (A,B,D) and control (C) fibroblasts. A: anti-actin, B: anti-phodrin, C,D: anti-vimentin.







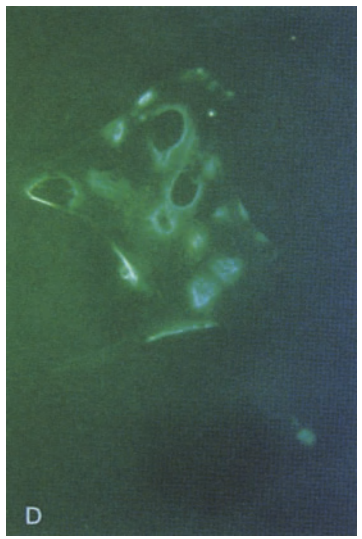
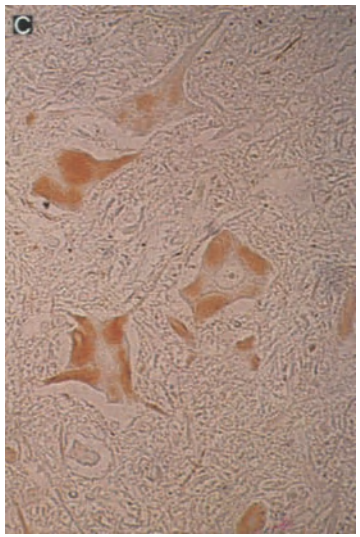
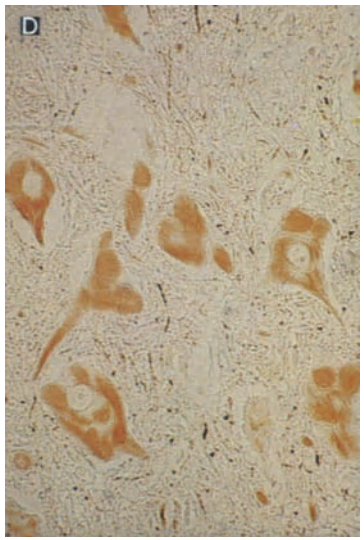


Fig. 4
Experimental neurofibrillary changes in rabbit brain. A: Electrophoretic pattern of crude cytoskeletal fraction, B: Bodian staining, C: immunostaining with anti-NF200P, D: immunostaining with anti-NF160P.





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A UNIFYING HYPOTHESIS ON THE ETIOLOGY OF ALZHEIMER'S DISEASE

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INTRODUCTION

In recent years, it has become increasingly evident that the fundamental scientific issues concerning the etiology of Alzheimer's disease (AD) should focus on the search for mechanisms of cell dysfunction and selective neuronal loss. It is now well established that the structural and functional changes in neurons occur in specific regions of the AD brain.

However, to date we still do not know the precise mechanisms of cell death in AD or why specific types of cells in particular brain regions are vulnerable. We do not know what the relationships are, if any, between the presence of abnormal proteins and neuronal function. The rapid pace of research, during the last few years, has begun to provide promising leads concerning several possible mechanisms of selective neuronal loss in AD. Some of the leading candidates for causes of cell death include: the presence of amyloid proteins, infectious agents, toxins, lack of trophic factors, cell membrane changes, and glucose metabolism.

The presence of large numbers of neuritic plaques and tangle-bearing neurons is the hallmark of AD. Recently, considerable progress has been made in understanding the protein chemistry of these neuropathologic lesions in AD. The location of the gene responsible for one of the amyloid precursor proteins has been identified, and the chemistry of paired helical filaments (PHF) is better understood now then a few years ago. However, it is still not clear whether the amyloid proteins of the plaques have any direct role to play in the etiology of AD. The presence of these abnormal proteins is not unique to AD. It is not clear whether the formation and accumulation of these abnormal proteins initiate the neuronal degenerative process, or whether they are the end-product of a disease process caused by some other agent. It is very likely that they may be coincidental to the disease process. We still do not know how these proteins affect cellular function. At present there is no clear or direct evidence that these abnormal proteins play a role in cell death. If they do, we need to understand the mechanisms by which this occurs.

At present, no single known etiologic factor under study can

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fully account for the clinical picture of AD and the post mortem pathological markers of the disease. We propose that, as a working hypothesis, the role of any single etiologic factor needs to be examined in the broader context of other ideas concerning the etiology of this disorder, e.g.: genetics, infective agent, metabolic disorder, blood-brain barrier changes, neurochemical deficits exposure to toxins, and the presence of abnormal proteins. This broader view is necessary because of the strong possibility that AD may not be due to a single event or an insult but is brought about by a series of different events over a long period in the life of the patient.

To understand the etiology of AD, it might be important to examine evidence showing a relationship between a particular variable, such as toxins and AD, in the context of other preceding critical physiological events during the lifetime of the patient, which may have predisposed the vulnerability of the brain to the disease.

The purpose of this paper is to re-evaluate, in light of new evidence, the calcium hypothesis of brain aging and Alzheimer's disease as a possible mechanism for selective neuronal death. In brief, the calcium hypothesis suggested that cellular mechanisms, which maintain the homeostasis of cytosolic $[Ca^{2+}]^1$, play a key role in brain aging; and that sustained changes in $[Ca^{2+}]i$ homeostasis could provide the final common pathway for selective cell death in AD (Khachaturian, 1984).

In recent years, it has become increasingly apparent that Ca^{2+} functions as a nearly universal messenger system for extracellular signals to regulate cell function in a variety of cells (Cheung, 1979; Carvahlo, 1982; McGraw et al., 1982; Rassmussen, 1986). There is abundant evidence that this calcium-mediated-signaling system changes in the aging nervous system (Landfield et al., 1989). The critical issues for studies of brain aging concern the questions of how and what cellular changes lead to disruption of calcium-mediated-signal transduction process and disstablization of calcium homeostasis within the cytosol. The crucial challenge is to find out the mechanism by which such changes come about. At present, we do not know how aging affects the processes that regulate the intracellular concentration of Ca^{2+}. It is not clear whether Ca^{2+} concentration change themselves or are the result rather than a cause of other pathogenic effects. But, it is clear that any significant and long-lasting change in the normal functioning of Ca^{2+} transport systems, pumps, buffers, or storage systems that help maintain the homeostasis could influence the delicate balance of Ca^{2+} concentration in the cytosol with serious consequences. Many of the age-related changes in brain function, and those associated with degenerative processes of AD, ultimately have to be accounted for either on the basis of altered neuronal functioning and or cell death. The calcium ion mediated signaling system and regulation of calcium ion homeostasis appear to be the final common pathway for such cellular changes. The calcium hypothesis is intended to be an alternative candidate in the search for a unifying explanation of selective cell loss in AD. It provides a reasonable framework to link a number of discrete age-related changes observed in the nervous system and AD associated pathology of the brain.

Normally the resting intracellular free calcium ion concentration $[Ca^{2+}]_i$ within a neuron is maintained between 10^{-8}M and 10^{-7}M. Until recently the idea of studying mechanisms by which intracellular free Ca^{2+} concentration are regulated would have been an unrealistic task. But important advances in several areas, such as patch-clamp recording, availability of furo-2 and quin-2 dyes for measuring Ca^{2+}, and a better

understanding of the biochemistry of inositol triphosphate (IP_3)/diacylglycerol (DAG), have made it possible to study the cellular mechanism of Ca^{2+} regulation (Miller, 1988). It is now well established that Ca^{2+} serves as a signal for numerous neuronal functions such as: control of neurotransmitter release, neuronal membrane excitability, serves as second messenger, and as a third messenger. Ca^{2+} can lead to the induction of a series of genes which encode proteins involved in transcriptional regulation. In addition, it regulates neuronal plasticity and growth of soma, neurites, growth cones, and terminal buttons. It also regulates housekeeping neuronal metabolic activity, e.g. phosphorylation reaction, axoplasmic flow, and proteolytic activity. It is also involved in trimming of dendrites and dendritic spines, possibly other pathological conditions, and neuronal death.

The central role of Ca^{2+} in cellular signal process involves a vast array of time scales ranging from millisecond, seconds, and minutes; to hours, days, and years. The longer time periods are particularly relevant for explaining the role of free calcium ion concentration within cytosol = $[Ca^{2+}]_i$ homeostasis in AD and age-associate changes in neuronal functioning and cell death. The complex interaction between the duration of calcium $[Ca^{2+}]_i$ change (i.e., $\triangle$ T) and the relative amount of the deregulation in the concentration of Ca^{2+} (i.e.. $\triangle$ $[Ca^{2+}]_i$), is critical for age-associated changes in neuronal functioning. In this revised version of the calcium hypothesis, I propose that a very small subtle change in free Ca^{2+} concentration but sustained over a long period, i.e. years, is likely to cause the same neuronal damage as that induced by a large change in $[Ca^{2+}]_i$ over a shorter period i.e., minutes or hours. This relationship between $[Ca^{2+}]_i$ and time can be expressed as:

$$\triangle[Ca2+]_i \text{ X } \triangle \text{ T} = \text{K}$$

so that when T is **small** i.e., milliseconds, a **large** $\triangle[Ca^{2+}]_i$ has the same consequence as a **small** $\triangle[Ca^{2+}]_i$ when $\triangle$ T is **large** i.e. years.

$$\textbf{large } \triangle[Ca^{2+}]_i \text{ X } \textbf{small } \triangle \text{ T} = \textbf{small } \triangle[Ca^{2+}]_i \text{ X } \textbf{large } \triangle\text{T}$$

The hypothesis proposes that a small change of what might appear to be an insignificant increase in $[Ca^{2+}]_i$ or a sustained but small disruption in Ca^{2+} homeostatic process over the period of several years may lead to age-associated changes in cell functioning.

The regulation of cytosol free calcium ion, at extremely low concentration relative to the concentration of calcium ion outside the cell, is dependent on several complex mechanisms. Disruption or a change in the efficient operation of any of these calcium homeostasis maintaining systems could lead to the age-associated changes in the cytosol $[Ca^{2+}]_i$. There is some evidence that the systems regulating Ca^{2+} homeostasis include: Ca^{2+} channels (both voltage-sensitive and receptor-operated types), buffering by calcium binding proteins, sequestration by cytoskeletal organelle, and energy-dependent calcium extrusion pumps. Evidence also exists that the efficiency of these systems is affected by the aging processes (Khachaturian, 1989).

While it was previously known that there are several alternative mechanisms through which the regulation of cytosolic $[Ca^{2+}]^1$ can be disrupted, this paper suggests that the role of membrane changes in a cascade of events, which might lead to disruption of $[Ca^{2+}]^1$ homeostasis, is a critical interceding event in cell death and AD pathology. The mechanisms by which the assembly, structure, and dynamics of membrane constituents, including proteins, change in aging and pathological conditions are important topics for understanding

aging and cellular mechanisms of $[Ca^{2+}]^i$ regulation. In addition, studies of membrane structure, dynamics and function are essential to a better understanding of the mechanisms by which intracellular messengers mediate neuromodulation. Critical issues for studies of brain aging concern questions of how and what cellular changes may lead to destabilization of calcium homeostasis within the cytosol and/or disruption of calcium mediated signal transduction processes. A crucial challenge is to determine the mechanism(s) that produce such changes. At present, we do not know the precise details of the processes that regulate intracellular concentration in $[Ca^{2+}]^i$ pathological conditions.

Studies of membrane molecular structure and dynamics are critical to our understanding of mechanisms by which cytosol Ca^{2+} homeostasis might be altered as part of the aging process, because maintaining the delicate balance of Ca^{2+} concentration between 10^{-8}M and 10^{-7}M within the cytosol is dependent on the normal function of various channels, extrusion pumps, storage, and buffering systems. These complex systems used by a cell to maintain a low cytosol $[Ca^{2+}]_i$ require the efficient operation of highly specific Ca^{2+} binding cites on various proteins and complexes of membrane associated proteins. It is becoming clear that changes in the synthesis and turn over of the membrane phospholipids can have a profound effect on the operations of membrane bound proteins. It is possible that the Ca^{2+} disruption in homeostasis are secondary to changes in membrane structure and dynamics, and energy metabolisms.

The mechanisms by which the assembly, structure, and dynamics of membrane constituents, including proteins, change in aging and pathological conditions are important topics for understanding aging and cellular mechanisms of $[Ca^{2+}]_i$ regulation. In addition, studies of membrane structure, dynamics, and function are essential to a better understanding of the mechanisms by which intracellular messengers mediate neuromodulation, thus providing the scientific basis for developing rational treatment strategies for devastating disorders of aging such as AD.

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ABNORMAL PHOSPHOLIPID METABOLISM IN NEURODEGENERATIVE DISEASES: ELEVATIONS IN GLYCEROPHOSPHOCHOLINE AND GLYCEROPHOSPHOETHANOLAMINE LEVELS IN BRAIN OF ALZHEIMER'S DISEASE BUT NOT IN DOWN SYNDROME PATIENTS

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INTRODUCTION

Among the numerous neurotransmitter abnormalities described in brains of patients with Alzheimer's disease (AD), the decrease in the activity of choline acetyltransferase [the acetylcholine (ACh)-synthesizing enzyme] was first identified (Bowen et al., 1976) and remains the most robust. The cholinergic deficit in is strongly correlated with the cell loss (McGeer et al., 1984) and senile plaques (Perry et al., 1987) characteristic of AD, and probably contributes to the amnesia that is so prominent in this disorder. Brains of patients with Down syndrome (DS) have pathological features of AD by the fourth decade of life (Coyle et al., 1988, for a recent review) and develop cholinergic deficits similar to those in AD (Yates, 1983); Any theory that attempts to explain the vulnerability of cholinergic neurons in AD or DS should take into account their unique dual requirement for choline: all cells need choline for incorporation into phosphatidylcholine (PC), a structural component of biological membranes, but cholinergic neurons also need choline for ACh synthesis (Blusztajn and Wurtman, 1983). In disorders like AD, in which the loss of cholinergic neurons presumably causes localized deficiencies in cholinergic tone, surviving neurons may undergo a net degradation of their membrane phospholipids in order to supply sufficient choline to support augmented ACh synthesis and release (Maire and Wurtman, 1985; Ulus et al., 1989). In support of this hypothesis, we now report that concentrations of major metabolites of PC [glycerophosphocholine (GPC)] and of phosphatidylethanolamine (PE) [glycerophosphoethanolamine (GPE)] are significantly increased in AD brains.

MATERIALS AND METHODS

Source of Brain Tissue

Brain tissue was obtained from the Massachusetts Alzheimer's Disease Research Center (ADRC) Tissue Resource Center (Director, Dr. E. Tessa Hedley-Whyte). Only cases with definite and uncomplicated AD were used [criteria as proposed by Khachaturian (1985)]. Control brains were from subjects who died without clinical evidence of neurological or psychiatric illness, and whose brains were normal upon neuropathological examination. All DS brains had confirmed histopathological characteristics of AD. The ages at death and times from death to brain collection were similar in both groups (Table 1).

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Tissue from the following regions was used:

1. temporal cortex (area 20/21)
2. parietal cortex (area 40)
3. lateral cerebellar cortex
4. caudate nucleus

The temporal and parietal lobes were selected because they correspond to sites of histologic change in AD and are thought to be involved in some of the behavioral manifestations of AD. The caudate nucleus and cerebellum were selected because these regions do not exhibit overt AD-type pathology. Frozen brain tissues from each region were thawed to -20°C and dissected on a cold plate into 100-200 mg. samples.

Table 1. Characteristics of the Control and Alzheimer's disease groups.

	Age (years)	*Postmortem* time (hours)	Males	Females
Controls	70.0 ± 3.0	17.0 ± 3.8	10	2
Alzheimer's	74.8 ± 2.9	11.2 ± 6.1	8	7
Down's	64.0 ± 3.8	10.2 ± 2.1	2	3

Ages and *postmortem* times are means ± SE.

Tissue extraction

Each brain sample was weighed and extracted in 20 volumes (w/v) chloroform/methanol (2:1 v/v). Tissue was initially homogenized in methanol, then chloroform was added and washed with 2/3 volume 50% methanol/water. The phases were separated by centrifugation, transferred to separate tubes, and dried under a vacuum.

Determination of GPC

The aqueous phase of the brain extract was reconstituted in water, filtered, and an aliquot equivalent to 20 mg of tissue subjected to a modification of the HPLC procedure described by Liscovitch *et al.* (1985). GPC was purified on a normal phase column (Pecosphere-3C Si, 4.6 x 83 mm; Perkin-Elmer, Norwalk, CT) using a linear gradient elution consisting of 2 buffers: buffer A, containing acetonitrile/water/ethanol/acetic acid/1.0 M ammonium acetate (800:127:68:2:3 v/v), and buffer B (same components (400:400:68:53:79 v/v) from 0 to 100% buffer B with a slope of 5%/minute, started 6 minutes after injection with a flow rate of 1.5 ml/min and a column temperature of 55 °C. Fractions with a retention time of 13.5-15 minutes (GPC) were collected, pooled and dried. GPC samples were reconstituted in 6 M HCl, hydrolyzed to free choline at 90 °C for 1 hour and then dried. Dried residues containing choline of hydrolysates of GPC were assayed for choline as described by Goldberg & McCaman (1973).

Determination of GPE

GPE was measured in aliquots (equivalent to 2-5 mg of tissue) of the aqueous phase following derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) (Cunico et al., 1986). Samples were incubated with 2 mM FMOC solution in 0.1 M sodium bicarbonate pH 8.0 and the excess of FMOC was extracted with pentane. Derivatized compounds were separated on a reverse phase HPLC column (Biophase ODS 5 µm, 250 x 4.6 mm; Bioanalytical Systems, Inc., West Lafayette, IN) using a concave gradient elution consisting of two buffers: buffer A containing 0.2 M sodium citrate-0.005 M tetramethylammonium chloride (pH 2.85) and acetonitrile (3:1 v/v), and buffer B containing 0.2 M sodium citrate-0.005 M tetramethylammonium chloride (pH 4.5) and acetonitrile (1:3 v/v). The concave gradient (from 0 to 100% buffer B) commenced at the injection and lasted for 70 min, followed by a 15 min reequilibration with buffer A. The flow rate was 1.4 ml/min and the column temperature was 30 °C. The derivatives were detected with a spectrofluorometer using 264 nm excitation and 340 nm emission.

RESULTS

GPC concentrations were increased in all brain regions from AD patients relative to controls (Table 2). This increase ranged from 1.7 to 2.3 fold. GPC levels in brain samples from DS patients were similar to those of controls (Table 2). Elevations in the amounts of brain GPE were also observed in samples from AD patients when compared to controls or DS (Table 3). As in the case of GPC, GPE levels did not differ between DS and controls (Table 3).

Table 2. Glycerophosphocholine in human brain.

	Glycerophosphocholine (nmol/g weight)		
	Control	AD	DS
Area 20	661 ± 126	1510 ± 180	785 ± 138
Area 40	640 ± 58	1314 ± 145	700 ± 102
Caudate/Putamen	1014 ± 189	1699 ± 225	891± 109
Cerebellar Cortex	732 ± 102	1524 ± 160	773 ± 154

The results are reported as means ± SEM. Statistical analyses by ANOVA and Duncan's multiple range test showed that the values in AD group were higher than in controls or DS in all brain regions ($p<0.05$).

Table 3. Glycerophosphoethanolamine in human brain.

	Glycerophosphoethanolamine (nmol/g weight)		
	Control	AD	DS
Area 20	701±79	1002±84	522±69
Area 40	635±44	1008±61	790±112
Caudate/Putamen	872±64	1061±62	785±95
Cerebellar Cortex	888±64	1154±85	740±80

The results are reported as means ± SEM. Non-parametric statistical analyses of the data (at 0.05 level) showed that the values in AD group were higher than DS in all brain regions but area 40 and were higher than control in all regions but are 20.

DISCUSSION

Comparison of the *postmortem* concentrations of the metabolites of the two major brain phospholipids, PC and PE, in control subjects, AD, and DS patients revealed dramatic differences. Levels of two catabolic intermediates, GPC and GPE, were increased in AD relative to controls or DS patients. Since the amounts of phosphodiesters (GPC, GPE) have been reported as stable in human brain tissue *postmortem* (Perry et al., 1981; Pettegrew et al., 1987), the changes described here most likely reflect *antemortem* conditions.

Previous studies have also demonstrated abnormalities in levels of phospholipid precursors and metabolites in AD brains. Elble et al. (1989) reported elevated choline concentrations in cerebrospinal fluid of AD patients. Using ^{31}P nuclear magnetic resonance technique, Pettegrew et al. (1988) found elevated concentrations of phosphomonoesters (i.e. the sum of phosphoethanolamine and phosphocholine) in AD brain, however the same authors reported that phosphomonoesters levels were inversely correlated with the number of senile plaques and were not different from those present in control brains in the intermediate to late stages of AD (Pettegrew

et al., 1988a). [In our preliminary studies, the sums of phosphoethanolamine plus phosphocholine levels were not significantly different in AD from those in control subjects (data not shown)]. We found that levels of GPE were higher in AD brains than in age-matched controls. Our results confirm and extend previous reports, based on ^{31}P nuclear magnetic resonance technique, that GPC levels were significantly elevated in several regions of brains of AD patients (Barany et al., 1985; Pettegrew et al., 1984). Although both GPC and GPE levels are elevated in AD, increases in GPC concentrations are more pronounced. The elevations in GPC levels were found in the brain regions rich in senile plaques and neurofibrillary tangles (cortex) as well as in regions normally devoid of this overt AD-pathology (caudate, cerebellum). Recent studies, which utilized immunohistochemical techniques, showed, however that accumulation of β-amyloid occurs in the latter regions of AD brains as well (Selkoe, 1989; Ogomori et al., 1989). Thus our observations suggest that abnormal phospholipid metabolism may be quantitatively similar in many brain regions while accumulation of abnormal proteins is more pronounced, but not limited to, cortex. Since neuronal degeneration appears to be more pronounced in the cortical areas as compared to caudate or cerebellum in AD, our data indicate that accumulations of GPC and GPE are not an epiphenomenon of cell death, but rather a fundamental aspect of pathophysiology of AD. Increased levels of GPC and GPE might result from decreased activity of phospholipase D, the enzyme that catalyzes release of choline from PC (Kanfer et al., 1986), concomitant with increased rates of PC hydrolysis via a phospholipase A_2 (PLA_2) and lysophospholipase-mediated pathway which generates GPC and GPE. The recent report by Farooqui et al (1988) that lysophospholipase activity is increased many-fold, and by Kanfer and McCartney (1986) that phosphocholine hydrolysis is diminished in AD brains,supports this hypothesis.

Membrane abnormalities affecting cells within and outside of the central nervous system have been reported in AD and raise the possibility of a systemic defect in phospholipid metabolism. [In is worth noting that the β-amyloid precursor peptide is presumed to be a transmembrane protein whose proteolytic processing may be aberrant in AD (Selkoe, 1989). This processing may be affected by abnormal membrane properties]. Zubenko et al .(1984) first reported that membrane fluidity in platelets of patients with AD was abnormal; this finding has been confirmed in a large series of patients (Zubenko et al., 1984; Hicks et al., 1987) and in studies of hippocampal membranes (Zubenko, 1986). The changes in platelet membrane fluidity were attributed to the proliferation of the internal membrane system (Zubenko et al., 1987), and associated with a lower cholesterol to phospholipid ratio (Cohen et al., 1987). Abnormal fluidity in platelet membranes has been observed in a subset of AD patients with characteristic clinical symptoms (Zubenko et al., 1987). The pattern of this abnormality within families of the AD probands was consistent with a fully penetrant autosomal dominant trait (Zubenko et al., 1987;1988). Other evidence for membrane abnormalities in tissues outside of the brain includes: lack of the normal age-related decline in platelet adenylate cyclase activity (Ebstein et al., 1986); increases in erythrocyte choline concentrations, accompanied by reductions in choline uptake (Miller et al., 1986); reductions in colchicine-induced concanavalin A capping in lymphocytes (Dujindan-Van den Berge et al., 1986); and increases in total cell calcium, associated with decreases in cytosolic free calcium and with reduced spreading in cultured skin fibroblasts (Peterson and Goldman, 1986; Peterson et al., 1986).

GPC and GPE levels in DS brains were similar to those of age-matched, elderly controls. These data indicate that despite similar cerebral pathological manifestations of AD and DS, the properties of phospholipid metabolism distinguish these diseases. If, as we hypothesize, the cholinergic lesion in AD is associated with the abnormal phospholipid metabolism, then similar lesion in DS may develop without phospholipid involvement by a different mechanism (DS, which results from the presence in cells of three copies of genes residing on chromosome 21, affects many metabolic pathways throughout patients' lifetime). However it is also possible that abnormal phospholipid metabolism is present in DS but it is not expressed as accumulations of GPC and GPE.

Taken together, the data point to a general involvement of cellular membranes in the pathophysiology of AD, and suggest that the disease process is widespread and not confined to a subset of CNS neurons. Whether abnormalities in phospholipid metabolism are the primary lesion or represent secondary manifestations of the disease is currently unknown. In either case cholinergic neurons would be especially vulnerable to damage because they alone use choline for

two purposes, PC and ACh synthesis. Under normal conditions, phospholipid turnover in cholinergic cells may be faster than in other neurons because they hydrolyze some of their PC to supply choline for ACh synthesis (Maire and Wurtman, 1985; Ulus et al., 1989; Blusztajn et al., 1987). Under abnormal conditions in AD, phospholipid degradation mediated via PLA_2 and lysophospholipase may be increased. This accelerated catabolism may transcend the cells' ability to resynthesize membranes and eventually compromise the structural and functional integrity the neurons. As more cholinergic cells die, some of the surviving neurons may fire faster in order to maintain cholinergic transmission, perhaps hydrolyzing more PC to supply choline for ACh synthesis (Wurtman et al., 1985) thus leading to irreversible changes.

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ALTERATIONS IN CATECHOLAMINE AND PEPTIDE NEURONS IN THE LOCUS COERULEUS IN DEMENTIAS OF ALZHEIMER'S AND PARKINSON'S DISEASE

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Abstract

A differentiation can be made between the locus coeruleus (LC) in normal brain, in Alzheimer's disease (SDAT) and Parkinson's disease (PD) for diagnostic purpose, based on the findings concerning the morphological alterations of the tyrosine-hydroxylase-immunoreactive neurons, the topographical distribution of neuron loss within the length of the LC, and, to some extent, the total reduction in cell number. A reduction of total neuron numbers of the LC of up to 87.5% as compared to age-matched controls is found in SDAT. In PD cases, the neuronal morphology is generally more severely altered than in SDAT cases. The neuron loss is more severe than in SDAT (up to 94.4%). Simultaneously there is an increase in peptide containing neurons (galanin and neuropeptide Y) in SDAT compared to normal controls. The consequences of these findings will be discussed.

Introduction

Senile dementia of the Alzheimer's type (SDAT) is neuropathologically characterized by severe cortical atrophy and cell loss as well as a high index of dementia as measured by numbers of neurofibrillary tangles (NFT) and neuritic plaques (NP) in neocortex and hippocampus. In addition, several subcortical afferent projection systems are disturbed in the disease, namely those based on acetylcholine, norepinephrine (NE) and serotonin. The occurrence of extrapyramidal signs in SDAT suggests involvement of dopaminergic pathways in some cases. Investigations of the functional role of the locus coeruleus-NE system in SDAT have previously focused on the study of the locus coeruleus (LC) cellular neuropathology and measurements of NE content in the various cortical projection areas of the LC. Quantitative investigations using the neuromelanin pigment as a marker for NE neurons have demonstrated a reduction of neuron numbers in the LC in most cases of SDAT with a high incidence of neuropathologic markers like NFT, NP and, occasionally, Lewy bodies in the remaining neurons. Recent studies using catecholamine biosynthetic enzymes have also demonstrated a loss of LC-NE neurons in SDAT, though with different results as to the total neuron numbers counted in control and SDAT cases. This cell loss from the LC was reported to be topographically arranged. NE-level, dopamine-beta-hydroxylase (DBH)-activity and the

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

levels of several other NE markers have been shown to be decreased in LC projection areas both in ante-mortem biopsies and in post-mortem brain tissue indicating a deficiency of the NE-transmission in SDAT. Correlation between cortical plaque formation and cortical NE-levels and LC neuron loss in the anterior and central regions of the LC known to project to these areas in animals have been reported. Also, the severity of cortical plaque incidence and the degree of reduction of LC neuron number has been observed to be correlated, though no direct correlation between the severity of dementia and the extent of LC damage has been demonstrated. A recent study, however, has shown a positive correlation of the occurrence of depression in SDAT and the decrease in LC neuron number.

Lesions of brainstem nuclei including the LC, with neuropathologic changes such as Lewy bodies and NFT in PD have been described many years ago and Parkinsonian state and LC-lesions similar to those found in the disease are caused by the administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the macaque monkey. Even though PD is principally a disorder of locomotion, it is now generally accepted that in a number of patients progressive mental impairment occurs in the course of the disease. Some authors have reported dementia in more than 50% of cases of PD. According to the responsiveness to L-dopa treatment it has been postulated that "two separate disorders can be distinguished in PD: an exclusive motor disorder occurring in a younger population with a longer and more "benign" course and a better response to L-dopa; and another, a motor followed by a cognitive disorder occurring in an older population with a more fulminant course and a poorer response to L-dopa". There has been a controversy over the distinction of a "cortical" dementia found in SDAT and a "subcortical" dementia present in PD patients. Several authors have claimed that in neuropsychological tests the dementia of SDAT, characterized mainly by aphasia, amnesia, agnosia and apraxia, can be distinguished from that found in PD patients, where the dementia is characterized by slowness of mental processing, forgetfulness, impaired cognition, apathy and depression, while no psychopathological difference between demented PD and SDAT patients was found by others. Some investigators have suggested that the dementia in PD displays a pattern of impairment typical for a lesion of the frontal lobe and a laterality of the disease has been suggested based on the finding that patients with greater disease involvement on the left side of the body showed greater neuropsychological impairment than those more affected on the right body side. The question whether the incidence of cortical plaques and tangles is correlated to the severity of dementia in PD is still also somewhat controversial. Early reports have shown more frequent occurrence of NP and NFT in demented PD patients than in non-demented, suggesting coincidental SDAT in these patients. Other authors could not demonstrate a positive correlation between NFT and NP formation and dementia, but reported a severe cell loss in the LC more frequently in demented PD patients than in those without symptoms of dementia. A correlation between the coeruleo-cortical NE-system and dementia has also been suggested based on modifications in the number of adrenergic receptors in demented PD patients.

Material and methods

Normal controls included the brains of 11 patients, 4 male and 7 female, ranging in age from 43 to 89 years, with no clinical history of neurological or psychiatric disease as confirmed by postmortem gross and microscopic neuropathological examination. Vital data and selection criteria for all control cases were described in detail (1). Data assembled in three paradigm control cases in the age group of the

patients in the Alzheimer's and Parkinson's groups served as control values for quantitative analyses. Appropriate levels of normal mental function in patients was shown by results of between 22 and 26 from a possible 30 points in the last available mini-mental status tests. In the group of senile dementia of the Alzheimer's type (SDAT) cases the brains of 8 patients that had been clinically diagnosed were studied, 2 male and 6 female cases with ages ranging from 71 to 85 years. Cases of dementia due to other neurological disorders, such as ischemia, multiple infarcts, Pick's disease etc were excluded. Postmortem delays ranged from 3 to 16 hours, with postmortem delays of 5 hours and less in 5 of the cases. Counts of neuritic plaques and neurofibrillary tangles were made on Bodian-silver-stained preparations. For all cases in this group, the counts yielded moderate to high indices of neurofibrillary plaques and tangles in the examined areas, which is indicative of SDAT. Seriously impaired mental function in these patients was indicated by a score of 0 to 5 points in the last available mini-mental status tests.
In the study of cases with Parkinson's disease 7 diagnosed patients were included, ranging in age from 76 to 90 years, three male and four female cases. Clinically, two of the patients had PD responsive to L-dopa treatment without symptoms of dementia (P-D), five patients had histories of rapidly progressive dementia, with onset 2-3 years before death. Of these five demented patients, three were responsive to treatment with L-dopa (P+D); two were atypical and their Parkinsonian symptoms did not respond to L-dopa treatment (P+D/L-dopa nonresponsive). The postmortem delays ranged from 3.5 to 21 hours, and was less than 7 hours in four of the cases. The clinical diagnosis of Parkinson's disease was confirmed at autopsy by both gross and microscopic neuropathological examination. The substantia nigra showed considerable cell loss, loss of pigmentation, numerous Lewy bodies, and gliosis pathognomic of Parkinson's disease in every case. Counts of neurofibrillary tangles and neuritic plaques were performed as described for the SDAT cases and yielded indices slightly higher than in the age-matched control and non-demented Parkinson's cases for the demented Parkinson's patients. The last available mini-mental status test scores were 22 to 26 for the non-demented Parkinson's disease group and 11 to 16 for the demented patients.

Fixation and immunocytochemistry

The protocols used for fixation of the studied brainstems and immunocytochemistry were described in detail in preceeding papers (1,2).

Computer-assisted quantitative morphological analyses

The computer system and the recording procedures used have been described in detail (1,2). Briefly, the immunocytochemically stained serial brainstem sections were reassembled in the correct anatomical order, and the LC outline in the coronal plane, its rostral and caudal borders were determined, and its rostrocaudal length on both sides of the brainstem was calculated. For the computer-assisted measurements of morphological parameters of TH-immunoreactive LC neurons, and for the mapping and counting of neurons and the three dimensional reconstruction procedure for the analysis of neuron distribution in the LC an IBM-AT-mouse based user-interactive image analysis system with the Cellmate program (Bioquant, Tenn.) was used. Outlines of individual cell somata and dendritic arbors were recorded for calculations of soma areas and dendritic arbor length. Plots of these recordings served to illustrate alterations in individual neuron morphology. To ensure comparability all quantitative measurements of neuronal parameters were carried out on immunoreactive neurons

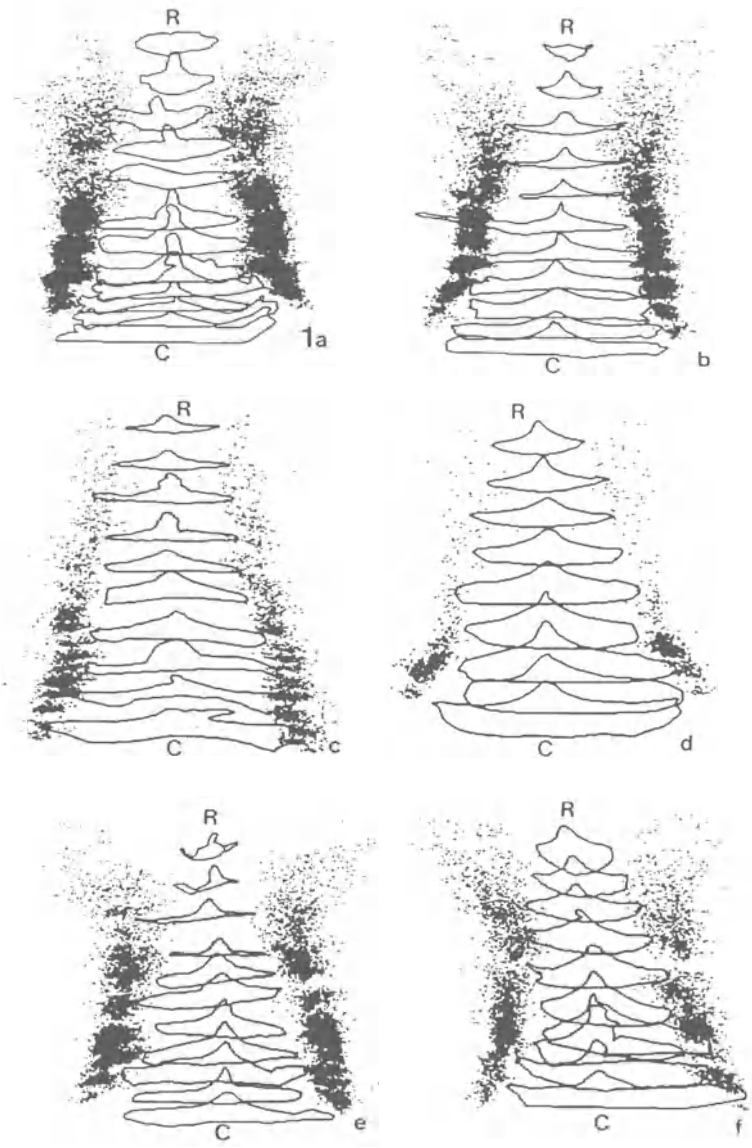

Fig. 1. Three dimensional computer reconstruction of the LC of a younger control case, 55 yr, (a); an older control case, 78 yr, case (b); a case of mild SDAT with comparatively little cell loss, 78 yr, case (c); a severe SDAT case with extreme cell loss, 77 yr, case (d); a PD case without dementia, 76 yr, case (e); and a PD case with dementia, L-dopa non-responsive, 82 yr, case (f). The reconstruction is viewed from dorsal, shifted in a 25^{0} angle from the plane of the figure. R = right, L = left LC. The outline of the fourth ventricle is drawn on every fourth section, and each TH-immunoreactive neuron on all the recorded sections is marked by a dot. Note the cell loss which occurs mainly in the rostral part of the older control case (b) as compared to the younger control case (a). Note also the high neuronal loss present predominantly in the rostral and middle parts in both SDAT cases (c) and (d). In the PD case with dementia/L-dopa non-responsive (f) cell loss is present throughout the nucleus.

stained with the PAP-method. Cell counts were performed on all reassembled sections of one TH-immunoreacted series of sections by cursor-marking the localization of whole cell bodies using different symbols signifying different morphological classes of cells (see below) in all focus levels throughout the entire extent of the LCs of both sides of the brainstem. TH-immunoreactive cells of the locus subcoeruleus and the pars cerebellaris loci coerulei were recorded but not counted. Neuron numbers on partially damaged sections were approximated either from the numbers counted on immediately adjacent TH-immunoreacted sections or from those counted in the contralateral LC of the same section and the ipsilateral LCs of the preceeding and following sections of the same series. Total neuron numbers were calculated by the computer for the entire LCs and, by their differing recording symbols, differentiated according to the four neuron classes: Large multipolar (LM), large "bipolar" (LB), small multipolar (SM) and small "bipolar" (SB) neurons. The recordings of the reassembled sections were then aligned to match as closely as possible the situation in the intact brains, and an image of the three dimensional distribution of the neurons was created by the computer.

Results

Identification of NE-producing neurons is done by immunocytochemical demonstration of two NE biosynthetic enzymes, tyrosine hydroxylase (TH) and DBH, and immunoreactions are visualized by the peroxidase-antiperoxidase (PAP) and immunogold-silver-staining (IGSS) methods. It is demonstrated that the reactions with antisera against TH and DBH yield equivalent results and that both immunocytochemical visualization methods allow detailed analysis of neuronal morphology. The neurons of the human LC fall into four distinct classes: large multipolar neurons with round or multiangular somata (LM), large elliptical "bipolar" neurons (LB), small multipolar neurons with round or multiangular somata (SM) and small ovoid "bipolar" (SB) neurons. Though most of the neurons contain neuromelanin pigment, some of the neurons of the larger type lack pigmentation. Dendritic arborization in all neuron types is extensive and computer-assisted quantitative measurements of the neuronal structure parameters soma size, dendritic arbor length, surface area and volume are given. Comparison of neuronal morphology in different age groups shows that even though the soma areas of LC neurons of all four classes are decreased in older normal adult brain, the dendritic arborization is equally extensive. Detailed mapping of the immunoreactive neurons and computer-assisted three dimensional reconstruction of the LCs are used to analyze the morphology of the nucleus as a whole. According to cellular distribution patterns, the LC is divided into rostral, middle and caudal parts with neurons scattered over a large area rostrally, tightly clustered in the middle and very densely packed in the caudal part. Small neurons predominate in all parts, but the relative contribution of larger cells decreases in a rostro-caudal direction. Small bipolar neurons are the most frequent cells of the caudal part and display distinct dorsomedial-ventrolateral orientation. These general morphological characteristics are the same in all age groups, but cell density in rostral and middle parts is decreased in old age, while the relative frequency of large cells is increased especially in the rostral LC. No age-dependent decrease in nuclear length is observed. Assessment of neuron numbers documents a cell loss of 27% to 55% in older adult brains. Cell loss is topographically arranged, being highest in the rostral part, lower in the middle and virtually absent in the caudal part. Quantitative assessment of the distribution of the different morphological neuron classes confirms the observa-

tions mentioned above, suggesting that especially in the rostral part of the LCs of older adult brains loss of smaller cells is comparatively higher than of larger cells. The computer-generated three dimensional reconstruction provides the possibility of visualizing LC shape and cell distribution closely approximating the situation in the intact brain and facilitates the detection of morphological differences of the LCs in individual brains (see Fig. 1a-f). After the studies of the controls, a detailed qualitative and quantitative investigation of the morphology and distribution of the NE neurons in the human locus coeruleus in two classes of neurodegenerative disorders involving dementia, the senile dementia of the Alzheimer's type (SDAT) and Parkinson's disease (PD) is undertaken. In SDAT, the four basic LC neuron classes found in the normal human brain are recognizable in the remaining cells, but the cell somata are generally larger, the cell bodies are swollen and misshapen, and the dendrites are forshortened and thick and less branched than in neurons of control LCs. Quantitative analysis confirms the qualitative observations. The reduction of absolute numbers of LC-NE neurons in paradigm cases of SDAT and PD as compared to controls are shown in the table below.

Table 1

Case	Age	Sex	Neuron number x 10^3
Control	79	m	47.5
Control	78	f	40.9
SDAT (mild)	78	m	34.0
SDAT (severe)	74	f	18.8
SDAT (severe)	77	f	5.7
P	76	f	31.1
P+D	83	f	23.3
P+D/L-dopa nonresponsive	79	m	2.5

A reduction of total neuron numbers of the LC of between 3.5% and 87.5% as compared to age-matched controls is found in SDAT. This neuron loss is topographically arranged: in the rostral part of the LC, the reduction is greatest, being more than 28% in the case least affected in this part, and 97% in the case most severely affected. The middle part is less, and the caudal part least affected by cell loss in all cases. The average rostrocaudal nuclear length in SDAT cases is reduced as compared to controls (13 mm and 14.9 mm respectively). In PD cases, the neuronal morphology is generally more severely altered than in SDAT cases. The four neuron classes are hardly distinguishable, the cell bodies are swollen, and frequently contain Lewy bodies. The dendrites are short and thin, and arborizations are reduced or virtually absent. In the neuropil surrounding the remaining neurons cell remnants and masses of extraneuronal pigment are found. The neuron loss is more severe than in SDAT (26.4% - 94.4%).While cell reduction varies within each group, a difference in the topographical arrangement of cell loss can be recognized between P+D and P+D/L-dopa non-responsive: in P+D the rostral part is predominantly affected, while in P+D/L-dopa non-responsive the neuron loss is equally great or greater in the middle and caudal part. The average rostrocaudal length in PD cases is less than in SDAT and the controls (12.4 mm).

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THE ROLE OF NEURONAL MEMBRANES DETERIORATION IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE: AN ULTRASTRUCTURAL PERSPECTIVE

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INTRODUCTION

Senile dementia of the Alzheimer's type (SDAT) is diagnosed on the basis of well known histopathological alterations which are present, but at a much lesser extent, also in the brain of normal aged people. Since the difference seems to be only quantitative, some authors support the contention that subtle neurochemical alterations accumulate in the brain of old individuals and may ultimately show a clinically evident pathology (Roth, 1985; Arendt and Bigl, 1987; Bertoni-Freddari, 1988a). Cellular membranes, while being important permeability barriers, are also directly involved in several important processes such as active transport of molecules, hormonal and immunological stimulation, transmission of the nervous impulse, etc.. During aging, changes in membrane basic constituents (phospholipids, cholesterol and proteins) lead to impaired cellular performances and this fact appears to be particularly true for the membranes of postmitotic cells, like neurones, which have the same age of the organism to which they belong (Oestreicher et al., 1986). In nerve cells a proper substitution of damaged molecules at their membranes is a necessary prerequisite to preserve specific functions such as ion homeostasis and action potentials, thus any delay and/or mistake in the turnover of altered constituents of neuronal membrane may result in serious impairments of cellular performances and in a potential threat to nerve cell longevity. In the present paper we report the results of a quantitative morphometric study undertaken to seek changes in synaptic membranes and mitochondria during physiological aging and SDAT.

SYNAPTIC CHANGES IN A TARGET AREA OF SDAT PATHOLOGY: THE HIPPOCAMPUS

Senile plaques (SP) and neurofibrillary tangles (NFT), the characteristic hallmarks of SDAT, seem to be more or less widespread in the demented brain (Hardy et al., 1986), however discrete areas of the

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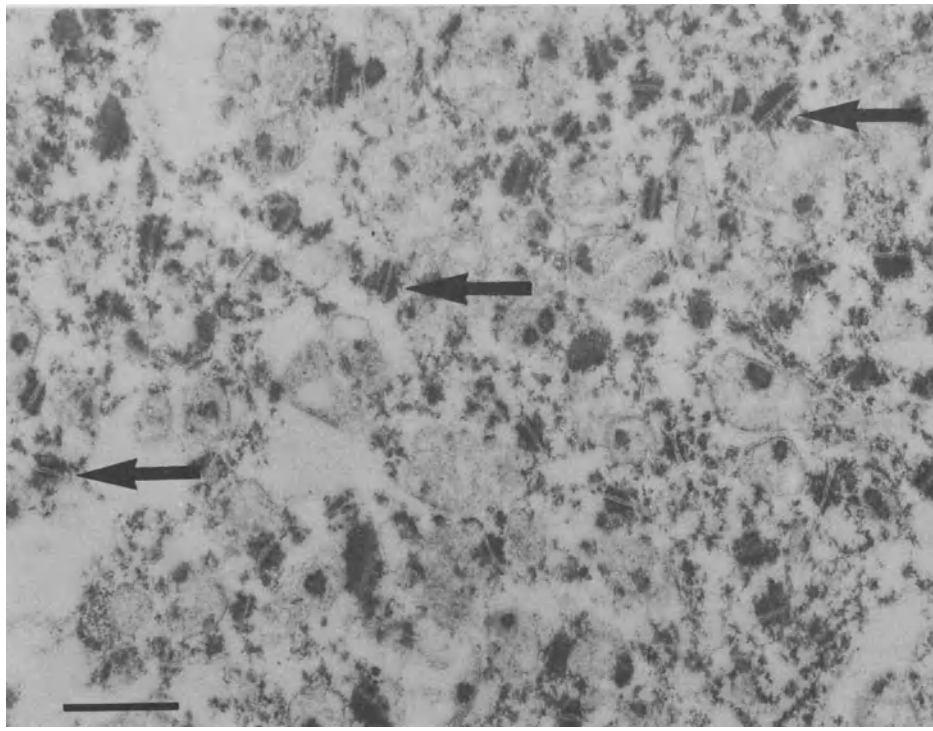

Fig. 1. E-PTA stained synaptic junctions (arrows) in the cerebellum of SDAT patient. Bar = 1 μm.

CNS (i.e. association areas in the cerebral cortex, hippocampus, amigdala and the basal nucleus) are so abundant in such alterations that the postmortem diagnosis of SDAT is based on the numerical density of SP found in one of these areas (Roth, 1985). In addition to such a zone-specific vulnerability, the cholinergic system appears to be very sensitive to SDAT histopathological alterations (Perry and Perry, 1980). Taking into account these well demonstrated changes, the dentate gyrus supragranular layer, an area of the hippocampal formation receiving cholinergic inputs from the septum, was investigated. Adult (mean age: 55.8 years), normal old (mean age: 81.4 years) and SDAT (mean age: 83.6 years) right hippocampi from autopsied patients were sampled for our studies. According to our previous papers (Bertoni-Freddari et al., 1988b; 1989), glutaraldehyde fixed tissue blocks were stained by means of the ethanol phosphotungstic acid (E-PTA) technique which represents a selective procedure to evidentiate synaptic junctions against the background (Fig. 1). A computer-assisted analysis of the electron microscopic negatives allowed us to measure the following parameters: surface density (Sv), i.e. the total area of the synaptic contact zones in a unit volume of tissue; the average area of a single synapse considered as a circular disk (S) and the numerical density (Nv) of the synapses, i.e. the number of contacts in a unit volume of tissue. In the same samples used for this electron microscopic investigation, quantitative morphometry was performed in semithin sections to measure the numerical density of dentate gyrus granular cells. As shown in Table

TABLE 1. MORPHOMETRIC PARAMETERS OF HIPPOCAMPAL DENTATE GYRI. MEAN (± S.E.M.)

	SYNAPSES			GRANULAR CELLS	
	Sv ($\mu m^2/\mu m^3$)	S (μm^2)	Nv (No.Syn./μm^3)	Nv ($x10^3/mm^3$)	Syn./Neur. ($x10^3/mm^3$)
ADULT	0.1798 (0.0097)	0.1149 (0.0041)	1.5991 (0.1331)	135.40 (8.31)	11.81 (0.83)
OLD	0.1399■ (0.0025)	0.1756■ (0.0096)	0.8308■ (0.0424)	92.19■ (8.13)	9.96■ (0.31)
DEMENTED	0.1083● (0.0081)	0.1780 (0.0098)	0.6270● (0.0270)	102.16 (9.10)	6.14● (0.58)

● Statistically significant vs. the old group.
■ Statistically significant vs. the adult group.

1, synaptic ultrastructural features are seriously affected by aging and SDAT. In a comparison between adult and old group, Nv and Sv significantly decrease whereas S increases. Old and age-matched SDAT patients showed the same synaptic average size (S), but Nv and Sv were significantly decreased in the demented patients. The number of granular cells is stable comparing physiological with pathological aging, but it is significantly decreased vs. adult value. The synapse to neurone ratio decreases by 15.6% and 48% in old and demented patients, respectively, when the adult value is considered 100%. Since the results regarding the number of synapses per neurone are independent from actual factors such as tissue shrinkage due to experimental processing, age and/or pathology, they help to define synaptic efficacy from a morphological point of view and support that the reported impairment in the dentate gyrus synaptic ultrastructure represents per se a prominent feature of old and demented CNS. In interpreting these data, we want to stress that synaptic junctions, although well defferentiated areas of the neuronal membrane, are very dynamic structures capable of adaptive response to environmental stimuli and experiential framework. Synaptic Nv and S appear to be inversely related to each other and both contribute to maintain the constancy of the total synaptic contact area, i.e. Sv (Hillman and Chen, 1984; Bertoni-Freddari et al., 1988b; 1989). By considering these three parameters per group of patients, we can get a reliable index of the morphological rearrangements occurring at synaptic contact zones in both the conditions investigated. In this context, the increase in S deserves particular interest since it may represent a compensative phenomenon to balance the decreased Sv brought about by the consistent reduction of Nv. This assumption, although speculative, finds support in an analysis of the synaptic population performed by a percentage distribution of S which excludes the possibility that the

TABLE 2. MORPHOMETRIC PARAMETERS OF CEREBELLAR GLOMERULI. MEAN (± S.E.M.)

	SYNAPSES			MITOCHONDRIA	
	Sv ($\mu m^2/\mu m^3$)	S (μm^2)	Nv (No.Syn./μm^3)	Vv ($\mu m^3/\mu m^3$)	Nv (No.Mit./μm^3)
ADULT	0.2095 (0.0136)	0.1032 (0.0040)	2.0286 (0.0834)	0.1381 (0.0036)	1.207 (0.0311)
OLD	0.1831 (0.0155)	0.1646■ (0.0137)	1.1294■ (0.0370)	0.1123■ (0.0035)	0.533■ (0.018)
DEMENTED	0.1231● (0.0118)	0.1526 (0.0066)	0.8303● (0.1033)	0.0888● (0.0029)	0.372● (0.013)

● Statistically significant vs. the old group.
■ Statistically significant vs. the adult group.

expanded contact zones in aging and SDAT undergo perforation and splitting into smaller junctional areas (Bertoni-Freddari et al., 1989).

SYNAPTIC AND MITOCHONDRIAL CHANGES IN A CNS AREA REPORTED TO BE FREE OF SDAT HYSTOPATHOLOGY: THE CEREBELLUM

Although in some cases of familial and presenile Alzheimer's disease amyloid plaques have been found in the cerebellar cortex (Pro et al., 1980; Azzarelli et al., 1985), this zone of the CNS is largely reported to be unaffected by SDAT pathology (Roth, 1985). In order to check whether the changes in synaptic ultrastructure observed in the hippocampus of normal old and SDAT patients, represent ubiquitous alterations involving the CNS as a whole, the same study on synaptic contact areas was performed in the cerebellar cortex of the same patients sampled for the hippocampal investigation. The anatomical model we chose for this study is the cerebellar glomerulus: a cell free zone in the granular layer of the cerebellar cortex which is innervated by mixed fibers (mostly gabaergic, serotoninergic and cholinergic). Since it is easily identified both at optic and electron microscopic level, and it is a discrete area in the cerebellar cortex, the glomerulus was very suitable also to perform morphometric measurements on the mitochondrial population of nerve endings from the same samples used for the synaptic studies. The aim of this latter investigation was twofold: first, to get data on energy-producing cellular organelles subserving synaptic transmission, and second, to check whether, in addition to synaptic junctions, membranes of different origin undergo deteriorative changes as a consequence of aging and SDAT. In the glomerulus the mossy fiber swells up and makes synaptic contacts with the dendrites of the surrounding granular cells, therefore the mitochondria we studied must be considered as organelles actively participating in energy production for the synaptic functions. This is an important point to mention since

physiological differences have been demonstrated between mitochondria from neuronal pericaria and synaptic regions (Harmon et al., 1987). Cerebellar tissue blocks were processed either as described for the studies on the hippocampal synapses or according to the conventional electron microscopic procedures. The mitochondrial parameters calculated by means of our computer-assisted image analyser were: the volume density (Vv) i.e. the total volume of mitochondria in a unit volume of tissue, and the numerical density (Nv), that is the number of mitochondria per unit volume of tissue. In the cerebellum (Table 2), we found that the average size of the single contact is the same in old and SDAT groups, but is significantly increased when compared with adult values. Nv undergoes a significant decrease both during physiological aging and SDAT. At variance with the hippocampal results, we did not find a significant difference between adult and old Sv, whereas the 33% decrease between SDAT and age-matched controls was significant. During physiological aging mitochondrial Vv and Nv underwent a significant decrease which was also demonstrated comparing SDAT vs. age-matched control patients. Despite from literature data the cerebellum is reported to be a CNS area unaffected by SDAT pathology, the present findings support that synaptic and mitochondrial ultrastructure is seriously deteriorated in old and demented subjects. According to the above discussed concepts on hippocampal synaptic plasticity, we envisage compensative phenomena also in the cerebellar cortex. Namely, the non significant difference in Sv between adult and normal old group may represent a successful compensation brought about by S. Although at a lesser extent, this reactive capacity of the nerve cells appears to be still present in the SDAT group, but fails to recovery completely Sv. With regard to the results on synaptic mitochondria, we want to point out that, at variance with E-PTA stained synapses, postmortem delay in collecting autoptic samples plays some role in mitochondria preservation, thus only those organelles displaying well preserved cristae were scored by our image analyser. The data shown in Table 2 support that changes in mitochondrial morphology are associated or may contribute to the reported alterations in metabolism related to energy production during aging and SDAT (Meier-Ruge et al., 1984; Sims et al., 1987). In particular, the decrease in Vv may be interpreted in terms of a reduced potentiality to produce energy, whereas the decrease in Nv as a reduced capability to cope with increased energy demands.

CONCLUSION

If we consider synaptic junctions and mitochondria as sensitive membrane models, our present data support that the deterioration of neuronal membranes may play a crucial role in the age-related impairment of brain functions and in the pathogenesis of SDAT. The occurrence of such alterations in the cerebellar cortex demonstrates that membrane damage should be considered as a ubiquitous event in the CNS of old and demented patients. Since in the SDAT group we found that synaptic and mitochondrial membrane changes proceed far beyond the alterations observed in the age-matched controls, it is reasonable to postulate the existence of a neuronal membrane deterioration threshold between normal

aging and SDAT in the development of the demented state (Bertoni-Freddari, 1988a). The recent findings on the involvement of a protein spanning the neuronal membrane in the pathogenesis of senile plaques (Marx, 1989), lends further support to the above assumption.

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EXPERIMENTAL SYNAPTIC DEGENERATION AS A MODEL FOR THE PATHOGENESIS OF ALZHEIMER'S DISEASE: MONOCLONAL ANTIBODIES AND A PROTEIN KINASE INHIBITOR BLOCK SYNAPSE FORMATION AND MAINTENANCE BETWEEN CULTURED CNS NEURONS

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INTRODUCTION

A "tracing circuit" model has been proposed[1], where in neuronal circuits are maintained by activity-dependent elimination of ex-circuit synapses and resultant sprouting of in-circuit axonal terminals, a process that might correspond to human memory. Since synaptic and neuronal degeneration[2], even following abnormal synapse formation[3,4], represents the possible pathogenesis of the dementia of Alzheimer's disease, the search for unknown molecules involved in the maintenance and formation of synaptic contacts is a promising approach to understanding of the disease.

Monoclonal antibodies (MAb) raised against NGF-differentiated PC-12 cell surface molecules were screened for their effect on the formation and maintenance of synaptic contacts. Multi-site Ca^{2+} fluorometry[5] revealed that long-term applications of some MAbs blocked synaptogenesis between rat hippocampal neurons in culture[6].

A membrane impermeable protein kinase inhibitor (K-252b) appeared to block synapse formation between cerebral cortical neurons in culture[7]. This result suggests that a specific phosphorylation of surface proteins on the synaptic membrane by an ecto-protein kinase is involved in the activity-dependent formation and maintenance of cortical synapses.

MATERIALS AND METHODS

Cells were dissociated and cultured by a modification of the method of Banker and Cowan[8]. Cerebral cortices and hippocampi were dissected from 18-day fetal rats. Small pieces of the tissue were digested with 0.02% papain. After dissociating, the cells were suspended in a medium consisting of 5 % newborn calf serum (Nakashibetsu Serum Center), 5 % heat-inactivated horse serum

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(Gibco) and 90 % Dulbecco's modified Eagle's medium (DMEM,Gibco). The final cell suspension was plated on poly-L-lysine coated coverslips. Cultures were maintained in a 7 % CO_2 atmosphere at 37°C. The medium was changed every 2-3 days.

Optical monitoring of excitatory synaptic activity was carried out as follows. A small amount of fura-2 AM was applied to the cultured cells. After washing of unincorporated dye, the fluorescence intensities from cultured neurons loaded with the Ca^{2+} indicator were recorded using video-assisted,multi-site fluorometry equipment[15], by a previously described method.

Monoclonal antibodies were chosen from a library[16] against cell surface antigens of nerve growth factor(NGF)-differentiated pheochromocytoma(PC-12) cells. Protein kinase inhibitor K-252b was isolated from the culture broth of Nocadiopsis sp. K-290.[9] The agent was dissolved in a small volume of dimethyl sulfoxiside (DMSO) and diluted by the incubation solution.

RESULTS AND DISCUSSION

Synaptic activity of the cultured CNS neurons

Cultured neurons from cerebral cortex and hippocampus of rats developed spontaneous oscillation of fluorescence intensity corresponding to excitatory synaptic activity[5]. After 5 days in culture, changes of fluorescence intensity become significant. This type of spike-like change of intracellular Ca^{2+} has been shown to be caused by electrical excitation of neurons. Oscillation of fluorescence intensity were observed simultaneously in most of the neurons. As already established in hippocampal neuron culture[5], this oscillation in the culture indicates the formation of functional synaptic contacts, since application of tetrodotoxin or of a glutamate receptor antagonist, 2-aminophosphonovaleric acid (APV), reversibly inhibited the increase of fluorescence intensity (data not shown). Spontaneous excitation of pace-maker neurons in culture is transmitted to other neurons through functional synapses, so that all the neurons in the synaptic network excite almost simultaneously. Therefore, it is quite reasonable to use synchronization of the oscillations in fluorescence intensity as an indication of synapse formation.

Application of monoclonal antibody during the culture

In preliminary experiments using hippocampal neuron cultures, clonic application of a monoclonal antibody (PCH 47-67) against PC-12 cell surface antigens[16], abolished synchronization of the oscillations in fluorescence intensity (Fig. 1). Most other monoclonal antibodies at similar concentrations did not interfere with synapse formation in vitro in control sister cultures. These data suggest that the monoclonal antibody inhibited the formation of functional synapses[5] in the culture. The cell surface antigen, which might be one of the functional molecules for synapse formation between CNS neurons, is under investigation.

Application of a protein kinase inhibitor during the culture

In control cultures, with or without comparable amount of DMSO, significant oscillation of the fluorescent intensity was commonly observed after 6-8 days in culture (Fig. 2,A). In the continuous presence of protein kinase inhibitor K-252b (0.2 μM) in the sister culture, the oscillation was not observed after the

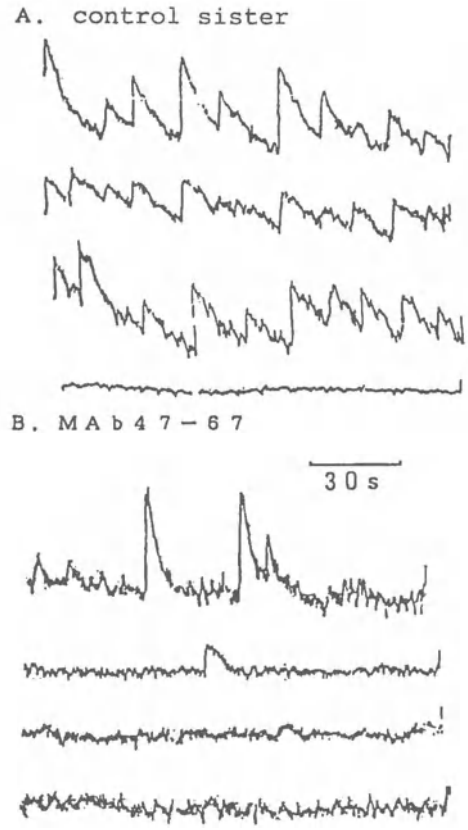

Fig. 1 Effects of a monoclonal antibody (PCH 47-67) on synapse formation[6].

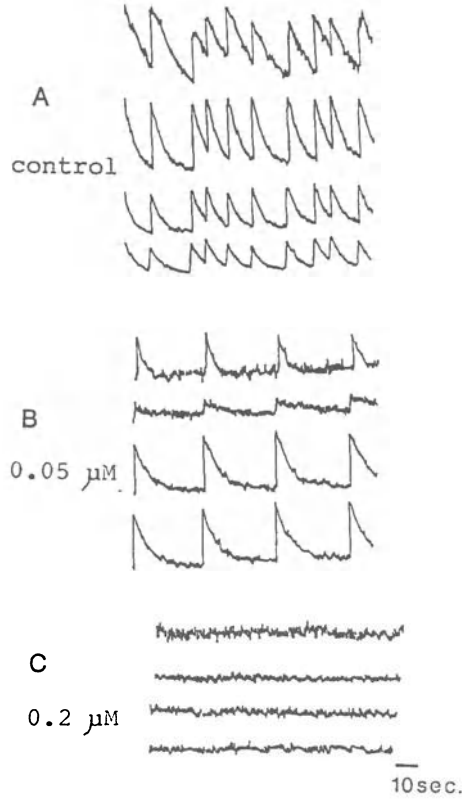

Fig. 2 Effects of a protein kinase inhibitor(K-252b) on synapse formation[7].

same culture period. The frequency of oscillation was lowered when 0.05 μM K-252b was applied (Fig. 2,B). Since a small number of neurons showed spike-like increases of fluorescence intensity in the culture treated with 0.2 μM K-252b, the inhibitor does not seem to inhibit the spontaneous activity of pace-maker neurons[7]. The data strongly suggest that the protein kinase inhibitor blocked synaptogenesis between cerebral cortical neurons.

Recent study has indicated that the inhibitor K-252b does not permeate cell membrane. This means that the site of action of K-252b is the outer surface of the cell membrane. It has been reported that neuronal membrane has an ecto-protein kinase, i.e. a protein kinase whose substrate site resides on the outer surface of the membrane, which phosphorylates N-CAM-like cell adhesion proteins[10]. The most probable target of the protein kinase inhibitor K-252b is this type of ecto-protein kinase. Another ecto-protein kinase which is regulated by gangliosides has recently been discovered[11]. The source of substrate for these enzymes is ATP released in the extracellular space. It has already been shown that ATP and adenine nucleotides are released upon stimulation from presynaptic terminals, presumably by exocytosis of synaptic vesicles which contain a high concentration of ATP as a common constituent[12,13].

The possible involvement of ecto-protein kinase in synapse formation provides as attractive molecular mechanism for the selective stabilization of the most heavily used synaptic inputs, from which a considerable amount of ATP is released by high frequency stimulation[14]. This sort of morphological plasticity at synapses, which can persist for a relatively long period, might be an important process in the establishment of long-term memory in the human brain[1].

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PATHOBIOCHEMICAL ASPECTS OF PARKINSON'S DISEASE AND DEMENTIA OF ALZHEIMER TYPE

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INTRODUCTION

Parkinson's disease (PD) and dementia of Alzheimer type (DAT) are both progressive neurodegenerative processes. The outstanding characteristics of both diseases are neuron loss in the neurotransmitter synthesizing areas and a decline in neurotransmitter content in those areas, which are the targets of these neurons, although degenerations differ between both diseases.

In the present paper, alterations in the cholinergic and monoaminergic systems as well as in the phopholipid (PL) and cholesterol (Ch) content and changes in thermostability, enthalpy and "membrane fluidity" in PD and DAT are described. Similarities and differences between these brain disorders will be outlined. The pathomechanisms which might underlie the degenerations in PD and DAT are discussed.

MORPHOLOGICAL CHANGES

Brain aging is accompanied by a loss of neurons with concomitant gliosis of variable extent in different brain regions. In normal brain aging there appears to be a loss of neurons in cortical areas, including the neocortex, hippocampus, amygdala and cerebellum (for review see Coleman and Flood, 1987). Additionally, certain brainstem regions also lose neurons in aging, i.e. the locus coeruleus, dorsal motor nucleus of the vagus, substantia nigra, suprachiasmatic nucleus, medial preoptic area and the nucleus basalis of Meynert (Coleman and Flood, 1987). The reduction in neuronal density is in the range of 10 to 60% from early adulthood to late old age (Coleman and Flood, 1987), while little or no reduction in cortical neuronal density occurs before the age of 50 (Brody 1955, 1979).

In DAT a much more severe reduction in neurons in both neocortex and hippocampus is observed (Ball, 1984), with a significant decrease of 40 to 46% in large neurons of various neocortical areas (Schechter et al., 1981). In DAT there is a loss of melanin containing noradrenergic

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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neurons of the locus coeruleus by 52 to 80% (Vijayashankar and Brody, 1979; Bondareff et al., 1982; Mann et al., 1982, 1984, 1986; Chui et al., 1986; Ichimiya et al., 1986; Moll et al., 1989), a significant decline in the number of cholinergic neurons in the nucleus basalis of Meynert by 44 to 75% (Perry et al., 1982; Rossor et al., 1982; Arendt et al., 1983; Tagliavini and Pilleri, 1983; Whitehouse et al., 1983; Jellinger and Riederer, 1984; Rogers et al., 1985; Chui et al., 1986; Ichimiyya et al., 1986; Mann et al., 1984, 1985, 1986; Moll et al., 1989), a decline in serotoninergic neurons of the dorsal raphe by 56% (Moll et al., 1989), of nucleus tegmentalis dorsalis by 12% (Mann et al., 1984, 1985) and of nucleus centralis superior by 37% (Tabaton et al., 1985). In substantia nigra reduction of dopaminergic neurons by 6 to 27% (Mann et al., 1984, 1985, 1987; Tabaton et al., 1985; Chui et al., 1986; Moll et al., 1989) is the lowest one of all neuromediator sytems observed in DAT. The dopaminergic neurons of the ventral tegmentum, which are the only dopaminergic neurons which project to cortex, are reduced by 43 to 61% (Mann et al., 1987).

In PD the tremendous loss of dopaminergic neurons in the substantia nigra (Tretiakoff, 1919) has long been known, while other neurotransmitter systems like the noradrenergic, serotoninergic, and cholinergic system are also affected in PD (for review see Jellinger, 1989). The noradrenaline synthezising area of locus coeruleus shows a depletion of pigmented neurons ranging from 50 to 80% with 75 to 80% loss of melanin (Jellinger, 1989). The serotoninergic neurons ascending from dorsal raphe nucleus decline by about 45% and 4.5% of these neurons contain Lewy bodies and 6.5% have neurofibrillary tangles (Jellinger, 1987). In PD cell depletion of nucleus basalis of Meynert, which is the major cholinergic input into the neocortex, amygdala and hippocampus (Mesulam and Mufson, 1984), ranges from 32 to 77% with a mean of 50 to 60% (Jellinger, 1989).

In normal aging and DAT there is a progressive increase in the number of senile changes, i.e. senile plaques and neurofibrillary tangles in neocortex and hippocampus, with predilection for the allocortex (Ball, 1977, 1984). In elderly patients and in many PD patients, a highly significant correlation has been found betwen the presence and severity of dementia and the amount of neurofibrillary tangles, and less significant with the number of senile plaques present in the neocortex and hippocampus (Ball, 1984, Wilkock and Esiri, 1982, Boller et al., 1979, Hakim and Mathieson, 1979; Riederer and Jellinger, 1983; Jellinger et al., 1983).

NEUROCHEMICAL AND NEUROBIOCHEMICAL CHANGES

As neurotransmitter synthesizing areas are affected in normal aging as well as in DAT and PD, but with varying degree, one would expect a decline in neurotransmitter content in target areas, when compensation mechanisms are not active.

NEUROTRANSMITTER SYNTHESIZING AND METABOLIZING ENZYMES

In normal aged persons, with regard to catecholaminergic functions, a severe reduction of tyrosine hydroxylase was noted for substantia nigra (-90%; Coté and Kremzner, 1983), caudate nucleus (-50 to -73%; Coté and Kremzner, 1983; Birkmayer and Riederer, 1983) and putamen (-83%; Coté and Kremzner, 1983). During life-span dopamine decreases to 50% of the controls in striatal regions (Carlsson and Winblad, 1976; Riederer and Wuketich, 1976). Dopa decarboxylase reduces by 70 to 80% in

striatum, 88% in substantia nigra, 53% in frontal cortex, and 11% in hypothalamus (Coté and Kremzner, 1983). While monoamine oxidase A (MAO-A) increases by 18% in striatum, 100% in substantia nigra and 22% in frontal cortex, the rise in MAO-B activity is even higher with values 60% over controls in caudate nucleus, 80% in putamen, 75% in substantia nigra and 70% in frontal cortex (Coté and Kremzner, 1983; Kornhuber et al., 1989). Significant increases in caudate nucleus and hypothalamus have also been reported by Carlsson (1981). While the main metabolite of the dopamine pathway, homovanillic acid (HVA), was not changed in these areas (Carlsson, 1981; Gaspar and Gray, 1984), one must conclude that normal aging is associated with a continous increase in the dopamine(DA)/HVA turnover, which leads to a decline in DA/HVA-ratio for example in the caudate nucleus between the ages of about 20 to 95 years from 0.44 to 0.23 (Coté and Kremzner, 1983). This is in contrast to PD with severe reduction of DA and a smaller decrease in HVA as consequence of a progressed degeneration process. Here both DA and HVA basically are correlated to neuronal loss in substantia nigra (Bernheimer et al., 1973). The HVA/DA-ratio increases up to 290% (Javoy-Agid et al., 1984).

NEUROTRANSMITTER SYSTEMS

Choline acetyltransferase (CAT) was measured as a marker for the cholinergic system . CAT-activity declines in both PD (54 to 75%; striatum, hippocampus and neocortex; Ball, 1984) and DAT (up to 91% for the temporal lobe, Moll et al., 1989) as compared to normal aged controls.

Looking upon the dopaminergic system, PD and DAT clearly can be differentiated when comparing striatal DA content [PD: about 10% of controls (Hornykiewicz, 1976); DAT: about 90% of controls (Jellinger und Riederer, 1984)]. In other brain areas the loss in DA in PD varies, beeing greatest in globus pallidus (-78%; Rinne et al., 1973, 1979; Birkmayer and Riederer, 1983), substantia nigra (-83%; Javoy-Agid et al., 1984; Rinne et al., 1973, 1979; Birkmayer and Riederer, 1983; Bobkobza et al., 1984), ventral tegmental area (-60%; Javoy-Agid et al., 1984), thalamus (-35%; Rinne et al., 1973), hypothalamus (-45%; Javoy-Agid et al., 1984; Rinne et al., 1973, 1979), nucleus accumbens (-62%; Javoy-Agid et al., 1984; Hornykiewicz, 1980; Bobkobza et al., 1984) and amygdaloid nucleus (-60%; Javoy-Agid et al., 1984).

In DAT great variations in DA content ranging from 45% over controls (Yates et al., 1979) to a 46% reduction (Winblad et al., 1982) in caudate nucleus and 30% over controls (Adolfsson et al., 1979) to a 34% reduction (Mann et al., 1980) in putamen have been reported, while our own data (Moll et al., 1989) show a reduction in hippocampus by 53% and amygdaloid nucleus by 51%. The DA metabolite HVA is reduced in neocortex (frontal lobe: 61%; parietal lobe: 63%; temporal lobe: 60%) and hippocampus by 55%. The nigrostriatal dopaminergic system, which is mainly affected in the pathogenesis of PD, is not affected at all in DAT (caudate nucleus, DA: 113%, HVA: 107%; putamen, DA: 99%, HVA: 94% of controls).

Noradrenaline has been found to be reduced in PD as compared to controls in the caudate nucleus, 46% (Birkmayer and Riederer, 1983) to 63% (Rinne et al., 1973); putamen, 56% (Birkmayer and Riederer, 1983); basal ganglia, 40% (Hornykiewicz, 1976) with slight changes in globus pallidus (79 to 83%; Rinne et al., 1973; Birkmayer and Riederer, 1983). The decrease seems to be similar in substantia nigra (31 to 48% of controls: Hornykiewicz, 1980; Rinne et al., 1973; Birkmayer and Riederer, 1983), locus coeruleus (57% of controls: Hornykiewicz, 1980),

nucleus accumbens (40% of controls: Hornykiewicz, 1980; and 62% of controls: Birkmayer and Riederer, 1983), hippocampus (39% of controls: Scatton et al., 1983), thalamus (36% of controls: Rinne et al., 1973; and 70% of controls: Birkmayer and Riederer, 1983), hypothalamus (73 to 118% (Javoy-Agid et al., 1984; Hornykiewicz, 1980; Rinne et al., 1973, Birkmayer and Riederer, 1983) frontal cortex (22% of controls: Scatton et al., 1983), cerebral cortex (34% of controls: Rinne et al., 1973) and cerebellar cortex (75% of controls: Rinne et al., 1973).

Some observations of neurotransmitter decline were made for the serotoninergic system, where in PD serotonin (5-HT) declines by 24 to 59% in various brain regions (Javoy-Agid et al., 1984; Rinne et al., 1974; Scatton et al., 1983; Birkmayer and Riederer, 1983) and 5-hydroxyindole acetic acid (5-HIAA) by 11 to 35% (Rinne et al., 1974; Scatton et al., 1983; Birkmayer and Riederer, 1983), while sometimes increase in 5-HIAA content was observed (Birkmayer and Riederer, 1982). In DAT decline of the serotoninergic system is more pronounced than in PD. In agreement with the literature (Gottfries et al., 1986; Reinikainen et al., 1988) we found a significant reduction of 5-HIAA in the hippocampus by 66% as compared to controls (Moll et al., 1989). In cortical regions reduction in 5-HT is 55 to 70% and reduction in 5-HIAA is about 55% (Moll et al., 1989).

Summarizing the data from morphological and neurochemical changes, one must conclude that in PD both substantia nigra and locus coeruleus are heavily damaged to about the same extent, whilst dorsal raphe nucleus and nucleus basalis of Meynert are only moderately affected. The degeneration of the substantia nigra is accompanied by a heavy loss of dopamine, but the decrease in noradrenaline does not adequately reflect the neuron loss in locus coeruleus when compared to the dopaminergic degeneration. Loss of serotonin in some brain regions is about the same as noradrenaline, although cell loss in the dorsal raphe nucleus is lower as cell loss in locus coeruleus. Moderate cell loss in nucleus basalis of Meynert is correlated with a moderate decline in CAT activity in PD.

In contrast only a slight loss of neurons in substantia nigra was found in DAT. Also degeneration of locus coeruleus, dorsal raphe nucleus and nucleus basalis of Meynert is moderate with loss of neurons being slightly more pronounced in the locus coeruleus and nucleus basalis of Meynert. Neurochemistry is well-correlated to neuropathological findings. No significant reduction in dopamine was found, coinciding with the very moderate damage of substantia nigra. While noradrenaline deficit mirrors directly the neuron loss in locus coeruleus, serotonin decline is slightly higher by comparable neuron loss in the dorsal raphe nucleus. The tremendous decline in CAT activity in DAT is comparable to the decline of dopamine in PD and here seems to be the outstanding characteristic of DAT. These findings are not reflected when comparing the synthezising areas, nucleus basalis of Meynert and substantia nigra. Neuron loss in nucleus basalis of Meynert in DAT (70%) is less pronounced than neuron loss in substantia nigra in PD (90%).

MEMBRANE CONSTITUENTS AND MEMBRANE STABILITY

Nucleus basalis of Meynert, the major cholinergic system projecting to neocortex, amygdala and hippocampus, was analyzed by differential scanning calorimetry (Riederer et al., 1989). In DAT thermostability significantly decreases from 81 to 68 °C ($p=0.0072$; Wilcoxon rank sum test) as compared to controls, reflecting either neuronal loss and/or changes in lipid-protein composition within these area. In PD patients

substantia nigra shows, as well, a decrease in enthalpy as a reduction in thermostability as compared to controls (H:-43.7±16.0 J/g (controls), +82.3±43.9 J/g (PD), p=0.0095, Wilcoxon rank sum test; thermostability: 57.9±6.5 °C (controls), 48.4±4.0 °C (PD), p=0.038, Wilcoxon rank sum test; Riederer et al., 1989). Again these alterations indicate a neuronal loss and/or changes in lipid-protein composition. They also may reflect increased lipid peroxidation which was found in substantia nigra of patients with PD by Dexter et al. (1989).

Our analysis of phospholipids (PL), cholesterol (Ch) and lecithin (PC) content reveal that our conclusions on changes in lipid-protein composition are in line with these suggestions.

In PD brains (n=8) we analyzed putamen for Ch, PL and PC content. There was a decrease in PL in this brain region (28.8 ± 1.0 mM (44 controls); 22.8 ± 0.4 mM (8 PD); $p < 0.0033$, Wilcoxon RST). However, Ch and PC were unchanged (all data as mean ± s.d.). In DAT there is a loss of total protein (54% of controls) in the nucleus basalis of Meynert and in the frontal cortex (78% of controls). The increase of lecithine (130%), Ch (126%), PL (131%), triglycerides (150%) in this preliminary study (3 controls matched for age and post mortem time to 3 DAT) is not significant. Further and extended studies are performed to ensure these changes. Moreover, a detailed mapping of PL-compounds and fatty acids may give solid evidences for the observed significant changes in thermostability and enthalpy of nucleus basalis of Meynert.

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CHARACTERIZATION OF ALZHEIMER NEUROFIBRILLARY TANGLE (NFT) EPITOPES AND THE BINDING SITE FOR THE REGULATORY SUBUNIT (RII) OF cAMP-DEPENDENT PROTEIN KINASE II IN HUMAN MICROTUBULE-ASSOCIATED PROTEIN 2 (MAP-2)

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Monoclonal antibodies (mAbs) generated against enriched protein preparations of Alzheimer neurofibrillary tangles (NFT) have been used as probes to study the structure of the proteins associated with NFT[1]. Anti-NFT antibodies have been found to cross react with purified neurofilaments, ubiquitin, tau and MAP-2 suggesting that NFT represent a derangement of the neuronal cytoskeleton. The question remains, however, whether these proteins are integral components of NFT or have regions of homology with NFT proteins. To address this question we screened a human brain cDNA expression library with a pool of mAbs raised against SDS-extracted NFT and have isolated several cDNA clones[2]. One of these clones detects 6 kb and 9.5 kb transcripts in human neuroblastoma mRNA preparations[2]. Sequence analysis of the cDNA has determined that the 1.7 kb insert encodes 569 amino acids of the projection arm of MAP-2[3].

To map the NFT epitopes within the MAP-2 protein the cDNA insert was subcloned into pATH 11. This vector utilizes the trp E promoter and expresses a portion of the trp E gene product, anthranilate synthetase, fused to the MAP-2 peptides. Plasmid-containing E. coli HB101 cultures were induced with indoleacrylic acid and produced approximately 20% of the total cellular protein as fusion protein. Expressed proteins were subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were then incubated with various NFT mAbs. A polyclonal anti-MAP-2 serum (generously supplied by Dr. Richard Vallee) was used as a control. Visualization was by 4-chloro-1-naphthol. By subcloning successively smaller restriction fragments into the pATH 11 vector we were able to assign the epitopes for four anti-NFT mAbs to a 51 amino acid region in the projection arm of the MAP-2 protein. The sequence which encodes this region is located between nucleotides 282 and 438 of the insert and is flanked by Alu I restriction sites (Figure 1).

As shown in Figure 2, all four anti-NFT mAbs recognize the Alu I-Alu I encoded fusion protein. The polyclonal anti-MAP-2 antibody did not react with this fragment. The amino acid sequence of this region is

EPSDQKEKESEKQSKPGEDLKHAALVSQPETTKTYPDKKDMQGTEEEKAPL.

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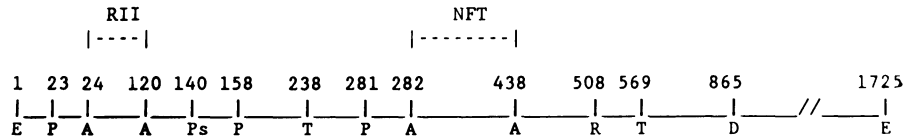

Figure 1. Restriction map and sizes of cDNA clones used for expression of human MAP-2 fragments. The restriction map indicates only those sites used for subcloning and expression. The restriction sites used are as follows: E, Eco RI; P, Pvu II; A, Alu I; Ps, Pst I; T, Taq I; R, Rsa I; D, Dra I. The number above each restriction site indicates the position of the first nucleotide of the enzyme recognition sequence. Position 1 is the Eco RI site in the linker GAATTCCGX, where X indicates the first nucleotide within the MAP-2 cDNA clone.

The MAP-2 epitopes which bind the anti-NFT mAbs are unique among the MAP-2 epitopes thus far described in that they are not solubilized by SDS extraction of tangle preparations and appear to be integral components of NFT. At least three of these mAbs recognize unique sites within the MAP-2 fusion protein. These epitopes may consist of contiguous amino acid sequences or possibly of sites generated by secondary structure. The 51 amino acid region in MAP-2 does not have extensive sequence homology with other cellular proteins sharing epitopes with NFT, such as tau, or any of the vast array of amino acid sequences filed in the data bases. Although two of the antibodies, mAb 635 and mAb 636 recognized both MAP-2 and tau[4], two other antibodies, mAb 322 and mAb 39, did not recognize human or bovine tau, indicating that reactivity with the 51 amino acid region in the MAP-2 fusion protein was not solely due to cross-reactivity with tau. Monoclonal antibodies 322 and 39 show distinct reactivities. Immunofluorescent staining

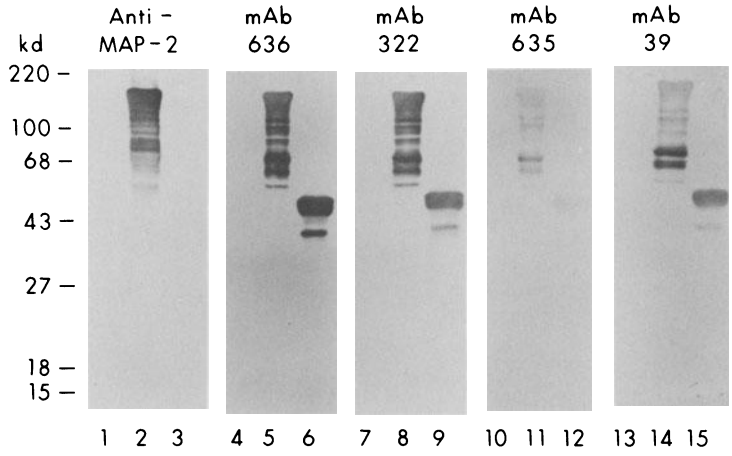

Figure 2. Western blots of fusion proteins recognized by anti-NFT antibodies. Lysates from HB101/pATH 11 were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were incubated with the indicated antibodies and visualized with 4-chloro-1-naphthol. Lanes 1, 4, 7, 10 and 13, no cDNA inserts; lanes 2, 5, 8, 11 and 14, 1709 bp MAP-2 cDNA; lanes 3, 6, 9, 12 and 15, Alu I-Alu I (282-438) insert. (Reproduced with the permission from J. Neurosci. Res.[3])

of HeLa cells was seen using mAb 322 but not using mAb 39[4]. The determinant in NFT that is recognized by mAb 39 is highly stable to formalin fixation. In contrast, the determinants recognized by the other three mAbs are destroyed by formalin[4].

Prior to this study, mAb 39 had not been observed to recognize any normal protein and was believed to be specific for NFT. The ability of mAb 39 to recognize the partial human MAP-2 fusion protein under the denaturing conditions used in the Western blotting suggests that the epitope it detects may be a feature of the primary sequence of human MAP-2. Monoclonal antibody 39 did not recognize the human MAP-2 fusion protein under the non-denaturing conditions used for the detection of bacteriophage clones on nitrocellulose. It reacted more strongly with the fusion protein containing the 51 amino acid region than with fusion protein containing the entire 569 amino acids, suggesting that, even under denaturing conditions, the epitope for mAb 39 may be partially hidden in the larger fusion protein. The hypothesis that the 51 amino acid region where the NFT epitopes lie is not exposed in native MAP-2 is consistent with the finding that the polyclonal anti-MAP-2 antibody fails to recognize this portion of MAP-2, although it recognizes epitopes on either side of this region[2]. These findings suggest that either the conformation of MAP-2 is highly altered in NFT or, more probably, that only a fragment of MAP-2 is present or exposed.

Our results indicate that the outer region of the human MAP-2 projection arm shares multiple epitopes with NFT. These epitopes are present in a large proportion of NFT[4] and are resistant to detergent extraction. The epitopes appear to be attributable to the primary sequence of MAP-2, and are in two cases not detectable in tau and in one case has been localized to paired helical filaments (PHF) by immunoelectron microscopy[5]. The data provide the strongest evidence to date that a portion of the MAP-2 molecule participates in NFT formation and justify a search for MAP-2 sequences in future biochemical analyses of PHF.

MAP-2 binds to several cytoskeletal and cytosolic proteins and these interactions may be involved in regulating normal cellular functions within neurons. It is possible that changes in the binding characteristics of MAP-2 to some or all of these proteins occur in brain tissue of individuals with Alzheimer disease. Immunohistochemical techniques have demonstrated that the regulatory subunit (RII) of type II cAMP-dependent protein kinase and MAP-2 co-localize in normal brain[6]. Chymotryptic digests of MAP-2 protein showed that the projection arm of MAP-2 retained the cAMP-dependent protein kinase activity[7]. To better understand the interaction of MAP-2 with cAMP-dependent protein kinase, we have identified the region of the MAP-2 protein which binds RII. Figure 3 is an autoradiogram of a nitrocellulose filter containing fragments of MAP-2 protein expressed in bacteria and probed for RII-binding activity. Binding of RII was seen for all MAP-2 fragments encoded by clones containing nucleotides 24-120 of the 1.7 kb cDNA (lanes 4-6, 8-11). The sequence of this 31 amino acid region is

DRETAEEVSARIVQVVTAEAVAVLKGEQEKE.

Proteins expressed in bacteria containing only the first 29 amino acids of the above sequence also bound RII (not shown). This region of MAP-2 is identical in the human and mouse proteins indicating that the RII-binding site is evolutionarily conserved. Because MAP-2 is phosphorylated by cAMP-dependent protein kinase, it is reasonable to assume that MAP-2-RII complexes are involved in the network of delicately balanced interactions that regulate cellular functions in neurons. It is not yet known if there are alterations in the association of RII with MAP-2 or whether there are changes in the MAP-2 phosphorylation state in NFT. Further studies on the interaction of RII and MAP-2 in NFT may provide new insights regarding cytoskeletal structure and function in Alzheimer disease. Supported by NIH grants AG-6803, AG-1136, DK-27736 and American Cancer Society grant NP-377.

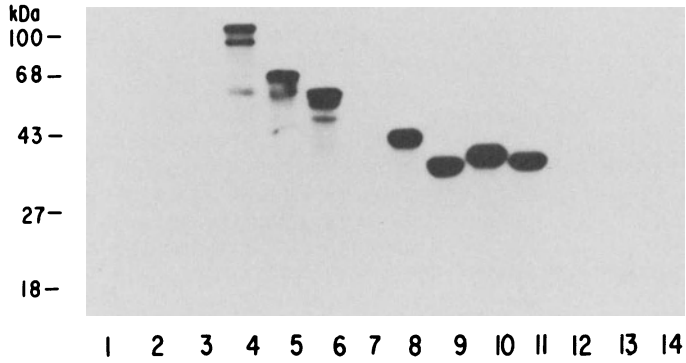

Figure 3. Autoradiogram of RII binding to MAP-2 fusion proteins. The MAP-2 restriction fragments were subcloned into pATH 11 or pUC 9 and transformed into E. coli. Expressed proteins were resolved in SDS-gels, transferred to nitrocellulose and sequentially incubated with purified bovine heart RII, anti-RII mAb 40, rabbit anti-mouse IgG and ^{125}I-protein A. Lane 1, MW markers; lanes 2 and 3, pUC 9 and pATH 11, no insert; lanes 4-6, pATH 11, bases 1-1725, 1-865, 1-508; lane 7, pUC 9, bases 865-1725; lanes 8-14, pATH 11, bases 1-238, 1-140, 23-158, 24-120, 158-281, 282-438, 438-569. (Reproduced with permission from Neuron[8])

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IDENTIFICATION AND CHARACTERIZATION OF MODIFIED FORMS OF TAU IN BRAINS WITH ALZHEIMER'S DISEASE

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In Alzheimer's disease affected brain, neurons are characterized by bundles of paired helical filaments (PHF) in perikarya and neurites (1). Antibodies raised against PHF enriched preparation were shown to react with microtubule associated protein tau, and antibodies to tau bind PHF (2,3,4). With few exceptions most of the antibodies to other cytoskeletal proteins such as neurofilament proteins or microtubule associated protein 2 have no reactivity with PHF. These observations lead to the suggestion that tau is a major antigenic component of PHF (5). PHF enriched preparations prepared by some methods were insoluble in SDS (6,7), while by other methods were soluble (8). Peptide fragments generated by proteolytic digestion of PHF enriched samples contained amino acid sequences similar to the carboxyl terminal half of the tau proteins (7,9). The reaction of PHF to two anti-tau antibodies has been demonstrated to require a pretreatment of PHF with phosphatase (2,10). These findings provided the basis for the suggestion that tau is a component of PHF and that the phosphorylation of PHF-related tau is different from normal tau. However, it is uncertain whether the abnormality of PHF-related tau is limited to phosphorylation, and if all regions of the tau molecule are incorporated into PHF. Additional information on the biochemical and immunochemical properties of PHF proteins is important for studying the abnormality in PHF.

Using a method which requires Sarkosyl treatment of the 15,000 x g supernatant of brain homogenate (11), a PHF enriched preparation was prepared. These filaments readily dissolved in 2% SDS and upon gel electrophoresis separated into several protein bands. Three of these proteins, which migrated to 60, 64, and 68 kDa, have molecular masses similar to the PHF polypeptides reported earlier (10). These proteins, referred to as PHF-related tau, were tested in our studies against various anti-tau antibodies whose epitopes have previously been determined to be distributed across a wide region of the tau molecule from near the N terminus to the C terminus (12). All anti-tau antibodies except a monoclonal antibody named Tau-1 reacted with the 60, 64 and 68 kDa proteins. These proteins, unlike normal tau, reacted with Tau-1 antibody only after incubation of the PHF preparation with alkaline phosphatase. The results indicate that the entire tau molecule is incorporated into PHF and are in agreement with previous suggestion that the PHF-related tau differs from most if not all of the normal tau in phosphorylation.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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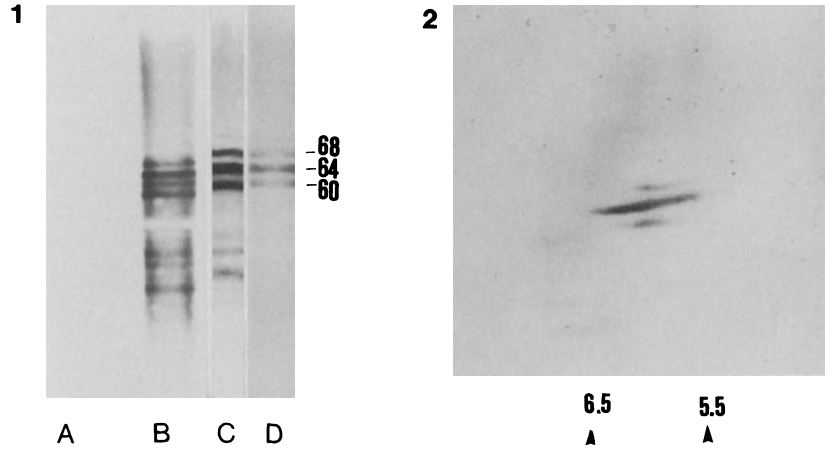

Fig. 1. PHF enriched-preparation contains proteins reactive with tau-reactive antibodies. (A), (B) & (C) are stained with Tau-1. (D) Stained with Ab 636.7. (A) No enzyme treatment. (B) The preparation, before electrophoresis, has been treated with alkaline phosphatase. (C) Blotted proteins have been treated with phosphatase.

Fig. 2. Immunoblot of two dimensional gel of PHF-related tau with Tau 46.

Besides anti-tau antibodies we found that a monoclonal antibody named Alz 50 recognized PHF-related tau (Fig. 1). Alz 50 was generated by immunization of mice with Alzheimer brain homogenate (13). Although this antibody bound poorly to neurons in normal brain, it has been shown in our previous studies to react with human and animal tau on Western blot (14). These results raise the possibility that the Alzheimer brain antigen responsible for eliciting Alz 50 antibody may be derived from PHF-related tau.

The PHF-related tau migrated slower than normal human tau on gel electrophoresis. Unlike PHF-tau which appears as three bands on immunoblots, normal human tau is composed of four to five closely migrating bands. The difference between the slowest migrating isoform of PHF-related tau and that of normal tau is about 2-3 kDa in molecular mass. Incubation of PHF-related tau with alkaline phosphatase, besides uncovered the Tau-1 site, affected the electrophoretic mobility of these proteins. Instead of three bands, the dephosphorylated PHF-tau appears as four bands (Fig 1), suggesting that the enzyme treatment may be able to convert the PHF-related tau to normal tau. Further studies using two dimensional (2-D) gel electrophoresis, however, indicate this may not be the case. 2-D gel analysis showed the pI of PHF- related tau between 5.5-6.5 (Fig 2). This is quite different from the pI of normal tau which ranges from 6.0 to 8.0. Alkaline phosphatase treatment of PHF preparation had only very little effect on the pI of PHF-related tau. The failure of restoring the pI of PHF-related tau raises the possibility that alterations other than phosphorylation may also be involved in the processing of normal tau to PHF-related tau. Identification of such changes would be fundamental for understanding the formation of PHF.

In considering the role of tau in PHF formation there is a question which concerns the spatial arrangement between different tau regions in PHF,

and the significance of various tau regions in the structural stability of PHF. Other investigators, using pronase digestion or CNBr cleavage have documented the firm association of a region of tau with PHF. This region corresponded to microtubule binding domains. To study the significance of other regions, we used immunogold labelling method. In preliminary studies an anti- tau antibody to an epitope at the N terminal region (amino acid residues 82-120 of fetal human tau) was found to bind poorly to PHF which had been incubated briefly (2 min) with chymotrypsin (10 ug/ml). In comparison, the binding of an antibody to epitope located at the C terminal region (amino acid residues 315-352) were only slightly affected (Fig. 3). Incubation of PHF with a higher concentration of chymotrypsin or trypsin was able to reduce or abolish the binding of PHF with antibody to the C end of tau. Both antibodies bound well to the untreated PHF. The enzyme treated PHF retained the ultrastructural characteristics of PHF. The results of these studies indicate that the N terminal region of the tau molecule is more accessible or susceptible to proteolysis and is most likely more peripheral than the C terminal region to the PHF proper. Although the N and possibly the C terminal regions of tau may not be essential for maintaining the structural stability of PHF, these regions may play a key role in determining whether the tau molecule is to function as promoter and stabilizer for microtubule assembly or for PHF formation. In this regard, it is interesting to note that more phosphorylated tau is less capable of promoting microtubule assembly, and is less flexible than normal tau (15, 16).

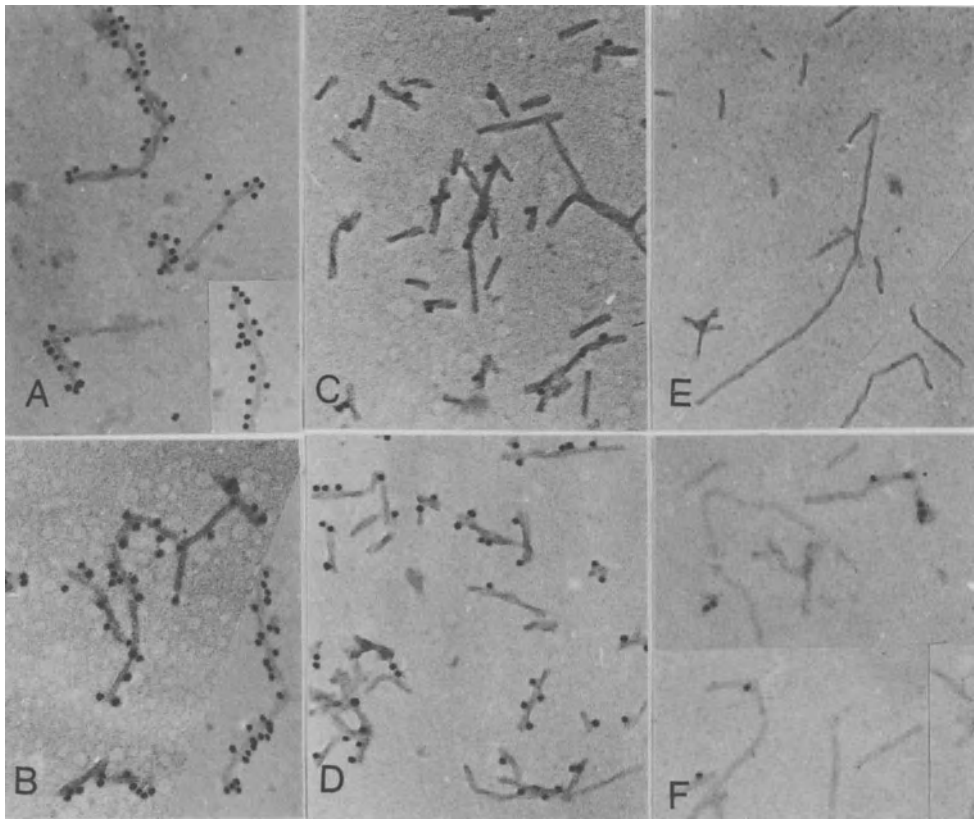

Fig. 3. Immunogold labelling of PHF with Tau 14 (A, C, E) and Tau 46 (B, D, F). (C) & (D) Treated with chymotrypsin, (E) & (F) treated with trypsin.

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PHOSPHORYLATION OF TAU PROTEIN WITH A NOVEL TAU PROTEIN KINASE FORMING PAIRED HELICAL FILAMENT EPITOPES ON TAU

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INTRODUCTION

In aged human brain and particularly in Alzheimer's disease brain, paired helical filaments (PHF's) accumulate in the neuronal cells. Recently, it was discovered that tau protein is a component of PHF. Tau protein is one of the brain-specific, microtubule-associated proteins (MAP's) and promotes the formation of microtubules in vitro and in vivo. Several groups found using antibodies that the tau in PHF is highly phosphorylated. Y. Ihara et al.[1] showed that anti PHF polyclonal antibodies contain an antibody reacting with phosphorylated tau (p-tau) but not with dephosphorylated tau (dp-tau). Grundke-Iqbal et al.[2] reported that monoclonal antibody to tau, tau-1, reacted with PHF, and reacted even more strongly with dephosphorylated PHF. Normally, tau is associated with microtubules and easily solubilized. However, tau in PHF is unusually insoluble, thus giving rise to the following question: how is tau incorporated into PHF to become an insoluble form? We assume that the phosphorylation of tau is the cause of PHF formation. To study the mechanism underlying accumulation of PHF's, we purified and characterized a novel protein kinase that phosphorylates tau protein to form PHF epitopes, and studied properties of tau phosphorylated by the kinase.

PROPERTIES OF THE PROTEIN KINASE

The p-tau in PHF had slower electrophoretic mobility on SDS-polyacrylamide gel electrophoresis than dp-tau. By detecting the activity inducing a mobility shift of tau as an index, we partially purified[3] the protein kinase from rat or bovine brain microtubule proteins. Human tau phosphorylated by the kinase reacted with the antibody to PHF prepared by Ihara et al.[1]. The kinase specifically phosphorylated tau and MAP2 among many proteins in the brain extract. The kinase also phosphorylated histone H1 but not casein. This enzyme is one of the protein serine/threonine kinases and is independent of well-known second messengers. The phosphorylation of tau by this enzyme was stimulated by tubulin under conditions conducive to microtubule formation, suggesting that the phosphorylation of tau could occur concomitantly with microtubule formation in brain. Since this kinase was usually bound to tau but not directly to tubulin, the enzyme is associated with microtubules through tau. From these properties related to tau, this kinase is designated as tau protein kinase.

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IDENTIFICATION OF THE PHOSPHORYLATION SITES ON P-TAU

For identification of the phosphorylation sites on the p-tau, tau phosphorylated with this kinase using [γ-^{32}P]ATP as a phosphate donor was digested by endoproteinase Lys-C to produce three labeled fragments, K1, K2, and K3. These three fragments were sequenced. The K2 fragment overlapped with the Tau-1 site[4] known to be one of the phosphorylation sites in PHF. This result further strengthens the possibility that tau protein phosphorylated by tau protein kinase is incorporated into PHF. As shown in Fig. 1, tubulin-binding sites on tau were located between K1 and K3 sites, while the K2 site was located in the sequence neighbouring the N-terminal side of K1. No phosphorylated sites were found on the tubulin-binding domains of tau, leading us to the idea that the interaction of tau with tubulin could induce the conformational changes on tau accessible to attack by the kinase.

These phosphorylation sites have a common sequence, SerPro or ThrPro. Other groups reported that the phosphorylation site on PHF has a SerPro sequence[4-6]. Their results correspond to ours. Well-known kinases recognizing such a sequence are growth-associated histone H1 kinase and neurofilament-specific kinase related to axonal outgrowth.[7] We consider tau protein kinase to be a member of such kinase family, and to play an important role in neurite outgrowth.

These phosphorylation sites are in homologous sequences between MAP2 and tau[8] as shown in Fig.1, indicating the phosphorylation sites are important for some function of MAP's though the sites are not tubulin-binding ones. These sites are not in tau fragments easily solubilized from PHF by proteolysis,[9] but the proteolysis-resistant residue reacts with antibodies against PHF, showing that these sites are quite insoluble even after proteolysis. The phosphorylation may induce the insolubility.

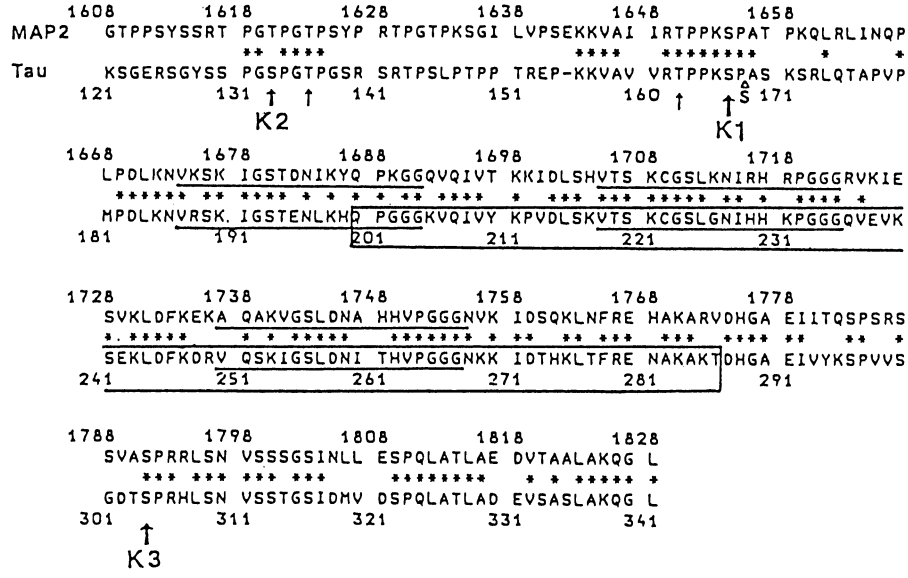

Fig. 1. Phosphorylation sites on p-tau. Arrows indicate the phosphorylation sites. This figure also shows homology between COOH-terminal sequences encoding MAP2 and tau.[8] Asterisks denote homology. Three 18-amino acid repeats (tubulin-binding sites) are underlined. The boxed area corresponds to the sequence of a fragment obtained from PHF.[9]

PROPERTIES OF P-TAU

We also studied certain properties of the p-tau. After phosphorylation, the elution position of K3 was retarded on reverse phase-HPLC, suggesting the phosphorylation of K3 increases its hydrophobicity in spite of incorporation of phosphate. K3 was further digested by endoproteinase Asp-N, and the digest was chromatographed on the reverse-phase column. Three phosphopeptides were eluted at different positions, but their sequences and phosphorylation sites were identical. This suggests that the phosphorylation at this site may induce a chemical modification. These abnormal behaviours might be related to the insolubility of PHF.

DISCUSSION

The biological significance of the formation of the PHF epitope on tau by this kinase will now be discussed. If the phosphorylation of tau by this kinase occurs concomitantly with microtubule formation and is stimulated in AD brain, we can infer that microtubule formation is stimulated in AD brain. This inference is consisitent with the findings that neurotrophic activity in AD brain extracts is significantly higher than in control brain extracts,[10] and that sprouting responses occur in AD brain.[11] Since this increased level of neurotrophic activity in AD brain appears not to compensate for neural death, neurite growth in AD brain may be abnormal and phosphorylated tau may be aggregated into PHF's. Moreover, Saitoh et al.[12] reported that phosphorylation of a 60-kDa protein is stimulated in AD brain. It is unknown whether their kinase is the same as our enzyme or not. The mechanism through which the phosphorylated tau is incorporated into PHF's remains unknown, but it would appear that this protein kinase plays an important part in the mechanism.

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ABERRATION OF VIMENTIN ARRAY IN FIBROBLAST FROM FAMILIAL ALZHEIMER'S DISEASE

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INTRODUCTION

The change of cytoskeletal systems in Alzheimer brain has been speculated (Takeda,1989). In the brain there are three main intermediate filaments; neurofilaments in the neuron, glial filaments in the astrocyte, and vimentin fibers in the mesenchymal cell. There are reports suggesting the change of intermediate filaments in Alzheimer' disease. Neurofilament protein is over phosphorylated (Sternberger,1985). Neurofilament protein shares antigenic epitopes with neurofibrillary changes of Alzheimer's disease (Miller,1986). The experimental neurofibriilary changes produced by aluminium intoxication in rabbit brains are composed of accumulated neurofilament fibers (Takeda,1984). Higher serum antibody titer against glial fibrillary acidic protein is reported with Alzheimer patients. In this report cultured fibroblasts from Alzheimer patients are studied. The decreased attaching ability of the cell to the stratum and the derrangement of vimentin fibers is studied by fluorescent immunostaining.

MATERIALS AND METHODS

The fibroblasts were cultured from the biopsied cutaneous tissue from 6 Alzheimer's disease patients and the 4-15 passages cells were used. The diagnosis of Alzheimer's disease was based on clinical observations and all of the cases are probable Alzheimer's disease according to the diagnostic guideline developed by the NINCDS-ADRDA Workshop (McKhann,1984). The cases whose family history disclosed more than three members of presenile dementia were classified as familial cases.The cells were maintained in Dulbeccos's modified essential medium with 10% fetal calf serum under 5% CO_2 at 37°C. Before the tissue biopsy the nature of the study was fully explained to the patients or the patients family and the agreement for the incooperation into the study was confirmed.

Assay for Cell Attaching Efficiency

The fibroblast in the passage of 4-8 were harvested by incubating in 0.02% EDTA and 0.25% trypsin for 8 minutes and the harvested cells were plated onto the dish (3.0×10^5/dish). The number of the floating cells were counted and the number of the attached cells were calculated after 10, 20, 30, and 60 minutes incubation.

Immunofluorescent Study

The fibroblast grown on polylysine-coated cover glass was fixed by 4% paraformaldehyde for 10 minutes and immunostained with the first antibody (anti-vimentin IgG(Amersham), anti-actin IgM (Transformation Res.), anti-tubulin IgM (Transformation Res.), anti-fodrin IgG(Shinnihon), anti-fibronectin IgM (Tago) for 2 hours and then with the FITC-labeled anti-mouse IgG+IgM for 2 hours and observed under Nikon fluorescent microscope.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Western blotting

Crude cytoskeletal fraction was obtained from harvested fibroblasts, electrophoresed on 7.5% gel, transfered to nitrocellulose membrane, and immunostained with the first antibody and then with the second peroxidase-labeled anti-IgG.

RESULTS

Characteristics of Alzheimer Fibroblast

The Alzheimer fibroblasts showed no difference in the appearance under phase microscope. They showed the same growth curve showing the same doubling time, 40-48 hours, with the control fibroblast in proliferating phase (Fig.1). When Alzheimer fibroblasts were maintained in the serum-depleted medium for more than 10 days, however, they showed difference in appearance. While the control fibroblast became round and detached from the dish easily, the Alzheimer fibroblast remained attached to the dish with abundant protrusions.

Efficiency of Cell Atachment

The efficiency of the cell to attach to the dish withing the limited period was compared between Alzheimer and control fibroblasts. Within 10 minutes 72% of the control fibroblast attached to the dish, while only 27% of Alzheimer cells has completed attachment (Fig. 2). The difference in the attaching efficiency was observed until 60 minutes incubation, but most of Alzheimer cells attached to the dish after 6 hours incubation.

Immunofluorescent Image of Alzheimer Cytoskeleton

After incubating the cells under the serum-depleted medium for more than 10 days, the fibroblast were immunostained with anti-vimentin, actin, phodrin, and fibronectin. The immunofluorescent staining with anti-actin (Fig.3A,B),anti-phodrin (Fig.3C,D), and anti-fibronectin (Fig.3E,F) showed no different distribution of the corresponding cytoskeleton-related protein arrangement. The staining with anti-vimentin revealed the significant difference in the distribution of fibers between Alzheimer and the control fibroblasts (Fig.3G,H). While the control cells showed the highly ordered arrangement of vimentin fibers, Alzheimer fibroblast showed the unique aberration of vimentin fiber arrangement. The even distribution of vimentin fibers were no more observed and the vimentin fibers were stained strongly in some regions but week in other regions. The continuity of the vimentin fibers seemed to be maintained. Colcemide, a reagent affecting vimentin arrangement, caused the capping of vimentin fibers in Alzheimer fibroblast as well as in the control cells.

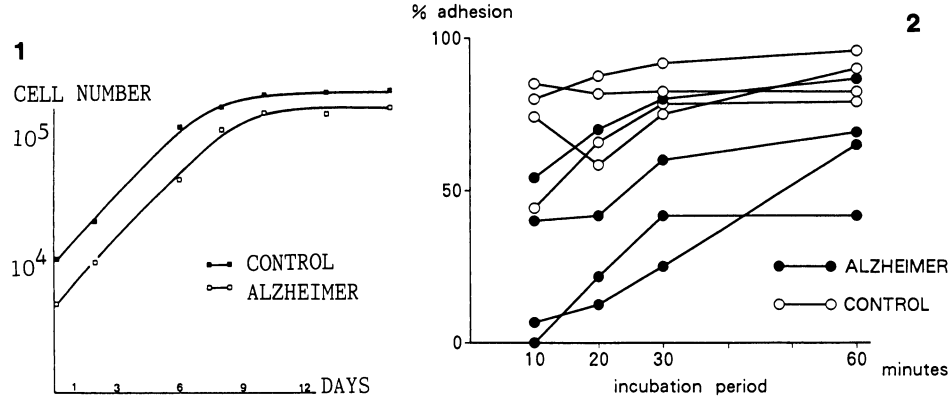

Fig. 1. Growth curve of Alzheimer (□—□) and control (■—■) fibroblast.

Fig. 2. Attaching efficiency of Alzheimer and control fibroblasts.

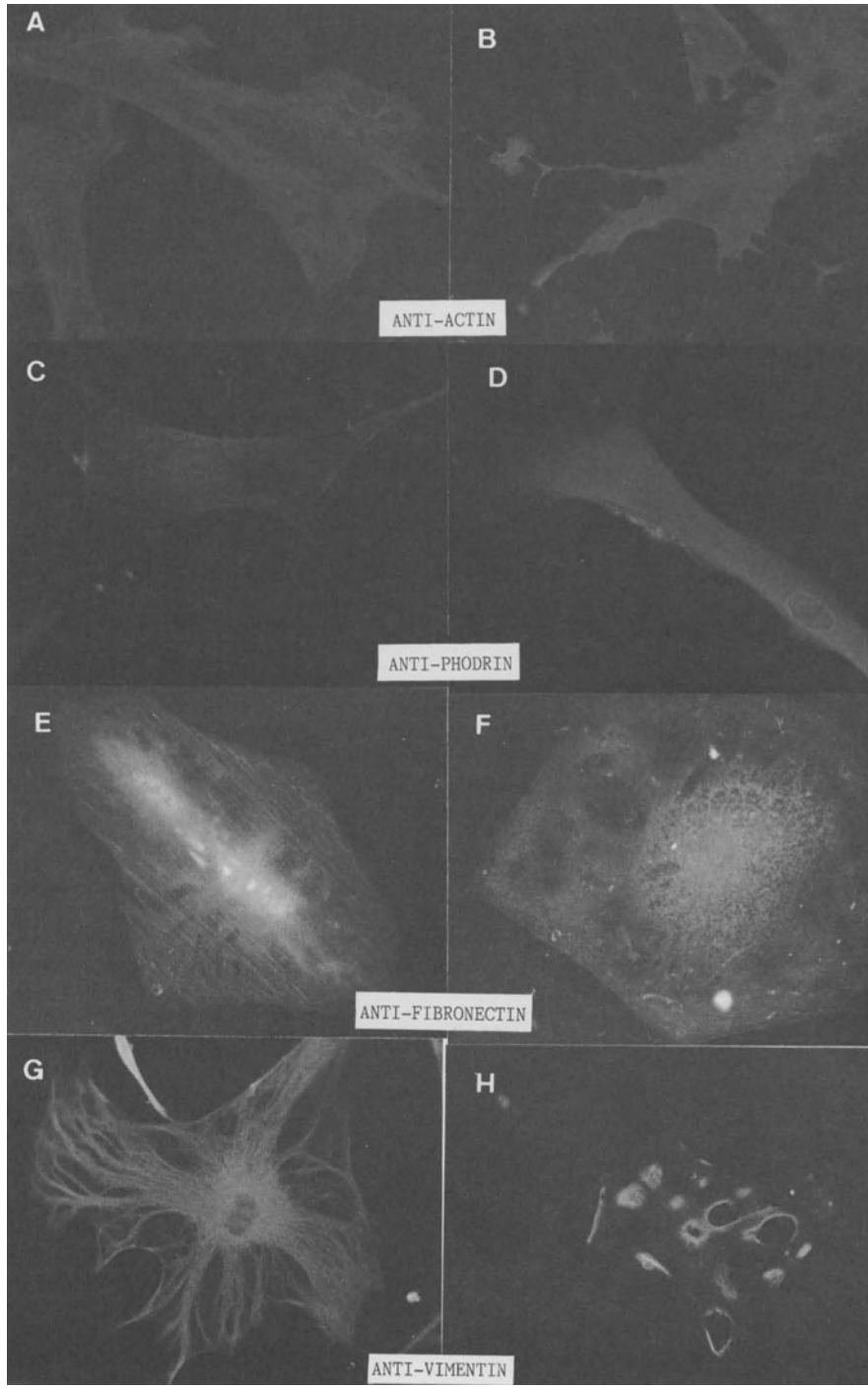

Fig. 3. Cytoskeletal arrangement observed by immunofluorescence of the control (A,C,E) and Alzheimer fibroblast (B,D,F). A,B: anti-actin. C,D: anti-phodrin. E,F: anti-fibronectin. G,H: anti-vimentin.

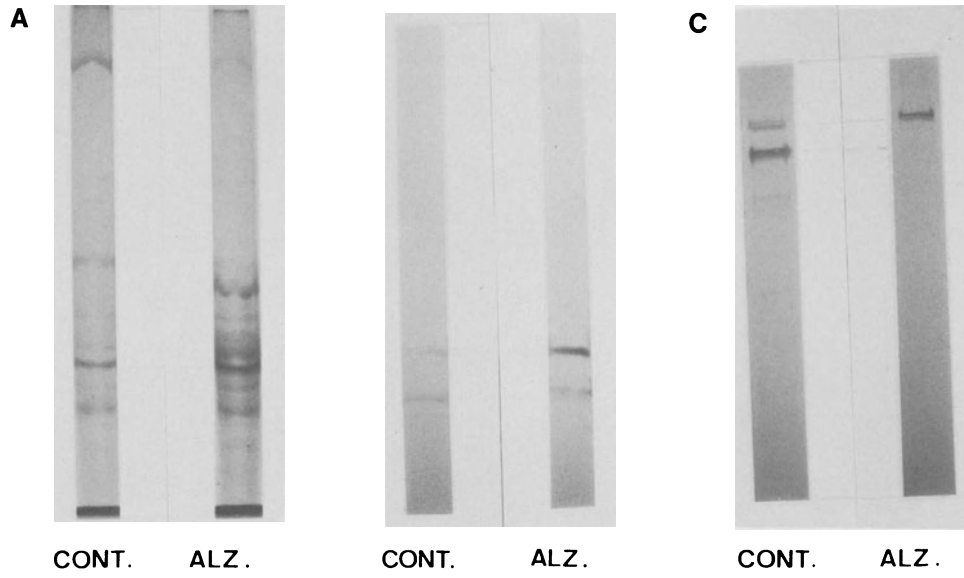

Fig. 4. Crude cytoskeletal protein from Alzheimer and control fibroblasts. A: Coomasie brilliant blue staining, B: anti-vimentin immunostaining, C: anti-phodrin immunostaining.

Western Blotting

The crude cytoskeletal protein fraction was electrophoresed and studied by Western blotting with anti-vimentin and anti-phodrin. Though vimentin from Alzheimer fibroblast showed the same molecular size with that of the control cells, the phodrin molecule from Alzheimer fibroblast showed the higher molecular weight than that of the control cells (Fig 4).

DISCUSSION

Under the proliferating phase the Alzheimer fibroblast showed the same appearance and the same growth rate, but they definitely showed the difference in the attaching efficiency to the petri dish within the limited period. The loss of the cell attachment to the plate may indicate the change in the surface structure of Alzheimer cells. Though Alzheimer cells showed no difference in vimentin arrangement in the proliferating phase, they showed the unique derrangement of vimentin fiber distribution by immunofluorescent staining. This aberration observed may be interpreted as the attachment sites of vimentin bundles to the plasma membrane is modified showing some aggragated attaching sites with more concentrated vimentin density. By Western blotting study, the change in phodrin molecule was strongly indicated in Alzheimer fibroblast. The change in phodrin molecule is plausible because it is the protein underlying the plasma membrane connecting the cytoskeletons and the plasma memebrane. Phodrin is well known to be phosphorylated and the phosphorylation of the molecule may be functionaly regulating the self-interaction of phodrin molecules and/or interaction of phodrin to other cytoskeletal proteins.

We speculate the aberration of vimentin fiber distribution observed in Alzheimer fibroblast may be functionlaaly related with the modified phodrin molecules in Alzheimer fibroblast. The aberration of intermediate fiber cytoskeleton may be the common underlying pathogenetic process in Alzheimer's disease.

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REGIONAL PHOSPHOLIPID PROFILE OF ALZHEIMER'S BRAIN:

^{31}P and proton NMR spectroscopic studies of membrane lipid extracts

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INTRODUCTION

The phospholipids constitute the major component of brain lipids accounting for greater than 60 % of total brain lipids. Most of the brain phospholipids are associated with the membrane system (1,2). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), and ethanolamine plasmalogen (EP) are the six main brain membrane phospholipids. The relative ratio of these six phospholipids (phospholipid profile) is known to exhibit regional specificity (1-4). Gray matter is especially rich in PC, while in white matter the proportion of PE is increased. While PS and PI are distributed throughout the brain in relatively even proportions, SM and EP reflect myelin rich structures. The phospholipid profile of the various regions of the brain are remarkably consistent and alterations in the profile reflect abnormalities in the membrane system. In this study, we investigated regional membrane phospholipid profiles of four major cortical areas (frontal, parietal, temporal, and occipital) in four cases of autopsy proven Alzheimer's disease using phosphorus-31 (^{31}P) nuclear magnetic resonance (NMR) spectroscopy and acidified lipid extraction of brains (3). Proton NMR spectroscopy was also performed to investigate the relative levels of unsaturated fatty acids.

MATERIALS AND METHODS

Lipid extraction

Four fresh frozen brains of pathologically proven Alzheimer's disease and normal controls (54 to 71 years old) were obtained from consented autopsy studies. Various cortical regions were blocked (1 g wet brain each) and homogenized in a Potter-Elvenhjem tissue grinder in 2 ml of ice cold water. Total lipid extracts were obtained from the homogenate by the modified Folch method with acidification (3,5,6) as briefed below.

9 ml of chloroform: methanol 2:1 (v/v) was added to the homogenate which was then washed with 0.3 ml of 6N HCl. The solution was subsequently vortexed with 14 ml of chloroform for two minutes and 3 ml of 0.05N KCl for two minutes. After centrifugation at 1,000 rpm for 20 minutes, the bottom layer was filtered, washed with 3-4 ml upper phase, vortexed for 5 minutes and then centrifuged at 1,000 rpm for 20 minutes. The upper phase was discarded and the remaining lower phase was evaporated to dryness under nitrogen to prevent oxidation of polyunsaturated fatty acids.

^{31}P Spectroscopy

The brain extracts dissolved in 2:1 chloroform-methanol were studied using a Nicolet NMR System NT-360. ^{31}P spectra of each sample contained in a 10 mm NMR tube were obtained at 145.8 MHz using a bilevel proton decoupling sequence at 26°C (recycle time 2.1 sec). Homogeneity over the sample volume was maximized using a 10 mM sodium phosphate (mono base) standard.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Subsequently, the shimming conditions of each sample were verified over the proton line width of the methyl group of methanol. The shimming conditions of the samples which showed an abnormal phospholipid profile on ^{31}P spectra were re-assessed after data accumulation to ascertain for possible error due to changes in shimming conditions. Because of the limited number of brains studied and difficulty in standardizing various variables, a quantitative correlation between the histopathologic findings and NMR spectroscopic phospholipid profile analysis was not attempted.

Proton Spectroscopy

The brain extracts dissolved in 2:1 deuterized chloroform-methanol were studied using a Nicolet NMR System NM-500. Proton spectra of each sample contained in a 5 mm NMR tube were obtained at 499.88 MHz using a one pulse sequence (recycle time 2 sec) at 25°C. The Lorentzian corrected heights of each resonance were used for quantification.

RESULTS

The following figure summarizes representative ^{31}P spectra of each cortical area in four cases of Alzheimer's disease. Spectra of occipital cortex in all four cases showed a normal phospholipid profile pattern with resonances resolved in appropriately narrow line widths. Spectra of frontal cortex in cases 1 and 4, parietal cortex in cases 1 and 2, and temporal cortex in cases 3 and 4 showed significantly decreased signal to noise ratio and severe disruption of the normal phospholipid

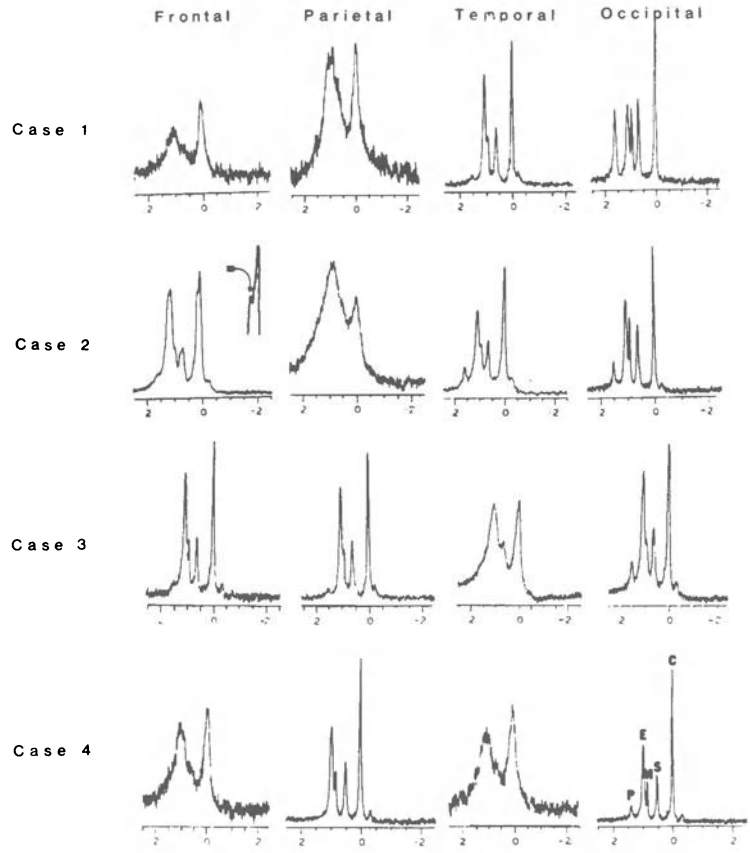

Fig. 1. **^{31}P NMR spectra of Alzheimer's brains.**
Phospholipid identification is shown in the spectrum of occipital cortex of case 4. P: plasmalogen, E: phosphatidylethanolamine, M: sphingomyelin, S: phosphatidylserine, C: phosphatidylcholine.

resonance pattern and broadening of resonance lines. Phospholipid contents in these cortices are thought to be significantly reduced. A spectrum of frontal cortex in case 2, however, showed minor broadening without significant reduction in signal to noise ratio. Additionally, an unknown resonance was clearly resolved downfield to the PC resonance (arrow). The latter findings suggest the presence of abnormal phospholipid species.

The following figure is a representative proton spectrum of acidified lipid extracts of Alzheimer's brain. The chemical shifts were referred to TMS. Chemical shift assignments are schematically shown in the figure. Table I summarizes double to single bond ratios from seven samples of cortical blocks from Alzheimer's brain which showed an abnormal ^{31}P spectrum and normal controls. The double bond to single bond ratio was significantly increased in Alzheimer's brain ($p < 0.001$, t-test).

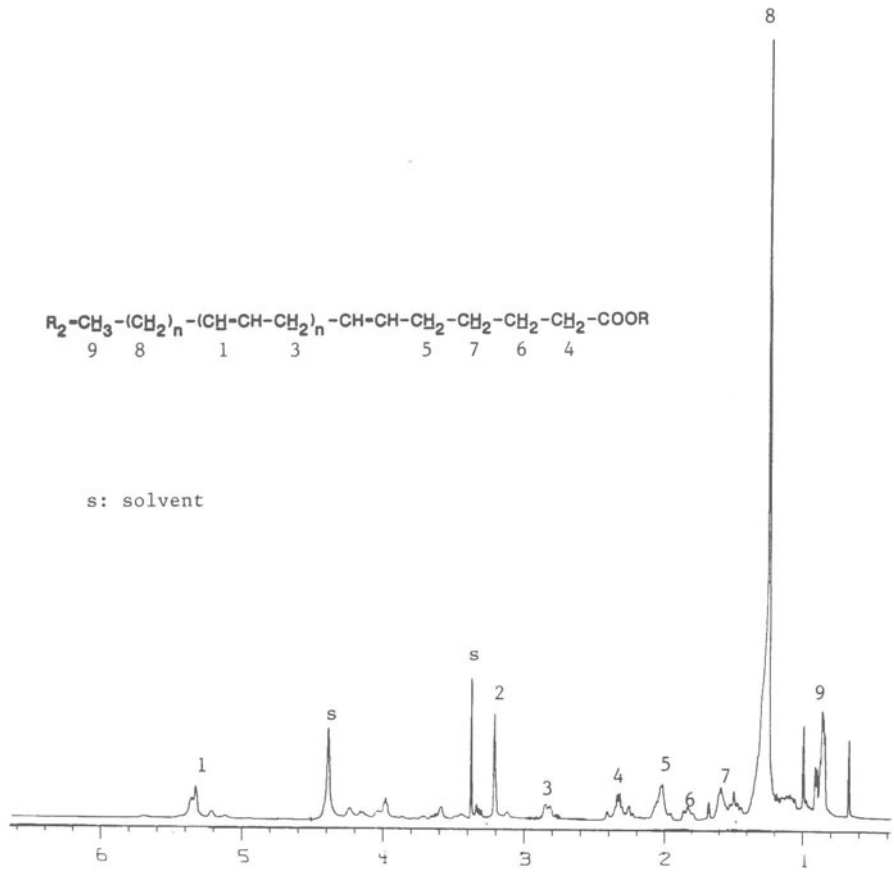

Fig. 2. **Proton NMR spectrum of Alzheimer's brain.**
Chemical shifts are assigned schematically to the corresponding fatty acid chain protons. The double to single bond ratio was determined by the ratio between the Lorentzian corrected heights of resonance 1 and 8.

Table I

Double bond to single bond ratio

Normal controls (n=7)	0.025±0.001*
Alzheimer's cortex (n=7)	0.039±0.002**

*SEM, ** $p < 0.001$ (t-test)

DISCUSSION

The ^{31}P NMR spectroscopic abnormalities presented in this study are qualitative rather than quantitative. Nevertheless, the data clearly indicated that significant changes in membrane phospholipids can occur in the frontal, temporal, and parietal cortices in Alzheimer's disease. The phospholipid profile of occipital cortex, which is believed not to be affected in Alzheimer's disease, consistently retained a normal profile. As shown in case 1, the gray matter of severely atrophic cortex in Alzheimer's disease showed almost complete disruption of the phospholipid profile. Only a gross resonance pattern enveloping the PC and PE resonances is retained and the signal to noise ratio is significantly reduced. This broadening and low signal to noise ratio may simply be accounted for by loss of total phospholipids due to the severe atrophy resulting in lesser NMR sensitivity. On the other hand, as illustrated in case 2, an unidentified resonance can be resolved in addition to the diffuse broadening of the resonance line without significant changes in signal to noise ratio. Potential technical factors contributing to the broadening of resonance lines in NMR spectroscopy were effectively excluded as the causes of the broadening of the resonance lines in this study (7). Shimming conditions for each sample were carefully assessed. The extraction method virtually eliminated the possibility of contamination of the samples by a paramagnetic substance. The solvents used for each sample were identical between studies therefore eliminating possible solvent effects. The temperature, spin rate, and decoupler power all remained constant between the studies. The hardware configuration of the spectrometer used was identical in all the studies. Therefore, the observed minor broadening and resolution of the unidentified resonance cannot be totally explained based on a reduction in the total brain phospholipids only. Rather, the findings suggest that the observed broadening in mildly atrophic cases is in part due to a clustering of multiple resonances which have chemical shifts similar to those of the corresponding normal phospholipid resonances. One of the plausible changes in the phospholipids which produces only minimal changes in ^{31}P chemical shifts, and, therefore, the broadening of ^{31}P spectra as discussed above is alteration in the side chain fatty acid components. The proton spectroscopic data presented here indicated that an increase in unsaturated fatty acids may play a role.

Several studies on phosphomonoesters and phosphodiesters in Alzheimer's disease have shown changes in the cellular concentration of the intermediary metabolites of membrane phospholipids in Alzheimer's brain (8-10). Activities of phospholipase D, one of the catabolic enzymes of membrane phospholipids, have also been shown to be reduced in Alzheimer's brain (11). The present study on regional membrane phospholipid profiles clearly demonstrated region specific abnormalities in the membrane phospholipids in Alzheimer's brain. An increase in unsaturated fatty acids may play a role in the genesis of the membrane phospholipid profile abnormalities. Further characterization of the membrane phospholipid abnormalities, especially side chain fatty acid profiles, by conventional quantitative methods such as thin layer chromatography (TLC) and gas liquid chromatography (GLC) is warranted.

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AGE-RELATED CHANGES IN THE RATE AND COMPOSITION OF THE SLOW AXONAL TRANSPORT

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INTRODUCTION

In contrast to the fast axonal transport which remains fairly constant throughout the development and aging of the animal, the rate of slow axonal transport has been shown to decrease progressively with age (Komiya, 1980). A parallel decline in the regeneration rate after nerve injury and a significant elongation of the initial delay before the onset of active regeneration has also been observed (Komiya, 1981). From the analysis of transported cytoskeletal proteins in sciatic nerves of young adult rats, we have shown that these proteins existed in the axon as two types of polymers, stable and dynamic, which could be distinguished biochemically (Tashiro & Komiya, 1989). In this study, occurrence of tubulin in these two forms in the aged animal was investigated in relation to its transport in order to elucidate the underlying changes in the organization of the axonal cytoskeleton.

MATERIALS AND METHODS

Radioactive Labelling and Axonal Transport of Cytoskeletal Proteins

Male albino Wistar rats, 7 week-old (young adult) and 80 week-old (aged), were used. Under ether anesthesia, L-[^{35}S]methionine (800-1400Ci/mmol; New England Nuclear, Boston) concentrated by lyophilization (25μCi in 0.2μl) was injected into the anterior horn area of L_3-L_5 spinal cord twice on each side. One to 12 weeks after injection, sciatic nerve and ventral root were dissected out, frozen on a plastic plate, and cut into 6mm consecutive segments.

Fractionation of Labelled Cytoskeletal Proteins

For each time point, a pair of nerves from one animal was processed together as described in Fig.1. A pair of 6mm segments from identical positions was frozen in liquid nitrogen and crushed to fine powder in a stainless steel mortar with a hammer operated by compressed air, and homogenized in 2.5ml of ice-cold Triton-buffer containing 1% Triton X-100, 50mM Tris (pH 7.5), 25mM KCl, 1mM $MgCl_2$, 5mM EGTA and 5mM DTT. The homogenate was layered onto 3ml of Triton-buffer containing 0.25M sucrose in addition and centrifuged at 100,000 g for 1hr. Proteins in the

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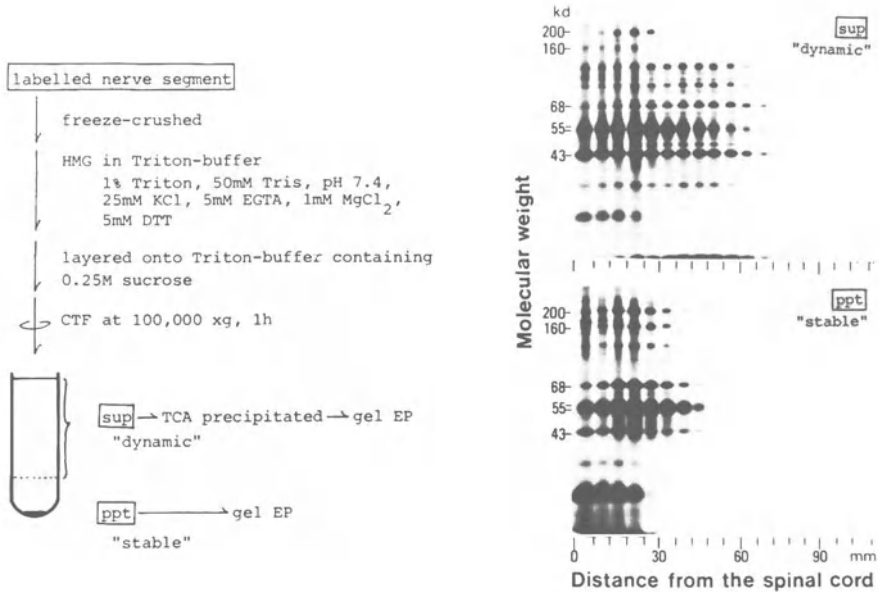

Fig.1 Fractionation of transported cytoskeletal proteins into dynamic and stable sub-populations. Result of fractionating the labelled proteins in the sciatic nerve segments obtained 2w after radioactive labelling of the spinal cord of a 7w-old rat is shown on the right.

resulting supernatant were precipitated with 10% TCA. Labelled proteins in both supernatant and precipitate fractions were separated by SDS-PAGE and visualized by fluorography (Fig.1). For the measurement of radioactivity, the bands corresponding to tubulin, actin and three neurofilament subunits were cut out from stained gels and radioactivity in each gel piece was determined by liquid scintillation counting following extraction with Soluene 350 (Packard Instrument Inc., Downers Grove, IL).

RESULTS AND DISCUSSION

Axonal Transport of Tubulin in the 7w-old Rat

As shown in Fig.1, labelled tubulin and actin were found both in the soluble and insoluble compartments after extraction with 1% Triton at 4°C. Tubulin in the insoluble fraction was not only resistant to low temperature but also to other microtubule depolymerizing conditions such as mM concentrations of Ca^{2+}, representing a distinct sub-population of stabilized polymer specific to the mature axon (Tashiro & Komiya, 1989). In the motor fibers of the young adult rat, such stably-polymerized tubulin amounts to 60% of total tubulin. Tubulin in the soluble fraction was transported apparently faster than the stably-polymerized tubulin.

When transport patterns of soluble and insoluble tubulin were analyzed separately at different time intervals after labelling as shown in Fig.2, time-dependent broadening of the tubulin wave and the appearance of a faster migrating component ahead of the main wave became evident, especially in the case of soluble tubulin. Separation of these two rate components was complete at 3 weeks after labelling (Fig.2, middle panel). The slower main wave contained most of stably-polymerized tubulin together with neurofilament proteins, while the faster component was enriched in soluble tubulin.

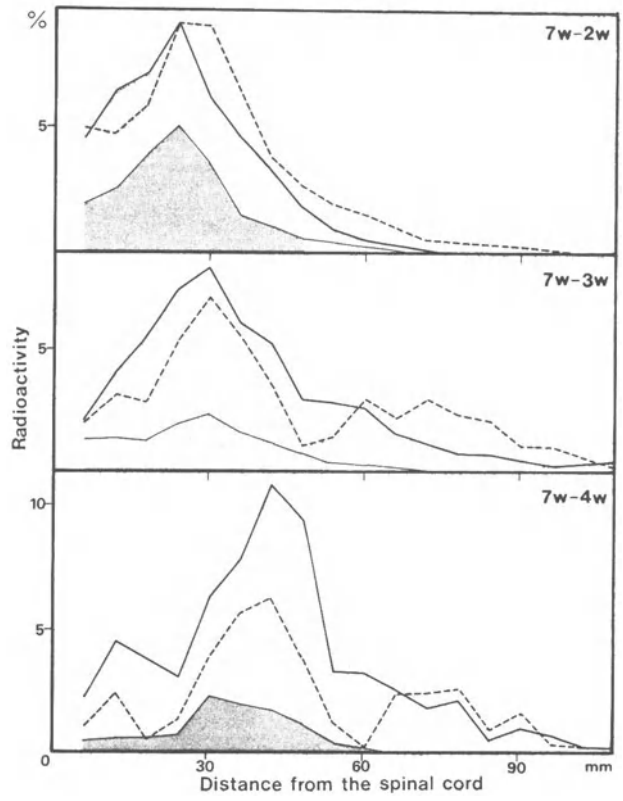

Fig.2 Axonal transport of tubulin in the sciatic nerve of the young adult rat (7w) 2, 3 and 4 weeks after radioactive labelling of the spinal cord. Radioactivity associated with stably-polymerized (——) and dynamic (-----) tubulin is as % of total tubulin radioactivity in the whole length of nerve. Neurofilament(68k)-associated radioactivity (▭) is expressed in proportion to that of tubulin.

Two sub-components of slow axonal transport have been defined originally in the optic nerve; group V or SCa with the slowest rate containing neurofilament proteins and tubulin, and a slightly faster group IV or SCb containing actin together with some cytoplasmic proteins (Willard & Hulebak, 1977; Black & Lasek, 1980; Tytell et al., 1981). The two rate components observed above seem to correspond to SCa and SCb in the sciatic motor fibers. Since tubulin and actin were present both in SCa and SCb, the two components in this system do not correspond to the movement of two discrete structural networks, the neurofilament-microtubule network and the actin-based network, as has been proposed. Rather, differences in solubility of the two components strongly suggest that they arise from the existence of two interconvertible states in cytoskeletal polymers, the stably-polymerized state and the dynamic state (Tashiro & Komiya, 1989).

Slow Axonal Transport in the 80w-old Rat

Two major differences were observed in slow axonal transport in the aged animal compared to that in the young animal. The first was a large decrease in transport rates of both stably-polymerized and dynamic forms of tubulin (Fig.3). Even at later time points (6-12w), the dual wave

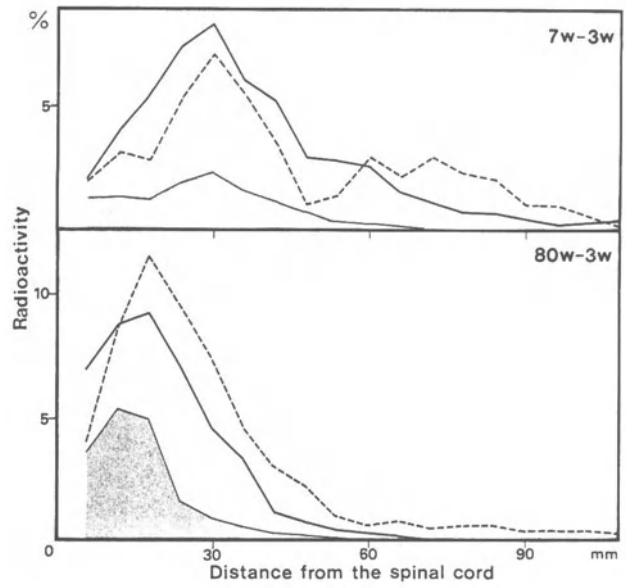

Fig.3 Comparison of tubulin transport in the sciatic nerve of the aged rat (80w) with that of the young adult rat (7w) 3 weeks after radioactive labelling of the spinal cord.

pattern of tubulin transport did not develop in the aged animal. Migration of the neurofilament proteins were even more severely retarded so that they remained almost stationary at segments 4-5 located at the exit of the motor axon from the backbone. The other feature was a progressive decrease in the proportion of stable form during the course of transport.

The analysis of rates and solubilities of transported tubulin such as described above has raised the possibility that subunits or smaller soluble fragments of cytoskeletal polymers rather than the network of polymers are the actual transport forms (Tashiro & Komiya, 1989). Selective inhibition of tubulin transport by the local application of taxol also suggested that the existence of dimer-microtubule equilibrium was essential for tubulin to be transported (Komiya & Tashiro, 1988). This was further confirmed by the recent morphological study which demonstrated that tubulin dimers were transported and added onto pre-existing polymers at their distal ends (Okabe & Hirokawa, 1988). The present results suggest that transport in the aged animal is severely retarded possibly due to impairment of the interconversion between the stable and dynamic forms and the local depolymerization-polymerization cycle.

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ASTROGLIAL GENE EXPRESSION IN THE HIPPOCAMPUS FOLLOWING PARTIAL DEAFFERENTATION IN THE RAT AND IN ALZHEIMER'S DISEASE

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Astrocytes play important roles in the complex neuronal and glial response to brain injury and pathological neuronal loss. Also referred as gliosis, this process is usually characterized by extensive astroglial hypertrophy and proliferation. In addition, reactive astrocytes undergo numerous cytological, biochemical and histochemical changes, including accumulation of vimentin, glycogen, increased oxidoreductive enzyme activity and most notably, increased accumulation of glial fibrillary acidic protein (GFAP) (Bignami and Dahl, 1976). Reactive astrocytes appear to be involved in healing responses to neuron injury by scavenging myelin and neuronal debris (Lee et al., 1977) and by releasing soluble proteins in their microenvironment which often have neurotrophic properties.

The role played by astroglia during neuronal regeneration in adult brain appears to be critical to neuronal plasticity. The early increase in hippocampal GFAP immunoreactivity following entorhinal cortex lesion is a good example of controlled reactive gliosis (Gage et al., 1988, Poirier et al., 1988). Neuronal regeneration is especially important in a disease like Alzheimer's disease (AD), where compensatory sprouting and reactive synaptogenesis is known to occur in the hippocampus of certain AD patients but not in others (Geddes et al., 1985; Hyman et al., 1987; de Ruiter and Uylings, 1987; Flood and Coleman, 1987). When present, the reorganization of the neuronal input in the hippocampus of AD patients resembles closely what has been described in rats with entorhinal cortex lesion (for review, see Cotman and Anderson, 1987). Consequently, we used the ECL rat model of reactive synaptogenesis to mimic and characterize the molecular changes that take place in the hippocampus of AD patients.

Recent studies using *in vitro* translation assay of total RNA from control and ECL rats indicate that at least seven hippocampal mRNAs showed altered prevalence 14 days after ECL (Poirier et al., 1989). The molecular weight of these translation products varies from 7 to 49 Kd. Among the most noticeable we noted the presence of the GFAP (with a molecular weight of 48-50 Kd, pI 5.5) and a 35-37 Kd polypeptide (pI 5.6), which has a migration profile similar to the apolipoprotein E polypeptide reported to be increased following peripheral nerve injury (Muller et al., 1986). The magnitude of changes prompted us to construct and differentially screen a cDNA library of hippocampal RNA extracted at the midpoint of maximal reactive synaptogenesis in order to isolate and characterize responsive transcripts.

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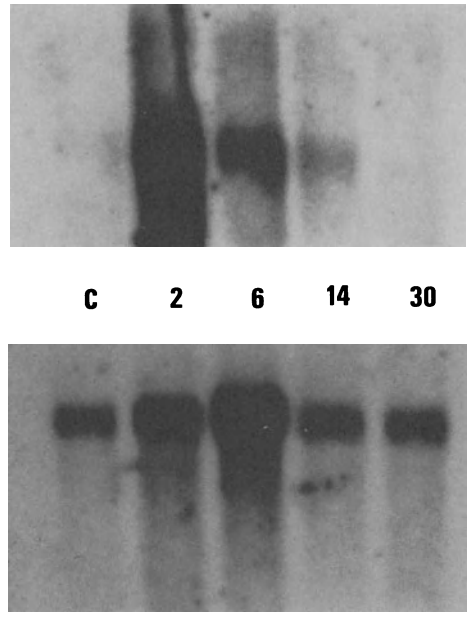

Fig. 1. Northern blot analysis of hippocampal GFAP (top) and apo E (bottom) mRNA prevalence following ECL. C: Control RNA; 2-30 : Lesioned

Seventy thousand plaques containing inserts were screened using a differential hybridization protocol and nine clones showing differential expression were isolated (Poirier et al., 1989). These 9 clones were sequenced and identified as either: the glial fibrillary acidic protein, the alpha*1*-tubulin or more interestingly, the apolipoprotein E (apo E).

The synthesis and accumulation of apo E in periphery during sciatic nerve degeneration/regeneration (Mahley, 1988) lead us to hypothesize that apo E mRNA regulation plays a role in determining the ability of CNS axons to regenerate and/or sprout following injury or neuropathology. Apo E, which is extremely critical for the transport of cholesterol to regenerating peripheral neurons (Mahley, 1988), binds to numerous brain cell types of astroglial origin (Pitas et al.,1987;Boyles et al., 1985).However, indication of its presence by immunocytochemistry does not necessarily establish that apo E is synthesized by immunopositive cells. Thus, to determine the cellular origin of the apo E mRNA changes in the deafferented hippocampi, an *in situ* hybridization for apo E mRNA was combined with immunocytochemistry for GFAP or macrophages on the same sections. Results indicate that apo E mRNA was largely restricted to GFAP-labeled astrocytes present in the denervated molecular layer of the dentate gyrus (not shown), but not over macrophages. These evidences confirm that astrocytes, in addition to macrophages in periphery, synthesize apo E.

A time course analysis of the gene expression of apo E and GFAP reveals rather dissimilar regulations (Fig. 2). Analysis of apo E and GFAP mRNA induction by Northern analysis shows that GFAP is markedly increased two days after the lesion (>10 fold) and return to control level by the 30th day post-lesion. On the other hand, apo E mRNA which lag behind by several days does not peak until the sixth day post-lesion (>6 fold), returning to control level by the 30th day post-ECL.

The hippocampal GFAP induction coincides with the early phase of glia proliferation and terminal degeneration (0-3 days) (Gall et al., 1979; Scheff et al., 1980), whereas the apo E increase (around 6 days) appears to coincide with the accumulation of dendritic polyribosomes in the denervated hippocampal neuropil, maximal ^{3}H-leucine incorporation in granule cell dendrites and acute neuronal sprouting (for review, see Steward, 1987).

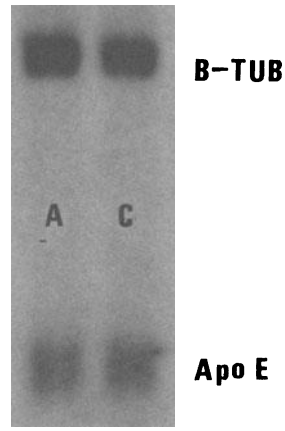

Fig. 2. Northern blot of hippocampal apo E mRNA in Alzheimer's disease. Each lane contains 3 ug of pooled total RNA from 3 AD (A) and 3 control (C) individuals. B-TUB: Beta-tubulin.

Ultrastructural studies of the molecular layer of the dentate following ECL show that throughout the 2-11 days post-lesion astrocytes progressively engulf both presynaptic terminals and preterminal axons (Lee et al., 1977). These results suggest that once neuron-derived particles are metabolized by reactive astrocytes, a large astroglial store of lipids is formed, providing a convenient and readily retrievable pool for membrane synthesis of precursors used in the assembly of myelin and neuronal sprouts. In such a model, it is conceivable that apo E is responsible for the distribution of cholesterol among sprouting neurons (commissural/associational neurons or septal neurons) and post-synaptic granule cells.

Preliminary data on apo E mRNA prevalence in the hippocampus of AD patients show that, unlike the ECL rat model, AD patients are unable to induce apo E in response to entorhinal and hippocampal neuronal loss (Fig. 3). These results contrast significantly with the GFAP gene expression which show, as it is the case for ECL rats, an increase of its mRNA prevalence in AD (Poirier et al., 1988). These data suggest that while the brains of AD patients are able to induce astrocytic reactivity in response to neuronal loss through GFAP increase, those same patients appear unable to induce the synthesis of apo E in response to deafferentation. The loss of cholesterol transport in AD could explain why many research groups failed to observe hippocampal terminal proliferation and reactive synaptogenesis in many AD patients (Represa et al., 1988; Flood and Coleman, 1987; de Ruiter et al., 1987). Studies are underway to characterize the apo E response in a larger sample of AD and control individuals.

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BLOOD-BRAIN BARRIER DISTURBANCE IN PATIENTS WITH ALZHEIMER'S DISEASE IS RELATED TO VASCULAR FACTORS

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INTRODUCTION

One of the theories of the pathogenesis of Alzheimer's disease (AD) is that blood-brain barrier (BBB) dysfunction is the primary event (for review see 1,2). Also in aging, BBB dysfunction has been suggested (for review see 2). AD is associated with aging, i.e., it is more frequent in higher age. Many other diseases are in the same way associated with aging and thus expected to occur together with AD quite frequently. Some of these age-associated diseases, for instance hypertension, diabetes mellitus and various manifestations of arteriosclerosis have an important effect on the cerebral vasculature and the BBB function (2,3). Thus, when examining the BBB function in aging and AD, the coexistence of other diseases suspected of interfering with the BBB function should be taken ino account.

MATERIAL AND METHODS

In the study, 118 patients with AD, males/females: 42/76, mean age (± SD) 71.8 ± 7.3 years, with diagnoses according to the NINCDS criteria (4), were included. The patients underwent a thorough examination, including CT scans, which in no case revealed other findings than atrophy (no infarcts were found). 51 patients had early onset AD (onset before the age of 65), and 67 late onset AD (onset at the age of 65 or over).
The controls consisted of 50 healthy individuals, males/females: 28/22, mean age (± SD) 71.5 ± 10.8 years. Inclusion criterion was "Mini-Mental State" score of 28-30.
In the AD group, clinical vascular factors were recorded: mild hypertension (n=24), mild non-insulin-dependent diabetes (n=3), and mild ischemic heart disease (n=27). In all, 44 patients had clinical vascular factors.
In 41 AD patients, also participating in a study of clinical symptomatology (5), an evaluation of the most pronounced symptomatology was made. The patients were moderately to severely demented. Patients with mild dementia were not included, since in this stage, no clear regional symptomatology has developed (6). Parietal AD (n=23) was defined as prominent parietal lobe symptoms (sensory aphasia, visual agnosia, visuospatial

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dysfunction and apraxia). Non-regional AD (n=18) was defined as obvious clinical evidence of memory impairment and general cognitive involvement (difficulties in abstract thinking and problem-solving, reduced logical/analytical ability) without or with only mild parietal symptomatology. By lumbar puncture, 12 ml of CSF was collected. The albumin ratio (CSF-albumin/serum-albumin) was used as an indicator of BBB function. For statistical comparisons between groups, the Wilcoxon 2-sample test was used for quantitative and the chi-square test for qualitative variables, and the Spearman correlation coefficient was used for correlations.

RESULTS

The AD group showed significantly ($p<0.01$) higher mean albumin ratio than the control group (Table 1). There was no significant difference in mean albumin ratio between the late onset AD group (n=67), albumin ratio (± SD) 7.1 ± 3.1, and the early onset AD group (n=51), albumin ratio (± SD) 6.5 ± 2.5.
In no group did the albumin ratio differ between sexes. In the AD group, there was no relation between albumin ratio and severity of the disease. The correlation between age and albumin ratio was 0.22 in the AD group and 0.26 in the control group.
When AD patients with vascular factors (n=44) were compared with the group without such factors (n=74), the former were found to have a significantly ($p<0.0001$) higher mean albumin ratio (Table 1). When AD patients without vascular factors (n=74) were compred with controls, no significant difference in mean albumin ratio was found (Table 1). When comparing parietal AD (n=23) with controls, no significant difference in albumin ratio was found, in contrast to the higher ($p<0.0001$) albumin ratio in the non-regional AD group (n=18) compared with controls (Table 2). The non-regional AD group was older, mean age (± SD) 77.9 ± 6.6 years, than the parietal AD group, mean age (± SD) 60.3 ± 8.6 years ($p<0.0001$), and had a higher frequency of clinical vascular factors, 10/18 (56%) versus 4/23 (17%) ($p<0.05$).

Table 1. COMPARISON BETWEEN AD PATIENTS WITH AND WITHOUT VASCULAR FACTORS AND CONTROLS

	n	ALBUMIN RATIO (mean + SD)
AD	118	6.8 ± 2.9
AD-vasc. fact.	44	8.1 ± 3.6
AD-no vasc. fact.	74	6.1 ± 2.0
CONTROLS	50	5.7 ± 2.1

AD vs controls; $p<0.01$
AD-vasc. fact. vs controls; $p<0.0001$
AD-no vasc. fact vs controls; NS ($p=0.21$)
AD-vasc. fact. vs AD-no vasc. fact.; $p>0.0001$

DISCUSSION

Indirect evidence for defective BBB function in AD has been demonstrated in some postmortem studies in the form of findings of extravasated plasma proteins in brain parenchyma of AD patients (1,2). Some CSF studies (7,9) have shown a BBB damage in AD, others have not (9,10). The finding that AD patients without vascular factors did not differ significantly from controls in albumin ratio (Table 1) suggests that AD does not involve BBB dysfunction. Previous CSF studies of BBB function in AD have not registered vascular factors, which may explain the inconsistent findings (7,8,9,10). The frequency of vascular factors does not exceed what is expected in AD (for review see 11). In the present study the clinical vascular factors in AD patients were of low severity and regarded as insufficient to produce dementia all by themselves, and no AD patient had cerebral infarctions.

The absence of correlation between age and albumin ratio in the control group (aged 52-85) suggests that the BBB function does not decline with age, at least not within this age range.

In summary, this study suggests that the BBB dysfunction found in AD is related to vascular factors and not a consequence of the disease itself, i.e., that neither pure AD nor normal aging is associated with a decline in BBB function. However, among AD patients fulfilling the NINCDS criteria for probable AD, a group of patients with mild BBB dysfunction emerge, characterized by higher age, concomitant vascular disease and less regional (=less parietal) symptomatology.

The BBB is situated in the brain capillaries, the structural basis being the tight junctions between the endothelial cells (2). In absence of other factors explaining BBB-damage (tumours, infarctions, etc.), the BBB damage found may be related to damage to the small cerebral vessels.

Table 2. COMPARISON BETWEEN PARIETAL AD, NON-REGIONAL AD AND CONTROLS.

	n	ALBUMIN RATIO	Sign.
PARIETAL AD	23	6.8 ± 2.4	ns
CONTROLS	50	5.7 ± 2.1	
NON-REGIONAL AD	18	8.4 ± 3.7	p<0.0001
CONTROLS	50	5.7 ± 2.1	

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AN EARLY CHANGE OF NEUROFIBRILLARY TANGLE FORMATION

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INTRODUCTION

Neurofibrillary tangle (NFT) is a pathological hallmark of Alzheimer's disease (1). NFT's are fibrous masses in neuronal perikarya and proximal neurites, and usualy hematoxylinophilic on H&E stain and very argyrophilic on silver impregnation (intracellular tangles). They are not static lesions and show evolutionary changes from intracellular to extracellular location. Extracellular NFT's are eosinophilic and less argyrophilic, and often are regarded as tombstones of neurons or ghost tangles (2,3). However, little has been known about initial changes of NFT formation. In this communication, we describe that there is an early change of NFT formation demonstrable by the modified Bielschowsky (Hirano) method.

MATERIAL AND METHOD

The modified Bielschowsky preparation (4) of many aged brains has been reviewed carefully with special attention to argyrophilic structures.

Two cases (71 and 72 year old men) of diffuse Lewy body disease with mild senile changes and a case (63 year old woman) of Alzheimer's disease with marked senile changes were selected for the immunohistochemical study.

Six micron sections of formalin-fixed, paraffin-embedded hippocampus were dephosphrylated and immunostained with a monoclonal antibody to tau protein, Tau-1 (5). The bound immunoglobulins were detected by the avidin-biotin complex method using 3-amino-9-ethylcarbazole as chromogen. After immunoreactive structures were photographed, bound antibodies were removed by immersion in glycine-HCl buffer (pH2.2) for 1 hour, and the sections were restained by the modified Bielschowsky method. Argyrophilic material was examined carefully and compared with immunoreactive structures.

RESULT

Numerous intracellular and extracellular NFT's were observed in a case of Alzheimer's disease by the modified Bielschowsky method. In addition to rare intracellular NFT's, many neurons containing diffuse or finely granular, argyrophilic material were found in 2 cases of diffuse Lewy body disease with mild senile changes. Unlike typical intracellular NFT's which showed strong fibrillary immunoreactivity with Tau-1, these neurons reacted

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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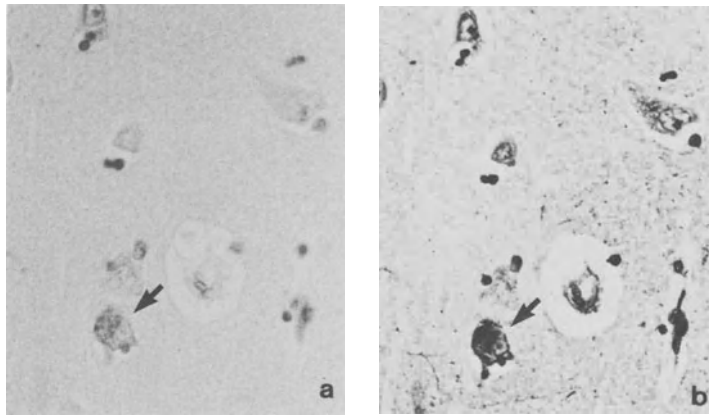

Fig. 1 A neuron with finely granular, argyrophilic material (b) shows diffuse or finely granular, weak immunoreactivity with Tau-1 (a). (prosubiculum of a case with diffuse Lewy body disease, a: Tau-1, b: mod. Bielschowsky, x100)

weakly with the antibody, and the immunoreaction products were distributed in a diffuse pattern (Fig. 1). Extracellular NFT's were poorly decorated with Tau-1.

DISCUSSION

This study demonstrates that in brain from aged subjects, some neurons have argyrophilic properties different from that of intracellular or extracellular NFT's. This type of neuron was found more frequently in brains with fewer NFT's suggesting that it may represent neurons at an early stage of NFT formation. This possibility was examined by immunostaining with Tau-1. The antibody has previously been shown to detect epitopes associated with paired helical filaments, straight filaments, granular material or ribosomes in Alzheimer brain tissue (6,7). Our finding of Tau-1 immunoreactivity in the diffuse argyrophilic neurons, therefore, may be indicative of cytoskeletal changes in these neurons. Weak Tau-1 immunoreactive neurons have recently been observed by other's in Alzheimer brains, as well (6). According to Bancher et al (6), these neurons contain "stage 0 tangles". The colocalization of the diffuse argyrophilic structures and the weak Tau-1 positive elements observed in our study suggests that the modified Bielschowsky method (Hirano) is an useful method for evaluating the evolutionary changes of NFT's.

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MONOCLONAL ANTIBODIES AGAINST PAIRED HELICAL FILAMENT IN ALZHEIMER'S DISEASE AND PARKINSONISM-DEMENTIA COMPLEX OF GUAM

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INTRODUCTION

Neurofibrillary tangles (NFT) are one of the most prominent pathological changes seen in the brains of Alzheimer's disease (Alz) patients. Under the electron microscope these NFT are revealed to be composed of accumulations of paired helical filaments (PHF). These structures are also observed in Parkinsonism-dementia complex of Guam (PD), and some other diseases besides Alz.

The two proteins tau and ubiquitin are reported to be integral components of PHF. But the nature of the other constituents of PHF have remained as a matter of controversy. The difficulty in purifying and solubilizing PHF convincingly does not allow us to analyze PHF directly.

To elucidate the components of PHF and the process of PHF formation, we raised monoclonal antibodies (mcAb's) against native PHF and detergent-insoluble PHF from Alz and PD, and characterized them.

MATERIALS AND METHODS

Preparation of immunogen

Detergent-insoluble PHF and amyloid were prepared from PD or Alz brain by the methods of Solkoe et al.[1] and Masters et al.[2] Native PHF were also prepared from Alz brain homogenate by the method of Yen et al.[3]

Production of monoclonal antibodies

McAb's against PHF were established by the conventional method. Balb/c mice were immunized with immunogen prepared by the above methods. All mcAb's were screened and checked by immunofluorescence (IF) with frozen sections of Alz brain and PD brain.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Characterization of mcAb's

Class of immunoglobulin (Ig) was determined by the Ouchterlony method and titer of ascites was determined by IF. To characterize these mcAb's, we performed an immunohistochemical study, enzyme-linked immunosorbent assay (ELISA), and Western blot analysis as described below.

1)Immunohistochemical study. Immunohistochemical study was carried out by IF with frozen sections; and a modified ABC method (ABC), with paraffinized sections of brain. Brain sections were obtained from 5 cases of Alz, 3 cases of PD, 3 cases of Down's syndrome, and 3 cases of aged-matched healthy subjects.

2)ELISA. To identify the epitope recognized by each mcAb, ELISA and Western blot analysis were performed. Neurofilaments (H,M,L;PROGEN BIOTECHNIK), heat-stable MAPs, and ubiquitin (SIGMA) were used as antigens for ELISA. Heat-stable MAPs were prepared from bovine brain by a modification of the method of Weingarten et al.[4]

3)Western blot analysis. Brain homogenate, partially purified PHF, neurofilaments (NF), heat-stable MAPs, and ubiquitin were used as antigens. Well-characterized anti-NF, anti-tau, and anti-ubiquitin mcAb's were kindly provided by Drs. Sternberger, Kosik, and Ihara respectively. They were used as positive controls for antigen/antibody reactions in the ELISA and Western blot analysis.

RESULTS

Twenty-one mcAb's against PHF were established. Thirteen of them were obtained by immunization of Balb/c mice with PHF from PD; and 8, with PHF from Alz. Their Ig class was IgG(k) or IgM(k). Antibody activity (IF) was positive up to as $5x10^3$ -$1x10^4$ dilution of ascites.

1)Immunohistochemical study

All these mcAb's stained NFT and they were classified into 3 groups according to immunostaining property. Group 1 mcAb's stained large NFT and neuritic filaments (Fig a. IF, frozen section of PD brain stained with GP 1C9 ; Fig b. ABC, section of Alz brain stained with GP 1C9; degenerated neurites around senile plaques were also stained). Group 2 mcAb's stained relatively small NFT, neuritic filaments, and fine filaments (Fig c. ABC, section of Alz brain stained with Am 719/1). Group 3 mcAb's reacted with NFT only (Fig d. ABC, section of Alz brain stained with GP 1D8).

2)ELISA

These mcAb's showed considerable variation in reactivity with our antigen system. There were several mcAb's that recognized NF, heat-stable MAPs, or ubiquitin. Some of them recognized both NF and heat-stable MAPs, and others recognized either NF or heat-stable MAPs. The epitopes recognized by 9 of the mcAb's remained undetermined by our

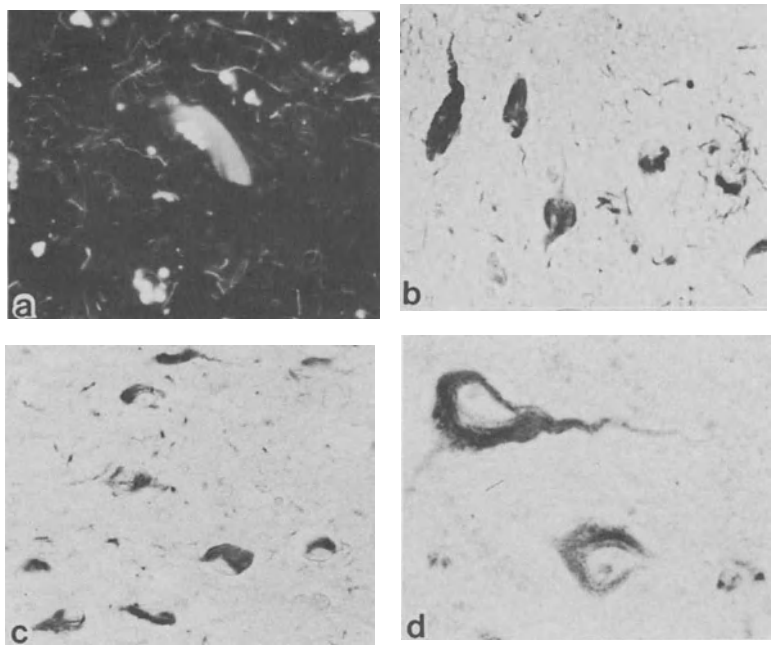

Fig.1 a-d. Immunostaining of frozen section of PD brain or paraffin embedded sections of Alz brain with mcAb's.

antigen system. There was a correlation between the three staining patterns classified by IF and those obtained by ELISA. McAb's that recognized NF, heat-stable MAPs, or ubiquin were classified into Group 1 or 2. The 9 mcAb's, that reacted with none of the epitopes examined by ELISA, were classified into Group 3, the one with NFT staining only.

3)Western blot analysis

Findings of Western blot analysis using NF and tau were consistent with results of ELISA. Am 135/1, GP 193/2, and GP 823/3, whose epitopes remained undetermined by ELISA, recognized 15 kd, 55 kd, and 60 kd bands, respectively, when Alz and PD brain homogenate was used as antigen.

DISCUSSION

McAb's against native PHF from PD have been established for the first time. These mcAb's are valuable to analyze the constituents of PHF in various diseases. Tau and ubiquitin were identified as components of PHF by immunohistochemical and biochemical studies.[5,6,7] We have confirmed that tau and ubiquitin immunoreactivities are in PHF of Alz, PD, and Down's syndrome.

Whether or not NF are involved in PHF formation remains obscure even now. Nukina et al.[8,9] reported that mcAb's against NF that stained NFT recognized phosphorylated epitopes of NF and also phosphorylated epitopes of tau due to crossreactivity. In our study, some mcAb's recognized both NF

and tau; but, on the other hand, several others recognized only NF or tau. Although precise epitopes of these mcAb's are not elucidated, the possibility can not be excluded that NF is an internal component of PHF.

The epitopes of 9 mcAb's that clearly stained only NFT remained undetermined. Among these, 3 recognized 15-60 kd bands in the Western blot analysis. The findings suggest that these mcAb's recognize unknown epitopes other than tau and ubiquitin.

Many mcAb's were established in our study. Their epitopes were variable or are still undetermined. Further investigation about the detailed epitopes of these mcAb's is still needed. But these mcAb's seem to be helpful for us to understand the nature of PHF and the process of PHF formation in various diseases.

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PATTERNS OF VULNERABILITY OF MESOSTRIATAL NEURONS

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INTRODUCTION

Of the major neurological diseases that affect the basal ganglia, Parkinson's disease is by far the most common. The cause of idiopathic Parkinson's disease has remained obscure, resisting attribution to either a genetic or an environmental factor. Yet the hallmark of this condition is well known: a marked, and often profound, loss of neurons in the substantia nigra pars compacta (Hassler, 1938). Attention accordingly remains focused on searching for characteristics of the nigral pars compacta neurons that might hold clues to the etiology underlying the development and progression of Parkinson's disease. These characteristics include the synthesis and metabolism of dopamine, the accumulation of large amounts of intraneuronal neuromelanin, and the vulnerability of these neurons, at least in some experimental conditions, to retrograde degeneration after lesion of their terminals in the neostriatum (Rosegay, 1944, Bedard et al., 1969; Imai et al., 1988). We focus here on two animal models that demonstrate a shared vulnerability of the midbrain dopamine-containing neurons and their striatal projections: the weaver mutation in the mouse, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in the monkey.

THE MUTANT MOUSE WEAVER

Weaver is an autosomal recessive mutation, first characterized by its effects on the cerebellum, that also leads to a profound loss of dopaminergic mesostriatal neurons beginning in the postnatal period; dopaminergic neurons of the mesolimbic system are largely, though not entirely, spared (Schmidt et al., 1982; Roffler-Tarlov & Graybiel, 1984; for review see Roffler-Tarlov & Graybiel, 1987). By postnatal day 90, the loss of tyrosine hydroxylase (TH)-immunoreactive neurons reaches nearly 70% in the substantia nigra pars compacta (A9), over 50% in the retrorubral area (A8), but only 26% in the

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ventral tegmental area (A10), as determined by Triarhou et al. (1988) using the nomenclature of Hökfelt et al. (1984).

The weaver mutation has highly selective effects on the dopaminergic innervation of the striatum (Graybiel et al., 1990, and references therein). The loss of TH-immunoreactive neuropil follows a gradient from profound loss in the dorsolateral caudoputamen to relative preservation in the ventrolateral and ventral caudoputamen, where the remaining TH-immunopositive innervation becomes contiguous with strong immunostaining of parts of the ventral striatum. This striatal pattern is consistent with pathologic findings in the mesencephalon. The nigral complex projects to the striatum proper through a dorso-ventral inversion (Fallon & Moore, 1978). Within cell group A9, the weaver mouse shows a profound loss of neurons ventrally, and partial preservation of a narrow dorsal tier of neurons that might be expected to innervate the ventral part of the striatum. There is also partial preservation of the substantia nigra's pars lateralis, which provides dopaminergic innervation to the lateral striatum (Fallon & Moore, 1978), and relative preservation of cell group A10, which projects most strongly to ventral striatum (Graybiel et al., 1990).

In the normal mouse, TH-immunostaining in the early postnatal period is characterized by clusters of strongly TH-immunoreactive fibers--the well-known dopamine islands--which appear in a weakly stained surround. By maturity the TH-immunostaining of the striatum is nearly uniform. Remarkably, the islandic pattern appears in young weaver mice despite the later loss of dopamine in these mutants. In adult weavers, the zones with remaining striatal TH-immunostaining show a compartmental pattern: although the overall immunostaining is markedly diminished, many pockets of very faint immunoreactivity appear within the surrounding matrix of TH-immunopositive neuropil. We have proposed that this pattern reflects differential loss of putative striosome- and matrix-projecting neurons of the murine nigral complex (Graybiel et al., 1990). This could be accounted for by partial preservation of the A8 region, and of the lateral and dorsal A9 regions (Gerfen et al., 1987; Jimenez-Castellanos & Graybiel, 1989), whereas the ventral A9 tier and the cell-dense medial region of the pars compacta, projecting to striosomes, are nearly completely lost.

The weaver mouse also shows a marked deficit in high-affinity dopamine uptake in synaptosomal preparations, a defect that appears at postnatal day 7, before any abnormality of dopamine content is detected in the striatum and before major loss of dopaminergic perikarya is evident in the mesencephalon (Roffler-Tarlov et al., 1990). This deficit of dopamine uptake is currently the earliest biochemically detectable marker for the weaver mutation, and further, this deficit is proportionally more severe than the loss of dopamine. In the adult weaver caudoputamen, dopamine uptake is reduced to about 20% of control values, while dopamine content is approximately 30% of control; in the nucleus accumbens, the content of dopamine is unchanged from control values, whereas high-affinity dopamine uptake in vitro is reduced by 30% (Roffler-Tarlov et al., 1990).

MPTP AS A STRIATAL AND NIGRAL NEUROTOXIN

MPTP, administered systemically, can cause severe and, at high doses, permanent damage to the mesostriatal dopaminergic system in many higher mammals, including cats, monkeys, and humans (for review see Langston, 1989). The cascade of events leading to this dopaminergic lesion can be pharmacologically interrupted at several steps. Conversion of MPTP, which can cross the blood-brain barrier, to its toxic metabolite 1-methyl-4-phenylpyridine (MPP+), can be blocked by monoamine oxidase inhibitors such as pargyline and deprenyl. The dopaminergic lesion can also be interrupted by blockade of dopamine uptake sites, presumably preventing the uptake of MPP+ into dopaminergic terminals or neurons (for references. see Langston, 1989; Kopin & Markey, 1988). Finally, the neurotoxicity of MPTP in the primate is partially blocked by administration of chloroquine, which may interfere with the binding of MPTP or MPP+ to neuromelanin within dopaminergic perikarya and thus reduce its cumulative toxicity in these neurons (D'Amato et al., 1987).

New evidence, if compiled from studies in several species, suggests that MPTP-induced damage to striatal terminals shows both overall gradients and local heterogeneity. Turner et al. (1988) have used classical silver staining techniques to show that, in the dog, MPTP leads acutely to a heterogeneous distribution of terminal damage in the caudoputamen. Terminal degeneration is found prominently in the matrix compartment, rather than in striosomes. We have shown that dopamine uptake sites, labeled autoradiographically with tritiated mazindol in a closely related species, the cat, are preferentially distributed in the matrix compartment (Graybiel & Moratalla, 1989). Moreover, both in cat and

primate, uptake-site concentrations were notably higher in the dorsal caudoputamen than in the ventral caudoputamen or ventral striatum, as first observed in the rat by Marshall (1988). Thus, the early toxin-induced changes at nigrostriatal terminals show a notable similarity to the regions with the highest catecholamine uptake-site concentrations in normal monkeys and cats.

Terminal damage, however, as seen by silver staining methods, can only be assessed a week or more after an initial lesion. Prominently matrical terminal damage might reflect the anterograde effects of preferential damage to matrix-projecting mesostriatal neurons after MPTP administration, rather than a direct primary effect of the toxin at mesostriatal terminals. Although the mesencephalic lesions reported in the primate MPTP literature vary, ventral and medial to middle parts of the nigral pars compacta seem to be most severely damaged, with intermediate damage in the A8 region, and relative sparing of the ventral tegmental area (see, e.g., Kitt et al., 1986; Schneider et al., 1987; German et al., 1988). However, Deutch et al. (1986) reported that nigral cell loss was most severe in the A8 region. It is not yet clear whether these patterns fit the distribution of early striatal terminal damage, visualized by silver techniques. In the monkey, however, analysis of TH-immunostaining in the striatum demonstrates a marked gradient of loss of TH-immunoreactive terminals, with the most severe damage in the dorsolateral caudate-putamen, and better preservation of TH-immunoreactivity in the ventromedial caudate-putamen and in the nucleus accumbens (German et al., 1988; Quinn et al., 1990). Biochemical measurements of dopamine levels in MPTP-treated primates show a concordant pattern, yielding most severe depletion in the dorsolateral caudate-putamen (Ellsworth et al., 1987a, 1989; German et al., 1988), and in the medial substantia nigra pars compacta (Schneider et al., 1987; Ellsworth et al., 1987b), with relative sparing of dopamine in the ventral tegmental area and lateral pars compacta.

A pronounced dorsolateral-to-ventromedial gradient of striatal damage has also been reported on the basis of the astrocytic reaction observed in the striatum of MPTP-treated cats by immunohistochemical staining of glial fibrillary acidic protein (GFAP) (Schneider et al., 1988). Strömberg et al. (1986) found that GFAP induction after MPTP was most pronounced in the dorsal caudoputamen in the mouse, and found marked GFAP induction in the substantia nigra as well, but Schneider et al. (1988) reported a homogenous induction of GFAP in the mouse striatum and no induction in the mouse substantia nigra. It would be interesting to learn whether GFAP-intense striatal astrocytes were compartmentalized in alignment with the degenerating terminals seen by Turner et al. (1988); however, the factors triggered by degenerating terminals that mediate astrocyte proliferation could be sufficiently diffusable to blur compartmental boundaries revealed by silver staining.

COMMENT: WEAVER, MPTP, AND THE MESOSTRIATAL SYSTEM IN PARKINSON'S DISEASE

Two questions underlie this comparative analysis of the neuropathology of the mesostriatal dopaminergic system in the weaver mouse and in the MPTP-lesioned animal. First, can common patterns of vulnerability be found in these models and in Parkinson's disease? Second, could a common factor, or combination of characteristics, underlie any shared pattern of vulnerability? The latter question invokes the possibility of a common final pathway to vulnerability, which might implicate characteristics of terminals, neuronal perikarya, or other factors in the special vulnerability of the mesostriatal system in each of these pathologic conditions.

As described above, in both the weaver mutant mouse and the MPTP-treated primate or cat, the loss of TH-immunoreactivity is most profound in the dorsolateral quadrant of the striatum, and this loss becomes less severe along a ventromedial axis directed toward the nucleus accumbens. In the weaver mouse, a defect in dopamine uptake, measured in vitro, is evident before any detectable loss of striatal dopamine, of striatal TH-immunostaining, or of TH-positive cell bodies in the nigral complex. The overall pattern of terminal loss in weaver mice parallels the general distribution of dopamine uptake sites, determined by autoradiography; the greater vulnerability of dorsal than ventral striatum in MPTP-treated monkeys also corresponds to the dopamine uptake-site distributions in this species; and the matrical terminal degeneration in the acutely MPTP-treated dog corresponds to the compartmental distribution of dopamine uptake sites in the cat.

Could a lesion of striatal dopaminergic terminals be the primary insult in both models? In the MPTP-treated primate, loss of TH-immunostaining can be much more dramatic in the striatum than in the nigral perikarya, and absolute dopamine deficits are usually more severe (up to 99%) in the

striatum as well. In the mouse and rat, at least under certain conditions, MPTP can induce a sharp loss of striatal dopamine, with contrastingly little loss of nigral perikarya (Ricaurte et al., 1986; Willis & Donnan, 1987; Vacca-Galloway et al., 1988; but see also Heikkila et al., 1985; Heikkila et al., 1989). It seems likely that MPP+, taken up through the dopamine uptake site, may be directly toxic to the striatal dopaminergic terminals, and when MPTP is administered directly into the striatum, both terminal loss and a retrogradely-directed degeneration of nigral perikarya ensue (Imai et al., 1988). It is still unclear whether a model for Parkinson's disease based on primary terminal degeneration, and secondary neuronal degeneration, would apply well to the human mesostriatal system (Graybiel, Hirsch, & Agid, 1990). On the one hand, Forno (1983) reported relatively little retrograde degeneration is seen in the human substantia nigra even after massive loss of striatal fibers and target cells by infarction. On the other hand, there is no reason to doubt that retrograde axonal transport occurs in the human, so that some forms of retrograde-induced degeneration could occur.

The weaver mutation, the MPTP model in primate or cat, and Parkinson's disease all reveal a general pattern of vulnerability in which neurons of the substantia nigra pars compacta are more severely affected than neurons of the adjacent ventral tegmental area, and further, in which the ventral pars compacta is more severely affected that its dorsal region (for the human see e.g. Hassler, 1938; Hirsch et al., 1988; Gibb et al., 1990). No currently identified marker discriminates this part of cell group A9 from the adjoining parts of cell groups A8 or A10. Differences in neuromelanin may be directly important, in that melanized neurons are preferentially lost in these regions in Parkinson's disease (Hirsch et al., 1988), and several studies suggest that in normal aging, there is preferential loss of the most heavily melanized neurons (for review, see Barden, 1981). On the other hand, murine dopaminergic neurons do not contain detectable neuromelanin (reviewed in Barden, 1981), and Gibb et al. (1990) have reported that ventral, lightly melanized neurons of A9 are lost earliest in the course of Parkinson's disease. A critical point that needs clarifying is whether there is preferential loss of lightly or heavily melanized TH-positive neurons in the MPTP-treated primate. No such analysis exists, because, although human dopaminergic neurons are often visibly heavily melanized, cat or monkey neuromelanin is essentially occult without special stains (Barden, 1981). We have recently developed a reliable method that combines immunohistochemistry for TH with a sensitive modified Fontana stain for neuromelanin (Quinn & Graybiel, 1990), and this technique may shed light on whether there is differential vulnerability of neuromelaninized neurons in the primate MPTP-induced lesion (Quinn et al., in progress). Finally, markers other than dopamine or melanin may link the nigral subgroups which are lost in the weaver mouse, MPTP-treated monkey, and the patient with Parkinson's disease. For example, two distinct calcium-binding proteins have recently been reported in dopaminergic neurons of the rat's substantia nigra, calbindin-28k and protein 10 (Gerfen et al., 1987; Winsky et al., 1989). The presence or absence of proteins such as these could be a link to the vulnerability of subsets of nigral dopaminergic projection systems. Clearly, apparent selective vulnerability of neuronal populations may require a conjunction of several such neuronal characteristics; but the opportunity now is at hand to screen for such factors both in parkinsonian models and in Parkinson's disease itself.

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COMPLEX I DEFICIENCY IN PARKINSON'S DISEASE AND MPTP-INDUCED EXPERIMENTAL PARKINSONISM

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INTRODUCTION

It has been considered that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)-induced parkinsonism is the best model available at present of Parkinson's disease. Elucidation of the mechanism of the neuronal degeneration in this model will contribute to the studies on the pathogenesis of Parkinson's disease. In this communication, we will present our data on the mechanism of the neuronal degeneration in MPTP-induced experimental parkinsonism and a preliminary observation on Parkinson's disease.

THE MECHANISM OF THE NEURONAL DEGENERATION IN MPTP-INDUCED PARKINSONISM

We noticed a structural similarity between 1-methyl-4-phenylpyridinium ion (MPP^+), a toxic metabolite of MPTP (1), and nicotinamide adenine dinucleotide (NAD^+), a cofactor widely used in oxidation-reduction reactions. We thought that MPP^+ might interfere with the reactions utilizing NAD^+. We first studied the effects of MPP^+ on the activities of mitochondrial respiratory enzymes. We used mitochondria prepared from whole mouse brains assuming that most of the cerebral mitochondria would behave in a similar way to MPP^+. The selectivity of the MPTP-toxicity against the nigral neurons was shown to depend on the active uptake of MPP^+ through the dopamine-uptake site (2). As long as the concentration of neuronal MPP^+ reaches a certain point, most of the neurons may be damaged.

We prepared mitochondrial suspensions from C57/BL mouse brains according to the method of Ozawa et al. (3), and the activities of mitochondrial Complex I, II, III and IV were assayed as described before (4,5). We found inhibition of Complex I by MPTP and MPP^+ (Table 1). Rather high concentration of MPTP or MPP^+ (mM order) was necessary to obtain those amounts of the inhibition. In animals treated with MPTP, the cerebral concentration of MPP^+ was reported to be much lower (micromolar order) (6). Therefore, we thought initially that this inhibition might not account for the toxicity of MPP^+. In 1987, Ramsay and Singer (7) reported the presence of an active uptake mechanism for MPP^+ in the hepatic mitochondria. Their report prompted us to investigate the effect of the preincubation of mitochondria on the inhibition of Complex I by MPP^+.

We incubated mitochondria prepared from C57/BL mouse or Fisher 344 rat

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Table 1. The Effect of MPTP and MPP^+ on the Activities of Mitochondrial Complex I, II, III and IV

	Control	MPTP(2mM)	MPP^+(2mM)
Complex I	100 %	34.1 %	59.8 %
Complex II	100	85.3	79.3
Complex III	100	86.5	104.5
Complex IV	100	93.2	95.5

Expressed as % of the activity of the respective controls, Means of the duplicate assays, Cited from reference (5),

brains with substrates of the TCA cycle and ADP with or without MPP^+. We measured oxygen consumption using a Clark-type oxygen electrode. Then, an aliquot of the mitochondrial suspension was transferred to a cuvette, and the activity of Complex I was assayed spectrophotometrically (8,9). In this experiment, a much lower concentration of MPP^+ (0.05 mM) was found to be effective to inhibit the mitochondrial respiration and the activity of Complex I. When glutamate and malate were used as the substrates to support the mitochondrial respiration, the oxygen consumption of the state 3 and the state 4 respirations were markedly inhibited by MPP^+ (Table 2). The activity of Complex I and the amount of ATP formed from ADP were also significantly reduced by MPP^+. On the other hand, the mitochondrial respiration supported by succinate was not inhibited by MPP^+. Succinate is oxidized to fumarate by Complex II. On the other hand, NADH formed from the oxidation of glutamate and malate is oxidized by Complex I. Therefore, the inhibition of the mitochondrial respiration supported by glutamate and malate strongly suggests inhibition of Complex I by MPP^+. The inhibition of the activity of Complex I was actually obtained by a low concentration of MPP^+, the concentration which could be found in animals treated with MPTP.

We also studied effects of MPP^+ on NAD^+- or $NADP^+$-linked dehydrogenases in the TCA cycle (10). Only the alpha-ketoglutarate dehydrogenase complex was significantly inhibited by MPP^+ (Table 3). Alpha-ketoglutarate dehydrogenase is considered to be the rate-regulating enzyme in the TCA-cycle (11). Therefore, the inhibition of this enzyme may cause deleterious effects on the mitochondrial respiration.

Table 2. The Effect of MPP^+ on the Mitochondrial Respiration, the Activity of Complex I and the ATP Synthesis

Substrate	Glutamate + Malate		Succinate + Rotenone	
	Control	MPP^+	Control	MPP^+
State 3	180 ± 25	21 ± 4*	209 ± 27	196 ± 14
State 4	38 ± 5	11 ± 3*	67 ± 27	63 ± 5
Complex I	57 ± 13	27 ± 6*	55 ± 6	52 ± 6
ATP formed	637 ± 23	210 ± 31*	641 ± 27	666 ± 31

Mean ± SEM (n = 4), Units: State 3 and 4 = nanoatom O utilized /min/mg protein, Complex I = nanomole NADH oxidized/min/mg protein, ATP = nanomoles formed from ADP (750 nanomoles added), * : $P < 0.001$.

Table 3. The Effect of MPP^+ on the NAD^+- or the $NADP^+$-linked Dehydrogenases in the TCA-cycle

	Control	MPP^+(2 mM)
Isocitrate DH (NAD-linked)	269.2 ± 47.4	253.3 ± 41.5
Isocitrate DH (NADP-linked)	97.0 ± 11.7	89.2 ± 16.7
Glutamate DH (NADH-linked)	550.7 ± 62.6	537.4 ± 47.8
Glutamate DH (NADPH-linked)	329.1 ± 24.3	307.8 ± 41.8
Alpha-ketoglutarate DH complex	32.9 ± 5.9	18.5 ± 3.3*
Malate DH	4625 ± 568	4607 ± 886

Expressed as nanomoles of respective nucleotide oxidized or reduced/min/mg protein, Mean ± SEM (n=4), *: $P < 0.01$, DH = dehydrogenase, Cited from reference (10).

Then, we investigated the effect of MPTP on the mitochondrial respiration ex vivo. We gave MPTP subcutaneously to mice, and the cerebral mitochondrial respiration was studied 5 to 6 hours after the last dose of MPTP (Table 4). The detail of the experimental procedure is reported elsewhere (12). The state 3 respiration and the activity of Complex I were significantly inhibited in mice treated with MPTP. The total amount of ATP synthesized was normal, however, the rate of the synthesis was significantly reduced. These inhibitions were completely prevented by the pretreatment of the mice with pargyline. Pargyline alone did not affect the mitochondrial respiration, the activity of Complex I or the amount of ATP synthesized.

From these results together with observations in the literature, the mechanism of the neuronal degeneration in MPTP-induced experimental parkinsonism may be summarized as follows. MPTP is taken up into astrocytes where MPTP is oxidized to MPP^+ by monoamine oxidase B (13). Then, MPP^+ is concentrated in the nigrostriatal dopaminergic neurons by the active uptake through the dopamine uptake site (2). Then, MPP^+ is further concentrated into mitochondria. According to the recent observation, the difference in the concentration of MPP^+ between the inside of mitochondria and the surrounding tissue may reach 800-folds (14). Within mitochondria, MPP^+ inhibits the mitochondrial respiration by inhibiting the activities of Complex I and the alpha-ketoglutarate dehydrogenase complex resulting in the energy

Table 4. The Effect of MPTP on the Cerebral Mitochondrial Respiration, the Activity of Complex I and the ATP Synthesis Ex Vivo

	State 3	ATP formed	Complex I
Control	128 ± 16*	645 ± 36	182 ± 32*
MPTP	66 ± 6	638 ± 35	115 ± 14
Pargyline	134 ± 31	689 ± 28	165 ± 32
Pargyline+MPTP	140 ± 15	636 ± 62	182 ± 14

Mean ±SEM (n = 4). Units: State 3 = nanomole O_2/min/mg protein with glutamate + malate as substrates, ATP = nanomoles ATP formed from 750 nanomoles of ADP, Complex I = nanomole NADH oxidized/min /mg protein, *: $P < 0.01$

crisis. This appears to be the most likely mechanism of the neuronal degeneration in MPTP-induced experimental parkinsonism.

STUDIES IN HUMAN MATERIALS

Biochemical Assays

Studies on MPTP-induced parkinsonism prompted us to investigate mitochondrial functions in Parkinson's disease. We first studied post-mortem changes in the mitochondrial respiratory enzymes in mouse brains. Mice were decapitated and the brains attached with the skulls were left at a room temperature for 6 hours and at 4°C for another 6 hours. The brains were taken out of the skulls 2, 4, 6, 8 and 12 hours after the decapitation, and frozen on a plate of dry ice to simulate the handling of autopsy brains. Activities of Complex I, II, III, IV, glutamate dehydrogenase and malate dehydrogenase were relatively stable for as long as 12 hours after the decapitation. Activities of the alpha-ketoglutarate dehydrogenase complex and $NADP^+$-linked isocitrate dehydrogenase diminished to approximately two thirds of the respective initial activities in two hours after the decapitation, however, the subsequent declines in the activities were small.

Then, we studied the postmortem changes in the above enzyme activities in the frontal cortices from autopsied patients died of various disorders. Brain death cases were excluded. Activities of the alpha-ketoglutarate dehydrogenase complex could not be measured in the autopsy materials. There were no significant correlations between the length of the postmortem period before freezing the brains and the activities of the enzymes studied except for Complex I. A significant negative correlation was noted between the activity of Complex I and the duration of the postmortem period. No significant correlation was noted in any of the enzymes studied between the activity and the age. (Most of our patients were above 50 years of age.)

Then, we measured the activities of these enzymes in the striata of 5 patients with Parkinson's disease and 5 controls. The activity of Complex I in Parkinson's disease was 33 ± 22 nanomole NADH oxidized/min/mg/protein, and that of the control 43 ± 34. The activity was lower in Parkinson's disease, however, the difference did not reach the statistical significance. On the other hand, the activity of Complex III was 206 ± 45 nanomole cytochrome C reduced/min/mg protein in the control, and 133 ± 50 in Parkinson's disease. The difference was statistically significant at the 5 % level. Activities of other enzymes studied were normal.

Immunoblotting Studies

We performed immunoblotting studies using specific antibodies against Complex I, III and IV in the next step. The antibodies against Complex I and III were generous gifts from Professor Takayuki Ozawa (Department of Biomedical Chemistry, University of Nagoya), and that against Complex IV from Professor Takeshi Satoh (Department of Neurology, Juntendo University). The methods are described elsewhere (15). Mitochondria were prepared from the striata and the substantia nigra, however, the amount of mitochondria prepared from the substantia nigra was not enough to obtain reliable data. Therefore, results on the striata are shown in Fig. 1. In four out of the 5 patients with Parkinson's disease, at least 4 subunits of Complex I were significantly reduced. One of the 4 subunits with decreased staining appeared to be either the 33-kDa subunit of the hydrophobic fraction or the 30-kDa subunit of the iron sulfur fraction. The other band with decreased staining appeared to be the 24-kDa subunit of the flavoprotein fraction. Another two bands with decreased staining appeared to be the 24- and the 25-kDa subunits in the hydrophobic fraction. Further studies are necessary for more exact assignment of these subunits, however, partial deficiencies

in Complex I seem to be one of the most important biochemical abnormalities in Parkinson's disease. The Complex III and IV subunits appeared normal.

DISCUSSION

Since the discovery of MPTP-induced parkinsonism, it has been postulated that Parkinson's disease may be caused by a chronic exposure to an unknown toxin. Most of the people may be exposed to such a toxin, suppose it exists. Then, a question arises why certain persons are afflicted with Parkinson's disease, and the majorities are not? Our assumption is that there may be a constitutional difference between those who get Parkinson's disease and those who don't. We want to elucidate if such a difference exists or not. Deficiencies in the subunits of Complex I that we found may represent examples of fundamental abnormalities, however, because Parkinson's disease is not a hereditary disease, they may be secondary changes. It is also possible that the chronic exposure to an unknown toxin may have caused such deficiencies. Recently, Schapira et al. (16) reported diminished activity of Complex I in the substantia nigra of patients with Parkinson's disease, and Bindoff et al. (17) reported decreases in the activities of Complex I, II and IV in muscle mitochondria from patients with Parkinson's disease. In addition, Parker et al. reported a decrease in the activity of platelet Complex I at the third Annual Symposium on Etiology, Pathogenesis and Prevention of Parkinson's Disease held in New Orleans in 1989. All of these findings have to be confirmed by further studies, however, the mitochondrial Complex I deficiency appears to be the most promising starting point to elucidate pathogenesis of Parkinson's disease. Further studies on this line are in progress.

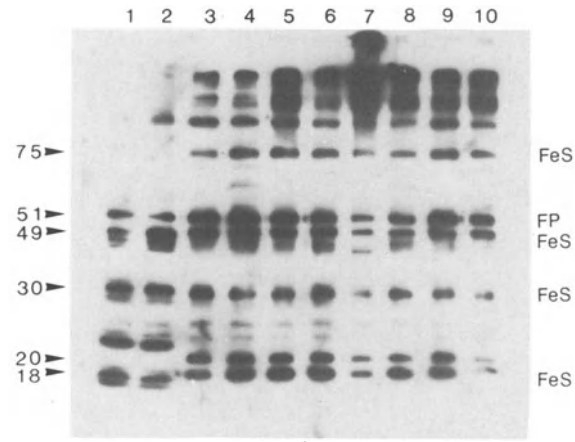

Fig. 1. Subunit analyses of Complex I in striatal mitochondria from Patients with Parkinson's Disease, The lane 1 represents purified Complex I, the lane 2 bovine heart mitochondria, the lane 3 to 5 control patients and the lane 6 to 10 patients with Parkinson's disease.

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BIOCHEMICAL REACTIONS LEADING TO PARKINSONIAN SYMPTOMS ELICITED BY MPTP

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Expression of the neurotoxicity of MPTP and of its analogs is initiated by a two-step oxidation of the corresponding pyridinium, catalyzed by monoamine oxidase (MAO) A or B. The initial enzymatic oxidation to the dihydropyridinium level is rapid, the second slower. Conversion of the dihydropyridinium to the pyridinium also occurs by spontaneous air oxidation and by chemical disproportionation. The latter is significant only at relatively high concentrations of the dihydropyridinium and seems to be negligible at neutral pH and concentrations of the dihydropyridinium likely to occur *in vivo*. A study of over 25 MPTP analogs[1] has shown that, depending on the structure of the MPTP analog, oxidation is predominantly catalyzed by MAO A, or by MAO B, or both may be able to process the tetrahydropyridine. Thus, while MPTP is primarily bioactivated by the B enzyme, 2'-CH_3-MPTP is rapidly oxidized by both, and 2'-Ethyl-, 2'-*n*-Propyl-, and 2'-Isopropyl-MPTP are poor substrates for MAO B but excellent ones for MAO A. Neurotoxicity *in vivo* reflects the specificities of MAO A and B. While deprenyl, the selective inhibitor of MAO B, blocks the neurotoxicity of MPTP,[2,3] clorgyline, the A-selective MAO inhibitor, but not deprenyl, prevents the neurotoxic action of the 2'-Ethyl analog in black mice, and both deprenyl and clorgyline are needed to protect against 2'-Methyl-MPTP.[4] It is clear that either type of MAO may play an important role in the expression of the neurotoxicity of environmental tetrahydropyridines.

The next step leading to destruction of the nigrostriatal cells is widely believed to be uptake of the pyridinium by the synaptic dopamine reuptake system.[5] This is both a means for concentrating the toxic oxidation product of MPTP and for pumping it to the stroma. In the striatal neuron neuromelanin binds MPP^+ very tightly: this might serve as transient storage[6] of the pyridinium until it is pumped into the mitochondria, where the final events occur. Snyder's laboratory favors the view that sequestration of MPP^+ by the neuromelanin in the substantia nigra helps explain the susceptibility of these cells to MPTP.[7,8]

Two main hypotheses have been proposed for the eventual cause of the destruction of dopaminergic neurons by MPP^+. The first is the "oxidative stress" hypothesis, which was based on the structural similarities of MPP^+ and the toxic herbicide Paraquat.[9] The latter is reduced in biological systems and its reoxidation generates superoxide, and, eventually, hydroxyl radicals which damage or kill the cell. Indeed, there is growing evidence

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of the increased formation of oxygen radicals being responsible for the progressive decline in the number of nigrostriatal cells on ageing.[10,11] Knoll[11] has, in fact, suggested that the prolongation of the life span of rats, as well as retention of their sex drive and learning ability when treated regularly with deprenyl, may be ascribed to blocking the oxidation of dopamine by glial MAO B.[8] Unfortunately, however, there is no evidence that the toxicity of MPP^+ is the result of superoxide generation. Earlier claims that glutathione and other antioxidants protect against MPTP or MPP^+ were shown to be erroneous on careful examination.[12-14] Further, unlike Paraquat, MPP^+ cannot be reduced under cellular conditions because of its low redox potential (-1.07V) and, hence, cannot generate superoxide.

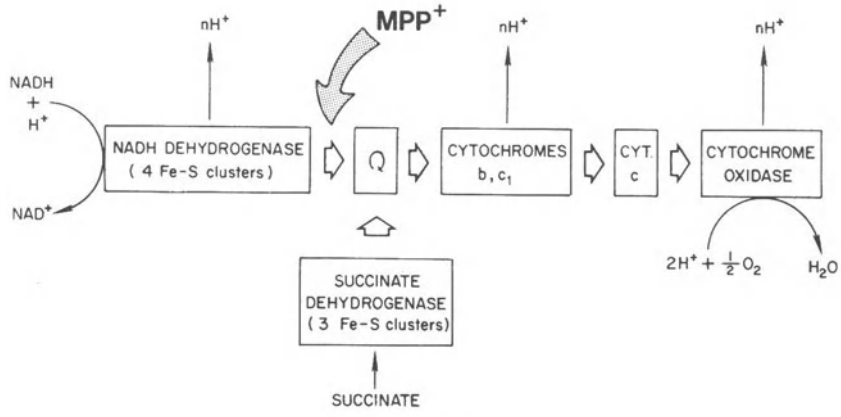
In contrast, there is growing evidence for the correctness of the other hypothesis, that nigrostriatal damage results from inhibition of mitochondrial NADH dehydrogenase by the toxic pyridines. The mitochondrial hypothesis was first proposed by Nicklas et al.[15] on the basis of the observation that ADP-stimulated (State 3) respiration on NAD^+-linked substrates such as glutamate and malate, in brain and liver mitochondria was severely inhibited by pre-incubation with 0.2 to 0.5 mM of MPP^+, while respiration on succinate was unaffected. The effect of MPP^+ on respiration was time-dependent: the longer the pre-incubation with MPP^+, the more extensive was the block.

Although this experiment was easily confirmed, its relevance to the neurotoxicity of MPP^+ seemed uncertain, because the concentration required to inhibit NADH oxidation in intact mitochondria was an order of magnitude higher than that measured in post mortem samples of the nigrostriatum of MPTP-treated black mice[16] and monkeys. (The latter was, of course, a very rough figure, since MPP^+ formation does not cease when the animal is sacrificed.) A more puzzling observation was that, on inverting the inner membrane by sonication of the mitochondria, so as to expose the NADH binding site to the outside, the effect of MPP^+ on NADH oxidation disappeared and could then only be seen at much higher - several millimolar - concentrations.[17,18] In confirmation of this, Complex I and inner membrane preparations showed I_{50} values of $\sim$ 4 mM for inhibition by MPP^+, but in contrast to mitochondria, the effect was instantaneous.[19]

The paradox was resolved when it was discovered [17,20] that mitochondria rapidly accumulate MPP^+ against a concentration gradient, reaching 10 to 20 mM concentrations in the matrix at low external concentration. The uptake is energized by the potential gradient of the membrane, so that valinomycin+ K^+ abolish it. Because the electrogenic uptake exhibited saturation kinetics, considerable structural specificity toward the pyridinium, and an energy of activation typical of mitochondrial carriers, we first thought that the transport was carrier-mediated. Subsequent findings convinced us that the effects noted could be explained without postulating a carrier. A particularly revealing finding was that tetraphenylborate (TPB^-), a substance known to facilitate the movement of cations across membranes, greatly potentiates the inhibition of mitochondrial respiration by MPP^+.[21]

The concentration of MPP^+ reached in the mitochondria as a result of the potential gradient of the membrane is sufficient to block NADH dehydrogenase completely. The inhibition site seems to be at the junction of NADH dehydrogenase and CoQ_{10}, the same region where rotenone, barbiturates, and piericidin A combine.[6] (Scheme 1) Since the reoxidation of NADH dehydrogenase via the respiratory chain is blocked, ATP synthesis ceases. Once the ATP pool is depleted, the membrane collapses and cell death ensues rapidly.

Since the demonstration in Heikkila's laboratory[22] that in mouse brain neostriatal slices MPP^+ causes the accumulation of lactate and alanine, as expected from the inhibition of NADH oxidation by the mitochondrial respiratory chain much evidence has accumulated in support of the mitochondrial



Scheme 1

hypothesis. Thus, the stereotaxic administration of MPP^+ to the nigrostriatum of rodents similarly causes extensive lactate accumulation. More recently, Sanchez-Ramos et al.[23] have summarized impressive evidence favoring Complex I as a target. Perhaps the most interesting among these was the observation that pentothal and other barbiturates increased the survival of MPP^+ treated dopaminergic neurons in cell culture, as expected from the hypothesis that MPP^+, barbiturates, and rotenone inhibit at the same site. In contrast, a variety of antioxidants had no protective effect, thus providing further evidence against redox cycling of MPP^+ and free radical formation, as suggested in the literature.[24] Moreover, the same investigators have also reported[25] that the superfusion of rat striatal slices with MPP^+ causes extensive oxidation of the cytochromes, as it must if the flux of reducing equivalents from NADH via its dehydrogenase to the cytochromes is blocked. This effect was antagonized by mazindol, an inhibitor of dopamine and of MPP^+ uptake, showing that the action of MPP^+ was specific.

Our recent studies have focused on the terminal events in mitochondria. It has been demonstrated[18] that of all MPP^+ analogs tested, 4-phenylpyridine is the most potent inhibitor of NADH oxidation in inner membrane (ETP) or Complex I preparations, but it has very little effect on State 3 respiration in mitochondria. This is because, being neutral at intracellular pH, it is not concentrated by the electronegative gradient of the inner membrane, but must diffuse passively across the membrane, never exceeding the concentration prevailing in the cytoplasm.

Table 1 compares the inhibitory effect of 18 synthetic analogs of MPP^+ on NADH oxidation in isolated membranes and in intact mitochondria, the latter being measured as respiration on glutamate and malate. There is a good correspondence in the inhibitory potencies in the two systems, particularly if one considers that neither the IC_{50} nor the K* values are simple, linear functions of the concentration of the inhibitor, but are empirical expressions. There are some exceptions. Thus, like 4-phenylpyridine, some of the compounds tested (e.g., N,N-diMe-PTP^+, MPPyrimidinium$^+$, 2'-Cl-MPP^+) are more potent inhibitors in submitochondrial particles than in intact mitochondria. A likely explanation is that such compounds, though positively charged, accumulate only to a limited extent in mitochondria or that their accessibility to the hydrophobic inhibitor site in intact mitochondria is restricted. Studies with TPB^-, and experiments on the uptake of MPP^+ analogs support these expectations.[19,26] The data in Table 1 demonstrate that there is significant structural specificity in the interaction of pyridine derivatives with the "rotenone site" in the Complex I region.

Table 1. Inhibition of Mitochondrial Respiration and NADH Oxidation by MPP^+ Analogs.

	NADH Oxidase Activity in Isolated Inner Membranes		Mitochondrial Respiration on NAD^+-Linked Substrates	
	IC_{50} (mM)	Relative Inhibition	K^* ($mM^{-1}min^{-1}$)	Relative Inhibition
MPP^+	4.0 ∿ 1.4	100	0.629	100
2'-Me-MPP^+	4.1	98	0.547	87
2'-Et-MPP^+	1.0	400	0.477	76
2'-Cl-MPP^+	0.7	571	0.448	71
2'-OMe-MPP^+	1.2	330	1.05	167
3'-Me-MPP^+	1.7	235	1.56	248
3'-Br-MPP^+	2.3	174	0.631	100
3'-Cl-MPP^+	3.0	133	0.574	91
3'-OMe-MPP^+	2.6	154	0.564	90
3'-F-MPP^+	5.5	73	0.355	56
4'-Me-MPP^+	0.4	1000	2.50	397
4'-F-MPP^+	2.0	200	0.360	57
PPP^+	2.4	167	0.56	89
$MeCP^+$	4.8	83	0.626	100
Me-4-BzP^+	3.0	133	0.16	25
Me-3-BzP^+	4.8	83	0.388	62
N,N-diMe-PTP^+	3.0	133	<<< MPP^+	
MPPyrimidinium$^+$	0.7	571	<<< MPP^+	
$MePyrP^+$	7.4	54	0.095	15

*K = Empirical expression relating inhibitor concentration to the time required to reach 50% inhibition of respiration, calculated from the slope of Dixon plots of (I) versus $1/t_{0.5}$. PPP^+ = N-propyl-4-phenylpyridinium; $MeCP^+$ = N-methyl-cyclohexylpyridinium; N,N-diMe-PTP^+ = N,N-dimethyl-4-phenyl-tetrahydropyridinium; MPPyrimidinium = N-methyl-4-phenylpyrimidinium; $MPyrP^+$ = N-methyl-pyridinylpyridinium; Me-4-BzP^+ and Me-3-BzP^+ = N-methyl-4-benzyl (or 3-benzyl) pyridinium. From Ramsay et al.[19]

Table 2. Effect of TPB^- on the Inhibition of NADH Oxidase Activity[a] of Inverted Particles by MPP^+ or 4-Phenylpyridine.

Inhibitor	Concentration (mM)	Inhibition (%) Without TPB^-	With 10μM TPB^-
MPP^+	0.5	0	34
	1.0	15	60
4-Phenylpyridine	0.2	48	48
	0.5	93	94
	1.0	100	100

[a]The NADH oxidase activity of ETP (0.02 mg/ml) was measured spectrophotometrically at 340 nm and 30°C in 50 mM NaPi pH 7.5, in the presence of the inhibitor after preincubation with the inhibitor for 5 min at 0.8 mg protein/ml. From Ramsay et al.[19].

Recent studies in collaboration with Dr. Heikkila's laboratory have yielded some very interesting results.[19,26,27] Fig. 1 shows that 10μM TPB^- dramatically lowers the time required to block State 3 respiration in liver mitochondria by 50μM MPP^+: the inhibition is virtually instantaneous with TPB^- present, but is only partial (80%) even after 30 min. in its absence. Analysis of the phenomenon[19,26,27] revealed that the effect of TPB^- is only partly due to increasing the energy-dependent uptake of MPP^+: the increase in uptake is 2.6-fold at apparent equilibrium, not nearly enough to account for the dramatic effect shown in Fig. 1. It was discovered (Table 2) that 10μM TPB^- also greatly enhances the inhibition of NADH oxidase by MPP^+ in isolated inner membranes. Hence, TPB^-, probably by ion pairing, increases the rate of penetration of the inhibitor to a crevice in the NADH dehydrogenase molecule where the inhibitory site is located.

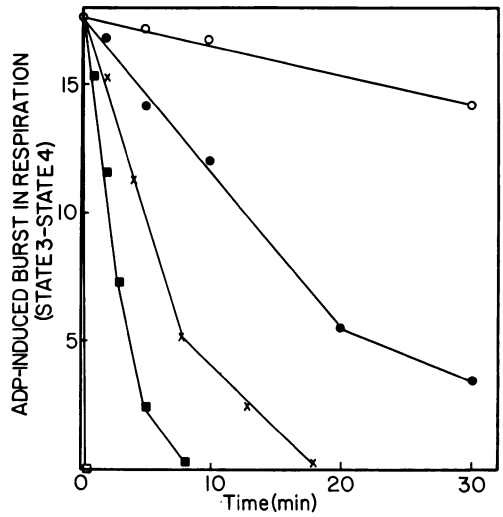


Fig. 1. Time course of the development of inhibition of State 3 respiration by MPP^+ and the effect of TPB^- thereon. Rat liver mitochondria were incubated in State 4 at 1 mg protein/ml in the presence of various concentrations of MPP^+ with glutamate-malate and buffer at 25°C as in previous work (9). At the time indicated ADP was added to 0.25 mM concentration and the rate of O_2 consumption recorded. The ordinate represents the increased respiration induced by ADP. Symbols: (○), no MPP^+; (●), 50 μM MPP^+; (X), 200 μM MPP^+; (■), 500 μM MPP^+; (□), 10 μM TPB^- plus 50 to 500 μM MPP^+. TPB^- (10 μM) alone has no effect on the control rate of respiration.

Such enhancement is seen only with cationic inhibitors: inhibition by the neutral 4-phenylpyridine molecule is unaffected. In contrast, inhibition in ETP preparations by N,N-diMe-PTP^+ is greatly enhanced by TPB^- and the equilibration of this molecule across the mitochondrial membrane is even more enhanced. This suggests that the reason why this compound (and others like it) does not block mitochondrial respiration is in part slow uptake by the mitochondria, in part limited penetration once inside the mitochondria to the

inhibition site in the Complex I region. In intact mitochondria access to this site may be even more restricted than in isolated membrane preparations.

These recent studies thus revealed another step in the series of complex biochemical events from the passage of MPTP across the blood-brain barrier to neuronal destruction.

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ENZYMOLOGICAL ASPECTS OF THE ACTIONS OF THE PARKINSONISM-INDUCING NEUROTOXIN MPTP

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INTRODUCTION

The role of the B-form of monoamine oxidase (MAO-B) in converting the compound MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) to neurotoxic metabolites is well-established from studies on the protection afforded by selective inhibitors of that enzyme (for reviews see Singer et al., 1987; Kinemuchi et al., 1987). The neurotoxic metabolite appears to be the 4-electron oxidation product, the 4-phenylpyridinium ion (MPP(+)). It has been proposed that the action of this compound as an inhibitor of mitochondrial oxidative phosphorylation from NADH may account for its neurotoxicity (Nicklas et al., 1985; Ramsay et al., 1986).

MPP(+) is an inhibitor of oxidative phosphorylation in mitochondria from all mammalian tissues so far examined and it has been proposed that the specific dopaminergic neurotoxicity arises from the ability of these nerve terminals to take up and concentrate this inhibitor (see Singer et al., 1987; Kinemuchi et al., 1987 for reviews). A role for the intermediate MPDP (1-methyl-4-phenyl-2,3-dihydropyridine), formed during the oxidation of MPTP, in its neurotoxicity has, however, not been definitely excluded. It has been suggested that this compound may play a catalytic role in the generation of toxic free-radicals (see Trevor et al., 1986). There have been conflicting reports as to whether oxidative tissue damage accompanies the neurotoxicity of MPTP (Corongiu et al., 1987; Gotz et al., 1989).

It is difficult to study the effects of MPDP in isolation since it is a highly reactive compound which can disproportionate to MPTP plus MPP(+) (see Kinemuchi et al., 1987) as well as being oxidised to MPP(+) by MAO.

THE OXIDATION OF MPTP

The oxidation of MPTP to MPDP and MPP(+) by MAO-B is a self-limiting process in that MPTP is a mechanism-based inhibitor (suicide substrate) of the enzyme. The process can be represented by the overall scheme:

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where E represents MAO-B, E - I represents the irreversibly inhibited species and (E.MPDP)* represents an intermediate complex which can either breakdown to yield product, or react to form the irreversibly inhibited species (Tipton et al., 1986). A possible chemical mechanism whereby this irreversible inhibition may take place has recently been proposed (Ottoboni et al., 1989).

This mechanism-based inhibitory scheme can be characterised by an apparent Km value for the overall process (K'), an intramolecular rate constant for the inhibitory process (k) and a partition ratio (r), which represents the number of mol of product formed per mol of enzyme at total inactivation. Analysis of the kinetics of inhibition of rat liver MAO-B has allowed these values to be calculated (Tipton et al., 1986) and these are shown in Table 1. We have recently extended these studies to investigate the inhibition of ox liver MAO-B, purified by the procedure of Salach et al. (1979), by MPTP. The kinetic parameters obtained are shown in Table 1.

The self-catalysed inhibition of MAO-B during the oxidation of MPTP imposes an upper limit on the amount of MPDP and MPP(+) that can be formed following MPTP administration. This may contribute to the species differences in sensitivity to the neurotoxicity of MPTP which have been reported (see Kinemuchi et al., 1987 for review). For example the levels of MAO-B in the rodent brain have been shown to be significantly lower than in human brain (Fowler and Strolin-Benedetti, 1983) and such species are relatively insensitive to the specific neurotoxicity of MPTP (Jenner and Marsden, 1986). The activities of MAO-B have also been shown to increase with age in several species (see Fowler et al., 1980) and this might contribute to the age-dependent increase in the sensitivity of rodents to the neurotoxicity of MPTP.

The observation that inhibitors of presynaptic dopamine uptake protect against the neurotoxic effects of MPTP and MPP(+) (for reviews see Singer et al., 1987; Kinemuchi et al., 1987) indicate that MPP(+) formation must occur outside the dopaminergic nerves. This is consistent with the observation that MAO-B is absent from human caudatal dopaminergic synaptosomes (O'Carroll et al., 1987). However that enzyme is present in nordrenergic and serotoninergic synaptosomes (O'Carroll et al., 1987; Thorpe et al., 1987). This suggests that MPTP diffusing into those nerve endings might result in MPP(+) formation occuring within them. Such in situ formed MPP(+) might be trapped within these nerves, because of its positive charge, and this might thus be expected to lead to some noradrenergic and serotoninergic neurotoxicity. However our own studies (Sullivan and Tipton, 1988) have indicated that MPTP does not readily penetrate the synaptosomal membrane and this factor may contribute to the relative insensitivity of such neurones to the neurotoxicity of MPTP. The MPP(+) formed outside the dopaminergic nerve terminals is presumably produced in glial cells which contain relatively high activities of MAO-B (O'Carroll et al., 1987). The mechanism by which this charged metabolite escapes from these cells remains to be elucidated.

The lack of any significant role for MAO-A in the neurotoxicity of MPTP is illustrated by the failure of selective inhibitors of that enzyme to protect against its neurotoxic effects (see Singer et al., 1987; Kinemuchi et al., 1987). MPTP is a poorer substrate for MAO-A than it is for MAO-B (see Tipton et al., 1986) and it may be that the slower rate of MPP(+) formation due to the activity of this enzyme form may not result

Table 1. Parameters for the Mechanism-Based Inhibition of MAO-B by MPTP and PTP.

Enzyme Source	Compound	K'(μM)	k(min^{-1})	r
Rat Liver	MPTP	6.2	0.032	17100
Ox Liver	MPTP	15.3	0.015	1590
Ox Liver	PTP	2.6	0.01	413

Data were obtained at 37°C and at pH 7.2. Those for rat liver MAO-B are from Tipton et al. (1986).

in its accululation to toxic levels. However, further work is required to identify the mechanisms involved.

THE OXIDATION OF PTP

The analogue of MPTP lacking a methyl group on the heterocyclic nitrogen, 4-phenyl-1,2,3,6-tetrahydropyridine (PTP), has been reported not to be neurotoxic (Irwin et al., 1986). However its fully-oxidised derivative, the 4-phenylpyridinium ion is apparently neurotoxic when directly injected into the brain (Snyder and D'Amsto, 1985). Studies on the kinetics of PTP oxidation by ox liver MAO-B have shown it to resemble MPTP in being a "suicide substrate" of the enzyme. The kinetic parameters calculated are shown in Table 1.

Compared with MPTP, PTP acts as a better inhibitor than substrate of the enzyme, as can be seen by comparing the partition ratios given by these two compounds. The smaller amount of product formed per mol of enzyme may contribute to the lack of neurotoxicity of this compound. However a more striking difference between MPTP and PTP emerged from these studies. The formation of MPDP and MPP(+) during the oxidation of MPTP can be readily followed spectrophotometrically by monitoring the increase in absorbance at 340nm and 280nm, respectively (Tipton and Sullivan, 1989). However when the oxidation of PTP was monitored the formation of the dihydropyridine species (PDP) was not followed by any significant formation of the corresponding pyridinium ion (PP(+)).

Thus the lack of neurotoxicity of PTP might be related to its failure to give rise to any significant amounts of the 4-phenylpyridinium ion. The ready formation of the dihydropyridinium intermediate might, however, suggest that the corresponding 1-methyl-4-phenyl-2,3-dihydropyridine does not play a major role in the acute neurotoxicity of MPTP.

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BIOCHEMICAL STUDIES ON PREDISPOSITION TO PARKINSON'S DISEASE

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After the L-dopa revolution in the treatment of Parkinson's disease (for review, see ref.1), there was a decade of consolidation, characterized by small but important variations on this therapeutic theme. However, in 1979, another great leap forward occurred when Davis et al. (2) clearly delineated the beginnings of the MPTP story. The sequence of events is now well known. Following that original publication, there was little interest until the phenomenon was rediscovered by Langston and his colleagues (3) in 1983, and the whole thing caught fire. Thus, a prodrug, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), had been discovered and shown to be neurotoxic only when converted by monoamine oxidase (MAO) B (4) to its quaternary ammonium derivative, 1-methyl-4-phenylpyridinium (MPP+). Administration of MPTP to man (2,3), or monkey (5) but not to rat (6) results in the best simulation of idiopathic Parkinson's disease that we possess, which responds characteristically to L-dopa (5). The MPTP-treated monkey fails to develop the characteristic nigrostriatal lesions if pretreated with an MAO B inhibitor (7,8), a major signpost for what was to follow.

The MPTP model is considered to be particularly important because most available evidence points to an environmental cause of Parkinson's disease. The findings of Ward et al (9), that there is no increased incidence of the illness in identical twins of proven cases, has been widely quoted, suggesting that the likelihood of genetic predisposition is low. One possible environmental cause, a putative viral aetiology, has been intensively investigated over the years (e.g. ref.10) with negative results. Ebmeier et al (11) have recently failed to replicate the claims of Mattock et al (12) that the development of Parkinson's disease is related to successive influenza pandemics of 1890-1930.

Parkinsonism may, then, be viewed as a final common pathway resulting from a variety of neurotoxins which interfere with the integrity of the nigrostriatal system and its output (13). Thus, carbon monoxide, manganese, carbon disulphide and arsine have all been incriminated, although there is not such a clear cause and effect relationship as with MPTP. Whether an environmental pollutant is responsible for the idiopathic disease, perhaps deriving from some industrial process and therefore revealing itself only at the time of the industrial revolution, has been widely postulated (e.g. ref.14). If James Parkinson's description of the illness (15) in 1817 really was the first - although

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this is disputed by some (16) - then this arrived remarkably late on the scene. A possible role of industrial effluents receives some support from epidemiological studies. Thus, in door-to-door surveys, exposure to industrial chemicals in China appears to be associated with an increased risk of Parkinson's disease, whereas village life and pursuits are associated with a low incidence (17). Similar door-to-door surveys revealed that blacks in Nigeria have a much lower prevalence ratio than blacks in the United States (18).

MPTP, the first and most powerful of the protoxins so far identified, already has a substantial number of toxic analogues (e.g. refs. 19-21) and their tally in the literature increases daily. Some of these analogues, indeed, are better substrates of MAO A and lead to neurotoxicity in a mouse model which can only be blocked by deprenyl together with the MAO A inhibitor, clorgyline (22). However, it should be mentioned that MAO B tends to predominate in the human striatum (23), whereas MAO A is relatively more important in the rodent (24).

Where are we to search for this unknown toxin - if it really exists? Perhaps we ought not to limit our scrutiny to industrial effluents and the like : items of food, such as the tomato (25), the use of which became widespread only at the beginning of the 19th century, should be considered. Pyridines are extremely common dietary constituents - caramelized sugar, for example, contains many different types (14). Peppermint and spearmint (26), and roasted cocoa (27), contain 3-phenylpyridine, whilst both 2- and 3-phenylpyridine are found in tea (28). Perry et al. (29) have shown that, despite their resemblance to MPTP, neither of these compounds, nor their N-methyl derivatives, are neurotoxic in mice.

How shall we narrow our search? If we do assume that the protoxin for which we seek is a substrate of MAO B, this enzyme may then be used to screen suspect compounds (19). MPTP-like compounds which eventually prove to be neurotoxic are characterized by a 4-5 double-bond in the pyridine ring, a methyl group attached to the nitrogen atom and a substitution in the 4-position (30). MPP+ is taken up avidly by adrenal medullary chromaffin granules (31,32), at least as well as noradrenaline (33); cytosolic concentrations and eventual mitochondrial accumulation are determined by the capacity of the saturable uptake transporter (34) on the chromaffin vesicle. Thus, Langston (35) has suggested that the adrenal medulla might act as a reservoir for the slow release of an MPP+-like neurotoxin into the body. It seems unlikely, however, that quaternary ammonium compounds of this type, to which the blood-brain barrier is quite impermeable, could gain access to the brain from the periphery. Even so, it may well be that the concentrating ability of the adrenal medulla for this type of compound should be utilized in any search for unknown compounds which might be enriched in this site. Such adrenal medullary uptake of this type is very similar to that observed in brain synaptosomes (36), where MPP+ is taken up by the dopamine uptake system. When synaptic vesicles are saturated, cytosolic concentrations and mitochondrial accummulation presumably occur. A sequence of this type may well be responsible for the mitochondrial respiratory deficit, the complex I deficiency, noted in the substantia nigra of parkinsonian patients (37).

Other possibilities, of course, might be invoked to account for the nigral cell death observed in parkinsonian patients. What are the chances of adventitious hydroxylation of dopamine occurring, giving rise to the neurotoxic 6-hydroxydopamine (38)? This might result from a deficit of superoxide dismutase (39). It is true that an increase of particulate,

manganese-dependent, mitochondrial superoxide dismutase has been observed in the parkinsonian substantia-nigra (40) but the finding may well be drug-induced : terminal parkinsonians are likely to have been in receipt of a complex cocktail of drugs. There is evidence that administration to the rat of at least one drug likely to be a constituent of this cocktail, (-)-deprenyl, can result in a very highly significant increase in superoxide dismutase (41). Perhaps this effect contributes to what may well be the ability of deprenyl to limit further progression of Parkinson's disease, even though it has always been assumed that any such effect stems from MAO B inhibition.

In a retrospective study, Birkmayer et al. (42) were able to show that patients treated with L-dopa plus decarboxylase inhibitor, together with deprenyl, lived significantly longer than those not in receipt of deprenyl. Very recently, Tetrud & Langston (43), in the first prospective study of this problem, demonstrated that deprenyl significantly postponed the necessity to institute L-dopa treatment in parkinsonians. A much larger study along these lines, the DATATOP trial (44), seems likely to provide a definitive answer to the question shortly. This trial also includes an evaluation of the antioxidant, vitamin E. It is of interest that some evidence does exist suggesting that a diet rich in the vitamin is protective against the development of Parkinson's disease (45).

As mentioned above, the generally accepted view as to how deprenyl might limit the progression of Parkinson's disease - and perhaps even prolong life in general (46) - is via MAO B inhibition. Even so, it should be remembered that other mechanisms may come into play; the drug is known to prevent amine reuptake (41) and certain drugs possessing this property, such as nomifensine (but not others - see ref.30), have the ability to protect monkeys against MPTP (47).

The use of long-term deprenyl in Parkinson's disease has generally been predicated on its ability to block the conversion of environmental protoxin to toxin. However, Hornykiewicz (48) has recently expressed doubts about this concept. He noted that, in idiopathic Parkinson's disease, the caudal subdivisions of the substantia nigra, which project to the putamen, are more affected by the degenerative process than the rostral, which project to the caudate and are more affected by MPTP pretreatment. He points out, though, that if an endogenously generated neurotoxic substance were responsible, to be generated in situ, it would accumulate in those parts of the nigra with the highest density of dopaminergic neurones i.e. the caudal region. Such an endogenous agent might well be tetrahydroisoquinoline (THIQ) which is synthesized in the parkinsonian brain in particular (49,50) and can be N-methylated to a protoxin (51) which is oxidized by MAO B, at about 3% of the rate of MPTP oxidation (52) to a neurotoxic quaternary ammonium compound. THIQ, which produced parkinsonism in primates on systemic administration (53) (or, more likely, its N-methyl derivative) selectively inhibits complex I of the brain mitochondrial electron transport system (54). As mentioned earlier, such a deficit has been noted in the parkinsonian substantia nigra (37).

If Parkinson's disease does derive from the action of some environmental or endogenous neurotoxin, it is obvious that all subjects exposed to it are not equally affected. It is quite conceivable, despite the twin studies of Ward et al (9) referred to earlier, that the action of such a toxin will only be manifested in the presence of previous genetic susceptibility. Indeed, as Sandler (55) has pointed out, the identification of peripheral biochemical differences may in future allow

the presymptomatic diagnosis of Parkinson's disease, thus making possible early institution of deprenyl therapy. Steventon et al. (56), for instance, have recently provided evidence of a deficiency in detoxication pathways involving sulphur metabolism. Thus, affected subjects may be unable to detoxicate toxic environmental compounds of this type. The same group (57) have identified a significant decrease of red cell thiolmethyltransferase in parkinsonians (and high activity in motorneurone disease). This enzyme, in fact, may be responsible for the detoxication of intestinal hydrogen sulphide (H_2S) (58). H_2S intoxication can lead to an encephalopathy and, rarely, to basal ganglia damage (59). Presymptomatic diagnosis of the disease is, of course, perfectly feasible with PET scan (60,61), although this procedure is so costly and labour intensive that it cannot be considered a mass screening technique.

The question of why some individuals develop Parkinson's disease whilst close relatives do not is open to other investigative approaches. Since the first description of an endogenous urinary MAO inhibitor (62), denominated tribulin (63), there have been intensive studies, showing that increased production of this activity is related to stress/anxiety, both in rat (64) and man (65,66). Tribulin is widely distributed in the body, with particularly high activity being present in the rat superior cervical ganglion (67). Considerable amounts of it are present in brain and its concentration is also increased by stress (68). It may well be that such an endogenous MAO inhibitor controls MAO activity in vivo (69). A major proportion of tribulin activity has now been unequivocally identified as isatin (2,3-dioxyindole) (70) and it is of particular interest that this substance is predominantly a competitive inhibitor of MAO B. It is well know, of course, that MAO B activity in the brain rises with age. Very recently, however, Ueki et al. (71) have been able to show that tribulin output also rises with age in control subjects - and it is possible that a normal production of this inhibitor regulates MAO B, preventing free oxidative deamination of any MPTP-like substance presented either in the environment or endogenously. How successfully is a matter for further investigation - in one mouse strain, at least, Walsh & Wagner (72) have been able to demonstrate a highly significant correlation between brain MAO B activity, which increases with age and degree of brain lesion induced by MPTP treatment. Can it be, then, that Parkinson's disease is a tribulin-deficiency disease? This possibility was first foreshadowed by Yong and Perry (73) but they were unable to find data in support, possibly because of drug interference. We ourselves, in a very preliminary evaluation of this question (71), were unable to find an increase with age in tribulin output in parkinsonians compared with that observed in normal subjects. We are investigating this possibility more intensively at the present time in a larger number of drug-free patients with Parkinson's disease.

The possible role of isatin itself, the major constituent of tribulin, is particularly interesting. As mentioned, its concentration in the body is responsive to such stimuli as stress and anxiety. And yet, we now have preliminary evidence that isatin is generated by gut flora (in preparation). Urine samples from germ-free rats contained significantly less of it than either conventional controls or germ-free rats into which microorganisms had been reintroduced. Thus, if an isatin deficiency really lies at the heart of Parkinson's disease, it may stem from some abnormality of gut flora. There are numerous precedents for endogenously generated substances being employed by the body in its normal functioning e.g. vitamin K which, when supplied by the gut flora, prevents haemorrhagic disease of the newborn (74) and benzodiazepines, which have been detected in brain material collected fifteen years before these compounds were first synthesized (75) and may well derive from plant or bacterial sources (76,77).

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PARKINSOISM PRODUCED BY AN ENDOGENOUS SUBSTANCE, TETRAHYDROISOQUINOLINE, IN MONKEYS AND MICE

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INTRODUCTION

The discovery by Langston et al[1]. that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a side product of meperidine, produced symptoms quite similar to those of Parkinson's disease in humans promoted research toward elucidation of the etiology of Parkinson's disease. MPTP, however, can not be a causal substance to produce Parkinson's disease, since MPTP does not exist in the natural environment. Barbeau et al[2]. suggested that paraquat, a herbicide having a similar chemical structure to MPTP, might cause Parkinson's disease, since a high incidence of the disease was observed in an agricultural area where paraquat was used compared with that in other areas they surveyed. This suggestion, however, can not be true, as the disease had already been described 1817 by James Parkinson when such an industry product did not exist.

On the other hand, Hirata and Nagatsu[3] have extensively screened substances with a similar structure as MPTP and found from in vitro as well as in vivo experiments that tetrahydroisoquinoline (TIQ), whose structure is shown in Fig. 1, might be a candidate to produce parkinsonism.

Recently, we administered TIQ to monkeys and mice and found that TIQ produced remarkable motor disturbances that were reversed by levodopa administration, biochemical changes in the substantia nigra of the monkeys, and neuropathological changes in the substantia nigra of the mice.

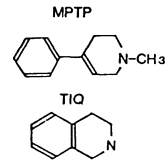

Fig. 1. Structures of MPTP and TIQ

PRESENCE OF TIQ IN THE BRAIN AS WELL AS IN FOODS

Gas chromatography-mass spectrometry has demonstrated the presence of TIQ in the brain of rats as well as humans[4-6]. In rats the TIQ concentrations were about 6 ng/g wet weight of tissue in the spinal cord and also 2 ng/g in the brain, while those in the lung, intestine, and liver were about 1 ng/g or less. The concentrations of TIQ in the frontal cortex of the brains of the control subjects and the patients with

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

Table 1. Concentrations of TIQ in Various Foods

Sample	Concentration of TIQ (ng/g)	Sample	Concentration of TIQ (ng/g or ng/ml)
Cheese	5.2	Yolk of boiled egg	1.8
Banana	2.2	White of boiled egg	2.2
Broiled sardine	0.96	Wine	0.56
Broiled beef	1.3	Beer	0.36
Flour	0.52	Whisky	0.73
		Milk	3.3

Parkinson's disease were 0.86 ± 0.23 and 0.54 ± 0.20 ng/g, respectively, and the corresponding values for the caudate nucleus were 0.64 ± 0.24 and 0.25 ± 0.08. Although the TIQ concentration tended to be lower in the Parkinson's disease than that in the controls, no definite conclusion could be drawn because of the low sample numbers.

Aside from its presence in the human body, TIQ has also been detected in cheese, wine, and cocoa[7]. Furthermore, Niwa et al[8]. recently found that TIQ was distributed widely in a variety of foods (see Table 1).

BEHAVIORAL CHANGES ASSOCIATED WITH LONG-TERM ADMINISTRATION OF TIQ IN MONKEYS

Behavioral changes produced by long-term administration of TIQ were studied in monkeys[9]. Four common marmosets were studied: A, a 2.1-year-old male; B, a 2.8-year-old female; C, a 2.5-year-old male; and D, a 2.6-year-old female. Saline alone was injected subcutaneously once a day for 16 days into A and B; and TIQ at 50 mg/kg, into C and D. Since TIQ does not dissolve in saline, a suspension of 1 g of TIQ in 20 ml of saline was made by shaking the container just before injection.

The behavioral changes were monitored by a conventional video-tape recorder, and videos were taken daily and evaluated according to the scores. Vertical and horizontal shifts were evaluated by checking of the animals, abilities to climb the wall of the cage and to move or jump on the floor, respectively. Motor functions of the face, trunk, limbs, and hands and feet were also scored, as were responses to people or food. Pathological motor symptoms such as lying down, moving with legs dragging, tremor, and muscular hypertonicity were carefully analyzed. Each item was evaluated by 5 grades from 0 to 4 such that the greater the motor dysfunction or severity of symptoms, the higher the value. Thus, a total score of 60 would indicate the worst possible condition. Monkeys A and B, the saline-injected, both had a score of 0, whereas C and D, the animals treated with TIQ for 16 days, showed scores of 44 and 39, respectively. The presence of akinesia in C and D was confirmed, as was tremor of 4-6 Hz in the legs, arms, and trunk. Hypertonus of the rigidity type was also present in C and D, being more marked in C and generally more severe in the legs. Thus these changes of behavior and motor functions led us to the conclusion that TIQ produces parkinsonism in these primates.

For biochemical study, all 4 animals were sacrificed under deep ketamine-induced anesthesia on the 16th day of daily administration of either saline or TIQ. Each brain was immediately removed, and kept at -80°C until biochemical analysis could be performed. To determine dopamine (DA), biopterin (BP), and tyrosine hydroxylase (TH) activity, we homogenized brain samples in 200 µl of 0.25 M sucrose, and a 30-µl aliquot of the homogenate was used for the assay of DA and BP.

DA was isolated by alumina absorption and measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD)[10]. Total BP was measured by a method using HPLC with fluorescence detection[11]. TH activity in 20 µl of the homogenate was determined by an HPLC-ECD method[12]. The incubation mixture (total volume, 200 µl) contained 0.2 M acetate buffer (pH 6.0), 50 µg of catalase, 1 mM (6RS)-methyltetrahydropterin, 0.1 M mercaptoethanol, 0.2 mM L-tyrosine, and the homogenate. Control incubation contained 0.1 mM 3-L-tyrosine and 0.2 mM D-tyrosine. Incubation was carried out at 37°C for 10 min, and DOPA formed was isolated by double columns of Amberlite CG-50 and alumina and measured by HPLC-ECD.

Biochemical data on DA and total BP concentrations and on TH activity in the substantia nigra and striatum were thus obtained. The reductions in DA and BP concentrations and TH activity were more pronounced in the substantia nigra than in the striatum. When C and D were compared with A and D, respectively, for the changes in the substantia nigra, the DA and BP concentrations were seen to be reduced to approximately 30% and 20% in both C and D; and TH activity, to 76% in C and to 30% in D. These results show that administration of TIQ resulted in depletion of DA and BP and a drop in TH activity. In D, both DA and BP concentrations and TH activity decreased to a similar extent; but in C, the reduction in TH activity was moderate compared with the reduction in DA.

Quite similar results were also obtained in 7 squirrel monkeys in which TIQ (20 mg/kg/day) was administered for 60 to 104 days and was discontinued for 7 days after the final injection. In these monkeys levodopa (40 mg/kg) was administered orally. In 5 monkeys thus treated there was marked improvement of the motor disability scores; the improvement in each monkey was 82%, 56%, 54%, 44%, and 39% and reached the maximum at about 60 to 90 minutes after the levodopa administration.

NEUROPATHOLOGICAL CHANGES IN MESENCEPHALIC DOPAMINERGIC NEURONS OF MICE ASSOCIATED WITH LONG-TERM ADMINISTRATION OF TIQ

Ogawa et al[13]. studied morphological changes in the brains of mice following chronic administration of TIQ. Male 8-week-old C57BL/6J mice were used. TIQ was injected subcutaneously for 70 days at a dose of 50 mg/kg/day. Twenty-four hours after the last injection of TIQ, the mice were anesthetized with sodium pentobarbital and their brains were perfused transcardially for 5 min with ice-chilled fixative containing 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Following removal of the brain, immersion fixation was carried out for 24 h at 4°C in the same fixative. After washing of the tissue with 0.1 M phosphate buffer containing 15% sucrose, sections were cut at a 20-µm thickness with a freezing microtome. Immunostaining of free-floating sections was carried out by the peroxidase-anti-peroxidase (PAP) method. The primary antibody used in the present study was rabbit anti-tyrosine hydroxylase (diluted 2000 times). The specificity of this antibody was confirmed previously[14]. In the control mice, soma of the nerve cells located in the pars compacta of the substantia nigra or SNc (A9 cell group), as well as those in the ventral tegmental area or VTA (A10 cell group), and their processes were intensely stained with anti-TH antibody (Fig. 2, A,B,C). In the TIQ-treated mice, however, TH-like immunoreactive (THLI) neurons in the SNc were markedly decreased in number (Fig. 2D,E). The VTA also showed a moderate reduction in the number of THLI neurons (Fig. 2D,F). Although normal-looking THLI neurons could be observed in all of these areas in the TIQ-treated mice, their numbers were markedly reduced. On the other hand, when cresyl violet staining was performed,

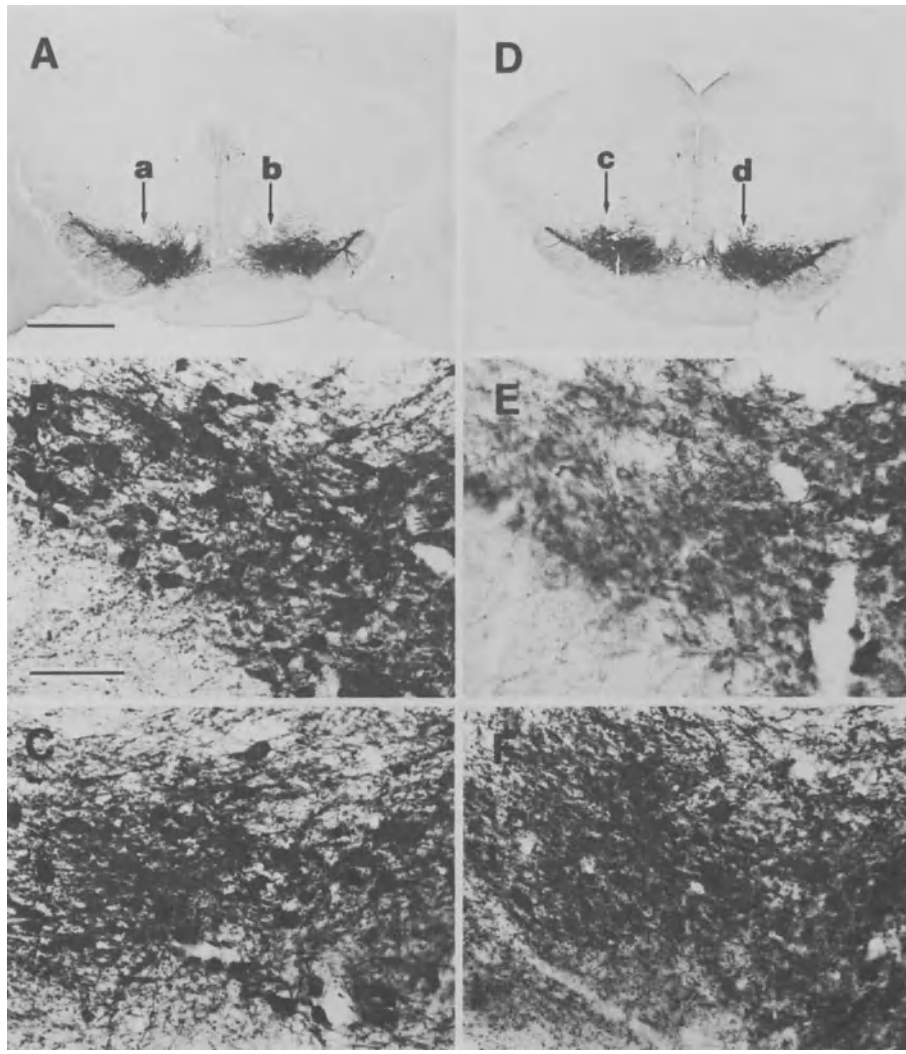

Fig. 2 Tyrosine hydroxylase (TH)-like immunohistochemistry of sections of the mesencephalon of a control (A,B,C) and of a TIQ-treated mouse (D,E,F) specifically showing the SNc and VTA regions. (B) Magnification of A at the area indicated by arrow a. (C) Magnification of A at the area indicated by arrow b. (E) Magnification of D at the area indicated by arrow c. (F) Magnification of D at the area indicated by arrow d. Note the marked decrease in TH-like immunoreactivity of SNc cells (E). A moderate decrease is also seen in the cells of the VTA (F). A and D, X 12. Bar = 1000 µm in A,B,C,E and F; X 128. Bar = 100 µm in B.

the numbers of neurons in the SNc and VTA of the TIQ-treated mice were almost the same as those of the control mice. In the TIQ-treated mice, some of the nerve cells located in the SNc looked somewhat smaller than those of the control mice. The THLI processes in all areas also remained normal.

In 3 controls and 3 TIQ-treated mice, the number of THLI neurons in the SNc was counted in 20-μm-thick sections processed for TH immunoreactivity. The sections were selected every 200 μm (5 mice) and every 80 μm (1 mouse). THLI neurons were counted for the bilateral SNc in each case. We used the following formula to estimate the total number of THLI neurons: Total counts = sample counts X p, p being the period at which sections were sampled. The mean ± SD of the number of THLI neurons in the SNc was 10190 ± 1812 in control mice (n=3), while it was 4671 ± 1069 in the TIQ-treated mice, the reduction rate being 56%. TH consists of both active and inactive forms and both forms are stained by this immunohistochemical method. Therefore, dopamine neurons not stained by anti-TH antibody in our experiment were severely damaged in terms of their ability to produce TH protein. However, the neurons themselves were preserved. Thus, we conclude that TIQ did not lead to neuronal death under our experimental conditions. It is known that following even MPTP administration to young adult mice, recovery from damage occurs both biochemically[15,16] and morphologically[17]. Since the mice we used were 8-week-old young adults, we must now evaluate the effect of TIQ on aged mice.

SUMMARY

TIQ has been shown to exist in the brain endogenously and to be distributed widely in foods. Long-term administration of TIQ produced levodopa-reversible motor disturbances similar to those seen in parkinsonism, decreases in concentrations of dopamine and total biopterin, and a drop in tyrosine hydroxylase activity in the substantia nigra of monkeys. Similar administration to mice produced a marked decrease in TH-positive neurons of the substantia nigra. The cell death, however, could not be comfirmed in these neurons by cresyl violet staining. Now we are studying the effect of TIQ in aged animals.

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TETRAHYDROISOQUINOLINE ALKALOIDS IN NEURODEGENERATIVE DISORDERS - INFLUENCE OF DRUG TREATMENT

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The presence of various 1,2,3,4-tetrahydroisoquinoline alkaloids in the human brain has been firmly established[1-5] and the possible involvement of these alkaloids in the etiology of some neurodegenerative disorders, such as Parkinson's disease, or in the craving for alcohol has been the object of many investigations and hypotheses.[4-8]

Preliminary results suggest that brain 1,2,3,4-tetrahydroisoquinoline (TIQ) levels might be higher in parkinsonian patients than in controls.[4] Symptoms similar to parkinsonism were produced by administration of TIQ to marmosets,[7] in line with the similar effects on mitochondrial respiratory functions[9] caused by TIQ and MPP^+ (N-methyl-4-phenylpyridinium ion), the neurotoxic metabolite of MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Analogously with the first step in the formation of MPP^+ from MPTP, N-methyl-TIQ was shown to be oxidized by monoamine oxidase into N-methylisoquinolinium ion,[10] an inhibitor of tyrosine hydroxylase as MPP^+.[11] However, in contrast to what was reported with MPP^+, striatal DA concentrations remained unchanged in the surviving marmoset, out of 4, after repeated administration of N-methyl-TIQ.[6]

Besides the possible neurotoxic role of phenylethylamine-derived alkaloids, such as TIQ, it has also been suggested that alkaloids derived from dopamine (DA), such as salsolinol (1-methyl - 1,2,3,4-tetrahydro - 6,7-isoquinolinediol, Sal), tetrahydropapaveroline (1-[(3,4-dihydroxyphenyl)methyl]-1,2,3,4-tetrahydro-6,7-isoquinolinediol, THP), and/or various alkaloids resulting from further metabolism of THP, might be the cause of choreic movements in Huntington's disease and of the on-off phenomenon in parkinsonian patients on L-dopa medication.[8,12] In addition, Sal, THP, and THP-derivatives have long been suspected to play a role in the addictive property of alcohol (see[13] for review). Regarding Sal, no difference in Sal concentrations was found in various brain structures from controls and alcoholics with ethanol in blood at autopsy,[1,3] whereas significantly decreased brain Sal concentrations were reported when alcoholics without blood ethanol were compared to controls.[1,2]

Sal[14] and TIQ[15] are present in high concentrations in some foods and beverages. While lack of agreement exists on whether Sal can enter the brain when given peripherally,[8] increased rat brain concentrations of TIQ were measured after intraperitoneal administration of TIQ.[15]

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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In this paper, we report on the urinary excretion of Sal in humans. These studies have been carried out to investigate whether the daily urinary output of Sal may reflect brain DA levels in parkinsonians and patients with degenerative dementia compared to controls. Since Sal exists as R and S enantiomers, concentrations of both isomers were measured. Moreover, as the biosynthesis of Sal in alcoholics and healthy subjects might follow different pathways, the enantiomeric composition and daily excretion of Sal were also determined in alcoholics. If Sal actually plays a role in the craving for alcohol, there is no evidence that both isomers should produce the same effect.

SALSOLINOL BIOSYNTHESIS IN HEALTHY SUBJECTS

In humans, the biosynthesis of Sal has been suggested to occur by condensation of DA with acetaldehyde,[16] or with pyruvic acid followed by decarboxylation.[17] We have established that the R enantiomer of Sal is the predominant or the only isomer present in the urine of healthy volunteers.[14,18] The presence of 1,2-dehydrosalsolinol (1-methyl-3,4-dihydro-6,7-isoquinolinediol, DSal) (Figure 1) in the urine of healthy volunteers was recently demonstrated (Dostert et al., unpublished results). Measurements of Sal and DSal levels in the urine of healthy subjects before and after administration (3 x 62.5 mg/day) of Madopar (L-dopa + inhibitor of peripheral aromatic L-amino acid decarboxylase) for 7 days led us to the conclusion that the biosynthesis of Sal in healthy volunteers does occur by condensation of DA with pyruvic acid according to Figure 1. There is apparently no alternative pathway. The stereoselective reduction of DSal into (R)-Sal seems to be the key step in the formation of Sal in normal subjects. It is worth noting that the enzymatic system(s) able to reduce the C=N bond in DSal was found to be missing or not functional in some subjects. It might also be that, in these subjects, another metabolic pathway, such as an oxidative one, predominates and masks the reductive step.

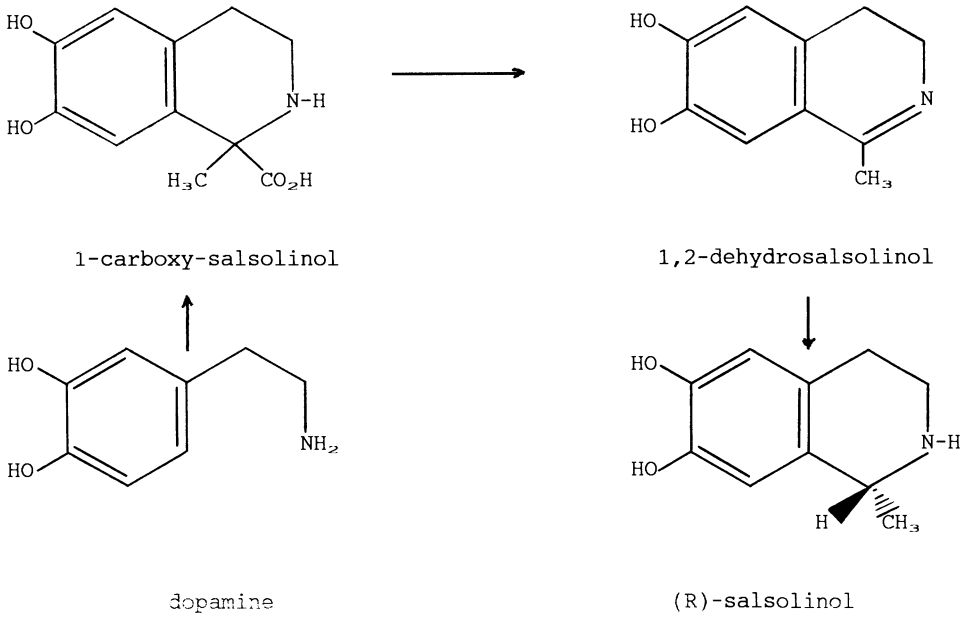

Figure 1. Biosynthetic pathway of (R)-salsolinol in healthy subjects.

URINARY EXCRETION OF (R)-AND (S)-SALSOLINOL IN PARKINSONIAN PATIENTS

In a first study,[19] using a GC/MS method (estimated limit of detection: 10 pmol/ml), the urinary excretion of total (R + S) Sal was found to be significantly lower in non-treated parkinsonian patients than in controls, being 39 ± 7 and 197 ± 54 nmol/day, respectively. Administration of Madopar to the parkinsonians for 7 days resulted in a dramatic increase in urinary Sal excretion (1409 ± 148 nmol/day).

In a second study,[20] conducted in non-treated parkinsonians with apparently more severe parkinsonism than the subjects of the first study, the urinary concentrations and daily excretion of (R)-and (S)-Sal and of DSal (free + sulfoconjugate) were measured before and after administration of Madopar (3 X 62.5 mg/day) for 7 days. Concentrations of Sal enantiomers and DSal were determined using an HPLC method with electrochemical detection, after derivatization with a chiral reagent in the case of Sal; limit of detection: 14 pmol/ml and 10 pmol/ml for the enantiomers of Sal and DSal, respectively. Neither the R nor the S enantiomer of Sal was detected before administration of Madopar. After Madopar, measurable concentrations of (R)-and (S)-Sal were found in the 24-h urine of all parkinsonian patients. The R/S ratio varied from 0.7 to 1.4, indicating that similar amounts of both isomers are excreted when the amount of DA available for Sal formation is artificially increased. Before Madopar, DSal (free + sulfoconjugate) was detectable in most patients, but the amounts excreted were markedly less than those of total DSal in normal subjects. The fact that the urinary excretion of Sal and probably DSal is largely reduced in parkinsonians compared to controls has to be seen in the light of the results reported by Ohta et al.,[5] who found significantly decreased concentrations of 1-methyl-TIQ in frontal cortex of parkinsonians compared to controls.

URINARY EXCRETION OF (R)-AND (S)-SALSOLINOL IN PATIENTS WITH DEGENERATIVE DEMENTIA

In biochemical studies of Alzheimer's disease and senile dementia of the Alzheimer's type, the dopaminergic system has received little attention. Findings on DA in Alzheimer's patients with histological confirmation are controversial; most studies, however, suggest that DA neurons are not or little affected.[21]

The urinary concentrations of (R)-and (S)-Sal were measured in 12 patients with degenerative dementia.[22] While (S)-Sal was never detectable, the R enantiomer was detectable in the urine of 5 patients. The presence of (R)-Sal in the urine of 5 out of 12 demented patients seems to indicate that, contrary to non-treated parkinsonians, the formation of (R)-Sal is not totally impaired in patients with degenerative dementia.

The mean daily urinary excretion of DSal (free + sulfoconjugate) was similar in the demented patients of this study and in the non-treated parkinsonians of the above mentioned second study, being 13.3 ± 12.3 and 16.1 ± 16.0 nmoles (mean ± S.D.), respectively. That the two groups of patients had similar daily output values of DSal, whereas (R)-Sal was detectable in demented patients but not in non-treated parkinsonians, suggests that the reduction of DSal into Sal is missing or is not, or poorly, functional in parkinsonians but still working in demented patients. The similar urinary output of DSal in the two groups of patients, in amounts largely higher than the amount of Sal excreted in demented patients, also suggests that brain DA-rich regions might be equally affected in de novo parkinsonians and severely demented patients.

URINARY EXCRETION OF (R)-AND (S)-SALSOLINOL IN ALCOHOLICS

In a first study,[23] we measured the daily urinary excretion of (R)-and (S)-Sal in healthy volunteers, collecting the urine in two fractions: from 9 a.m. to 5 p.m. and from 5 p.m. to 9 a.m. None of them drink wine at lunch. The S enantiomer was only present during the period 5 p.m. - 9 a.m., and only in the urine of those who drink regularly a substantial amount of alcoholic beverages during the evening meal.

In another study the daily urinary excretion of (R)-and (S)-Sal was measured in 6 chronic alcoholics.[24] Urines were collected after 1 or 2 days of hospitalization. Both the S and the R enantiomers were found in the urine of 4 out of the 6 subjects. In one subject, only the R enantiomer was detectable, whereas in another subject both enantiomers were undetectable. In this latter subject, however, urinary concentrations of DSal were similar to those of the other subjects. The absence of Sal in the urine of this subject confirms that the reduction of DSal can be impaired in some individuals, and suggests that, also in alcoholics, the biosynthesis of Sal does not occur by condensation of DA with acetaldehyde. There was no relationship between the presence of alcohol in blood and the urinary excretion of either the R or the S enantiomer of Sal. The subject without Sal in urine had alcohol in blood at his admission in the hospital.

DISCUSSION AND CONCLUSION

There is increasing evidence that phenylethylamine-derived alkaloids, which, more easily than polyhydroxy-alkaloids derived from DA or noradrenaline can cross the blood-brain-barrier and accumulate in the brain, may play a role in the etiology of neurodegenerative diseases.

DA-derived alkaloids, such as Sal, THP and THP-derived compounds, have not been suspected to be involved in the etiology of neurodegenerative disorders, although they might play a role in the incidence of side-effects.[8] Concerning Sal, there is strong evidence that its biosynthesis occurs in humans by condensation of DA with pyruvic acid, leading to the formation of only the R isomer under normal conditions. The significantly decreased urinary excretion of Sal in parkinsonians with respect to controls suggests that measurement of Sal excretion might be used for the early detection of Parkinson's disease. However, it must be kept in mind that the last step in the formation of Sal is apparently impaired in some individuals. In parkinsonian patients under L-dopa therapy, as in most alcoholics, not only the R but also the S enantiomer of Sal is present in urine. There are controversial data on the ability of Sal to bind to opiate receptors.[25-28] However, (S)-Sal was more potent than (R)-Sal in inhibiting the contractile response elicited by electrical stimulation of the myenteric plexus of the guinea-pig ileum,[28] which contains opiate receptors virtually identical to the μ-receptor of the central nervous system. A low incidence of alcohol use in Parkinson's disease patients has been noted.[29] Is there any relationship between the formation of (S)-Sal in L-dopa treated parkinsonians and abstinence from alcohol?

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CHRONIC L-DOPA THERAPY IN PARKINSON'S DISEASE: CAN IT ACCELERATE DEGENERATION OF NIGROSTRIATAL DOPAMINERGIC NEURONS?

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INTRODUCTION

In Parkinson's disease, there is a selective degeneration of the neuromelanin-containing dopaminergic neurons in the pars compacta with anterograde loss of the ascending nigrostriatal axonal projections and their terminal arborizations in the caudate and putamen nuclei. The cause of the progressive death of dopaminergic neurons is yet unknown. Among others, it may be due to an excessive intraneuronal production of cytotoxic free radicals. The dopaminergic neurons are particularly prone to free radical formation which occurs via two main processes, i.e., breakdown of dopamine by the enzyme monoamine oxidase and synthesis of the pigment neuromelanin. Under normal physiological circumstances, there is probably adequate protection of these neurons by the natural intracellular mechanisms that scavenge and neutralize the cytotoxic free radicals. Theoretically, in Parkinson's disease there may be a basic failure of one or more of such defense mechanisms which leads to abnormal intraneuronal accumulation of cytotoxic free radicals and consequently to cell death. Another hypothetical possibility is that there is an overproduction of such harmful products, in excess of the neutralizing capacity of the protective apparatus, induced by exposure to MPTP-like neurotoxins derived from the external environment[1] or through an endogenous metabolic derangement.

The majority of patients with Parkinson's disease are currently treated with L-dopa. In the parkinsonian striatum, at least part of the systemically-administered L-dopa is taken up and converted to dopamine by the surviving nigrostriatal nerve-terminals.[2] It is well-known that auto-oxidation of L-dopa itself generates a variety of potentially-toxic free radical species.[3] Also, the continuous oxidative deamination of the dopamine formed from exogenous L-dopa by monoamine oxidase produces superoxides both intra- and extra-neuronally. Theoretically, therefore, prolonged administration of L-dopa in Parkinson's disease may also be toxic, and accelerate the degeneration of the remaining nigrostriatal neurons. This could occur particularly, but not necessarily, if such surviving neurons are already vulnerable due to a pre-existing damaging free radical stress associated with the basic (yet obscure) cause of the illness. Thus, it is possible that chronic "bombardment" of the brain with L-dopa therapy might alter the predetermined course of Parkinson's disease and accelerate its progression.

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The question whether long-term L-dopa therapy may precipitate the deterioration of Parkinson's disease is extremely important not only for academic-scientific purposes but for very practical reasons. There is a constant debate within the neurological community as to whether L-dopa should be started early and immediately upon diagnosis even in mild cases, or whether it should be postponed for the later, more severe stages of the disease when the patient really needs its full benefit for functional and social reasons.[4,5] The main basis for this controversy is that it is still undetermined whether declining efficacy and response fluctuations associated with long-term L-dopa therapy is due to disease progression or to the chronic administration of L-dopa itself. If it is found that L-dopa indeed accelerates loss of the surviving nigrostriatal neurons, it would provide a strong argument against early initiation of this treatment and for delay for the more advanced stages of the disease. It would also support a role for chronic L-dopa therapy in the development of loss of responsiveness.

In a previous study,[6] we found that prolonged (for 18 months) oral administration of very large doses of L-dopa did not damage nigrostriatal neurons in mice. However, in this experiment, the animals were normal with intact dopaminergic projections and we could not rule out the possibility that in Parkinson's disease the surviving nigrostriatal neurons are particularly susceptible to the toxic effect of L-dopa. We, therefore, examined in mice whether the integrity of their nigrostriatal neurons is affected by chronic L-dopa administration if such neurons are rendered vulnerable by treatment with the dopaminergic neurotoxin MPTP.

Methods and Results

We first examined whether acute administration of L-dopa can cause increases in striatal dopamine concentrations after destruction of nigrostriatal nerve terminals by MPTP. C57 black mice were injected with MPTP (40 mg/kg, s.c.). One week later, MPTP-injected animals and controls were injected either with saline or with L-dopa (50 mg/kg, i.p.). one hour after pretreatment with carbidopa and decapitated one hour later. Striated levels of L-dopa, dopamine (DA) and its major metabolite dihyrdoxyphenylacetic acid (DOPAC) were measured in this and following experiments using HPLC-EC.

Table 1. Utilization of Exogenous L-dopa in Mouse Striatum After Destruction of Nigrostriatal Nerve-Terminal by MPTP

	DOPA	DA	DOPAC
	[ng/mg protein] [means±S.E.M]		
Control	1.3±0.5	132±10	6.3±0.4
L-dopa	9.7±2.3	200±12	34.6±5.4
MPTP	0.8±0.5	44±5	4.7±0.2
MPTP + L-dopa	14.1±1.2	111±6	43.5±2.9

Treatment with MPTP produced marked depletions in striatal DA levels ($p<0.001$; t-test) indicating degeneration of nigrostriatal nerve-endings by the neurotoxin. Relatively lesser reductions were observed in DOPAC concentrations suggesting increased DA turnover by surviving dopaminergic neurons. Acute challenge with L-dopa increased DA and DOPAC concentrations in both control and MPTP-treated mice indicating continued utilization of exogenous L-dopa in striatum despite partial but massive destruction of dopaminergic neurons.

To examine the effects of chronic L-dopa administration, mice were injected once with saline or MPTP (40 mg/kg, s.c.). From 24 hours later, control and MPTP-treated mice were injected i.p., once daily for 30 days, with a mixture of L-dopa (50 mg/kg) and carbidopa (10 mg/kg). Animals were decapitated after an additional washout period of 10 days after last injection.

Table 2. Effect of Chronic Administration of L-dopa on Striatal DA and DOPAC Levels in Mice Challenged Once with MPTP

	DA [ng/mg protein]	DOPAC [means±S.E.M.]
Control	114±4	6.6±0.4
Chronic L-dopa	125±5	7.2±0.3
MPTP	63±4	4.4±0.3
MPTP + Chronic L-dopa	65±3	3.7±0.5

In animals given chronic L-dopa, DA and DOPAC levels in striatum were similar to those in controls indicating that dopaminergic nerve-terminals remained intact even after sustained "bombardment" with L-dopa. MPTP produced about 50% decrease in striatal DA concentrations. In MPTP-treated mice, striatal DA decrements were not further amplified by chronic administration of L-dopa indicating that it was not harmful to DA neurons even if they were made vulnerable by an acute pretreatment with MPTP.

It can be argued that although damage to dopaminergic nerve-terminals induced by a single challenge with MPTP persists for several months, the neurotoxin is rapidly washed out from rodent brain and vulnerability of the neurons to further oxidative stress such as caused by chronic L-dopa may be rather short-lived and not last throughout the month of L-dopa administration. Mice were therefore given subthreshold doses of MPTP (2 mg/kg, s.c.) once daily for 30 days, alone or combined with L-dopa and carbidopa (50 and 10 mg/kg, respectively, i.p.) and decapitated after 10 additional days of washout. Table 3 shows the results of this experiment. Chronic administration of MPTP at small daily doses did not produce any reductions in striatal DA and DOPAC levels indicating that dopaminergic neurons were not damaged by this treatment. Likewise, chronic combined administration of L-dopa and subthreshold doses of MPTP were not harmful to nigrostriatal nerve-endings.

Table 3. Effect of Combined Chronic Administration of L-dopa and Subthreshold Doses of MPTP on DA and DOPAC Concentrations in Mouse Striatum

	DA [ng/mg protein]	DOPAC [means±S.E.M.]
Control	150±11	9.9±0.4
Chronic MPTP	163±8	10.3±0.5
Chronic L-dopa + MPTP	176±13	11.0±0.6

CONCLUSION

Chronic administration of L-dopa is not toxic to and does not damage dopaminergic neurons in mice even after these are made vulnerable by MPTP given as an acute single pretreatment challenge or by repeated subthreshold doses combined with L-dopa. Study suggests that if a

similar situation prevails in humans, prolonged treatment with L-dopa does not accelerate degeneration of surviving nigrostriatal neurons and progression of the illness.

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IRON-MELANIN INTERACTION IN SUBSTANTIA NIGRA AS THE NEUROTOXIC COMPONENT OF PARKINSON'S DISEASE

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INTRODUCTION

In spite of the fashion to implicate environmental (e.g. MPTP-like) or endogenous (6-hydroxydopamine like) neurotoxins or an interaction between such factors and ageing as a process for nigra-striatal dopamine neuron loss, resulting in Parkinsonian syndrome, the primary cause of the idiopathic Parkinson's Disease (PD) remains unknown. The nigra-striatal (SN) dopamine neurons of basal ganglia are very sensitive to many chemical insults, some of which have endogenous origin. Among these are the generation of oxygen free radicals formed from H_2O_2, generated by auto-oxidation and oxidative deamination of dopamine to melanin and deaminated products, respectively. The basal ganglia is endowed with highly active systems for scavenging of oxygen radicals. Among these are glutathione, glutathione peroxidase, superoxide dismutase and ascorbate, known to be present in relatively high concentrations. Theoretically a reduction in any of these could be highly damaging to the dopamine neurons (1). However, biochemical reactions which would promote the excessive formation of cytotoxic oxygen free radicals and which in turn can be highly damaging to the cell as a resultant lipid peroxidation should also be considered. Thus, ultimately, the balance between production and disposition of free radical may be the important factor. This has led a number of investigators to implicate oxidative stress as the primary cause of PD (2,3).

The abnormality of transition metal metabolism in inducing oxidative stress and their role in oxygen free radical formation is well documented and established. Of the metals so far examined for their role in PD, manganese has received some publicity because of the Parkinson-like sydrome reported in manganese miners (4). By contrast, iron, more than any other metal, has been cited as being involved in oxygen free radical formation and induction of lipid peroxidation processes in systemic organs (5). Until recently its role in the central nervous system function and dysfunction has received little attention (6). For the past 15 years we have been examining the role of iron in brain

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function. This is warranted by the fact that it has a unique and selective distribution in brain regions, with globus pallidus and nigra having the highest concentration. Furthermore its brain concentration is under a fine control, where both deficiency and excess can be damaging to the brain function (6).

THE ROLE OF IRON IN OXIDATIVE STRESS IN THE SUBSTANTIA NIGRA OF PARKINSON'S DISEASE

Membrane lipid peroxidation, resulting from generation of oxygen free radicals via H_2O_2 is the process by which cell membranes are thought to degenerate as a result of cytotoxic events due to chemical insults or the presence of free tissue iron (5). Although the exact role of iron in lipid peroxidation is not fully understood, nevertheless it is well established. Both Fe^{2+} and Fe^{3+}, or their chelated forms, can participate in oxygen free radical formation and may either initiate, or promote lipid peroxidative processes under a variety of diverse conditions. The attention has been focussed on the iron catalyzed formation of cytotoxic hydroxyl (0H.) radical as the important step for initiation of lipid peroxidation and iron catalyzed decomposition of lipid hydroxperoxides (LOOH) as the driving force in this reaction (7). The formation of the perferryl or ferryl iron species and the absolute ratio of Fe^{2+} to Fe^{3+} and transition of iron between its two valancies are thought to be important for initiation of lipid peroxidation (8). Furthermore, it has been postulated that it is oxygen radicals generated at or on the membrane by iron that are responsible for iron initiated lipid peroxidation. The suggestion has been made that iron, which initiates or participates in lipid peroxidation, may actually be bound or solubilized by membrane lipid (). At this point it is important to remember that the major fraction (70%) of iron is myelin-bound. In any case, a central role for the participation of iron in lipid peroxidation and oxidative stress can no longer be questioned or ignored (9).

This phenomenon may be directly relevant to the whole pathophysiology of nigra-striatal dopamine neuron degeneration in PD, where in substantia nigra (SN) a significant increase of iron and lipid peroxidation have been reported on several occasions. Intracranial, intra-amygdaloid or intraventricular injection of Fe^{2+} or Fe^{3+} salts do indeed cause substantial <u>in vivo</u> lipid peroxidation and neurodegeneration (10).

Our studies have clearly demonstrated not only an increase in total iron but also a change in the ratio of Fe^{2+}:Fe^{3+} from 3:1 in control to 1:1 in Parkinsonian substantia nigra. The fact that ferritin content is unaltered suggests the presence of free cytotoxic iron. This fact, together with an altered ratio of Fe^{2+}:Fe^{3+}, indicates the presence of oxidative stress and lipid peroxidation (3,11). One recent report has demonstrated an increase of basal lipid peroxidation in SN (12).

Lipid peroxidation will proceed with either Fe^{2+} or Fe^{3+} provided that a mechanism exists to facilitate the interconversion of iron between its redox states (8). This can be achieved via interaction with H_2O_2 and chelation of iron by endogenous chelators e.g. ADP and melanin. Figure 1 provides illustrative pathway for the existence of such

systems in the SN, where the state of Fe^{2+} can be shifted in favour of Fe^{3+} in the presence of H_2O_2 generating systems resulting in OH. radical formation and lipid peroxidation.

Monoamine oxidase (MAO) B initiated oxidative deamination of dopamine and the autooxidation of dopamine to melanin would generate H_2O_2. MAO B is known to be increased with aging and a positive feed back effect of H_2O_2 on MAO-B activity has been noted (13). The overall resultant effect would be a viscious circle for further H_2O_2 generation (Figure 1). In normal circumstances, H_2O_2 in the brain is inactivated by glutathione peroxidase an enzyme requiring the rate limiting substrate, reduced glutathione (GSH). Both catalase and peroxidase are absent from the brain, making the GSH pathway for H_2O_2 elimination as the primary route. However, in PD significant reduction of GSH and ascorbate in SN has been reported by us (3). In this circumstance, H_2O_2 can interact with increased Fe^{2+} and drive the formation of OH. through the iron-catalyzed Haber-Weiss reaction (Fenton reaction) and shift the redox state of iron in favour of Fe^{3+}.

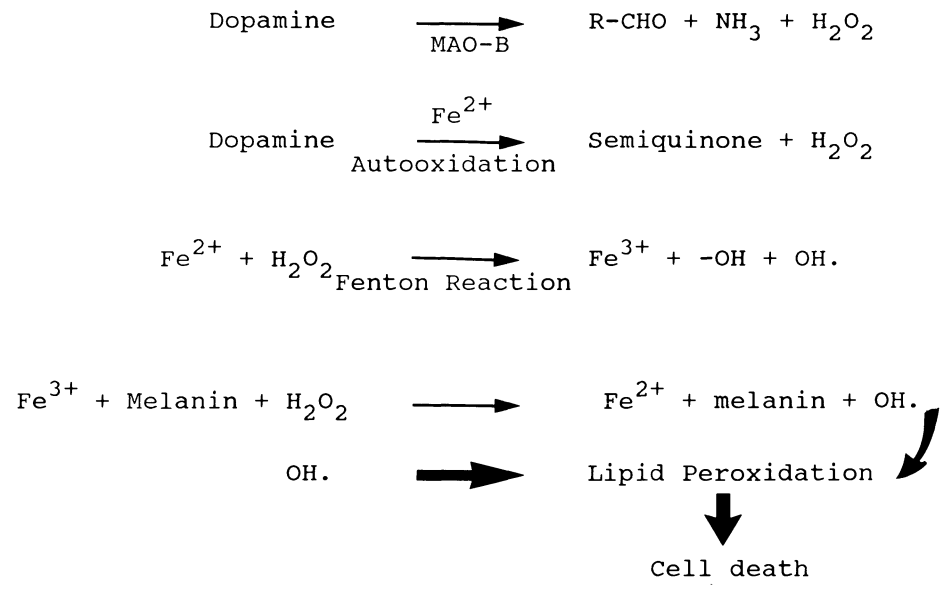

Figure 1. Reaction pathway of dopamine oxidation and the formation of lipid peroxides.

In normal circumstances iron is stored in its inactivated form either bound to ferritin or haemosiderin such as that found in the liver. The unaltered ferritin and the significant increases of iron noted in SN would suggest that the high amount of iron present in SN is either chelated or bound to other proteins or small soluble molecules. The most logical choice for such a molecule in the SN could be melanin or neuromelanin, present in relatively high amounts in SN. The chelation of iron, namely Fe^{3+}, by melanin can serve as a catalept for the conversion between its two redox states.

It is generally accepted that melanin is an effective radical scavenger and protects the nigra-striatal dopamine neurons from biochemical insults. However, this notion needs to be reconsidered because of recent observations of Mann and Yates (14) who showed that the more heavily pigmented neurons of SN appear to be preferentially lost in PD and during the course of aging, when both iron and melanin are know to increase. In comparing the SN of control and Parkinsonian brains Hirsch et al. (15) have demonstrated the greater vulnerability of the population of neurons containing neuromelanin to the neurodegenerative process of PD. Their studies also showed a direct relationship between the distribution of pigmented neurons normally present and the distribution of cell loss in the SN of individuals dying with the disease.

The metal chelating properties of melanin (16) make it an important candidate to alter the amount, rate of formation and distribution of reactive hydroxyl radical (OH.) generated by the increase of iron in the SN (17). The substantial amount of free catechols present in the polymer nature of melanin acts as an efficient chelator. With low concentration of Fe^{2+}, melanin decreases the yield of OH.. By contrast melanin substantially increases the rate of OH. production if the predominent form of iron is Fe^{3+}. This has been attributed to the ability of melanin to reduce the chelated Fe^{3+} to Fe^{2+} in the presence of H_2O_2 (Figure 1). Hydroxyl radical (OH.) production in the presence of melanin is significantly greater if the Fe^{3+} is chelated (18). Thus, depending on the state of iron, melanin can either increase or decrease the production of OH.. Therefore, the conditions necessary for the participation of neuromelanin in lipid peroxidation and neurodegeneration exists in the SN of Parkinsonian brain. The questions uppermost in mind are why the SN should accumulate such high iron content and whether the iron is deposited in the neuromelanin containing dopamine neurons in association with melanin, thus making the neurons more vulnerable to neurodegeneration.

IRON-MELANIN INTERACTION AND LIPID PEROXIDATION

Our studies have now demonstrated two binding sites for $^{59}Fe^{3+}$ on synthetic dopamine-melanin (DA-M), with K_D values of 13 nM and 200 nM. The protein component of neuromelanin does not make a contribution to iron chelation. The binding (chelation) of $^{59}Fe^{3+}$ by DA-M can be inhibited by drugs which act as iron chelators. Among such compounds tested (U74500A, desferrioxamine, chlorpromazine, 8-hydroxyquinoline, 0-phenanthroline, 2,2-dipyridyl), U74500A and desferrioxamine were the most potent inhibitors with IC_{50} values of 10 and 60 nM respectively.

While Fe^{3+} potentiates the basal lipid peroxidation in the presence of rat cortical homogenates, DA-M is inhibitory. By contrast, DA-M does not prevent Fe^{3+} induced lipid peroxidation and at higher concentrations it potentiates Fe^{3+} induced lipid peroxidation. Iron chelators that inhibit the binding of $^{59}Fe^{3+}$ to melanin also block iron-melanin induced lipid peroxidation. These findings are considered to be relevant to the functional binding of Fe^{3+} to melanin and may be important as an explanation for the specificity of neurodegeneration of melaninized nigra striatal dopamine neurons in PD. At present no evidence is available to evaluate the presence of iron in melaninized

neuron and whether the increase of iron is within such neurons.

Iron itself may also contribute to the formation of melanin, since dopamine increased the binding of $^{59}Fe^{3+}$ to dopamine melanin. It is well recognized that trace amounts of transmission metals (Fe^{2} and Cu^{2+}) can oxidize unreactive catecholamines to reactive semiquinones which eventually form the melanin. Indeed, one aspect of iron over-load in subjects given iron therapy is darkening of the skin, due to the formation of melanin. This process has been shown to result also in excessive lipid peroxidation in skin fibroblasts (19). Iron chelation therapy with desferroxamine or 3,4-dihydroxybenzoic acid not only depigments the skin (melanin reduction) but significantly reduces lipid peroxidation.

In the final analysis it is possible that PD could be related to siderosis of SN and thus iron chelation may be one logical approach to prevention of oxidative stress and cell death. Among the iron chelators so far described the 21-amino steroid compound (e.g. U74500A) appears to be most promising, since these compounds are lipophylic and cross the blood brain barrier (20).

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DOPAMINE IMMUNOCYTOCHEMISTRY IN THE NIGROSTRIATAL SYSTEM OF PRE- AND POSTNATAL MICE

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INTRODUCTION

Brain tissue transplantation is now used for the functional recovery of experimental parkinsonian animals and parkinsonian patients. For example, grafts of catecholamine(CA)-containing neuronal cells, such as those in fetal brain tissues from the substantia nigra(SN) and ventral tegmental area(VTA), were able to abolish the abnormal rotating movement in animal models of hemiparkinsonism[1]. Tyrosine hydroxylase-like immunoreactivity (TH-LI) in the SN and VTA of fetal brain has been generally used to assess the functional activity of the grafts. However, even though TH-LI is demonstrated in the brain tissues used for grafting, aromatic L-amino acid decarboxylase(AADC)- and dopamine(DA)-LI should also be proved to be present. In order to confirm at what stages of development the SN and VTA tissues are most appropriate for brain grafting, we obtained mouse brain taken during prenatal and postnatal development and examined the neurons containing CA-synthesizing enzymes immunocytochemically using specific antisera to TH, AADC, and DA[2].

MATERIALS AND METHODS

C57BL/6J mice were used for experiments from embryonic day 12(E12) to adult. All antisera were raised in rabbits and well characterized in our laboratory[2]. The immunocytochemical procedure used was described elsewhere[2].

RESULTS AND DISCUSSION

Advanced appearance of TH- and AADC-LI and delayed appearance of DA-LI in the mesencephalon

TH- and AADC-immunoreactive(IR) cells were observed at E12. From E14, DA-IR cells also started to be detected in the mesencephalon(Fig.1). From E16, TH-, AADC-, and DA-LI became stronger, reaching their maximum during the postnatal/adult period. In contrast, medial forebrain bundles(MFB) from SN to caudate-putamen(C-P) were traced clearly for TH and AADC but faintly for DA(Fig.1).

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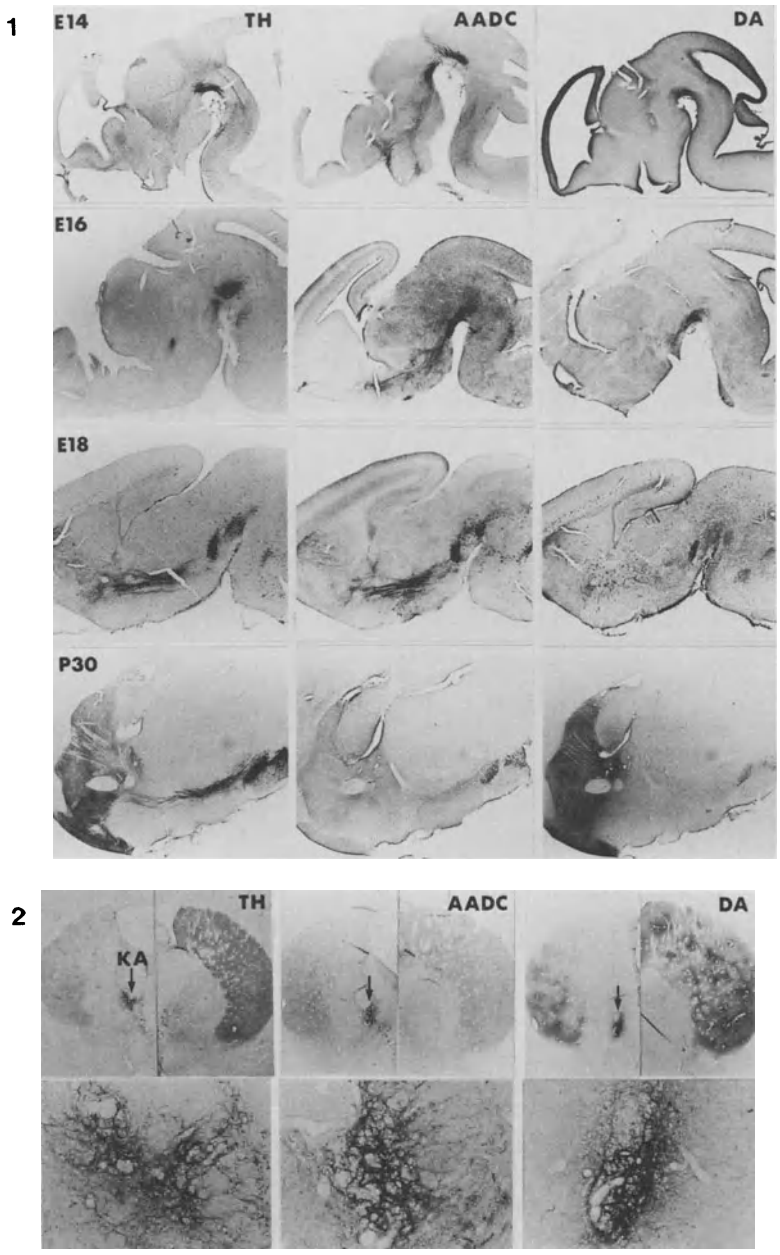

Fig.1. Photomicrographs of adjacent sagittal sections of the mouse mesencephalon stained for TH, AADC, and DA. The SN and VTA show intense staining for TH and AADC but faint staining for DA at E14. From E16, all three markers, TH, AADC, and DA, are stained stronger, reaching their maximum intensity in the postnatal/adult period. In contrast, the MFB from SN to C-P is clearly observed for TH and AADC but is very obscure for DA.

Fig.2. Photomicrographs of adjacent frontal sections of mouse MFB, 2 days after KA injection(0.2 nmol/0.5 μ l) into the MFB of an adult mouse. TH-, AADC-, and DA-IR fibers are accumulated in the proximal part of the MFB(arrow). Note very weak IR terminals in the corresponding area of a C-P comparing with that in the non-lesioned C-P. The lower panel shows enlarged photomicrographs of the respective lesioned MFB shown above.

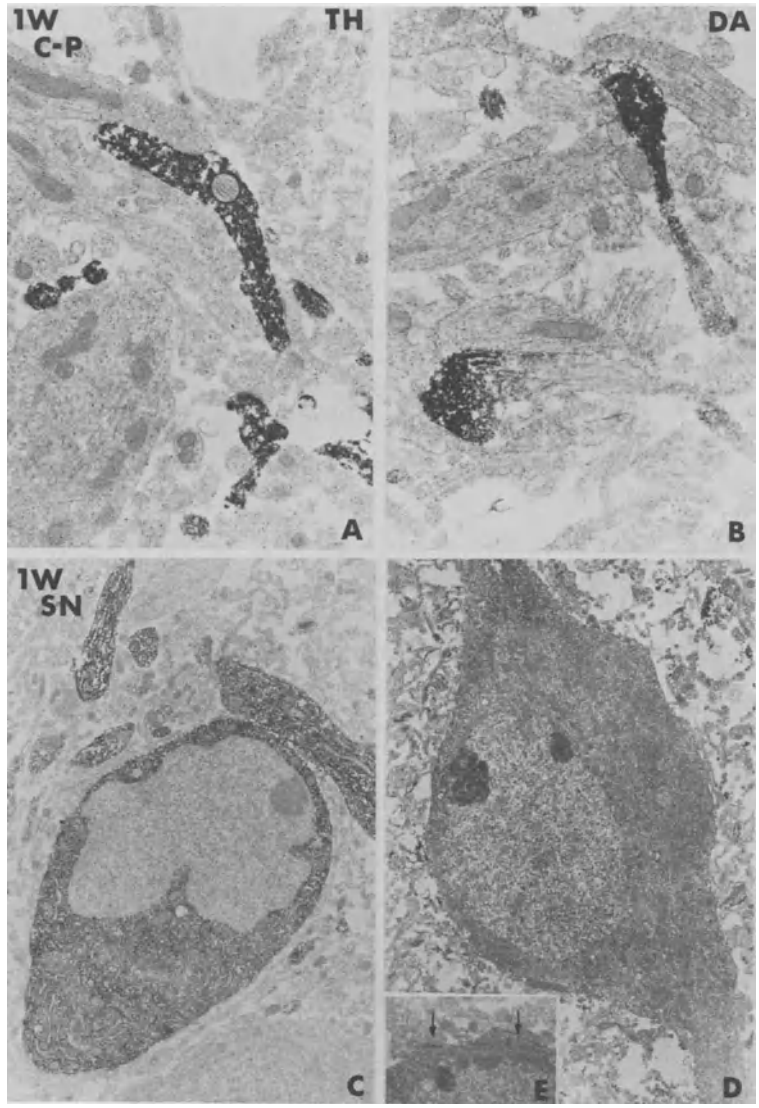

Fig.3. Ultrastructural localization of TH and DA in the striatum(A and B) and SN(C, and D,E) of a 1-week-old MPTP-treated mouse. Small and round synaptic vesicles show TH-(A) or DA-LI(B) in axon terminals of the C-P. TH-LI is traceable clearly along the axons, but DA-LI is only observed just in the axon terminals. In the SN, both TH- and DA-LI are localized evenly throughout the cytoplasm. No degeneration is observed in the nucleus or in mitochondria. A few synaptic contacts of non-catecholaminergic terminals (E, arrow) are noted on the soma of DA-IR neurons(D).

Axoplasmic transport

We also identified accumulation of TH-, AADC-, and DA-LI in fibers from SN to C-P in the kainic acid(KA)-lesioned proximal part of the MFB(Fig.2).

Acute toxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MTPT)

A single injection of a relatively high dose(free base, 2.8 mg/kg) of MPTP into 1-week-old mice reduced TH-, AADC-, and DA-LI in the C-P. In contrast, changes in TH-, AADC-, and DA-IR density were not observed in the cell bodies of the SN and VTA. These results are similar to the subacute effects of MPTP on DDY mice[3] and to the transplacental effects of MPTP on C57BL mice[4].

Ultrastructure

DA immunoreactivities in the C-P and SN were ultrastructurally examined. In the C-P of MPTP-treated mice, the DA axon terminals contained TH-(Fig.3A) or DA-LI(Fig.3B) in small and round synaptic vesicles. Usually TH-LI could be traced clearly along processes of the axons but DA-LI was only observed just in axonal terminals after MPTP treatment. Synaptic contacts were rarely exhibited on dendritic shafts and spines. In the SN, immunoreactivity for TH and DA was localized evenly throughout the cytoplasm(Figs.3C,D). No degeneration of nuclei and mitochondria was seen. A few synaptic contacts of non-catecholaminergic terminals (Fig.3E) were noted on the soma of DA-IR neurons(Fig.3D).

SUMMARY

1) TH- and AADC-LI appeared first at E12 followed by DA-LI at E14 in the neurons of the mouse mesencephalon.
2) Axoplasmic transports of TH-, AADC-, and DA-LI in fibers from SN to C-P were identified in KA-lesioned MFB.
3) Acute toxic effects of MPTP manifested as a reduction in TH-, AADC-, and DA-LI were observed in the C-P, but not in the SN. Ultrastructurally DA axon terminals contained DA- and TH-IR synaptic vesicles.

These results indicate that DA is a transmitter in a large portion of the SN and VTA neuronal population.

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THE EFFECTS OF N-METYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) ADMINISTRATION TO MATERNAL MICE ON THE CATECHOLAMINE SYSTEM IN THE BRAIN OF POSTNATAL MICE

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INTRODUCTION

It is well-known that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a reduction in the number of dopaminergic neurons in the nigro-striatal system of primates and rodents.[1-3] The molecular basis of the neurodegeneration by MPTP has been intensively studied and the toxicity was found to be ascribed to its oxidative product, 1-methyl-4-phenylpyridinium ion (MPP^+).[4] The sensitivity of mice to MPTP is age-dependent[5]; systemic administration of MPTP in young mature mice could not induce a long-lasting reduction in the dopamine (DA) level.[6] Recently in our laboratory, MPTP was found to be transported from the maternal mouse to the fetus through the placenta, for MPTP and MPP^+ were detected in the brains of fetal mice.[7] MPP^+ was reported to destroy the dopaminergic cells cultured from the rat embryonic mesencephalon,[8] but the *in vivo* effect of MPTP on animals in the fetal stage has never been reported.

In this study, the activity of tyrosine hydroxylase [tyrosine, tetrahydropteridine : oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2, TH] and catecholamine (CA) content were examined in the brains of postnatal mice to determine the effect of prenatal exposure to MPTP on the DA system in developmental state. Prenatal administration of MPTP through mother mice induced prolonged reduction of DA synthesis with transient increment of TH activity. The significance of effects of MPTP on the DA system in developmental state is discussed in relation to characteristic biochemical reaction of DA neurons in fetus to MPTP or environmental compounds with neurotoxicity similar to MPTP.[9]

MATERIALS AND METHODS

MPTP hydrochloride and sodium 1-heptanesulfonate were purchased from Aldrich Chemical Co. MPP^+ was from Funakoshi Pharmacentical Co. All other chemicals were of analytical grade.

Examination of the transport of MPTP to prenatal mice through mother mice

Pregnant C57BL/BYA mice (30-45 g, 12-20 weeks old) were separated into two groups. To one group MPTP dissolved in distilled water (30 mg/kg body weight) was injected intramuscularly at the 18th day of gestation, and the animals were sacrificed at 1, 3, 6, 12, and 24 hr after injection. The maternal and fetal brains were rapidly removed and frozen on dryice.

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To the other group, MPTP was intramuscularly injected at a lower dose, 5 mg/kg body weight, at the 14th or 18th day of gestation. The brains were obtained 3 hr after injection. MPP^+ contents in the brain were determined as follows; The whole brains of the prenatal mice and mother mice were homogenized with 9 v/wet weight of 10 mM potassium phosphate buffer, pH 7.4. Aliquots of homogenates were mixed with the same volume of 0.1 M perchloric acid and centrifuged. MPTP and MPP^+ contents of the supernatant were measured fluorimetrically by the previously reported HPLC method.[10]

Effect of systemic prenatal administration of MPTP to mother mice on TH activity and CA concentration in the postnatal mouse brain

To follow the developmental changes in TH activity and CA content, we removed whole brains of prenatal and postnatal control mice on the 16th day of gestation and at 0, 7, 14, and 28 days of life and determined them. To examine the effect of MPTP, the chemical (5mg/kg body weight/day) was injected into pregnant mice for 7 days between the 12th and 18th day of gestation. To controls was injected the same volume of distilled water. On days 7 and 28 after birth, the whole brains of the postnatal mice were isolated, and on days 28 of life, the striatum was also isolated. Brains were homogenized with 10 ml of 10 mM potassium phosphate buffer, pH 7.4, per gm of brain. The amounts of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were determined using HPLC. TH activity was determined based on measurement of the amount of DOPA formed from L-tyrosine by use of HPLC with electrochemical detection as reported previously by Nagatsu et al.[11]

Protein concentrations were estimated by the method of Bradford.[12]

Statistical analysis of mean differences were done by Student's *t*-test.

RESULTS AND DISCUSSION

To follow the transfer of MPTP from mother mice to fetuses, we determined MPTP and MPP^+ concentrations in the fetal brains , after injection of MPTP (30 mg/kg weight) into the mother mice. Definite amounts of MPP^+ were detected in the brains and reached the maximum at 3 hr after MPTP injection and decreased to half of the maximal concentration at 12 hr after injection. MPP^+ could not be detected 48 hr after injection. The MPP^+ concentration in the brains of fetuses whose mothers had been injected with lower doses of MPTP (5 mg/kg weight/day) was 3.52 $\pm$ 0.59 (pmol/mg protein) at 3 hr after injection. There was no significant difference in the MPP^+ content of the fetal brains when the chemical was introduced on the 14th or 18th day of gestation, 3.27 $\pm$ 0.19 or 3.67 $\pm$ 0.12 (pmol/mg protein), respectively.

In brains of control mice, TH activity, expressed as specific activity (moles of L-DOPA formed/min/mg protein), increased with age to about 4-fold on the day of the birth over that on the 16th day of gestation , and on the 28th day reached almost 90% of the activity in the adult brain, which is consistent with the results reported previously.[13]

The effects of systemic MPTP administration to pregnant mice on the TH activity in the brains of newborn mice were then examined. As summarized in Table 1, at 1 week of life, the specific activity of TH (moles/min/mg protein) increased significantly in the whole brains of the mice prenatally exposed to MPTP compared with that of controls. Although TH specific activity increased to 163% of that of controls, the total activity and the DA content in the brain were reduced to 92% and 77% of the control value, respectively. The contents of HVA and DOPAC in the brains were significantly decreased.

At the age of 4 weeks, both the total and the specific activity of TH in the whole brain of mice exposed to MPTP decreased to 78% and 69% of that of the controls, as shown in Table 1. In the striatum, the effects

Table 1. Effect of systemic prenatal MPTP administration on TH activity in the whole brain of newborn mice

Age (week)		n	Tyrosine hydroxylase: Specific activity (pmol/min/mg protein)	Tyrosine hydroxylase: Total activity (pmol/min)
1 w	Control	5	3.01 ± 0.16	74.0 ± 2.5
	Experiment	8	4.90 ± 0.22*	63.9 ± 4.7
4 w	Control	5	5.74 ± 0.22	416 ± 15
	Experiment	6	4.50 ± 0.15**	208 ± 15***

Data values are mean ± S.E.M.
* $p<0.05$, ** $p<0.005$, *** $p<0.001$

of MPTP administration were more marked than in the whole brain. TH activity and DA content in the striatum were reduced to 40% and 77% of the control values as reported by Mayer *et al.*[14]

The results reported here show that MPTP injected into a pregnant mouse is transported into the fetal brain through the placenta and fetal blood-brain barrier. MPTP itself was detected in the fetal brain, but at present, it is not certain whether MPP^+ detected in the fetal brain is produced *in situ* there or is produced in maternal or fetal tissues other than brain and then transported into the fetal brain. Our data show also that MPTP or MPP^+ given during fetal life induces a prolonged reduction in TH activity in the nigro-striatal system that is still evident even after birth. The reduction in TH activity may be due to the cell loss of dopaminergic neurons caused by the prenatal exposure to MPTP. A transient but definite increase in the specific activity of TH was observed at one week after birth. Considering that the total TH activity was reduced markedly, the increase may be due to an increase in the TH homospecific activity (expressed as units of the activity/amount of TH protein) in remaining neurons of the degenerating dopaminergic system, which may be comparable to the increase in TH homospecific activity seen in the human brain at the early stage of Parkinsonism. The molecular basis of the increase in TH homospecific activity has not been well clarified, but may be due to an increase in the phosphorylation of the TH protein, which increases the enzyme activity markedly.

The MPTP amounts used for the systemic prenatal administration were much lower than those used commonly for experiments on aged animals. In addition, the MPP^+ content in the fetal brains was only one sixth of that in the maternal brain. These facts indicate that in the prenatal period dopaminergic neurons are very sensitive to MPTP and that the damage will last even after birth.[14] In aged-animal models, the increased neurotoxicity of MPTP is considered to be associated with increased activity of monamine oxidase, especially that of the type B. However, in the case of the fetal brain, some other mechanism, such as an undeveloped blood-brain barrier, may be involved to account for the increased and prolonged neurotoxicity of MPTP. Prenatal exposure to any environmental compound with neurotoxicity, such as MPTP, may induce the cell death of specific neurons in some localized regions of the fetal brain and thus may elicit neuro-degenerative disease.[15]

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MITOCHONDRIAL ABNORMALITIES IN THE NIGRAL NEURONS OF CRAB-EATING MONKEYS WITH EXPERIMENTAL PARKINSONISM

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INTRODUCTION

Experimental parkinsonism has been induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1,2,3,4-tetrahydroisoquinoline (TIQ) in several primates, and leads to an animal model of Parkinson's disease.[1-4] The pathological change observed in the MPTP-treated monkeys is selective neuronal damage in the substantia nigra. Neurotoxic effects of MPTP on the nigral neurons are possibly due to inhibition of mitochondrial oxidation by some MPTP metabolite.[3] The detailed pathomechanism of mitochondrial abnormalities, however, remains ill defined.

The present study describes mitochondrial changes found in the nigral neurons of crab-eating monkeys with MPTP- and TIQ-induced parkinsonism.

MATERIAL AND METHODS

Twelve female adult crab-eating monkeys (*Macaca fascicularis*) were used for the study, of which six were administered intravenously a daily dose of MPTP at 1.0 to 1.3 mg/kg for 14 days,[3] three were subjected to a daily subcutaneous injection of TIQ at 100 mg/kg for 20 days, and the other served as a control. Under general anesthesia, the brains were fixed by perfusion through the aorta with 2.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4. Samples from the substantia nigra were post-fixed in 1.0% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope.

RESULTS

All the monkeys treated with MPTP developed bradykinesia, muscular rigidity, occasional intermittent tremor, and finally fell into a profound akinesia. The TIQ-treated monkeys had a slight decrease in spontaneous movement during the period, whereas the controls showed no unusual signs.

Sections of the brain revealed no gross difference in nigral pigment

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between the treated monkeys and the controls. Histological changes in the substantia nigra of the MPTP-treated monkeys were represented by neuronal necrosis, extracellular pigmentation, and histiocytic infiltration, followed by neuronal loss, melanophagia, and astrocytosis. No Lewy bodies were noted. Ultrastructurally, in the MPTP-induced parkinsonism the affected neurons showed a vacuolar change in the perikaryon with nuclear chromatin clumping and nucleolar disintegration. Mitochondria underwent a distortion of the cristae and contained electron-dense inclusions within the matrix. These inclusions were flocculent (Fig. 1) and sometimes spherical in shape (Fig. 2). The flocculent inclusions were smaller in size than the spherical ones, and the latter were also observed in the controls. Abnormal mitochondria were located close to the distended

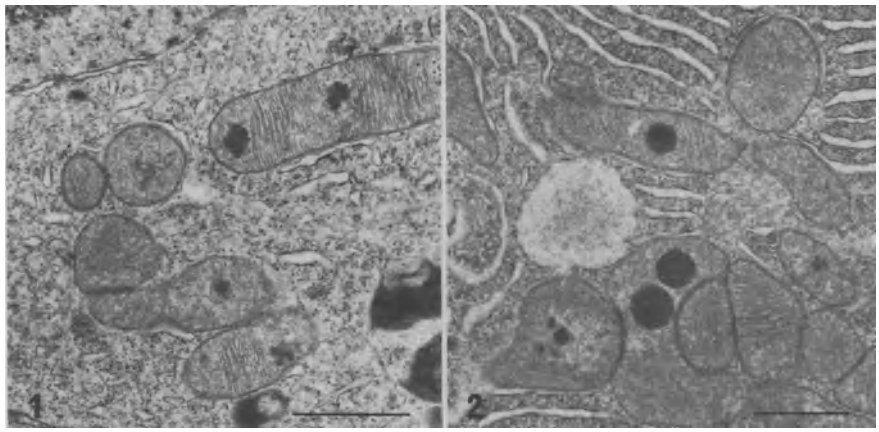

Fig. 1. Mitochondria in a nigral neuron of an MPTP-treated monkey show a distortion of the cristae and electron-dense flocculent inclusions within the matrix. The bar indicates 1-μm.
Fig. 2. Spherical inclusions in other mitochondria. 1-μm bar.

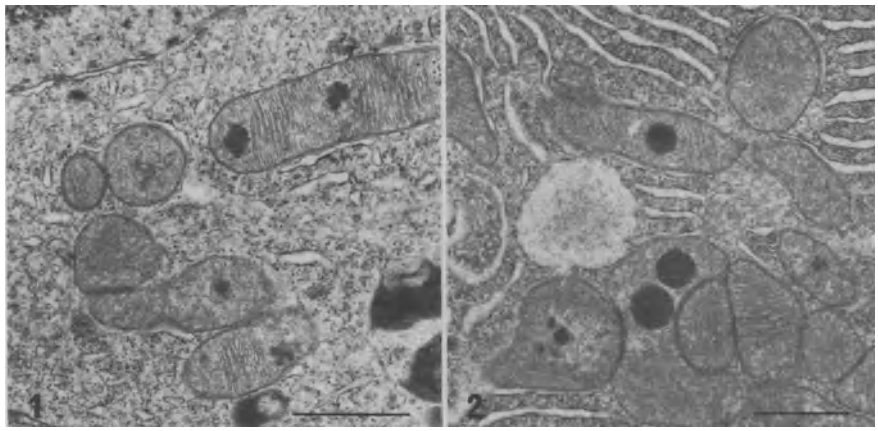

Fig. 3. Occasional mitochondria are partially covered with periodic dots and filaments on the outer membrane. 0.5-μm bar.
Fig. 4. At one pole of a longitudinally sectioned mitochondrion, the parallel filaments intersect mutually to from a lattice-like network of filaments. 0.5-μm bar.

Golgi vesicles. Occasionally, the mitochondria were partially covered with periodic dots and parallel filaments on the outer membrane (Fig. 3) The dots gradually elongated to a rod-like shape and finally transformed into the filaments. At one pole of a longitudinally sectioned mitochondrion, the parallel filaments intersected mutually to form a latticene of filaments (Fig. 4). Abnormal mitochondria frequently appeared in a group, with the dots and filaments intermediating in the gap between the outer membranes of apposing mitochondria (Fig. 5).

In the TIQ-treated monkeys the nerve cells of the substantia nigra were slightly atrophic with a depletion of pigment granules. Mitochondrial abnormalities were less remarkable. The flocculent inclusions were found only on rare occasions (Fig. 6).

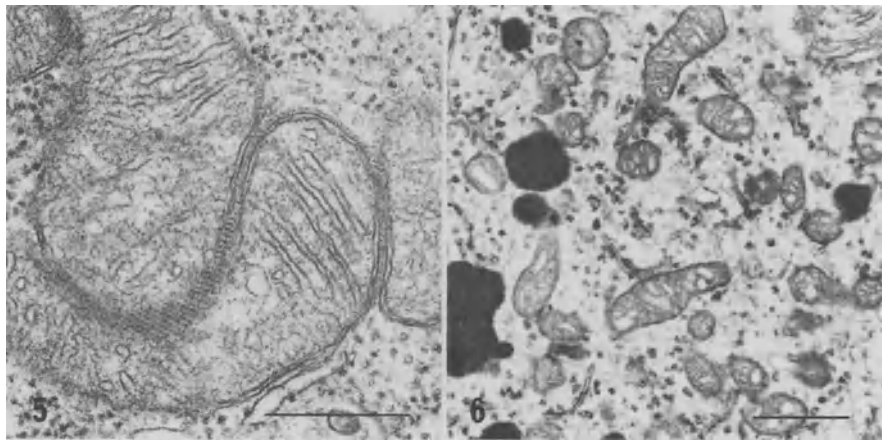

Fig. 5. Dots and filaments in the gap between the outer membranes of apposing mitochondria. 0.5-μm bar.
Fig. 6. In the TIQ-treated monkey the mitochondrial abnormalities are less remarkable and the flocculent inclusions are found rarely. 1-μm bar.

DISCUSSION

In the crab-eating monkey with MPTP-induced parkinsonism the mitochondrial changes observed in the nigral neurons were characterized by a distortion of the cristae associated with the occurrence of intramatrical inclusions, flocculent and sometimes spherical in shape. The spherical inclusions in neuronal mitochondria have been reported in the substantia nigra in some neurological conditions in humans without parkinsonism, and in healthy primates as well as in the controls of this study; so they seem to be non-specific.[3] The intramatrical flocculent inclusions have been suggested to represent an insoluble precipitation of ions, abnormal protein depositions, focal changes in conformation of matrix proteins, or accumulation of nucleic acids.[6] The flocculent inclusions in this study may be comparable to the precipitated substance related to MPP^+, a major metabolite of oxidation of MPTP that has been postulated to be the compound responsible for inhibition of NAD-linked oxidation in mitochondria.[7] The presence of a lattice-like network of filaments on the outer membrane of abnormal mitochondria is unique,[8] and we consider it to be a pathological configuration related to MPTP-induced parkinsonism, and to be different from the stubby mitochondria that have been previously reported in cases of amyotrophic lateral sclerosis.[9] Its significance and morphogenesis remain to be clarified.

On the other hand, the TIQ-treated monkeys showed less remarkable mitochondrial abnormalities in the nigral neurons than the MPTP-treated monkeys. TIQ, an analogue of MPTP, has been identified in the normal brain and its concentration is increased in the parkinsonian brain.[10] Tyrosine hydroxylase activity and dopamine concentration have been reported to be decreased in TIQ-treated animal.[4] Thus, TIQ is a candidate as an endogenous neurotoxin to induce parkinsonism. From the results in this study, TIQ appears to be less toxic than MPTP to the nigral neurons.

SUMMARY

In the crab-eating monkey with MPTP-induced parkinsonism, the nigral neurons underwent a necrotic damage associated with mitochondrial abnormalities represented by a distortion of the cristae and the occurrence of intramatrical inclusions. The flocculent inclusions can be attributed to inhibition of mitochondrial oxidation by an MPTP metabolite. A unique configuration was found in some mitochondria; that is, the outer membrane was partially covered with a lattice of filaments. In the nigral neurons treated with TIQ, an analogue of MPTP, mitochondrial abnormalities were less remarkable than in the MPTP-induced parkinsonism.

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L-DOPA-INDUCED FACILITATION OF DOPAMINE RELEASE VIA PRESYNAPTIC β-ADRENOCEPTORS IN STRIATAL SLICES FROM MPTP-TREATED C57BL MICE: EVALUATION OF THE ACTION OF L-DOPA IN ANIMAL MODEL FOR PARKINSONISM

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L-3,4-Dihydroxyphenylalanine (DOPA) is the most effective therapeutic agent for Parkinson's disease and is believed to act exclusively via its conversion to dopamine (DA). However, we proposed that DOPA itself may act as an endogenous neuroactive substance.[1-3] To asses this proposal, we employed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)-treated C57 black (BL) mice as a model for parkinsonism, and examined the effect of L-DOPA in this system by observing striatal slices from these animals.

EVALUATION OF MPTP-TREATED C57BL MOUSE MODEL FOR PARKINSONISM

Adult C57 BL male mice were injected with MPTP (30 mg/kg, i.p., twice daily for 5 days). At selected times after the final injection, the tissue contents of catecholamines in the brain removed from these mice were measured by HPLC-ECD. In the striatum, the MPTP administration decreased by 70 % DA and DOPAC contents 10 - 20 days after the last injection, with no reductions in levels of NA and DOPA. The decrease in DA and DOPAC tended to recover partially 30 - 40 days after the final administration.[4]

Locomotor, pole, and traction tests were performed after the final MPTP or saline injection. The procedures for these tests were as described previously.[4,5] Decreases in the locomotor activity and impairment of limb movements scored by both pole and traction tests were clearly seen in the MPTP-treated mice for 10 to 20 days after the withdrawal (Table 1).

Table 1. Behavioral impairment in C57 BL mice after the completion of MPTP administration.

Treatment	n	Locomotor activity	Pole test	Traction test
Saline	20	123 ± 20.6	7.3 ± 1.2	2.1 ± 0.5
MPTP	20	35 ± 6.7*	4.2 ± 0.7*	1.1 ± 0.3*

Behavioral tests were done for 10 ~ 20 days after the final injection of MPTP or saline, as described.[4,5] Data are mean ± S.E.M. of n estimations. * $P < 0.01$, compared with saline, Student's t-test.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table 2. Density/mm^2 of neurons in the substantia nigra of C57 BL mice after the completion of MPTP injections.

Group	n	Neuron density (no./mm^2)
Saline	10	232.3 ± 19.5
MPTP		
Undamaged	14	209.3 ± 27.4
Damaged	11	90.2 ± 15.2**
Total	25	156.9 ± 39.3*

Coronal sections of the zone compacta and the zone reticulata were prepared 1 month after the final injection of MPTP or saline. Mean ± S.E.M. of n estimations *P < 0.05, **P < 0.01, compared with saline.

Light microscopic observation done 1 month after MPTP treatment revealed that the brain region affected by the chemical was the substantia nigra. No pathological changes were seen in the other regions including the striatum, cerebral cortex, cerebellum, and brain stem. The lesions of the substantia nigra could be divided into two groups. One group showed damages with the number of neurons markedly decreased (Table 2); and the remaining neurons were mostly shrunken and their cytoplasms were pyknotic. The other group was superficially undamaged. Electronmicroscopically, however, the neurons in both groups showed various abnormalities.[4] Some pathological changes were also observed in the striatum. The regions other than the substantia nigra and striatum showed no pathological changes.

These results show the validity of the MPTP-treated C57 BL mice as a model for parkinsonism.

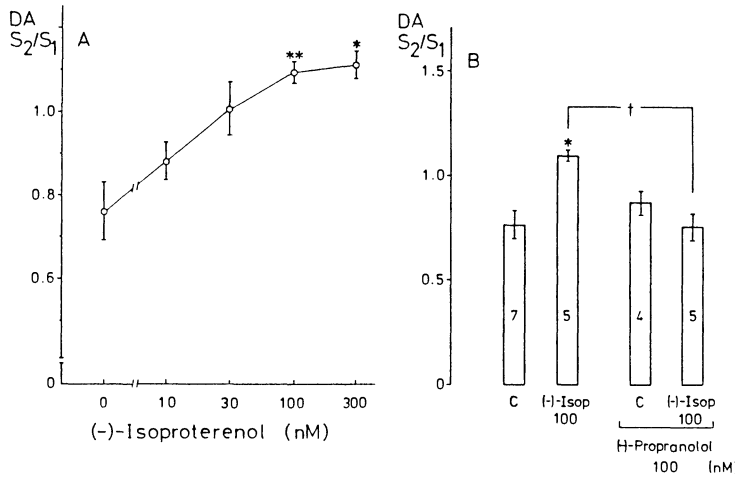

Fig. 1. A: Facilitatory effects of (-)-isoproterenol on the impulse-evoked release of endogenous DA from striatal slices from non-treated C57 BL mice. Each value represents the mean ± S.E.M. from 3 - 7 estimations. *P < 0.05, **P < 0.01, compared with control at 0 nM, unpaired Student's t-test (two-tailed). B: antagonism by (-)-propranolol against (-)-isoproterenol [(-)-Isop]-induced facilitation of the DA release. Columns and vertical bars show means ± S.E.M., and the numbers of estimations are shown in columns. *P < 0.01, compared with corresponding control (C). †P < 0.01, compared with (-)-isoproterenol alone. Other details are as in A.

Table 3. Effects of L-DOPA on spontaneous release, impulse-evoked release, and tissue content, of DA in striatal slices from MPTP-treated C57 BL mice

L-DOPA (nM)	n	Sp_2/Sp_1 ratio	S_2/S_1 ratio	DA content (pmol/mg wet wt.)
None	11	0.58 ± 0.08	0.80 ± 0.06	19.3 ± 4.00
1	5	0.40 ± 0.03	1.07 ± 0.18	20.3 ± 2.70
3	5	0.61 ± 0.05	1.36 ± 0.19*	16.0 ± 2.20
10	4	2.45 ± 0.95*	0.99 ± 0.07	16.0 ± 5.30
30	11	7.14 ± 1.11*	0.66 ± 0.05	28.1 ± 4.40
100	5	21.05 ± 5.34**	2.17 ± 0.65*	41.9 ± 1.00**

Two weeks after the final injection of MPTP or saline, striatal slices were prepared for superfusion experiments. Tissue content was measured at the end of experiments. Each value represents mean ± S.E.M. from n estimations. *P < 0.05, **P < 0.01, compared with control, unpaired Student's t-test (two-tailed). Other details are as in text.

EFFECTS OF L-DOPA ON THE IMPULSE-EVOKED RELEASE OF ENDOGENOUS DA IN STRIATAL SLICES FROM MPTP-TREATED C57BL MICE

In superfused striatal slices prepared from MPTP- or saline-treated mice at 2 weeks after the final injection, biphasic impulses (2 Hz, 2 ms, 25 V, 3 min) were given twice, 60 (S_1) and 90 (S_2) min, after the start of superfusion. (-)-Isoproterenol and L-DOPA were applied 15 min before S_2. Pretreatment with (-)-propranolol was initiated at the start of superfusion. The effects were evaluated by the spontaneous release ratio, Sp_2/Sp_1, and the evoked release ratio, S_2/S_1.

In striatal slices from MPTP-non-treated mice, (-)-isoproterenol at 10 - 300 nM concentration-dependently facilitated the evoked release of DA without modifying Sp_2/Sp_1 (Fig. 1A). (-)-Isoproterenol (100 nM)-induced facilitation was completely antagonized by 100 nM (-)-propranolol (Fig. 1B), indicating the presence of presynaptic β-adrenoceptors to

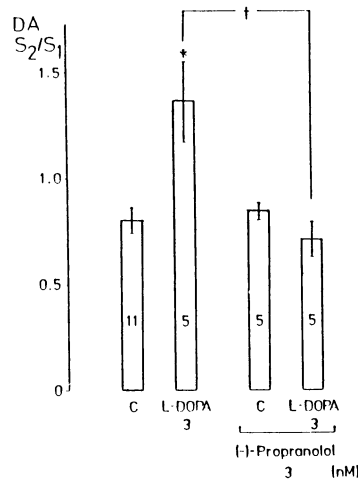

Fig. 2. Antagonism by (-)-propranolol against L-DOPA-induced facilitation of the evoked release of DA in striatal slices from MPTP-treated C57 BL mice. *P < 0.05, compared with corresponding control (C). †P < 0.05, compared with L-DOPA alone, unpaired Student's t-test (two-tailed).

facilitate the release of striatal DA. These findings are consistent with those obtained with rat brain slices.[1,2,6]

In striatal slices from MPTP-treated C57 BL mice, the evoked release and the tissue content of DA decreased by approximately 50 %, compared with corresponding values for the saline-treated mice (data not shown).

The minimum concentration of L-DOPA required to increase the spontaneous DA release was 10 nM, and this increase was concentration-dependent at 10 - 100 nM L-DOPA (Table 3). This increase seems to be due to conversion of L-DOPA to DA, because it was completely prevented by a dopa-decarboxylase inhibitor in rat brain.[1,2]

The lower concentrations of L-DOPA, 1 and 3 nM, facilitated the evoked release of DA in a concentration-dependent manner without increasing the spontaneous release and tissue content (Table 3). L-DOPA at 10 and 30 nM tended to decrease the evoked release of DA from the peak facilitation. L-DOPA at 100 nM again facilitated the evoked release, and increased the DA content to the level of the saline-treated mice (41.0 ± 4.3 pmol/mg). The increases in the evoked release and tissue content of DA at L-DOPA 100 nM are probably due to the conversion of L-DOPA to DA.

L-DOPA (3 nM)-induced facilitation was antagonized by (-)-propranolol at 3 nM (Fig. 2). This action seems to be due to L-DOPA itself, since similar actions were seen even under the inhibition of dopa-decarboxylase in rat brain slices.[1,2] These results indicate that L-DOPA facilitated the evoked release of DA via presynaptic β-adrenoceptors, which is in agreement with findings in brain slices from normal rats.[1,2] This facilitation appears to be a primary action of L-DOPA rather than the conversion to DA in MPTP-treated C57 BL mice, an animal model for Parkinson's disease.

CONCLUSIONS

We established neurochemically, behaviorally, and neuropathologically MPTP-treated C57 BL mice as a model for parkinsonism. In the striatal slices from this model animal, the primary action of L-DOPA appears to be facilitation of DA release via presynaptic β-adrenoceptors. This finding may give a new insight into L-DOPA therapy in Parkinson's disease.

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FUNCTIONAL ALTERATIONS IN STRIATAL CHOLINERGIC AND STRIATO-NIGRAL GABA-ERGIC NEURONS FOLLOWING 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) ADMINISTRATION

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INTRODUCTION

It has been well documented that systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) to humans, monkeys, and mice induces various motor disturbances resembling Parkinson's disease due to the destruction of nigro-striatal dopaminergic neurons.[1-3] Concerning the mechanism of the neurotoxicity, it is considered that the 1-methyl-4-phenylpyridinium ion(MPP^+), which is converted from MPTP by monoamine oxidase type B in cerebral glial cells and accumulates in the cell body and/or terminals of the nigro-striatal dopaminergic neuron[4,5], induces a significant decrement of dopamine content associated with the inhibition of tyrosine hydroxylase in the striatum,[6,7] a significant loss of nerve cells due to the binding to neuromelanin,[8,9] and the inhibition of NADH-ubiquinone oxidoreductase[10] in the substantia nigra.

On the other hand, it is well known that the nigro-striatal dopaminergic neuron is suppressively modulated by the striato-nigral γ-aminobutyric acid(GABA)-ergic neuron.[11,12] Furthermore, this dopaminergic neuron has been found to modulate suppressively cholinergic interneurons in the striatum.[13,14] These facts indicate that interactions of the above three neuronal systems are involved in the extrapyramidal regulatory mechanism on motor co-ordination derived from the basal ganglia.[15,16] These reports also suggest that the destruction of nigro-striatal dopaminergic neurons by MPTP may induce functional changes in striatal cholinergic interneurons as well as in striato-nigral GABAergic neurons.

In this study, therefore, we examined possible functional alterations in the striatal cholinergic and striato-nigral GABAergic neurons following MPTP administration to mice.

MATERIALS AND METHODS

Male mice of the dd strain (20-30 g), given free access to laboratory chow and tap water, were intraperitoneally injected with MPTP hydrochloride (20-60 mg/kg), which was dissolved in saline, for 5 days (once each day, between 10:00 a.m. and 11:00 a.m.). At 3 days after the last injection, the mice were sacrificed by focused microwave irradiation (5KW for 0.7sec.) to the head or by decapitation. Brains were removed immediately and dissected according to the method of Glowinski and

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Iversen.[17] They were then sliced in oxygenated Krebs-Ringer bicarbonate buffer with a microslicer. The contents of catecholamines, neuroactive amino acids, and acetylcholine were determined using a high-performance liquid chromatograph(HPLC) system according to the methods of Wagner et al.,[18] Ida and Kuriyama,[19] and Potter et al.,[20] respectively. Enzyme activities of L-glutamic acid decarboxylase(GAD), GABA-transaminase(GABA-T), choline acetyltransferase(CAT), and acetylcholinesterase(ACh-E) were assayed by the trapping method of $[^{14}C]CO_2$ formed from [^{14}C]L-glutamic acid,[21] the measurement of fluorescence of NADH formed from NAD,[22] a radioenzymic method using [^{14}C]acetyl CoA,[23] and a colorimetric method using dithiobisnitrobenzoic acid,[24] respectively. The [^{3}H]GABA and [^{3}H]choline uptakes into slices were measured according to the methods of Kuriyama et al.[25] The release of [^{3}H]GABA from slices was measured by the method described by Kuriyama et al.[25] The metabolic turnover rate of GABA in the brain was determined by the method of Loscher[26]. In this case, the GABA content was measured spectrofluorometrically according to the method of Graham and Aprison.[22] The specific bindings of [^{3}H]spiperone to dopaminergic D_2 receptors and [^{3}H]quinuclidinyl benzilate(QNB) to muscarinic cholinergic receptors in a particulate fraction prepared from the striatum were assayed according to the respective methods of Grigoriadis and Seeman[27] and Yamamura and Snyder.[28] The specific bindings of [^{3}H]muscimol and [^{3}H]flunitrazepam to $GABA_A$ and benzodiazepine receptors as well as the binding of [^{3}H]t-butylbicycloorthobenzoate to chloride channels coupled with $GABA_A$ and benzodiazepine receptors were also determined by the methods of Ito and Kuriyama[29] and Lawrence et al.[30] with the use of a particulate fraction prepared from the midbrain. Immunohistochemical stainings using specific GABA antibody and specific antibody for $GABA_A$ receptor complex were performed by the ABC method described by Takasu et al.[31] and by the PAP method outlined by Taguchi et al.[32]

RESULTS AND DISCUSSION

Administration of various doses of MPTP induced a significant decrease in dopamine content in the striatum. This alteration was found at the dose of 20 mg/kg, and increasing doses of MPTP induced a further drop in dopamine content. Although the alteration of GABA in the midbrain was not significant at 20 mg/kg, MPTP at 50 mg/kg induced a significant decrease in GABA in the midbrain. To determine if this decrease was localized in a particular area of the midbrain, we used immunohistochemical staining procedures with specific GABA antibody. In slices obtained from the midbrain of control mice, the strongest staining sites were found in the substantia nigra followed by the periventricular

Table 1. Effect of MPTP(50 mg/kg) administration on dopamine(DA), choline, and acetylcholine(ACh) contents in striatum and on L-glutamic acid and GABA contents in striatum and substantia nigra(S.N.)

	DA	Choline	ACh	L-Glutamic acid		GABA	
	Striatum	Striatum	Striatum	Striatum	S.N.	Striatum	S.N.
Control	7.0±0.86	66.6±4.47	15.0±3.18	0.93±0.05	6.7±0.2	0.29±0.02	3.0±0.10
MPTP	2.7±0.64*	65.7±9.04	15.3±2.81	0.97±0.06	5.0±0.5*	0.29±0.01	2.6±0.08*

a,μmol/g w.w., b,nmol/g w.w.
*$p<0.05$, significantly different from each control value.

Table 2. Effect of MPTP(50 mg/kg) administration on activities of GAD, GABA-T, CAT, and ACh-E in striatum and substantia nigra(S.N.)

	GAD		GABA-T		CAT	ACh-E
	Striatum	S.N.	Striatum	S.N.	Striatum	Striatum
Control	622±37.8	321±18.1	634±44.7	795±39.9	44.1±1.79	15.6±0.59
MPTP	678±21.8	318±42.1	736±26.2	722±36.8	48.6±4.42	16.9±0.91

a,nmol/mg protein/hr, b,μmol/mg protein/hr

gray and hypothalamus. On the other hand, the administration of MPTP at 50 mg/kg selectively induced a weaker staining in the substantia nigra than was found in the control without altering the intensity in other cerebral regions including the striatum. Furthermore, chemical determination of GABA content also revealed that the administration of 50 mg/kg of MPTP induced a selective and significant decrease in GABA in the substantia nigra (Table 1). A significant decrease in L-glutamic acid content was also noted in the substantia nigra. To clarify the cause of the decrease of GABA, we then examined the activities of GAD and GABA-T. As seen in Table 2, MPTP(50 mg/kg) administration, however, did not induce any alteration in GAD activity in the striatum or substantia nigra determined in the presence or absence of added pyridoxal-5-phosphate(PLP). Furthermore, MPTP did not change the kinetic parameters for GAD including the Km values for L-glutamic acid and PLP. GABA-T activities in the striatum and substantia nigra also did not show any changes following MPTP administration (Table 2). In addition, MPTP administration did not induce any alteration in [^{3}H]GABA uptake or release in the substantia nigra. On the other hand, we found that MPTP(50 mg/kg) administration induces a significant decrement of the in vivo turnover rate of GABA in the substantia nigra (Fig.1). This result indicates that a functional decrease of the GABAergic neuron, which projects to the substantia nigra, is induced following MPTP administration.

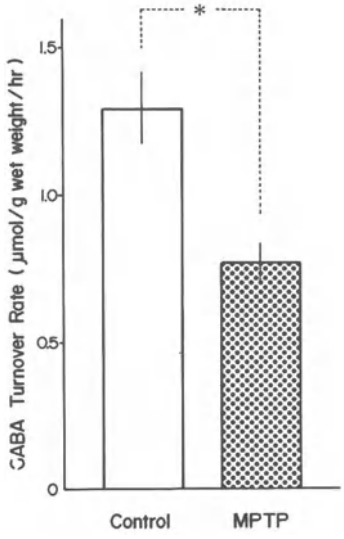

Fig. 1 Effect of MPTP administration on in vivo turnover rate of GABA in the substantia nigra. Mice received an intraperitoneal injection of aminooxyacetic acid (AOAA, 30 mg/kg) at 3 days after the last injection of saline or MPTP (50 mg/kg) and were then sacrified by microwave irradiation at 3 hr after the administration of AOAA. For the determination of GABA, see MATERIALS AND METHODS. *P<0.05, significantly different from control value.

Table 3. Effect of MPTP(50 mg/kg) administration on bindings of various [^{3}H]ligands to particulate fractions from striatum and midbrain

	Striatum		Midbrain		
	[^{3}H]Spiperone (0.5 nM)	[^{3}H]QNB (0.2 nM)	[^{3}H]MUS (5.0 nM)	[^{3}H]FLU (0.5 nM)	[^{3}H]TBOB (2.0 nM)
Control	5.4±0.44	98.6± 2.16	510±24.6	118±18.4	6.3±3.60
MPTP	8.5±0.55*	59.2±10.18*	549±28.9	105±17.2	24.8±3.98*

MUS, Muscimol; FLU, Flunitrazepam.
Each value is expressed as fmol/mg protein.
*P<0.05, significantly different from each control value.

We also examined the effect of MPTP administration(50 mg/kg) on choline and acetylcholine contents in the striatum (Table 1). MPTP administration, however, did not induce any significant alterations of choline and acetylcholine contents there. Furthermore, the activities of CAT and ACh-E in the striatum were not altered following the MPTP administration (Table 2). Similarly, no alteration in the striatal [^{3}H]choline uptake was noted in MPTP-treated animals.

It is well known that an alteration in the content of a neuroactive substance may induce a change in the binding capacity of its respective neurotransmitter receptor. Therefore, we examined the effect of MPTP administration on the receptor binding of various [^{3}H]ligands to particulate fractions obtained from the striatum and midbrain (Table 3). In the striatum, we found that [^{3}H]spiperone binding to dopamine D_2 receptor and [^{3}H]QNB binding to muscarinic receptor were significantly increased and decreased, respectively, following MPTP administration. In the midbrain, [^{3}H]TBOB binding to chloride channels, but not [^{3}H]muscimol binding to $GABA_A$ receptor and [^{3}H]flunitrazepam binding to benzodiazepine receptor, was significantly increased following MPTP administration. To determine the regions possessing the increased [^{3}H]TBOB binding in the midbrain, we examined immunohistochemically the effect of MPTP administration on immunoreactive sites for $GABA_A$ receptor complexes containing the chloride channel using specific antibody raised against the purified $GABA_A$ receptor complex. In these immunohistochemical studies, we found that MPTP administration induced an increase in the staining intensity in the substantia nigra. These results strongly suggest that a functional decrement in muscarinic receptor and increment in $GABA_A$ receptor complex may occur in the striatum and substantia nigra, respectively. Furthermore, these results also suggest that a down-regulation of muscarinic receptor in the striatum and an up-regulation of $GABA_A$ receptor complex in the substantia nigra may be induced following MPTP administration.

In summary, the MPTP-induced motor disturbances that resemble those of Parkinson's disease may be induced not only by the functional suppression of nigro-striatal dopaminergic neurons but also by the functional decrement in the striato-nigral GABAergic neuron. Furthermore, possible involvement of a functional alteration in striatal cholinergic interneurons as a consequence of the decreased function of striato-nigral GABAergic neurons is also suggested.

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HEMIPARKINSONISM IN MONKEYS AFTER UNILATERAL STRIATUM INFUSION OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP)

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INTRODUCTION

Systemically administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin inducing parkinsonism, is biotransformed into 1-methyl-4-phenylpyridinium ion (MPP^+), which then enters dopaminergic neurons via the dopamine uptake system to destroy nigral cells.

Although MPP^+ uptake is more prominent in nerve terminals than in nerve cell bodies, terminal degeneration alone is not enough to lead to the death of the neuron. Either MPP^+ is retrogradely transported to the cell bodies after being taken up at the nerve terminals, or the dopamine uptake sites on the cell bodies and their dendritic processes are responsible for the toxin directly entering the neuron (Langston and Irwin, 1986).

Perhaps in favor of the latter, Snyder and D'Amato (1986) have pointed out that the differential sensitivity of norepinephrine and dopamine cell bodies may stem from variations in the catecolamine innervation of the locus ceruleus and substantia nigra. The locus ceruleus is densely innervated by catecholamine terminals that should accumulate MPP^+ and thus protect the locus ceruleus neurons from neurotoxicity. In contrast, no such protection is available for the dopaminergic cells of the substantia nigra that receive only a few catecholamine terminals.

In an attempt to clarify this question and to construct a model for pure hemiparkinsonism, we administered MPTP directly into the unilateral striatum, caudate nucleus or putamen, of crab-eating monkeys via an Alzet osmotic minipump (Imai et al., 1988).

MATERIALS AND METHODS

Adult male crab-eating monkeys (Macaca fascicularis) weighing 3-5 kg were used for this study. Under anesthesia by intramuscular injection of ketamine (30 mg/kg), each monkey was placed in a stereotaxic frame, and stereotaxic ventriculography was done to improve the accuracy of cannula placement. A stainless steel, L-shaped cannula (0.6- or 0.8-mm outer diameter) was implanted in the head of the unilateral caudate nucleus or in the putamen at the same antero-posterior position as the implantation in the caudate nucleus. The cannula was connected by polyvinyl tubing to an Alzet 200-µl osmotic minipump that had been filled with 0.4-4 mg of MPTP HCl in physiological saline.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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After the pump had been wetted by placement in the subcutaneous tissue of the hind neck, it was placed into final position and delivered its content for 14 days. The numbers of monkeys used are listed in Table 1. Three monkeys served as controls; one was infused with the vehicle alone in the unilateral caudate nucleus, and the other 2 were infused with 4 mg of MPTP HCl in the unilateral thalamus.

During up to 6 months following minipump implantation, neurological symptoms and signs were checked frequently and recorded on videotape. Apomorphine (0.1-0.2 mg/kg) was administered intramuscularly, and its effects were also monitored by videotaping, especially for quantification of circling.

Table 1. Numbers of Monkeys Used for Various Infusion Sites/Drug Concentractions

Infusion Site	MPTP HCl (mg)			
	4	1	0.4	0
Caudate Nucleus	5 (H 5, THH2)	1 (THC1)	5 (H 3, THC1)	1 (H 1)
Putamen	1		2	
Thalamus	2 (H 1)			

H: Histology
THH: TH Immunohistochemistry
THC: TH Chemistry

For histological studies, all monkeys were deeply anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally and perfused transcardially with heparinized physiological saline followed by perfusion with ice-cold 4% paraformaldehyde in phosphate buffer. The brain was rapidly removed, and placed and kept in the same cold fixative overnight. Frontally sliced blocks were dehydrated and embedded in paraffin, and cut at 4-6 μm. Two sets of tissue were obtained: one set for Nissl and Kluver-Barrera stains, and another set of adjacent sections for tyrosine hydroxylase (TH) immunohistochemistry. For demonstration of the immunoreactivity of TH, deparaffinized tissue sections were incubated with a rabbit antiserum against TH purified from bovine adrenal medulla. The sections were then processed by the avidin-biotin method employing diaminobenzidine, and were lightly counterstained with hematoxylin.

For neurochemical analysis, monkeys were deeply anesthetized with pentobarbital sodium given intraperitoneally and decapitated. The brain was removed and frozen rapidly in dry ice, and kept at -70°C in a freezer until used. The brain was sliced frontally into blocks of 5-6-mm thickness at 0°C. The required brain regions including caudate nucleus, nucleus accumbens and putamen were punched out with a special needle. Tissues were homogenized in ice-cold 0.32 M sucrose solution. An aliquot for determination of dopamine concentration was deproteinized and injected into an HPLC equipped with a fluorodetector. TH activity was measured by the method of Nagatsu et al. (1979).

RESULTS AND DISCUSSION

Monkeys Administered 4 mg of MPTP into the Caudate Nucleus

Behavior. Within a week after the start of MPTP infusion, each monkey exhibited a flexed posture and hypokinesia of the contralateral limbs, and spontaneous mild circling toward the MPTP-treated side. After treatment with apomorphine a striking reversal of the circling motion occurred. These behavioral disturbances continued to increase for 3 months and then reached a plateau. No parkinsonian tremor at rest was observed in any of the monkeys.

Histology and Chemistry. The MPTP infusion site was confirmed to be in the center of the head of the caudate nucleus. On the control side, homogeneous TH immunoreactivity, indicating the dopamine nerve terminals, was seen throughout the striatum. TH staining of the MPTP-treated side of the striatum showed that there was almost no immunoreactivity in the caudate nucleus and dorsal putamen, but moderate to nearly normal immunoreactivity in the region from the ventromedial putamen to the nucleus accumbens. In the midbrain, the number of TH-immunoreactive neurons in the substantia nigra (SN) of the treated side was markedly reduced along the entire rostrocaudal and dorsoventral extent relative to that of the control side. On the other hand, TH-immunoreactive neurons and fibers in the ventral tegmental area of Tsai(VTA) on the treated side did not show any marked change. Both TH activity and dopamine concentration in the caudate nucleus and putamen on the MPTP-infused side were also markedly reduced relative to those of the untreated side, but they were preserved in the nucleus accumbens, parallel to the histological finding.

Discussion. The above-mentioned findings clearly showed that hemiparkinsonism was produced in the monkey after unilateral caudate nucleus infusion of 4 mg of MPTP. MPP^+ uptake at the dopamine terminals alone and then retrograde axonal transport to the cell bodies seems to be sufficient to destroy SN dopamine neurons in the primate. Why did a diffuse SN cell loss occur after a local intracaudate infusion? Area of intrastriatal diffusion of MPTP would depend on the amount of the toxin infused.

Monkeys Administered 0.4 mg of MPTP into the Caudate Nucleus

Behavior. No clear hemiparkinsonism occurred in this series. No flexed posture of the contralateral upper limb, no hypokinesia of the limb and no spontaneous circling were observed. After treatment with apomorphine, however, a mild circling away from the MPTP-treated side appeared.

Histology and Chemistry. Both TH activity and dopamine concentration in the caudate nucleus on the drug-treated side were markedly reduced, but in the putamen they were reduced only near the infusion site. Histologically in the midbrain the SN on the treated side showed a partial cell loss, mainly rostrally, and clusters of spared cells.

Monkeys Administered MPTP into the Putamen

4 mg of MPTP. Behaviorally, the sole monkey used exhibited a clear hemiparkinsonism, virtually the same as seen in those animals given 4 mg of MPTP into the caudate nucleus. Apomorphine-induced circling away from the MPTP-treated side was also observed.

0.4 mg of MPTP. Behaviorally, the two monkeys showed a slight hemiparkinsonism, that is, no flexed posture but a mild hypokinesia of the contralateral upper limb, so that reaching for food was limited to the

uninvolved limb. After treatment with apomorphine, however, no circling away from the MPTP-treated side was observed.

CONCLUSION

Hemiparkinsonism was produced in the monkey after unilateral caudate nucleus or putamen infusion of MPTP. MPP^+ uptake at the dopamine nerve terminals alone and then retrograde axonal transport to the cell bodies seems to be sufficient to destroy nigral dopamine cells in the monkey. Infusion of 4 mg of MPTP into the cuadate nucleus produced an almost total nigral dopamine cell loss. Infusion of 0.4 mg of MPTP into the caudate nucleus produced a partial nigral cell loss, mainly rostrally, and clusters of spared cells were seen. It seems that the threshold to produce hemiparkinsonism by MPTP infusion is lower in the putamen than in the caudate nucleus and that the threshod to produce apomorphine-induced circling is lower in the caudate nucleus than in the putamen.

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CYCLOSPORIN A ENHANCES NEUROTOXICITY OF N-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) IN MICE

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INTRODUCTION

N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a well-known neurotoxin that elicits symptoms very similar to those seen Parkinsonism in humans[1,2] and causes specific neurodegeneration in the nigro-striatal system in primates[3] and rodents.[4] The molecular basis of the neurotoxicity of MPTP has been extensively studied.[5,6] Recently we reported that activities of tyrosine hydroxylase [tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2, TH] and L-3,4-dihydroxyphenylalanine decarboxylase (aromatic L-amino acid decarboxylase, aromatic L-amino acid carboxylyase, EC 4.1.1.28) were markedly reduced in the striatum of C57BL/6N mice after 8 days of MPTP administration.[7] In addition, a marked reduction in dopamine (DA) content was observed in the striatum. On the other hand, cerebrospinal fluid obtained from Parkinsonian patients showed a marked reduction in beta 2-microglobulin,[8] which suggests that some disorder of the immune system may be involved in the pathogenesis of Parkinsonism. The present study was undertaken to study the effect of cyclosporin A (CsA), an immune suppressor, on the toxicity of MPTP to assess the contribution of the immune system to the pathological changes seen in the dopaminergic and noradrenergic systems in parkinsonism.

MATERIALS AND METHODS

MPTP was purchased from Aldrich Chemical (Milwaukee, WI, U.S.A.). MPTP was dissolved in phosphate-buffered saline (PBS) and injected (30 mg/kg body weight/day) into C57BL/6N mice (male, 30 week-old, 20-25 g weight). CsA was donated by Sandoz, and was used intravenously in solution form (0.25 g/5 ml of solvent). The solution was diluted with PBS and injected into mice (4 mg/kg/day, subcutaneously); solution without MPTP served as the control. After daily administrations of MPTP and CsA for 8 days, the mice were killed after a further 8-day, drug-free interval. The brains were removed immediately and placed on ice, as reported previously.[9] The striatum and hypothalamus were dissected and homogenized in 0.32 M sucrose. TH activity was determined by measurement of DOPA produced from L-tyrosine by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD).[10] DA contents were quantitatively assayed by HPLC with ECD.[11] Total biopterin amounts were

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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determined by HPLC with fluorescence detection.[12] Protein content was determined by the method of Bradford,[13] with bovine γ-globulin as standard.

RESULTS

As summarized in Table 1, after 8 days' administration of MPTP, a marked reduction in TH activity was observed in the striatum, compared with the control injected with saline alone. This eduction in TH activity by MPTP was enhanced by CsA injected together with MPTP. On the other hand, CsA alone did not affect the activity of TH in the striatum. The DA content in the striatum was reduced by MPTP, and also the reduction in DA content was more dominant in mice injected with CsA in combination with MPTP.

In the hypothalamus, TH activity was not so markedly reduced by MPTP alone as in the striatum, as summarized in Table 1. In contrast, TH activity was reduced significantly in mice administered with MPTP in combination with CsA: to 30.3 % of the control value. TH activity was not reduced by injection of CsA alone. DA content in the hypothalamus was also reduced significantly in mice treated with MPTP combined with CsA.

Biopterin contents (a cofactor of TH) in the striatum and hypothalamus were measured, and these results are also shown in Table 1. The total biopterin contents in the striatum and hypothalamus were reduced by administration of MPTP alone, and the reduction was enhanced by co-administration with CsA.

DISCUSSION

As reported in our previous paper[7] and confirmed here again, MPTP reduces TH activity and total biopterin contents in the striatum. This reduction in activity is ascribed to a lower TH protein content, as shown by enzyme immunoassay,[14] and suggests degeneration of dopaminergic neurons

Table 1. Effects of MPTP and CsA on TH activity and DA and biopterin contents in the striatum and hypothalamus

	n	TH activity (pmol/min/mg protein)	Dopamine (pmol/mg protein)	Biopterin (pmol/mg protein)
Striatum				
Control	4	350 ± 46	461 ± 21	3.79 ± 0.36
CsA	4	323 ± 50	459 ± 59	3.81 ± 0.81
MPTP	4	109 ± 20*	101 ± 19*	2.01 ± 0.26*
MPTP + CsA	4	68.4 ± 12.2*	78 ± 14.9*	1.53 ± 0.11*
Hypothalamus				
Control	4	77.5 ± 21	21.1 ± 4.9	2.47 ± 0.18
CsA	4	54.5 ± 15	27.1 ± 3.9	2.28 ± 0.49
MPTP	4	54.5 ± 9.0*	18.8 ± 4.7*	1.20 ± 0.16*
MPTP + CsA	4	23.5 ± 3.8*	8.2 ± 1.6*	0.917 ± 0.339*

Each value represents mean and SD of duplicate measurements of each sample (*n*: number).

* Significant difference from control, $p<0.001$, by Student's *t* test.

Control mice were injected with saline alone, and the respective amounts of Cs A and MPTP administered daily were 4 mg/kg and 30 mg/kg per day for 8 days. All animals were sacrificed 8 days after the last injection.

in the striatum. Enhancement of the neurotoxicity of MPTP by CsA might be due to increased degeneration of dopaminergic neurons.

Severe toxicity to the central nervous system with neurological symptoms such as tremors, neuralgia, and convulsions were observed in patients treated with cyclosporin.[15,16] However, CsA was not detected in the cerebrospinal fluid and has been considered not to be transported into the brain across the blood-brain barrier in man[17] or mouse.[18] On the other hand, in experimental chronic viral infection of the central nervous system of mice with mouse hepatitis virus 3 (MHV3), CsA was found to increase the rate of acute death and viral titers in the brain when cyclosporin treatment was started at the same time as the virus infection.[19] When the treatment was started two weeks after virus infection, CsA inhibited chronic MHV3-induced infection of the central nervous system. The discrepant effects of CsA treatment on MHV3 infection suggest that CsA may increase acute cytopathogenic lesions in the brain caused by virus infection and that it may have a beneficial effect on chronic immune responses. The latter effect was also shown by its inhibitory effect on the expression of experimental allergic encephalitis.[20] The cumulative effect of CsA on MPTP-induced lesions in the dopaminergic neurons may be due to its effect on the immune system or may be dependent on MPTP toxicity. The molecular basis of the enhanced toxicity of MPTP by CsA awaits further study.

CONCLUSION

This study was done to assess if neurotoxicity of N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is potentiated by co-administration of cyclosporin A (CsA), an immunosuppressant agent, to C57BL/6N mice. Our results show that the neurotoxicity of MPTP is potentiated and not only dopaminergic but also noradrenergic neurons are also impaired when Cs A is co-administered. Since both types of neurons are known to be affected in Parkinson's disease,[21] co-administration of MPTP and Cs A to mice may produce a parkinsonian model similar to human Pakinson's disease.

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EFFECTS OF VARIOUS DRUGS ON BEHAVIOR AND STRIATAL DOPAMINE CONTENTS IN MPTP-TREATED MICE EXPOSED TO STRESS

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INTRODUCTION

Although the symptoms of patients with Parkinson's disease are worsened by various stresses, a phenomenon known as stress-induced akinesia[1,2] the pathophysiology and therapy of stress-induced akinesia has not been clarified. When the stress is mild, symptoms are easily improved by an increase in the dosage of 1-3,4-dihydroxyphenylalanine (L-DOPA). However, when patients with Parkinson's disease are exposed to severe and long-term stress and the symptoms are highly aggravated, L-DOPA treatment is less effective. We previously reported that, due to a decreased dopamine (DA) level and enhancement of DA turnover, 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP)-treated mice became remarkably akinetic after stress[3] and that imipramine (IMP) and diazepam (DZP) were clinically more effective than L-DOPA in improving the condition.[4]

The present study was undertaken to examine the efficacy of various drugs (IMP, DZP, morphine, and naloxone) in MPTP-treated mice exposed to immersion immobilization stress as an experimental model of stress-induced akinesia in Parkinson's disease.

MATERIALS AND METHODS

Experimental design

Male C57BL/6J mice weighing 20-30g (6-8 weeks old) were used in the present experiment. The mice were kept in an animal room for at least one week prior to the beginning of the experiments. The animals had free access to food and water, and were kept in a room having a 12hr/12hr light/dark cycle. In our preliminary study, we described a regimen of MPTP (Aldrich) intraperitoneal injections which produced a depletion of striatal DA greater than 80 %. This regimen consisted of daily doses of 30mg/kg of MPTP for three days. The control animals were injected intraperitoneally with an equal volume of physiological saline. The mice were divided into four subgroups as follows: MPTP-treated group (MPTP group), saline-treated group (saline group), MPTP-and stress-treated group (MPTP+stress group) and saline- and stress-treated group (saline+stress group). Each group consisted of five mice.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Additional groups of mice were administered MPTP one week prior to IMP (a gift from CIBA-GEIGY CO.), DZP (a gift from Takeda K.K.), morphine (MOR a gift from Prof. Kimishima), or naloxone (NAL, a gift from Sankyo K.K.) treatment. All drugs were dissolved in physiolosical saline and injected intraperitoneally. A doses of IMP at 5, 10, or 20 mg/kg, of DZP at 2, 5, or 10 mg/kg, of MOR at 3 or 6mg/kg, or of NAL at 5mg/kg was administered once before the immersion immobilization stress was applied. We established the following groups: MPTP+stress+IMP (5mg/kg), MPTP+stress+IMP (10mg/kg), MPTP+stress+IMP (20mg/kg), MPTP+stress+DZP (2mg/kg), MPTP+stress +DZP (5mg/kg), MPTP+stress+DZP (10mg/kg), MPTP+stress+MOR(3mg/kg), MPTP+ stress+MOR (6mg/kg), and MPTP+stress+NAL (5mg/Kg).

Stress

The mice were exposed to immersion immobilization stress for 15 hours in a water bath whose temperature was kept at 25°C, as described by Takagi et al. This stress model is thought to correspond clinically to severe stress.

Locomotor activity

One mouse from each group was placed separately in a plastic cage and its locomotor activity was measured with an Animex meter (MUROMACHI KIKAI CO., LTD.) after the stress loading every 30 minutes over a 7-hr period. Locomotor activity was measured three times in each group.

Determination of brain DA, DOPAC, and HVA contents

The mice were sacrificed by decapitation at 0 hr, 3hr, 6 hr, 24 hr, and 7 days after stress. The brains were rapidly removed, and both the cerebral cortex and the striatum were dissected on ice.

After dissection, these brain areas were weighed, homogenized in a 1-ml volume of 0.1 N perchloric acid containing 2 μl of 3,4-dihydroxybenzylamine (DHBA) as an internal standard, and centrifuged at 15,000 X g for 20 min. The supernatant was stored at -20 °C until assayed.

Concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanilic acid (HVA) in the supernatant were determined by high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) according to the method of Mayer and Shoup,[6] with minor modifications.

The DA index was calculated as (DOPAC+HVA)/DA in two brain lesions. The results were analyzed statistically by analysis of variance (ANOVA).

RESULTS

All mice exposed to stress developed acute gastric mucosal lesions, and no mice died from loading stress. In IMP-, DZP-, and MOR-pretreated groups obvious gastric ulcers were few, but there were superficial gastric lesions such as hemorrhage or erosive gastritis.

Locomotor activity

Locomotor activity of IMP (5mg/kg)-, DZP (2mg/kg)-, NAL (5mg/kg)-pretreated mice decreased, whereas that of IMP (10, 20mg/kg)-, DZP (5, 10 mg/kg)-, and MOR (3, 6 mg/kg)- pretreated ones did not.

Results of the experiment on DA, DOPAC, and HVA contents and DA index in the striatum at 0 hour are summarized in Table 1.

Immediately after stress, the striatal DA content of the MPTP+stress group showed a significant reduction when compared with the MPTP group ($P<0.01$). In the 5 and 10 mg/kg IMP-, 2 and 5 mg/kg DZP-, and 5 mg/kg NAL-pretreated groups, striatal DA contents were significantly reduced as well.

Table 1. Striatal DA, DOPAC, and HVA levels (ng/mg) and (DOPAC+HVA)/DA ratio in mice at 0 hour after stress.

	D A	DOPAC	HVA	(DOPAC+HVA)/DA ratio
Saline	9.886±3.287	0.736±0.263	0.874±0.253	0.168±0.033+
saline+stress	8.852±0.422	0.753±0.109	1.150±0.186	0.215±0.026+
MPTP	3.809±0.626	0.314±0.044	0.506±0.085	0.217±0.024
MPTP+stress	2.018±0.387**	0.308±0.057	0.431±0.132	0.373±0.074**
MPTP+stress +IMP 5mg/kg	2.283±0.492**	0.311±0.069	0.453±0.124	0.352±0.111*
MPTP+stress +IMP 10mg/kg	2.405±0.643*	0.251±0.054	0.469±0.170	0.290±0.096
MPTP+stress +IMP 20mg/kg	3.555±0.702	0.403±0.124	0.419±0.119	0.227±0.036
MPTP+stress +DZP 2mg/kg	2.153±0.610**	0.331±0.152	0.412±0.057	0.359±0.072*
MPTP+stress +DZP 5mg/kg	2.508±0.305*	0.361±0.124	0.485±0.058	0.334±0.037*
MPTP+stress +DZP 10mg/kg	3.452±0.223	0.360±0.034	0.457±0.094	0.236±0.024
MPTP+stress +MOR 3mg/kg	3.360±0.844	0.215±0.086	0.515±0.085	0.218±0.008
MPTP+stress +MOR 6mg/kg	3.906±0.610	0.225±0.067	0.566±0.118	0.206±0.041
MPTP+stress +NAL 5mg/kg	1.972±0.785	0.252±0.086	0.698±0.242	0.605±0.314*

N=5 Values indicate the mean±SD
** P<0.01] vs.MPTP group
* P<0.05]
+ P<0.05 vs.saline group

Determination of brain DA, DOPAC and HVA contents

In the 20mg/kg IMP-, 10 mg/kg DZP-, 3 and 6 mg/kg MOR- pretreated groups, striatal DA contents were not significantly reduced.

Striatal DOPAC and HVA contents were not significantly different among the groups.

Results of DA, DOPAC, HVA contents and DA indexes of the cortex at 0 hour are summarized in Table 2. In this brain resion stress did not affect the parameters significantly, except in the case of the (DOPAC+HVA)/DA ratio.

Table 2. Cortical DA, DOPAC, and HVA levels (ng/mg) and (DOPAC+HVA)/DA ratio in mice at 0 hour after stress.

	D A	DOPAC	HVA	(DOPAC+HVA)/DA ratio
Saline	1.041±0.112	0.057±0.062	0.230±0.034	0.279±0.070
Saline+stress	0.900±0.099	0.067±0.030	0.223±0.063	0.325±0.079
MPTP	0.305±0.053	0.039±0.012	0.104±0.020	0.472±0.108
MPTP+stress	0.280±0.091	0.040±0.027	0.145±0.053	0.717±0.267*
MPTP+stress +IMP 5mg/kg	0.259±0.090	0.047±0.020	0.127±0.037	0.743±0.376*
MPTP+stress +IMP 10mg/kg	0.264±0.080	0.059±0.028	0.111±0.031	0.640±0.234
MPTP+stress +IMP 20mg/kg	0.303±0.150	0.070±0.010	0.147±0.017	0.496±0.274
MPTP+stress +DZP 2mg/kg	0.264±0.052	0.035±0.005	0.128±0.019	0.623±0.044*
MPTP+stress +DZP 5mg/kg	0.288±0.082	0.032±0.011	0.122±0.020	0.533±0.048
MPTP+stress +DZP 10mg/kg	0.251±0.038	0.038±0.012	0.113±0.015	0.576±0.039
MPTP+stress +MOR 3mg/kg	0.302±0.070	0.049±0.006	0.091±0.020	0.479±0.087
MPTP+stress +MOR 6mg/kg	0.317±0.146	0.059±0.017	0.131±0.028	0.654±0.201
MPTP+stress +NAL 5mg/kg	0.257±0.155	0.038±0.021	0.103±0.020	0.764±0.229*

N=5 Values indicate the mean±SD
* P<0.05 vs.MPTP group

In a time-course study, the striatal DA content of the MPTP+stress group recovered 24 hours after stress to the level of the MPTP group. Striatal DA contents in 5 mg/kg IMP-, 2mg/kg DZP- and 5mg/kg NAL-pretreated groups recovered 24 hours after stress to the level of the MPTP group like the MPTP+stress group, and those in the 10 mg/kg IMP- and 5 mg/kg DZP- pretreated groups recovered even more quickly, by 3 hours after stress loading.

DISCUSSION

Pretreatment with IMP, DZP, or MOR limited the stress-induced decrease in locomotor activiy and striatal DA level. Pretreatment with IMP at 10 or 20 mg/kg, DZP at 5 or 10 mg/kg, or MOR at 3 or 6 mg/kg was effective, but pretreatment with IMP at 5 mg/kg, DZP at 2 mg/kg, or NAL at 5 mg/kg was not effective.

In this study, the inhibition by IMP of DA uptake might cause a limitation in the enhancement of DA turnover. The mode of action of DZP may be to act on the GABAnergic system via benzodiazepine receptors and thus limit the release of DA in the striatum.

MOR is partly involved in the stress process, and NAL has an inhibitory effect on cathecholamine synthesis. MOR and NAL may affect DA levels in the striatum and cortex, and play critical roles in behavioral as well as emotional responses to stressful stimuli.

CONCLUSION

IMP, DZP, and exogenous opioid peptides like MOR are effective drugs to prevent or treat stress-induced akinesia in patients with Parkinson's disease.

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ACUTE EFFECTS OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) ON BODY TEMPERATURE OF VARIOUS STRAINS OF MICE

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INTRODUCTION

Systemic administration of the neurotoxin MPTP to experimental animals induces the selective destruction of the nigrostriatal dopamine (DA) system. This MPTP-induced toxicity requires conversion of MPTP to its active metabolite, 1-methyl-4-phenylpyridine (MPP^+), by the mitochondrial enzyme monoamine oxidase B-form (MAO-B).[1] This selective MPTP neurotoxicity is accounted for by active MPP+ accumulation into DA neurons via the high affinity DA uptake system.[1] The mitochondria, in addition to being the site for MPP+ formation, may also be an important target of MPP^+ neurotoxicity, since MPP^+ inhibits mitochondrial respiration, resulting in prevention of ATP formation with consequent cell death in the DA system. The MPTP-treated primates represent the best model currently available for study of the etiology of Parkinson's disease.

We earlier reported findings that a single systemic or direct injection of MPTP or MPP^+ into albino mouse (ddY) brain markedly changed the body temperature, and we viewed this as an acute pharmacological effect.[2] For example, a single systemic MPTP administration caused marked hyperthermia, mediated by peripheral cholinergic functions, followed by subsequent long-lasting, centrally mediated hypothermia. As noted earlier and above, for MPTP neurotoxicity, primates are more sensitive than rodents; and, in addition, the sensitivity to MPTP varies from species to species as well as between different strains of mice.[3-5] Even in animals with low sensitivity, MPTP treatment results in differences in striatal content of DA and its metabolites, in DA accumulation by striatal synaptosomes, and in cell loss, depending on the strains of mice used, doses, duration of treatment, and dosing intervals.[6]

The present study was undertaken to clarify whether various strains of mice also show different sensitivity to a single MPTP injection in terms of the thermal effects.

MATERIALS AND METHODS

In this study, six different strains of male mice, ddY, BALB, ICR, C57BL, B6C3F1, and C3H, weighing 25 ± 2 g, were used. Changes in rectal

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temperature were recorded as described previously.[2] Each group of ten mice with rectal temperature between 37.0-38.0 °C were selected and then maintained 1 hr to acclimatize them to ambient environment in a temperature-controlled room before injection of drug(s). Contents of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in mouse hypothalamus were determined by HPLC with ECD.[7] MPTP was injected at a fixed dose of 24 mg/kg (i.p.).

RESULTS

Fig. 1 shows the time courses of changes in rectal temperature caused by a single MPTP injection (24 mg/kg, i.p.) into six different albino and pigmented mouse strains. The temperature was recorded at various times over a 7-hr period. This MPTP dose remarkably changed rectal temperature in two phases; in the first an increase occurred and in the second, a decrease. In general, the MPTP-induced hyperthermic effect was more marked in albino ddY, BALB, and ICR mice, but the subsequent hypothermia was more marked in the pigmented C57BL, B6C3F1, and C3H mice. Pretreatment of these six strains of mice with

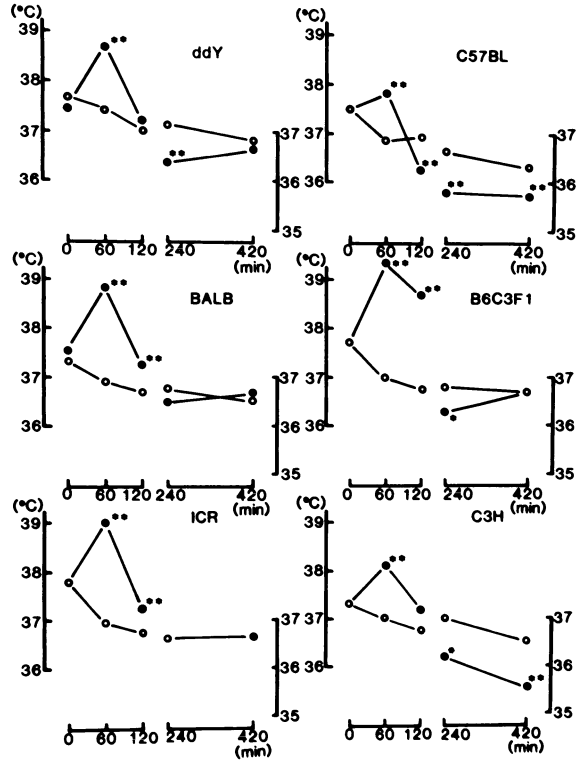

Fig. 1. Effects of i.p. administration of MPTP (24 mg/kg) on mouse rectal temperature in the six different strains. Rectal temperature was determined after MPTP administration at the various times indicated. ○ : Saline (n=10), ● : MPTP24mg/kg (n=10). *:p<0.05, **:p<0.01 significant difference from saline-treated control.

a quarternary derivative of atropine methylbromide (20 mg/kg, i.p.), an anti-cholinergic agent with predominantly peripheral effects, prevented only the initial hyperthermic effect, but not the subsequent hypothermia. In contrast, pretreatment with an MAO-A inhibitor, clorgyline (1 mg/kg, i.p.), MAO-B inhibitor , l-deprenyl (5 mg/kg, i.p.), or a catecholamine re-uptake inhibitor, nomifensine (2.5 mg/kg, i.p.), failed to prevent MPTP-induced hyperthermia in all six strains tested. In contrast to the MPTP injection, MPP^+ (12 mg/kg, i.p.) caused only hyperthermia, but the i.c.v. injection (10 µg/mouse) caused only hypothermia in both albino (ddY) and pigmented (C57BL) mice.

In spite of no prevention of hyperthermia, l-deprenyl, clorgyline and nomifensine prevented MPTP-induced hypothermic effect in both mouse strains. This MPTP-induced hypothermia was also completely prevented by a 5-HT re-uptake blocker, fluoxetine (20 mg/kg, i.p.). In ddY mice, the systemic MPTP injection did not significantly change DA, DOPAC, and HVA levels in the hypothalamus during the initial hyperthermic phase (assayed 60 min after the injection), whereas an increase in DA levels to 183 % was found for C57BL mice. In contrast to DA content, hypothalamic 5-HT levels greatly increased to 195 % and 167 %, respectively, in these two mouse strains. At the hypothermic stage (240 min after MPTP injection), hypothalamic DA and its main metabolites did not change significantly in either strain, but the levels of 5-HT and its metabolite were markedly changed compared with those of the saline control (ddY mice: 5-HT; +51 %, 5-HIAA; -39 %, C57BL mice: 5-HT; +15 %, 5-HIAA; -36 %).

DISCUSSION

In agreement with previous findings,[2] the present results show that a single injection of MPTP into various strains of mice produced marked hyperthermia, and subsequent hypothermia. In MPTP-treated albino (ddY) and pigmented (C57BL) mice, hyperthermia was completely blocked by a quarternary derivative of atropine methylbromide, an anti-cholinergic drug with peripheral effects but with little central action. This anti-cholinergic drug, however, failed to block the hypothermic effect, indicating the possibility that these two thermic effects might be mediated by separate mechanisms or at different sites by the same mechanism. The initial hyperthermic effect was also produced by systemic MPP^+ administration in both ddY and C57BL mice, and clorgyline and l-deprenyl failed to block MPTP-induced hyperthermia. Thus, the MPTP-induced hyperthermia may result from elevated peripheral heat production, probably mediated by activated cholinergic functions. Moreover, unlike neurotoxicity to the DA system, the MPTP-induced hyperthermic effect is not dependent on MPP^+ formation, since both clorgyline and l-deprenyl, at the dose which in vivo almost selectively and completely inhibits either MAO-A or -B activity alone,[8] could not antagonize the hyperthermia induced by MPTP. Thus, both MPTP and MPP^+ per se induce the hyperthermic effect.

MPTP-induced hypothermia was more marked in the pigmented C57BL, B6C3F1, and C3H mice than in the albino ddY, BALB, and ICR mice. These findings agree with the previous results that, among different strains of mice, the neurotoxic effects of MPTP are more marked in pigmented than in albino mice.[4] However, there is no clear correlation between the higher neurotoxic effects in pigmented animals and differences in MAO-B activity or in DA uptake rate between species, strains, or animals of different age.[5] Likewise, there is no correlation between different intra- and extraneuronal MAO-A and -B activities and different sensitivities of C57BL and NMRI mice to MPTP toxicity.[6] In this study, MPTP-induced hypothermia in C57BL, but not albino ddY mice, was blocked by either

clorgyline or l-deprenyl at the dose selectively inhibiting only either MAO-A or -B activity alone. Thus, in spite of this selective, hypothermic action of MPTP in pigmented mice, the precise mechanism by which MPTP acts on mouse body temperature remains unclear.

The catecholamine uptake blocker nomifensine weakly, and the 5-HT uptake blocker fluoxetine strongly, prevented this hypothermic effect induced by MPTP. Also, MPTP did not significantly change hypothalamic DA and its metabolite levels, but greatly changed 5-HT and its metabolite levels. Taken together, it seems likely that MPTP-induced hypothermia may result from changes in hypothalamic 5-HT and its metabolite levels, but not from changes in DA and its metabolites, although the precise effects of these changes on temperature remain unclear.

In conclusion, although the exact mechanism of MPTP action on central thermoregulation remains to be clarified in different strains of mice, the present results demonstrated that significant thermic effects were more markedly induced by MPTP in pigmented C57BL, B6C3F1, and C3H mice than in albino ddY, BALB, and ICR mice.

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EFFECT OF MPP^+ AND TIQ ON CULTURED RAT MIDBRAIN DOPAMINERGIC NEURONS

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INTRODUCTION

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induces Parkinson's disease in humans, monkeys and some animals (1,2). MPTP was oxidized to 1-methyl-4-phenylpyrdinium ion (MPP^+) by monoamine oxidase (3). Nagatsu and Hirata reported that endogenous candidate of pyridinium compound, tetrahydroisoquinoline (TIQ) decreased tyrosine hydroxylase (TH) and 3,4-dihydroxyphenylacetic acid in mice striatum (4). In the current investigation, effect of MPP^+ and TIQ on cultured rat midbrain dopaminergic neurons were studied, using cell or organotypic culture systems.

MATERIALS AND METHODS

Cell Culture of Fetal Rat Midbrain

Whole or ventral midbrain, dissected aseptically from fetal (16 day gestation) Wistar rat, were disociated by incubation at 37°C in 0.25% trypsin and 0.1% DNase. The tissues were sedimented (1,500 rpm, 3 min) following the addition of cold horse serum. The pellet was resuspended in the culture medium and further dissociated by trituration through a plastic pipette. The single viable cells were counted and plated into 48-well plates at a density of 3.0×10^5 cells in 500 µl of medium for tyrosine hydroxylase (TH) staining, or 1.5×10^6 cells/35 mm dish for [^{3}H]-dopamine ([^{3}H)-DA) uptake experiment. The neurons were grown in the culture medium at 37°C under 5% CO_2 with medium change twice a week. After one week in culture, the medium was removed and changed to a drug containing one. Then medium and drugs were changed every three days for another 8 days. The employed culture media were Eagle's minimum essential medium (EMEM) supplemented with either 10% fetal bovine serum (FBS, Filtron), 10 mg/ml glucose, 350 µg/ml glutamine, 2 mg/ml sodium bicarbonate, 50 U/ml penicillin and 85 U/ml streptomycin.

Organotypic Culture of Fetal Rat Midbrain

Ventral midbrains were dissected as above mentioned. The tissue was cut into 1 mm^3 cube and sedimented gently. Sedimented clamps were resuspended with culture medium and plated into 35 mm dish at a density of

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one embryonic ventral midbrain per dish. The neurons were grown in the culture medium at 37 °C under 5% CO_2. The schedule of drug administration was the same as the cell culture experiment. The organotypics were exposed to drugs for 8 or 15 days and [^{3}H]-DA uptake abilities were measured.

TH Staining

The whole midbrain neurons cultured were fixed and treated with mouse monoclonal anti-TH antibody (diluted x200) and stained with ABC Kit (Vectastain PK 4002, CA., USA) as manufacturer's recommendation. The stained neurons were counted in about 10% of the culture area and expressed as cells/cm^2.

[^{3}H]-DA Uptake

The whole or ventral midbrain neuron cells, or organotypic ventral midbrains were incubated with 50 nM [^{3}H]-DA (New England Nuclear, 10 Ci/mmol) in modified PBS (6 mg/ml glucose, 0.75 mM $CaCl_2$, 0.75 mM $MgCl_2$ and 0.1 mM pargyline) for 20 min at 37 °C. The cells were scraped with 1 ml of 0.2% Triton X-100. After the addition of 10 ml of scintillator (Aquasol 2), they were gently stirred and radioactivities were counted.

Drugs

MPP^+ and TIQ were synthesized by authors (S.O. and M.H.). They were dissolved with culture medium and sterilized with 0.2 μm filter. In this condition, MPP^+ was very stable. The purity of TIQ was checked with thin layer chromatography following methylene chloride extraction. Mouse monoclonal anti-TH-antibody was a kind gift (Prof. H. Hatanaka, Osaka Univ., Japan). Other chemicals used were the highest purity available commercially.

RESULTS

Rat embryonic whole midbrain cells were exposed to MPP^+ or TIQ for 8 days after 7 days incubation. Then the cultured neurons were stained with monoclonal anti-TH antibody and surviving TH-positive neurons were counted. MPP^+ at the doses of 1 or 10 μM did not alter the number of TH-positive neurons. TIQ (100 or 1000 μM) also did not have any effect on TH-positive surviving neurons. At higher drug concentrations, MPP^+ 100 μM or TIQ 1 mM, all the cells were dead nonspecifically and peeled off from the culture plates.

Rat embryonic whole midbrain cells were treated with MPP^+ for 8 days after 7 days incubation. Then the [^{3}H]-DA uptake abilities of the cultured neurons were examined. MPP^+ at the doses from 0.1 to 10 μM did not cause any significant change. Therefor we employed ventral midbrain culture in order to concentrate the TH-positive neuron population. However also in this condition MPP^+ (1, 10 μM) did not decrease the uptaken [^{3}H]-DA amount. Higher MPP^+ dose (100 μM) was again completely toxic to the all cultured cells.

Rat embryonic ventral midbrain was cultured organotypically under MPP^+ exposure for 8 days after 7 days incubation. Then the [^{3}H]-DA uptake abilities were examined. MPP^+, 0.1 and 1 μM, decreased the amount of uptaken [^{3}H]-DA dose-dependently, and the decrement was statistically significant at 1 μM of MPP^+ (Fig. 1).

Ventral midbrain derived from embryonic rat was cultured organotypically. TIQ was included in the culture medium for 8 or 15 days after 7 days incubation. During the 8 days TIQ treatment at 1000 μM, the amount of uptaken [^{3}H]-DA decreased about 70% (Fig. 2). Moreover when the drug treatment were prolonged to 15 days, the effect of TIQ became more potent

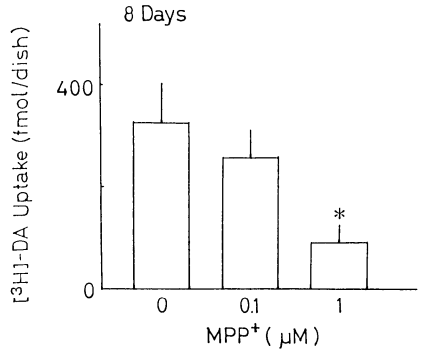

FIG. 1 Effect of MPP^+ on [^{3}H]-DA Uptake in Ventral Midbrain Organotypic Culture
MPP^+ was treated for 8 days after 7 days preincubation. Bars indicate S.E.M.
*:P<0.05 (ANOVA)

and clearer. TIQ caused remarkable decrease of [^{3}H]-DA uptake dose-dependently, and the minimum effective concentration was 10 uM (Fig. 2).

DISCUSSION

The toxic effect of MPP^+ was successfully confirmed in the [^{3}H]-DA uptake experiment only in organotypic culture condition. Thus this organotypic culture system would be an useful method for the evaluation of drug toxicity. In the cell culture system, however, MPP^+ did not exert any specific effect on either the number of TH positive neurons or [^{3}H]-DA uptake. Several reasons could be considered for this difference between organotypic and cell culture condition. First, trypsinization and/or mechanical pipetting to dissociate single cells from tissue might cause severe cell damage. Therefore there is possibility that fragile dopaminergic midbrain neurons were already dead during the culture procedure. Moreover TH positive neurons decreased during culture period. For example, 500-600/cm^2 TH positive neurons surviving after 7 days preincubation de-

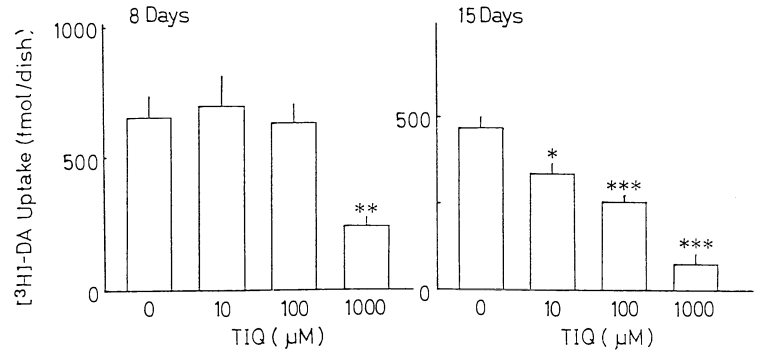

FIG. 2 Effect of TIQ on [^{3}H]-DA Uptake in Ventral Midbrain Organotypic Culture
TIQ was administered for 8 (left) or 15 (right) days after 7 days preincubation. Bars indicate S.E.M. *:P<0.05, **:P<0.01, ***:P<0.001 (ANOVA)

creased to 100 cells/cm^2 in 8 days culture without any drug treatment. This decrement of TH positive neurons could not be rescued when the culture medium was changed into EMEM containig 25% FBS and 5% chick embryo extract (prepared in our laboratory). Second, in organotypic culture condition, neurons could get sufficient trophic support from the surrounding glial cells. Thus we could observe cell functions in organotypic culture which are more similar to in vivo than in cell culture.

The effect of TIQ was less than that of MPP^+. This might be due to the lack of N-methylation in TIQ molecule. Prolonged TIQ exposure, 15 days, brought more potent effect on [^{3}H]-DA uptake. Thus, it was suggested that the toxicity of TIQ was not acute but rather subacute or accumulating. Because of its accumulating toxicity, the validity of TIQ as a endogenous parkinsonism inducer was further supported.

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ASSAY SYSTEM FOR NEUROTOXICANTS CAUSING PARKINSON'S DISEASE: 1-METHYL-4-PHENYLPYRIDINIUM ION (MPP^+) INHIBITS SURVIVAL OF CULTURED NEURONS FROM SUBSTANTIA NIGRA OF FETAL MONKEY(Macaca fascicularis)

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INTRODUCTION

Most neurotoxicological research is carried out using living rodents (mice and rats). This has three main drawbacks. The first is the fundamental difficulty of extrapolating these results to human toxicity. However, obtaining statistical data on living non-human primates is practically impossible because of its high cost. The second is the killing of a great number of living animals in opposition to recent animal protection movements. The third is the difficulty of molecular and cellular studies *in vivo*. To avoid or decrease these scientific and social problems, it is worthwhile exploring the possibility of using *in vitro* preparations such as brain slices[1] or cultured neurons[2].

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes degeneration of dopaminergic neurons in substantia nigra leading to Parkinsonian syndrome only in primates, human[3] and monkey[4], suggesting that MPTP-like neurotoxicants, for example tetrahydroisoquinoline[5], are the cause of Parkinson's disease. However, no reliable *in vitro* system has been reported for the assay of specific cytotoxicity on dopaminergic neurons in primate substantia nigra. We have succeeded in culturing tyrosine hydroxylase(TH)-positive neurons from the substantia nigra of fetal monkey. The continuous presence of 1-methyl-4-phenylpyridinium ion (MPP^+) in such cultures decreased the survival of neurons to 2-25% of sister control cultures. Cultures of monkey neurons are convenient *in vitro* experimental systems not only for the detection of primate-specific neurotoxicity but also for studies of the pathogenesis of primate-specific neural diseases both at the molecular and the cellular levels.

MATERIALS AND METHODS

Primary culture of substantia nigra n eurons from fetal monkeys

Pregnant cynomolgus monkeys(Macaca fascicularis) were anesthetized and the fetuses (77-101 days of gestation age) taken out by Caesarean

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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section. An area including substantia nigra was dissected out and frozen in Ringer's solution containing dimethyl sulfoxide using liquid nitrogen[6]. The tissue was digested with 0.015 unit/ml papain in Ca^{2+}, Mg^{2+}-free, phosphate-buffered saline(CMF-PBS) for 15 min at 37 C. After separation of dissociated cells, the papain digestion was repeated. The cells were mechanically dissociated and dispersed in culture medium. The culture medium consisted of Dulbecco's modified Eagle medium containing 5% newborn calf serum, 5% horse serum and 1 mM sodium pyruvate. The cell suspension was plated on a poly-L-lysine coated coverslip as described[7] and cultured in a humidified atmosphere of 93% air plus 7% CO_2. MPP^+ was included in the medium during the culture period, which was 7 days.

Immunocytochemistry

Cells were rinsed 3 times with CMF-PBS and fixed for 30 min in freshly prepared 4% paraformaldehyde in CMF-PBS at room temperature. After several rinses with CMF-PBS, CMF-PBS containing 0.3% Triton X-100, 0.5% of fetal bovine serum (FBS) and 0.1% of sodium azide was applied to the cells for 2 days at 4 C. After a brief wash with CMF-PBS, non-specific binding sites were blocked by FBS(5%) in CMF-PBS for 10 min at room temperature. The cells were incubated for 5 days at 4 C with antiserum against tyrosine hydroxylase (TH)[8] (1:4000). After rinsing , the cells were stained by the avidin-biotin-peroxidase technique using the Vectastain ABC kit. Peroxidase activity was visualized by incubation with a 0.1 M phosphate buffer containing 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) and 0.01% hydrogen peroxide.

RESULTS AND DISCUSSION

Culturing of neurons from fetal monkey

We succeeded in culturing monkey substantia nigra neurons from 77-101 days fetus. A considerable number of living neurons on the glial layer

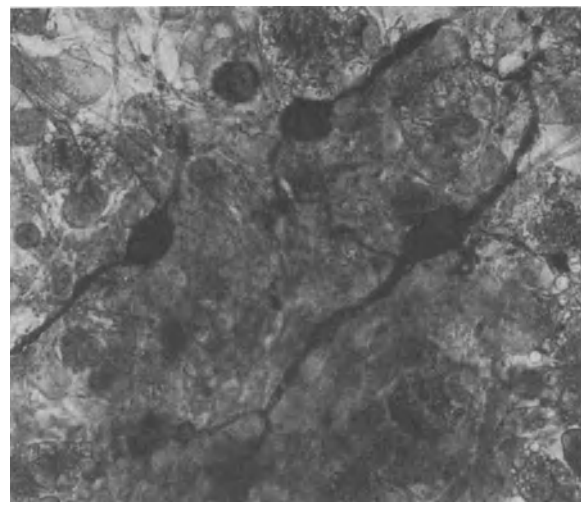

Fig. 1 Immunocytochemistry of cultured neurons from an area including substantia nigra of fetal monkey. Cells were stained with anti-rat tyrosine hydrooxylase antibody by ABC method. See details in Material and Method section.

were observed under phase-contrast microscopy after 7 days in the culture. Most, if not all, of the neurons could be stained by anti-bovine brain microtubule associated protein (MAP)II antibody (data not shown). Some of the neurons were also immunostained by anti-rat TH antibody and their morphology appeared very similar to that of mature human substantia nigra neurons (Fig 1).

Effects of MPP^+ on the survival of cultured neurons

Application of MPP^+(10 μM) during the entire period of the culture decreased the number of surviving neurons which were stained by MAP II antibody (Fig.2). The number of surviving neurons varied from 2% (77 days fetus) to 25% of the control sister cultures (101 days fetus). It appeared that the cultured neurons of monkey substantia nigra were much more sensitive to MPP^+ than those from rat dopamine-containing cells (PC-12) which did not significantly degenerate even in the presence of 100 μM MPP^+[9]. More detailed study of the specificity of the action of MPP^+ on monkey substantia nigra neurons will be published elsewhere.

These preliminary data suggest that cultured neurons from monkey substantia nigra can be used not only as a practical assay system for neurotoxicants causing Parkinson's disease, but also as a good experimental preparation for the molecular and cellular study of its pathogenesis. We believe that similar cultures of human substantia nigra neurons are technically possible using the method outlined in this paper, although ethical problems would arise concerning the use of human fetuses.

ACKNOWLEDGEMENT

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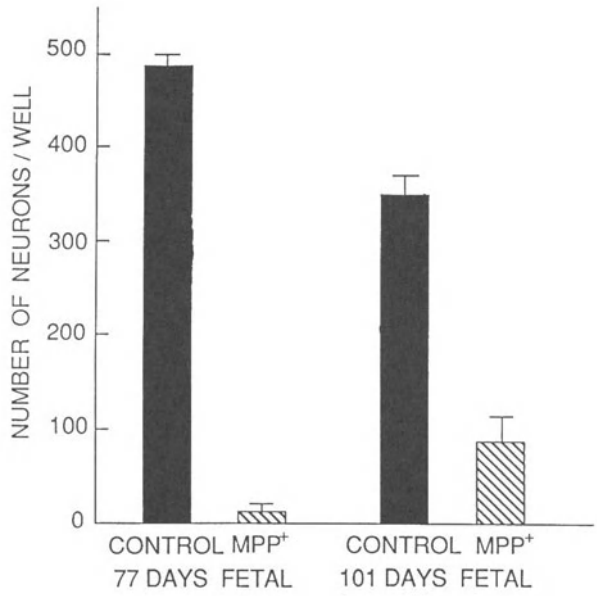

Fig. 2 Effects of MPP^+ on the survival of cultured neurons.

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THE EFFECT OF TETRAHYDROISOQUINOLINE ON THE MITOCHONDRIAL RESPIRATION

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INTRODUCTION

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism has been considered as the best model available at present of Parkinson's disease (1-4). The neurotoxic effect of MPTP on the nigrostriatal dopaminergic neurons resides in the inhibition of the mitochondrial respiration by 1-methyl-4-phenylpyridinium ion (MPP^+), an oxidation product of MPTP, resulting in the energy crisis of those neurons (5-11). Since the discovery of MPTP and MPP^+, it has been proposed that (an) exogenous or (an) endogenous MPTP-like substance(s) may be the cause of Parkinson's disease. 1,2,3,4-Tetrahydroisoquinoline (TIQ) has emerged as one of such candidates (12). TIQ was shown to inhibit the state 3 respiration of the mitochondria (13). This effect of TIQ on the mitochondrial respiration is similar to that of MPP^+. In this communication, we report effects of TIQ on the enzyme-protein complexes in the electron transport system and on the respiratory enzymes in the tricarboxylic acid (TCA) cycle.

MATERIALS AND METHODS

Mitochondria were prepared from whole brains of 2- to 3-month-old male C57/BL mice, and the activitiès of Complex I, II, III and IV, and alpha-ketoglutarate dehydrogenase complex (KGDHC), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (ICDH) activities were assayed spectrophotometrically with or without TIQ. Mitochondrial suspensions were frozen and thawed once before biochemical assays. TIQ was added at a final concentration of 5 mM. The details of the methods were reported previously (7,8,10,11). The results were subjected to the Student's t-test.

RESULTS

Figure 1 summarizes the effect of TIQ on Complex I, II, III and IV. Five mM of TIQ markedly inhibited the activity of Complex I to 30 % of the control ($P<0.001$). On the other hand, TIQ was of no effects on Complex II, III and IV.

The effect of TIQ on the enzymes studied in the TCA cycle are summarized in Figure 2. The activity of KGDHC was significantly inhibited to 62%

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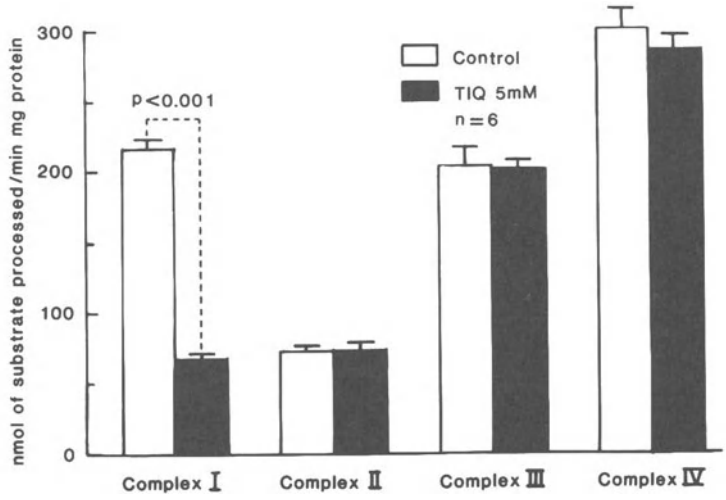

Figure 1. The effect of 1,2,3,4-tetrahydroisoquinoline (TIQ) on the electron transport system, Mean ± SEM (n=6), the concentration of TIQ = 5 mM.

of the control (P<0.001). The activities of NAD^+- and $NADP^+$-linked ICDHs were slightly reduced to 88 % and 84 % of the control, respectively. Inhibitions by TIQ of Complex I and KGDHC were dose-dependent. The IC_{50} of TIQ against Complex I was 2.25 mM, and that against KGDHC 8 mM (data not shown).

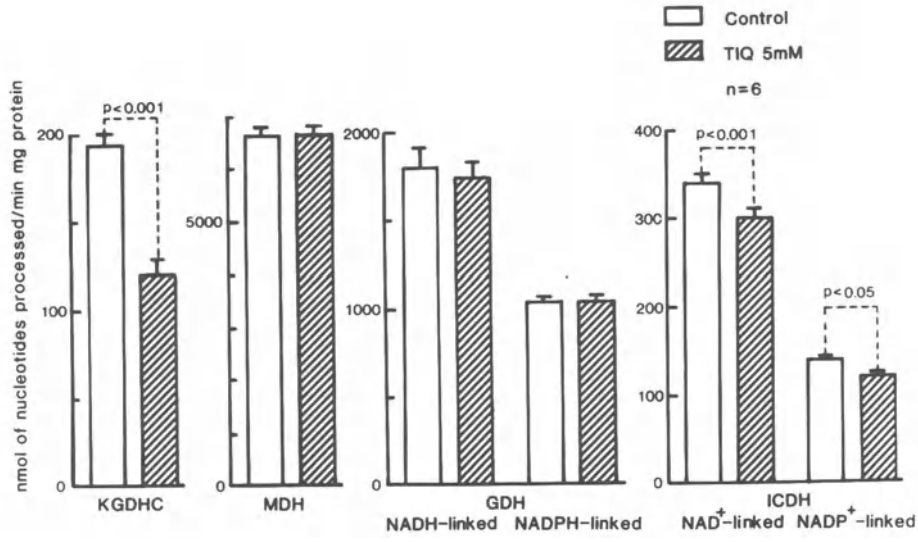

Figure 2. The effect of 1,2,3,4-tetrahydroisoquinoline (TIQ) on the tricarboxylic acid cycle dehydrogenases, Mean ± SEM (n=6), the concentration of TIQ=5 mM, KGDHC=alpha-ketoglutarate dehydrogenase complex; MDH=malate dehydrogenase; GDH=glutamate dehydrogenase; ICDH=isocitrate dehydrogenase.

DISCUSSION

Tetrahydroisoquinoline has a structure closely related to MPTP, and has been proposed as one of the candidates for endogenous neurotoxins which may cause Parkinson's disease (12). The presence of TIQ was shown in rat (14) and human brains (15,16). In addition, a long-term administration of TIQ was shown to produce parkinsonism and loss of dopamine content and tyrosine hydroxylase activity in the nigrostriatal regions in primates (17).

We reported inhibition by TIQ of the state 3 respiration, the Complex I activity and the ATP synthesis in the intact mitochondria prepared from mouse brains (13). In the present study, we found inhibition of the Complex I activity in the mitochondrial electron transport system, and of KGDHC in the TCA cycle by TIQ. As the inhibitory effects on NAD^+- and $NADP^+$-linked ICDHs were small, they appear to be of little significance.

These inhibitory characteristics of TIQ on the mitochondrial respiration are quite similar to those of MPP^+. An active uptake system for MPP^+ has been found in the hepatic mitochondria (18). However, it has not yet been shown whether an active uptake system for TIQ exists or not.

It appears to be important to search for substances which are actively taken up into mitochondria to inhibit the mitochondrial respiration for the elucidation of the pathogenesis of Parkinson's disease.

Acknowledgements

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DETECTION OF TETRAHYDROISOQUINOLINE, A PARKINSONISM-RELATED COMPOUND, IN PARKINSONIAN BRAINS AND FOODS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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INTRODUCTION

After 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was discovered to cause parkinsonism in humans, monkeys, and mice,[1-2] Nagatsu et al. screened various compounds structurally similar to MPTP for neurotoxicity by assaying the inhibition of tyrosine hydroxylase activity in tissue slices of striatum in situ, and found that both pyridinium and phenyl rings were essential for the effects and that N-methyl-1,2,3,4-tetrahydroisoquinoline (N-Me-TIQ) and 1,2,3,4-tetrahydroisoquinoline (TIQ) could be candidates of endogenous or environmental factors that cause Parkinson's disease.[3]

TIQ was recently discovered in rat brain,[4] as well as in parkinsonian and normal human brains.[5] Repeated administration of TIQ to marmosets caused accumulation of TIQ in their brains[6] and induced a parkinsonian state with a reduction in tyrosine hydroxylase, dopamine, and total biopterin concentrations in the substantia nigra.[7] TIQ has also been detected in cheese, wine, and cocoa,[8] thus suggesting a possible origin of TIQ in human brains.

In this present study we demonstrated that TIQ is commonly present in various foods, and also quantified TIQ levels in parkinsonian and normal human brains.

METHODS

Chemicals

TIQ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); heptafluorobutyric anhydride (HFBA), from Gaskuro Kogyo Inc. (Tokyo, Japan); and 1,3,4,5,6,7,8-heptadeutero-isoquinoline (d_7-isoquinoline), from MSD Isotopes (Montreal, Canada).

A mixture of 1,3,5,6,7,8-hexadeutero-1,2,3,4-tetrahydroisoquinoline (d_6-TIQ) and 1,3,4,5,6,7,8-heptadeutero-1,2,3,4-tetrahydroisoquinoline (d_7-TIQ) was synthesized from d_7-isoquinoline.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Sample preparation

To quantify TIQ levels in the brains of 4 patients with Parkinson's disease and 4 patients without neurological disease, we homogenized frontal lobe tissue (1g) for 30 sec at 0 °C with 5 ml of 0.4 M perchloric acid containing 0.1% EDTA and 0.1% ascorbic acid. The homogenate was spiked with 50 ng of a mixture of d_6-TIQ and d_7-TIQ as an internal standard, and then centrifuged at 10000g for 20 min at 4 °C. The supernatant was transferred to a glass tube, and mixed with 10 ml of diethyl ether. The aqueous phase was adjusted to pH 12, and extracted with 10 ml of dichloromethane. The organic phase was extracted with 10 ml of 0.1 M hydrochloric acid containing 0.1% EDTA and 0.1% ascorbic acid. The aqueous phase was adjusted to pH 12 and extracted with 10 ml of dichloromethane. The organic phase was dried over anhydrous sodium sulfate, and the filtrate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 40 µl of ethyl acetate-HFBA (1:1,v/v) and derivatized at 70 °C for 30 min.

To quantify the TIQ levels in cheese (5 g), banana (20 g), broiled sardine (20 g), broiled beef (20 g), flour (20 g), the yolk of boiled egg (20 g), and the white of boiled egg (20 g), the food was homogenized for 30 sec at 0 C in 5 volumes of 0.4 M perchloric acid containing 0.1% EDTA and 0.1% ascorbic acid. The homogenate was spiked with 50 ng of a mixture of d_6-TIQ and d_7-TIQ as an internal standard, and processed as stated above.

To quantify the TIQ levels in wine (20 ml), beer (20 ml), whiskey (20ml), and milk (20ml), the beverage was spiked with 50 ng of a mixture of d_6-TIQ and d_7-TIQ as an internal standard, and then 5 volumes of 0.4 M perchloric acid containing 0.1% EDTA and 0.1% ascorbic acid was added. The solution was then processed in a similar manner as stated above.

Gas chromatography - mass spectrometry (GC/MS)

A Shimadzu GC-9A gas chromatograph combined with a double focusing mass spectrometer (Shimadzu 9020-DF) was used. The gas chromatograph was equipped with an OV-1-bonded, fused silica capillary column (25m x 0.25 mm I.D.) and a moving needle-type solventless injector. The injection temperature was 280°C, and the column temperature was programmed from 130°C to 160°C at 3°C/min. Electron-impact ionization (EI) mass spectra were recorded at an ionizing energy of 70 eV, ion source temperature of 250°C, trap current of 60 µA, and accelerating voltage of 3 kV.

Quantification of TIQ by GC/MS

The TIQ levels in the brains and various foods were quantified by use of selected ion monitoring (SIM). The mass numbers used for SIM were m/z 328: $(M-H)^+$ion of HFB-derivatized TIQ, m/z 329: M^+ion of HFB-derivatized TIQ, m/z 335: M^+ ion of HFB-derivatized d_6-TIQ, and m/z 336: M^+ ion of HFB-derivatized d_7-TIQ. A calibration line relating the concentration of TIQ to the peak-height ratio of TIQ at m/z 329 to the internal standard (d_6-TIQ) at m/z 335 was obtained from the SIM chromatograms.

RESULTS

Fig. 1 shows the EI mass spectra of the HFB-derivatized TIQ (a), and the HFB-derivatized mixture of d_6-TIQ and d_7-TIQ (b) used as the internal standard. Fig. 2 shows the SIM chromatograms of (a) HFB-derivatized TIQ, (b) control brain, and (c) parkinsonian brain. The concentration of TIQ in the parkinsonian brains, 13.2 (SD 1.3) ng/g, was not significantly increased as compared to normal human brains, 11.0 (SD 5.7) ng/g.

Table 1 Concentrations of TIQ in foods.

Concentration of TIQ (ng/g or ng/ml)			
Cheese	5.2	Banana	2.2
Broiled sardine	0.96	Broiled beef	1.3
Flour	0.52	Boiled egg yolk	1.8
Boiled egg white	2.2	Wine	0.56
Beer	0.36	Whiskey	0.73
Milk	3.3		

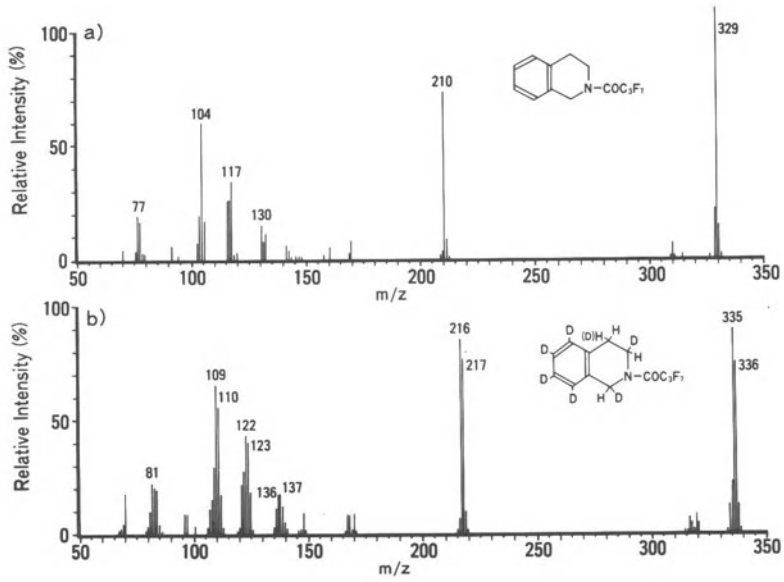

Fig. 1 EI mass spectra of HFB-derivatized TIQ (a) and HFB-derivatized mixture of d_6-TIQ and d_7-TIQ (b).

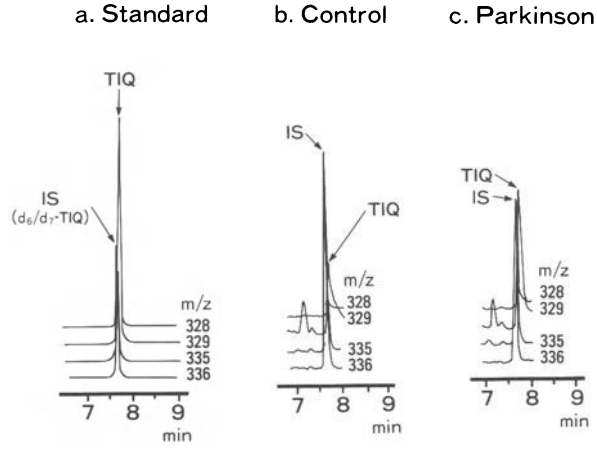

Fig. 2 SIM chromatograms of HFB-derivatized TIQ (a) and HFB-derivatized extracts from control brain (b) and parkinsonian brain (c).

The concentrations of TIQ in various foods are shown in Table 1 as the means of two to three samples. TIQ was present at high concentrations especially in cheese, milk, boiled egg white and yolk, and banana.

DISCUSSION

We quantified TIQ in the parkinsonian and normal human brains, and could not find any significant increase in the concentration of TIQ in the parkinsonian brain as compared with that in the normal brain. However, the pathological change of Parkinson's disease is localized to the nigrostriatum. In an attempt to clarify the pathological role of TIQ in Parkinson's disease, TIQ levels in the nigrostriatum of patients with Parkinson's disease should be compared with those in the normal nigrostriatum.

We detected TIQ in various foods such as cheese, milk, banana, boiled egg, broiled beef, broiled sardine, whiskey, wine, flour, and beer. The origin of TIQ in the brains could be from foods, since TIQ can easily pass through the blood-brain barrier and may accumulate in the brain over a long period. The effects of its accumulation in the brain in relation to Parkinson's disease in human beings remain for further study. It should be noted that the concentrations of TIQ in foods are very low as compared with the very high concentration of TIQ required to produce parkinsonian symptoms in the monkey brain. Therefore, TIQ in foods may not be related to the occurrence of Parkinson's disease, unless it remains stable during long term accumulation.

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BIOSYNTHESIS OF N-METHYLISOQUINOLINIUM ION, A POTENT INHIBITOR OF CATECHOLAMINE METABOLISM, FROM 1,2,3,4-TETRAHYDROISOQUINOLINE THROUGH N-METHYL-1,2,3,4-TETRAHYDROISOQUINOLINE IN HUMAN BRAIN

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INTRODUCTION

The discovery of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a neurotoxin that elicits symptoms very similar to those of parkinsonism in humans[1] indicates that similar compounds may accumulate in human brain and cause neurodegeneration of the central nervous system. In human brain, 1,2,3,4-tetrahydroisoquinoline (TIQ) was found and its amount was increased in a parkinsonian brain.[2] TIQ produced parkinsonism in primates by systemic administration, with a reduction in dopamine and biopterin contents and in the activity of tyrosine hydroxylase [TH; tyrosine, tetrahydropteridine : oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] in the nigro-striatal region.[3] Of the derivatives of TIQ, the N-methylisoquinolinium ion ($NMIQ^+$) has a chemical structure similar to that of an oxidative product of MPTP, N-methyl-4-phenylpyridinium ion (MPP^+). $NMIQ^+$ is a potent inhibitor of TH in rat striatal slices,[4] and of monoamine oxidase [MAO; monoamine : oxygen oxidoreductase (deaminating), EC 1.1.3.4].[5] More recently, in a rat clonal pheochromocytoma cell line, PC12h, as a model of dopaminergic neurons, $NMIQ^+$ inhibited *in vitro* and *in vivo* activity of TH, MAO, and aromatic L-amino acid decarboxylase [aromatic L-amino acid carboxy-lyase, EC 4.1.1.28].[6] These results suggest that $NMIQ^+$ may be an endogenous inhibitor similar to MPP^+. To confirm the biosynthesis of $NMIQ^+$ from TIQ in human brain, N-methylation of TIQ was studied, using an enzyme sample prepared from human brain. This paper reports the synthesis of NMTIQ by an N-methyltransferase in human brain and its oxidation into $NMIQ^+$ by MAO. The enzymatic studies on the two steps of reactions were discussed in relation to the effect of TIQ derivatives on catecholamine metabolism in human brain.

MATERIALS AND METHODS

Materials: Human brain frontal cortex was homogenized with 10 vol. (ml) of 10 mM potassium phosphate buffer, pH 7.4, /wet weight (g) of tissue, and used as a source of an N-methyltransferase. As sources of type A and B MAO, human brain synaptosomal mitochondria were used.[7] TIQ was purchased from Aldrich; S-adenosyl-L-methionine (SAM) and pargyline, from

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

Sigma. $NMIQ^+$ was kindly donated by Dr. Y. Hirata; deprenyl by Dr. J. Knoll. Clorgyline was obtained from May and Baker.

Assay for N-methyltransferase activity and HPLC analysis of NMTIQ: Brain homogenate was incubated with 100 μM TIQ, 50 μM SAM, and 100 μM pargyline in 100 mM Tris-HCl buffer, pH 8.25, at 37°C for 60 min. After alkaline treatment, NMTIQ was extracted with dichloromethane. NMTIQ was quantitatively analyzed using HPLC with electrochemical detection.[8]

Assay for oxidation of NMTIQ into $NMIQ^+$: Oxidation of NMTIQ was assayed by measurement of the amount of $NMIQ^+$ produced from NMTIQ. NMTIQ was incubated with type A and B MAO samples in 50 mM potassium phosphate buffer, pH 7.4, at 37°C for 20 min, after preincubation with 1 μM deprenyl or clorgyline at 37°C for 15 min to differentiate type A and B activities. The reaction was terminated by addition of perchloric acid, and the sample was applied to an HPLC apparatus, equipped with a fluorescence detector. The fluorescence intensity at 380 nm was measured with excitation at 335 nm.[9]

RESULTS AND DISCUSSION

N-Methylation of TIQ

After a 60-min incubation of the enzyme sample with TIQ, a definite amount of NMTIQ was detected by HPLC. The formation of NMTIQ was linearly dependent on the protein amount and the reaction time up to 90 min. The methyltransferase activity had its optimum at pH 8.25. The activity required S-adenosyl-L-methionine (SAM) as a methyl donor, as shown in Fig. 1. The value of Michaelis constant, K_m, and the maximal velocity, V_{max}, in terms of SAM were 5.11 ± 1.69 μM, and 7.31 ± 0.21 pmol/min/mg protein, respectively. The values of K_m and V_{max} in terms of TIQ were 20.9 ± 5.5 μM and 7.98 ± 1.21 pmol/min/mg protein, respectively. In subcellular fractions of frontal cortex, a major part of the N-methyltransferase activity was found in the soluble, cytosolic fraction, 2890 pmol/min (88% of the total activity), followed by the microsomal fraction (187 pmol/min, 5.7% of the total).

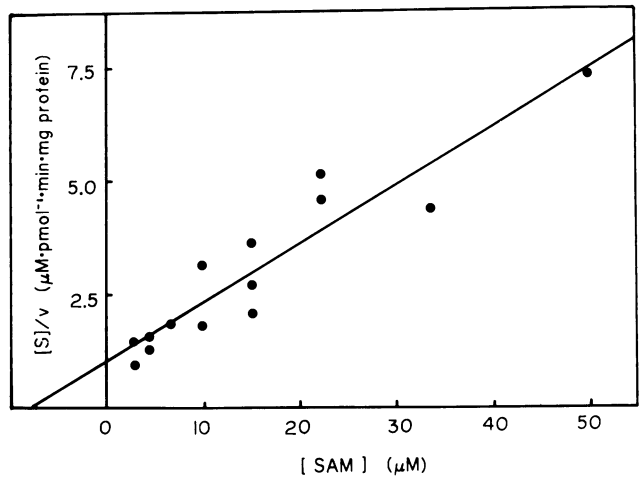

Fig. 1. Effect of SAM concentration on the reaction velocity of an N-methyltransferase from human brain. The data were plotted according to Woolf.

Oxidation of NMTIQ into $NMIQ^+$

After incubation of NMTIQ with MAO in human brain synaptosomal mitochondria at 37°C for 20 min, the sample was analyzed by HPLC. Production of $NMIQ^+$, which ion was found to have strong fluorescence with excitation and emission maxima at 335 nm and 380 nm, respectively, was dependent on the reaction time and the amount of MAO. The oxidation was linear with the reaction time up to 20 min. The pH-activity profile of NMTIQ oxidation by type A MAO in human brain synaptosomal mitochondria showed its optimum about pH 7.25. The K_m and V_{max} value of type A MAO were higher than the values of type B in human brain synaptosomes: 571 ± 25 μM and 0.286 ± 0.057 pmol/min/mg protein, and 463 ± 43 μM and 0.159 ± 0.033 pmol/min/mg protein, respectively.

These data show that the two steps of the biosynthesis of $NMIQ^+$ from TIQ via NMTIQ may occur in the brain, as shown in Fig. 2. N-Methyltransferase activity is detected in mammalian liver, kidney, and lung, however, the activity in human brain has not been well clarified, and at present we don't know whether N-methyltransferase in human brain is the same as the enzymes detected in other tissues. Ansher *et al.* reported formation of MPTP and MPP^+ by N-methylation of 4-phenyl-1,2,3,6-tetrahydropyridine and 4-phenylpyridine by an N-methyltransferase,[10] that may be comparable to that forming NMTIQ from TIQ, as reported here. TIQ found in human brain is supposed to arise from the nonenzymatic Pictet-Spengler reaction, which involves the condensation of β-arylethylamine with a carboxyl compound. Furthermore, the presence of TIQ was confirmed in foods.[11] It is not yet certain whether TIQ detected in human brain is formed by *in situ* condensation of amines with aldehydes or is derived from foods, because TIQ was found to be transported through the blood-brain barrier into brain.[12]

1,2,3,4-Tetrahydroquinoline and tetrahydroisoquinoline derivatives were previously reported not to bind to MAO.[13] The data presented here show that the N-methyl derivative of TIQ was oxidized by MAO, which indicates that the presence of a methyl residue at the nitrogen atom seems to be essentially required for the binding to MAO. Oxidation of N-methyl derivative of TIQ may be comparable to the formation of MPP^+ from MPTP by MAO. NMTIQ is a substrate for both type A and type B MAO, both of which are now known to be important in the oxidation of MPTP-like molecules.[14]

This work was supported by a Grant-In-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan.

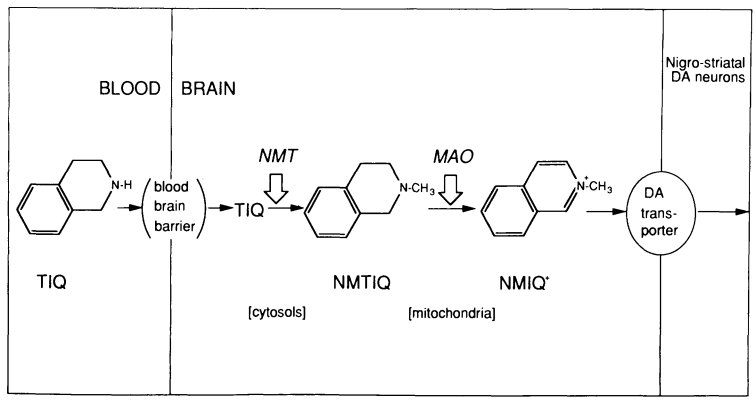

Fig. 2. Formation of $NMIQ^+$ ion in the brain.

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IMPORTANCE OF 1-METHYLTETRAHYDROISOQUINOLINE (1MeTIQ) IN PARKINSON'S DISEASE

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INTRODUCTION

The existence of endogenous 1-methyl-1,2,3,4-tetrahydro-isoquinoline (1MeTIQ) accompanied with 1,2,3,4-tetrahydro-isoquinoline (TIQ) in the rat brain has been confirmed in our previous research.[1] The possibility of TIQ and 1MeTIQ intake from some foods was also pointed out.[2] Recently, the neurotoxic properties of TIQ have been discussed, especially with regard to Parkinson's disease.[3-7] In spite of structural similarity to TIQ, there has been no study for the role of 1MeTIQ, the structure of which is shown in Fig. 1, in Parkinson's disease, except for our work that confirmed a decrease in 1MeTIQ content in parkinsonian brain.[8]

In this paper, we report a significant change in the 1MeTIQ content in the brains of idiopathic parkinsonian and parkinsonian model animals, and the protecting effect of 1MeTIQ on the parkinsonian model animal.

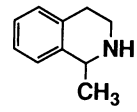

Fig. 1 Structure of 1-methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ).

RESULTS AND DISCUSSION

To investigate 1MeTIQ content in the brain of the parkinsonian model animal, we used male C57BL/6 mice (9-10 weeks old) treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) at 30 mg/kg i.p., for 4 days. As shown in Fig.2, the 1MeTIQ content was significantly decreased in the brains of MPTP-treated mice, a well-established parkinsonian model.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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As was already shown earlier,[8] the 1MeTIQ content was also markedly reduced in the parkinsonian brain, particularly in the frontal lobe (Fig. 3). This result corresponds well to that found in the model experiment, in other words, the reduction of 1MeTIQ content in the brain occurred in both idiopathic Parkinson's disease and the parkinsonism model.

Because the aging process is frequently involved in parkinsonism, we investigated the relationship between aging and 1MeTIQ content in normal human brain and in parkinsonian ones (Fig.4). The aging process contributed to the decrease in 1MeTIQ content in both parkinsonian and control brains.

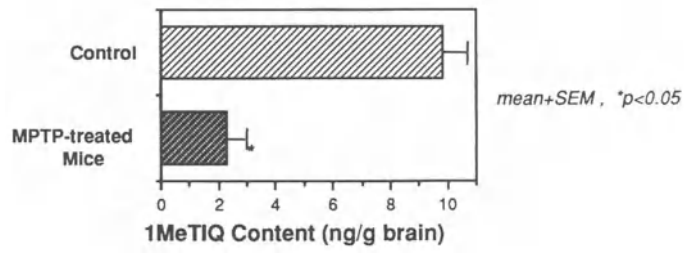

Fig. 2 1MeTIQ Content in MPTP-treated Mice

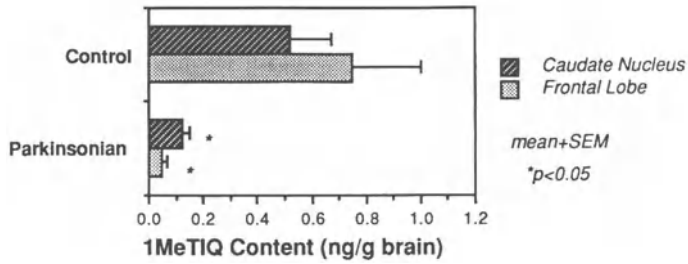

Fig. 3 1MeTIQ Content in Human Brain

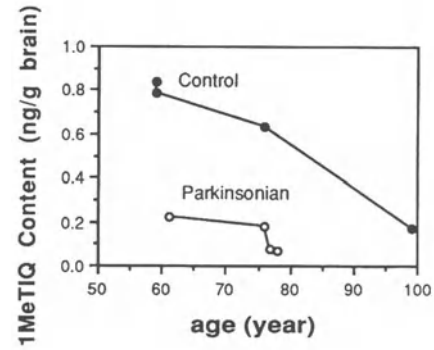

Fig. 4 Relationship between aging and 1MeTIQ content in human brain

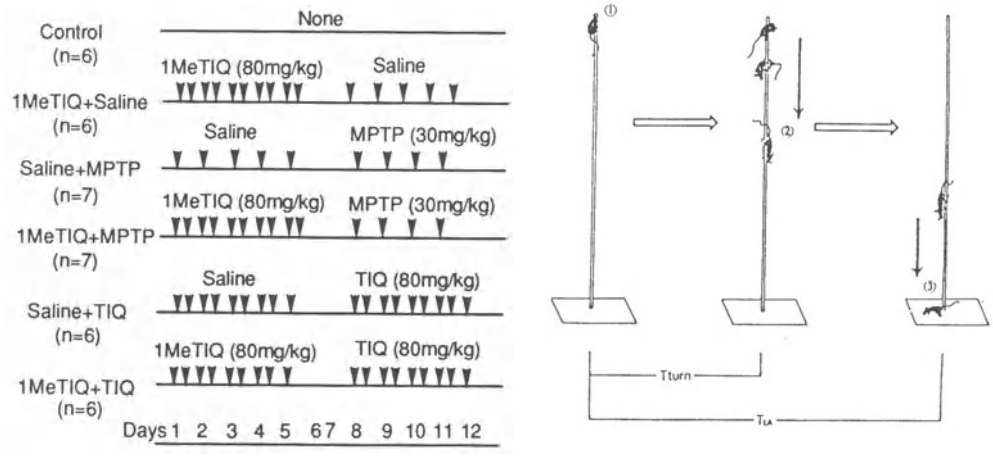

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Fig. 5 Procedure for the pole test
C57BL male mice (9-10 weeks old, i.p.)

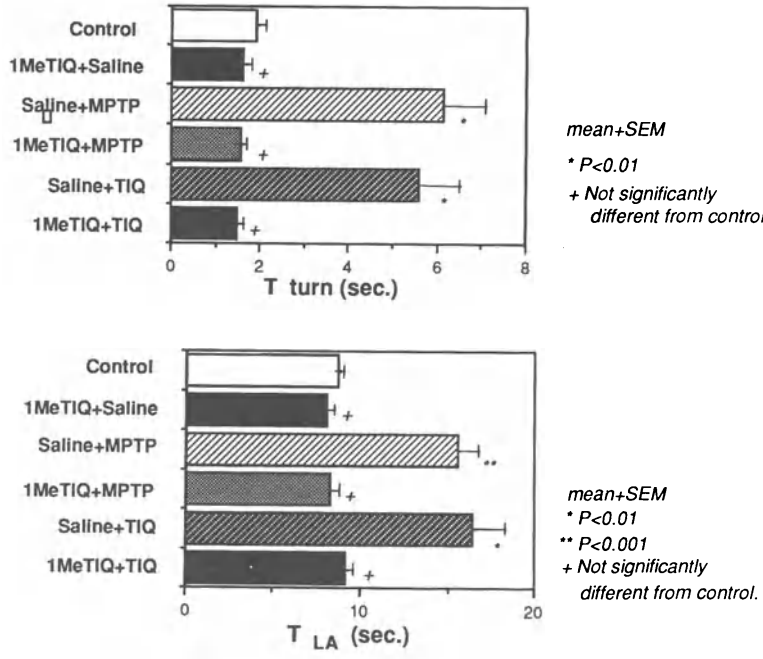

Fig. 6 T turn and T LA of the pole test

From above results, 1MeTIQ in the brain is a possible candidate of the small-molecular marker for parkinsonism and aging, and it can be presumed that 1MeTIQ plays a role in protecting the brain from parkinsonism and aging processes. Therefore, the pharmacological effect of 1MeTIQ on parkinsonism was also studied. Dosage schedules of 1MeTIQ, TIQ, and MPTP for C57BL/6 mice (9-10 weeks old) are shown in Fig.5. The pharmacological activity was evaluated by the "pole test" method developed by Ogawa et al..[9] This method is very useful for the evaluation of bradykinesia. As shown in Fig. 6, the parkinsonism, especially bradykinesia, induced by MPTP and TIQ was completely recovered with pretreatment with 1MeTIQ. Thus, 1MeTIQ, an endogenous substance related to Parkinson's disease, appears to be a promising compound for use as an anti-parkinsonism agent. Further investigation of its pharmacological effects are now under way.

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FORMATION OF A NOVEL AND NEUROTOXIC TETRAHYDROISOQUINOLINE DERIVATIVE, 1,3-DIMETHYLTETRAHYDROISOQUINOLINE (1,3DiMeTIQ), A CONDENSATION PRODUCT OF AMPHETAMINES AND ACETALDEHYDE IN VIVO

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INTRODUCTION

d-Amphetamine and alcohol are generally thought to be mutually antagonistic with respect to their pharmacological action. However the toxic effect of d-amphetamines is not appreciably reduced by the concomitant administration of alcohol.[1] It is reported that the combined abuse of methamphetamine and alcohol causes a significant increase in psychosomatic disorders than the abuse of methamphetamine only.[2] We hypothesized that a novel metabolite of amphetamines may be involved in such disorders and proposed that 1,2,3,4-tetrahydroisoquinoline (TIQ) and 1-methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ) are formed by ring cyclization of endogenous phenylethylamine.[3-5] In the present study, we examined the possibility that 1,3DiMeTIQ could be formed from exogenous amphetamines, an analog of phenylethylamine, by cyclization with acetaldehyde, a metabolite of ethanol (Fig. 1).

We investigated the in vivo formation of 1,3DiMeTIQ in rats under chronic ethanol treatment with repeated administration of amphetamine or methamphetamine. We also observed the behavioral abnormality induced by 1,3DiMeTIQ injection and measured the amounts of biogenic amines in the brain.

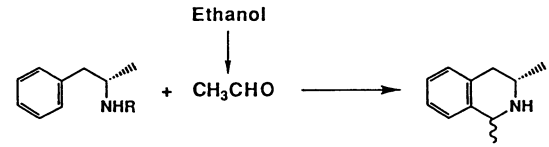

Fig. 1 Proposed mechanism of 1,3DiMeTIQ formation.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Fig. 2 shows reconstructed GC-MS total ion chromatogram and electron impact mass spectra of HFB derivatives of authentic 1,3DiMeTIQ. The identification of the diastereomeric isomers in rat brain was based on the presence of peaks with a retention time and at a mass number (m/z) corresponding to the diastereoisomers of HFB derivative of authentic 1,3DiMeTIQ using high-resolution GC-SIM (R= 5000). Fig. 3 shows the high-resolution GC-SIM chromatogram of HFB derivatives of 1,3DiMeTIQ in the rat brain under chronic ethanol treatment with repeated administration of d-amphetamine (over 3 weeks).

The concentrations of 1,3DiMeTIQ in rat brain and plasma were determined by GC-SIM at a low resolution (R=500)

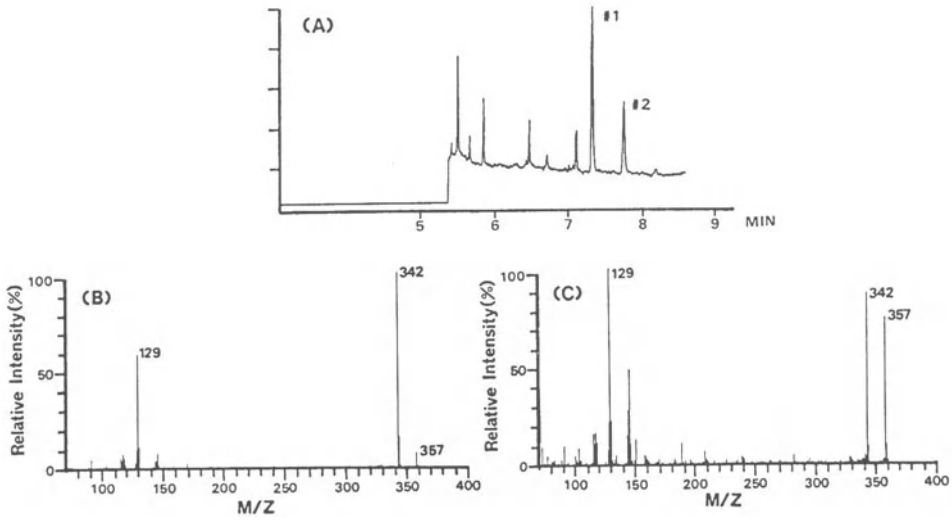

Fig. 2. Reconstructed GC-MS total ion chromatogram (A) and electron impact mass spectra of diastereoisomers of HFB derivatives of 1,3DiMeTIQ : (B) peak 1, (C) peak 2.

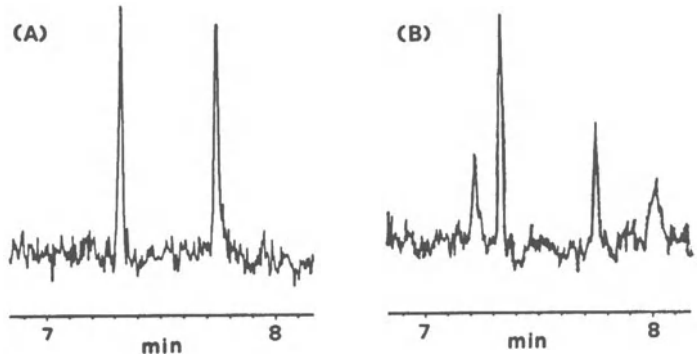

Fig. 3. GC-SIM chromatogram at m/z 357.096 of HFB-derivatized (A) authentic 1,3DiMeTIQ, (B) extract from the brain of a chronic alcoholism rat with repeated amphetamine administration.[6]

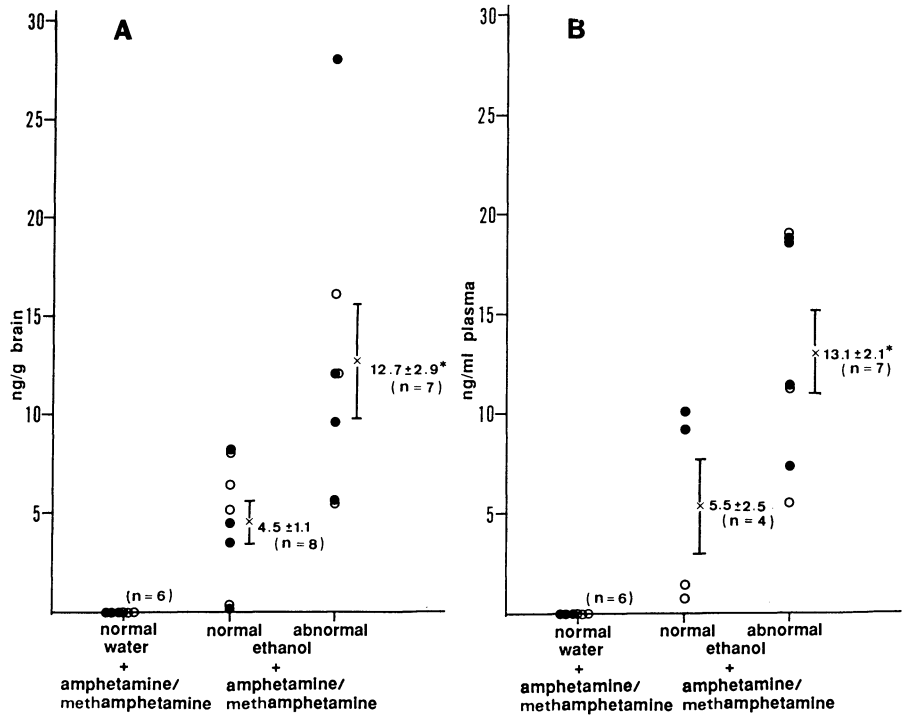

Fig. 4 Relationship of behavior abnormality and the concentration of 1,3DiMeTIQ in brain (A) and plasma (B).[6]
Each point was obtained by measurement of the content of 1,3DiMeTIQ in different animals. Numbers of animals are shown in parentheses. The other values are the mean± S.D. ●, amphetamine-treated, ○, methamphetamine-treated. *p< 0.05 compared with normal rats treated with ethanol-amphetamines.

RELATIONSHIP BETWEEN BEHAVIOR ABNORMALITY AND THE CONCENTRATION OF 1,3DIMETIQ IN BRAIN AND PLASMA

We confirmed that 1,3DiMeTIQ content was related to behavioral abnormalities such as tremor, curvature of the back, stereotypy, drooling, and hypersensitivity(Fig.4).

NEUROTOXIC EFFECT OF 1,3DIMETIQ

Behavioral alterations were observed following a single administration (i.p.) of 45, 90, or 135 mg/kg 1,3DiMeTIQ·HCl. The rats at each dose level manifested similar behavioral alterations such as tremors, curvature of the back, staggering gait, and akinesia. At high doses, intense tremors, prostration, Straub tail, rotation of the tail, and acute death were observed. The level of 1,3DiMeTIQ was measured in the brains of rats that showed behavior abnormality after injection of 50 mg/kg 1,3DiMeTIQ·HCl, which was not lethal but was a high dose. The concentration of 1,3DiMeTIQ in such animals was 84.2± 30.9 μg/g wet brain, n = 5 (mean± S.D.). 1,3DiMeTIQ was not detected in the brain of saline-treated rats (n = 5).

The concentrations of biogenic amines were measured with Neurochem analyzer(ESA Inc., Bedford, MA). As shown in Fig.5, 1,3DiMeTIQ inhibited every monoamine oxidase in this metabolic pathway and markedly decreased tryptophan hydroxylase activity.

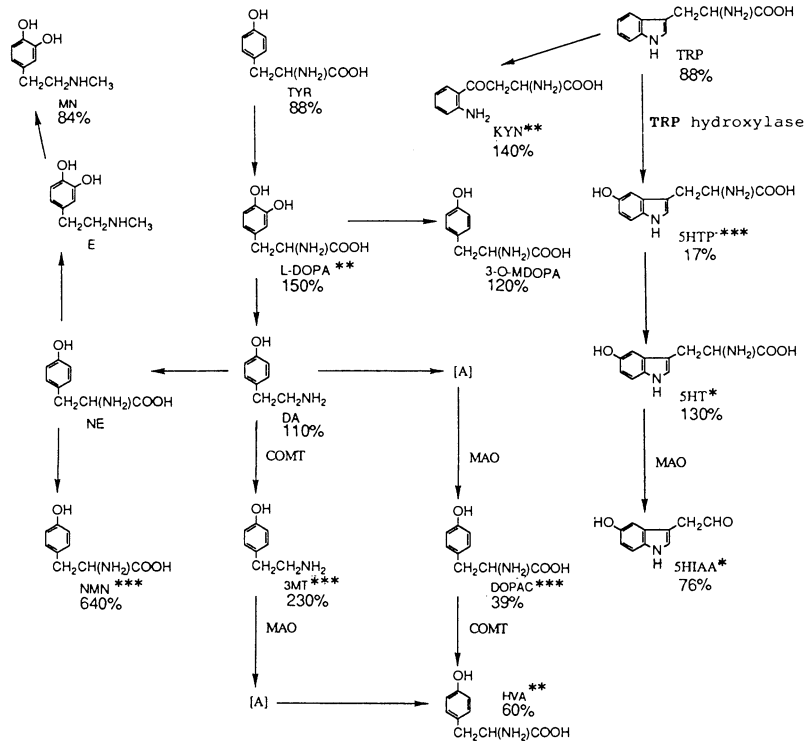

Fig. 5. Effect of toxic doses of 1,3DiMeTIQ on the dopaminergic and serotonergic metabolism in rat brain.
Each value represents percent of control. n = 4, control ; n = 5, 1,3DiMeTIQ *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test.

Since 1,3DiMeTIQ is neurotoxic tetrahydroisoquinoline derivative, we will pay attention to the role of 1,3DiMeTIQ, a novel metabolite of amphetamines, in psychosomatic disorders by the combined abuse of amphetamines and ethanol.

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TRYPTOPHAN METABOLITES IN PARKINSON'S BRAIN

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INTRODUCTION

Quinolinic acid (QA) is an intermediate in the kynurenic pathway from tryptophan (TRP) to nicotinic acid. Recently, QA has been demonstrated to be increased in the aging rat brain[1] and to act on neurons as an endogenous excitotoxin.[2] Kynurenic acid (KYA), another product of this pathway, has been shown to antagonize excitotoxic amino acids and QA.[3] Based on these findings, it has been proposed that changes in the balance between QA and KYA are related to the pathogenesis of several neurodegenerative disorders.[4] Indeed, Schwarcz et al.[2] reported that QA produced striatal lesions similar to those of Huntington's disease, and Beal et al.[5] also found the formation of KYA was reduced in Huntington's striatum, whereas Moroni et al.[6] failed to find any increase in QA in Alzheimer's cortex.

Parkinson's disease (PD) is a neurodegenerative disease characterized clinically by tremor, akinesia, and rigidity, and pathologically by marked neuronal loss in the substantia nigra. Since the pathogenesis of PD still remains unknown, attention should be paid to endogenous substances such as QA. Therefore, in the present study, we investigated tryptophan metabolites including those of kynurenic pathway to elucidate the etiology of PD.

MATERIALS AND METHODS

Pars compacta of the substantia nigra (SN) and putamen (PT) of control and PD brains were obtained from the Brain Tissue Resource Center. The mean age and post-mortem interval are shown in Table 1. There was no statistical significance between controls and PD. Five of 7 cases received L-dopa therapy.

Samples thus obtained from SN and PT were weighed and sonicated on ice for 3 x 10 seconds. After centrifugation (12,000 rpm. 30 min) at 4°C, aliquots of supernatants were injected into a HPLC system. The principle of this new HPLC system has been described in a previous

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table 1 Clinical Findings of Control and PD Cases

	Age at Death (yrs)	Number of Cases Sex Distribution	Post-mortem Interval (hrs)
SN			
Control	69.00±7.77	7 (M6, F1)	16.80±3.21
PD	72.00±2.14	5 (M5, F0)	10.90±4.26
PT			
Control	70.50±3.91	12 (M6, F6)	8.74±2.34
PD	71.50±1.93	6 (M6, F0)	8.20±3.03

paper.[7] In brief, it is a HPLC system with 16 electrochemical detectors and a gradient system to increase selectivity. We can easily identify many compounds at the same time by using three coulometric sensors for electrochemical resolution and peak identification. The mobile phase and time line of the methods for analysis are listed in Table 2. Method 2 was developed in order to measure accurately small amounts of KYA, 3-hydroxykynurenine (3-OHKY) and 3-hydroxyanthranilic acid (3-OHAN). Protein concentration was measured by the method of Lowry et al.[8]

Table 2. Methods for Analysis.

		Method 1	Method 2
COLUMN		15cm x 4.6 mm $3\mu C_{18}$	8cm x 4.6 mm $3\mu C_{18}$ x 2
FLOW		1 ml/min	1.2 ml/min 0.8 ml/min
MOBILE PHASE			
	A	0.1M KH_2PO_4 (pH3.1)	0.05M NA_2HPO_4 (pH6.26)
	B	0.1M KH_2PO_4 (pH3.24) in 50% MeOH/50%H_2O	0.05M NA_2HPO_4 (pH6.26) in 50% MeOH/50% H_2O
TIME LINE			
	MIN	0 16 16 39 49 54 63	0 16 35 60 70
	%B	0 0-6 24 64 100 100	5 5 15 100 100
ARRAY		1 ch: 0mv, 16ch: 900mv increment: 60mv	1 ch: 0mv, 16ch: 1050mv increment: 70mv

RESULTS

The concentrations of TRP metabolites are shown in Table 3. The concentrations of TRP and serotonin (5HT) in the SN were lower in PD cases than in controls. Although the concentrations of metabolites in the kynurenic pathway showed no significant changes in the SN of PD, those of kynurenine (KYN) and KYA were decreased to 60% of the control values; in contrast, those of 3-OHKY and 3-OHAN were increased to 300% and 350% respectively of controls. Therefore, the KYN/3-OHKY ratio in the SN of PD was significantly low, while the KYN/KYA ratio was almost equal to that in the SN of controls. In the PT of PD, the results obtained were similar to those in the SN. The concentrations of TRP and 5HT showed a significant decrease in the PT of PD. The concentrations of KYN and KYA were also decreased to 70% of controls. On the other hand, those of 3-OHKY and 3-OHAN were increased to 190% and 180%, respectively, of the controls. The KYN/3-OHKY ratio in the PT of PD lowered to 53% of that in the PT of controls, although there was no statistical significance to this difference.

Table 3 Tryptophan Metabolites[a] in Parkinson's Brains

	SUBSTANTIA NIGRA (COMPACTA)		PUTAMEN	
	CONTROL=7	PD=5	CONTROL=12	PD=6
TRP	826.13±68.31	451.21±30.27***	848.34±84.63	486.18±30.73**
5HT	86.67±12.56	57.61±10.68*	28.81± 3.28	13.66± 1.42**
KYN	54.89±10.92	34.73± 1.55	53.16± 9.09	38.13± 2.60
KYA	1.63± 0.41	0.96± 0.21	5.59± 1.98	3.86± 0.37
3-OHKY	1.38± 0.44	4.53± 2.02	2.16± 0.61	4.16± 1.49
3-OHAN	0.07± 0.01	0.26± 0.06	0.11± 0.05	0.20± 0.08
$\frac{TRP}{5HT}$	10.82± 1.68	9.46± 1.78	32.40± 3.43	37.16± 3.83
$\frac{TRP}{KYA}$	17.97± 2.54	13.02± 0.81	20.18± 3.03	13.15± 1.34
$\frac{KYN}{KYA}$	50.51±14.84	43.99± 7.86	12.72± 2.49	10.31± 1.06
$\frac{KYN}{3\text{-}OHKY}$	52.03±11.19	16.35± 6.29**	37.72± 8.52	19.92± 7.32
$\frac{3\text{-}OHKY}{3\text{-}OHAN}$	17.83± 3.29	15.37± 3.85	37.00± 8.56	28.05± 4.82

[a]Concentrations of neurochemicals are expressed as pmol/mg protein±SEM.
*:$p<0.05$, **:$p<0.01$, ***:$p<0.001$ compared with control.

DISCUSSION

The concentrations of neurochemicals in the SN and the PT of controls presented here were for the most part consistent with those given in previous papers.[5,9] The decrease in TRP and 5HT levels in PD brain shown in this study were also reported by Scatton[10] and Sparks.[11] Such a decrease is thought to reflect a loss of serotonergic neurons in PD.

Since Coyle et al.[12] produced striatal lesions with kainic acid similar to those of Huntington's disease, much attention has been paid to the relation between the pathogenesis of neurological disorders and neuroexcitotoxins, especially endogenous ones. In PD, although the etiology is not elucidated yet, Nagatsu and Yoshida[13] have proposed the possibility that PD might be caused by the endogenous substance tetrahydroisoquinoline. In this study, we found an abnormality in the kynurenic pathway in that the KYN/3-OHKY ratio was significantly decreased in the SN of PD cases, whereas the KYN/KYA ratio was not changed, compared with that in the SN of controls. This result suggests that the metabolic pathway from KYN toward QA may be more enhanced than that toward KYA in the basal ganglia of PD patients. As QA has been demonstrated to have the character of an excitotoxin, this imbalance in the kynurenic pathway over the long term may contribute to the death of neurons in the SN and result in the onset of PD. It would be interesting to investigate in the future the enzymatic activities of the kynurenic pathway in PD patients.

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A PARALLEL RELATIONSHIP BETWEEN PARKINSON'S DISEASE AND AN EXCESS OF S-ADENOSYLMETHIONINE-DEPENDENT BIOLOGICAL METHYLATION IN THE BRAIN

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INTRODUCTION

The primary symptoms of Parkinson's disease (PD) are resting tremors, bradykinesia and muscular rigidity, due to degeneration of the dopaminergic nigrostriatal pathway. Dopamine (DA) is depleted in the neostriatum[1] and melanin pigments in the substantia nigra (SN)[2]. The relative concentration of the DA metabolite, homovanillic acid (HVA) (HVA/DA ratio), also was reported to be increased in the neostriatum[3] and urine[4,5] of PD patients. An agent that resembles another methylated metabolite of DA, 3,4-dimethoxyphenyl-ethylamine (DIMPEA), was shown to be excreted in the urine of PD patients[5,6]. The levels of serotonin (5-HT) and norepinephrine (NE) are also decreased and the activity of acetylcholine (Ach) is increased in the brain of PD patients.

There may be more wide spread neurological aberrations in PD than usually thought, because degenerative changes[7,8,9,10,11,12,13,14,15] were reported in several non-nigrostriatal areas of PD patients. This suggest that PD may be due to one or more marginally specific toxic metabolites that can impair the nervous tissues, in general. An excess of S-adenosyl-methionine (SAM) may play such a toxic metabolic role, because several sub-strates and products of SAM-dependent biological methylation were identified with PD. The increased HVA/DA ratio and the DIMPEA-like substance found in the urine of PD patients indicate that the methylation of DA increased in PD. Furthermore, the depletion of NE and 5-HT may be related to methylation; and because some by-products of methylation are hypokinetic[5,6] and cyto-toxic, methylation may also explain the movement disorders as well as the neuronal destruction in PD. In addition, SAM has been shown to cause tremor[16]. A hypothesis is presented to show a common biological basis for the symptoms and pathology of PD and the biochemical and pathological changes that can occur as a consequence of excesses in SAM-dependent biological methylation. Such a hypothesis will explain the motor disturbances, the biochemical aberrations, the neurological degeneration and the mechanism of action of l-dopa in parkinsonism; both when it is effective and when it losses its effectiveness. To test this hypothesis we injected SAM into the brain of rats and observed the effects on motor functions, neuronal degeneration and the immunoreactivity of tyrosine hydroxylase.

MATERIAL AND METHODS

Sprague Dawley male rats were acclimatized for at least one week in a

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room with 12 hr. light and 12 hr. dark cycle and water and food supplied *ad libitum*. Under chloral hydrate anesthesia (400 mg/kg) a stainless steel guide cannula was stereotaxically placed for injection into the lateral ventricle of each rat. The placement of the cannula, with reference to bregma was 1.4 mm lateral, 0.5 mm caudal and the tip extended to the inner surface of the cranium. After 2 days recovery, injections were made in the lateral ventricle, 5 mm from the surface of the cranium. The chloride, iodide and the toulenesulfonate salts of SAM as well as S-adenosyl-L-homocysteine (SAH) were injected. Five µl of phosphate buffered saline (PBS), pH 7.4, was injected as control.

Groups of rats that received single injections of SAM (1 µmol/rat) were observed for the presence of tremors and for other changes in motor functions. The rats were sacrificed 1 hour or 4 days post-injection and their brains studied for tissue damage and for changes in tyrosine hydroxylase. Another group received daily injections for 4 days and were sacrificed 6 days after the last injection. One µmol was given on the first and 2 µmol each for the subsequent 3 days. For the histochemistry the rats were reanesthetized and transcardially perfused with cold PBS followed by 4% paraformaldehyde in PBS. The brains were removed and placed in cold 15% sucrose, prepared in PBS, and kept at 4 degrees C for about 24 hr.. The brains were then frozen in powdered dry ice and stored at -78 degrees C. Thirty µM sections were prepared in a cryostat and mounted on gelatin chrome-alum coated slides. A set of the slides was stained with cresyl violet or thionin, another was reacted for the determination of TH immunoreactivity (TH-IR)[17].

RESULTS

When injected into the lateral ventricle SAM caused marked impairments of motor functions. The dominant effects were tremors, hypokinesia, rigidity, rotation and abnormal posture (Fig. 1). The onset of tremors was about 1 min. and occurred mainly in the snouts and the extremities (Fig. 1,A). The rats did not move freely, but made attempts before movements were achieved. Circling

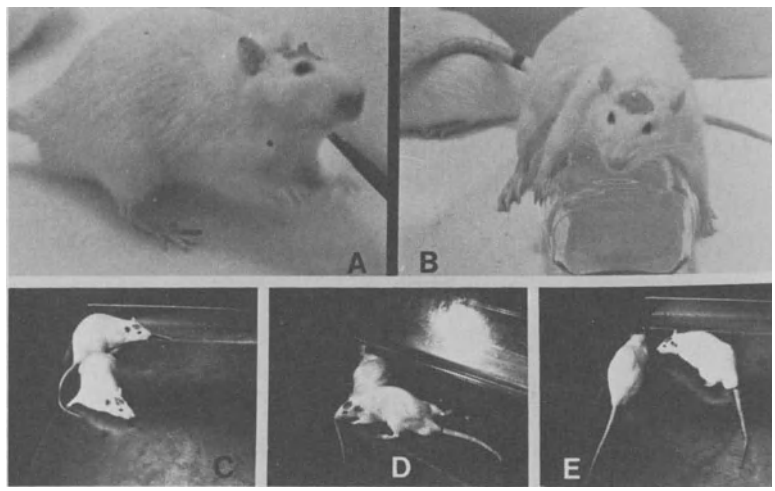

Figure 1 illustrates the motor abnormalities caused by SAM. A shows a rat during tremors; note the blurred forelimbs and snout in A, indicative of resting tremor. B shows "motor freezing". C highlights the rotational behaviors, D the abnormal posture and E the recovery of the rat.

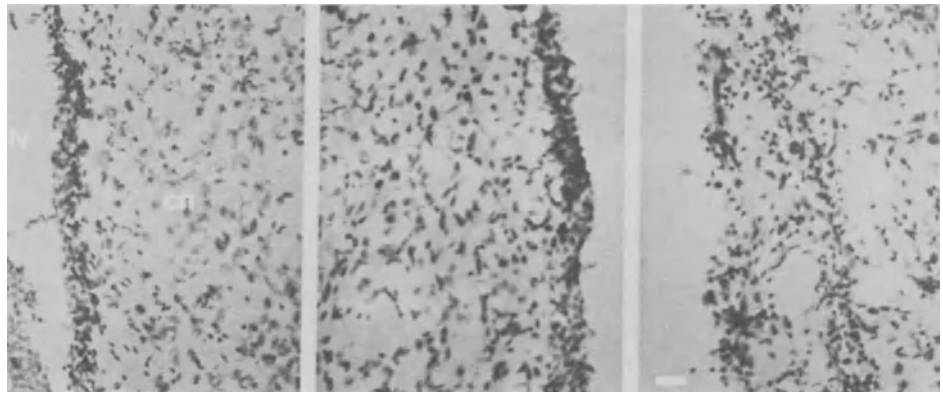

Figure 2 shows thionin-stained caudate nucleus (cn) of rats sacrificed 4 days post-injections. The left represents the control cn, the middle and the right the contralateral and ipsilateral cn of the SAM-injected brain. Note the disrupted ependymal cell layer of the ventricle on the ipsilateral, and the presence of dense Nissl substances in both sides of the SAM-injected brain. lv=lateral ventricle. Bar= 50 µm.

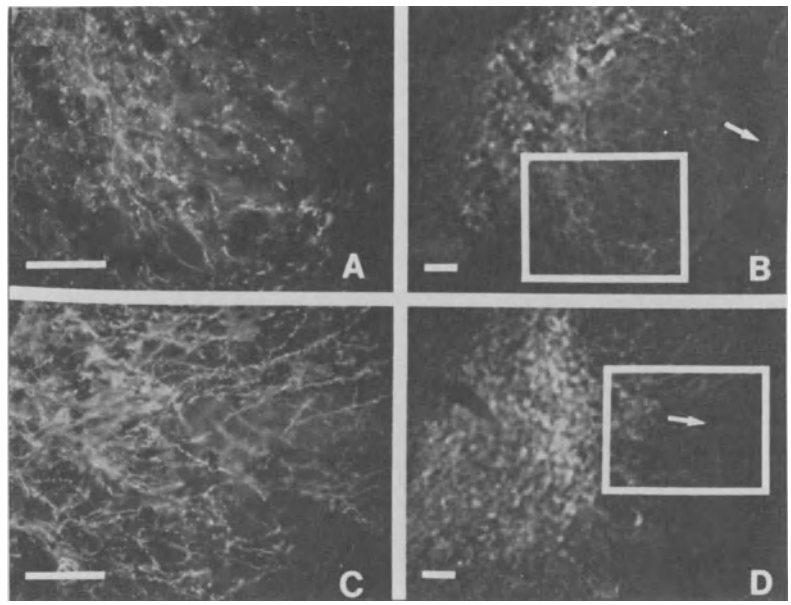

Figure 3 shows a decrease in tyrosine hydroxylase immunoreactivity (TH-IR) in the SN of SAM- (A, B) as compared to PBS- (C, D) injected rats. A and C highlight the boxed areas in B and D, respectively. Note also the disruption of the profile of the TH-IR containing fibers in A as compared with C. Bars: = 50µm.

or rotatory movements, mainly contralateral to the injection site, were observed. The animals showed increased acuity to sound and touch and moved violently to such sensory interventions. After about 40 min. the intensity of the tremors subsided and the animals exhibited less inclination to move. When propped the rats remained in the abnormal position (Fig. 1,B) for extended periods. The effective dose-spectrum for SAM was narrow. Slight motor abnormalities were achieved with an injection of 0.25 µmol/rat and very severe effects involving seizures were achieved with 1.5 µmol/rat. All the salts of SAM caused parkinsonian-like motor impairments in the rats. SAH did not cause such effects. The rats appeared to be fully recovered within about 2 hr.. following the injection of SAM, but showed more tolerance to a subsequent dose given 24 hr. later. Rats that were sacrificed 4 days after receiving a single injection of SAM showed visible tissue disruption to the ependymal cell layer proximal to the injected lateral ventricle and adjacent to the caudate nucleus (CN) (Fig. 2). Tyrosine hydroxylase (TH) immunoreactivity (IR) in the substantia nigra was decreased in the SAM-injected rats (Fig. 3) and more dramatic on the injected side. The TH-containing fibers seemed to be degenerating, evidenced by a disordered (more bead-like) profile in the SAM-injected rats SN (Fig. 3 A, C). Cellular and TH changes in the rats that were sacrificed 1 hr. post-injection were not detected. The SN of the rats that were injected for 4 consecutive days and sacrificed 6 days after the last injection showed a reduction in area, accumulation of phagocytic cells and a decrease in the population of the larger neurons (Fig. 4). These findings are indicative of degeneration in the SN.

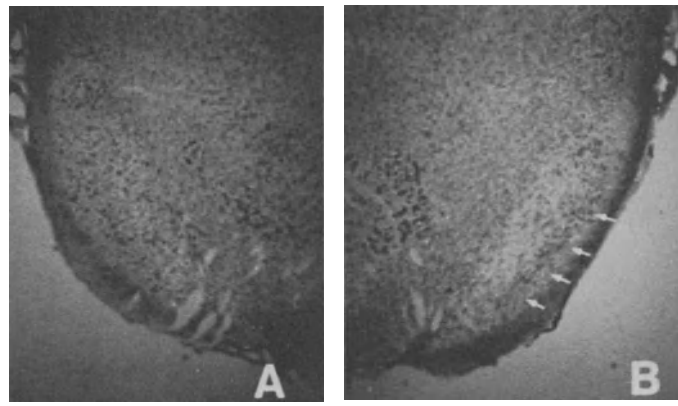

Figure 4 shows that degeneration occurred in the SN of the SAM-injected rat. Note the accumulation of a longitudinal cluster of phagocytic cells (arrows) and the absence of larger neurons on the ipsilateral as compared to the contralateral side. The arrows also indicate the ventrolateral orientation.

DISCUSSION

Although the time frame for these studies were short, as compared to the slow development of PD, the SAM-induced aberrations in the rats were strikingly similar to the symptomatology of PD. Tremors, rigidity, hypokinesia and abnormal posture are the major symptoms of PD. SAM induced all of these symptoms in the rat. Tremors occurred mainly in the snouts and forelimbs of the rats. In humans PD tremors affect mainly the lips, hands and fingers. The motor freezing that occurred in human parkinsonism may be similar to the "propped" freezing that was demonstrated in the rats.

The reported depletion of DA, NE, 5-HT and melanin and the increase in HVA/DA ratio and probably DIMPEA in PD may be related to a SAM-dependent increase in the methylation of DA, NE (Fig. 5) and 5-HT. SAM is probably the limiting factor in these reactions and there is evidence to support this proposal. Therefore, an increase in SAM will drive the methylation reactions forward and will probably deplete DA, NE and 5-HT and increase the methylated metabolites of these biogenic amines. The methylated amines may be partly responsible for the physical impairments observed in PD because DIMPEA[5,6] and various other methylated amines[18] caused hypokinesia in experimental animals. The increased methylation will shunt tyrosine and dopa, the likely precursors for melanin, from melanin synthesis toward the catecholamines methylation pathways (Fig. 5). This may explain the depletion of melanin in PD patients. The SN damage and the decrease in TH may be similar to that which also occurred in PD. The differences may be due to the specific placement, the high concentration and the acuteness of the action of SAM.

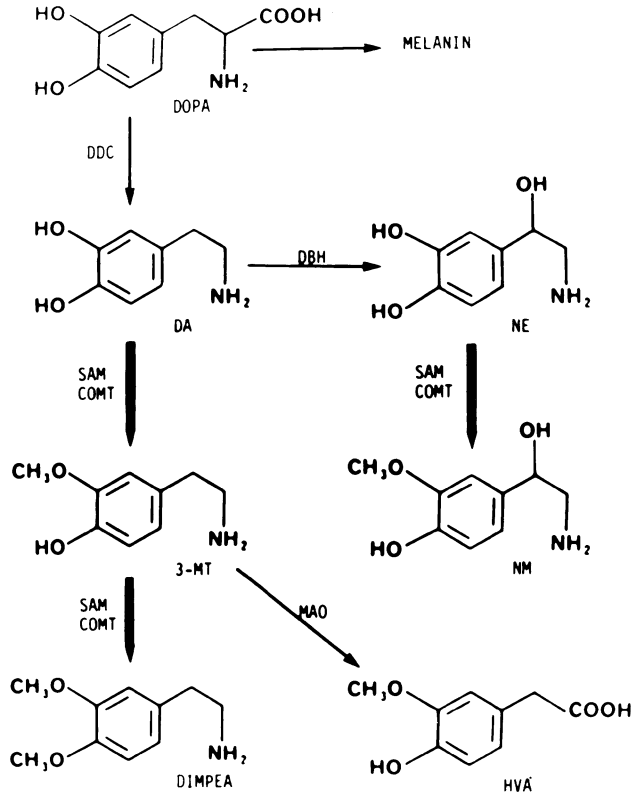

Figure 5 depicts the reaction of SAM with DA, 3-methoxy tyramine (3-MT) and NE and illustrates how 3-MT, DIMPEA and the ratio of HVA/DA may be increased in PD, and how DA, NE and melanin pigments may be decreased. An increased methylation may shunt tyrosine and l-dopa toward the methylation pathways and away from the synthesis of melanin. The heavy arrows show the pathways that may be influenced significantly by SAM. DDC = dopa decarboxylase, COMT = catechol-O-methyltransferase, DBH = dopamine betahydroxylase, MAO = monoamine oxidase.

This study indicated that excessive SAM-dependent methylation may be involved in the cause of PD. For a non-specific metabolite like SAM to cause specific and focal physiological effects, as occurred in PD, the relative susceptibility of the patient's nigrostriatal system (NS) ought to play a very important role. The fact that the NS is subservient to finely tuned, high-gain and visible functions, such that slight interference with its order of operation will be easily manifested, is one factor that may predispose the system. The NS seems to be under constant demands by the modalities that it subserves, is probably more metabolically active and is enriched with molecules, e.g. biogenic amines, with which SAM will interact. In addition, the PD patients NS may initially contain a low number of dopaminergic cells so that damage to the cells will more likely cause impairments. The close proximity of the CN to the ventricle may subject the NS cell terminals to SAM, should it accumulate in the CSF.

The hypothesis that excess SAM may be involved in PD further suggests that L-dopa, in its course of action, may be effective in treating PD not only by increasing dopamine but, more importantly, by depleting SAM[19], through the reaction of SAM with l-dopa[20] as well as with the newly synthesized DA. Maybe that is why l-tyrosine, the immediate precursor of dopa, and in spite of a mere fifty percent reduction of tyrosine hydroxylase in some PD cases, cannot relieve the symptoms of PD, because of the absence of a meta-hydroxyl group that causes l-tyrosine to react poorly with SAM. The suggestion that SAM may be increased in PD also infers that its reaction with dopa during treatment will be similar to the reaction with the catecholamines (CA) during the course of the disease; thus methylated CA will be produced during the disease as well as during treatments with l-dopa. Since some methylated CA are physiologically active it is suggested that methylated CA may contribute to the symptoms of PD as well as to the complications and contraindications observed during l-dopa therapy. Consequently, based on this report new therapeutic approaches may involve decreasing methylation while increasing the DA concentration. To decrease the SAM-dependent methylation, inhibitors of the synthesis of SAM, potent methyl acceptors and a reduction in the intake of methionine, the precursor of SAM, are suggested. It follows, therefore, that an abundance of methionine in protein-rich diets rather than the competition of dietary amino acids for the uptake of l-dopa may explain the low compliance to l-dopa therapy in the presence of high protein intake in PD patients[21,22].

ACKNOWLEDGEMENTS

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DOPA AND DOPAMINE CAUSE THE DESTRUCTION OF CULTURED NERVE CELLS IN THE PRESENCE OF IRON: POSSIBLE MECHANISM OF THE NIGRAL DEGENERATION IN PARKINSON'S DISEASE

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INTRODUCTION

Melanine-containing nerve cells in the brainstem are regularly and distinctively involved in Parkinson's disease. Most prominent nerve cell loss is found in the substantia nigra, the most important dopaminergic center. The pathogenesis of this condition is still unknown in spite of extensive approaches from many aspects. All hypotheses for the nigral degeneration seem lacking in convincing evidences (Barbeau, 1984). On the other hand, biochemical analyses have revealed that the substantia nigra from parkinsonian subjects contains more ferric ion and lipid peroxide, and less polyunsaturated fatty acid than those from control subjects (Riederer et al., 1989; Dexter et al., 1989). These findings support the idea that melanine-containing nerve cells are destroyed by lipid peroxidation of the cell membrane, and that dopamine, its metabolically related compounds and iron take part in initiation of the pathologic process of the disorder.

In this communication, we report that dopa and dopamine cause cultured nerve cell death in the presence of iron and postulate that there is little or no participation of active oxygen species in this toxic process. Furthermore, the lipid peroxide is considered to be formed when neurons are exposed to dopa or dopamine along with iron. We suggest that dopa (dopamine) and iron-induced lipid peroxidation play a significant role in the nigral degeneration in Parkinson's disease.

MATERIALS AND METHODS

Nerve Cell Culture: The method of dorsal root ganglion (DRG) nerve cell culture has been described in detail elsewhere (Tanaka et al, 1989). In brief, DRGs obtained from 5 week-old BALB/c male mice were dissected free of meningeal tissue and dissociated by treatment with 2 mg/ml collagenase in L-15 medium for 60 min followed by trituration in chemically defined serum-free medium. Nerve cell cultures were initiated by seeding 200 μl of the dissociated cell suspension (6000 cells/ml) into individual wells of a 96-well collagen-coated microculture plate. All cultures were maintained at 37℃ under a water-saturated atmosphere of 95% air-5% CO_2.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Exposure of Cultured Nerve Cells to Dopa or Dopamine: Cultures were maintained for 24 hours. Then the medium was replaced by 200 μl of physiological saline with 25 mM HEPES (pH 7.4) containing the following reagents: A; 0.5 mM dopa, B; 0.5 mM dopa and radical scavengers (2.5 μM superoxide dismutase and 40 μg/ml catalase), C; 0.5 mM dopa and 1 mM deferoxamine mesylate, D; 1 mM deferoxamine mesylate, and control experiments(CTL); no addition. To check whether cultured nerve cells were detached from the wells, which might be caused by dopa-induced degeneration of collagen used to coat microculture plates, wells pretreated with reaction A were also applied to control experiments (PTW). In experiments for investigation of cytotoxicity induced by dopamine, 0.5 mM dopa in the reaction mixture mentioned above was replaced by 1.0 mM dopamine. After incubation for 60 min at 37℃, the reaction mixture in each well was replaced by 200 μl of fresh culture medium and the cultures were again maintained for further 3 days.

Enzyme Immunoassay for Neurofilament Protein: Nerve cell cultures were fixed with 4% paraformaldehyde for 2 hrs and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Following incubation with 10% fetal calf serum in PBS overnight, cultures were incubated with a 1:1000 dilution of rabbit serum against neurofilament protein (200K) for 60 min, washed three times with PBS, and incubated with a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody for 60 min. Cultures were then washed three times with PBS and finally incubated with 200 μl of peroxidase substrate system ABTS for 30 min. Aliquots of the reaction mixtures were then transferred to a fresh microculture plate and optical density (O.D.) was measured at 410 nm. To confirm the correlation between the number of surviving nerve cells and O.D., cultures with various cell densities were maintained for 4 days under the same condition as mentioned above and then were subjected to enzyme immunoassay.

Demomstration of Lipid Peroxide Formation in Nerve Cells: Cerebral cortices of new-born Wistar rats were collected in a rinse solution containing 0.02% EDTA followed by incubation with 0.25% trypsin-200 U/6ml DNase I at 37℃ for 5 min. Cells were dissociated in 25 mM HEPES in physiological saline. Reaction mixture contained nerve cells (3.7 X 10^8 cells/2ml) with or without 0.5 mM Fe^{3+}-ADP complex, 0.5 mM dopa, 3 mM deferoxamine mesylate, 2.5 μM SOD-40 μg/ml catalase. Fe^{3+}-ADP complex was prepared as described by Sugioka and Nakano (1982). Volume of each reaction mixture was 2 ml. After incubation for 60 min at 37 ℃, thiobarbituric acid-reacting substance (TBARS) was quantified by the method reported by Ohkawa et al.(1979) with partial modification and was expressed as amount of malondialdehyde (MDA) formed (nmoles/mg protein).

RESULTS

EIA of NF: NF levels expressed as O.D. were significantly correlated with the number of surviving nerve cells ($r=0.987$, $P<0.001$). This means that O.D. obtained by EIA is a reliable index of the number of nerve cells surviving in tissue culture, so EIA was used to evaluate the amount of spared nerve cells in the following experiments.

Effects of Exposure to Dopa and Other Reagents (Fig.): O.D. was markedly decreased by exposure to dopa or dopamine, but were returned to the control level by addition of deferoxamine mesylate, a powerful iron-chelating agent. Deferoxamine itself showed no neuronotrophic effect. These results indicates that dopa and dopamine cause nerve cell death in the presence of iron in the culture. Addition of a sufficient amount of SOD and catalase did not alter the cytotoxic effects induced by dopa (dopamine) and iron. This indicates the lack of participation of superoxide and hydroxyl radicals in the process. Because the number of nerve cells cultured in wells pretreated with the medium and dopa (dopamine) were not decreased in comparison with control culture, degeneration of collagen used for coating does not participate in the O.D. decrement by exposure to dopa (dopamine) and iron.

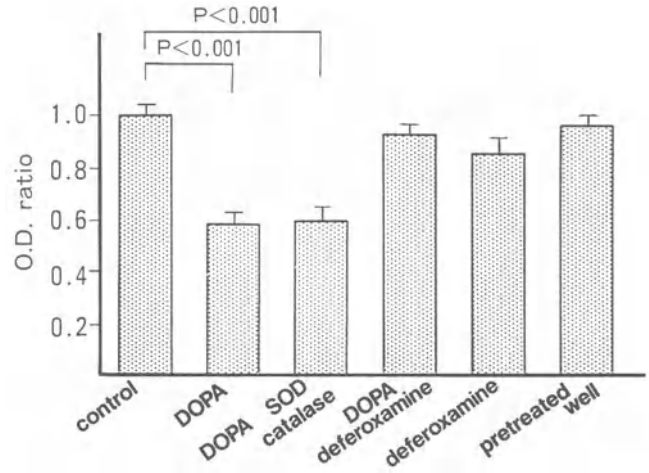

Fig. 1. Effects of Exposure to Dopa and Other Reagents: Dopa caused a significant reduction of O.D. ratio to control cultures (an index of surviving nerve cells). Destruction of cultured nerve cells was prevented by addition of deferoxamine mesylate, but was not altered by addition of the radical scavenging enzymes. Exposure to dopamine also showed cytotoxicity essentially identical with this (data not shown).

Lipid Peroxidation Induced by Dopa and Iron: 9.63 nmoles/mg protein of TBARS was formed only when cortical nerve cells were exposed to dopa in the presence of iron-ADP complex. Addition of deferoxamine completely abolished the formation of TBARS. Incubation with each of these compounds alone did not generate TBARS in the cells. The radical scavenging enzymes showed no preventive effect on lipid peroxidation. Dopa and iron induced neuronal lipid peroxidation in vitro.

DISCUSSION

This study showed that dopa and dopamine caused cultured nerve cell death in the presence of iron and that these substances also induced lipid peroxidation in cortical neurons in vitro. In this study, SOD and catalase were found to reduce neither the destruction of nerve cells nor the formation of lipid peroxide. These results justifiably lead us to consider the lipid peroxidation of the cell membrane responsible for the cultured nerve cell death.

Of the potentially important substances for etiology of Parkinson's disease, active oxygen species, especially hydroxyl radicals, were postulated to be easily generated and to be injurious to neurons in the substantia nigra (Barbeau, 1984). The present in vitro study, however, shows that hydroxyl radicals and superoxide are not produced or have no important role in neuronal cell death. In experiments with liposomes prepared from phospholipids extracted from rat liver microsomes, ferric ion significantly promoted a peroxidative cleavage of unsaturated fatty acids after coordinating with dopa or dopamine (in submission, data not shown). Such lipid peroxidation was shown to be induced without participation of any active oxygen species.

Dopamine and related compounds are potentially cytotoxic. Graham et al. (1978) concluded that 6-hydroxydopamine should kill cells through the production of active oxygen species, while for dopamine and dopa the reaction of quinone oxidation products with nucleophiles should also contribute to their cytotoxicity. In contrast to us, they paid no attention to iron in the reaction system, but attached importance to cytotoxic compounds probably formed through autoxidation of dopa and dopamine.

In the culture we used in the present study, nerve cells were not derived

from a dopaminergic center. Dopa (and dopamine) exogenously added and iron included in the culture evoked a harmful reaction. These facts suggest that the substantia nigra is at risk of dopa (and dopamine) and iron-induced cell damage because of the abundance of these substances in the area. We suppose that this cytotoxicity causes the nigral degeneration in Parkinson's disease due the lack of some factors preventing lipid peroxidation. Further studies are required to elucidate what triggers the destructive process.

Many investigators are now intensively exploring exogenous toxic substances to cause the nigral degeneration like MPTP without any successful outcome. We stress that potentially causative factors exist endogenously in noraml subjects as shown in the present study.

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FOOD-DERIVED HETEROCYCLIC AMINES AS POTENT INHIBITORS OF CATECHOLAMINE METABOLISM

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INTRODUCTION

Following the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine as a neurotoxin that produces symptoms similar to those of parkinsonism in humans,[1] many naturally-occurring or synthesized compounds have been proposed as putative neurotoxins. Among carboline derivatives, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) are produced by pyrolysis of tryptophan in food.[2] It was found from *in vitro* and acute *in vivo* experiments that Trp-P-1 and Trp-P-2 reduced DOPA formation in rat striatal tissue slices and were potent inhibitors of monoamine oxidase [MAO; monoamine : oxygen oxidoreductase (deaminating), EC 1.4.3.4].[3] To examine chronic *in vivo* effects of Trp-P-1 and Trp-P-2 on catecholamine metabolism, was used a clonal rat pheochromocytoma cell line, PC12h, as a model of dopamine (DA) neurons. The cells were cultured in the presence of Trp-P-1 and Trp-P-2 for 6 days, and their effects on activity of the enzymes involved in catecholamine metabolism, such as tyrosine hydroxylase [TH; tyrosine tetra-hydropteridine : oxygen oxidoreductase (3-hydroxylase), EC 1.14.16.2] and aromatic L-aminoacid decarboxylase (AADC; aromatic L-aminoacid carboxy-lyase, EC 4.1.1.28), were examined. Using PC12h cells, we also examined whether these amines may be taken up into DA neurons selectively.

This paper describes that Trp-P-1 and Trp-P-2 were taken up into PC12h cells by the transport system specific for dopamine, accumulated in cells, and reduced the enzyme activity of TH and AADC. The inhibition of catecholamine metabolism by these carcinogenic heterocyclic amines was discussed in relation to their possible role in the brain as naturally-occurring and food-derived inhibitors of catecholamine metabolism.

MATERIALS AND METHODS

Materials

PC12h cells were cultured in the presence of Trp-P-1 or Trp-P-2 for 6 days, and the culture medium was changed at the 4th day of culture.[4] The cells were harvested, washed with phosphate-buffered saline (PBS), suspended in 10 mM potassium phosphate buffer, pH 7.4, and sonicated. Trp-

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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P-1 and Trp-P-2 were purchased from Wako; L-DOPA, DA, and noradrenaline, from Sigma; NSD-1055 and pyridoxal-5-phosphate (PLP), from Nacalai Tesque. Serotonin came from E. Merck. Nomifensine was kindly donated by Hoechst; mazindol, by Sandoz; and sulpiride by Fujisawa.

Assay of enzyme activities

TH activity[5] and AADC activity toward L-DOPA[6] were assayed using HPLC with electrochemical detection.

Assay for the uptake of Trp-P-1 and Trp-P-2 into PC12h cells.

PC12h cells were harvested, washed, and suspended in modified Krebs-Ringer solution. The cell suspension was then incubated with Trp-P-1 or Trp-P-2 at 37°C for 20 min, and then was washed by centrifugation with PBS.[4]

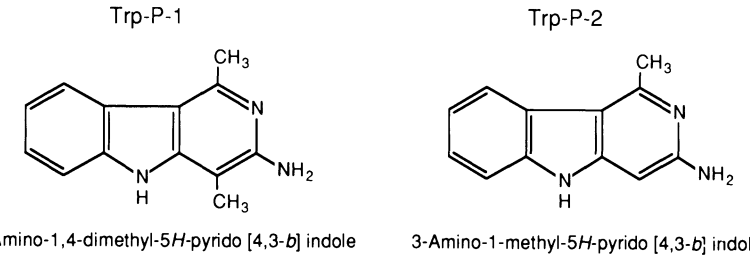
Assay for the amounts of Trp-P-1 and Trp-P-2

The concentrations of these amines in PC12h cells were quantitatively measured by HPLC: an HPLC apparatus was connected to a spectrofluorometer and the fluorescence intensity at 400 nm was measured with excitation at 310 nm. An Asahi reversed phase ODP-50 column was used and the mobile phase was 25 mM sodium phosphate buffer, pH 2.0, to which acetonitrile was added to 18% [4].

RESULTS AND DISCUSSION

Figure 1 shows the chemical structures of Trp-P-1 and Trp-P-2. After 6 days' culture in the presence of Trp-P-1 or Trp-P-2, definite amounts of amines were recognized in the PC12h cells. TH activity in the cells was markedly reduced by culture in the presence of these amines, as summarized in Table 1. Reduction in TH activity was observed at concentrations higher than 10 nM; and with 10 μM Trp-P-1 and Trp-P-2, TH activity was almost negligible. Table 1 also shows marked reduction of AADC activity with Trp-P-1 or Trp-P-2, and Trp-P-1 inhibited AADC activity more profoundly than Trp-P-2. These heterocyclic amines inhibited the AADC activity independent or dependent of PLP added into the reaction system. On the other hand, the amounts of cell protein were reduced only with 10 μM Trp-P-1.

The uptake of these heterocyclic amines in PC12h cells was next examined. The uptake was dependent on the incubation time and the



3-Amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*] indole

3-Amino-1-methyl-5*H*-pyrido [4,3-*b*] indole

Fig.1. Chemical structures of Trp-P-1 and Trp-P-2.

concentrations of amines added. The uptake velocity was saturable at higher amine concentrations and followed the Michaelis-Menten equation. The K_m and V_{max} values of the uptake of Trp-P-1 and Trp-P-2 into PC12h cells were 326 ± 57 μM and 786 pmol/min/mg protein for Trp-P-1 and 265 ± 19 μM and 618 ± 43 pmol/min/mg protein for Trp-P-2, respectively. The effects of catecholamines and other compounds on the uptake of Trp-P-1 and Trp-P-2 are shown in Fig. 2. DA and serotonin inhibited the uptake of both the amines, but noradrenaline did not. Mazindol and nomifensine, specific inhibitors of DA uptake, reduced the uptake markedly, while an antagonist of D_2 receptors, sulpiride, did not affect the uptake.

Table 1. Effects of Trp-P-1 and Trp-P-2 on the activities of TH and AADC.

Cells cultured with		Enzyme activity (nmol/min/mg protein) TH	AADC
Trp-P-1	10 nM	0.65 ± 0.03	0.07 ± 0.02
	100 nM	0.58 ± 0.08	0.03 ± 0.01
	1 μM	0.50 ± 0.03	0.02 ± 0.01
	10 μM	not detected	not detected
Trp-P-2	10 nM	0.53 ± 0.03	0.46 ± 0.03
	100 nM	0.42 ± 0.06	0.22 ± 0.01
	1 μM	0.46 ± 0.02	0.02 ± 0.01
	10 μM	not detected	0.01 ± 0.01
Control		0.75 ± 0.04	0.57 ± 0.03

Each value represents the mean and SD.
AADC activity was measured in the presence of 5 μM PLP.

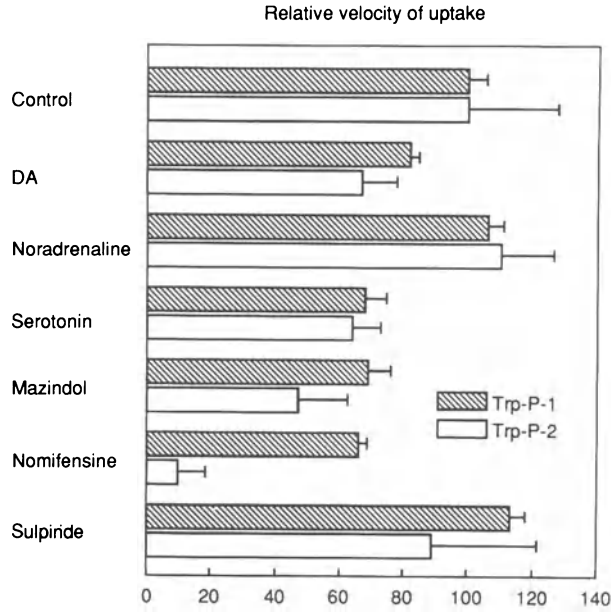


Fig. 2. Effects of various compounds on the uptake of Trp-P-1 and Trp-P-2.

The data show that Trp-P-1 and Trp-P-2 reduced the enzyme activity of TH, the rate-limiting enzyme of catecholamine biosynthesis, and of AADC. AADC activity was more sensitive than TH activity to these amines. The enzyme activities of these enzymes were measured under the optimal conditions for their activity, with high concentrations of substrate and co-factors, such as (6R)-L-*erythro*-5,6,7,8-tetrahydrobiopterin for TH activity or PLP for AADC activity. So, the reduction in enzyme activity may be due to a reduction in the enzyme amount in the cells. It should be emphasized that reduction of the enzyme activities was observed at the concentrations lower than the concentration required to reduce the amount of total cell protein.

Trp-P-1 and Trp-P-2 were detected in plasma of normal human subjects,[7] which may be derived from foods, because these heterocyclic amines are found in various kinds of cooked meat or fish.[2] In addition, transport of Trp-P-1 into the brain through the blood-brain barrier was confirmed by intravenous injection into mice.[8] Our data showed that these heterocyclic amines were taken up into PC12h cells by the DA transport system, which indicates that in the brain they may be taken up into DA neurons in specific regions of the brain, such as the nigro-striatal system. Inhibition of the uptake of these amines by serotonin suggests that they may be taken up also into serotonergic neurons.

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SENILE CHANGES IN THE HUMAN OLFACTORY BULBS

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INTRODUCTION

Little attention has been paid to the morphological changes in the olfactory bulbs in patients with dementia of the Alzheimer type, despite the fact that rhinencephalo-limbic structures, which receive fibers directly from the olfactory bulbs, are known to be severely affected.[1-4] Recently, Pearson et al.[4] raised the possibility that the olfactory tracts provide a portal of entry to the brain for any putative pathogenic agent(s) that induces senile plaque formation and/or Alzheimer's neurofibrillary tangles (NFT).

In this study, we examined the pattern of extension of Alzheimer's NFT and senile plaques in the olfactory bulbs.

MATERIALS AND METHODS

The olfactory bulbs and the cerebrum in 100 routine autopsy cases (deceased persons from 33 to 94 years old) including dementia of the Alzheimer type were examined by light and electron microscopy.

For light microscopy, 10% formalin-fixed olfactory bulbs and stalks were embedded and sectioned horizontally along the long axis. Sections were stained with H&E, thioflavin S, or Bielschowsky silver impregnation , or processed for immunohistochemistry using polyclonal antibodies to (1) a synthetic peptide of residues 1-28 of β-protein,[5] (2) tau (provided by Yasuo Ihara), or (3) GFAP. Immunohistochemistry was performed using the avidin-biotin-peroxidase (ABC) method (Vector Lab., U.S.A.). To enhance the immunoreactivity of amyloid deposits, we pretrated with 99% formic acid for 5 minutes.

For electron microscopy, 1% glutaraldehyde/4% paraformaldehyde-fixed specimens and 10% formalin-fixed specimens of olfactory bulbs from five cases were refixed in 1% osmium tetroxide and embedded in Epon.

RESULTS

Alzheimer's neurofibrillary tangles (NFT) in olfactory bulbs

Alzheimer's NFT were identified by Bielschowsky silver impregnation and tau immunostain (Fig.1). NFT were first observed in the anterior

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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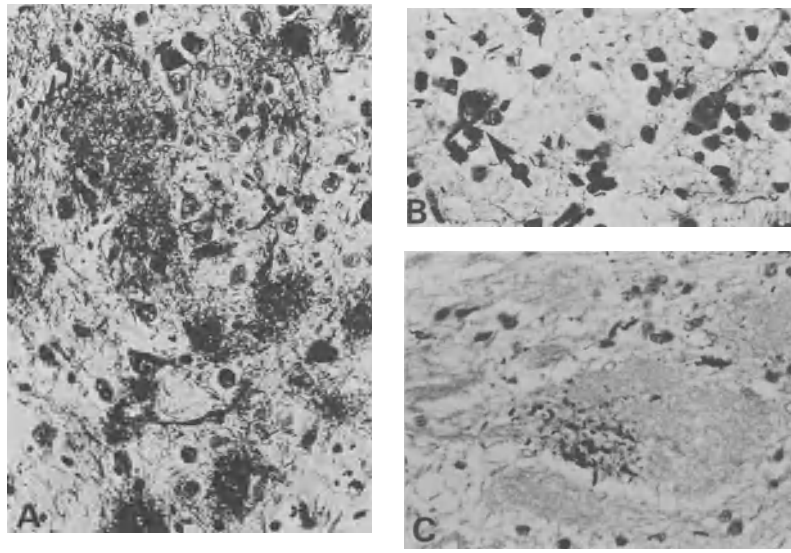

Fig. 1. A:Many Alzheimer's NFT and senile plaques seen in the anterior olfactory nucleus in Alzheimer's disease(Bielschowsky stain, X160). B:NFT in a mitral cell(arrow)(Bielschowsky stain, X400). C:β-protein-positive fibrils in the olfactory glomerulus(β-protein+hematoxylin, X200).

olfactory nucleus in specimens older than 60, and the incidence rose with age. The incidences were approximately 20 % in the 50th decade, 30 % in the 60th, 65 % in the 70th, and 100 % (4/4 cases) in the 80th. NFT were positively correlated with the quantity of senile changes (NFT and senile plaques) in the temporal cortex, as shown in our previous report.[3] In cases in which many NFT were observed in the anterior olfactory nucleus, they were seen widely in tufted cells but rarely in mitral cells (Fig. 1B). Tau-positive and silver-impregnated fine fibrils were also observed in the olfactory glomerulus.

So-called eosinophilc tangles were seen by GFAP immunostain in the anterior olfactoy nucleus in one case of Alzheimer's disease.

Senile plaques in olfactory bulbs

Senile plaques were identified by Bielschowsky silver impregnation and β-protein immunostain (Fig. 1). Senile plaques in the olfactory bulbs were found in 18 out of the 100 cases. Senile plaques in the olfactory bulbs first appeared in the anterior olfactory nucleus after the appearance of many senile plaques in the cerebral cortex. The plaques in the anterior olfactory nucleus were seen as primitive or diffuse types. Typical plaques were rarely seen. Cerebral amyloid angiopathy was not observed in the olfactory bulbs. In the advanced stages, irregularly shaped accumulations of β-protein-positive fibrils were distributed widely in the molecular layer, mitral cell layer, and olfactory glomerulus (Fig. 1C).

In the cases in which many NFT and senile plaques were seen in the olfactory bulbs, many such lesions were also seen in the cerebral cortecies, but there was no cases in which senile changes were predominantly in the olfactory bulb.

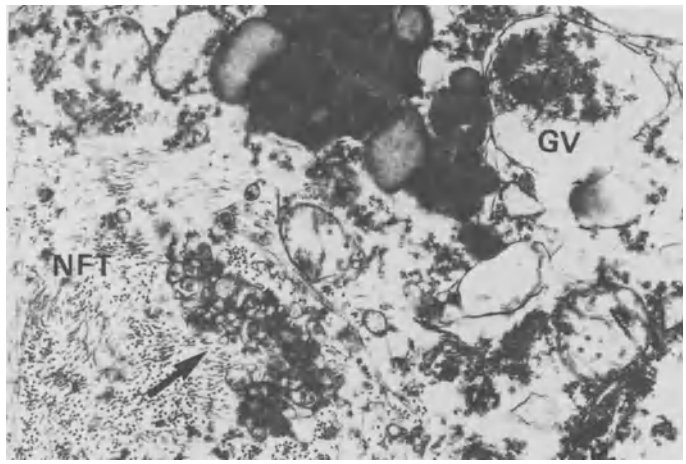

Fig. 2. Amorphous materials with tubular structure (arrow), Alzheimer's neurofibrillary tangles (NFT), and granulovacuolar bodies (GV) were observed in the same neuron in the anterior olfactory nucleus (X21,000).

Electron microscopy

Examination by electron microscopy disclosed that NFT in the anterior olfactory nucleus consisted of a mixture of straight and twisted tubules. Granulovacuolar bodies and Hirano bodies were also observed. Fine structures corresponding to eosinophilic tangles were also seen. Amorphous materials with tubular structure, NFT, and granulovacuolar bodies were all observed in the same neuron in the anterior olfactory nucleus of Alzheimer's disease (Fig. 2).

DISCUSSION

Alzheimer's NFT and senile plaques in the olfactory bulbs were previously studied using silver staining.[1-3] The recently developed β-protein immunostain used here is not only a very specific but also a highly sensitive method for the detection of amyloid deposits in the brain.[5]

Recently, deficits in odor detection and discrimination have been added to the list of the signs of Alzheimer's disease,[6] and anatomical studies suggest that the olfactory pathway may be involved early in the illness.[4,7] It is important to pinpoit the location of the initial formation of plaques and tangles. Mann et al.[8] compared the incidence and distribution of senile plaques and NFT in the olfactory bulbs, amygdara, and hippocampus of many cases with Alzheimer's disease and Down's syndrome, and they showed that of the three, olfactory bulbs were the least affected in both diseases. So, they suggested that the olfactory bulbs are affected later in the course of the disease. Our data also show that the olfactory bulbs are not the earliest sites of senile plaque formation. From our results, patterns of extension of senile plaques and NFT in the olfactory bulbs are considered as follows: NFT appeared in the anterior olfactory nucleus in persons who were about 60 years old at the time of death, and the incidence rose with age. Senile plaques were found first in the ante-

rior olfactory nucleus after the appearance of many senile plaques in the cerebral cortex. In the advanced stages, NFT were also observed in tufted cells and mitral cells, senile plaques were distributed widely in the molecular layer, and β-protein-positive structures extended up to the olfactory glomerulus. NFT in Alzheimer's disease have been reported in the anterior olfactory nucleus and in tufted cells but not in mitral cells.[1,2] In our examination, NFT were also rarely seen in mitral cells.

The fine structure of senile changes in the olfactory bulbs was reported earlier by us.[3] NFT in the olfactory bulb consist of the same fine structures as in the cerebellum. Amorphous materials with tubular structure are mimetic to the inclusions reported in aged rat brains.[9]

SUMMARY

The patterns of extension of Alzheimer's neurofibrillary tangles (NFT) and senile plaques in the olfactory bulbs were examined in 100 autopsy cases by use of the modified Bielschowsky method and by immunohistochemical stainings with polyclonal antibodies against tau and the synthetic peptide of residues of β-protein. Some of them were also examined by electron microscopy. NFT were observed in the anterior olfactory nucleus in specimens from persons older than 60 at the time of death, and the incidence rose with age. Senile plaques were first found in the anterior olfactory nucleus after the appearance of many such plaques in the cerebral cortex. In the advanced stages, NFT were also observed in tufted cells and mitral cells, and senile plaques were distributed widely in the molecular layer and olfactory glomerulus.

In conclusion, the olfactory bulbs are not the earliest sites of senile plaque formation.

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LARGE NEURONS IN THE NEOSTRIATUM AND BASAL NUCLEUS OF MEYNERT: SIMULTANEOUS DECREASE IN ALZHEIMER'S DISEASE

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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in middle and late life. Among the histologic features found in AD, Alzheimer's neurofibrillary tangles (NFTs) are present in great profusion in the cerebral cortex with relative sparing of the occipital lobe and paracentral gyri, and are present in the basal nucleus of Meynert (bnM), hypothalamus, and tegmental nuclei of the rostral brain stem. Morphometric studies revealed loss of neurons in these areas.

Neurochemical investigations of AD have demonstrated a severe deficiency in the activity of choline acetyltransferase (ChAT) in the neocortex, hippocampus, and bnM. The activity of ChAT was also moderately reduced in the caudate nucleus in AD, and the patients frequently show extrapyramidal signs, such as muscle rigidity, hypokinesis, or tremor. The reduced ChAT activity in the neostriatum of AD has been considered as a consequence of the dysfunction of the afferent cholinergic thalamostriate system, with sparing of the intrinsic cholinergic neurons of the neostriatum. The neostriatum, which has a high concentration of cholinergic neurons, as does the bnM, has not been precisely examined in AD.

On the other hand, progressive supranuclear palsy (PSP) is also a fatal neurological disorder in adults, and it combines the clinical features of a supranuclear ophthalmoplegia, pseudobulbar palsy, dysarthria, dystonic rigidity of the neck and upper trunk, and dementia. The histologic appearance of the PSP brain consists of loss of neurons and fibrillary gliosis in the globus pallidus, subthalamic nucleus, bnM, red nucleus, substantia nigra, tectum and tegmentum of the brain stem, and dentate nucleus, and of the occurrence of NFTs containing 15-nm wide straight tubules in the remaining neurons in these areas.

In the neostriatum in PSP, the activity of ChAT has been reported to be decreased. Neuropathologically the presence of NFTs, gliosis, and occasional neuronal loss in the neostriatum has been described in PSP. Actually, however, the neostriatum in PSP has not yet been precisely scrutinized quantitatively.

The aim of this study was to evaluate the quantitative changes of the neurons in the neostriatum of AD and of PSP, and to elucidate whether the large neurons, which are supposed to be cholinergic, in the neostriatum and bnM show a correlative decrease in AD and PSP.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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MATERIALS AND METHODS

Seven patients with AD (age: 59-79 years, average: 67.9 years) and 6 with PSP (age: 62-82 years, average: 69.2 years) showing typical clinical histories and neuropathological findings, and 6 control subjects (age: 57-80 years, average: 68.8 years) without dementia, ophthalmoplegia, or rigidity, were examined. Patients with complications of anoxic or ischemic episodes, severe liver dysfunction, intracranial mass lesion, or cerebral infarcts, and those who were being treated with anticonvulsants or anticancer agents were excluded. The brains were fixed in 10% formalin, and coronally sliced tissues were embedded in paraffin.

For the quantification of neostriatal neurons, two Klüver-Barrera (KB)-stained, 10-μm sections of the caudate head and putamen were made 20 μm apart at the level of the nucleus accumbens and mammillary body. The sectional area of the caudate head or putamen was measured using KB-stained sections projected on the platen of a digitizer at a 4-fold magnification.

In order to elucidate the basis of neuronal degeneration in terms of decrease in volume, the relation between cell body and nuclear areas was investigated. Cell body and nuclear areas of 40 neurons in the caudate head and putamen of each case were measured by a digitizer with a drawing tube. Regression lines were calculated to illustrate differences or similarities between the cell body and nuclear areas of the control, AD, and PSP cases.

Based on these findings, we regarded the area of the nucleus as a marker for degenerative changes in the neostriatal neurons in AD and PSP. We defined in this study that large neurons are the neurons with a nuclear area greater than 101 μm^2, and small neurons, less than 100 μm^2.

The measurement of the small neurons was taken from 0.5-mm^2 areas of the upper and lower portions of both caudate head at the level of the nucleus accumbens and putamen at the level of the mammillary body, under 1000-fold magnification. The data were multiplied by the sectional area of the caudate head or putamen to obtain the converted size distribution of every 20 μm^2 of the area of the nucleus of neurons in the whole sectional area of the caudate head or putamen. The total number of small neurons was taken as the sum of those from each of the two sections.

The number of large neurons is small compared with that of small neurons. In order to comprehend the changes of the large neurons more accurately the entire caudate head and putamen of the two sections were surveyed under 400-fold magnification. The size distribution of every 20 μm^2 of the nuclear area of the large neurons was made, and the sum of the total number of the large neurons was calculated from the two sections. Statistical evaluation was done using the t-test.

For the quantification of the large neurons in the bnM, two KB-stained 10-μm sections of the bnM 100 μm apart were prepared at the level of the infundibulum, which is considered to be the level showing the maximum number of such neurons. The sphere of the bnM and the large neurons were defined according to the criteria reported previously. In the present study, the large neurons in the bnM are defined as neurons with their shortest diameter of the perikarya larger than 25 μm. Statistical evaluation was done using the Mann-Whitney U-test for comparison of the numbers of large neurons between AD or PSP patients and the control subjects, or X^2-test for evaluation of the ratio of decrease of large neurons between the caudate nucleus and putamen of AD or PSP and control subjects initially, and then between those in the neostriatum and bnM of the subjects.

General observation was also performed using successive serial hematoxylin-eosin, Bodian, Holzer, phosphotungstic acid hematoxylin, periodic acid-Shiff and Congo red, and anti-tau immuno-stained sections.

RESULTS

General observations revealed severe loss of large neurons in the neostriatum in AD and PSP patients. The remaining large neurons in those patients showed shrinkage, faint Nissl substance, increased amount of lipofuscin, delicate fibrils, or nuclear eccentricity with NFT accumulation. The number and cytologic features of the small neurons looked normal. The number of large neurons in the bnM of AD was severely reduced, and the remaining neurons frequently showed NFTs (1,2).

The sectional area of the caudate head and putamen was well preserved in AD and PSP. Cell body and nuclear areas of large as well as small neurons in AD and PSP patients were positively correlated. Regression coefficients were correlated to those of control, AD, and PSP subjects (1,2).

The number and size distribution of the small neurons showed no significant change in either AD or PSP. However, the average numbers of large neurons in the caudate nucleus and putamen in AD patients were 33% and 30% of those in the controls, and the corresponding numbers in PSP patients were 42% and 30% of those in the controls. The decreases in large neurons showed a quite marked correlation between the caudate nucleus and putamen in both AD and PSP patients (Table)(3).

The average numbers of large neurons in the bnM were respectively 29% and 87% of those in the controls (O) in patients with AD and PSP (Table). A significant correlation existed between the decrease in the number of large neurons in the neostriatum and bnM ($P<0.01$) in AD (▲), but not in PSP (*), as shown in the figure (3).

Table1. Loss of neurons in the caudate head and putamen.

	No. of small neurons		No. of large neurons		
	Caudate	Putamen	Caudate	Putamen	B.n. Meynert
Control	54122±12269	51492± 9195	217±55	218±65	925±156
AD	48512± 6638	44155± 7465	72±45*	66±46*	264±155*
PSP	49251±13433	53377±11763	90±19*	66±22*	808±228

The values indicate mean ± S.D. *P<0.01 comparison with control

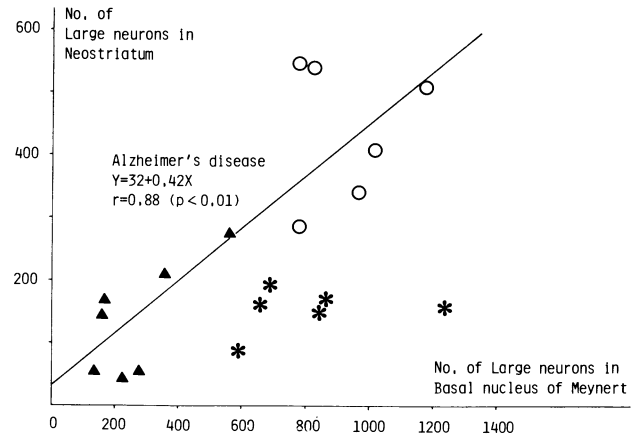

Figure1. Correlation between numbers of large neurons in neostriatum and basal nucleus of Meynert.

DISCUSSION

General observation of the neostriatum in AD and PSP patients revealed that the degeneration of large neurons in the neostriatum and, bnM in these diseases may have close relation to the occurrence of NFTs.

In the present study, cell body and nuclear areas of neurons in the neostriatum of control, AD, and PSP cases were significantly positively correlated, and a significant decrease in the number of large neurons and good preservation of the number of small neurons were revealed. These findings might indicate that the nucleus and cytoplasm of the large neurons show simultaneous and parallel morphological changes such as shrinkage or breakdown in the neostriatum of AD and PSP (1,2).

In the neostriatum, ChAT and acetylcholine esterase have been revealed in the large neurons, and it is suggested that most of the cholinergic neurons exert their action as interneurons and that some of the large neurons make up the efferent neurons of the neostriatum.

The degrees of decrease in large neuron number in the caudate nucleus, putamen and bnM were quite similar in AD, and the decreases were statistically correlative. Thus the large cholinergic neurons in the neostriatum and bnM were considered to degenerate simultaneously in an equal ratio in AD. On the other hand, no such correlation was apparent in PSP (3).

The large cholinergic neurons in both the neostriatum and basal forebrain, including the bnM, diagonal band of Broca, and septal nucleus, are those which exclusively possess nerve growth factor (NGF) receptors within the cerebrum. In the basal forebrain, it has been established that NGF is an essential protein for the development and survival of cholinergic neurons and that depletion of NGF induces degeneration of these neurons. In addition, the NGF receptor-immunoreactive cells appear to degenerate in the basal forebrain of aged subjects. On the other hand, the biological effects on NGF on the neostriatal cholinergic neurons have been shown to include promotion of ChAT activity and prevention of neuronal degeneration following treatment with ibotenic acid.

Since synchronous elevation of the amount of NGF in both the bnM and neostriatum occurs following suction lesioning of the cerebral neocortex and after hypoxic ischemic brain injury, it appears that a close relationship exists between the neocortex and the large cholinergic neurons in both the neostriatum and bnM through the mediation of NGF.

It has been reported that NGF is produced exclusively by astrocytes and that the production is promoted by some kinds of catecholamines. In order to investigate the pathogenesis of AD, it seems necessary to clarify the mechanism of the linkage between degeneration in the neostriatum and in the bnM, and to elucidate the neuron-astrocyte interaction in the formation of NGF for maintenance of the cholinergic neurons.

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A COMPARISON OF "PURE" ALZHEIMER'S DISEASE AND ALZHEIMER'S DISEASE PLUS VASCULAR LESIONS (MIXED DEMENTIA) IN THE ELDERLY

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INTRODUCTION

It is widely accepted that senile dementia of the Alzheimer type and dementia due to vascular lesions are the two most common kinds of dementia in the elderly. However, the clinical criteria required to differentiate these two major progressive dementias are still unreliable (1, 2, 3). Furthermore, it has been shown (4, 5) that there were difficulties in classifying patients based on morphologic findings. Validation studies by Tierney et al (6) and Davous et al (7) demonstrated that depending on the neuropathological criteria applied, the same patients could be classified as Alzheimer's disease or mixed dementia. In order to investigate further the concept of mixed dementia, we established a comparison of neurological, neuropsychological and pathological findings in 2 groups of patients prospectively studied and referred independently by the neuropathologist as "pure" Alzheimer's disease (AD) and Alzheimer's disease plus vascular lesions or mixed dementia (MD).

SUBJECTS AND METHODS

In the present work, we have included 38 subjects after neuropathological examination. Their mean age at death was 85 years. There were 1 male and 37 female. Two groups have been defined according to pathological criteria previously described (8) : 1) patients with "pure" AD (N=23) and 2) patients with AD plus vascular lesions or "mixed" dementia (MD) (N=15). These patients had been prospectively studied before death. All of them had been submitted to the Mini Mental State Examination (MMSE) of Folstein et al (9) and to a standardized neurological examination (10). A quantified neuropsychological study was available in the less demented patients (N=17) (11). The major vascular risk factors were systematically investigated in all cases. All the patients submitted to a CT scan (N=24) had an ischaemic score < 4 (12). Of the 23 AD cases, 22 had been clinically diagnosed as AD. Only 2 of the 15 MD cases received this clinical diagnosis, the other 13 being defined on clinical grounds as AD in 10 and multi-infarct dementia in 3.

Statistical Analysis was performed with the Student's t test and x2 test with Yate's correction for small numbers.

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RESULTS

The mean weight (± SD) of the brains was 1100 g ± 132 in the AD group vs 1086 g ± 153 in the MD group. The mean age at death was higher in the MD group (88,8 ± 3,9) than in the AD group (84,5 ± 7.9). The duration of follow-up was 3.5 ± 2 y for AD patients and 2.2 y ± 1.6 for MD patients. No statistical differences were found between the 2 groups for these 3 parameters.

Vascular risk factors (VRF)

The 2 groups could not be differentiated by the frequency of VRF. These were relatively rare in view of the old age of the patients. No history of stroke was reported in any patient. 12 of 23 AD cases and 8 of 15 MD cases were free of any VRF. The most frequent VRF in the AD group was hypertension (5 cases). It was present in 2 MD cases. The other VRF were found only once or twice in each group. 4/23 AD cases and 1/15 MD cases had 2 VRF associated. No patient in any group had 3 or more associated VRF. (Table I).

Table I. Vascular risk factors

	AD (N=23)	MD (N=15)	P
History of stroke	0	0	NS
Hypertension	5	2	NS
Myocardial infarct	2	0	NS
Angina	2	2	NS
History of cardiac failure	2	0	NS
Atrial fibrillation	0	2	NS
Conduction block	2	0	NS
Diabetes	1	0	NS
Peripheral arteriopathy	1	2	NS

Neurological and neuropsychological examinations

The mean duration between clinical examination and death was 11.8 months. One patient in each group had a stroke before death. The MMSE scores were lower in the AD group (7.5 ± 5.6) than in the MD group (10.7 ± 3) but not significantly different. Abnormal neurological findings were found in a similar proportion of patients in both groups as shown in Table II.

Table II. Major abnormal neurological findings

	AD (N=23)	MD (N=15)	P
Gait or stance abnormality	7	7	NS
Motor weakness	0	0	NS
Frontal release signs	8	6	NS
Extra-pyramidal signs	17	10	NS
Babinski's sign	2	1	NS
Myoclonus	2	1	NS
OKN abolition	9	5	NS
Incontinence	17	7	NS

OKN : optokinetic nystagmus

A detailed neuropsychological study was available in the less demented patients, 10 AD and 7 MD cases. The scores of memory, language, praxia and gnosia were similar in both groups as shown in table III.

Table III. Neuropsychological scores (Mean ± SD)

	AD (N=10)	MD (N=7)	P	Normal* (N=20)
Memory	61.1 ± 12.2	61.2 ± 9.5	NS	92 ± 9
Language	60.6 ± 35.7	68.8 ± 25	NS	9.3 ± 6.7
Praxia	29.6 ± 14.9	29.1 ± 11	NS	5 ± 3
Gnosia	35.7 ± 11.1	36.8 ± 4.1	NS	10 ± 7

* Normal values for age - matched control patients (ref. 11)

Neuropathological findings

Senile plaques were counted in 6 cortical areas and in the hippocampus of both hemispheres, using the Thioflavine S staining. There was no significant difference between the mean number of plaques in the left and right hemispheres in both groups. The mean number of plaques was significantly higher in the frontal cortex and in the parieto-occipital cortex of AD cases compared with MD cases although there was no significant difference between the 2 groups in the temporal cortex and the hippocampus (Table IV).

Table IV. Mean density/mm2 (± SD) of senile plaques

	AD (N=23)	MD (N=15)	P
Frontal cortex	9.4 ± 5.2	6 ± 3.4	< .05
Temporal cortex	12 ± 7.8	7.9 ± 4.5	NS
Pariéto-occipital cortex	10 ± 4.8	7.1 ± 3.2	< .05
Hippocampus	10 ± 4.8	8 ± 4.4	NS
Total	10.5 ± 4.8	7.1 ± 2.4	< .05

Neurofibrillary tangles (NFT) were counted semi-quantitatively in the same areas. In 13 AD cases, the neocortex was almost free of NFT, i.e. the density was 1/mm2 or less in all areas studied, except the hippocampus. In 5 AD cases, the density of NFT was 10/mm2 or more in the neocortex, the highest level located in the temporal lobes. The 5 other AD cases had 2-10 NFT/mm2 in the neocortex. In MD, only 1 case had more than 1 NFT/mm2 in the neocortex. In the hippocampus, the density of NFT was > 10/mm2 in most ca-

Table V. Vascular lesions

	AD (N=22)	MD (N=15)	P
Atheroma of great vessels	4	6	NS
Capillary hyalinosis	9	13	< .01
Amyloid angiopathy	15	11	NS
Infarctions	1	15	< .001
White matter lesions	0	5	< .02

ses in both groups. The mean density of hippocampal NFT was higher in AD than in MD patients (respectively 15 $\pm$ 6 vs 11.2 $\pm$ 8.2) but the difference was not significant.

Vascular lesions : the results are summarized in Table V.

One patient in each group had a recent infarction of large volume (300-400 ml) related to a stroke which happened shortly before death. As previously defined, no ischaemic foci were found in the other 22 AD patients. Softenings were present in 15/15 MD cases with a volume of infarction varying from 10 to 200 ml (mean 30 ml). They were cortical in 1 case, subcortical in 9 cases and mixed cortical and subcortical in 5 cases. Amyloid angiopathy was common in both groups. MD patients had significantly more frequent capillary hyalinosis and white matter lesions. Atheroma of the great vessels appeared more frequent and more severe in the MD group than in AD but the difference was not significant.

Subcortical lesions. The subcortical structures from which originate the major cholinergic, dopaminergic, noradrenergic and serotoninergic pathways showed significant lesions with neuronal loss, SP and NFT in the nucleus basalis, Lewy bodies in the locus coeruleus or in the S. nigra. The frequency of these lesions is indicated in Table VI.

Table VI. Frequency of subcortical lesions

	AD (N)	MD (N)	
Nucleus Basalis	16/17	13/14	NS
S. Nigra	6/22	4/14	NS
Locus Coeruleus	17/23	4/14	$< .01$
Raphe nuclei	6/22	2/14	NS

The lesions of the locus coeruleus were significantly more frequent in the AD group.

COMMENTS

These results are consistent with previous findings showing that there is a substantial overlap between degenerative and vascular dementia in the elderly (4, 13). In the present study, no significant differences in age, vascular risk factors, neurological signs or type of intellectual impairment were observed between AD and MD patients. It is of interest that no history of stroke was reported in the later group and that hypertension, considered to be a major VRF in the elderly, was equally frequent in both groups. The ischaemic score never predicted MD, suggesting that it might not be very reliable for this diagnosis.

The most clear-cut findings which distinguished both groups were purely morphologic : AD patients had a higher number of plaques and NFT in the neocortex and a more severe neuronal loss in the locus coeruleus, whereas MD patients had softenings, more frequent small vessel disease and white matter changes. However, all patients had significant lesions of the Alzheimer type and it is therefore likely that AD was the primary dementing illness in these two groups since MD patients had rather limited vascular lesions. It is possible that these lesions contributed to the clinical syndrome in relation to their location rather than to their volume but the extent of this contribution is difficult to assess since no significant dif-

ference in the neurological signs and neuropsychological deficits was observed.

In conclusion, there is much discrepancy in the literature on vascular and mixed dementias (14, 15, 16). Our results suggest that the concept of mixed dementia is of questionable usefulness until it is proven that it is related to specific clinical signs or vascular lesions.

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CHOLINE ACETYLTRANSFERASE AND SUBSTANCE P LEVEL IN ALZHEIMER'S DISEASE AND MULTI-INFARCT DEMENTIA

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INTRODUCTION

Neurochemical studies on dementia of Alzheimer type (SDAT) have indicated changes not only in the cholinergic system but also an involvement of other transmitters such as those of the noradrenergic, dopaminergic, and serotonergic systems. For substance P the results have not been consistent, though both decreased and unchanged levels of substance P have been reported. The purpose of this study was to measure substance P-like immunoreactivity and choline acetyltransferase activity in brains from SDAT patients and from patients with multi-infarct dementia (MID).

MATERIALS AND METHODS

The SDAT group consisted of eight patients: 7 males, 1 female; mean age of 78 ± 2 years; mean duration of the disease of 5 ± 0.6 years. The diagnosis SDAT was decided on clinical (DSM-III) and histopathological grounds. The MID group consisted of 11 patients (6 males, 5 females; mean age of 78 ± 2 years) with a mean duration of the disease of 5 ± 0.7 years. Nine patients (3 males, 6 females ; mean age of 73 ± 3) with no neurological or psychiatric illness comprised the control group. At autopsy frontal cortex, hippocampus, amygdala and caudate nucleus were dissected.

The tissue was frozen at -70°C and then pulverized in liquid nitrogen. Pulverized tissue was added to approximately 10 volumes of 1 M acetic acid at +90 °C. The samples were cooled on ice for 5 min and homogenized in a Teflon/glass homogenizer. After another 5-min heating at +90 C the samples were centrifuged at 2000 g for 20 min. Separation of substance P from substance P C- and N-terminal fragments was done according to the method of Sakurada et al.[1]

The choline acetyltransferase (ChAT) activity was determined according to the radioenzymatic method.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

RESULTS AND DISCUSSION

As shown in Table 1, the ChAT activity in the hippocampus was reduced markedly both in the SDAT (-60 %) and MID (-53 %) groups. These findings are in agreement with those of Perry et al.,[2] although the decrease in ChAT activity of the MID group was less pronounced in their study.

Table 1. Description of the cases

	Control	Alzheimer	Multi-infarct Dementia
Age (yrs)	73 ± 3	78 ± 2	78 ± 3
Sex	6F/3M	1F/7M	5F/6M
Duration (h) Time death-autopsy	38 ± 4	27 ± 5	41 ± 6
Brain Weight (g)	1282 ± 35	1395 ± 34	1325 ± 36
Art.scl.[a]	5+/3++	4+/2++	5+/5++
ChAT (nkatal/g) hippocampus	1.62 ± 0.11	0.65 ± 0.10***	0.76 ± 0.11***

[a]Degree of arteriosclerosis in basal vessels, according to Gottfries et al.[3]
***p<0.001 when compared with control.

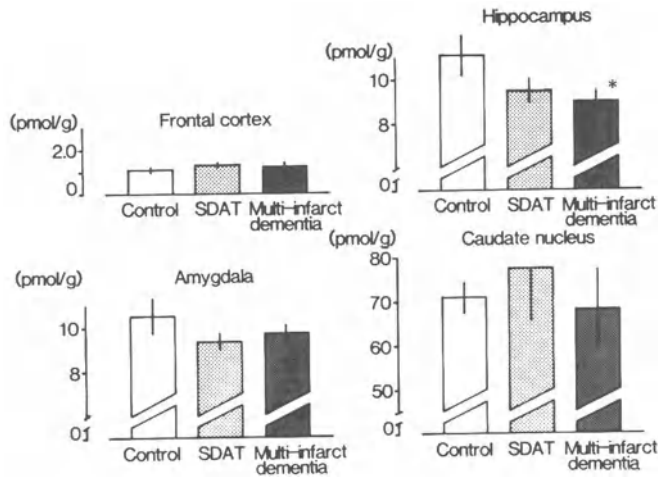

Fig. 1 Substance P-like immunoreactivity (pmol/g tissue) in different brain regions of patients with SDAT and multi-infarct dementia.
*p<0.05 when compared with control.

Substance P-like immunoreactivity was measured in four brain regions. A marked regional difference was noted in control brains with highest content in the caudate nucleus, intermediate in the hippocampus, amygdala and lowest in the cortex. In this study, ion-exchange chromatography was used to eliminate contamination of substance P by precursors and fragments, which might cross-react with substance P antiserum. As seen in Fig. 1, a significant reduction in substance P-like immunoreactivity was found in hippocampus (-35 %) of the MID group, while no significant change was found in the SDAT group. As noted in Fig. 1, however, there was a tendency towards lower values in the hippocampus and amygdala of the SDAT group in comparison with those of the control group, but the differences were not significant.

When the data for substance P-like immunoreactivity was plotted against ChAT acivity in a coordinate system, a positive correlation was noted in the hippocampus of the dementia groups, especially in the MID group. In SDAT the muscarinic receptors seem to be preserved,[4] and even a receptor compensatory mechanism has been suggested.[5] On the other hand, in MID a decreased number of muscarinic receptors in the hippocampus has been recorded.[6] This was also the case for chronic alcoholics where parallel decreases in ChAT activity and number of muscarinic receptors were measured.[7] These changes might indicate a more unselective and general damage to the nerve terminal as compared with that in SDAT. The finding of a significantly reduced substance P-like immunoreactivity in MID also points in that direction. Interestingly, the coexistence of ChAT and substance P in brain has been reported.[8]

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A STUDY ON POSTMORTEM CHANGES IN VASOPRESSIN mRNA IN RAT BRAIN USING A NON-ISOTOPIC METHOD

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INTRODUCTION

Recent studies in Alzheimer type dementia(ATD) disclosed a depletion of several neuropeptides in postmortem brains. With the progress of molecular biology, we can also study changes in neuropeptides messenger RNA(mRNA) to interpret the depletion in ATD brains. In general, radioactive probe has been used in the hybridization technique. However, we already reported in situ hybridization histochemistry (ISHH) method using a biotinylated oligonucleotide probe(Arai et al.,1988). In the present study, we tried to establish a method of dot and northern blot analysis using non-radioactive probes for quantification of mRNA.

When we measure RNA content in human brains obtained from autopsy, however, one of the important problems lies in the evaluation of postmortem changes. In several studies, the RNA stability was investigated in postmortem brains by OD260 measurement. These studies described that RNA's have extensive stabilities in the postmortem period. On the other hand, Taylor et al.(1986) suggested the range of defined RNA's detected by hybridization techniques might be influenced by the postmortem period.

In the present study, therefore, the postmortem changes in vasopressin mRNA were also investigated in rat brains obtained at various postmortem periods as a model of autopsied human brains using Northern blot analysis with non-isotopic probes.

MATERIALS AND METHODS

An oligonucleotide probe for vasopressin mRNA (30mer)was made by a Applied Biosystems DNA synthesizer and biotinylated by the terminal deoxynucleotide transferase method with a Terminal Labeling Kit(Enzo Inc.). The probe was complementary to nucleotides cording for the the first 10 amino acids of the glycopeptide region of the vasopressin/neurophysin precursor.

Male adult Sprague-Dawley rats were used, and were divided into two groups. One group was maintained under normal conditions. The other group was given 0.34M (2%wt/v) sodium chloride up to 1 week for salt loading(dehydration). Rats were deeply anaesthetized and then sacrificed.

To prepare various postmortem conditions, we kept the rats at room temperature (25 ℃) for 0,8,24 hours after sacrifice. Then, the brain was removed and the hypothalamic region was separated from the whole brain. The tissue was homogenized in 4M guanidium thiocyanate solution. Total RNA was extracted by the methods of Chirgwin et al.(1979)and Yoshikawa et al.(1988). Amounts of RNA were quantified by OD260 absorption, and the purity was certified by the ratio of OD260/280.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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The amounts of vasopressin mRNA was quantified by dot blot analysis. RNA was blotted onto a nitrocellulose membrane(Millipore) using a BRL hybridot manifold. The membrane was prehybridized for 4 hrs at 30℃ in 50% formamide, 1× Denhardt's solution (containing 0.1% each of Ficoll 400,polyvinylpyrrolidone, and bovine serum albumin), 5 ×SSC, 50mM sodium phosphate buffer (pH 6.5), 0.1% sodium dodecyl sulphate(SDS), 0.5% dextran sulphate, 250 μg/ml sermon sperm DNA, and 32 μg/ml yeast tRNA. This was replaced with fresh hybridization solution containing the biotinylated oligonucleotide probe at a concentration of 50-100ng/ml. The membrane was hybridized for 36-40 hrs at 30℃.

Northern blot analysis was performed for the detection of vasopressin mRNA hybridization signal. Total RNA (10 μg) was loaded onto a 1.2% agarose gel and electrophoresed. The RNA was transferred to a piece of nitrocellulose membrane and then hybridized as described above.

Visualization of vasopressin mRNA was made with the use of a BluGENE nucleic acid detection system(BRL). The membrane was washed two times for 10 minutes each time in 2 ×SSC, two times for 10 min each time in 0.2×SSC, and finally two times for 30 min each time in 0.1 ×SSC at 42 ℃. Final color development was according to the manufacturer's information. The amount of specific mRNA was measured by a MCID system(Imaging Research).

The method of ISHH was described elswhere(Arai et al.,1988).

RESULTS

Total RNA yields from rat hypothalami ranged from 0.68 to 1.15 μg/mg tissue, and were not related to the salt loading and postmortem delay.

Vasopressin mRNA in the hypothalamus was dramatically increased in the salt-loaded rats compared with the normal rats, as expected(Fig.1). This finding was confirmed by the result of Northern blot analysis(not shown).

Concerning the postmortem change, vasopressin mRNA could be detected in the rat brains obtained at 8 hrs postmortem, but not in the brains obtained at 24 hrs postmortem (Fig.2). Almost the same results were obtained in the ISHH experiments using rat models(not shown).

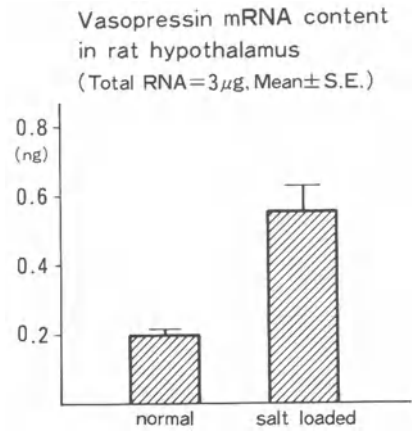

Fig.1. The results of dot blot analysis
Normal rats (n=4). Salt-loaded rats (n=4).

Fig.2. Northern blot analysis for vasopressin mRNA .
Right lane: rat brain with an 8-hr postmortem delay.
Left lane: rat brain with a 24-hr postmortem delay.
Total RNA = 15 μg

DISCUSSION

Commonly, radioactive probes are used in the hybridization technique. However, non-isotopic method has several advantages compared with the isotopic method. 1)The non-isotopic method can be handled more easily than the routine isotopic method that requires special facilities. 2)The non-isotopic method does not require autoradiography so that the visualization can be carried out more quickly. 3)Biotinylated probes are more stable than radioactive probes. The result of dot blot hybridization for vasopressin mRNA comparing salt-loaded rats with normal rats indicates that our method can be useful for quantification of neuropeptide mRNA.

Johnson et al.(1986) demonstrated mRNA's to have extensive stabilities in the postmortem period. They investigated rat and human postmortem brains with the method of A260 absorption and by Northern blot analysis. For the latter, they used probes for Thy-1, a glycoprotein found in synaptic junctions, B-tublin, a cytoskeletal protein, and the hamster scrapie PrP27-30 protein. On the other hand, Taylor et al. (1986) suggested a postmortem reduction in somatostatin mRNA in rat brain during postmortem delay. The present results concerning the postmortem changes in vasopressin mRNA support the findings by Taylor et al. The discrepancy among the studies might be explained by the different metabolism between the cytoskeletal protein mRNA's and the neuropeptide mRNA's. The present findings strongly suggest that neuropeptide mRNA's should be investigated in autopsied human brains as soon as possible after death.

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TOPOGRAPHICAL DISTRIBUTION, FINE STRUCTURE OF COMPONENT FIBRILS, AND IMMUNOREACTIVITY OF NEURONAL INCLUSIONS IN ALZHEIMER'S, PARKINSON'S, AND PICK'S DISEASES

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INTRODUCTION

Neurofibrillary tangles(NFT's), Pick bodies(PB's), and Lewy bodies(LB's) are filamentous neuronal inclusions that individually characterize different diseases. The distribution pattern, fine structure of the component fibrils, and immunoreactivity of these inclusions seem to share much in common with one another[1-4]. However, it has not been fully documented what similarity and dissimilarity can exist among them. It is not yet clear whether NFT's in cases of Alzheimer's dsease, Down's syndrome, and myotonic dystrophy show an identical immunoreactivity. The purpose of this study was to elucidate these points.

MATERIALS AND METHODS

The brain, spinal cord, and autonomic nervous system of the following autopsy cases were used in this study : 3 patients with Alzheimer's disease(65-y-o M, 58-y-o M, and 66-y-o F); 4 with Parkinson's disease(72-y-o F, 70-y-o M, 75-y-o M, and 38-y-o M with diffuse LB's); one[4] with Pick's disease with diffuse PB's; one with Down's syndrome, aged 45 years; and one with myotonic dystrophy, aged 61 years. Topographical distributions of each type of inclusion in the respective cases were investigated mostly on silver-impregnated preparations. They were mapped and compared. Distribution maps of the same disease were superimposed to obtain the distribution pattern and maximal range. Samples for electron microscopy were taken from each of the cases, mostly at autopsy, and fixed in 2.5 % glutaraldehyde in PBS and then in 1 % OsO4 solution. All the samples were embedded in epoxy-resin by the ordinary method. Thin sections were double-stained and observed. For immunocytochemistry, anti-human phosphorylated tau antibody(rabbit) and anti-bovine ubiquitin antibody(rabbit) were given to us by Dr. Y. Ihara(Tokyo Metropolitan Institute of Gerontology) and Dr. S. Tsuchida(Hirosaki University School of Medicine), respectively. It had been confirmed that they reacted with human phosphorylated tau and human ubiquitin, respectively. The avidin biotin peroxidase complex immunocytochemical method (VECTASTAIN KIT) was adopted.

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RESULTS

Topographical distributions of three types of inclusion are shown in Table 1. The distribution pattern and the maximal range of NFT's obtained were consistent with previous reports by others.[5,6] Topographical distribution of the case of Pick's disease with diffuse PB's was like that described in our recent publication,[4] which was in agreement with previous reports by others. Topographical distributions of the cases of Parkinson's disease, including one with diffuse LB's were seen in our earlier study,[7] and are consistent with previous reports by others.[8,9] Comparison of the maximal distribution ranges and patterns of the three

Table 1. Topographical distribution patterns of neuronal inclusions

	NFT's in Alzheimer dis.	NFT's in Down synd.	LB's in Parkinson dis.	PB's in Pick dis.
F_1, F_2, F_3	4-3	4	3	4
cingulate gyr.	4	4	4	4
orbital gyr.	4	4	2-3	3
rectal gyr.	4	4	2-3	3
olfactory bulb	4	4	?	3
T_1, T_2, T_3	3-4	4	4	4-3
amygdaloid	4	4	4	4
fusiform gyr.	4	4	4	4-3
parahippocampus	4	4	4	4
hippocampus	4	4	1-2	4
insula	4	4	4	4-3
claustrum	2	3	2	3
innominate subst.	3	3	3	3
hypothalamus	3	3	2-3	3
caudate	2	2	0	3-4
putamen	2-3	3	1-2	2
globus pallidus	2	3	0	2
thalamus	3	3	0	0-1
motor cortex	3-4	3-4	2-3	2
sensory cortex	3-4	3-4	2-3	2
parietal lobule, sup.	3-4	3-4	2-3	2
inf.	4	4	3	4
calcarine	3	3-4	0-1	0-1
occipital cortex	4	4	2-3	2-3
tectum	1	2	1	3-4
central gray	2	3	2-3	3-4
oculomotor nucl.	1-2	3	1	2
red nucl.	0-1	1	0	2
substantia nigra	1	2-3	3	2
reticular formation	2	3	2	2
dorsal raphe nucl.	2-3	3	2	2
superior central nucl.	2-3	3	2	3
reticulotegmental nucl.	2	2	1-2	2
locus coeruleus	1	3	3	2
pontine nuclei	0	2	0	2
dorsal vagal nucl.	0	1	2	2
inferior olive	0	0	0	0
arcuate nucl.	0	0	0	2
cerebellar cortex	0	0	0	0
cerebellar nuclei	0	0	0	0
anterior horn	0	1	1	1
posterior horn	0	0	0	0
intermediolateral nucl.	0	0	1	0
sympathetic ganglia	0	0	3	0

NFT's, neurofibrillary tangles; LB's, Lewy bodies; PB's, Pick bodies.
0, none; 1, a few; 2, several; 3, many; 4, numerous; ?, not examined

Table 2. Immunoreactivity of three types of filamentous neuronal inclusion.

	Neurofibrillary tangles			Pick	Lewy
	Alzheimer dis.	Down syndr.	Myotonic dystr.	bodies	bodies
Anti-ubiquitin	+	+	+	+	+
Anti-tau	+	+	+	+	-

types of inclusion showed a considerable similarity. Only a quantitative difference could be seen between the anatomical structures affected by NFT's and those by PB's, such as the red nucleus and tectum of the midbrain where occurrence of PB's was much greater than that of NFT's. Many PB's and LB's were also found in the structures where many NTF's occurred. The pattern and maximal range of LB distribution showed some similarity to those of NFT's. A qualitative difference between the two, however, was seen in that many LB's were found in the sympathetic ganglia, but only several in Ammon's horn (CA1), and none in the thalamus, red nucleus, and pontine nuclei, and vice versa for NFT's.

The purpose of the electron microscopic observations was to confirm that each type of inclusion had respectively the identical ultrastructure as had been described in each disease. NFT's were composed of massive paired helical filaments (PHF's) and, in part, of straight tubules. Each PHF measured about 20-25 nm in diameter at its widest, and was periodically reduced to 10 nm about every 80 nm. Individual straight tubules measured approximately 15 nm in diameter, as had been described by other authors.[10] PB's were composed of smooth-surfaced, straight, tubular filaments and/or of long-period constricted fibrils. Each straight tubular filament measured about 15 nm ± 3. Each long-period constricted fibril measured about 25 nm at the widest and was periodically reduced to 10 nm about every 160 nm. LB's in the cerebral cortex were composed of very fuzzy filaments measuring about 14 nm ± 4 in diameter. LB's in the brainstem were the same as those described by many authors.[7]

The results of immunocytochemistry of the three types of inclusion are given in Table 2.

DISCUSSION

The comparison of the maximal distribution patterns of three types of inclusion showed a considerable similarity. The similarity of the distribution pattern of PB's and NFT's has been pointed out by some authors.[4] From our results, only a quantitative difference could be seen between the anatomical structures affected by NFT's and those by PB's. Many PB's and LB's were present in the structures where many NFT's were found, except in Ammon's horn. A paucity of LB's in Ammon's horn(CA1) has also been shown in diffuse LB disease.[8] Distribution pattern of LB's has been investigated in the brainstem and in the cerebrum in Parkinson's[7,9] and diffuse LB disease.[8] In the light of these reports, the LB distribution pattern was found to show some similarity to that of NFT's. A qualitative difference between the two, however, lay in the occurrence of many LB's in the sympathetic ganglia, and only several in Ammon's horn(CA1), and none in the thalamus, red nucleus, and pontine nuclei. Electron microscopic observations revealed the characteristic fine structure of the component fibrils of each type of inclusion. The morphology of the individual fibrils was in agreement with the descriptions by other authors. A similarity of the component fibrils of PB's to those of NFT's can readily be acceptable. Major component fibrils of LB's were about 14-nm(± 4)

filaments with a fuzzy surface, which differ from those of NFT's or PB's. Monoclonal antibodies BF 10 and RT 97 are known to react specifically with 160-KD and 200-KD neurofilament proteins, respectively.[1,2] It has been shown that these two monoclonal antibodies label both NFT's of Alzheimer's disease and PB's, but not LB's.[1,2] The present study has showed that the antibodies to tau and ubiquitin label PB's, as well as NFT's in cases of Alzheimer's disease, Down's syndrome and myotonic dystrophy. Similar data were reported by Love et al.[3] LB's have been found to share epitopes with ubiquitin, but not with tau. This is consistent with the fine structural similarity of the component fibrils between NFT's and PB's, and with the less similarity of those between the former two and LB's. Ubiquitin is known to play an important role by conjugation to cellular proteins for their digestion by a specific, ATP-dependent protease. The demonstration of ubiquitin immunoreactivity in the three types of inclusion, in diverse disorders, leads us to the speculation that the ubiquitin accumulation in these inclusions results from its conjugation to proteins that are resistant to digestion rather than from defective proteolysis itself.[3] The partial similarity of the topographical distribution pattern, of the fine structure of component fibrils, and of the immunoreactivity of these inclusions may reflect a commonality of cell-metabolic responses to causal factors of these inclusions.

SUMMARY

Topographical distribution, fine structure of the component fibrils, and immunoreactivity of filamentous neuronal inclusions in Alzheimer's , Parkinson's, and Pick's diseases were studied to elucidate what similarity and dissimilarity existed among them. There was only a quantitative difference between the maximal distribution pattern of NFT's and that of PB's. A qualitative difference, however, was seen between that of NFTs and of LBs : many LB's occurred in the sympathetic ganglia, only several in Ammon's horn(CA1), and none in the thalamus, red nucleus, and pontine nuclei ; and for NFT's, vice versa. There was a considerable similarity of fine structure of the component fibrils of PB's to that of NFT's, whereas there was little such similarity between LB's and NFT's. This is consistent with the immunoreactivity of these inclusions :PB's as well as NFT's in cases of Alzheimer's disease, Down's syndrome, and myotonic dystrophy showed a positive reaction to anti-tau antibody, whereas LB's did not. The three types of inclusion all showed a positive immunoreaction to anti-ubiquitin antibody. In conclusion, a partial similarity of the topographical distribution pattern, of the fine structure of component fibrils, and of the immunoreactivity of these inclusions may reflect a commonality of cell-metabolic responses to causal factors of these inclusions.

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NEURONAL LOSS IN THE SUBSTANTIA NIGRA AND VENTRAL TEGMENTAL AREA IN PARKINSON'S DISEASE AND ALZHEIMER'S DISEASE

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SUMMARY

Regional neuronal loss in the substantia nigra (SN) and ventral tegmental area (VTA) was studied in relation to extrapyramidal symptoms and dementia in patients with Alzheimer's disease (AD) and Parkinson's disease (PD) and controls. The number of neurons in the substantia nigra was reduced both in AD and PD, although to a greater degree in the latter. In the VTA, reduction in neuronal counts was seen only in PD but not in AD. Nigral degeneration was related to extrapyramidal symptoms in both AD and PD. Interestingly, dementia was associated with neuronal loss in the medial SN in PD but not in AD. The results suggest that nigral degeneration may contribute as a subcortical component to the intellectual impairment in patients with PD but not in AD implicating the possibility of different pathophysiological bases of cognitive impairment in these diseases.

INTRODUCTION

The two most prominet brainstem areas rich in dopaminergic neurons are substantia nigra (SN) and ventral tegmental area (VTA). Neurons in the SN project to the striatum and together with the neurons in the VTA to cortical and limbic areas (1-4). Neurons in the SN show a topographical organisation in which the medial neurons project predominately to the caudete nucleus, whereas putamen receives its nigral input mainly from the lateral part(5). It has been suggested that the striatal nuclei may have separate functional roles, the putamen having a predominately motor role and caudate nucleus taking part in cognitive functions (6,7).

In order to see whether the degeneration of VTA or SN is related to extrapyramidal symptoms or dementia of the patients with AD and PD, the number of neurons in these brain areas were calculated and correlated with the clinical variabless of the patients. The results concerning nigral degeneration in PD have been published earlier (8) but the results are included to enable comparison between PD and AD. Also the results concerning neuronal loss in the VTA have been published elsewhere (9).

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PATIENTS AND METHODS

The neuronal densities were investigated in 27 patients with AD (mean age 79.8 ± 4.9 years) and 12 patients with idiopathic PD (74.0 ± 7.3 years) and 18 controls (77.6 ± 6.9 years). At autopsy, the brain was halved sagittally and the left half was stored deep frozen for biochemical analyses and the right half was fixed and subjected to neuropathological investigation. Due to the nearness of the VTA to the cutting line, samples from all of the patients were not representative and thus, the number of individuals in SN study is greater. The diagnostic criteria and the scoring of the severity of dementia and extrapyramidal symptoms has been described earlier (8,9).

SN degeneration was quantified using 5-um-thick Bielscowsky sections The pars compacta of the SN used for calculations was divided into four areas (from medial to lateral). Neurons hitting a rectangular test area delineated by an ocular grid were counted. The VTA was divided into two distinct neuron groups: central and paranigral. Three 20-um-thick sections stained with cresyl Violet were used to count the number of neurons. Student's t-test with Bonferroni correlation was used to compare individual patient groups with each other. The correlation of the clinical variables with the neuronal counts were determined by Spearman rank correlation test.

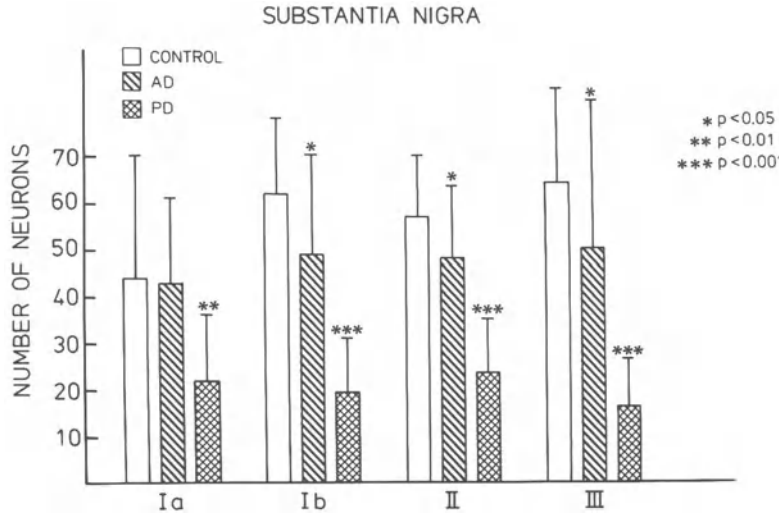

Figure 1. Number of neurons (mean + SD) in four areas of the substantia nigra (see text) in patients with Alzheimer's disease and Parkinson's disease. P-values refer to comparisons with controls.

RESULTS

In PD the reduction in the number of neurons in the SN was substantial (Figure 1), the decline being greatest in the lateral part. In AD the neuronal counts were moderately reduced, being 97%, 79% ($p<0.05$), 83% ($p<0.05$) and 78% ($p<0.05$) of the control values from the medial to lateral part of the SN respectively (Figure 1).

In PD the number of lateral neurons had a negative correlation with the hypokinesia and rigidity of the patients. Dementia was, however, more severe in those patients showing few neurons in the medial part of the SN ($r = -.77$, $p=0.004$). In AD no significant correlation was found between the number of nigral neurons and the degree of dementia. Of the extrapyramidal symptoms rigidity ($r = -.45$, $p<0.05$) and orofacial dyskinesias ($r = -.49$, $p<0.05$) were associated with reduced neuronal counts in the lateral part of the SN.

In patients with PD, the number of neurons in the central part of the VTA was reduced to 27% of control value ($p<0.05$) whereas a nonsignificant decline (to 66% of controls) was seen in the paranigral part of the nucleus. In AD the number of neurons in both parts of the VTA was comparable to that of the controls.

The degree of dementia of PD patients showed a tendency towards negative correlation with the neuronal count in the central VTA ($r = -.40$, $p<0.1$). In AD no clear-cut correlations were seen.

DISCUSSION

In this study, degeneration of the SN was found to be related to extrapyramidal symptoms and dementia in patients with PD and also to extrapyramidal symptoms in AD, whereas no such correlations were seen in the VTA.

In the present study, the overall loss of neurons in the SN and VTA in patients with AD was of the same degree that has been reported in previous studies (10-13). A much more greater deal of the neurons was found to be lost in PD. Indeed, it has been found that at least 80% of the dopaminergic input to the striatum must be lost before parkinsonian symptoms manifest (10).

VTA projects mainly to the frontal cortex and limbic areas (1-4). In the human brain SN, in addition to its striatal projections, contributes also to the cortical and limbic dopaminergic innervation. While the human VTA is almost rudimentary, it may be that in humans SN is at least partially involved in functions that are performed by VTA neurons in animals.

The present results suggest that nigral projections may contribute as a subcortical component to the cognitive impairment and dementia process seen in PD. However, no clear-cut association was found between nigral degeneration and intellectual impairment in AD. This may explain the differences seen in the pathophysiological bases and clinical manifestations of cognitive impairment in AD and PD.

ACKNOWLEDGEMENTS

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CHARACTERISTICS OF REACTIVE MICROGLIA IN ALZHEIMER'S AND PARKINSON'S DISEASE BRAIN TISSUE

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The brain has been believed to have an immunologically privileged status. Factors such as the absence of a lymphatic drainage, the restricted entry of globulins through the blood-brain barrier and a high tolerance to grafts appear to account for this view[1]. We suggest that brain responds in an appropriate fashion to immunological challenge and that microglia are resident representatives of the immune system. These cells express major histocompatibility complex (MHC) molecules, which are cell surface glycoproteins that play a significant role in the immune response. T-lymphocytes can interact only with cells expressing such MHC molecules. In Alzheimer's and Parkinson's disease brain, reactive microglia express the MHC class II molecule, HLA-DR[2,3]. HLA-DR is the major MHC glycoprotein involved in foreign antigen presentation to T helper/inducer lymphocytes.

MATERIALS AND METHODS

The brains of Alzheimer's and Parkinson's disease cases as well as cases without neurological complication were obtained within 2-14 hours of death. The brain blocks were fixed in a cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) pH 7.4 for 2 days. After cryoprotection, sections were cut on a freezing microtome at 30 μm thickness and collected in phosphate buffered saline containing 0.3% Triton-X100. Free floating sections were processed according to the immunohistochemical method described previously[2]. Some sections were counterstained with thioflavin S for demonstration of senile plaques[4]. Monoclonal antibodies used were; HB104 (American Type Culture Collection (ATCC)) for HLA-DR; anti-leucocyte common antigen (LCA) (Dakopatts); Leu-M5 (Becton-Dickinson) for p150/95; 32.2 (Medarex) for IgG Fc receptors (FcγR)I; 2E1 (AMAC) for FcγRII; Leu-3A (Becton-Dickinson) for CD4; and DK25 (Dakopatts) for CD8 molecules. Rabbit anti-glial fibrillary acidic protein (GFAP) (Dakopatts) was used to identify reactive astrocytes. Goat anti-type IV collagen (Chemicon) and mouse monoclonal HB120 (ATCC) for MHC class I antigen were used to label blood vessels in double immunostaining.

For immunoelectronmicroscopy, small tissue samples were fixed in cold 1% glutaraldehyde, 4% PFA in PB for 6-24 hours. Fifty μm vibratome sections were immunostained, postfixed in 1% osmium tetroxide and embedded in Epon. Ultrathin sections were counterstained with uranyl acetate and lead citrate, and examined with a Phillips 201 electron microscope.

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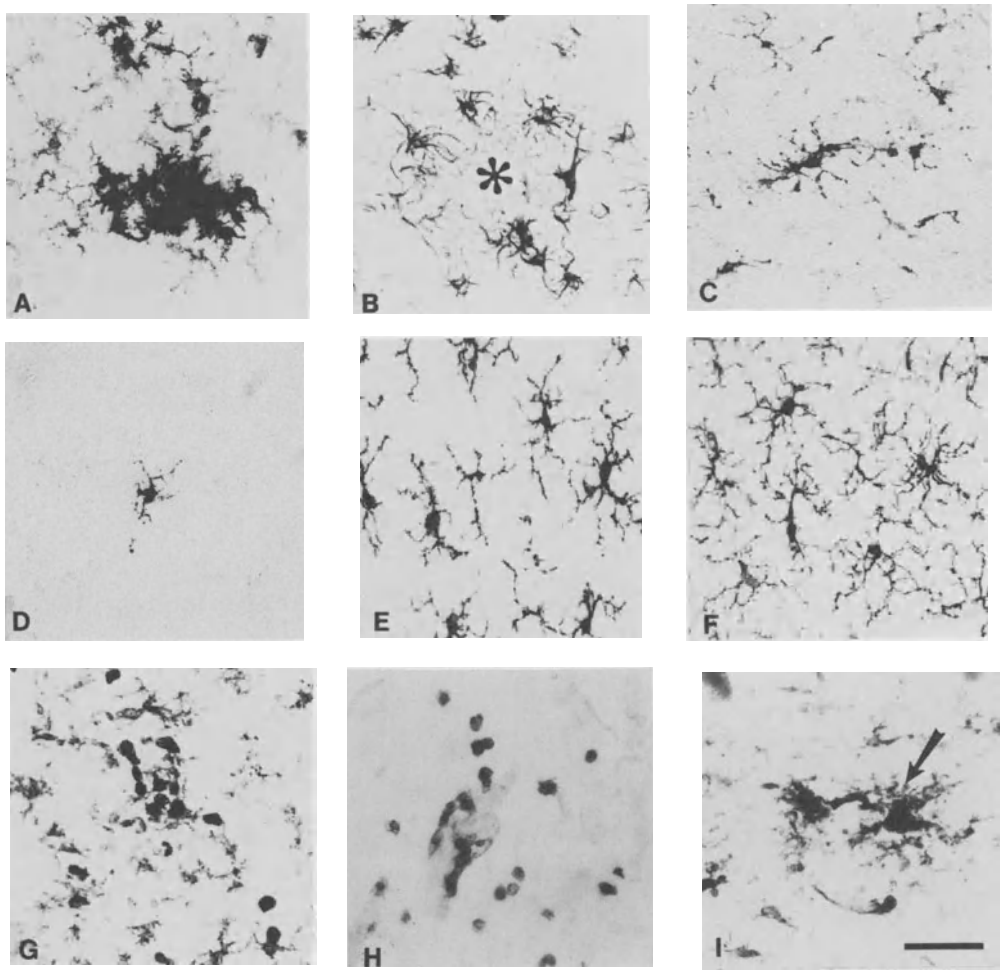

Fig. 1. A: HLA-DR staining of an Alzheimer's disease hippocampal section showing numerous reactive microglia. Arrow indicates a microglial agglomerate associated with a senile plaque. B: GFAP staining. GFAP positive astrocytes are gathered around the periphery of a senile plaque (*). C: HLA-DR staining of a control case white matter. HLA-DR positive cells show a morphology consistent with that of resting microglia. D: HLA-DR staining of a control case grey matter. Only a small number of microglia stain positively. E: FcγRI staining of an adjacent section of D. Numerous resting microglia stain positively. F: P150/95 staining of another adjacent section of D. Resting microglia stain positively. The majority of these FcγRI (E) and p150/95 (F) positive resting microglia are negative for HLA-DR (D). G: LCA staining of an Alzheimer's disease hippocampus. In addition to LCA positive microglia, a significant number of leucocytes are seen. H: Double immunostaining for CD8 and MHC class I antigen. CD8 positive lymphocytes are located not only in blood vessels but also in the brain parenchyma. Blood vessels are lightly labelled with MHC class I antigen. I: Parkinson's disease SN stained for HLA-DR. HLA-DR positive microglia congregate around degenerated melanin containing neurons (arrow). Such HLA-DR positive microglia are virtually absent in the SN of control cases. Scale bar = 50 μm.

RESULTS

As previously reported[2,3], antibody to HLA-DR strongly stained cells with a morphology consistent with that of reactive microglia originally described by del Rio Hortega. Double immunostaining for HLA-DR and GFAP established that GFAP positive astrocytes were negative for HLA-DR. In Alzheimer's disease cerebral cortex, a large number of reactive microglia were positive for HLA-DR in both grey and white matter. In grey matter, they frequently formed agglomerates (Fig. 1A, arrow) located in the center of senile plaques. GFAP positive astrocytes, by contrast, congregated at the margin of senile plaques (Fig. 1B).

In control cases, only small numbers of microglia expressed HLA-DR. They were more numerous in white (Fig. 1C) than grey matter (Fig. 1D). However, antibodies to LCA, p150/95, FcγRI and FcγRII consistently stained both resting and reactive microglia in control as well as neurological cases. Figures 1E and 1F illustrate FcγRI and p150/95 staining of sections adjacent to that of Figure 1D. Reactive microglia stained more intensely with each of these antibodies than resting microglia. Double immunostaining for HLA-DR and LCA revealed that HLA-DR positive microglia are a subpopulation of LCA positive cells (data not shown).

A significant number of LCA positive round cells were found in the affected area of Alzheimer's disease brain tissue (Fig. 1G). Of these LCA positive leucocytes, T-lymphocytes were further identified with staining for CD4 (T-helper/inducer) and CD8 (T-cytotoxic/suppressor)[5]. CD8 positive cells outnumbered CD4 positive lymphocyte. In fresh-frozen acetone fixed tissue, a small number of microglia stained faintly for CD4 (data not shown). CD4 and CD8 positive T-lymphocytes were observed marginating along the walls of blood vessels and invading the parenchyma (Fig. 1H).

In Parkinson's disease substantia nigra (SN), HLA-DR positive microglia were found in close proximity to melanin-containing dopaminergic neurons (Fig. 1I, arrow). The presence of melanin debris in cytoplasm of

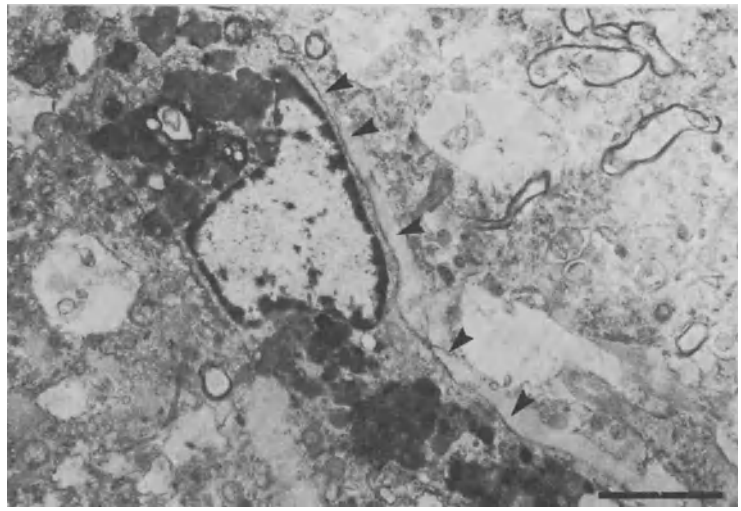

Fig. 2. Electron photomicrograph of Alzheimer tissue showing a HLA-DR positive cell. Arrow heads indicate immunoreaction product on the cell membrane. The cytoplasm contains numerous dense bodies. Scale bar = 4 μm.

some HAL-DR positive microglia indicated that they were phagocytosing the degenerated SN neurons. In the SN from a neurologically normal subject, such HLA-DR positive microglia were virtually absent.

Figure 2 illustrates the fine structure of a typical HLA-DR positive cell. HLA-DR immunoreaction product occurs on the outer cell membrane. The labelled cell has a nucleus with dark peripheral blocks of chromatin, which is typical of microglia. The cytoplasm contains abundant dense, highly insoluble lipids, consistent with the phagocytotic nature of these cells.

DISCUSSION

Reactive microglia are positive for HLA-DR in Alzheimer's and Parkinson's disease brain tissue. Both the light and electron microscopic observations indicate that HLA-DR positive microglia have phagocytic capability. Since a known function of HLA-DR is to present antigen to the T-lymphocytic system, such a result might suggest an involvement of the immune system in the pathogenesis of these neurological disorders. The occurrence of CD4 and CD8 positive T-lymphocytes in the affected area of Alzheimer's disease brain may support this notion[5]. Many previous reports have suggested the existence of humoral antibodies to neuronal elements in Alzheimer's disease[6]. Interaction between the T-lymphocytic system and activated phagocytotic cells expressing HLA-DR is considered to be an important route for production of specific antibodies.

The origin of microglia has been the subject of considerable controversy. In this study, we have demonstrated that microglia share a number of cell surface proteins with macrophages derived from blood monocytes. These are; LCA which appears on all haematopoietic cells except erythrocytes; FcγRI and FcγRII which mediate phagocytosis and are found on neutrophils and monocytes; p150/95 which belongs to a family of integrin receptors and is related to cell adhesion of monocytes. Activation of microglia may cause the upregulation of expression of these cell surface proteins. Some reactive microglia express HLA-DR and CD4 molecules which are also detected on macrophages. It seems reasonable to speculate that such phenotypic resemblances of microglia to macrophages indicate the monocytic origin of microglia.

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ALZHEIMER - PARKINSON DISEASE SPECTRUM AND DIFFUSE LEWY BODY DISEASE

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INTRODUCTION

Diffuse Lewy body disease (DLBD) is a recently recognized primary degenerative dementia that shares pathological features with Alzheimer's disease (AD) and with Parkinson's disease (PD) (5,6,10-13,16). Clinically, it is difficult to diagnose DLBD (2) and most patients come to autopsy with a diagnosis of AD or less commonly progressive supranuclear palsy (5), because of rigidity and axial dystonia in some patients, or Creutzfeldt-Jacob disease, because of myoclonus or periodic complexes on electroencephalograms in some patients (18). In the majority of subjects, cognitive decline overshadows motor abnormalities. Despite degeneration in the substantia nigra and diencephalon that is indistinguishable from PD, some patients have had no apparent extrapyramidal features.

Neuronal degeneration in the brainstem and diencephalon is associated with hyaline cytoplasmic inclusions, Lewy bodies, that are immunoreactive with antibodies to neurofilament and ubiquitin (1,14,19). In DLBD, Lewy bodies are more widespread than in PD. They are present in great numbers in the amygdala and limbic cortices, but are also detected in neurons in the association cortices of the neocortex. The inclusions in cortical neurons, like those in the brainstem and diencephalon, contain neurofilament and ubiquitin epitopes (5,6). Immunocytochemistry with antibodies to ubiquitin is, in fact, the most sensitive method to detect cortical Lewy bodies (6).

Almost every case of DLBD that has been reported has had varying degrees of associated changes of the ALzheimer type (12). This has taken the form of senile plaques (SP) with few or no neurofibrillary tangles (NFT), in most cases (5,6,13,16). Only a small number of juvenile cases of DLBD have been described (20). These subjects may have no SP or NFT. The nature of the SP in DLBD has been investigated with histochemical and immunohistochemical methods (6). They more closely resemble the SP in pathological brain aging than those in AD (3,4).

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Biochemical studies of DLBD have been limited (5). Changes in choline acetyl transferase activity and somatostatin-like immunoreactivity are similar to AD. In both AD and DLBD there is decrease cortical cholinergic and somatostatinergic markers. In fact, the cholinergic deficits tend to be more marked in DLBD than in AD. Hippocampal cholinergic and somatostatinergic markers are far more variable in DLBD than in AD, which may reflect the fact that the hippocampus is spared in some cases of DLBD (6).

The purpose of the current investigation was to determine the frequency of DLBD in a series of brains evaluated for aging and dementia and to determine the nature of Alzheimer type changes in these brains.

MATERIALS AND METHODS

We examined the brains of 216 individuals for the presence of Lewy bodies in the substantia nigra. At least one Lewy body was found in 44 cases. These cases were then subjected to ubiquitin immunostaining using an affinity-purified antibody to ubiquitin. Sections included the hippocampus in all cases and up to 5 additional neocortical sections. In 33 cases cortical Lewy bodies were detected. In 27 cases the cortical Lewy bodies were sufficiently widespread to warrant a diagnosis of DLBD.

In some brains, cortical NFT were also present. To distinguish cortical Lewy bodies from neurofibrillary tangles, sections were double immunostained with a mouse monoclonal antibody to Alzheimer NFT (19) and the rabbit antibody to ubiquitin. The sections were examined for the presence of SP and NFT with thioflavin S fluorescent microscopy. The number of SP was counted in 10x fields and NFT in 40x fields. An average of all the lesions in available association cortices was recorded. Sections from each case were also stained with Bodian's stain after first immunostaining the section with an affinity-purified rabbit antibody to beta-amyloid synthetic peptide. The beta-amyloid antibody immunostaining was performed after incubating the section in 90% formic acid for one hour. Other sections were stained with Bielschowsky's stain. Some brains were immunostained with monoclonal antibodies to Alzheimer NFT and neurofilament.

Ten brains were from individuals who were participants in prospective studies of aging and dementia at our institution. Formalin-fixed tissue from these brains were taken from the frontal lobe and hippocampus and sectioned at 40 micrometers thickness on a Vibratome. The sections were then immunostained with Alz50 using a previously published protocol (17). Immunoreactivity was scored as 0 to 3+ based upon the extent of neuronal staining, in addition to staining of SP, NFT and neuropil neurites.

RESULTS

In this series, all the cases of diffuse LBD had sufficient numbers of SP to warrant a diagnosis of AD. On the other hand, if only classical plaques were counted, few of the cases would have met these criteria. This is because SP in diffuse LBD were

so-called "diffuse" or "very primitive" plaques. Most were little more than an ill-defined deposit of amyloid with few if any neuritic elements visible on the Bodian stain (6). With ubiquitin immunostaining, however, one could detect a small number of ubiquitin-immunoreactive structures in some of the "diffuse" plaques. Recent studies have demonstrated that the ubiquitin-immunoreactive structures contain electron dense membranous bodies consistent with dystrophic neurites (7). They do not contain paired helical filaments.

A minority of diffuse LBD cases had neocortical NFT. On the other hand, NFT were more commonly detected in the hippocampus. This is reflected in the Alz-50 immunostaining. Some of the cases with little or no Alz-50 immunoreactivity in the cortex had substantial amounts of Alz-50 immunoreactivity in the hippocampus. In the hippocampus, the SP in diffuse LBD were also more likely to contain neurites that could be labeled with Alz-50 or with monoclonal antibodies to NFT.

Results of semiquantitative evaluation of the 44 cases and 10 additional AD cases is shown in Figure 1.

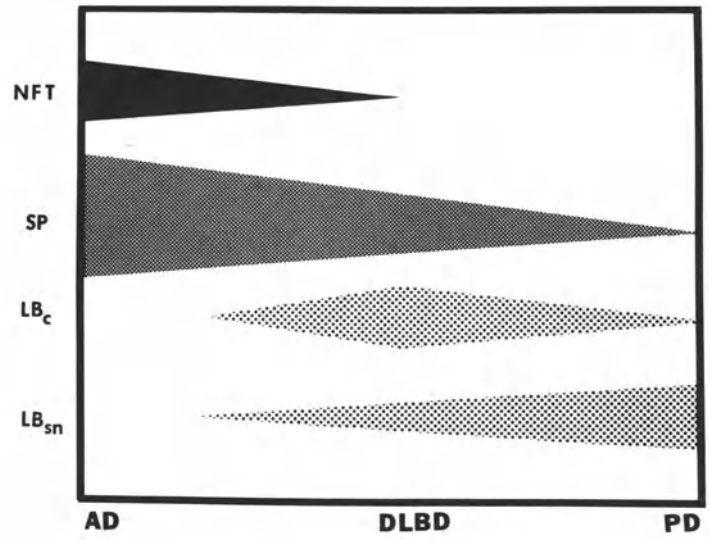

Figure 1. Alzheimer - Parkinson Disease Spectrum. NFT and SP- neocortical tangles and plaques; LBc - cortical Lewy bodies; LBsn - Lewy bodies in substantia nigra.

Data from Alz-50 immunocytochemical evaluation of prospectively studied subjects is shown in Table 1. It is clear that all AD cases have extensive cortical and hippocampal Alz-50 immunoreactivity, but that cases with PD or DLBD have far less immunoreactivity, especially in the neocortex. Some cases show Alz-50 immunoreactivity in the hippocampus in the absence of reactivity in the neocortex. This pattern is not seen in AD.

Table 1

CLINICAL FEATURES OF PROSPECTIVELY STUDIED SUBJECTS WITH RESPECT TO NEOCORTICAL PATHOLOGY AND ALZ-50IMMUNOREACTIVITY

	Blessed Test Score							Neocortex			Alz-50	
YR:	81	82	83	84	85	86	87	88	SP	NFT	LB	Hp	Ctx
PD					5	7	12	16*	2	0	4	0	0
Diffuse LBD													
"plaques only"													
				12	32	*			17	0	17	1+	0
	8	12		19		21		*	37	0	5	2+	1+
			11		14	21		*	50	0	6	3+	1+
"plaques and tangles"													
	10		20	26	*				12	2	3	1+	0
				30	30	32	32	*	50	8	7	3+	3+
							28	*	50	4	2	3+	3+
AD with Nigral LB													
			16		33	*			22	35	0	3+	3+
	10		30	31	32	32	32	33*	50	11	0	3+	3+
		33		33		*			50	10	0	3+	3+
				31	*				22	2	0	3+	3+

Blessed scores of more than 8 are consistent with dementia. Maximum score is 33. * = death.

DISCUSSION

Results of this study suggest that DLBD forms a middle ground in an Alzheimer-Parkinson disease spectrum. There are ample studies documenting Alzheimer changes in PD and of Parkinson changes in AD (8,9,15). Although there are some similarities with AD, it would seem premature to assume that DLBD is merely a variant of AD (11). The nature of the SP in DLBD more closely resembe the amyloid deposits seen in pathological brain aging, than the SP in AD. Furthermore, the absence of PHF-type neuritic degeneration and absence of Alz-50 immunoreactivity in the neocortex are ways in which DLBD differs from AD. The presence of Lewy bodies in cortical neurons is also rare in AD. At one end of the spectrum, however, are DLBD cases that share a number of histological (and clinical) features with AD, just as there are cases at the other end of the spectrum that closely resemble PD.

At present the defining feature of DLBD is widespread cortical Lewy bodies. We define "widespread" as Lewy bodies in neurons of the neocortex, in addition to the limbic cortices and the insular cortex. In cases with co-existing NFT, it is essential that cortical Lewy bodies be differentiated from NFT. We have found double staining with antibodies to Alzheimer NFT and ubiquitin useful in making this distinction.

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ACETYLCHOLINESTERASE HISTOCHEMICAL CHANGES IN THE CEREBRAL CORTEX OF ALZHEIMER'S DISEASE

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INTRODUCTION

A reduction in cortical choline acetyltransferase is one of the most widely demonstrated biochemical effects in Alzheimer's disease (AD)[1]. Acetylcholinesterase (AChE), the degrading enzyme for acetylcholine, is also reduced[2]. By application of a sensitive method for AChE-histochemistry we have recently demonstrated a strong association of AChE-positive axons with senile plaques[3].

In this study, many types of AChE change were identified in Alzheimer's cerebral cortex, including senile plaques and degenerating axons.

MATERIALS AND METHODS

Autopsied brains from 3 controls (average age 65.3) and 6 Alzheimer's cases (average age 75.8) were examined. The brains were perfused with phosphate-buffered saline followed by a chilled paraformaldehyde mixture. They were then post-fixed by immersion in 4% paraformaldehyde. Sections from several cortical areas, i.e. the frontal, parietal, occipital, temporal precentral andparahippocampal cortices, were cut on a freezing microtome and stained for AChE by our previously described method[3,4].

Adjacent sections were stained for tangles and senile plaques by a modified Bielchowsky's method and a modified thioflavin S method.

RESULTS

Brains fixed by perfusion within 10 hours of death gave satisfactory AchE histochemical results. Senile plaques stained positively. Staining of adjacent sections by Bielchowsky's method or by thilflavin S showed an identical morphology. However, double or triple staining by combinations of these three methods has not been successful.

Control staining was carried out by omission of the substrate (acetylthiocholine iodide) from the incubation medium, or by preincubation of the section in a solution containing the specific AChE inhibitor BW284c51 at 0.1 mM concentration. Such control staining, except for red

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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blood cells, was completely negative. The falsely positive red blood cells, which stained with DAB due to their peroxidase-like content, also appeared in control sections, and were easily differentiated from the specific AChE positive structures.

The abnormal AChE staining in AD tissue varied considerably from area to area of the cerebral cortex. The parahippocampal, parietal, and temporal cortices were the most severely affected. The precentral and occipital cortices were relatively less damaged but the basic neuropathological changes nevertheless appeared in all regions.

The size and distribution of senile plaques were almost identical by AChE staining compared with Bielschowsky's or thioflavin S staining. Amyloid cores and wisps of primitive or classical senile plaque material were both AChE positive (Fig. D, E). In the parahippocampal region, there was an extra ordinary, large sized primitive plaque mixed with cloudy precipitated material (Fig. C). Under higher magnification, this area consisted of AChE-rich fibrous deposites and diffuse staining. Only the fibrous structures appeared by Bielschowsky and thioflavin S staining.

The second typical change in AD cortex involved AChE positive axons. Fibers in control brain were small and varicose in nature and formed a dense network (Fig. A). The varicosities were 5-10 μm apart. In AD cases, the number of fibers was drastically reduced (Fig. B), particularly in the parietal and temporal cortices. Residual fibers showed randomly enlarged swellings (Fig. G), toutuosities, and sprouting (Fig. H). Many classical and primitive plaques positive for AChE appeared on the residual AChE fibers (Fig. E). Some amyloid cores had AChE positive fibers (Fig. E) passing through or terminating in them, giving the appearance that the plaques had built up around the fibers. Large size plaques in the parahippocampal gyrus also contained residual AChE positive fibers. A residual linear or reticular arrangement of AChE positive material remained even after disappearance of the fibers (Fig. C).

Neurofibrillary tangles were mostly AChE negative in the cerebral cortices and no glial elements were stained. Only clusters of large tangles were AChE positive in the entorhinal cortex (Fig. I). Additionally, a few small tangles were scattered throughout the other layers.

DISCUSSION

Senile plaques in the cortex of AD cases were similarly distributed by Bielschowsky's silver, thioflavin S and AChE staining. The AChE method revealed a close relationship of these plaques with cholinergic axons. Most of the axonal cortical AChE is localized in projections from the substantia innominata[3]. Hence, the strong reduction in AChE positive fibers is presumed to reflect loss of substantia innominata neurons[3,5].

The AChE positive axonal changes identified in AD suggests that factors promoting degeneration are widely and diffusely distributed in AD cerebral cortex and may have an affinity for AChE positive fibers.

The existence of AChE in senile plaques suggests a participation of this enzyme in their evolution. However, it is still uncertain as to the origin of the extracellular AChE, and why its reactivity is restricted to diffuse deposits and senile plaques. There are at least four possible sources of this extracellular AChE: 1) cholinergic or other AChE containing fibers; 2) cortical neurons which are cholinoceptive; 3) AChE diffusion from the seurm; and 4) glial cells.

There is a striking relationship between AChE-positive areas and those identified by antibodies to β-amyloid protein[6]. The possibility might be considered that AChE is somehow associated with the breakdown of β-amyloid protein precursor into β-amyloid protein itself.

Neurofibrillary tangles were mostly AChE negative. Thus, AChE does not seem to be associated with their formation.

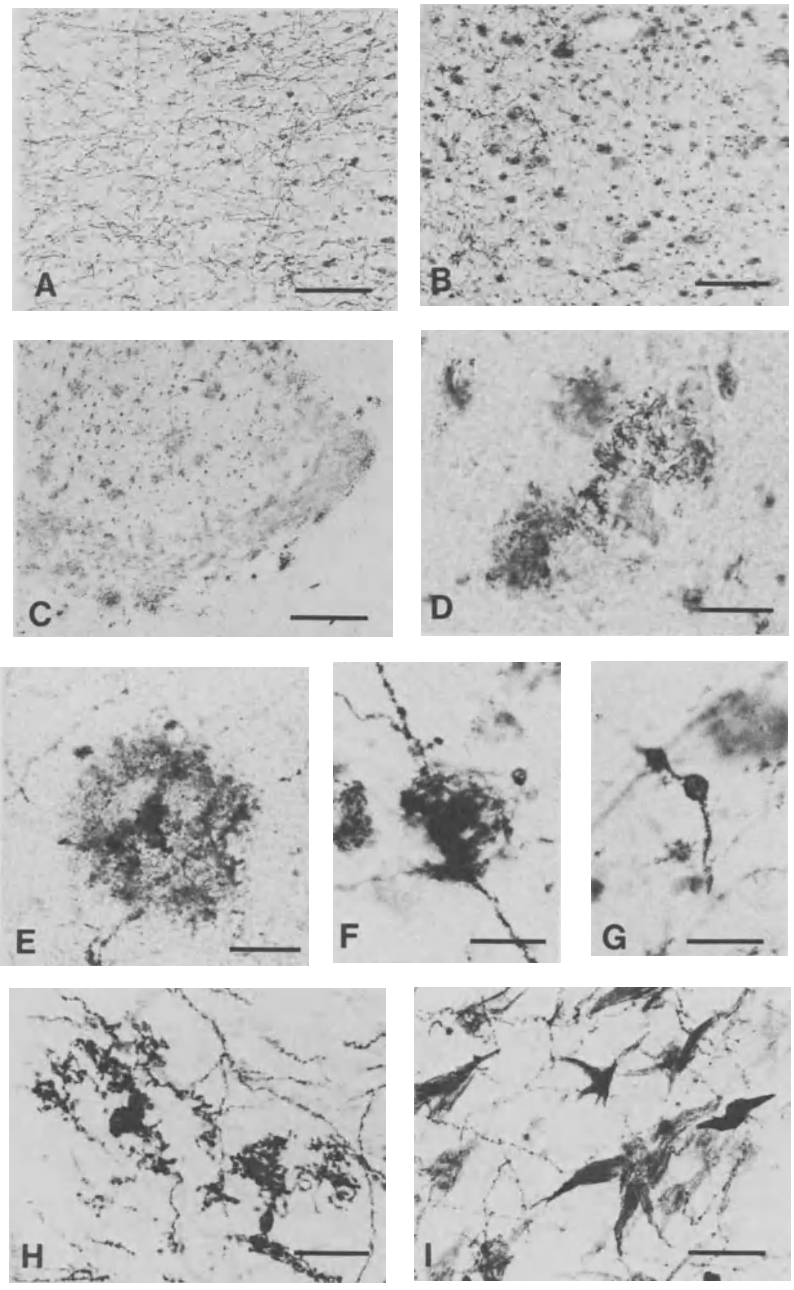

Fig. A. AChE axons in the prefrontal cortex of control. Bar=100μm (Fig. A-B).
Fig. B. AChE axons and senile plaques in the Alzheimer's prefrontal cortex.
Fig. C. An extraordinary large-sized AChE senile plaque in the Alzheimer's parahippocampal cortex. Bar=200μm.
Fig. D. Higher magnification of AChE senile plaques composed of fibrous and diffuse materials. Bar=25μm.
Fig. E. An AChE classical senile plaque with a core and surrounding wisps passed through by AChE axons. Bar=25μm.
Fig. F. AChE neurites with axons in a senile plaque. Bar=25μm.
Fig. G. AChE axonal baloonings in the Alzheimer's frontal cortex. Bar=10μm.
Fig. H. AChE axonal sprouting in the temporal cortex. Bar=25μm.
Fig. I. AChE neurofibrillary tangles in the entrohinal cortex. Bar=50μm.

Further work, particularly at the ultrastructural level, will be necessary to establish more firmly relationships between AChE positive extracellular deposits and AChE containing fibers.

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BASEMENT MEMBRANE COMPONENTS IN ALZHEIMER'S DISEASE

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INTRODUCTION

Characteristic features in dementia of Alzheimer type (DAT) include senile plaques, neurofibrillary changes and alterations of basement membrane components, although the changes may be either primary or secondary to neuronal degeneration or other pathologies. Laminin (LA) and type IV collagen (CO) are basement membrane components (Timple et al., 1979). A high laminin immunoreactivity has been reported in newly formed vessels in developing tissues, neural grafts (Shigematsu et al., 1989 a), in neoplasms and in excitotoxin induced lesions (Shigematsu et al., 1989 b). Therefore, immunohistochemistry using antibodies against LA or CO should be useful not only for demonstrating vascular structures in normal brains (Eriksdotter-Nilsson et al., 1986) but also for studying basement membrane alterations associated with various pathological changes. We have examined the immunoreactivity of the basement membrane components, LA and CO, in DAT brains.

MATERIALS AND METHODS

Four DAT brains (females aged 75, 81 and 85) and two controls (amyotrophic lateral sclerosis male aged 60 and Parkinson's disease female aged 72) were used for the present study. All DAT patients were diagnosed clinically as such and subsequent neuropathological findings using Bielshowsky's silver stain were compatible with the diagnosis. The brains were perfused through the internal carotid and vertebral arteries with 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.6) and postfixed in the same fixative. The cortical areas investigated included the frontal pole, superior temporal gyrus, precentral gyrus, and occipital gyrus. The postfixed blocks were soaked in 15% sucrose in 0.1M PB and were cut into 30- μm-sections on a freezing microtome. Sections were stained immunohistochemically for LA and CO, and by Bielschowsky's silver stain, using standard techniques.

RESULTS

The most prominent feature of the CO immunoreactivity was the intense staining of capillary vasculatures, fine spike-like sprouts called

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streamers and dilated, thin walled vasculatures (Fig. 1). LA immunoreactivity was also demonstrated in vasculatures but more weakly than CO immunoreactivity.Some senile plaques were observed in association with vasculatures and loaded with LA(Fig.2) and CO immunoreactivity (Fig.1 B). LA immunoreactivity was also demonstrated in neurons which had no or minimal neurofibrillary changes(Fig.3 A),whereas CO immunoreactivity was negligible in neuronal elements except for in several neurofibrillary tangles. Many corpora amylaceas were located along with the capillaries demonstrated by LA or CO immunohistochemistry (Fig.3 B).

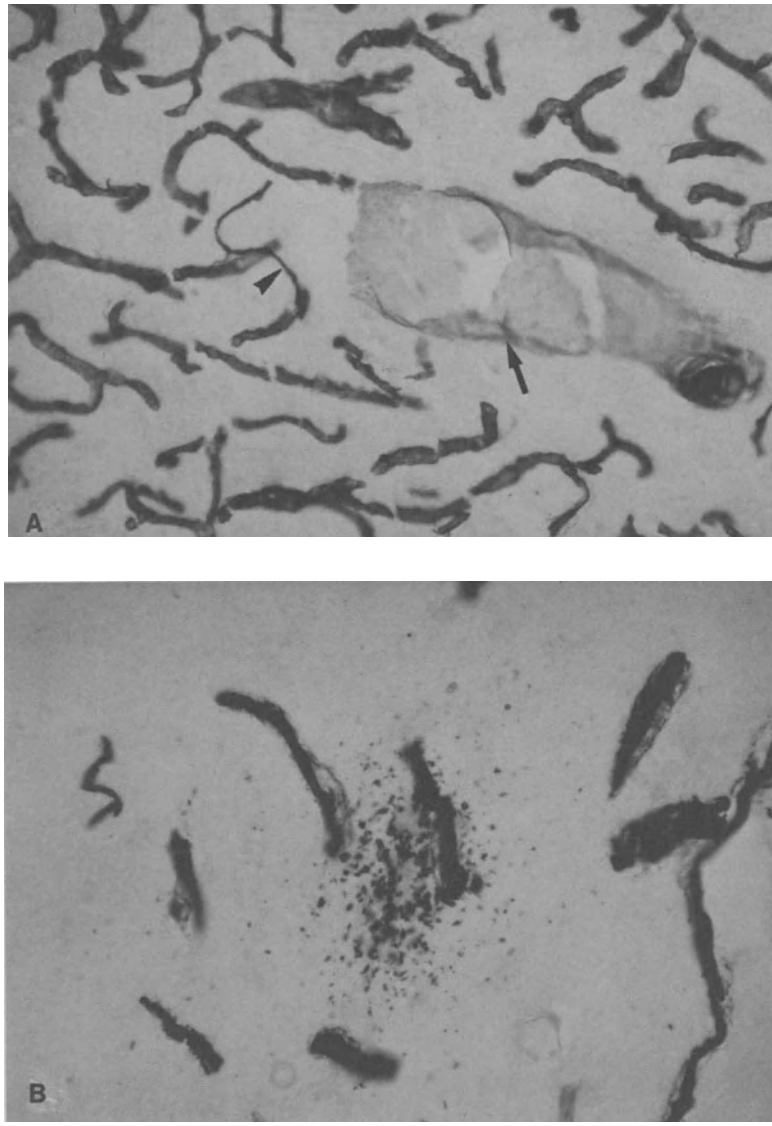

Fig.1. CO immunohistochemistry.
A) Intense staining of capillary vasculatures, a fine spike-like sprout called streamer (arrowhead) and dilated, thin walled vasculature (arrow) is seen.
B) A senile plaque is loaded with CO immunoreactivity

DISCUSSION

The non-collageneous extracellular matrix glycoprotein, LA, plays an important role in endothelial organization and behavior during the angiogenetic process (Foidart and Reddi, 1980). LA promotes the peripheral and central neurons. In neovascularization, LA is associated with actively migrating and proliferating endothelial cells, whereas the appearance of CO, another basement membrane component, correlates more with lumen formation and the maintenance of a differentiated endothelial cell phenotype (Form et al., 1986). Thus LA and CO are valuable makers for investigating neovascularizations or alterations of basement membranes in various pathological conditions.

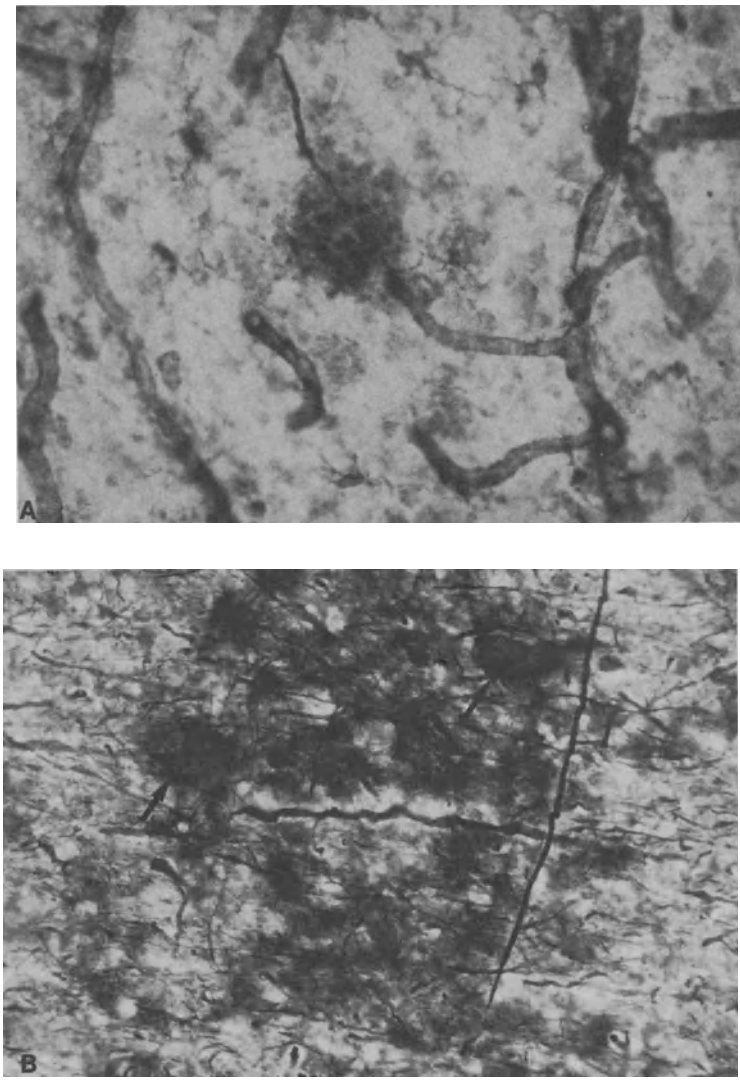

Fig.2. LA immunohistochemistry.
A) A senile plaque is associated with vasculatures loaded with LA immunoreactivity.
B) Vasculatures (arrows) are seen in a senile plaque (Counterstained by Bielshowski's silver).

In DAT, thickened and redundant capillary walls and basement membranes were reported (Scheibel et al., 1987; Athanikar et al., 1988) Electron microscopic examination revealed that all of the senile plaques contained at least some amyloid fibrils which seemed to be produced at the basement membranes of the capillary endothelial cells (Miyakawa et al. 1982; Higuchi et al., 1987). In the present study, senile plaques often, if not always, were associated with capillaries demonstrated by LA or CO immunohistochemistry and even had numerous spotty immunoreactivities against LA or CO.

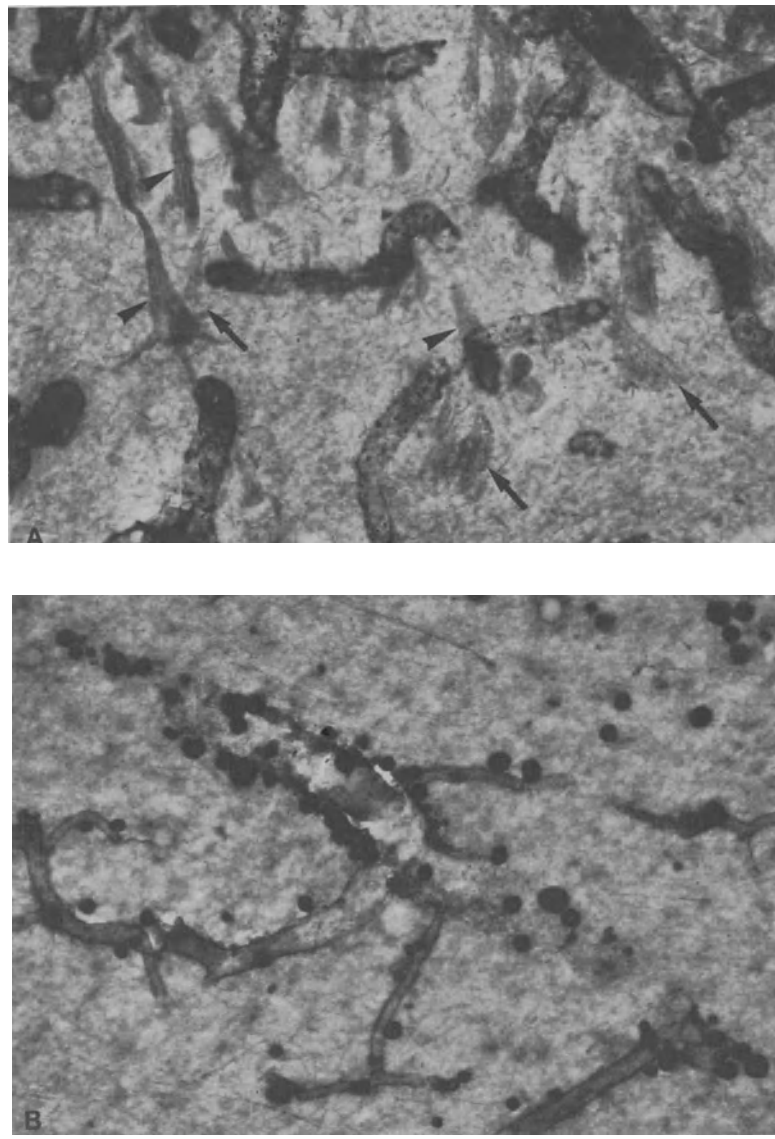

Fig.3. LA immunohistochemistry counterstained by Bielshowski's silver.
A) LA immunoreactivity is demonstrated in neurons (arrows) with no or with minimal neurofibrillary changes. Neurofibrillary tangles (arrowheads). B) Many corpora amylaceas are located along with capillaries.

Thus, alterations of basement membrane components may be involved in the pathogenesis of DAT, and LA and CO immunohistochemistries are useful in studying them.

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REGULATION OF CEREBRAL CORTICAL AND HIPPOCAMPAL BLOOD FLOW BY CHOLINERGIC FIBERS ORIGINATING IN THE NUCLEUS BASALIS OF MEYNERT AND SEPTAL COMPLEX

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INTRODUCTION

Intracerebral cholinergic fibers originating in the nucleus basalis of Meynert (NBM) and septal complex projecting to the cortex and hippocampus have been reported to degenerate in Alzheimer's disease as well as in aged people[1,2]. However, physiological function of these cholinergic fibers have remained obscure.

The cholinergic nervous system is very important for vasodilative function in autonomic nervous regulation of various peripheral organs. For example, cholinergic sympathetic nerve fibers innervating blood vessels in skeletal muscles are important for preparatory vasodilation before contraction of skeletal muscles[3], and cholinergic parasympathetic nerve fibers innervating cardiac coronary vessels are important for vasodilatation of the coronary vessels.

As concerns cerebral circulation, metabolic regulation of cerebral blood flow (CBF) has long been emphasized, but the importance of neural regulation has been relatively disregarded. We started the present experiment two years ago with the purpose of examining whether or not these intracranial cholinergic fibers can act as vasodilators in the cortex and hippocampus. The results demonstrate that activation of these cholinergic fibers releases acetylcholine (ACh) from the nerve terminals in the cortex and hippocampus, resulting in regional vasodilation and increase in regional blood flow in the cortex and hippocampus[4,5,6,7,8]. The results will be briefly introduced.

The experiments were performed on rats anesthetized either with urethane (1.1 g/kg) or with halothane (1.0%). The trachea was cannulated, and respiration was maintained by a respirator. The end-tidal CO_2 was kept constant at 4.0-4.5%, monitored by a gas analyzer (1H26, Nippondenki San-ei). Rectal temperature was maintained at 37.0-38.0 °C using a heating lamp and a pad. Systemic arterial blood pressure was

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monitored via a cannula inserted into the femoral artery. The animal was mounted in a prone position on a stereotaxic instrument (SR-5, Narishige). After craniotomy, either a coaxial metal electrode or a microsyringe of 0.3 mm outer diameter was inserted into the unilateral NBM or the septal complex for a focal electrical or chemical stimulation of that area.

RESULTS AND DISCUSSIONS

1. Response of cortical CBF measured by laser Doppler flowmetry following focal stimulation of the NBM [4,5]

Regional cortical CBF was continuously recorded using the laser Doppler flowmetry (LDF; ALF2100, Advance or BPM403, TSI). After craniotomy, the probe was placed on the parietal lobe with the dura either intact or removed. The blood flow within the space of approximately 1 mm^3 right below the recording probe of the LDF was continuously measured, and the flows measured by the LDF was proved to have a linear relationship with flows measured by the hydrogen clearance method[9].

Electrical stimulation of the unilateral NBM produced a stimulus strength-dependent increase in the ipsilateral cortical CBF, as demonstrated in Fig. 1A and summarized in Fig. 1B. The response was obtained in the ipsilateral frontal, parietal and occipital cortices. The response started

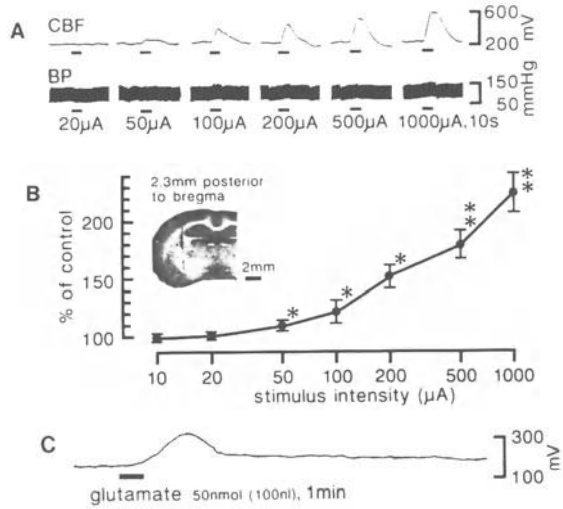

Fig. 1. The effect of focal stimulation of the unilateral NBM on CBF measured by LDF in the parietal cortex ipsilateral to the stimulation. A: sample recordings of the CBF and blood pressure after electrical stimulation of the NBM (B2.3, L3.7, V7.6) with parameters of 0.5 ms, 50 Hz, for 10 s, with various intensities as indicated below the black bars. B: specimen slice (inset) representing a transverse section of the right side of the brain at 2.3 mm posterior to the Bregma for the case recorded in A. The graph shows the relation between stimulus intensity (abscissa) and the increases in CBF (ordinate) in 6 rats. Each dot and vertical bar indicates mean ± S.E.M. * $P<0.05$; ** $P<0.01$; significantly differing from prestimulus control values using the paired t-test. C: sample recording of CBF in the parietal cortex following microinjection of L-glutamate (50 nmol/100 nl) into the NBM. (From Ref. 4)

within a few seconds following the onset of stimulation. The stimulation of the NBM did not always accompany changes in systemic arterial blood pressure. Namely, the response was not dependent on systemic arterial blood pressure.

As the electrical stimulation might stimulate neuronal soma as well as adjacent nerve fibers, sodium L-glutamate was microinjected into the unilateral NBM for 1 min to stimulate chemically the nerve cells in or near the NBM alone. The cortical CBF was increased following the microinjection of L-glutamate as demonstrated in Fig. 1C.

The response of increase in CBF by the electrical stimulation of the NBM was attenuated significantly after intravenous administrations of muscarinic cholinergic blocking agent atropine (0.5 mg/kg), and nicotinic cholinergic blocking agent mecamylamine (2 mg/kg). As the i.v. injection of mecamylamine produced a remarkable decrease in systemic arterial blood pressure, dopamine was infused i.v. to maintain the systolic arterial blood pressure above 100 mmHg, but the depressive effect of mecamylamine was still observed. Such evidence suggested a contribution of the cholinergic system, including both muscarinic and nicotinic receptors, to the present cortical vasodilative response.

2. Response of extracellular ACh in the cortex to focal stimulation of the NBM [6]

Extracellular ACh was collected using the microdialysis

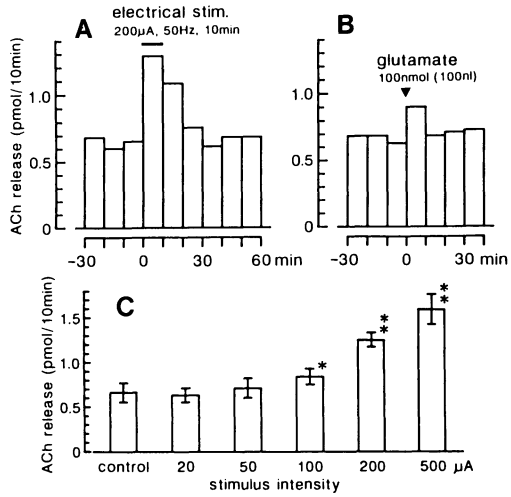

Fig. 2. The effect of focal stimulation of the unilateral NBM on extracellular ACh release in the parietal cortex ipsilateral to the stimulation. The amount of ACh release in the perfusate every 10 min, measured by the microdialysis technique, is plotted on the ordinate. A: electrical stimulation (200 μA, 0.5 ms, 50Hz) for 10 min, as indicated by the upper horizontal bar, in one rat. B: microinjection of L-glutamate (100 nmol/100 nl) for 1 min as indicated by the upper triangle, in another rat. C: effect of various intensities of electrical stimulation. Ach release in the perfusate during stimulation for 10 min was represented as the mean $\pm$ S.E.M. ACh response to the stimulation were compared with ACh during resting condition (control) using the paired t-test. *P<0.05, **P<0.01. (Modified from Ref. 6)

technique. A coaxial microdialysis probe (CMA/10, Carnegie Medicin) was inserted into the cortex. ACh was measured by the method of high-performance liquid chromatography using electrochemical detection. A focal electrical or chemical stimulation of the unilateral NBM produced an increase in extracellular ACh release in the ipsilateral cortex as demonstrated in Fig. 2A and B. The response of the extracellular ACh release following focal electrical stimulation of the NBM was current-dependent (Fig. 2C). The results suggested that the increased cortical ACh following the stimulation of the NBM acted as a potent vasodilator substance in the cortical vasodilating system.

3. Response of regional CBF in different brain areas following focal electrical stimulation of the NBM [7]

Regional CBF (rCBF) was measured according to the Kety's principle, using ^{14}C-iodoantipirine (^{14}C-IAP) as a tracer. Various brain regions were collected using the dissecting technique. The electrical stimulation of the unilateral NBM produced increase in regional CBF only in the ipsilateral cortices including the frontal, parietal and occipital cortices, but not in the ipsilateral diencephalon, midbrain, pons, medulla oblongata, cerebellum, and in all examined regions contralateral to the stimulation.

4. Response of hippocampal CBF measured by LDF following stimulation of the septal complex [8]

The regional hippocampal (Hpc) CBF was first measured continuously using the LDF. The probe of the LDF was inserted into the hippocampus. A focal electrical or chemical stimulation of the septal complex produced a current-dependent increase in Hpc CBF (Fig. 3). The Hpc CBF responses were elicited whether or not there were changes in systemic arterial blood pressure. A microinjection of L-glutamate in the septal complex produced an increase in Hpc CBF.

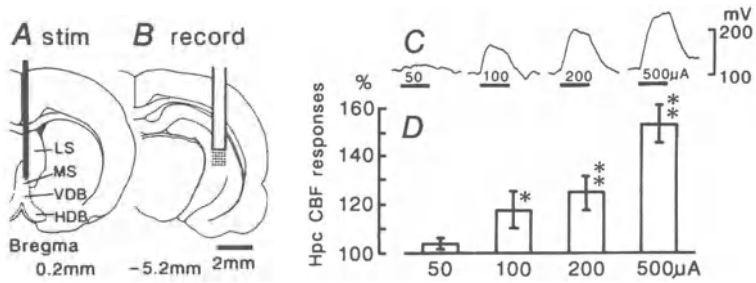

Fig. 3. Effects of focal electrical stimulation of septal complex on Hpc CBF. A: stimulating electrode inserted into the septal complex (B0.2, L0.2, V6.5). B: recording probe of the LDF inserted into the hippocampus (B5.2, L5.0, V5.0). Dotted area indicates the recording area. C: sample recordings of Hpc CBF after stimulation with parameters of 0.5ms, 50 Hz, for 30 s, with various intensities as indicated. D: relation between stimulus intensities (abscissa) and responses of Hpc CBF expressed as % of prestimulus control CBF (ordinate, n=9). Each column and vertical bar indicates mean ± S.E.M. * $P<0.05$; ** $P<0.01$; significantly differing from prestimulus control values using the paired t-test. (From Ref. 8)

The responses of increase in Hpc CBF upon injection of L-glutamate into the septal complex were not influenced by administration of atropine, but almost totally abolished by successive administration of mecamylamine, indicating that the L-glutamate-induced vasodilative responses of Hpc CBF were produced through activation of the nicotinic receptor. The responses of increase in Hpc CBF by electrical stimulation of the septal complex were not significantly influenced either after atropine alone, or after successive administration of mecamylamine, although in the latter case, the responses tended insignificantly to attenuate. This result is in contrast with the finding that the vasodilative effect of electrical stimulation of the NBM on cortical CBF was significantly attenuated by both muscarinic and nicotinic cholinergic blockers. These results suggested that non-cholinergic fibers originating in the septal complex, or fibers passing through the septal complex might contribute to the present vasodilative responses in the hippocampus elicited by electrical stimulation of the septal complex.

5. Response of extracellular ACh in the hippocampus to focal stimulation of the septal complex [8]

Focal electrical stimulation of the medial complex produced an increase in extracellular ACh release in the hippocampus (Fig. 4).

6. Response of regional CBF in different brain areas following focal stimulation of the medial septum [7]

The electrical stimulation of the medial septum increased regional CBF in Hpc, but not in other regions examined including cerebral cortices, diencephalon, midbrain, pons, medulla oblongata and cerebellum measured by the ^{14}C-IAP method.

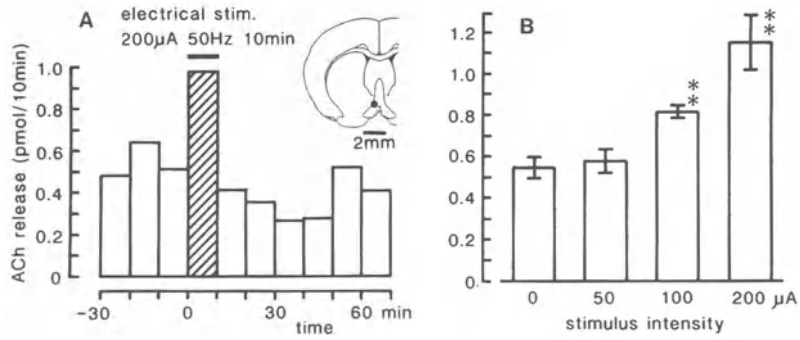

Fig. 4. Effect of focal electrical stimulations of the septal complex on extracellular ACh release in hippocampus. A: ACh release per 10 min (in ordinate) was consecutively measured (in abscissa) in one rat. Electrical stimulation (200 μA, 0.5 ms, 50 Hz) was applied for 10 min, as indicated by the upper horizontal bar. A closed circle in the diagram indicates the stimulated area. B: Effect of various intensities of electrical stimulation of the septal complex on ACh release (n=5 for each column). Ordinates (ACh release) in A and B are identical. Each column represents mean $\pm$ S.E.M. ACh release during the 10 min stimulation were compared with the prestimulus ACh release for 10 min using the paired t-test. **P<0.01. (From Ref. 8)

SUMMARY AND CONCLUDING REMARKS

In summary, activation of the intracranial cholinergic fibers originating in the NBM and septal complex releases acetylcholine in the cortex and hippocampus, which results in vasodilation and increase in regional cerebral blood flow in the cortex and hippocampus.

Such intracranial cholinergic vasodilator nerve fibers, however, do not belong to the autonomic nervous system according to the traditional definition by Langley, but interestingly, these fibers behave in the brain just like autonomic nerves do in peripheral organs. We need to further investigate the functional significance of this vasodilation. I would like to speculate at this moment on the analogy of this system to the peripheral sympathetic cholinergic preparatory vasodilative system in skeletal muscles which activates and supplies sufficient oxygen to muscles before the muscles start to contract. The intracranial cholinergic vasodilative system may have a similar preparatory meaning for supplying sufficient oxygen and glucose to nerve cells in the cortex and hippocampus just before the nerve cells in these areas start to activate. The development of dysfunction of some higher cognitive nervous functions in Alzheimer's disease may occur partly by degeneration of the cholinergic preparatory regulatory system for regional CBF.

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IMMUNE SYSTEM REACTION TO MODEL LESIONS OF RAT BRAIN

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Traditional ideas about how the immune system responds to injury and infection of neural tissue are now being revised, particularly with respect to intervention by T-lymphocytes. They require major histocompatibility complex (MHC) antigens for cellular recognition. Recent investigations have revealed the vigorous expression of HLA-DR, a MHC class II antigen by reactive microglia in Alzheimer's and Parkinson's disease as well as some other neurological disorders[1,2]. The major known function of HLA-DR is to present foreign antigen to T-helper/inducer cells. In affected areas of Alzheimer's disease brain, a significant number of T-lymphocytes have also been detected[3], indicating that the appropriate tissue elements for a cell mediated immune response are present. The purpose of this study was to investigate the response of immune components to controlled sterile lesions to rat brain. Such information should contribute to understanding data obtained in human degenerative neurological diseases.

MATERIALS AND METHODS

Male Wistar rats were used in this study. The lesions were induced by; (1) stab wound designed to cut underlying white matter of the cerebral cortex; (2) epidural application of kainic acid powder (0.8 mg) to the frontoparietal cortex; (3) intraventricular injection of kainic acid (0.2 ug in 0.5 ul of physiological saline); (4) 6-hydroxydopamine (6-OHDA) (8 µg in 4 µl of physiological saline) injection into the nigrostriatal pathway in the lateral hypothalamus. Rats were sacrificed after various survival periods. The brains were quickly removed and fixed in 2% paraformaldehyde, 1% picric acid in 0.1M phosphate buffer pH7.4 for 24 hours. After cryoprotection, 30 µm sections were cut on a freezing microtome. Immunostaining was performed according to the procedures described previously[4,5]. Monoclonal antibodies used were; OX42 (Serotec) to the C3bi receptor (CR3); OX18 (Sera-lab) to the common part determinant of MHC class I antigen; OX6 (Sera-lab) to the MHC class II antigen; OX1 (Sera-lab) to leukocyte common antigens (LCA); ED1 (Serotec) to a cytoplasmic antigen of monocytes and macrophages; W3/13HLK (Sera-lab) to pan-T cells and granulocytes; W3/25 (Serotec) to T-helper/inducer cells; OX8 (Serotec) to the T-cytotoxic/suppressor cells; and OX39 to interleukin II receptor (IL2R) (Chemicon). Rabbit anti-glial fibrillary acidic protein (GFAP) (Dakopatts) was used to identify astrocytes.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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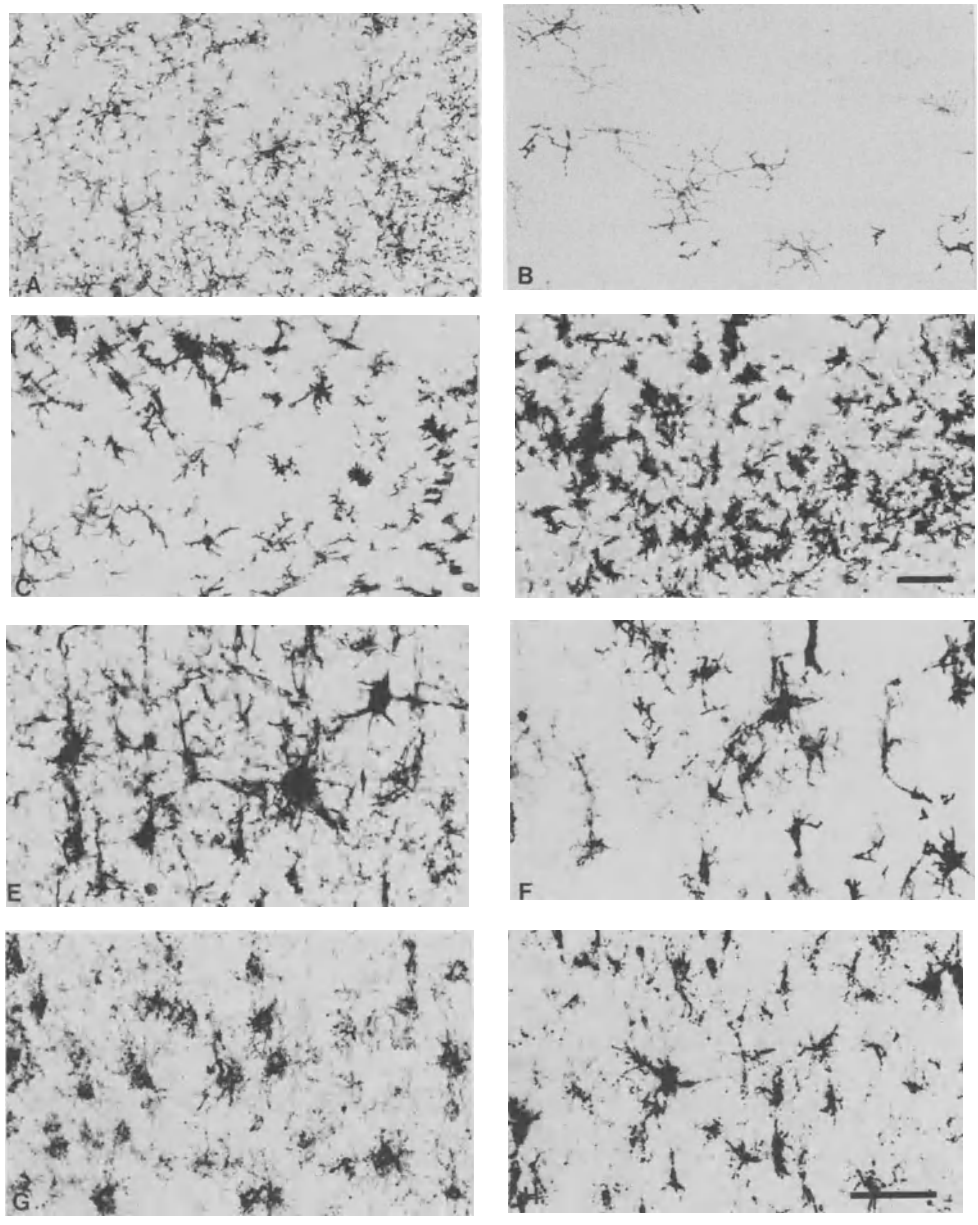

Fig. 1. (A) CR3 (OX42) positive microglia in a control rat hippocampal CA3. (B) MHC class II (OX6) positive microglia in a control rat internal capsule. (C)&(D) CR3 staining of hippocampal CA3 lesion. One day (C) and 6 days (D) after intraventricular kainic acid injection. Intensely stained reactive microglia have swollen cell bodies with short, thick processes. (E) MHC class I (OX18) positive microglia forming cellular agglomerates after the cortical stab wound. (F) Class II staining of a semiadjacent section of (E). (G) LCA (OX1) staining of the cortical lesion induced by epidural kainic acid. (H) ED1 positive microglia in the cortical lesion. A-D and E-H are the same magnification respectively. Scale bar = 50 μm.

RESULTS

In non-treated rats, CR3 (OX42) was detected on small glial cells with thin, highly branched long processes (Fig. 1A). The morphology was consistent with that of resting microglia as originally described be del Rio Hortega. MHC class I antigen (OX18) was detected on vascular endothelial cells and a small number of microglia in both grey and white matter. MHC class II antigen (OX6), LCA (OX1) and W3/25 were confined to a small number of microglia in white matter (Fig. 1B).

Figures 1C and 1D illustrate the microglial reaction in the hippocampal CA3 field after intraventricular kainic acid injection. CR3 positive microglia transformed themselves into reactive forms as early as 1 day after lesioning (Fig. 1C). They appeared hypertrophic with short, thick and poorly ramified processes. The expression of CR3 by reactive microglia increased dramatically, reaching a plateau in the 6-10 day period (Fig. 1D). Such reactive microglia also expressed MHC class I antigen. Class I positive reactive microglia appeared on day 1 or 2, increased in number and peaked in a week. Figure 1E illustrates MHC class I staining of reactive microglia following cortical stab wounds. They frequently formed cellular agglomerates around degenerated neurons. The microglial reaction was also observed along fiber tracts of degenerated neurons. This was particularly evident in the nigrostriatal pathway after 6-OHDA induced nigral lesions. Expression of MHC class II antigen by reactive microglia was first detected on day 4 or 5 post lesioning (Fig. 1F). Class I positive cells outnumbered class II positive cells at this time period in most types of the lesions. After 20 to 30 days, the number of MHC positive microglia began to decline. The microglial reaction subsided substantially after 3 to 5 months.

Reactive microglia were also positive for LCA (OX1), ED1 and W3/25. Expression of these antigens by microglial cells was first observed 4 to 5 days after lesioning. Figure 1G and 1H illustrates reactive microglia positive for LCA (1G) and ED1 (1E) following the kainic acid induced cortical lesions.

Infiltration of round cells into the lesioned brain parenchyma was visible during the early time period. These cells were positive for LCA, MHC class I and less frequently class II antigens. Although W3/25 and OX8 were originally described to recognize T-helper/inducer and T-cytotoxic/ suppressor cells respectively, these antibodies labelled a number of round cells of various sizes, as well. The majority of these round cells were presumably monocytes/macrophages (Fig. 2A and 2B). The presence of a small but significant number of T-lymphocytes was confirmed with W3/13HLK in the lesions at later stages (after about 1 week). At that time period, granulocytes, which also stained positively for W3/13HLK and were prominent in the lesioned areas on day 1 to 3, were substantially reduced or absent. Some of these round cells were also positive for IL2R.

This time limited response contrasted with that observed for reactive

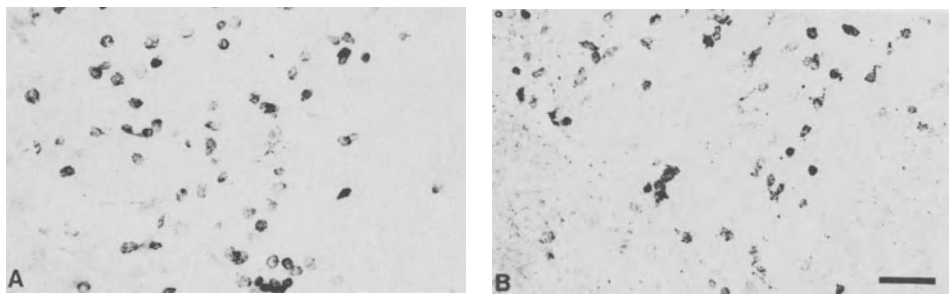

Fig. 2. (A) W3/25 staining of kainic acid induced hippocampal CA3 lesion. (B) OX8 staining of cortical stab wound. The majority of these round cells were considered to be macrophages. Scale bar = 50 μm.

astrocytes. GFAP positive astrocytes marked the lesioned area, forming glial scar tissue in later stages.

DISCUSSION

A panel of antibodies to proteins associated with immune system cells was applied to rat brain tissue. As reported previously[6,7], complement receptor CR3 (detected by OX42) was constitutively expressed by resident microglia. In white matter of control rat brain, detectable levels of MHC class I and class II antigens as well as LCA were also expressed on a small number of microglia. Although the significance of such antigen expression by resident microglia remains unknown, these results infer the phenotypic resemblance of resident microglia to monocyte/macrophage lineage. We have already reported that GFAP positive astrocytes never express these antigens in either control tissue or in lesioned rat brain[4,5].

In all types of brain lesions used in this study, expression of CR3 by reactive microglia was dramatically enhanced, which is consistent with other reports[6]. MHC class I antigen was expressed by reactive microglia in very early time periods, while expressions of class II antigen, LCA, ED1 and W3/25 by microglial cells began 4 to 5 days after lesioning. This delay might be the result of induction of appropriate synthetic pathways in microglia in the central nervous system. However, it could also be possible that reactive microglia expressing these antigens were all of recent monocytic origin and infiltrated into brain after injuries. The origin of brain macrophages and/or microglia has been a matter of considerable controversy. Obviously, further investigations are needed to clarify this issue, but phenotypic difference have not yet been identified and it is probable that they both belong to the monocyte phagocytic system.

T lymphocytes can interact only with cells expressing MHC antigens. Following the brain injuries, numerous reactive microglia were positive for MHC antigens. Therefore, the presence of T lymphocytes, although only in modest numbers, indicates that a classical immune response may take place in such brain lesions.

ACKNOWLEDGEMENTS

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COMPUTER-AIDED QUANTITATIVE ANALYSIS OF THE DISTRIBUTION OF CATECHOLAMINE-CONTAINING FIBERS IN THE PRIMATE PREFRONTAL CORTEX

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INTRODUCTION

The primate prefrontal cortex is densely innervated by midbrain dopamine (DA) neurons. Although the function of this mesocortical DA system is not known, it is believed that this catecholamine (CA) fiber system might be involved in certain mental disorders. In the present study, we investigated the CA innervation of the monkey prefrontal cortex by tyrosine hydroxylase (TH) immunohistochemistry, which is thought to be a reliable method to study cortical DA fibers.[1] The density of TH-containing fibers was measured by a computer-aided image analysis technique.

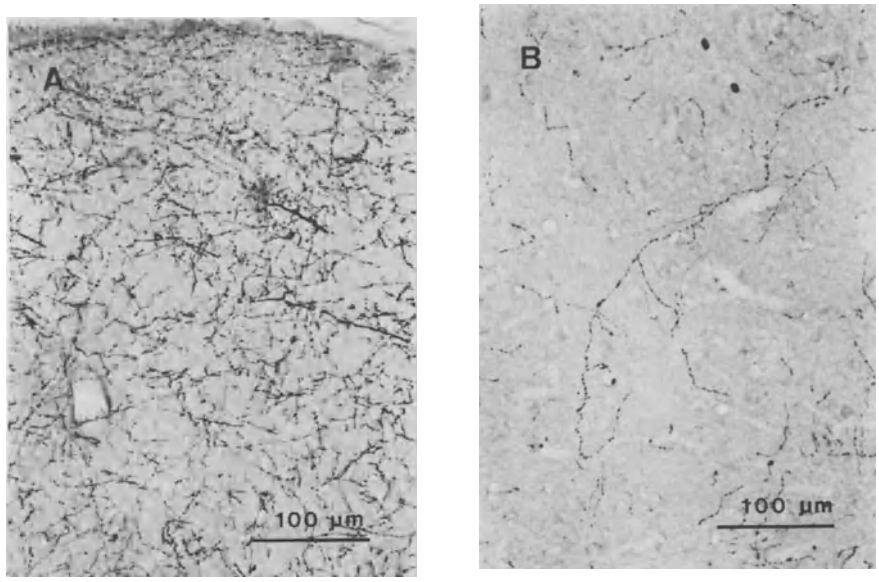

Fig.1. TH-immunoreactive fibers of area 24 (A) and area 46 (B). The density of fibers in A was measured and graded as 5, and the density of fibers in B was equal to grade 3 (see Fig.3).

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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MATERIALS AND METHODS

A) Materials

The Japanese monkey (Macaca fuscata) was deeply anesthetized with ketamine hydrochloride prior to perfusion with aldehyde fixative (Zamboni solution).[2] After postfixation, 20 μm-thick coronal sections were made with a cryostat, and were later processed for TH-immunohistochemistry. The sections were rinsed in 2% normal goat serum in 0.1M Tris-buffered saline, followed by overnight incubation in rabbit antiserum directed against bovine TH.[3] TH-immunoreactivity was visualized by a standard immunohistochemical procedure using avidin-biotin peroxidase complex (Vector) followed by a reaction in 3,3'diaminobenzidine-H_2O_2 solution.

B) Data analysis

Computer-assisted image analyzer, NEXUS 6400 (Kashiwagi Res.Co.),[4] was used to perform a quantitative study on the distribution of TH-immunoreactive fibers. We measured the density of TH-positive fibers in 323 areas located in 11 cortical regions, i.e., Walker areas 6,8,9,12,13,14, 24,25,32,45, and 46. The microscopic image was transferred to the NEXUS 6400 by means of a video camera (Victor KY-210), and was shown on a display monitor. We digitized the tissue image to black or white color image. In the present study, the density of fibers (density value, CAD) was obtained by measurement of the size of computed areas occupied by digitized TH-positive structures per 5800 μm^2 by NEXUS 6400. A detailed description of image processing will be given elsewhere (H.Matsumura, M.Fujii, S. Iritani, K.Satoh, and I.Nagatsu, in preparation). The statistical differences between areas were assessed by Student's t-test.

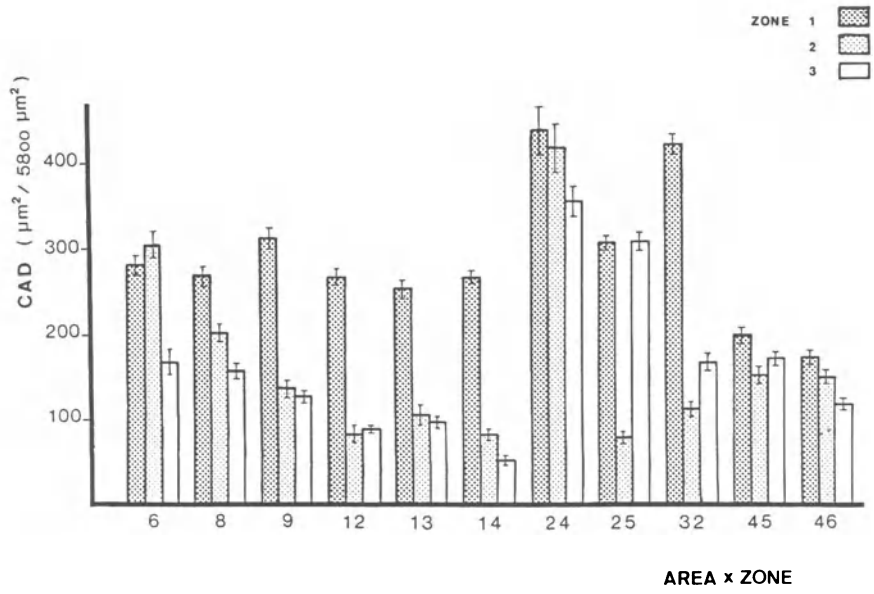

Fig.2. Density of TH-immunoreactive fibers in 11 cortical regions. Zone 1 represents the molecular layer; zone 2, layers II-IV; and zone 3, layers V-VI. The average of CAD values is shown with standard deviation.

RESULTS

The area occupied by TH-positive fibers varied extremely between cortical regions and between cortical layers. A high density of TH-immunoreactive fibers was observed in the cingulate cortex or area 24 (Fig.1A), and a low concentration in other cortical regions, e.g., area 46 (Fig.1B). Laminar distribution: We measured the density of TH-immunoreactive fibers in 3 different zones. In general, the molecular layer (zone 1) contained a large number of TH-immunoreactive fibers and exhibited the highest CAD values in the prefrontal cortex. CAD values ranged from 34 to 918 μm^2 (mean=302.5, SD=12.6). Middle and deeper layers contained a low-to-moderate density of fibers. CAD ranged from 11 to 875 μm^2 (mean=217.9, SD=13.6) in zone 2 (cortical layers II-IV), and from 6 to 636 μm^2 (mean=187.8, SD=11.6) in zone 3 (cortical layers V-VI). Using the t-test, we found significant differences between zone 1 and zone 2 ($F=1.53$, $p < .001$), and between zone 1 and zone 3 ($F=1.38$, $p < .001$). Difference in the laminar distribution by cortical regions: Figure 2 shows the differences in densities of TH-containing fibers in three zones of the prefrontal cortex. High CAD values were measured in zones 1 and 2 of area 24, and in zone 1 of area 32. Comparison of measurements of CAD values in zone 1 revealed that TH-immunoreactive fibers were extremely dense in area 24 and area 32 and that for zone 2, TH-positive fibers were densest in area 24. In zone 3, areas 24 and 25 were relatively rich in the fibers. In area 46, low CAD values were observed in all cortical layers. Diagrammatic representation of the density of TH-immunoreactive fibers in the prefrontal cortex (Fig.3): A high concentration of TH-containing fibers was observed in Walker areas 6, 9,24, and 32. Area 24 exhibited a unique pattern having a high concentration in all layers.

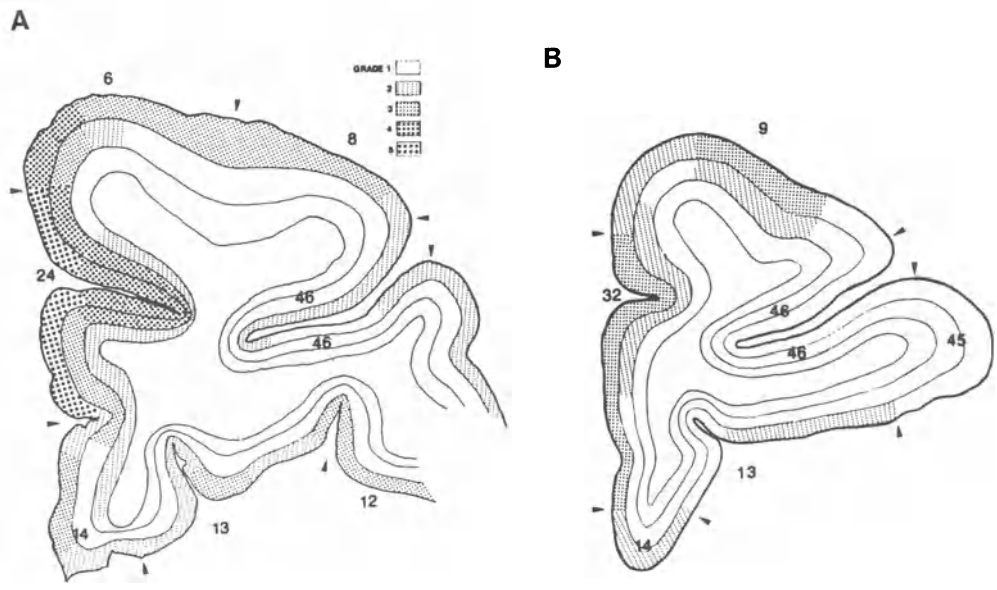

Fig. 3. Schematic representations of density of TH-containing fibers in the levels of prefrontal cortex (A,B). CAD values are graded into 5 degrees: Grade 1 represents CAD values of 0-150 μm^2 per 5800 μm^2; grade 2, 150-300 μm^2; grade 3, 300-450 μm^2 (Fig.1); grade 4, 450-600 μm^2; grade 5, more than 600 μm^2.

DISCUSSION

The present study illustrates the differences in the pattern of CA fiber distribution in the prefrontal cortex of the macaque monkey. Computed density values varied from layer to layer and from region to region. High densities were observed in the molecular layer of all prefrontal cortex areas. We ascertained that some areas had the characteristic pattern of laminar distribution as revealed by the quantitative densitometry of TH-immunoreactive fibers. Areas 24 and 6 showed a distribution pattern in which the fibers were dense in all cortical layers from the molecular layer to the deep layer. These distribution patterns suggest a functional specialization of the CA innervation of the prefrontal cortex.

In the prefrontal cortex, nearly all CA fibers shown by TH-immunoreactivity are thought to be DA fibers.[1] Further, Lewis and collaborators indicated that studies of the monoaminergic system in the nonhuman primate cortex might closely reflect their innervation patterns in the human cortex.[5] The present study revealed an extremely dense innervation of TH-containing fibers (probably dopaminergic) in the monkey prefrontal cortex. The observation suggests a functional specialization of DA innervation in the primate cerebral cortex, which might differ significantly from that of subprimates.

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NIGROSTRIATAL LOOP DISRUPTION IN PARKINSON'S DISEASE AND STRIATONIGRAL DEGENERATION

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INTRODUCTION

Parkinson's disease (PD) is known to involve degeneration of nigrostriatal dopaminergic neurons, resulting in a dopamine deficiency in the striatum.[1] On the other hand, parkinsonism has been described as a clinical manifestation of other extrapyramidal disorders, such as striatonigral degeneration (SND)[2] and progressive supranuclear palsy.[3] SND was first described as a distinct clinicopathological entity by Adams et al.,[2] and symptoms of pure SND are those of PD; while in the SND patients rigidity and slowness of movement of extremities are prominent.[2] In addition, it is noteworthy that SND has been suggested to be a true supranigral form of parkinsonism, in which the striatal lesion is supposed to procede the nigral involvement.[4]

Recent evidence has documented the existence of a marked subregional differentiation of the human striatum with regard to neurochemical mosaicism that relates to the efferent and afferent fiber systems, including fiber connections.[5] Accordingly, it is to be expected that regionally different patterns of striatal and nigral involvement may be associated with certain neurological deficits such as those seen in parkinsonism.

We performed a topographical study of the regional and subregional involvement of the striatonigral projection fibers and nigrostriatal dopaminergic neurons in the substantia nigra of patients with PD and SND. For this purpose, tyrosine hydroxylase and calcineurin (CaN), a Ca^{2+} calmodulin-regulated protein phosphatase, were used as markers. Tyrosine hydroxylase identifies catecholamine (dopamine)-containing neurons[6] and CaN serves as a marker for striatal, medium-sized, spinous neurons that send their axons to both the globus pallidus and substantia nigra.[7,8]

MATERIALS AND METHODS

Tissues used in this study were obtained from 11 patients with pathologically confirmed PD and 9 normal controls. Three autopsied patients with SND were also examined, as summarized in Table 1. Brain tissue was fixed in 10% neutral formalin for several weeks, sliced, and

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embedded in paraffin. Histological and immunocytochemical studies of the substantia nigra were done on 6-μm sections at the level through the superior colliculus and caudal red nucleus.

Affinity-purlfied rabbit antibody to CaN previously characterized and employed in other immunocytochemical studies was used as described before.[7,8] Rabbit antiserum to tyrosine hydroxylase (Eugene Tech. International Inc., Allendale, NJ) was used at a dilution of 1:200 in phosphate-buffered saline containing 3% bovine serum albumin. Incubation was carried out for 18 h at 4°C. Visualization of bound primary antibodies was done with the Vectastatin ABC Kit (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions.

We determined the total number of dopaminergic neurons in each section of the substantia nigra by counting those neurons containing both tyrosine hydroxylase immunoreactivity and neuromelanin pigment in their perikarya, using an eyepiece fitted with a grid at x100 magnification. The substantia nigra was divided into two parts, medial and lateral.

RESULTS

As compared with the number in controls, in PD patients there was an apparent depletion of dopaminergic neurons in the substantia nigra (Table 2), with the number of dopaminergic neurons in the lateral portion reduced to 31.4% of the control value. The reduction in the medial portion was less severe, corresponding to 49.5% of the control. These differences were considered statistically significant ($P<0.001$). In the substantia nigra of one case of PD, the dopaminergic neurons had almost completely disappeared in the lateral portion and only a small number of them were seen in the medial portion (Fig. 1A). By contrast, CaN immunoreactivity was densely distributed throughout the substantia nigra of patients with PD (Fig. 1B).

In all patients with SND, a marked loss of dopaminergic neurons was observed in the substantia nigra. The number of dopaminergic neurons in the lateral portion was consistently smaller than that in the medial portion (Table 1). This finding is illustrated by semi-quantitive plotting of dopaminergic neurons (Fig. 2A). It can be seen that dopaminergic neurons were more densely localized in the medial portion than in the lateral portion. Again, in all AND patients there was a marked depletion of CaN immunoreactivity in the lateral part of the substantia nigra (Fig. 2B), thus reflecting striatal degeneration, which was most evident in the lateral and caudal portions of the putamen.

DISCUSSION

Dopaminergic neurons in the lateral portion of the substantia nigra project primarily to the putamen, while the caudate nucleus is mainly innervated by the projection fibers originating from the medial portion of the substantia nigra.[1,9] The striatonigral projection fibers appear to be organized in a manner reciprocal to that of nigrostriatal fibers, implying the existence of a dopamine-related interconnecting loop between the striatum and the substantia nigra (i.e., the nigrostriatal loop).

This study demonstrated that in PD patients a marked depletion of dopaminergic neurons occurs in the substantia nigra, with the lateral portion being more severely affected. In relation to the reciprocal connection between the substantia nigra and the striatum, this finding may indicate that striatal dopamine deficiency is most significant in the

Table 1 Subdivisional Distribution of Dopaminergic Neurons in the Substantia nigra in Autopsied Patients with SND.

	Age	Sex	Cell Number/section	
			Medial Portion	Lateral Portion
Case 1	70	F	164	51
Case 2	72	F	67	20
Case 3	59	F	281	85

Table 2 Subdivisional Distribution of Dopaminergic Neurons in the Substantia nigra in Normal Controls and in Patients with PD.

	Cell Number (mean ± S.D.)		% of controls
	Controls (n=9)	PD patients (n=11)	
Medial Portion	323 ± 30	160 ± 66*	49.5
Lateral Portion	312 ± 39	98 ± 48*	31.4

*P<0.001 as compared with corresponding control region (Student's t-test).

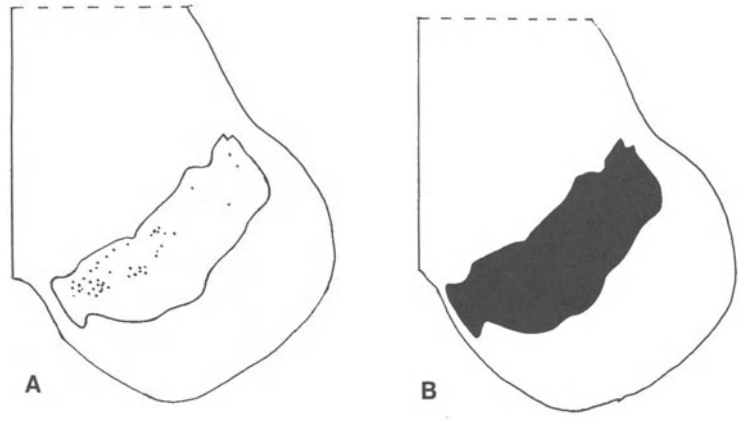

Fig. 1. Immunocytochemical findings in the midbrain from a patient with PD. (A) diagram of the distribution of dopaminergic neurons; (B) diagram of the distribution of CaN-immunoreactive materials. CaN immunoreactivity is densely distributed throughout the substantia nigra.

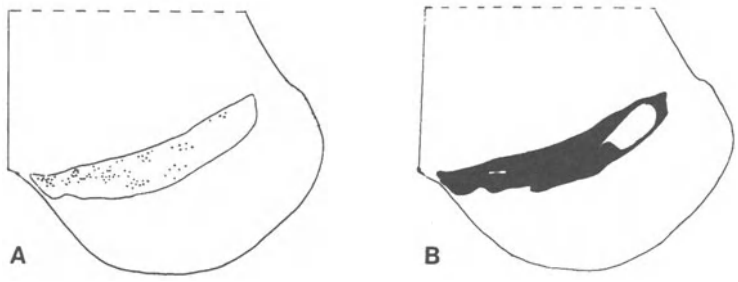

Fig. 2. Immunocytochemical findings in the midbrain from a patient with SND. (A) and (B) are diagrams of the distribution of the dopaminergic neurons and CaN-immunoreactive materials, respectively, in the substantia nigra of Case 2.

putamen, which interpretation would support the view that motor deficits of PD are for the most part a consequence of dopamine loss in the putamen.[1] On the other hand, CaN-immunoreactive striatonigral projection fibers were well preserved in the substantia nigra of patients with PD.

Also in SND patients a severe reduction in the number of nigrostriatal dopaminergic neurons was consistently observed in the lateral portion of the substantia nigra. Although this may explain the parkinsonism in SND patients, there exists additionally a marked depletion of CaN-immunoreactive striatonigral fibers in the lateral portion of the substantia nigra, reflecting putaminal degeneration. This could account for the parkinsonism of SND, because nigroputaminal dopaminergic inputs have no affect on the striatal function when they lose their target neurons in the putamen. In addition, the caudal portion of the putamen has been described to be the most affected with respect to striatal dopamine deficiency in PD patients[10] and to striatal involvement in SND patients.[2,4] Our results would indicate that parkinsonism, at least in PD and SND, may be due to the disruption of the functions of the putamen and the lateral portion of the substantia nigra, which have dense reciprocal connections as a part of the dopamine-related nigrostriatal loop.

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IMMUNOCYTOCHEMICAL STUDIES OF SUBSTANCE P AND MET-ENKEPHALIN IN THE GLOBUS PALLIDUS OF PROGRESSIVE SUPRANUCLEAR PALSY

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INTRODUCTION

The globus pallidus (GP) is considered to receive the afferent nerve fibers originating mainly from the striatum[1] and may play a role in expression of the striatal functions.[2] Substance P(SP) and Met-enkephalin (MEnk) are neuropeptides that have been demonstrated in the basal ganglia and appear to be part of the straitopallidal and striatonigral projections.[3,4] A study on the regional distribution of the peptides in the GP showed the internal segment to be rich in SP and an abundance of MEnk in the external segment.[3] Based on neurochemical measurements and on immunohistochemical studies, abnormalities in basal ganglia SP and MEnk have been reported in Huntington's disease (HD)[4-7] and Parkinson's disease (PD).[6-8] Progressive supranuclear palsy (PSP), a parkinsonian-like syndrome, is characterized by supranuclear ophthalmoplegia, axial dystonia, and pseudobulbar palsy.[9] Histologically, neuronal cell loss, gliosis, and neurofibrillary tangles involve mainly the globus pallidus, subthalamic nucleus, substantia nigra, and other brainstem tegmental nuclei. Few reports have appeared on SP and MEnk in the basal ganglia of PSP. In one study no significant alterations were observed,[10] and one case report showed some decrease in SP content in the external segment of the pallidum.[11] To ascertain whether some alteration is present in the striatal efferent nerve terminals, we performed an immunohistochemical study on the globus pallidus of PSP patients.

MATERIALS AND METHODS

Brain tissue was obtained at autopsy from four PSP patients and four neurologically normal control subjects. Postmortem brain tissues from the patients were fixed in 10% neutral formalin for 3 weeks, sliced coronally, and embedded in paraffin. Histologically, all PSP cases showed mild to severe neuronal loss with gliosis of the globus pallidus. Loss of neurons and gliosis were also prominent in the subthalamic nucleus and substantia nigra. Myelin pallor was seen in the globus pallidus. No apparent neuronal loss was found in the striatum. For immunocytochemical studies, 8-μm sections from the area of the basal ganglia were prepared. Rabbit antisera to SP (from Cambridge Research Biochemicals) and MEnk (from

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Immuno Nuclear Corporation) were used at dilutions of 1:1000 with overnight incubation at 4°C. Then the avidin-biotin complex immunoperoxidase assay was performed with the Vectastatin ABC Kit (Vector Labs.) using 3,3'-diaminobenzidine as chromogen. Imidazole was used to increase the sensitivity of the cytochemical reaction for peroxidase.

RESULTS

In the control cases positive staining for both SP and MEnk was found in fibers and terminals. The fibers showed numerous "pipe-shaped" and "ring-like" structures that appeared to consist of fine fibers lining the surfaces of dendrites. SP-like immunoreactivity was densely localized in the internal segment in contrast to that of MEnk, which was strong in the external segment (Fig. 1 a,c). Higher magnification revealed the "pipe-shaped" and "ring-like" structures outlining the dendrites and the cell surfaces of the pallidal neurons (Fig. 1 b,d). The regional distribution of SP and MEnk in the human globus pallidus were consistent with previous reports.

In the PSP cases the SP- and MEnk-like immunoreactivities were moderately to markedly reduced in the pallidal internal and external segments, respectively (Fig. 2 a,c). The "pipe-shaped" staining pattern described above was scarcely shown (Fig. 2 b,d), and in some places the immunoreactivity was almost completely lost. The MEnk-like immunoreactivity was relatively spared in the medial and dorsal portion in the external segment. The decrease in the immunoreactivity was proportional to the neuronal loss of the globus pallidus.

DISCUSSION

Recent immunohistochemical studies have shown SP and MEnk to be localized in the human striatopallidal pathway,[4] and they originate mostly from medium-size spiny neurons. Moreover, neurochemical subregional differences in SP and MEnk distribution have been reported in the globus pallidus.[4] Our results indicate that SP is mainly present in the internal segment of the globus pallidus (GPi), and MEnk, in the external segment of the globus pallidus (Gpe). These findings are consistent with those found in human brains by other investigators.[3,4,6,7]

In comparison to controls, the same general pattern of SP- and MEnk-containing fibers and terminals was seen in the PSP pateients. However, the staining intensity for both neuropeptides was moderately to markedly decreased in GPi and GPe, respectively. As far as we are aware, only two reports have appeared on the expression of SP and MEnk in PSP.[10,11] In one study of nine patients, a slight reduction (25-30%) in SP-like material in GPe and substantia nigra was detected by radioimmunoassay; but the decrease was not statistically significant.[10] With regard to MEnk, no alterations were noted in the PSP patients.[10] A slight diminution of SP levels in GPe and substantia nigra was described in a single report in which the same assay system was employed.[11] The results of our immunohistochemical study revealed a significant decrease in SP in the GPi and a severe reduction in MEnk in the GPe. The discrepancy between our results and those previously reported,[10,11] could be explained by the fact that our immunohistochemical procedure permits evaluation of the subbrgional distribution of these substances in some specific structures.

The globus pallidus may be considered as a modulator of striatal function and may be affected in neostriatal neurodegenerative disorders.

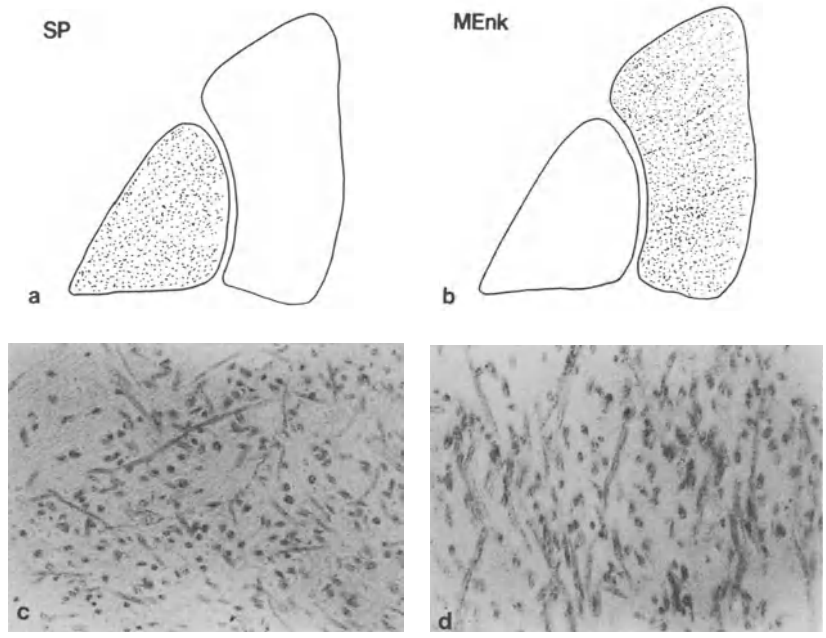

Fig. 1. Diagrammatic distribution of immunohistochemical staining for SP (a) in the internal pallidal segment (GPi) and for MEnk (b) in the external pallidal segment (GPe). Light microscopic findings of SP (c, X190) and MEnk (d, X190) in a control case.

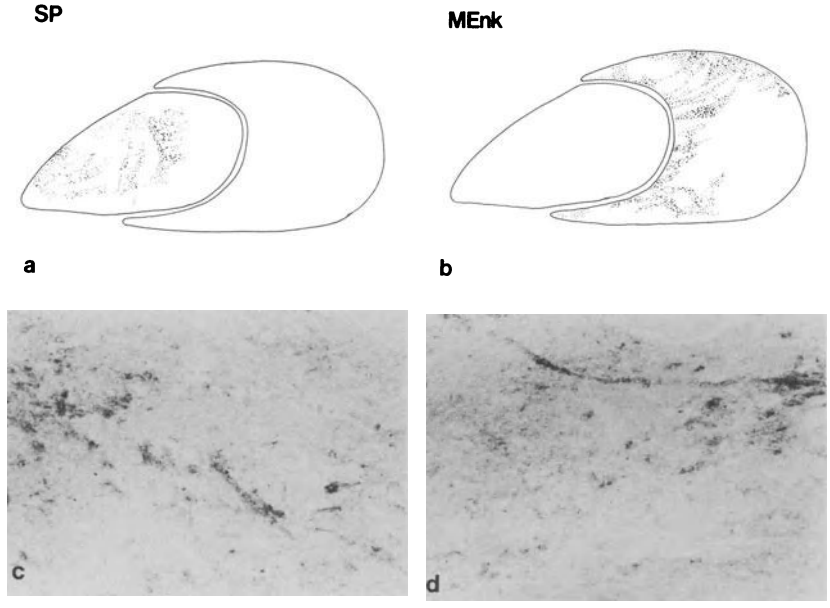

Fig. 2. Diagrammatic distribution of immunohistochemical staining for SP in GPi (a) and MEnk in GPe (b). Light microscopic findings of SP (c, X190) and MEnk (d, X190) in a PSP patient. The staining intensities of the SP- and MEnk-immunoreactivities were markedly decreased.

In patients with Huntington's disease and striatonigral degeneration, a marked loss of SP- and MEnk-like immunoreactivities was observed in the globus pallidus, probably secondary to the loss of projection neurons of putamen and/or caudate.[6, 7, 12] Neurochemical markers have proven to be useful in detecting the involvement of the pallidal afferent nerve fibers, the alterations of which are otherwise difficult to evaluate.[6, 7, 12] In PSP, while the projection neurons of the striatum appeared normal, loss of target neurons and myelin pallor was found in the globus pallidus. The decreases in SP- and MEnk-like immunoreactivities observed would indicate that neurochemical changes of the striatopallidal axon terminals are associated with the degeneration of the pallidal target neurons.

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HETEROGENEOUS DISTRIBUTION OF L-DOPA IMMUNOREACTIVITY IN DOPAMINERGIC NEURONS OF THE RAT MIDBRAIN

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INTRODUCTION

Behavioral and biochemical studies have supported the idea that the dopaminergic projection systems from the midbrain to the basal ganglia or to the limbic cortex could be a key role in motor or psychomotor functions.[1,2] Midbrain dopamine (DA) neurons were first histochemically identified by the Falck-Hillarp formaldehyde histofluorescence technique,[3,4] and later further analyzed by immunocytochemistry using antibodies against tyrosine hydroxylase (TH).[5] Recent advances in immunological methodology has enabled the production of antibodies against cathecholamines themselves.[6,7,8] Owing to these antisera, we could analyze the morphological status of DA or its precursor, L-DOPA, at the single cell level by immunocytochemical means.[8,9,10] In the present study, we report the localization characteristics of L-DOPA in midbrain DA neurons of the rat brain defined by specific and sensitive antiserum raised against L-DOPA, and the subdivision of the dopaminergic neuronal cell group by L-DOPA immunostaining.

MATERIALS AND METHODS

Male adult Sprangue-Drawley rats were used in this study. Under deep anesthesia, the rats were perfused with 0.1M phosphate buffer (PB) containing 5% glutaraldehyde and 1% sodium metabisulfite (SMB). After posfixation in the same fixative for 8h, brains were removed and cut with a vibratome. The sections were incubated with rabbit anti-L-DOPA serum (dilution 1:10000) for 4 days at 4 ℃ in 0.1M PB supplemented with 0.1% Triton X-100 and 1% SMB. Some sections were treated with well-characterized rabbit anti-dopamine serum[6] (1:10000), rabbit anti-TH serum[11] (1:10000), or rabbit anti-AADC[12] (1:5000). These sections were processed for the peroxidase anti-peroxidase method[13], as described previously.[10] The characteristics of anti-L-DOPA serum were detailed previously.[8,9] Furthermore, the immunocytochemical absorption test by preincubation of the antiserum against L-DOPA with the compound L-DOPA-G-BSA (L-DOPA and BSA conjugated with glutaraldehyde) revealed no immuno-

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staining, although preincubation with DA-G-BSA did not affect the immunostaining.

RESULTS AND DISCUSSIONS

The midbrain DA neurons form an continuous cell group in the substantia nigra or SN (A9 cell group), the ventral tegmental area or VTA (A10), and caudal ventrolateral tegmentum of the midbrain (A8).[14] L-DOPA

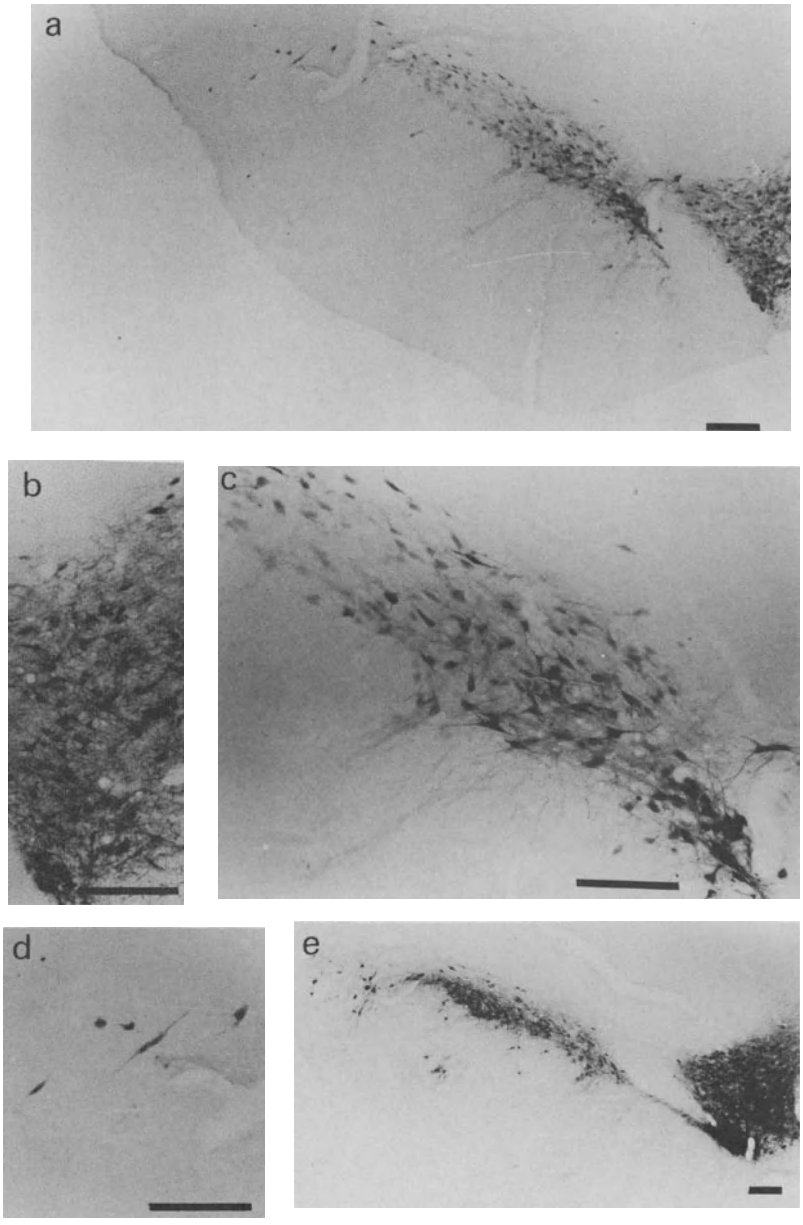

Fig. 1. Immunocytochemistry of L-DOPA (a,b,c,d) and dopamine (e) in the rat midbrain ventral tegmentum. (b),(c),(d) are high-magnification photomicrographs of (a). Bars=200 μm.

immunoreactivity was distinctly identified in neurons of these areas (Fig. 1), and L-DOPA-immunoreactive (IR) neurons showed a distribution similar to that of DA-IR neurons in the SN and VTA. Among these areas, intensity of L-DOPA immunostaining of cell bodies and fibers was highest in the VTA (Fig. 1a,b). In the pars compacta of the SN, there was a tendency for cells located in the medial part to have a more intense L-DOPA immunoreaction, whereas neurons of the central to the lateral part of the pars compacta only showed faint immunoreactivity (Fig. 1c). In the pars lateralis of the SN, the immunoreactivity in the neurons was more intense than that in the lateral part of the pars compacta, but less intense than that in the VTA (Fig. 1d). In the pars reticulata, the staining intensity of neurons was variable. In contrast to the L-DOPA immunoreactivity, DA immunoreactivity was intense in almost all neurons of the VTA and SN, and no prominent diversity of immunostainings were noted (Fig. 1e). In the TH- and AACD- immunocytochemistry, the immunostaining of each neuron was even and intense, and we could not detect any topographical differences.

A variety of immunoreactivity was also noted in the A8 cell group (Fig. 2a). In the rostral raphe linear nucleus, intensely labeled neurons were prominent, but in the central gray of the midbrain, most cells were weakly stained. However some solitary and very intensely labeled neurons were identified (Fig. 2b).

The heterogeneous intensity of catecholamine neurons was already mentioned in the pioneering works with Falck-Hillarp catecholamine fluorescence.[4] However, TH seems not to be involved in this heterogeneity.[5] In previous study, we presented this discrepancy between intensity of immunoreactive TH and catecholamine fluorescence in the A1/C1 area of the ventrolateral medulla oblongata.[15] In the present study, we demonstrate that neurons in the pars compacta of SN, particularly those in its central and lateral part, showed weaker L-DOPA immunoreactivity than VTA cells. Heterogeneous immunoreaction was not prominent in DA immunocytochemistry, and actually none in TH- or AADC- immunocytochemistry. The weak reactivity to L-DOPA in SN cells may be explained by more rapid decarboxylation of L-DOPA to dopamine by AADC, or less production of L-DOPA from tyrosine by TH, than occurs in the neurons in the VTA. The finding of the L-DOPA-IR cells with very high intensity located in midbrain central gray suggest the possible existence of cells forming L-DOPA as an end product. These cells were suspected to be present in the ventrolateral part of the hypothalamic arcuate nucleus.[9]

In summary, the present results have demonstrated the heterogeneous immunoreactivity of L-DOPA in midbrain dopaminergic areas. A variety of immunoreactivity of L-DOPA in DA neurons was also reported in the cat.[10] It is interesting to note that DA neurons that are located in the medial part of the pars compacta of the SN and VTA project not only to the caudate-putamen but also to the ventral striatum (nucleus accumbens and tuberculum olfactorium)[14,16] seem to correspond to the neurons showing stronger L-DOPA immunoreactivity. Functional classification of neurons

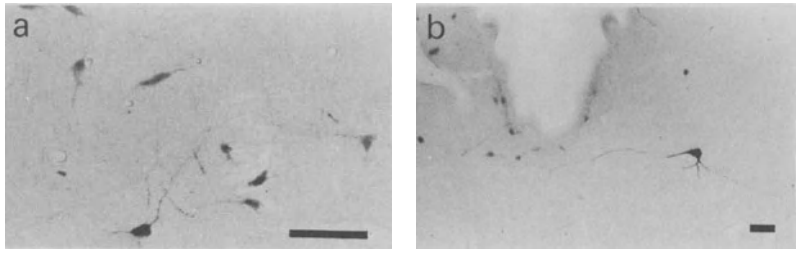

Fig. 2. L-DOPA immunoreactivity in A8 (a) and midbrain central gray (b). Bars=100 μm.

based on precursor substance may be beneficial to analyze metabolic characteristics of midbrain dopaminergic neurons at the cellular level.

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CHOLINERGIC SYSTEMS IN ALZHEIMER'S DISEASE, PARKINSON'S DISEASE AND PROGRESSIVE SUPRANUCLEAR PALSY

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I. INTRODUCTION

Although the localization of cholinergic neurones in the central nervous system has been studied in extensive detail (Table 1), little is known as yet about their connections, and still less about their functions. Information deriving from experiments with animal models applies to a large extent to human brain as well, but more specific data on the localization and possible functions of neurones in humans can be obtained by taking advantage of selective lesions in the brains of patients with neurodegenerative disorders. Some studies have been performed to determine the nature and severity of cholinergic lesions in these diseases. The data are limited, however, because the most specific method of evaluation available until recently was the in vitro measurement of the activity of choline acetyltransferase (ChAT), the enzyme catalyzing acetylcholine synthesis. This method, while quantitative, does not distinguish between enzyme contained in cell bodies or nerve terminals, and cannot detect, within small structures, inhomogeneous distributions of these neuronal elements, or irregularities in their loss under pathological conditions. It is now possible to visualize cholinerigc cell bodies, fibers and varicosites with immunocytochemical techniques using antibodies directed specifically against human ChAT. In addition, this type data can be quantified by computerized image analysis.

We will present a brief summary of the principal biochemical and immunocytochemical data concerning three neurodegenerative diseases, Alzheimer's disease, Parkinson's

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table 1.

CHOLINERGIC CELLS GROUPS*	STANDARD NOMENCLATURE	MAIN PROJECTION AREA
Ch 1	Medial septal nucleus	Hippocampus.
Ch 2	Vertical limb nucleus of the diagonal band of Broca	Hippocampus.
Ch 3	Lateral part of the horizontal limb nucleus of the diagonal band of Broca	Olfactory bulb.
Ch 4	Nucleus basalis of Meynert, globus pallidus, substantia innominata, nucleus of the ansa lenticularis, neurons within the boundaries of the diagonal band	Neocortex and amygdala.
Ch 5	Nucleus pedunculopontinus, neurons within nucleus cuneiformis and parabrachial area	Thalamus + + + + (anterior, lateral, reticular nucleus) ; substantia nigra+ + ; striatum + ; globus pallidus + ; lateral hypothalamus ; superior colliculus ; spinal cord.
Ch 6	Laterodorsal tegmental nucleus	Thalamus + + + + (anterior, lateral, reticular nucleus) ; substantia nigra + + ; striatum + ; globus pallidus + ; lateral hypothalamus; superior colliculus ; spinal cord.
Ch 7	Medial habenular nucleus	Interpeduncular nucleus.
Ch 8	Parabigeminal nucleus	Superior colliculus ; lateral geniculate nucleus.

* According to Mesulam et al, 1983.

disease and progressive supranuclear palsy (PSP), at three levels in the brain, the cerebral cortex, the basal ganglia and the brainstem. Possible mechanisms of compensation for cell loss and the functional consequences of the destruction of certain central cholinergic systems will be discussed.

II. CHOLINERGIC NEURONES IN NEURODEGENERATIVE DISEASES

A. Alzheimer's disease

1. Subcortico-cortical systems

Measurements of ChAT activity have shown that the greatest cholinergic deficiencies in patients with Alzheimer's disease are found the hippocampus followed by neocortical regions (table 2). The neurones projecting to these regions originate in the septum and the substantia innominata, respectively. Loss of cholinergic neurones has been reported in the nucleus basalis of Meynert (Whitehouse et al, 1981; see review in Tagliavini and Pilleri, 1984) and the septum (figure 1), but dissenting opinions have ben voiced (Pearson et al, 1983; Perry et al, 1982; Allen et al, 1988) has been evoked. Morphological images suggesting

Table 2. CHOLINE ACETYLTRANSFERASE ACTIVITY POST-MORTEM IN THE CEREBRAL CORTEX OF PATIENTS WITH ALZHEIMER'S DISEASE

1. ChAT activity is decreased by a mean of about 60% (Bowen et al; Davies and Maloney, 1976; Perry et al, 1977). Extreme values: -27% (Wilcock et al, 1982) to -95% (Henke and Lang, 1983).

2. The decrease in ChAT activity in the cerebral cortex (Rossor et al, 1982), particularly in the superficial layers of the cortical mantel (De Kosky et al, 1985), results from cell loss in the nucleus basalis of Meynert (Whitehouse et al, 1981).

3. ChAT activity is most severely decreased in the hippocampus (Henke and Lang, 1983), reflecting damage to the septohippocampal cholinergic pathway, confirmed by selective loss of ChAT-immunostained neurones in the septal area (Figure 1).

4. The decrease in cortical ChAT activity is more severe in patients with early onset of the disease (Bird et al, 1983). It attenuates with age at the time of death (Rossor et al, 1981). ChAT activity in the cortex of patients older than 79 at death are similar to those in control subjects of the same age.

5. The decrease in ChAT activity is accompanied by a decrease in acetylcholine synthesis and choline uptake, as demonstrated on pre-mortem cortical biopsies (Sims et al, 1983).

6. The decrease in ChAT activity correlates positively with the density of senile plaques (Perry et al, 1978; Mountjoy et al, 1984) and negatively with the number of muscarinic receptors in the cortex (Rinne et al, 1989).

7. The decrease in ChAT activity is also correlated with the severity of dementia measured by Mini-Mental tests (Perry et al, 1978; Wilcock et al, 1982, Ruberg et al, 1990). The lowest scores are obtained by patients with myoclonus (Bird et al, 1983).

decreased funtional activity of these neurones have also been presented (Mann and Yates, 1982).

Since cholinergic innervation suffers most in the hippocampus of patients with Alzheimer's disease, this structure was selected for detailed qualitative and quantitative immunocytochemical study with an antiserum against human ChAT (Ransmayr et al, 1989). It was determined that: a) there are no intrinsic cholinergic neurones in the human hippocampus, but that two different cholinergic systems innervate the structure, a major system originating in the septum innervating the hippocampus dorsally via the fimbria and fornix and a minor system arising in the temporal cortex; b) loss of cholinergic innervation is severe though variable in patients with Alzheimer's disease, and affects all substructures to the same extent; c) senile plaques containing ChAT-positive fibers or nerve terminals are distributed inhomogeneously, however, predominating in the outer two thirds of the stratum moleculare (fascia dentata) and the stratum pyramidale (subiculum and CA1).

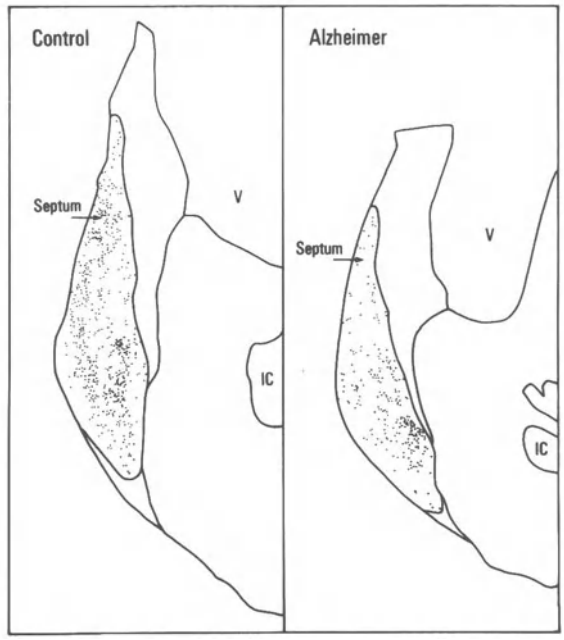

Figure 1.

Cholinergic neurons labelled with an anti-ChAT antiserum, in the septum of a subject with Alzheimer's disease and a control on a frontal section of the medial septum. Each point represents an immunolabelled cell body. The total number of cholinergic neurons in the septum is reduced by about 50% in Alzheimer's disease. V = ventricle; IC = internal capsule. (Lehéricy et al, in preparation).

2. Subcortical systems

ChAT activity is normal in most subcortical regions of patients with Alzheimer's disease, although decreases have been reported in the caudate nucleus, nucleus accumbens, amygdala, septum, anterior perforated substance, substantia innominata, anteromedian nucleus of the thalamus, and cerebellar cortex (Rossor et al, 1982). Cholinergic structures in the brainstem, like the tegmental pedunculopontine nucleus (Ch5) (Zweig et al, 1987; Jellinger, 1988) and the laterodorsal tegmental nucleus (Ch6) (Brandel, Malessa, Hirsch, Agid, in preparation) seem to be spared.

The distribution of cholinergic lesions in the brain of subjects with Alzheimer's disease is complex, and may be inhomogeneous within structures. An example is the striatum, where loss of cholinergic interneurones, identified with an antiserum against ChAT, is restricted to the ventral region, including the nucleus accumbens, where it reaches 60% (Lehéricy et al, 1989) (Figure 2). As would be expected, the density of the fibers projecting from these interneurones is also reduced. The lesion, which is specific to the ventral striatum, also seems selective for the cholinergic neurones, since those containing neuropeptide Y are not at all affected (Lehéricy et al, 1989).

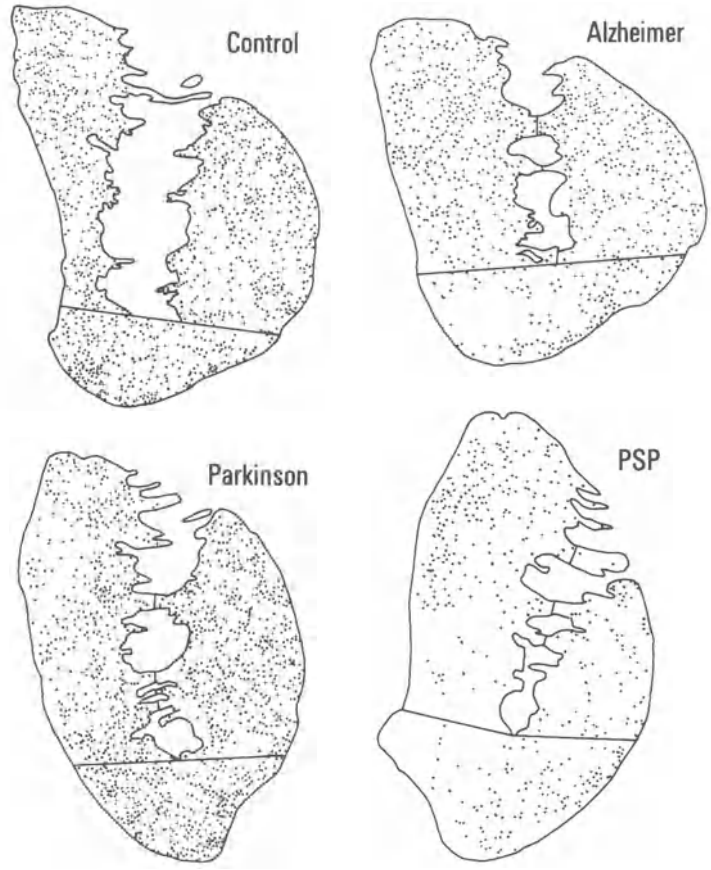

Figure 2.

Cholinergic neurons on frontal sections of the striatum from control subjects and patients with Alzheimer's disease, Parkinson's disease and PSP. Putamen to the right of the internal capsule, caudate nucleus to the left of the internal capsule, ventral striatum below the line. Each point represents a ChAT-positive cell body. Note: absence of cholinergic cell loss in Parkinson's disease; loss of cholinergic neurons in the ventral striatum in Alzheimer's disease; generalized loss of cholinergic neurons in PSP, predominant in medial part of ventral striatum. For detailed definition of structures and immunocytochemical techniques, see Hirsch et al, 1989 and Lehéricy et al, 1989.

B. Parkinson's disease

Decreased ChAT activity in the neocortex and hippocampus (Ruberg et al, 1982; Perry et al, 1985) and loss of neurones in the substantia innominata (Whitehouse et al, 1983), and probably in the septum, indicate that the innominatocortical and septohippocampal systems are lesioned in patients with Parkinson's disease. The degree to which ChAT activity decreases in the cerebral cortex is correlated with the severity of intellectual deterioration observed in the patients. A significant loss of activity can already be found in the frontal cortex and hippocampus of parkinsonian

patients who show no dementia (Dubois et al, 1983) (Figure 3).

ChAT activity is normal in the striatum of parkinsonian patients (Ruberg, 1982), unlike those with Alzheimer's disease. A preliminary observation has shown that the total number of cell bodies labelled with an antiserum against ChAT was similar in patients and controls (Hirsch et al, 1989) (Figure 2). In the brainstem, which has escaped study until now, we know, at most, that the density of cholinergic neurones in the tegmental pedunculopontine nucleus (Ch5) is decreased in certain patients (Hirsch et al, 1987; Jellinger, 1988), especially among those with extensive loss of dopaminergic neurones of the substantia nigra (Zweig, 1989).

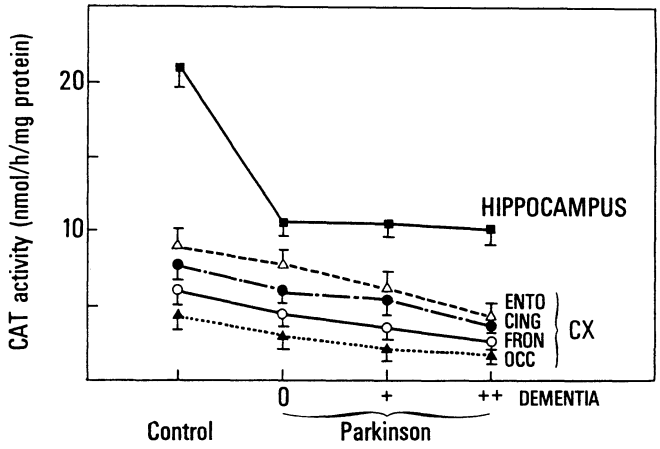

Figure 3.

ChAT activity in the hippocampus and four neocortical regions (entorhinal, frontal, cingulate, occipital) of 20 controls and 20 parkinsonian patients with varying degrees of intellectual impairment. 0 = no dementia; + = slightly demented; ++ severely demented (see Dubois et al, 1985).

C. Progressive supranuclear palsy

The septohippocampal and innominatocortical cholinergic neurons are probably affected in PSP, but less than in Parkinson's or Alzheimer's disease. ChAT activity is often found to be slightly, but not always significantly, reduced in cortical areas (Ruberg et al, 1985; Agid et al, 1986). This is consistent with neuropathological observations of moderate neuronal loss in the substantia innominata (Tagliavini et al, 1983). Other subcorticocortical systems (noradrenergic, serotoninergic, dopaminergic) seem to be intact (see review in Agid et al, 1986).

Although the cholinergic neurones which project to the

cortex suffer only to a limited extent in this disease, cholinergic interneurones in the striatum are severely affected. Large decreases in ChAT activity have been observed in the caudate nucleus, putamen, nucleus accumbens, internal and external pallidum, subthalamic nucleus, and substantia innominata (Ruberg et al, 1985), although no significant loss of ChAT activity in the basal ganglia was reported in another study (Kisch et al 1985). Morphological evidence shows that loss of cholinergic neurones in the striatum is generalized throughout the structure, but particularly apparent in the nucleus accumbens (Figure 2).

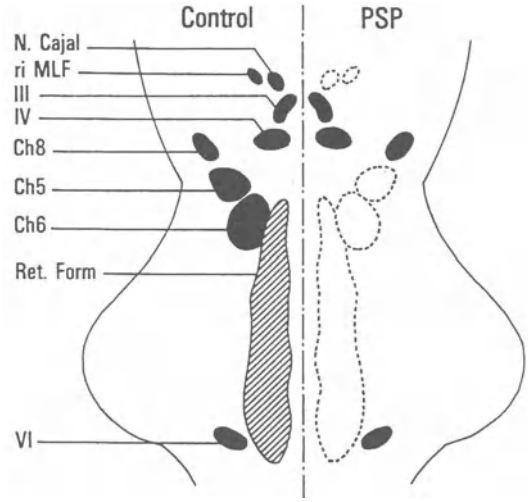

Figure 4.

Cholinergic neurones in the ponto-mesencephalic region of control subjects and patients with PSP. N. Cajal = nucleus interstitialis of Cajal; riMLF = rostral interstial nucleus of the medial longitudinal fasiculus; III, IV, VI = third, fourth and sixth cranial nerves; Ch8 = nucleus parabrachialis; Ch5 = tegmental pedunculopontine nucleus; Ret. Form = reticular formation. Black areas = rich in cholinergic neurones; hatched area = poor in cholinergic neurons; dotted line = loss of cholinergic neurones.

Cholinergic neurones are most severely affected in the upper brainstem (ponto-mesencephalic region) in PSP (Hirsch et al, 1987; Juncos et al, 1990): the tegmental pedunculopontine nucleus (Ch5), the laterodorsal nucleus (Ch6), the interstitial nucleus of Cajal and its annex the rostral interstitial nucleus of the medial longitudinal fasciculus (riMLF) (Figure 4). The nucleus parabrachialis (Ch8) is spared, however (Malessa, Hirsch, Agid, in preparation).

III. SIGNIFICANCE OF THE PARTIAL AND HETEROGENEOUS DEGENERATION OF CHOLINERGIC SYSTEMS IN NEURODEGENERATIVE DISEASES

A. Can loss of central cholinergic neurones be compensated?

Sprouting of cholinergic nerve terminals in the hippocampus of experimental animals has been reported after partial lesion of the septohippocampal pathway in the presence of nerve growth factor (Hefti et al, 1989), and after grafts of embryonic cholinergic neurons from the septal region into a hippocampus partially denervated by lesion of the fimbria (Segal et al, 1985). To our knowledge, no evidence of sprouting has been reported in neurodegenerative diseases affecting cholinergic neurones.

Physiological mechanisms of compensation after denervation have been detected in central dopaminergic neurones. Both presynaptic (hyperactivity of the remaining neurones) and postsynaptic (receptor hypersensitivity) components have been described (see Agid et al, 1987). The possibility that there is an increase in the synthesis and release of acetylcholine in cholinergic neurones spared by lesions has been inaccessible to investigation, since its metabolite choline, is a poor indicator of transmitter turnover, in that it is immediately reutilized for acetylcholine synthesis. Two recent post-mortem observations on patients with Alzheimer's disease suggest, however, that hyperactivity of cholinergic neurones in

Figure 5.

Abnormal cholinergic fiber labelled with an antiserum against ChAT, in the hilus of the hippocampus of a patient with Alzheimer's disease. Bar = 200μm. From Ransmayr et al, 1989, with permission.

lesioned structures may exist. A certain number of ChAT-positive nerve fibers in the hippocampus of these patients contained a series of enlarged bead-like varicosities (Ransmayr et al, 1989) (Figure 5) that were never observed in control subjects. These enlargements may contain accumulations of neurotranmitter. Compatible with this hypothesis, a normal density of binding sites for ^{3}H-vesamicol, an inhibitor of vesicular acetylcholine transport, was observed in the temporal cortex of patients with Alzheimer's disease and demented patients with Parkinson's disease, in spite of a 50% decrease in ChAT activity (Ruberg et al, 1990). Whether this reflects an increase in the number of synaptic vesicles or in their transport capacity in remaining nerve terminals, or a decrease in ChAT activity in otherwise intact nerve endings, remains to be determined. Should these morphological and biochemical observations indeed reflect a mechanism of compensation in cholinergic neurones, they would explain why non-demented parkinsonian patients, who have decreased levels of ChAT activity in certain regions of the cerebral cortex, show no evidence of a central cholinergic deficiency until challenged with otherwise subliminal doses of anticholinergic drugs (Dubois et al, 1987).

There have been a number of studies on acetylcholine receptors in neurodegenerative disorders, particularly Alzheimer's disease. Interpretation of the data is difficult, however, since measurements were made for the most part with dissimilar methods, on whole brain structures, from populations of patients that were not always well defined. Data concerning cholinergic receptors in cortical and subcortical regions of patients with Alzheimer's disease remain contradictory (Table 3). In summary, it seems that in most structures the number of muscarinic M1 type receptors increases. Since these are thought to be located post-synaptically, the increase may reflect denervation induced-hypersensitivity. M2 type receptors, on the other hand, may decrease in number, consonant with the notion that these receptors are preferentially located on cholinergic nerve terminals that degenerate in Alzheimer's disease. The same reasoning may apply to nicotinic receptors which are generally thought to decrease in cortical regions. In addition, it has been reported that the coupling of M1 receptors and its G-protein is impaired in the parietal cortex of patients with Alzheimer's disease (Smith et al, 1987). It is possible, even probable, that these biochemical changes are consequences of incipient damage to remaining cholinergic neurons rather than signs which point towards an etiology of the disease. The relative integrity of cholinergic receptors in the cortex of patients with Alzheimer's disease, in any case, augures well for the development of a substitutive treatment for the symptoms of Alzheimer's disease. Muscarinic receptors also seem to be unimpaired in cortical regions of patients with Parkinson's disease (Ruberg et al, 1982). Hypersensitivity of ^{3}H-QNB binding sites has even been observed in cases where cholinergic denervation in the frontal cortex and hippocampus was severe; i.e., in patients with dementia. This hypersensitivity is increased even further in patients who were treated for long periods of time with anticholinergic

drugs (Dubois et al, 1985). In PSP, where the cerebral cortex is essentially intact, the density of muscarinic receptors labelled with ^{3}H-QNB is similar in the frontal cortex of patients and controls (Ruberg et al, 1985).

Table 3. CHOLINERGIC RECEPTORS IN ALZHEIMER'S DISEASE

1. Muscarinic receptors

Most authors (White et al, 1977; Palacios 1982; Davies 1978; Shimohama et al, 1986) report that the density of muscarinic receptors (measured with ^{3}H-QNB or ^{3}H-atropine) is unchanged in the cerebral cortex. A decrease in muscarinic receptors is observed, however, in the nucleus accumbens, the amygdala (Rinne et al, 1985) and the hippocampus (Shimohama et al 1986; Reinikainen et al, 1987). In very severe cases, decreases are observed in the cerebral cortex (Reinikainen et al, 1987). An increase in ^{3}H-QNB binding is reported in a small number of patients in the frontal cortex and the hippocampus (Nordberg et al 1983).

The number of M1 type receptors does not seem to be modified either in the frontal cortex (Mash et al, 1985) or the hippocampus, whether measured with ^{3}H-QNB in the presence of carbachol (Rinne et al, 1985) or by autoradiography with ^{3}H-methyl-scopolamine (Probst et al, 1988).

Normal concentrations of M2 receptors are observed by autoradiography in the hippocampus (Probst et al, 1988) and various cortical regions (Lang and Henke, 1983). Decreased numbers are reported in the frontal cortex (Mash et al, 1985) and hippocampus (Rinne et al, 1985) when biochemical methods are used (^{3}H-QNB in the presence of oxotremorine). This suggests that M2 receptors are located on the cholinergic afferents to the cortex which degenerate in patients with Alzheimer's disease.

2. Nicotinic receptors

The density of nicotinic receptors is reported to decrease in cortical regions when measured with α-bungarotoxin (Davies and Feisulin, 1981), ^{3}H-acetylcholine in the presence of atropine (Whitehouse et al, 1986), ^{3}H-nicotine (Nordberg et al, 1986), or ^{3}H-N-methyl-carbamylcholine (Araujo et al, 1988), although no change is observed by Shimohama et al (1986) with ^{3}H-nicotine.

The density of nicotinic receptors in subcortical regions (striatum, globus pallidus, thalamus, nucleus basalis of Maynert) has been reported to be normal (Araujo et al, 1988), but decreases in the putamen and nucleus basalis of Meynert have also observed (Shimohama et al, 1986).

The density of muscarinic receptors in the striatum of patients with Alzheimer's disease seems normal, although decreased numbers have been reported in the nucleus accumbens and the amygdala (Table 3). The same is true for Parkinson's disease (Dubois et al, 1985) and PSP, unlike D2 receptors in the latter, which are significantly reduced in number (Ruberg et al, 1985). These observations suggest that the cholinoceptive cells of the caudate nucleus, putamen and nucleus accumbens are spared, at least to a large extent.

B. What are the functional consequences of the loss of cholinergic neurones?

The functional consequences of cholinergic denervation in the human brain evidently differ, depending on where it occurs: the cerebral cortex, the basal ganglia, or the brainstem.

The loss of cholinergic nerve terminals in the cerebral cortex is generally attributed to cell loss in subcortical regions: the septal region for the hippocampus, the substantia innominata for the neocortex. The presence of intrinsic cholinergic neurons in the human cerebral cortex needs to be demonstrated. There seems to be none in the hippocampus, however (Ransmayr, 1989). In Alzheimer's disease, the fact that ChAT activity and cholinergic innervation decrease relatively homogeneously in the neocortex (Rossor et al, 1982) and hippocampus (Ransmayr et al, 1989) respectively suggests that cholinergic dysfunction probably plays some role in the neuropsychological disorders observed in the disease. The significant correlation between the decrease in ChaT activity in the temporal cortex of patients with Alzheimer's disease and the degree to which their cognitive functions are altered when measured on a simple scale (the Blessed test) (Ruberg et al, 1990 - Figure 6) supports this notion. A large corpus of experimental

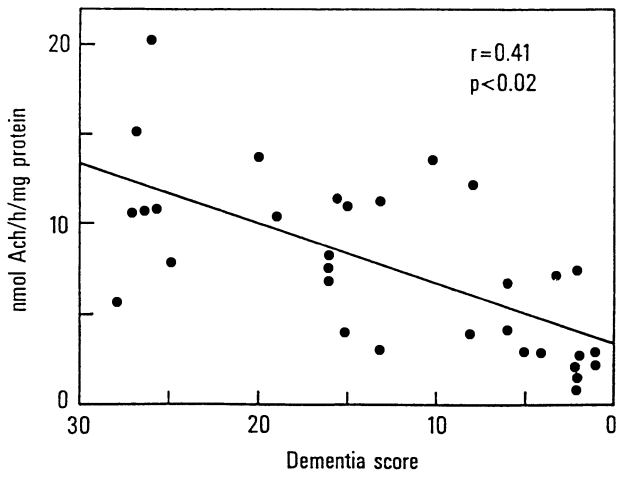

Figure 6.

ChAT activity in the temporal cortex of patients with Alzheimer's disease, as a function of their degree of intellectual deterioration. From Ruberg et al, 1990, with permission.

literature indicates that loss of cholinergic innervation in the hippocampus contributes to memory disorders whereas denervation of the neocortex impairs diverse complex cognitive functions, resulting, in particular, in the appearance of frontal lobe-type symptoms (see review in Agid et al, 1987). The subcortico-cortical cholinergic systems are also partially destroyed in patients with Parkinson's disease, especially in those in whom intellectual

deterioration is severe (Figure 3). Although the relative importance of the cortical cholinergic deficiency with respect to lesions of other ascending pathways (noradrenergic, dopaminergic, serotoninergic) is not perfectly clear, it evidently has something to do with memory impairment (Sadeh et al, 1982) and the appearance of frontal signs that can be recognized even in parkinsonian patients without patent dementia (Dubois et al, 1990). It is easy to understand, then, that the inconsiderate prescription of anticholinergic drugs to demented parkinsonian patients with severe central cholinergic deficiencies can provoque mental confusion and hallucinations (Desmet et al, 1982). In short, whatever the disease, even minor loss of cholinergic innervation to the cerebral cortex, must reasonably contribute to cognitive dysfunction, even if the primary cause is much more complex.

The integrity of the cholinergic neurones and their receptors in the striatum of patients with Parkinson's disease indicates that at least a part of the basal ganglia circuitry assuring output from the striatum remains intact. This is assumed to explain the efficacy of both L-DOPA and anticholinergic treatment, since the cholinergic interneurones are at least partly innervated by nigrostriatal dopaminergic neurones. This is not the case in PSP where cholinergic interneurones in the striatum degenerate as well (Figure 2), possibly explaining the absence of reactivity PSP patients to L-DOPA. The lesion is particularly severe in the ventral striatum (including the nucleus accumbens), a strategic link between the limbic system (amygdala and limbic cortex) and frontal motor regions. Neuronal loss in this structure may be expected to play some role in the cognitive disorders classically observed in this disease. This is also the case in Alzheimer's disease, where loss of cholinergic interneurones in the ventral striatum might be implicated in certain behavioural disorders, such as apathy or hyperactivity (Lehéricy et al, 1989).

The contribution of cholinergic lesions in the brainstem to the symptomatology of neurodegenerative dieases is not well known. Damage to these small cholinergic structures is particularly characteristic of PSP (Figure 4). Given the distribution of cholinergic fibers originating in the tegmental-pedunculopontine nucleus (Ch5), neuronal loss in this structure may contribute to motor dysfunction in PSP and certain cases of Parkinson's disease. Bilateral electrical stimulation of this region in the cat is known to impair locomotor activity (Fukushima et al, 1987). Neuronal loss in the laterodorsal tegmental nucleus (Ch6) which projects to the dorsomedian nucleus of the thalamus, a relay to the frontal cortex, might be implicated in certain aspects of the frontal syndrome associated with this disease (Agid et al, 1986). The interstitial nucleus of Cajal is essential for the coordination of eye, head and neck movements, and for head posture. In addition, it connects vestibular and vertical occulomotor nuclei (see Fukushima et al, 1987). The premotor signal for vertical saccades is known to originate in the riMLF (Büttner-Enever et al, 1982). Loss of the cholinergic neurons in these two nuclei is implicated without doubt in the supranuclear palsy of PSP patients, principal nosological criterion of the disease.

These anatomoclinical correlations, incomplete and few in number, have resulted in the introduction of cholinergic agonist treatment for central cholinergic deficiencies. One must remain prudent about the anatomico-clinical correlations, however, since the existence of cholinergic cell loss in a given structure does not exclude that other nerve cells may also be affected and responsible for the essential elements of the clinical picture.

IV. CONCLUSIONS

Numerous questions remain. What exactly is the functional state of the neurones apparently spared by the lesion? Is there sprouting of nerve terminals and dendrites when cholinergic neurones begin to degenerate? Does the cholinergic deficiency affect certain neurones more than others within a given brain structure? Does cholinergic denervation predominate in certain regions of the cerbal cortex (associative or other) or in the striatum (matrix or striosomes)? The list is not exclusive.

The most important question, but also the most difficult to answer, concerns the cause of cholinergic cell death. The loss of large cholinergic cells in the substantia innominata or septum was attributed to the fact that they belong to what has been termed the isodendritic core (Rossor, 1981). In other cases, the cholinergic cells that degenerate cannot be distinguished morphologically from those that are spared: the cells of Ch8 compared to those of Ch5 and Ch6, in PSP, for example. The observation that the cholinergic cells in the ventral striatum are manifestly more vulnerable than those in the dorsal striatum in Alzheimer's disease (Lehéricy et al, 1989) is particularly striking in this regard. One thing seems clear, however: it is not simply because these neurones are cholinergic that they degenerate. The reason for the selective vulnerability of certain groups of cholinergic neurones in the brain of patients with neurodegenerative diseases remains to be discovered.

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NEUROCHEMICAL ASPECTS OF PARKINSON'S DISEASE AND THE DEMENTING BRAIN DISORDERS: RELATION TO BRAIN AGEING

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INTRODUCTION

Alzheimer's disease (AD) as well as idiopathic Parkinson's disease (PD) are brain disorders of older age. This raises the obvious question: Are perhaps age-dependent brain neurotransmitter changes involved in the etiology and clinical symptomatology typical of these neurodegenerative disorders? The typical neurotransmitter-related symptoms in question are: dementia in AD and signs of motor disorder in PD.

DEMENTIA, CHOLINERGIC BRAIN AGEING AND ALZHEIMER'S DISEASE

At present, the dementia of AD is believed to be due, in large part, to a degeneration of cholinergic neurones which originate in the nucleus basalis-septum area and innervate the cerebral cortex and limbic forebrain (see Bartus et al.,1982; Coyle et al.,1983). This is supported by the fairly consistent and severe cholinergic deficit observed in postmortem AD brain (Pope et al.,1965; Bowen et al.,1976; Davies and Maloney,1976; Perry et al.,1977; for review, see Mann,1988). What makes this observation pathoetiologically relevant is the fact that reduction in cholinergic forebrain innervation is also found in the ageing brain (McGeer et al., 1984).

Neurodegenerative Disorders Other Than AD

In our study of the relationship between dementia, ageing and brain cholinergic systems we first looked at neurodegenerative disorders with dementia other than AD. These brain disorders included: Progressive Supranuclear Palsy (PSP; 5 cases); Dementia-Parkinsonism-Motoneurone Disease (DPMN; 1 case); Neuronal Intranuclear Inclusion Body Disorder (NINIB; 1 case); and adult Down Syndrome (DS; 2 cases). In addition, we examined the cholinergic system in the brain of patients with Olivopontocerebellar Atrophy (OPCA). In general, these conditions differed from one another both in respect to clinical symptoms and brain pathology. What the first 4 disorders had, however, in common was clinically disabling dementia; in addition, the first 3 conditions presented with parkinsonian symptomatology with marked to severe cell loss in the substantia nigra. As an index of the integrity of the brain cholinergic neurones we measured in several cortical and subcortical brain regions the activity of choline acetyltransferase (CAT).

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Table 1. Choline Acetyltransferase (CAT) Activity In Brain Regions Of Patients With Progressive Supranuclear Palsy (PSP), Olivopontocerebellar Atrophy (OPCA), Adult Down Syndrome (DS), Dementia-Parkinsonism-Motoneurone Disease (DPMN) And Neuronal Intranuclear Inclusion Body Disorder (NINIB): Comparison With Controls

Brain Region	PSP (mean ± sem)	% of control	OPCA (mean ± sem)	% of control	Adult DS single values	% of control	Controls (range)	DPMN	Controls (range)	NINIB
Cortex										
Occipital	0.55 ± 0.17	102	0.19 ± 0.03	34	0.43 0.18	45	0.29 - - 1.08	0.81	0.30 - - 1.00	0.32
Parietal	0.98 ± 0.24	131			0.25 0.09	16	0.38 - - 1.54	0.63	0.42 - - 1.71	0.45
Temporal	1.02 ± 0.26	110	0.38 ± 0.05	38	0.38 0.09	21	0.68 - - 1.76	0.94	0.60 - - 1.81	0.65
Putamen (rostral)	15 ± 4	71					35 - 51	32	18-32	19
Hippocampal gyrus	1.37 ± 0.63	114			0.75 0.34	30	0.65 - - 2.95	2.13	0.63 - - 2.75	0.95
Ammon's horn	1.74 ± 0.86	132	1.10 ± 0.14	58	0.90 0.15	27	0.79 - - 2.83	1.06	0.84 - - 2.77	1.20
Amygdala	3.34 ± 1.65	113			0.17 0.17	3	1.09 - - 7.86	1.63	1.04 - - 6.50	3.77

CAT activity expressed in nmol/mg protein/10 min. As controls served age-matched non-neurological patients. (Mean age for: PSP controls 71.5 years, n = 8; OPCA controls 54 years, n = 16; adult DS controls 58 years, n = 4-6; DPMN controls 62.0 years, n = 10; NINIB controls 64.0 years, n = 8.) Number of PSP patients: 5; number of OPCA patients: 11-16.For the single cases with DPMN and NINIB respectively, instead of % of control means the lowest and highest CAT values (ranges) are given. Data taken from: Kish et al., 1985a (PSP); 1985b (NINIB); 1987 (OPCA); Gilbert et al., 1988 (DPMN); and partly unpublished (NINIB; adult DS).

(1) Progressive Supranuclear Palsy. In our 5 cases of PSP (Kish et al.,1985a) the biochemical analysis revealed that despite severe dementia, the CAT activity in cortical, hippocampal and subcortical areas was within normal range (Table 1). From this we have to conclude that in our patients the cognitive impairment was not related to cholinergic brain changes.

(2) Dementia-Parkinsonism-Motoneurone Disease. Similar to PSP, in our case of DPMN (Gilbert et al.,1988) the CAT levels throughout the cerebral cortex, subcortical areas and hippocampal formation were within control range (Table 1). Therefore, the severe dementia present in this case could not be accounted for by loss of cholinergic forebrain innervation.

(3) Neuronal Intranuclear Inclusion Body Disorder. Analogous results were also obtained in the case with NINIB (Kish et al.,1985b) whose essentially normal levels of CAT in cortical, subcortical and hippocampal areas again left the severe dementia basically unexplained (Table 1).

(4) Adult Down Syndrome. It is known that DS individuals develop, later in life, regularly severe AD brain pathology. However, not all of them seem to develop clinically disabling dementia (see Wisniewski and Rabe,1986).

In agreement with the literature (Yates et al.,1980; 1985; Godridge et al.,1987), we found in two adult DS cases (age: 55 and 59 years) a marked brain CAT reduction which was as severe as in AD brain (Table 1). Assuming that not all adult DS patients become demented, the so far regularly found low brain CAT already suggests that brain cholinergic deficit can exist without disabling dementia. This point is further borne out by our results obtained in patients with OPCA.

(5) Olivopontocerebellar Atrophy. Confirming our initial results of a retrospective study (Table 1; Kish et al.,1987), in a recent prospective study we found in OPCA patients reduced cortical cholinergic deficiency which was comparable to the CAT loss in AD cortex (Kish et al.,1989); despite this, there was an absence of any significant disabling dementia as defined by DSM-III criteria.

Summing up our CAT results in non-AD neurodegenerative disorders with or without disabling dementia, it can be concluded that in the patients studied, reduction of cholinergic forebrain function was not an indispensable condition of dementia, and vice-versa dementia was not an inevitable result of pronounced brain (cortical) cholinergic deficit.

Brain Cholinergic Systems And Ageing

The intriguing results of our study regarding the role of cholinergic brain changes in dementia of non-AD degenerative disorders prompted us to examine the behaviour of the cholinergic forebrain systems during normal ageing. We studied this question by examining the effect of ageing upon brain CAT activity in 35 neurologically normal controls ranging in age from 2 months to 89 years. The results of our still ongoing study indicate that in the ageing brain loss of cholinergic neurone function seemed to follow a very specific, regionally selective pattern. Thus, CAT reduction was pronounced in the hippocampal gyrus/entorhinal area, Ammon's horn and dentate gyrus, much less so in neocortical brain areas and not at all present in the amygdaloid nucleus, the caudate nucleus and the putamen (manuscript in preparation). Since brain cholinergic neurones have been implicated in learning and memory, it is logical to expect that the marked CAT loss in the hippocampal formation during ageing may be related to the decline of memory function frequently observed in aged individuals. From this the idea suggests itself that the well documented memory and brain

CAT loss in patients with AD may be due, in large part, to accelerated ageing of the cholinergic neurones.

Alzheimer's Disease And The Ageing Of Brain Cholinergic Neurones

In agreement with a large number of published studies (for reviews, see Perry,1986; Mann,1988; Rossor,1988), we found in AD brain a marked CAT loss in a number of cortical and subcortical brain regions including the hippocampus (manuscript in preparation). However, a comparison between AD and the senescent brain shows that the respective patterns of CAT changes are not comparable. This is especially true for the CAT in the amygdaloid nucleus which was severely reduced in AD but not at all affected by ageing. In the caudate nucleus and putamen the changes in AD and ageing were actually in opposite directions (with older controls showing a trend towards increasing CAT levels). From this lack of correlation we would like to conclude that the loss of cholinergic innervation in AD brain represents a pathological process of its own, distinct from mechanisms operative under conditions of physiological or accelerated brain ageing.

PARKINSON'S DISEASE, AGEING OF BRAIN DOPAMINE AND DEMENTIA

Regarding the relationship between idiopathic PD, the brain dopamine (DA) system and ageing, again the question can be asked: Are perhaps the DA changes in PD brain the result of accelerated ageing of brain DA neurones? Such an "ageing hypothesis" is supported by neuropathological observations of an age-related loss of DA cell bodies in the compact zone of the human substantia nigra (McGeer et al.,1977).

In respect to PD and brain DA, it must be realized that in contrast to the variable relationship between dementia and loss of brain cholinergic neurones, severe loss of nigro-striatal DA is a very specific and regular change, being a precondition for the motor disorder of PD. In every case of idiopathic PD so far analyzed in our laboratory the striatal DA loss was clearly outside the control range, without any overlap between controls and PD cases (Hornykiewicz,1988; Hornykiewicz et al.,1989) (Table 2). This is in sharp contrast to several non-DA changes which are found in some but not all PD brains; for instance, the reduced CAT levels in PD brain distinctly overlap with control values (Smith et al.,1988) and therefore lack true specificity. The same can be said about many other non-DA changes, including noradrenaline, serotonin, amino acids and neuropeptides.

Table 2. Individual Striatal Dopamine (DA) Values In Idiopathic Parkinson's Disease (iPD) - Comparison With Controls

	Putamen				Caudate Nucleus			
	iPD		Controls		iPD		Controls	
Single DA values (µg/g)	0.13	0.22	4.96	6.79	0.57	1.07	3.76	4.46
	0.05	0.07	3.73	3.88	0.39	0.54	2.05	2.01
	0.10	0.26	3.60	5.62	0.25	1.05	2.25	4.16
	0.03	0.09	5.65	4.67	0.65	0.40	3.30	2.82
			4.33	3.49			3.52	2.41
Mean ± sem	0.12 ± 0.03		4.67 ± 0.34		0.62 ± 0.11		3.07 ± 0.28	
Range	0.03 - 0.26		3.49 - 6.79		0.25 - 1.05		2.05 - 4.46	

Aging Of Striatal Dopamine Neurones

In addition to the above observations on the specificity of striatal DA loss in idiopathic PD, we have recently found another specific change, namely a subregional pattern of DA loss in PD caudate and putamen (Kish et al., 1988). This pattern was characterized by rostro-caudal and dorso-ventral gradients (Table 3).

To examine whether normal ageing produces a similar effect, we studied the influence of ageing upon the striatal DA in 24 neurologically normal controls, ranging in age from 14 to 92 years.

Our data (manuscript in preparation) confirm previous observations of an age-dependent loss of DA in (whole) caudate and putamen (Carlsson and Winblad, 1976). Judged from the parallel course of the two regression lines, the magnitude of the DA reduction was identical for both striatal nuclei (60% DA loss at the mean age of 84 compared with mean age of 20).

On the subregional level, age-related DA loss produced a slight rostrocaudal gradient, with the caudal portions of both the caudate and the putamen somewhat more affected. Subdivision of each of the striatal slices in the dorso-ventral direction did not reveal any consistent gradients of DA loss.

Striatal Dopamine Loss: Parkinson's Disease versus Ageing

How do these data compare with the pattern of striatal DA loss in idiopathic PD? As summarized in Table 4, the patterns of striatal DA loss in normal ageing are not typical of the patterns observed in PD. In PD, but not in ageing, the DA reduction in putamen is much more severe than in caudate. Similarly, in PD, but not in ageing, the dorsal striatal subdivisions, especially in the caudate, are distinctly more affected than the ventral subdivisions. Finally, the rostro-caudal DA gradient produced by ageing does not mimic the gradient in the caudate in PD, where the

Table 3. Striatal Dopamine (DA) Gradients in Idiopathic Parkinson's Disease

Subregional DA gradients	Caudate µg/g (mean) ± sem)	% of control*	Putamen µg/g (mean) ± sem)	% of control*
Rostral	0.22 ± 0.05	7.6	0.17 ± 0.08	3.3
Intermediate	0.73 ∓ 0.22	18.3	0.06 ∓ 0.02	1.3
Caudal	0.91 ∓ 0.22	25.9	0.03 ∓ 0.01	0.5
Dorsal	0.35 ± 0.11	9.7	0.07 ± 0.04	1.4
Intermediate	0.73 ∓ 0.22	18.3	0.17 ∓ 0.08	3.3
Ventral	1.05 ∓ 0.17	29.6	0.48 ∓ 0.24	10.9

*For actual control values (n=10), see Kish et al., 1988. For the rostro-caudal gradient, DA values measured in the intermediate subdivision of slice nos.2,4 and 7 were used for caudate and 4,7 and 10 for putamen; for the dorso-ventral gradient, the DA values measured in slice no.4 were used both for caudate and putamen. Eight cases with idiopathic Parkinson's disease were studied.

Table 4. Striatal Caudate (CN) versus Putamen (PUT) Dopamine Patterns In Normal Ageing And Idiopathic Parkinson's Disease

DA Loss In	Ageing	Idiopathic Parkinson's Disease
(1)	PUT (whole) = CN (whole)	PUT (whole) > CN (whole)
(2)	Dorsal PUT, CN = ventral PUT, CN	Dorsal PUT, CN > ventral PUT, CN
(3)	Caudal PUT, CN > rostral PUT, CN	Caudal PUT > rostral PUT; caudal CN < rostral CN

rostro-caudal DA loss was in the opposite direction as in ageing. We conclude, therefore, that "accelerated" ageing cannot play a primary role in the etiology of PD.

Taken together, our results on brain DA as well as cholinergic systems indicate that accelerated or aggravated brain ageing by itself is not a primary cause of the cholinergic deficit in AD or the DA loss in PD. This does not exclude that ageing, if greatly aggravated, could prepare the ground for the corresponding disorders and thus - in an unspecific way - facilitate the development of the corresponding motor as well as cognitive disturbances.

Basal Ganglia Dopamine - Relation To Motor And Cognitive Functions

As already pointed out, the patients suffering of the non-AD and non-PD degenerative conditions, namely PSP, NINIB and DPMN, had both dementia and marked parkinsonian motor disturbances. It can be seen in Table 5 that the reduced striatal DA levels in these conditions were well in the parkinsonian range. They were sufficient to account for the parkinsonian features in the affected patients, thus further strengthening

Table 5. Interregional And Subregional Dopamine Loss In Striatal Nuclei Of Patients With Neurodegenerative Disorders With Parkinsonism And Dementia - Comparison With Idiopathic Parkinson's Disease

Striatal subdivision	Dopamine in % of control			
	Idiop.PD (8)	PSP (3)	NINIB (1)	DPMN (1)
Caudate (whole)	18.5	7.9	0.6	1.5
Putamen (whole)	2.2	5.7	0.4	5.7
Caudate subdivision				
- rostral	7.6	9.0	0.6	1.8
- intermediate	18.3	8.5	0.6	1.8
- caudal	25.9	7.0	0.3	1.2

Abbreviations: Idiop.PD = Idiopathic Parkinson's Disease; PSP = Progressive Supranuclear Palsy; NINIB = Neuronal Intranuclear Inclusion Body Disorder; DPMN = Dementia-Parkinsonism-Motoneurone Disease. Data taken from: Kish et al.,1985a (PSP; cases no.22,189 and 245); 1985b (NINIB); 1988 (idiop.PD); and Gilbert et al.,1988 (DPMN); number of cases in parentheses.

the specificity of striatal DA loss for the clinical expression of parkinsonism. In this respect, of the DA changes in the two striatal nuclei the always severe DA loss in all parts of the putamen is believed to be the most crucial change (see Kish et al., 1988).

In contrast to the satisfactory relationship between striatal DA loss and the parkinsonian symptoms, the disabling dementia present in these patients was - paradoxically - not accompanied by any cholinergic brain changes. The question arises: Did perhaps the striatal DA loss also play a role for the cognitive deficits in these patients?

Recent studies on the neurophysiology of the striatum have specifically implicated the caudate nucleus as part of a "complex", cortical-subcortical association loop system, subserving higher-level ("cognitive") functions (DeLong et al.,1983; Evarts et al.,1984).

This is interesting because in our study the most striking difference between those patients with PSP, NINIB and DPMN having dementia and parkinsonism on one hand and patients with idiopathic PD on the other hand was specifically the pattern of DA loss in the caudate nucleus - where, as shown in Table 5, the DA loss was, on average, not only severe but also quite diffuse, strongly affecting those parts of the nucleus which are much less affected in PD. In our opinion, these observations lend neurochemical support for the possibility of an involvement of caudate DA in cognitive function. In addition, they seem to reveal yet another aspect of the many faces of the neurochemistry of dementia.

CONCLUSIONS

(1) Marked brain cholinergic deficit does not necessarily result in disabling dementia. Disabling dementia can occur without marked brain cholinergic loss.
(2) Ageing is unlikely to be the primary cause of the cholinergic and dopaminergic deficits typically found in AD and PD respectively.
(3) Marked and diffuse DA loss in caudate nucleus may contribute to "cognitive" deficits found in some patients with basal ganglia involvement.

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NEUROPHARMACOLOGY AND FUNCTIONAL ANATOMY OF THE BASAL GANGLIA: EXPERIMENTAL MODELS FOR PARKINSON'S AND ALZHEIMER'S DISEASE

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BACKGROUND

The term Basal Ganglia refers to large subcortical nuclear masses considered to be derivatives of the forebrain. Significant progress in the understanding of the morphology and functional organization of the basal ganglia followed the demonstration and mapping of central monoamine neuron systems. Dopamine is an essential neurotransmitter in the basal ganglia. Indeed, the neostriatum is richly innervated by dopaminergic fibres originating in the pars compacta of the substantia nigra (Ungerstedt 1971, Lindvall & Björklund 1974), and this pathway constitutes a pivotal axis in the pathogenesis of Parkinson's disease. A deficit in dopamine transmission is the most constant abnormality found in *Parkinson's disease* (Hornykiewicz 1963). Classically the most conspicuous symptoms of Parkinson's disease include akinesia and bradykinesia, rigidity and postural abnormalities, which can lead to skeletal deformities. Indeed, we have recently reported that discret lesions of the basal ganglia produce deformities of the spinal cord and scoliosis in rats (Herrera-Marschitz et al. 1990d). These skeletal deformities are in turn the causes of further impairments of motor behaviour. Thus, brain and peripheral systems interact when producing motor behaviour, in such a manner that causes and effects are reciprocally influenced.

Alzheimer's disease is characterized by dysfunction and death of several neuronal populations, directly and/or indirectly associated with cerebral cortex and hippocampal formation. Several neurotransmitter systems seem to be involved in the production of symptoms associated with Alzheimer's disease. Indeed, there is evidence for changes in GABA, 5-hydroxytryptamine, noradrenaline and dopamine transmission (see Davies 1979, Terry & Davies 1980, Whitehouse 1987, Whitehouse et al. 1980, 1982), and recently, a role for galanin (Hökfelt et al. 1987) has been pointed out. However, the most constant changes are found in markers for acetylcholine and somatostatin. In Alzheimer's disease and senile dementia of Alzheimer type, there is a marked reduction of choline acetyltranferase activity and somatostatin in the cerebral cortex and hippocampus (Davies et al. 1980). Consistently, reduction of cortical choline acetyltransferase is accompanied by the disappearance of large cells from the basal nucleus of Meynert (Whitehouse et al. 1982), which constitutes the principal source of cholinergic fibres to the neocortex (Woolf et al. 1983, Mesulam and Mufson 1984). A reduction in number of cells in the nucleus basalis has been reported to be the primary feature of Alzheimer's disease (Witehouse et al. 1982, Coyle et al. 1983). However, after the report of a case of senile dementia of Alzheimer type showing a decrease in the size, but not in the number of cholineacetyltransferase positive cells in the nucleus basalis (Pearson et al. 1983), the possibility that nucleus basalis changes are secondary to cortical damage has been considered (Sofroniew et al. 1983, Cuello et al. 1986). Thus, we have developed a cortical devascularizing model in the rat in order to study retrograde changes af-

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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fecting forebrain cholinergic neurons, with the aim of creating an experimental model of Alzheimer's disease (see Maysinger et al. 1988, 1989).

Parkinson's and Alzheimer's diseases have been traditionally considered to be ethiologically and semiologically independent diseases. At present, there are, however, clinical and experimental evidence suggesting that overlap between both disturbances is common (see Hakim et al. 1979, Adolfsson et al. 1979, Boller et al. 1980, Cross et al. 1981, Epelbaum et al. 1983, Taylor et al. 1986, 1987; Brown et al 1986). The idea that cholinergic nucleus basalis-cortical and dopaminergic meso-telencephalic pathways are reciprocally influenced has received experimental support (see Boyter et al. 1984, Allen et al. 1985). In primates and rats, the nucleus basalis-cortical projection links directly and/or indirectly with meso-limbic dopamine projections (see Walaas 1981, Graybiel and Ragsdale 1983). Furthermore, the neocortex projects massively to the neostriatum and nucleus accumbens (see Graybiel and Ragsdale 1983, Herrling 1985). Indeed, there are neuronal loops connecting topographically the sensory-motor and limbic regions of the cortex with the dorso-lateral and ventro-medial striatum, which in turn project to the lateral preoptic area and substantia innominata. The nucleus basalis, which provides the major source of cholinergic innervation to the entire neocortical surface (Mesulam and Mufson 1984) exists within this region. Furthermore, there is a convergence of afferents from frontal cortex and substantia nigra onto acetylcholineesterase rich patches of the superior colliculus, one of the major outputs of the basal ganglia. The functional significance of these loops and their implications in the production of symptoms of Parkinson's and Alzheimer's diseases is not known, but their existence suggests interactions which may be relevant for the understanding and treatment of parkinsonism and senile dementia of Alzheimer type.

EXPERIMENTAL MODELS

In our work we study the interaction between different nuclei of the basal ganglia and the cerebral cortex. The effects of discret lesions and/or cerebral stimulations on transmitter release and behaviour are analyzed with behavioural, biochemical and histochemical methods. Advantage is taken of the fact that the main components of the basal ganglia are extensively uncrossed, which makes it especially suitable for studies analyzing the functional consequences of unilaterally enhancing or depleting putative neurotransmitters. In vivo release of catecholamines, adenosine and acetylcholine are studied with microdialysis and High Performance Liquid Chromatography (HPLC) coupled to electrochemical- (EC), ultraviolet- and EC-enzymatic reactor detector systems. Behaviour is studied, in rats, with the rotational model where dopamine neurons are unilaterally degenerated after 6OHDA lesions (Ungerstedt and Arbuthnott 1970) and, in mice, with motility meters (Ferre et al 1990).

The effects of putative antiparkinsonian drugs are analyzed in experimental models mimicking parkinsonism and Alzheimer's diseases (see above). Attention is also given to the treatments with trophic factors, mainly nerve growth factor (NGF) and the monosialoganglioside GM1. The 6OHDA rotational model (Ungerstedt and Arbuthnott 1970) constitutes an experimental prototype for studying Parkinson's disease. We have utilized this model to analyse new therapeutical strategies, including treatments with trophic factors. We are also looking for models of other neurodegenerative diseases, such as dementia of Alzheimer type. Two models have been proposed, one utilizing cortical devascularizing lesions to produce retrograde degeneration of neurons in the nucleus basalis, and another model where neurons in the nucleus basalis are directly lesioned.

MODULATION OF STRIATAL DOPAMINE RELEASE BY THE STRIATO-NIGRAL PATHWAY

We have found that, in the rat, striatal dopamine relase is differently modulated by striato-nigral GABA, dynorphin A, substance P and neurokinin A pathways. GABA and dynorphin A exert a negative feedback on striatal dopamine, while substance P and neurokinin A provide a positive feedback (Herrera-Marschitz et al. 1987, Reid et al. 1988,). Substance P and neurokinin A effects are conveyed via different receptors (Reid et al. 1990a,b,) and via different neuronal and metabolic (Herrera-Marschitz et al 1990c) pathways. Exogeneously administered substance P

may be cleavaged to a shorter active fragment (substance P (1-7)), which can have antagonistic properties against substance P (Herrera-Marschitz et al 1990c). The pharmacological properties of the C-terminal substance P fragment have also been studied (substance P (6-11)) (Reid et al. 1989).

Since the decrease in striatal dopamine release induced by nigral GABA or dynorphin injections into the nigra is followed by an increase in extracellular GABA levels (Reid et al. 1990b) dopamine may exert an inhibitory modulation on GABA neurons in the striatum. However, this action is complex since the D1/D2 dopamine agonist apomorphine and the selective D1 agonist SKF 38393 stimulate, while the D2 agonist pergolide inhibit striatal GABA release, suggesting that D1 and D2 dopamine receptors differentially regulate striatal GABA release and are stimulatory and inhibitory respectively (Reid et al 1990b).

MODULATION OF STRIATAL DOPAMINE RELEASE BY THE CORTICO-STRIATAL PATHWAY

There is evidence that striatal dopamine release is presynaptically modulated by a glutamatergic cortical input (Giorguieff et al. 1977) through direct axonal interactions between cortical glutamatergic and mesencephalic dopaminergic afferents. This hypothesis received some support from biochemical and histochemical studies showing direct intrastriatal axonal interaction. However, the majority of the striatal afferents from the cortex and substantia nigra make axodendritic synaptic contacts with striatal neurons, giving a basis for polysynaptic loops including GABA and/or acetylcholine neurons, by which cortical glutamate neurons also could modulate striatal dopamine release. We have recently found that cortical stimulation produces an increase in striatal acetylcholine and dopamine release (Herrera-Marschitz 1990). These effects, however, seem to be mediated by different glutamate receptors: kainate agonists produce an increase in striatal dopamine release, while NMDA agonists produce an increase in striatal acetylcholine release (Herrera-Marschitz et al 1990a).

DOPAMINE IN THE FRONTO-PARIETAL CORTEX OF THE RAT

We have presented evidence for dopamine terminals in the deep layers of the frontoparietal cortex of the rat (Herrera-Marschitz et al. 1989). In this region extracellular dopamine is found in a 1 nM range and can be increased by depolarization or amphetamine stimulation, and suppressed by mesencephalic 6OHDA lesions (Herrera-Marschitz et al. 1989). As in the striatum, nigral sub-stance P produced an increase in cortical DA release, while neurokinin A stimulated striatal dopamine release only (Reid et al. to be published). Differences in striatal and cortical dopamine function have also been found in studies measuring the mRNA expression of several neurotransmitters with in situ hybridization and RNA blots. We have found that GAD (a marker for GABA neurons), somatostatin and NPY mRNA gene expression increase in the striatum, but decrease in the cortex after dopamine deafferentation (Lindefors et al. 1989, 1990), suggesting therefore that dopamine has a different functional role in the striatum than that in the frontoparietal cortex.

CORTICAL AND STRIATAL ACETYLCHOLINE RELEASE

Extracellular levels of acetylcholine can be simultaneously measured in the cortex and striatum of rats (Maysinger et al. 1988). These levels are selectively stimulated by several pharmacological treatments and inhibited by specific lesions. A unilateral ibotenic acid lesion into the nucleus basalis produce a strong decrease in extracellular acetylcholine levels in the ipsilateral cortex, but not in the striatum (Herrera-Marschitz et al. 1990b). Unilateral decortication produce only a small decrease in cortical acetylcholine levels, which is in agreement with neuroanatomical evidence showing that acetylcholine mainly constitute an extrinsic system in the cortex,. Furthermore, we found that the lesions affect cortical and striatal dopamine levels as well, which probably reflects indirect functional interactions.

EFFECTS OF NEUROTROPHIC FACTORS ON BEHAVIOURAL, BIOCHEMICAL AND HISTOCHEMICAL CHANGES PRODUCED BY LESIONS

The idea that degenerative processes may be delayed or even reversed by exogeneously administered trophic factors such as NGF and GM1 is now accepted. NGF is

synthesized within target tissues of some peripheral and central neurons and can act on specific receptors. These receptors mediate the local action of NGF and its internalization, which in turn initiates retrograde transports to cell bodies. Thus, NGF can be used as a pharmacological tool to induce nerve growth and repair. NGF has been used to promote phenotypical transformation of cell grafts. Indeed, we have found that NGF can induce changes of chromaffin cells, transforming their endocrine-like feature into neuron-like features. The changes have been associated to the reversing of symptoms of experimentally induced parkinsonism (Strömberg et al 1985). The effects of NGF treatments on extracellular acetylcholine, dopamine and adenosine levels in the cortex and striatum of rats with unilateral devascularizing cortical lesions have also been studied (Maysinger et al. 1988, 1989).

The monosialoganglioside GM1 has been used to promote nerve growth and repair (Agnati et al. 1983). GM1 can prevent retrograde changes in nucleus basalis produced by cortical lesions (Cuello et al. 1986). GM1 can also stimulate the activity of cortical cholineacetyltransferase in regions adjacent to the lesions. We have extended these studies by analyzing the effect of decortication and treatments with GM1 and NGF on cortical and striatal acetylcholine, catecholamine and adenosine levels measured with microdialysis (Maysinger et al. 1988). We found that chronic administration of GM1 or NGF by an intraventricularly implanted guide cannula connected to an osmotic minipump reversed biochemical and morphological changes induced by the lesions (Maysinger et al. 1988, 1989). We have also applied GM1, microencapsulated in human serum albumin, to the devascularized cortex. It was found that microencapsulated GM1 promoted recovery of retrograde morphological changes in nucleus basalis magnocellularis and a caused a parallel increase in acetylcholine release. We concluded that topically applied neurotrophic factors may offer therapeutic possibilities in reversing damages after brain lesions.

MECHANISMS OF ACTIONS OF ANTIPARKINSON DRUGS

Most of the antiparkinsonian drugs are dopamine agonists. We have studied the selectivity of several such agonists on different receptor populations with the idea that receptor multiplicity may constitute a mechanism by which dopamine is affecting different neuronal pathways. Recent microdialysis studies support this hypothesis: In the striatum, dopamine exerts an inhibition of GABA release via D-2 receptors, while D-1 stimulation increases the release of GABA (see above).

We have previously found that methylxanthines may share some of the antiparkinsonian properties of dopamine agonists (Herrera-Marschitz et al. 1988). In recent studies we found evidence that methylxanthines potentiate the effects of dopamine by D-1 agonism (paraxanthine or caffeine) and by adenosine A-2 antagonism (theophylline, paraxanthine or caffeine) (Ferre et al. 1990).

CONCLUSIONS

The intention with this review has been to point at the powerful animal models that do exist for Parkinson's and Alzheimer's disease. These models have been used in combination with microdialysis to monitor transmitter release. The results show an ever increasing complexity of functional neural interaction through different transmitters and their receptors. However, in spite of the overwhelming complexity it is obvious that system after system can be analyzed in this way to provide knowledge leading towards future advances in the pharmacotherapy of Parkinson's as well as Alzheimers's disease.

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NEUROTRANSMITTER CHANGES IN ALZHEIMER-TYPE DEMENTIA AND THERAPEUTIC STRATEGIES

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INTRODUCTION

Neurotransmitter changes are one of the most important data when we think about the pathogenesis of Alzheimer-type dementia (ATD) and therapeutic strategies for ATD.

The cholinergic deficits have received much attention since 1976 when a severe depletion of choline acetyltransferase(ChAT) activity in ATD was reported by three British groups.[1] Administration of the precursors of acetylcholine(lecithin and choline) and acetylcholinesterase inhibitor(physostigmine) have been examined. However, the results were disappointing. Exceptionally, tetrahydroaminoacridine, which is also an acetylcholinesterase inhibitor but which has a longer half-life than physostigmine, was reported to be efficacious for the treatment of ATD, and is now being investigated more intensely.

In the present study, therefore, we focused on the factors that might explain the absence of a clear effect of the cholinergic drugs in the treatment of ATD, and how to interpret the depletion of peptides which is another interesting finding in ATD brains. According to these findings, therapeutic strategies were also discussed.

MATERIALS AND METHODS

Autopsied human brains obtained from ATD cases and age-matched normal controls with no neuropsychiatric disorders were examined in the present study. All of the brains used were verified neuropathologically.

Detailed clinical and postmortem data, and the methods for the determination of biochemical data, immunohistochemistry, and molecular biological techniques were already reported elsewhere.[2-5]

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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RESULTS AND DISCUSSION

Existence of changes in other neurotransmitter systems

The changes in concentrations of serotonin(5-HT) and its metabolite, 5-hydroxyindole acetic acid(5-HIAA) in ATD brains are shown in Fig.1, suggesting the involvement of the serotonergic system. This serotonergic damage was one of the most dramatic changes we have found in ATD.

Table 1 summarizes the neurotransmitter changes we have found in ATD. These findings clearly indicate that not only the cholinergic system but also other neurotransmitter systems are involved in ATD. ATD can be called a multi-neurotransmitter systems disorder.

Heterogeneity of the ATD group

According to neuropathological findings, the presenile-onset Alzheimer's disease and senile dementia of Alzheimer-type are usually included in the one category of ATD.

However, our study disclosed that the ChAT activity was severely depleted in the early-onset group, whereas the activity was almost normal in the late-onset group. The turnover rate in the serotonergic system was also decreased more clearly in the early-onset group than in the late-onset group. These data suggest that the ATD group is not a homogeneous one.

Functional level in the remaining neurons

In Parkinson's disease, it was reported that the remaining neurons in substantia nigra are compensationally hyperactive. How about the remaining neurons in Alzheimer-type dementia?

We examined the turnover rate in the dopaminergic and serotonergic neurons and discovered that the dopaminergic neurons are in a hyperactive state, but the serotonergic neurons are not. These findings suggest that the remaining neurons in the

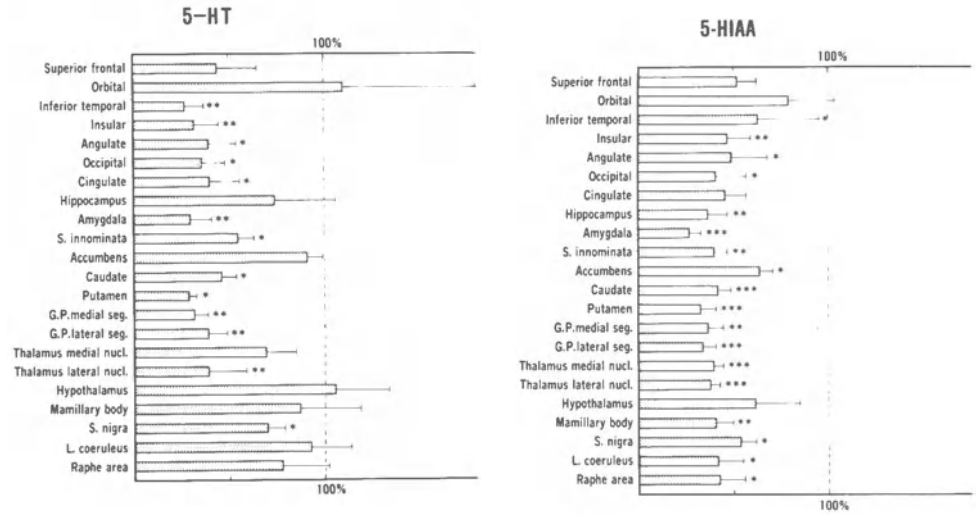

Fig.1. Concentrations of serotonin and 5-HIAA in ATD brains. All values(mean + SEM) are expressed as percent of the mean value of controls for each region.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 1. Neurotransmitter-related markers in ATD brains

Ach	choline acetyltransferase	↓
	acetylcholinesterase	↓
5-HT	5-HT	↓
	5-HIAA	↓
	tryptophan hydroxylase	↓
NA	noradrenaline	↓
DA	dopamine	→ or ↓
	homovanillic acid	→ or ↓
	tyrosine hydroxylase	→ or ↓
Amino acids	glutamate	↓
	other amino acids	→
SOM	somatostatin	↓
VIP	vasoactive intestinal polypeptide	→ or ↓

↓, significantly decreased compared with control brains; →, no significant difference between ATD and control brains

serotonergic system are damaged so severely that the compensational mechanism cannot wort properly. It seems important whether the remaining neurons work compensationally or not.

Inhibitory factors

A peptide named galanin was recently isolated from porcine intestine. Galanin was reported to inhibit acetylcholine release and to exist in the neurons in the brain.

We stained galanin-containing neurons in the hypothalamus in human brains by immunohistochemical methods. The number of the neurons was not decreased in the ATD brains(Fig.2). We also measured galanin-like immunoreactivity(GLI) in the cortical areas. The GLI seemed to be increased in the ATD brains, suggesting that this galanin may inhibit the cholinergic transmission in the ATD brains.

Changes in the postsynaptic site

Concerning receptor bindings, the muscarinic receptor binding was reported to be almost normal in ATD brains. However, abnormalities in nicotinic receptor binding and serotonergic receptor binding were reported in ATD brains.

In the postsynaptic site, we were also interested in the second messenger level, especially in the calcium ion metabolism. Several types of calcium-binding protein have been reported to exist in neurons, and one of them interesting to us is parvalbumin, which may have a role of buffering calcium ion in the cytoplasm. We stained control and ATD brains immunohistochemically with an antiserum against parvalbumin. The results

obtained from computerized image analysis disclosed that not only the number of the stained neurons but also the size of the neurons in the ATD brains were significantly decreased compared with those in the control brains(Fig.3). These findings suggest that the calcium buffering system in the postsynaptic site may also be damaged in ATD brains.

Neuropeptide changes

The content of somatostatin, corticotropin releasing factor (CRF), and other peptides have been reported to be decreased in ATD brains. However, what does the depletion mean?

It is still not clear whether the number of the peptide-containing neurons is decreased, or the synthesis rate is down regulated, or the terminals are in a hyperactive condition that caused accelerated release of the peptide. One of the best methods to interpret the condition is to examine the messenger RNA(mRNA) for the peptides. We have a good example for the third case to show a relation between neuropeptide and mRNA content. If salt is loaded in rats, vasopressin is released from the hypothalamus to decrease the osmotic pressure in the serum. However, immunohistochemical examinations disclosed that the vasopressin immunoreactivity was decreased in the hypothalamus of the salt-loaded rats, probably because vasopressin was released very rapidly. On the other hand, the vasopressin mRN content was dramatically increased in the hypothalamus of salt-loaded rats, suggesting the vasopressin-containing neurons are in a hyperactive condition. Therefore, although depletions of peptides have been reported in ATD, more data will be needed to understand the condition clearly.

However, before we analyze mRNA's in ATD brains, it is certainly necessary to check the postmortem stability of mRNA. We used rat models that simulated various postmortem conditions and studied postmortem changes in vasopressin mRNA in the hypothalamus. The Northern blot analysis of vasopressin mRNA dis-

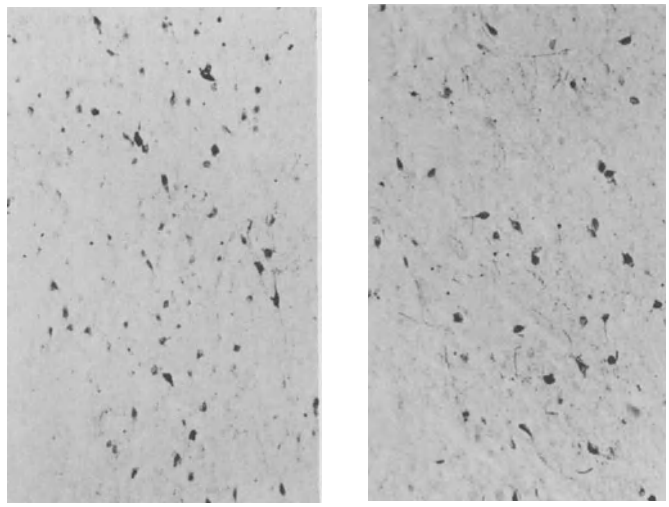

Fig.2. Galanin-containing neurons in hypothalamus.
Left, control brain; Right, ATD brain.

closed that the vasopressin mRNA could be detected in the brains obtained at 8 hrs postmortem, but not in those obtained at 24 hrs, suggesting that mRNA study can be made using autopsied brains obtained within 8 hours after death. We also used in situ hybridization histochemistry to examine the postmortem changes in vasopressin mRNA in rat brains, and obtained almost the same results.

Therapeutic strategies for ATD

The findings mentioned above give us many suggestions to develop new drugs for the demented patients.

Clinically, when a new drug is examined, it is important to select a homogeneous subgroup of ATD patients. Concerning the clinical stage, the patients at early clinical stage should be selected. Another point is what kind of scale should be used to evaluate efficacy. The demented patients show numerous symptoms. Therefore, a clinical scale, like the multidiscriminating scale of GBS reported by Gottfries et al.,[6] with which we can evaluate even a small change in any symptom, will be needed.

For the cholinergic drugs, agonists to the cholinergic receptors have not been examined intensely and should be developed. Drugs that accelerate the release of acetylcholine from terminals may also be interesting.

Moreover, since ATD is a multi-neurotransmitter systems disorder, a replacement therapy for several systems may be necessary. Cholinergic drugs and replacement therapy of noradrenalin, or serotonin, or glutamate should be examined. Several peptides might be also interesting for clinical trial. However, it should be emphasized that more data is needed to consider the therapeutic strategies concerning the neuropeptide changes. Further, a combined therapy of cholinergic drugs and an antagonist of galanin might be effective for the treatment of ATD patients.

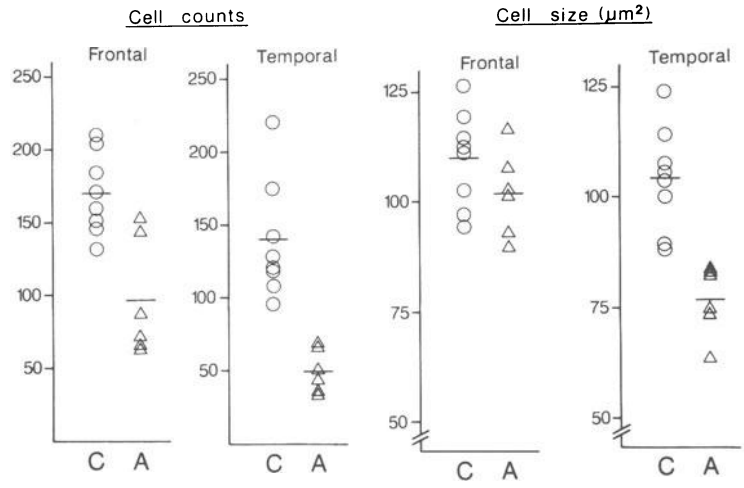

Fig.3. Total number and mean cell body size of parvalbumin-immunoreactive neurons. C, control brains; A, ATD brains; bar, mean value in each group.

ACKNOWLEDGEMENTS

We are particularly grateful to Profs.Y.Fukuda, S.Shirai, T.Nagatsu, and to Drs.M.Inui, S.Yoshino, T. Ishii, T.Moroji, S.Oyanagi, K.Kosaka, K.Ikeda, K.Kobayashi, Y.Ichimiya, N.Iwamoto Y.Makino, M. Minami, I. Noguchi, M.Kimura, and T.Takahashi for their generous help.

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IN VITRO EXPERIMENTAL APPROACH FOR DETERMINATION OF THE CENTRAL CHOLINERGIC NEURONAL ACTIVITY

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INTRODUCTION

Dysfunction of the central cholinergic neurons is observed in patients with Alzheimer's disease and other motor disorders. Drugs exerting effects on central cholinergic activities have been used for therapy in these diseases. Therefore, a simple in vitro experimental system for measurement of cholinergic neuronal activity is required for pathological and pharmacological studies, and for the screening of drugs potentially effective for the above diseases.

Cholinergic pathways in the CNS have been already clarified. The basal forebrain area, especially the basal nucleus of Meynert (BNM) and the medial septum (MS), are rich in large cholinergic cell bodies innervating the neocortical regions and the hippocampus (HIP), respectively. In the striatum, cholinergic interneurons with a short axon are densely present. We attempted to determine regional differences in the potency of acetylcholine (ACh) synthesis using slices of the rat brain BNM, MS, HIP, frontal cortex (FC) and caudate putamen (CP).

MATERIALS AND METHODS

Male Wistar rats (8-10 weeks old) were sacrificed by decapitation and the BNM, MS, HIP, FC and CP brain regions were quickly dissected out and sliced freehand (0.5 mm thickness and 8 to 30 mg weight) with a razor blade on an ice-cold plate. The slices were perfused with artificial cerebrospinal fluid (ACSF) or high-K^+ ACSF with the following composition (mM): NaCl 139, KCl 3.4 (in high-K^+ ACSF, NaCl 112.4, KCl 30), $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 21, NaH_2PO_4 0.6, glucose 10, saturated with 95% O_2-5% CO_2. Eserine (10 uM) was added to all media. First, the slices were perfused with high-K^+ ACSF for 30 min to deplete the intracellular releasable fraction of ACh. After high-K^+ perfusion, the slices were perfused with ACSF in the absence or presence of hemicholinium-3 (HC-3) and choline. After the ACSF perfusion, tissue ACh content was determined by radioimmunoassay for ACh. In some experiments, the perfusate was collected for determining the amount of released ACh. The procedures of radioimmunoassay have been described elsewhere (Kawashima et al., 1980, 1988, Suzuki et al., 1988, 1989 a, b). Tissue ACh content was expressed as nmol/g wet tissue.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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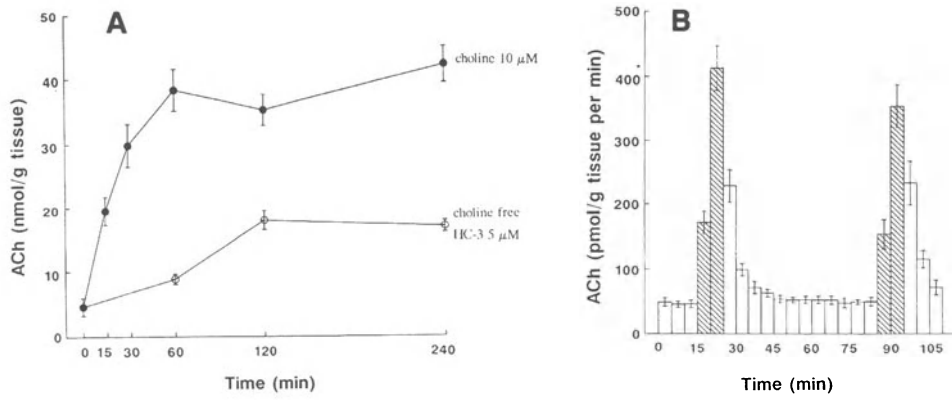

Fig. 1. A) Time courses of changes in acetylcholine (ACh) content in the rat hippocampal slices. Basal level of ACh content (see text) is represented as 0 time. (—●—) perfused with 10 uM choline containing ACSF. (—Θ—) perfused with choline-free, 5 uM HC-3 containing ACSF. B) ACh release from rat hippocampal slices after ACSF with choline perfusion (60 min). High-K^+ stimulation was applied in the hatched areas.

RESULTS AND DISCUSSIONS

In HIP, the basal level of ACh content (ACh content after high-K^+ perfusion for 30 min) was 4.6 nmol/g tissue, and the ACh content was increased by perfusion with ACSF (containing 10 uM choline, Fig. 1 A). The ACh content increased and reached a plateau level (38 nmol/g tissue) at 60 min of perfusion. These results indicated that the intracellular ACh content was maintained at a steady level. In the absence of choline and the presence of HC-3 (5 uM) perfusion, ACh content was increased at 60 min of perfusion and reached a plateau level (18 nmol/g tissue) with 120 min of perfusion. Some of the ACh was thus synthesized independently of the extracellular choline.

The spontaneous release of ACh from hippocampal slices after 60 min of ACSF perfusion was about 50 pmol/g tissue per min (Fig. 1 B). High-K^+ stimulation elicited marked ACh release (about 400 pmol/g tissue per min). Under the present experimental conditions, the amounts of spontaneous and high-K^+-evoked release of ACh in 1 min were about 0.1% and 1% of the total ACh content, respectively. We have found previously that high-K^+ stimulation (5 min duration) did not affect the ACh content (Suzuki et al., 1989 a). These results indicate that the amount of released ACh corresponds to a very small portion of the total ACh content, and that the decrease in ACh content produced by this release is immediately compensated by rapid synthesis in the neuron.

In this study, we also examined regional differences in the potency of ACh synthesis. The ACh content was increased during 60 min of ACSF perfusion (with 10 uM choline) in all areas examined. The amount of increased ACh content occurred in the order CP>MS>BNM>HIP>FC. On the other hand, the order of the increasing rate of ACh content after ACSF perfusion (increased ACh content converted into percentage of basal ACh content) was CP>HIP>FC>MS>BNM. These results indicate that, in the striatum, the potency of ACh synthesis is very high. In the BNM and MS, basal ACh contents were higher than those in the HIP and FC. However, the rates of increase in the BNM and MS were apparently lower than in other regions. These results can be explained by the existence of a large amount of

Table 1. Changes in acetylcholine content by ACSF perfusion in various regions of the rat brain

	Basal	ACSF (choline 10 uM) 60 min
CP	9.84 ± 0.94 (100)	107.63 ± 17.64 (993.8)
MS	11.95 ± 0.42 (100)	50.14 ± 8.01 (319.6)
HIP	4.64 ± 1.38 (100)	38.34 ± 3.21 (726.3)
BNM	12.91 ± 0.87 (100)	41.06 ± 6.43 (218.1)
FC	2.18 ± 0.17 (100)	17.89 ± 1.58 (720.6)

"Basal" indicates ACh content after high-K^+ perfusion. Other experimental procedures and abbreviations are described in the text. Values are mean ± SEM nmol/g tissue for 4-8 experiments. Values in parentheses represent increasing rates of ACh content after ACSF perfusion (percentages of amount of increased ACh content compared with basal level of ACh).

unreleasable ACh (represented as the basal level) in the BNM and MS. These data indicate that ACh can be synthesized also in cholinergic cell bodies of the basal forebrain and that ACh is not readily released in the basal forebrain region. The axonal transport of ACh has been observed in cholinergic neurons of Aplysia (Koike, 1984). The results of our present study suggest a possibility that ACh synthesized in the cholinergic cell bodies of the basal forebrain is transported axonally to terminals, e.g., the neocortex or hippocampus. In the HIP and FC, high rates of increase were observed. Although the ACh content in the FC was the lowest among the brain regions examined, the rate of increase was rather high. These data suggest that ACh content is not related to the potency of ACh synthesis in cholinergic neurons.

Drugs affecting central cholinergic activity have been developed for treatment of some motor disorders and Alzheimer's disease. These drugs can be classified as follows: 1) precursors of ACh; 2) compounds affecting ACh synthesis; 3) compounds affecting ACh release; 4) cholinesterase inhibitor; 5) postsynaptic acetylcholine receptor agonist or antagonist; 6) compounds affecting postsynaptic cell function (e.g., transmembrane control). The experimental system used in this study is useful for detecting the ability of such compounds to affect ACh synthesis and release. Thus, drugs classified as types 1) to 4) can be screened by this system. Regional selectivity of drug effects must be examined in order to develop drugs with specific pharmacological effects and to predict side effects. Our present system is thus suitable for estimating the effects of drugs on specific brain regions.

Most previous in vitro experiments have employed the method of preloading radiolabeled precursors of ACh (especially choline) for detection of ACh release. However, such methods cannot determine the absolute amount of ACh release and may not reflect the total cholinergic activity, since the manner of storage and release of ACh is not uniform in the cholinergic nerve (Whittaker, 1986). In contrast, we determined the

total amount of released and stored ACh by radioimmunoassay, thus, allowing determination of total presynaptic cholinergic activities. Our experimental system is expected to be useful for physiological and pharmacological studies as well as for screening drugs affecting on central cholinergic activity.

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PHARMACOLOGICAL PROFILE OF BRAIN GLUCOSE UTILIZATION OBTAINED BY MONITORING OF LACTATE RELEASE IN VIVO UNDER THE FREE MOVING CONDITION

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INTRODUCTION

In recent years, knowledge has been accumulated about dynamic roles of the brain in accordance with progress in science technology. For example, glucose uptake ratio, O_2 utilization, and blood flow rate in the CNS were obtained by autoradiography, positron emission tomography, and magnetic resonance imaging. From data obtained by these means and others, it was assumed that glucose utilization in the CNS was important not only as the basis of cell physiology but also for psychophysiological functions. It is also important to know the relationship between behavior and neuronal activities in several brain regions, in order to conduct basic research on Alzheimer's diseases, Parkinson's disease, and other neuropsychological diseases. And it would be essential for the research to investigate the sites of action of therapeutic drugs for those diseases on the background of the brain glycolytic pathway. But it was hard to observe both the brain glucose utilization and its behavior (cf. 2-deoxy-D-glucose method).

Lactate is a major end product of the glycolytic pathway. We observed extracellular lactate levels in the rat brain, using in vivo on-line brain microdialysis, to study brain glucose utilization during the pharmacological modification of neuronal functions. It was the purpose of this report to determine how neurotransmission (mainly via acetylcholine, dopamine, and serotonin) regulates glucose utilization in the CNS. The application of brain microdialysis for measurement of lactate release may provide a useful biochemical tool for the study of the regional glucose utilization and its behavior after neural stimulation. This method was introduced by E. M. C. Schasfoort et al.[1] to study the effect of stress on rat hippcampal lactate-release in the rat.

MATERIALS AND METHODS

Male Sprague-Dawley rats (250-330 g) were anesthetized with pentobarbital (50 mg/kg, ip.), and were implanted with unilaterally hand-made, U-shaped, dialysis probes (o.d., 0.3 mm; length, 7 mm; molecular cut-off, 50,000; donated by Asahikasei of Japan) into the medial prefrontal cortex (mpfc;

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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B:-2.7 mm, L:1.2 mm, V:3.5 mm under the dura) and/or striatum (str; B:0 mm, L:3 mm, V: 7.5 mm under the Bregma). Ringer's solution was perfused at 2.5μ l/min into the implanted dialysis probe 16-24 hours after its implantation.

In the first experiment, fractions were collected into cooled tubes every 20 min starting one hour after the start of perfusion in both the mpfc and str. Lactate and glutamate were measured by a fluorometric enzyme assay. In brief, 500 μ l of lactate assay solution (60 mM glycine-50 mM hydrazine buffer [pH 9.2], 5 U/l lactate dehydrogenase, 25 mg/l NAD^+) was reacted with 7.5 μ l samples for 45 min at room temperature. And in the case of glutamate, 500 μ l of glutamate assay solution (60 mM glycine-50 mM hydrazine buffer [pH 9.2], 5 U/l glutamate dehydrogenase, 25 mg/l NAD^+, 20 μ g/l ADP) was reacted with 10 μ l samples for 45 min at room temperature. And NADH in these samples was measured fluorometrically at 450 nm with excitation at 340 nm. We observed the effects of 9-amino-1,2,3,4-tetrahydroacridine HCl H_2O (THA; 5 mg/kg, i.p.) on both the lactate and glutamate releases in the rat mpfc and str in this experiment.

In the second experiment (on-line brain microdialysis measured on lactate), dialysates were mixed directly with a lactate enzyme solution (120 mM glycine-100 mM hydrazine buffer [pH 9.2], 10 U/l lactate dehydrogenase, 50 mg/l NAD^+, 2.5 μ l/min) in a T-tube, through which the mixture was passed for 10 min before reaching a fluorometer equipped with a flow-cell (820-FP Jasco of Japan, Fig.1.). We studied striatal lactate release by injection of apomorphine (APO; 0.03 mg/kg or 0.5 mg/kg, s.c.) or its vehicle (0.1 % ascorbate ml/kg, s.c.) in this system. And locomotor activities were measured by counting the number of times both forepaws crossed the bars, whose intervals were 10 cm, on a 50 x 50 cm square. This activity was assessed 5-10, 15-20, and 25-30 min after indicated injections. And we also monitored the effect of 5-methoxy-N,N-dimethyl tryptamine (5-MeODMT; 1 mg/kg and 2 mg/kg, s.c.).

RESULTS

The recovery of lactate and of glutamate was 28.6± 0.5 % (n=4) and about 35 %(n=2) in vitro, respectively (data not shown).

THA (5 mg/kg, i.p.) enhanced lactate-release in both rat mpfc and str (Fig.2 a,c). And at the same time glutamate-release increased, too (Fig.2 b,d). By on-line brain microdialysis, striatal lactate-release was reduced by the injection of 0.03 mg/kg APO and increased by the injection of 0.5 mg/kg APO in each of three experiments (Fig.3 a,b). And locomotor activities were observed to change in parallel with changes in the level of lactate-release elicited by each dose of APO. And 5-MeODMT (1 mg/kg and 2 mg/kg) increased the level of mpfc lactate-release (Fig.5).

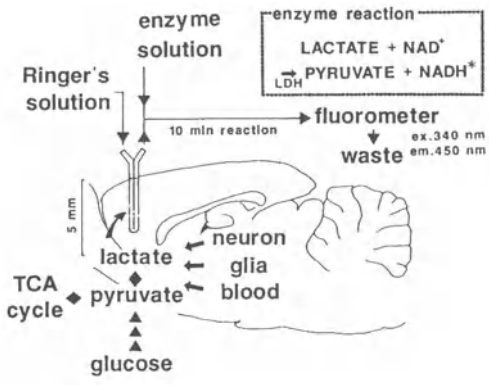

Fig.1. On-line brain micro-dialysis system.

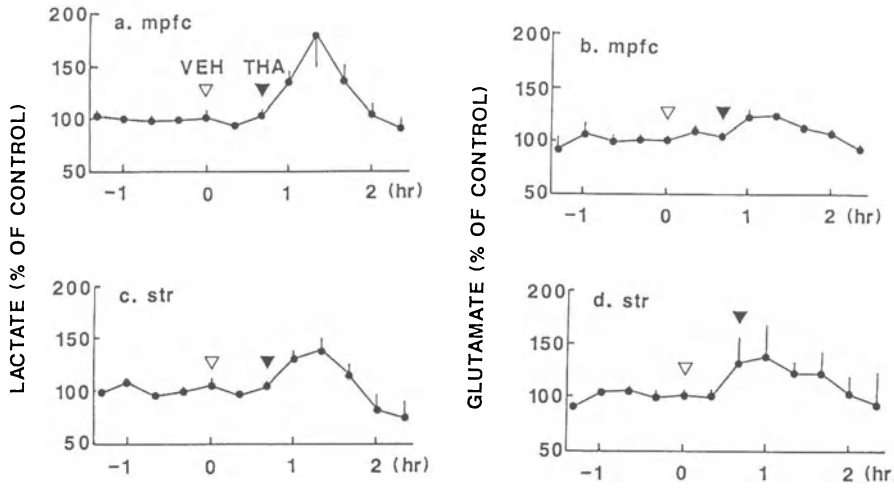

Fig.2. Effects of THA (5mg/kg, i.p.) on lactate- and glutamate-release in the medial prefrontal cortex and striatum. THA was dissolved in a vehicle (VEH) of 0.1% Tween 80. Each 100% value was calculated by the average of four fractions before the VEH injection. Concentrations of each 100% values: a 306± 11μM (n=24), b 7.89± 0.24μM (n=16); c 228± 7μM (n=24); d 7.07± 0.34μM (n=16). (a:lactate in mpfc, n=6 b:glutamate in mpfc, n=4 c:lactate in str, n=6 d:glutamate in, str, n=4)

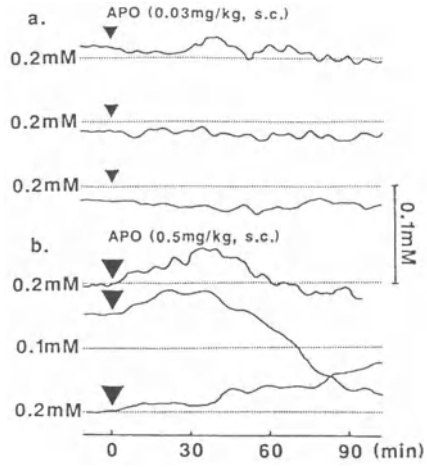

Fig.3. Effects of APO on striatal lactate-release measured by on-line brain microdialysis.

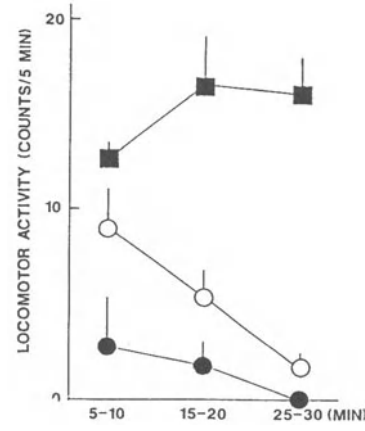

Fig.4. Effects of each dose of APO (○:vehicle-injected control, ●:0.03 mg/kg, and ■:0.5 mg/kg) on rat locomotor activity, n=5 (For details, refer to MATERIALS AND METHODS).

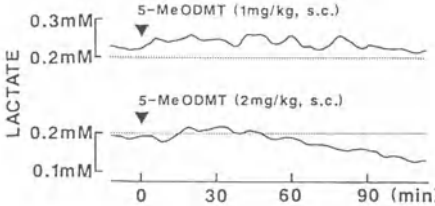

Fig.5. Effects of 5-MeODMT on mpfc lactate-release measured by on-line brain microdialysis.

DISCUSSION

There is no answer as yet to the source from which lactate and glutamate originate. But considering that the lactate and glutamate are produced in glucose metabolism, our results above and others (2-DG study, PET, MRI, and ref.[1]) suggest that several neurotransmissions modulate brain glucose utilization.

Much investigation has been made on the cholinergic dysfunction in Alzheimer's disease. It is impossible to clarify the clinical therapeutic effect of several cholinesterase inhibitors, like THA, on Alzheimer's disease.But we feel that it is important to accumulate knowledge about the cholinergic system in the CNS. What about brain glucose metabolisum in Alzheimer's patients? G. W. Small et al.[2] showed by PET a decrease in the cerebral glucose metabolic ratio in Alzheimer's patients. We have demonstrated here the effects of THA on the lactate- and glutamate-release in vivo. THA was reported to increase the level of acetylcholine (ACh) in the CNS, but we found that THA increased not only ACh levels but also the lactate and glutamate releases in rat mpfc and str in vivo (Fig.2 a-d). These results support the possibility that cholinergic neurotransmission regulates the glycolytic pathway in these areas. But with the therapeutic effect of THA in mind, we should note that the dose of 5 mg/kg of THA seemd to produce an extrapyramidal-like syndrome in our experiment.

Studying DAergic function of striatal glycolytic pathway and animal behavior by using APO (Figs;3 and 4), we obtained results substantially in agreement with already established facts. Low-dose APO (0.03 mg/kg), which stimulates autoreceptors of DAergic neurons, gave hypo-modulation of both striatal glycolysis and motoractivity. The high dose of APO (0.5 mg/kg), which does not cause stereotypical behavior, increased both parameters. But it is not so simple to explain the relationship between striatal DAergic neurons and motoractivity, because the results of Fig.2a suggest the variation of striatal glycolysis might have another role, while striatal DAergic neuron is generally considered to produce striatal cholinergic neuron tonic-inhibitory, as the therapeutic theory of drugs for parkinsonism.

The glycolytic effect of 5-MeODMT (Fig. 5) was studied preliminarily in view of the 5-HT dysfunction recently reported occur in Alzheimer's disease.

In summary, observation was made on the released lactate in rat brain using in vivo on-line brain microdialysis, to study the brain glucose utilization during the pharmacological modification of neuronal functions. The level of lactate release was modulated by the cholinergic and serotonergic afferents in the medial prefrontal cortex, whereas the striatal lactate release was regulated by nigrostriatal dopaminergic neuronal functions in parallel with changes in locomotor activity.

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BEHAVIORAL AND NEUROCHEMICAL EVALUATION OF STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RATS FOR VASCULAR DEMENTIA-ANIMAL MODEL

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The stroke-prone spontaneously hypertensive rat (SHRSP) is known to be an unique model of stroke because the lethal time course of SHRSP coincides well with that of patients with cerebrovascular lesions (Okamoto et al., 1974). The aim of the present study was to clarify the proposed possibility that SHRSP may be a vascular dementia-animal model. First, a study was carried out to evaluate stroke-related behavioral changes and learning ability using passive avoidance response. An attempt was also made to investigate the pathophysiology of SHRSP as an animal model for cerebrovascular lesions by determining neurotransmitter levels in cerebrospinal fluid (CSF).

MATERIALS AND METHODS

SHRSP and their genetic controls, Wistar Kyoto rats (WKY), were kindly donated by Prof. Dr. Okamoto, Department of Pathology, Kinki University School of Medicine.

In the stroke-related behavioral study, age-matched male SHRSP and WKY were subjected to a 12 hour light and dark alternation cycle. Ambulation and drinking activity counts were determined simultaneously with a Ambulo-Drinkometer (O'Hara & Co., Ltd., Tokyo). Ambulatory and drinking activity data were obtained at hourly intervals and subjected to various statistical analyses: unpaired t-test, analysis of variance and two-tailed t-test. The data were also subjected to autocorrelation and power spectral analysis. The average rhythm period (tau; τ_{LD}) was determined by power spectral analysis.

In the learning ability study, 12-week-old SHRSP and Wistar normotensive rats (NWR) were used. Rats were bred under a 12 hour light and dark alternation cycle and subjected to an one-trial step-through passive avoidance task. Each rat was allowed to habituate in a light chamber for 60 sec and to enter the dark chamber by removing a guillotine door. Three seconds after entering the dark chamber, the rat received a foot shock (75 V, 0.2 msec) for 3 sec. Twenty four hours before and after the acquisition trial, latency time was recorded up to a maximum of 600 sec. Retention trials were carried out for 5 days.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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For the determination of neurotransmitters and their metabolite levels in CSF, 15-weeks-old SHRSP and WKY were used. Rats were anesthetized with alpha-chloralose and urethane. CSF was collected via a polyethylene cannula which was inserted into the cisterna magna via the atlanto-occipital membrane under a microscope. CSF was allowed to flow from the cisterna magna into an iced microhematocrit tube. CSF catecholamine (CA), 5-HT and their metabolite (3, 4-dihydroxyphenylacetic acid; DOPAC, 4-hydroxy-3-methoxy-phenylacetic acid; HVA, 5-hydroxyindole-3-acetic acid; 5-HIAA) levels were measured using high performance liquid chromatography with an electrochemical detector (HPLC-ECD). Total CSF volume needed was less than 150 µl for each rat. Ten µl of unprocessed CSF was used for simultaneous determination of monoamines and their metabolites. When necessary, alumina extraction was performed for catecholamine assay. For ACh and choline (Ch) determination, CSF was collected into polyethylene tubings containing an acetylcholine esterase inhibitor, eserine, and an internal standard, ethylhomocholine. CSF was injected directly into a HPLC-ECD with an attached immobilized enzyme column.

RESULTS AND DISCUSSION

I. Stroke-related behavior in SHRSP

From an early age, SHRSP systolic blood pressure is significantly higher than that of WKY(Minami et al., 1987). SHRSP died from cerebral infarction, cerebral hemorrhage and their combination at an age of around 35 weeks old. On the other hand, in the control WKY whose average life span was 83 weeks old, senility was the most frequent cause of death.

Before stroke (15 weeks), ambulation and drinking counts of the SHRSP in the dark phase (82%) were higher than those in the light phase (18%). Both parameters were well synchronized with the light and dark alternation cycle. With aging, daily ambulation decreased while daily drinking activity increased in both SHRSP and WKY. Daily ambulation and drinking activity in 15- and 40-week-old SHRSP were significantly greater than those of WKY(Fig. 1).

In the SHRSP that died of cerebral infaction, the ambulatory activity in the light period increased abruptly followed by a

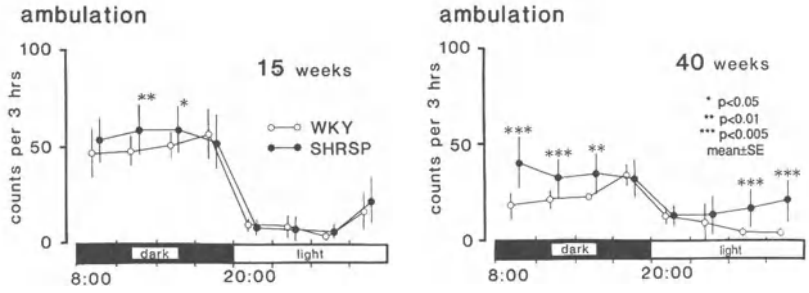

Fig. 1. Daily ambulation in 15 and 40 weeks old SHRSP and WKY. Values indicate ambulatory activity during continuous 3-hour periods.

desynchronization with the light and dark alternation cycles. Moreover, the SHRSP ambulatory activity was desynchronized with their water drinking activity. With regard to the behavioral changes in the SHRSP whose deaths were caused by cerebral hemorrhage, desynchronization with light and dark alternation cycles and with water drinking activity was also observed.

The behavioral changes of the SHRSP were analyzed by power spectral analysis (Minami et al., 1984). SHRSP before stroke (15 weeks) had a 24 hour "τ" value for both ambulation and drinking activity. However, at the onset of stroke, a much longer periodicity was observed in addition to the 24 hour periodicity in the SHRSP that died from cerebral hemorrhage. On the other hand, a circadian rhythm persisted in the WKY which was die from senility after reaching an age of 100 weeks.

These behavioral changes in ambulation and drinking activity including the disturbance of circadian rhythms before death in SHRSP may correspond to behavioral changes such as delirium-state observed in patients with dementia and point to the possibility that SHRSP may be a suitable vascular dementia-animal model.

II. Evaluation of learning ability using passive avoidance test

In 12-week-old NWR, the response latency to enter the dark compartment was 545.9±36.0 sec (mean±SE, n=7) in the first retention test which was carried out 24 hours after the acquisition trial. As compared with NWR, the response latency in SHRSP was significantly reduced (355.3±71.71 sec, n=6). A significant difference in latency time was noted untill the second retention test, and the response latency declined to that before the acquisition trial (23.1±7.1 sec in NWR and 30.8±0.6 sec in SHRSP) with successive retention tests(Fig.2).

The significant impairment in passive avoidance response observed in SHRSP may reflect an impairment of memory. However, further study is

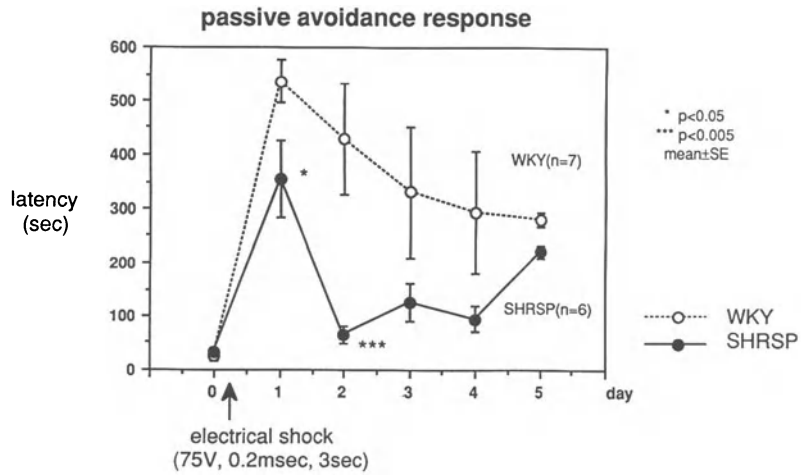

Fig. 2. Passive avoidance response in SHRSP and NWR.
Verticals; the mean latency of the response.
Abscissa; the retention trial. The first retention test was carried out 24 hours after the acquisition trial, followed by four further retention tests at 24-hour intervals.

needed to evaluate the learning ability of SHRSP, since there is a possibility that SHRSP may have a different threshold for pain than WKY.

III. Determination of neurotransmitters and their metabolite levels in CSF

CSF monoamine, ACh and their metabolites are determined with good reproducibility and high sensitivity (Matsumoto et al., 1989). In 15-week-old SHRSP, CSF norepinephrine (NE) concentration was significantly higher and CSF HVA and 5-HT levels were significantly lower than those in age-matched WKY. On the other hand, CSF ACh concentration was significantly lower in SHRSP than that in WKY.

The changes in CSF NE and ACh may reflect central noradrenergic and cholinergic activity, although pathophysiological significance of these findings was uncertain. It has been reported that the increase in CSF NE level was observed in patients with primary hypertension (DeQuattro et al., 1984). On the other hand, CSF ACh levels were found to correlate negatively with degree of dementia evaluated by Memory and Information Test (Davis et al., 1982). The changes in CSF NE and ACh observed in SHRSP might be involved in the pathogenesis of cerebrovascular lesions associated with hypertension.

The present study demonstrated that behavioral changes both in activities and rhythms and a memory impairment evaluated by passive avoidance response were observed in SHRSP. The changes in central noradrenergic and cholinergic activity were also suggested. These changes might reflect pathogenesis of cerebrovascular lesions caused by high blood pressure. From the present behavioral and neurochemical study, it is suggested that SHRSP may be a vascular dementia-animal model caused by cerebrovascular lesions.

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ACETYLCHOLINESTERASE OF CEREBRAL MICROVESSELS CHANGES IN ALZHEIMER'S DISEASE

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INTRODUCTION

Acetylcholinesterase (AChE) is histochemically demonstrable in capillary basement membranes of brain regions that contain relatively high AChE activity in their neuronal elements (Kreutzberg et al., 1975). Moreover, AChE might possibly be coupled with choline transport in microvessels isolated from the rat forebrain (Shimon et al., 1989). However, AChE activity has not been studied biochemically in microvessels isolated from human brains.

We earlier reported a method for isolation of microvessels from autopsied human brains (Tsuji et al., 1987). This isolation method for intracerebral microvessels enabled us to examine enzymes related to acetylcholine metabolism in human brain microvessels. In the present study we determined the activities of AChE together with properties of AChE in intracerebral microvessel preparations isolated either from control brain or from Alzheimer brain.

MATERIALS AND METHODS

Materials

Post-mortem human brains were obtained from 3 patients with non-neurological diseases and 3 patients with Alzheimer's disease. Both groups were matched for ages and autopsy delay. Brains were cut into 2 hemispheres at autopsy. One hemisphere was fixed in 10% formalin for pathological examination.

Isolation of microvessels and capillaries

Microvessels and capillaries were isolated according to the method described previously (Tsuji et al., 1987). The arachnoid membrane and pial arteries with perforating arteries were carefully removed. About 3-g pieces of the occipital lobe were cut out, minced and, sieved through a 250-um nylon mesh. The material on the mesh was resuspended in balanced solution, mildly homogenized, and sieved again, this time through a 73-μm nylon mesh. The material on this mesh was washed with 0.25 M sucrose and passed through a 150-μm mesh. The material retained on this mesh con-

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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sisted mainly of small arteries with attached capillaries (large microvessel fraction). The material that passed through the 150-μm mesh was put on a glass bead column to eliminate nonvascular elements and was designated as the capillary fraction (small microvessel fraction). We purified both fractions by centrifugation on discontinuous sucrose density gradients, 58,000 g for 60 min.

Centrifugation on sucrose density gradient

The tissue was thoroughly homogenized with a glass homogenizer in a buffer containing 0.01 M Tris-HCl (pH7.0), 1% Triton x-100, 0.05 M $MgCl_2$ and 1 M NaCl. The homogenate was centrifuged at 100,000 g for 60 minutes. The supernatant solution was collected and analyzed on a 5-20% linear sucrose density gradient of 4.5 ml on a 40% sucrose cushion, both prepared with the above buffer. Then 0.2 ml of the supernatant solution was layered onto the gradient. The gradient was centrifuged at 100,000 g for 17.5 hours and fractions (100 μl) were collected.

Digestion with collagenase

The supernatant solution of homogenized microvessels was incubated in the presence of 100 μg/ml collagenase at 37°C for 10 minutes and then layered on to a 5 to 20% linear sucrose density gradient.

Measurement

AChE assay was carried out spectrophotometrically using thiocholine as substrate, as described by Ellman et al. (1961), but somewhat modified (Nakano et al., 1986). The activity of γ-GTP was determined according to the method of Orlowski and Meister (1965) with a modification using γ-glutamyl-p-diethylaminoanilide as the substrate. The amount of cerebroside was measured as described by Hess and Lewia (1965).

RESULTS

Characterization of Vessel Fractions

The composition and purity of microvessel fractions were assessed by phase-contrast microscopy, activity of γ-GTP, and cerebroside content. A small quantity of capillaries was observed in the large microvessel fraction, but the predominant vessels were small arteries (50-200 μm diameter) containing smooth muscle. The small microvessel fraction consisted predominantly of capillaries without a muscle layer and only partially of small arterioles. Contamination with surrounding tissues or cell debris was scarce in either microvessel fraction.

AChE Activity

In control subjects, activities of AChE in the large and small microvessel fractions were significantly higher than the activity in the homogenate of brain parenchyma. In Alzheimer's disease, the AChE activity in the brain parenchyma was significantly lower than that in age-matched controls, while the AChE activity in cerebral large and small microvessels was significantly higher than that in the control (Table).

Analysis on Sucrose Density Gradient Centrifugation

The AChE in brain parenchyma of the occipital lobe was predominantly 10 S, suggesting the G4 form. Small peaks corresponding G2 and G1 forms were also observed, but AChE activity was not detected near the

bottom of the tube. The homogenate of microvessels contained an AChE species with a high sedimentation coefficient. All the peaks were inhibited completely by BW284c51, indicating the absence of nonspecific cholinesterase.

The supernatant solution of the microvessel homogenate was digested with protease-free collagenase. The activity of AChE disappeared in the fractions near the bottom of the tube. The peak of AChE was observed in fractions corresponding to 10 S, which suggests the conversion to G4 globular form. AChE activity in microvessels of Alzheimer brain was also found in fractions near the bottom of the tube, but the aggregated AChE was not easily converted to 10 S by the collagenase digestion (Fig.A, B).

Table 1. Acetylcholinesterase Activity

	S	L	H
control	6.54±0.51	8.89±0.63	4.28±0.34**
ATD	13.14±2.61 *	13.95±1.37 *	1.68±0.30

Acetylcholinesterase activity in small vessel fraction (S), large microvessel fraction (L), and in homogenate of brain parenchyma(H) obtained from control and Alzheimer brain (ATD). Data are expressed as the means ±SE. *p<0.001,**p<0.005

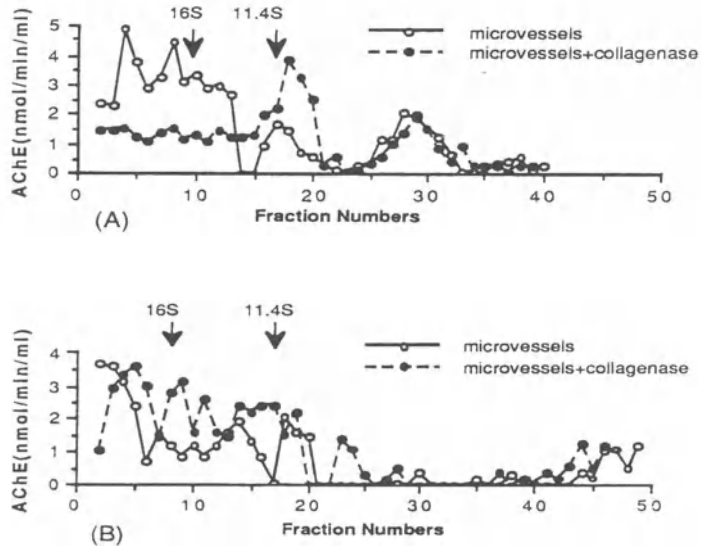

Figure 1. Sedimentation profiles of AChE molecular forms in the homogenate from brain tissue and microvessels of normal control (A) and Alzheimer's disease (B). Arrows indicate the positions of the marker enzymes; beta-galactosidase (16.0 S), and catalase (11.4 S). Broken lines represent AChE molecular forms of brain microvessels after collagenase treatment.

DISCUSSION

AChE has been demonstrated in cerebral capillaries of various animals, especially in basement membranes or in pinocytotic vesicles (Kreuztberg and Tôth, 1974). Electron microscopic observations have led to inference that AChE molecules secreted in the extracellular space from cholinergic terminals might be trapped in the basement membranes (Kreutzberg et al., 1979). The secreted form of AChE has been postulated to be globular (G4).

We demonstrated that large amounts of AChE in human brain microvessels showed a sedimentation coefficient different from that of A12 (16 S), representing several peaks larger than 16 S. Digestion with trypsin as well as collagenase failed to convert the gigantic molecule to A12 or other A type isozymes (A8, A4). Therefore, while the asymmetric form would be involved in the aggregate macromolecules of AChE, the macromolecules probably consist of globular AChE (G4) that constitutes by far the major AChE isozyme in the mammalian brain.

The present study of AChE in Alzheimer's disease indicated that the activity in brain microvessels was higher than that in the same vessels in control, normal brain; in contrast, the AChE activity in parenchyma was higher in the control brains. Moreover, properties of vascular AChE in the Alzheimer's brain might be different from those of the enzyme in normal brain, since the collagenase digestion was less effective to resolve AChE in microvessels of Alzheimer brain. It is important to study the mechanism explaining how the AChE molecule is bound tightly to components like vascular basement membranes, especially in Alzheimer's disease, because AChE appears also in the extracellular space such as in senile plaque (Mesulam and Moran, 1987).

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GENES OF HUMAN CATECHOLAMINE-SYNTHESIZING ENZYMES

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INTRODUCTION

Catecholamines (CA; i.e., dopamine, noradrenaline, and adrenaline) are neurotransmitters in dopamine, noradrenaline, adrenaline neurons in the brain and play important roles in Alzheimer's and Parkinson's diseases. CA are synthesized from tyrosine in the following pathway: tyrosine ⟶ 3, 4-dihydroxyphenylalanine (DOPA) ⟶ dopamine ⟶ noradrenaline ⟶ adrenaline. Thus, four enzymes catalyze the biosynthesis of adrenaline: (1)tyrosine hydroxylase (TH), (2)aromatic L-amino acid decarboxylase (AADC, or DOPA decarboxylase, DDC), (3)dopamine β-hydroxylase (DBH), and (4)phenylethanolamine N-methyltransferase. We have cloned and characterized cDNA's of TH, AADC, DBH, and PNMT genes and genomic DNA's of TH, DBH, and PNMT. We determined the nucleotide and deduced amino acid sequences of human CA-synthesizing enzymes.

HUMAN TYROSINE HYDROXYLASE (hTH)

TH (EC 1, 14, 16, 2)[1] catalyzes the hydroxylation of tyrosine to DOPA, the initial and rate-limiting step in CA biosynthesis. TH is a tetrahydropterin-dependent, iron-containing tetrameric monooxygenase, probably consisting of homologous subunits. Grima et al.[2] and we[3-5] found four types of hTH mRNA (types 1-4) by cDNA cloning, and determined their cDNA nucleotide sequences and the deduced amino acid sequences (Fig. 1). These mRNA's are constant for the major part, but are distinguishable from one another by the insertion/deletion of 12-bp and 81-bp sequences near the N-terminus. Fig.1 shows type-4 hTH cDNA, which contains a 12-bp plus 81-bp (93-bp) sequence (with both solid and dotted underlines) composed of the 12-bp sequence of cDNA from type-2 mRNA (with a dotted underline) and the 81-bp sequence from type-3 mRNA (with only a solid underline). Type-1 hTH mRNA is the shortest and lacks the 93-bp sequences, and contains the coding region of 1491 bp encoding 497 amino acids. The human TH gene is composed of 14 exons interrupted by 13 introns, and spans 8.5 kb.[5,6] The 12-bp insertion sequence in type-2 and -4 mRNA's is derived from the 3'-terminal portion of exon 1, and the 81-bp

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insertion sequence in type-3 and -4 mRNA's is encoded by exon 2. The N-terminal region is encoded by the 5'-portion of exon 1, and the remaining region from exons 3 to 14 are common to all four kinds of mRNA. There are two modes of alternative splicing. One is the alternative use of two donor sites in exon 1. The other mode of alternative splicing is the inclusion/exclusion of exon 2 in the spliced products, which determines expression of type-1,-2, or type-3,-4 mRNA's. Computer-assisted analysis of the secondary structure of the primary transcript led to the prediction of four stable hairpin-loops in introns 1 and 2. The selection of these 4 types of hTH mRNA can be explained by the hairpin-loop model. We assume the presence of trans-acting factors that stabilize the hairpin structure. Transcription of the TH gene is regulated in a tissue-specific manner and by inducible factors. The 5'-flanking region of the hTH gene contains the transcription factor *Sp* 1, which is known to activate the transcription of many mammalian genes, and the cyclic AMP-responsive elements for transcriptional activation of various genes. Types 1-4 hTH mRNA are expressed in COS cells;[7] and types 1 and 2, in C6 cells.[8,9] When these transfected cells were transplanted into rat brain, TH was expressed and DOPA was produced in the brain.[9] Such genetically engineered non-neuronal cells, that secrete a large amount of L-DOPA could be a good tool to clarify the precise mechanisms of the functional recovery obtained by grafts of catecholamine-containing neuronal cells.

HUMAN AROMATIC L-AMINO ACID DECARBOXYLASE (DOPA DECARBOXYLASE, hDDC)

DDC (EC 4, 1, 1, 28) catalyzes the second step in CA biosynthesis, i.e., decarboxylation of L-DOPA to dopamine.[10] DDC is a homodimer and requires one pyridoxal phosphate per subunit. We[11] isolated and characterized a cDNA clone encoding hDDC. A human pheochromocytoma cDNA library was screened by use of an oligonucleotide probe corresponding to a partial amino acid sequence of the enzyme purified from the human pheochromocytoma. The isolated cDNA clone encoded a protein of 480 amino acids with a calculated molecular mass of 53891 (Fig.2). The amino acid sequence, Asn-Phe-Asn-Pro-His-Lys-Trp, around a possible cofactor (pyridoxal phosphate) binding site was identical in human, *Drosophila*, and pig enzymes.

HUMAN DOPAMINE BETA-HYDROXYLASE (hDBH)

DBH (EC 1,14,17,1) catalyzes the third step of catecholamine biosynthesis, i.e., conversion of dopamine to noradrenaline. DBH is a copper-containing, ascorbate-requiring monooxygenase.[12] Lamouroux et al.[13] isolated a cDNA clone encoding human DBH and reported the nucleotide sequence and the deduced amino acid sequence. We[14] isolated two different mRNA types (types A and B) for human DBH and its genomic DNA, and showed that these mRNA's are generated through alternative polyadenylation from a single gene. Type A (2.7 kb) and type B (2.4 kb) encoded the same amino acid sequence and were different only in the 3'-untranslated region. Type A contained a 3'-extension of 300-bp at the end of the type B sequence (Fig.3). We also isolated the hDBH gene (approximately 23 kb); it was composed of 12 exons and existed as a single gene. Exon 12 encoded the 3'-terminal region of 1013 bp of type A, including the 300-bp sequence. Northern hybridization and S1 nuclease mapping experiments supported the conclusion that alternative use of two polyadenylation sites from a single DBH gene generates two different mRNA types, types A and B. The ratio of type A to B mRNA's in pheochromocytoma was approximately 1.0 to 0.2. We found possible transcription regulatory elements, TATA, CCAAT, CACCC, GC boxes, and glucocorticoid and cyclic AMP responsive element near the transcription initiation site of the DBH gene. The functional

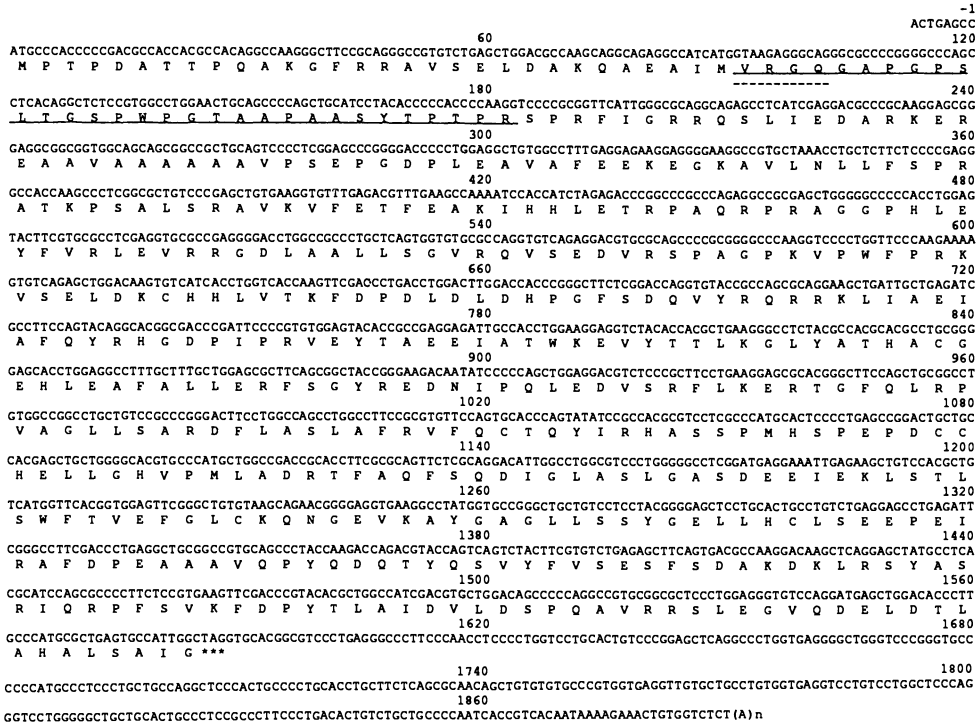

Fig. 1. Nucleotide sequence of cDNA encoding human tyrosine hydroxylase type 4 and deduced amino acid sequence.

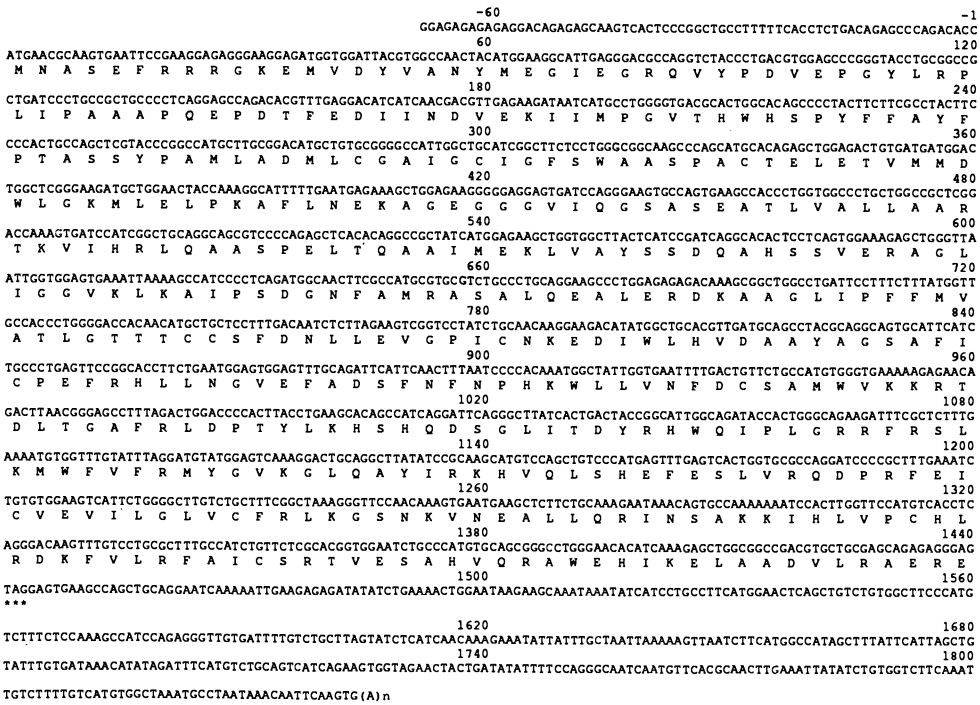

Fig. 2. Nucleotide sequence of cDNA encoding human aromatic L-amino acid decarboxylase and deduced amino acid sequence.

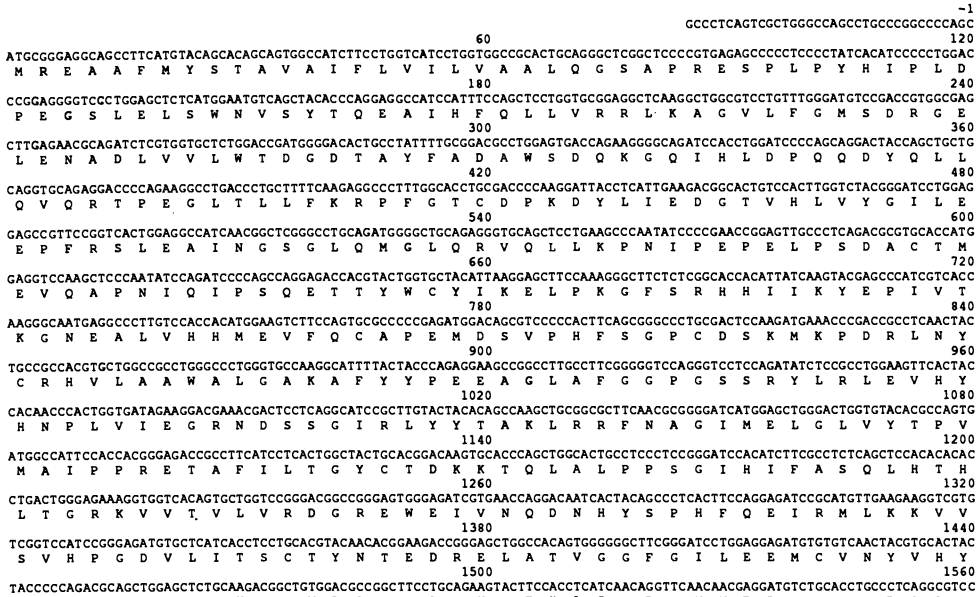

Fig. 3. Nucleotide sequence of cDNA encoding human dopamine β-hydroxylase type A and deduced amino acid sequence.

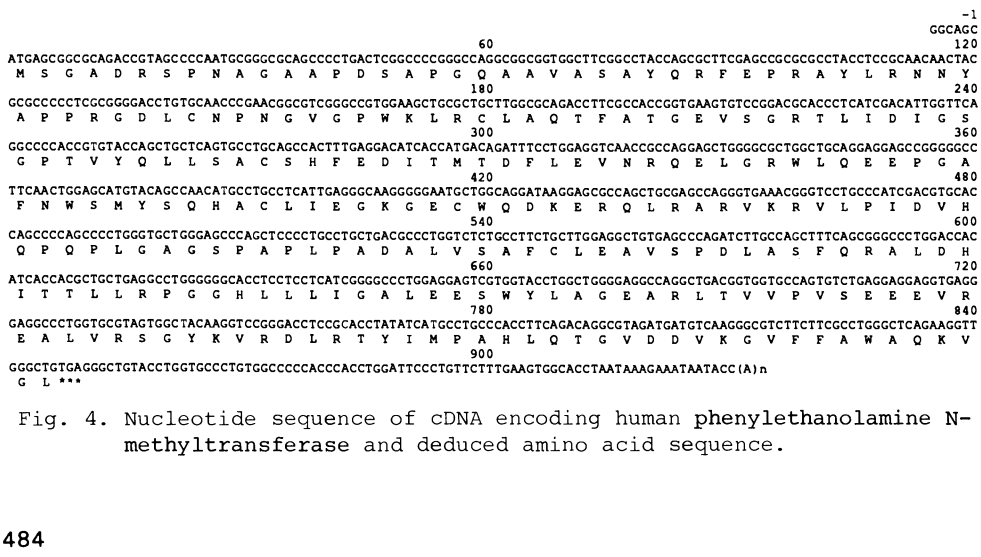

Fig. 4. Nucleotide sequence of cDNA encoding human phenylethanolamine N-methyltransferase and deduced amino acid sequence.

significance of the production of multiple mRNA's having different 3'-untranslated regions through alternative polyadenylation is not known. The 3'-untranslated region may be involved in mRNA stability and translational efficiency.

HUMAN PHENYLETHANOLAMINE N-METHYLTRANSFERASE (hPNMT)

PNMT (EC 2,1,1,28) is the terminal enzyme in CA biosynthesis, and catalyzes the formation of adrenaline from noradrenaline, using S-adenosyl-L-methionine as the methyl donor.[15] We reported the complete nucleotide sequence of hPNMT cDNA and the deduced amino acid sequence of the enzyme (Fig.4).[16] Determination of the nucleotide sequence revealed that hPNMT consists of 282-amino acid residues with a predicted molecular weight of 30853 Da, including the initial methionine. The amino acid sequence of the hPNMT was highly homologous (88%) with that of the bovine enzyme.[17] We also assigned the PNMT gene to chromosome 17[16]. Baetge et al.[18] and we[19] cloned the genomic DNA of hPNMT, and found the hPNMT gene to consist of three exons and two introns spanning about 2.2 kb. We[19] also observed for the first time the presence of a minor PNMT mRNA (type B, 1.7 kb) besides the major mRNA (type A, 1.0 kb) as reported previously.[16] Type-B mRNA carries an approximately 700 nucleotide-long untranslated region in the 5' terminus. This suggests that two types of mRNA are produced from a single gene through the use of two alternative promoters. The 5'-flanking region of the gene contains several consensus sequences for glucocorticoid responsive elements and *Sp* 1 binding sites.

CONCLUSIONS

Isolation and characterization of cDNA's of all four human catecholamine-synthesizing enzymes, hTH, hDDC, hDBH, and hPNMT, revealed the deduced amino acid sequences. Multiple mRNA's of hTH, hDBH, and hPNMT were discovered by cDNA cloning. Characterization of genes for human CA-synthesizing enzymes may clarify the molecular mechanisms underlying the differentiation and expression of catecholaminergic neurons, and the regulation of CA neurotransmitter biosynthesis, both of which are involved in Parkinson's and Alzheimer's diseases.

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GENE ACTIVITY AND NEURODEGENERATION IN THE EXTRAPYRAMIDAL SYSTEM: A PROGRESS REPORT ON MOLECULAR AND MORPHOLOGICAL CORRELATES

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Functional neuronal plasticity and synaptic remodelling appear to be consistent feature of aging brain (Coleman et al., 1986). However, even in the absence of overt neuropathology, heterogeneous atrophic changes may occur in the brain with advancing age, suggesting differential mechanisms on specific brain regions, even in the same anatomical structure. For example hippocampal dentate granule neurons apparently remain intact and show hypertrophy of dendritic processes and increased perikaryal size in the aged brain (Coleman and Flood, 1987). Nonetheless many others e.g., pyramidal neurons of the hippocampus (Ringborg 1966) and cerebral cortex (Peters et al., 1987) show reduced perikaryal RNA content and decreased size of their perikarya, nuclei, or nucleoli. In addition, the nucleolar shrinkage is less in human locus ceruleus (LC) than substantia nigra (s.nigra) neurons (Mann and Yates, 1979), further confirming the cellular selectivity of changes. These studies support the general concept that age-related neuronal atrophic changes is not an universal or inevitable characteristic of the senescence, but may be brain region, cell type, and species specific.

During Alzheimer's (AD) and Parkinson's (PD), neuronal compensatory changes may also occur. The increased dendritic arbor in dentate gyrus granule cells during AD, may reasonably be interpreted as a deafferentation response to perforant pathway deterioration (Geddes et al., 1985). In PD, although the remaining s. nigra dopaminergic (DAergic) neurons are atrophic with smaller nuclei (Mann and Yates, 1983), increased striatal dopamine (DA) release and turnover has been reported (Hornykiewicz and Kish, 1986).

It is possible that these phenomena could merely reflect the relative sparing of subgroups of neurons that are less susceptible to neurodegeneration. For example recent evidence suggest two biochemically distinct population of nigrostriatal DAergic neuron, based on the differential expression of a 28kd calcium binding protein (CaBP) (Gerfen et al., 1987). These subsets appear to differ in their susceptibility to nigral experimental neurotoxic lesions (Grimes et al., 1988). Other studies suggest that neurons containing NADPH-diaphorase may be selectively protected from the deleterious effects of neurodegenerative diseases such as AD and Huntington (Kowall et al., 1987). Although these studies suggest that some neurons may be more susceptible to degeneration than others, the temporal sequence and extent to which atrophic changes

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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in macromolecular biosynthesis may limit compensatory responses in the remaining neurons during neurodegeneration (as well as during normal aging) is still unknown.

We are exploring a rat lesion model for select aspect of nigral atrophic changes (including shrinkage of nucleoli), observed in the remaining nigral neurons in PD (Pasinetti et al., 1989). The nucleolar shrinkage in the remaining neurons of the s. nigra in PD (Mann and Yates, 1983) was particularly puzzling, because lesions of this pathway induce hyperactivity of the remaining neurons in young rats, as indicated by increased synthesis and release of dopamine and increased TH activity at the striatal terminals (Stachowiak et al.,1987, Zigmond and Striker, 1984).

Adults male rats were given unilateral 6-hydroxydopamine injection (6-OHDA) into s. nigra and sacrificed 9 months later. Measurements of striatal catecholamines showed major depletion of DA on the lesioned side, but increased striatal DOPAC/DA ratios, which indicates increased release of DA at the remaining striatal terminals. However, atrophic changes occurred in the remaining nigral DAergic cell bodies, which were atrophied just as seen in PD. In these measurements, DAergic neurons were identified by immunocytochemistry (ICC) with antisera to rat TH. The cell bodies, nuclei, nucleoli of TH immunoreactive neurons were about 30% smaller than in contralateral (intact) side. The smaller nucleoli imply decreased synthesis of ribosomes, which would be consistent with the gross loss of neuronal RNA reported in the s. nigra during PD (Mann and Yates, 1983). To establish the extent of change in neuron RNA, we examined two messenger RNA populations using <u>in situ</u> hybridization tyrosine hydroxylase (TH) and beta-tubulin mRNA. For these measurements, brain sections were prepared for ICC to TH, followed by hybridization to cRNA (complementary) antisense strand probes made in transcription vector. This approach allows us to assay specific mRNA in identified dopaminergic neurons. We found selective >50% loss of TH mRNA per neuron, while beta-tubulin mRNA tubulin was unchanged. Based on these finding, we predict a little discussed regulatory defect in PD: that the remaining DAergic nigral neurons become deficient in TH mRNA, with consequent limitations of TH enzyme production and its synthetic activity.

Opposite changes of the TH mRNA in its cell body and of DA synthesis and release at its striatal terminals imply a dichotomous regulation. Several mechanisms for these changes can be considered. It is possible that 6-OHDA has long lasting toxic effect that cause damage to the remaining neuron cell bodies, while allowing the terminals to manage some compensation. It is possible that this phenomena could merely reflect the relative sparing of a subgroup of neurons less susceptible to neurodegeneration.

We are extending these finding by examining TH mRNA prevalence in the remaining s. nigra DAergic neurons, striatal TH activity and striatal TH protein, DA, DOPAC content at various time after nigral 6-OHDA lesions. Recent data suggest earlier atrophic changes at 90 days post lesion. We found that the remaining nigral DAergic neurons were atrophic (-20%) and that the TH mRNA concentration in this neurons was reduced by 50% as compared with contralateral s. nigra dopaminergic neurons and intact animals. Moreover, we confirmed atrophic changes described at 270 days postlesion (Pasinetti and Finch, submitted)

In the nigrostriatal system, the loss of s. nigra neurons may compromise a subset of striatal neurons (either those projecting to s. nigra, or those innervated by s. nigra), which could finally feed forward through polysynaptic pathways to further compromise the already atrophied

s. nigra neurons. In support of this hypothesis, striatal neurons (MSI) in PD show atrophic changes (McNeill et al., 1988), while lesioning of the striatum in rats produces selective transneuronal degeneration in the s. nigra. In particular, we found that gross unilateral striatal ibotenate lesions induced an ipsilateral anterograde transneuronal degeneration of s. nigra reticulata glutamic acid decarboxylase (GAD)-mRNA containing neurons (Pasinetti et al., submitted). Since our data were obtained by in situ hybridization without combined ICC for GAD, we are not yet certain whether the phenomenon was due to loss of GAD-mRNA containing neurons, or to a dramatic decrease in GAD mRNA prevalence. Of particular interest was the apparent selective loss of DAergic neurons in the caudal portion of s. nigra compacta as suggested by quantification of nigral TH immmunopositive neurons. Although divergent reports (Olney, 1985) suggest no changes of the nigral DAergic population in similar lesioning protocol, we note that many of these reports lack any topographical localization and quantification of DAergic neurons following striatal lesions. The description of a dissociation of compartmentally organized striatonigral and nigrostriatal system on the basis of the expression of CaBp, suggest that a subset of nigral DAergic/CaBp neurons localized in the rostral tier of the nucleus is more resistant to neurotoxic insults (Grimes et al., 1988). It is worth mentioning that the rostral portion of the s. nigra compacta in our paradigm were the least affected by anterograde striatal lesion. The regulation of TH mRNA prevalence in the remaining DAergic neurons following striatal lesions will identify further correlation between neurotoxic lesions and denervation which we hypothesize alter gene expression and lead to further neurodegenerative cascades.

Apparently similar atrophy of basal forebrain cholinergic neurons also can be induced by decortication (Sofroniew et al., 1983). Moreover lesions that kill 30% of basal forebrain cholinergic nuclei in rats cause very slowly evolving neurodegenerative transynaptic cascades that lead to neuronal degeneration in the entorhinal cortex and hippocampus (Arendash et al., 1987); these changes gives a striking model for neuronal atrophy in these same pathways during AD. We need more information about the changes in macromolecular biosynthesis (specific RNA and protein species) in order to establish if neuron atrophy has the same final common regulatory pathway, e.g., in aging, in diseases like PD or AD, or after experimental deafferentation. Finally, we also note the potential bearing of these studies to investigate the role of neurotrophic factors during neurodegeneration. It will be important to learn how various drug treatment may influence the synthesis, prevalence and translation of different mRNA transcripts relevant to neuronal plasticity.

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Portions of these reviews of our work may be found nearly verbatim in other reports by the authors. This research was supported by National Parkinson Foundation and United Parkinson Foundation to CEF and GMP, and by NIA grant (AG-A607909-01) to CEF.

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ANALYSIS OF DOPAMINE AND NEUROPEPTIDES IN 6-OHDA-LESIONED RATS

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INTRODUCTION

Cholecystokinin (CCK) and dopamine (DA) coexist in the ventral tegmental area and medial substantia nigra neurons.[1] In the brain of Parkinson's disease patients, a prominent decrease in CCK content of the substantia nigra has been reported,[2] and it is thought that DA and CCK might be related to the etiology of Parkinson's disease. The precise functional interaction between DA and CCK, however, is not clarified. Based on the hypothesis that CCK has a functional role as one of the modulators of DA metabolism, ceruletide (CLT), which is a CCK-related decapeptide, is currently under trial for the treatment of diseases with involuntary movements including Parkinson's disease.[3] In the present study we attempted to clarify the mechanism of action of CLT and CCK with respect to their receptors as well as their effect on behavior in a Parkinson's disease model, namely, the 6-OHDA unilaterally lesioned rat.

MATERIALS AND METHODS

Male Sprauge-Dawley rats (250-300g) were used, and the experiments were performed twenty days following the stereotaxic injection of 6-OHDA into the right medial substantia nigra. For brain microdialysis, a U-shaped microdialysis probe was made from a hollow fiber and 23 gauge needle. Dialysis was done through the U-shaped end which was 4mm in length. The recovery rates of DA and its analogue were about 20-30% in vitro.

To analyze the effect of CLT on circling behavior in 6-OHDA-lesioned rats, we first calculated the number of circlings induced by intraperitoneal (i.p.) injection of Amphetamine (2mg/kg) or Apomorphine (2mg/kg). One hour after i.p. injection of CLT (50, 100, 200 or 400 μg/kg), Amphetamine (2mg/kg) or Apomorphine (2mg/kg) was injected i.p., and the number of circlings was calculated.

For analysis of DA release by brain microdialysis and HPLC with ECD, the rats were anesthetized with ether and fixed into a stereotaxic apparatus. The microdialysis probe was implanted in the left striatum. The probe was continuously perfused with physiological saline at a rate of

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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2μl/min, and dialysates were collected every 25 min and injected into the HPLC and ECD system for measurement of DA release. After the HPLC pattern became stable, Amphetamine (2mg/kg) was injected i.p. and DA release was measured. One hour after i.p. injection of CLT (200 μg/kg), the same dose of Amphetamine was injected i.p. and DA release was measured.

For autoradiographic analysis of CCK receptors, we decapitated the rats 30 days after surgery and removed and froze the brains immediately. Sections (30 μm) were cut with a cryostat and these were mounted on gelatin-subbedded slides. We made autoradiographic localization of CCK binding sites in the striatum and the nucleus accumbens using ^{125}I-Bolton-Hunter-CCK, as previously described.[4,5]

RESULTS

1. Analysis of circling behavior

CLT itself had no effect of inducing of circling behavior in the 6-OHDA-lesioned rats at any of the doses studied. The animals became inactive after CLT injection i.p.. CLT suppressed Amphetamine-induced circling toward the lesioned side in a biphasic dose-response manner. CLT also suppressed Apomorphine-induced circling toward the non-lesioned side in a similar manner (Figure 1). CLT had its the weakest effect at 100 μg/kg as compared with 50 and 200 μg/kg doses.

2. Analysis of DA release by microdialysis with HPLC and ECD

Amphetamine (2mg/kg i.p. injection) induced DA release from the striatum on the non-lesioned side; the release was about 7 times greater than that of basal release at 30-180 min after injection, and the DA level returned to the basal level 240 min after injection. A repeated similar dose of Amphetamine did not induce the same effect (data not shown). (CLT 200 μg/kg, i.p. injection) inhibited Amphetamine-induced DA release significantly.

3. Autoradiographic analysis of 6-OHDA-lesioned rat brain using ^{125}I-CCK

Autoradiographic analysis of section of 6-OHDA-lesioned rat brain containing the striatum and the nucleus accumbens showed no significant difference between the normal and the lesioned side.

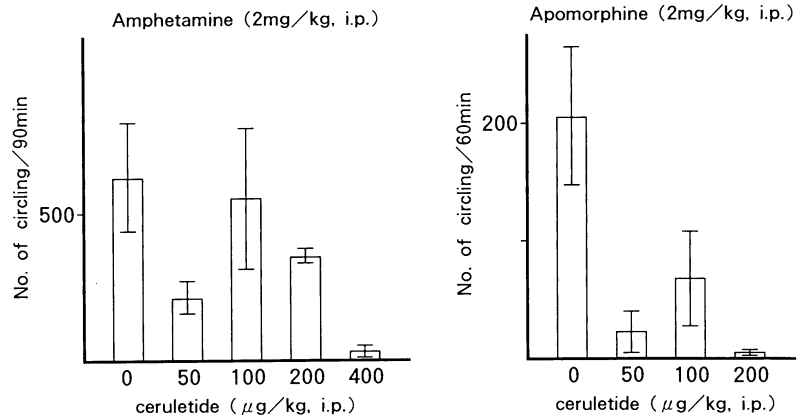

Fig. 1. Effect of ceruletide on Amphetamine or Apomorphine-induced circling behavior in 6-OHDA-lesioned rats.

DISCUSSION

As functional modulation of DA metabolism by peripherally administered CLT should be clarified prior to application of the drug to the treatment of diseases with involuntary movements including Parkinson's disease, we analyzed the effect of intraperitoneal injection of CLT on behavior in 6-OHDA-lesioned rats.

The suppressive effect of an i.p. injection of CLT on Amphetamine-induced circling behavior can be explained in at least two waysas shown in the left side of Figure 2: 1) CLT blocks the post-synaptic DA receptors directly; or 2) CLT inhibits DA release pre-synaptically. The observation of inhibition by CLT of Amphetamine-induced DA release suggests that CLT receptors exist on the pre-synaptic membrane and compete with the effect of Amphetamine.[6] It was already reported, based on a study using the microdialysis technique, that peripheral administration of CLT suppressed endogenous DA release.[7] But our data obtained by the microdialysis technique showed that peripheral administration of CLT inhibited even Amphetamine-induced DA release. CLT i.p. injection also suppressed Apomorphine-induced circling behavior.

In the 6-OHDA model, the number of DA receptors should be increased. Apomorphine is an analogue of DA. Low doses of Apomorphine inhibit DA cells presynaptically, but a sufficiently high dose of Apomorphine has a postsynaptic stimulatory effect and elicits behavioral responses.[8] We chose a high dose, i.e., 2mg/kg, to clarify the function of CLT at the post-synaptic membrane. Judging from the suppressive effect of CLT, the CCK receptors might have increased in number in the caudate and posterior medial nucleus accumbens on the lesioned side. Results of the autoradiographic analysis, however, did not show any increase in the number of CCK receptors in the striatum and the nucleus accumbens on the lesioned side. Increased CCK binding was also found in the nucleus accumbens following ventral tegmental lesions using 6-OHDA. Lack of any alteration in caudate CCK receptors following lesions of the nigro-striatal pathway have been reported.[9]

We conclude that CLT blocks the effect of Apomorphine via CCK postsynaptic receptors that may be different from DA receptors, as shown in the right side of Figure 2, and that the CCK receptors might exist on both pre- and post- synaptic membranes.

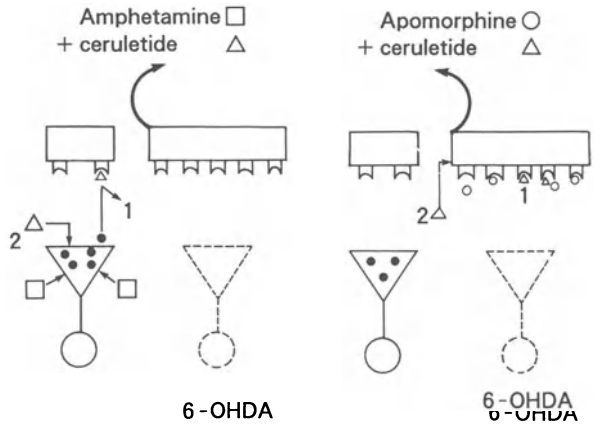

Fig. 2. Effect of ceruletide on Amphetamine- or Apomorphine-induced circling behavior in the lesioned rat. Dopamine, ●
1.Blocks postsynaptic DA receptors 1.Blocks postsynaptic DA receptors
2.Blocks presynaptic DA receptors 2.Blocks Apomorphine via CCK receptors

It has been reported that peptides can pass through the blood-brain barrier (BBB) to some extent.[10] However, CLT does not cross the BBB in measurable quantities as measured by conventional methods.[11] Although, controversial, in the lesioned animal model, or in aged patients, BBB should be damaged to some degree, and it is likely that CLT can pass through the BBB in sufficient quantity to manifest its effect. Judging from the biphasic effect of suppression of Amphetamine- and Apomorphine-induced circling behavior, the CLT receptors may be divided into low- and high-affinity types.[6,12] In the central nervous system, there are two different types of receptors, i.e., "central-type" and "peripheral-type".[6] However, further and more detailed investigations will be necessary to clarify the characteristics of CLT receptors and CLT itself.

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ENHANCEMENT OF IN VIVO DOPAMINE RELEASE FROM THE RAT STRIATUM BY 6R-L-ERYTHRO-5,6,7,8-TETRAHYDROBIOPTERIN AS STUDIED BY BRAIN DIALYSIS

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INTRODUCTION

6R-L-erythro-5,6,7,8-Tetrahydrobiopterin (6R-BH_4) is a natural cofactor for tyrosine hydroxylase, a rate-limiting enzyme for biosynthesis of catecholamines (Nagatsu et al., 1964). Since the concentration of 6R-BH_4 in the brain (Fukushima and Nixon, 1980) is low compared with Km values of tyrosine hydroxylase (Nelson and Kaufman, 1987), tissue levels of 6R-BH_4 are considered to regulate the activity *in vivo* of this enzyme. Our previous findings that intracerebroventricular administration of 6R-BH_4 resulted in enhanced tyrosine hydroxylation in the rat brain (Miwa et al., 1985) supported this possibility. In the same report, increases in the content of metabolites of dopamine, which is an index of the activity of dopaminergic neuron, was noted. Furthermore, administration of 6R-BH_4 has been reported to improve clinical symptoms of Parkinson's disease (Curtius et al., 1984). These data taken together suggest that 6R-BH_4 enhances release of catecholamine from nerve terminals. Therefore, in the present study, the effects of 6R-BH_4 on dopamine release *in vivo* was investigated using a brain dialysis method.

MATERIALS AND METHODS

Male Wistar rats (250-300 g) were anesthetized with diethylether and a dialysis probe was stereotactically implanted in the striatum. The location of the probe was confirmed by visual examination of the brain at the end of each experiment. The dialysis probe was continuously perfused at a flow rate of 9.5 µl/min with Ringer solution (147 mM NaCl, 4 mM $CaCl_2$ and 2.3 mM KCl, pH 6.1) and dialysates were collected every 20 min in micro test tubes containing 20 µl of 1 M perchloric acid, 10 mg/ml sodium bisulfite and 10 mM EDTA.

After dopamine levels in dialysates had reached a steady-state, 6R-BH_4 was administered by adding it to the perfusion fluid. The 6R-BH_4 solution for dialytic administration was prepared immediately before use and the pH of the solution was adjusted to 6.1 by adding NaOH solution. Usually, four or five 20-min control dialysates were collected before administration of 6R-BH_4. Dopamine measurements were then continued for an additional 2 h.

Dopamine collected in dialysates was assayed by HPLC with electrochemical detection after purification with an alumina batch method (Koshimura et al., in press).

The tyrosine hydroxylation *in vivo* was estimated as described previously (Hayashi et al., 1988).

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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6R-BH_4 in the perfusion fluid was assayed using HPLC with fluorescence detection according to the method of Fukushima and Nixon (1980) as modified by Miwa et al. (1985).

RESULTS AND DISCUSSION

Prior to the dialysis study, the recovery of dopamine through the dialysis membrane was determined. The probes were placed in Ringer solution containing various concentrations of dopamine (0.05, 0.5 and 5 μM), and were perfused with Ringer solution at a flow rate of 9.5 μl/min. Dialysates were collected every 20 min. The recovery of dopamine through the dialysis membrane (concentration in dialysate/concentration in surrounding fluid x 100%) was constant (about 5%) at this concentration range. Therefore, the data described beneath will be presented without any correction for recovery.

Fig. 1 shows the effects of 6R-BH_4 added to the perfusion fluid on the amount of dopamine collected in dialysates. The amount of dopamine collected in dialysates per 20-min period reached a steady-state 40 min after the start of brain dialysis and remained constant up to 240 min. Following dialysis of the striatum with Ringer solution containing various concentrations of 6R-BH_4, dopamine levels in dialysates increased dose-dependently. At 1.0 mM, the maximum dopamine levels in dialysates were about 8-fold greater than the control value.

To estimate the approximate concentration of 6R-BH_4 in the striatum in the vicinity of the dialysis probe, the recovery of 6R-BH_4 through the dialysis membrane was determined by the same method for the determination of the recovery of dopamine and was about 3% at the concentration range examined (0.25, 0.5 and 1.0 mM).

Since 6R-BH_4 is rapidly oxidized in the neutral solution (Kaufman, 1967), actual concentration of 6R-BH_4 in the perfusion fluid was determined. About 50% of 6R-BH_4 in the perfusion fluid remained unoxidized until 60 min after the start of perfusion of 6R-BH_4 at 1.0 mM.

In order to determine whether the increase in dopamine levels in dialysates resulted from an increase in dopamine release or from an inhibition of dopamine uptake mechanism, the effects of pretreatment with nomifensine, a specific inhibitor of dopamine uptake, on the 6R-BH_4-induced increase in dopamine levels in dialysates was examined (Fig. 2). Following intraperitoneal injection of various doses of nomifensine, dopamine levels in dialysates increased dose-dependently and reached a maximum at doses higher than 100 mg/kg, suggesting that dopamine uptake is completely inhibited at these doses. The increases in dopamine levels were observed up to 160 min after the injection. When 6R-BH_4 was added to the perfusion fluid following pretreatment with nomifensine (100 mg/kg), dopamine levels in dialysates further increased. These results suggest that the 6R-BH_4-induced increase in dopamine levels in dialysates is not the result of inhibition of dopamine uptake but the result of an increase in dopamine release.

The 6R-BH_4-induced enhancement of dopamine release *in vivo* was virtually abolished by pretreatment with tetrodotoxin (data not shown), which inhibits neuronal activity by blocking sodium channels in neuronal tissues. This result suggests that 6R-BH_4-induced dopamine release *in vivo* is dependent upon neural impulses reaching nerve terminals and is not the result of non-specific effects of 6R-BH_4 such as displacement of dopamine in the storage vesicle and destruction of nerve terminals.

In order to determine whether the 6R-BH_4-induced dopamine release resulted from an increase in the rate of dopamine biosynthesis or not, the effects of 6R-BH_4 on dopamine levels in dialysates was examined after pretreatment with α-methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase (Fig. 3). Even after intraperitoneal injection of 250 mg/kg of α-methyl-p-tyrosine, 6R-BH_4 induced an increase in dopamine levels in dialysates. To confirm that activity of tyrosine hydroxylase *in vivo* is com-

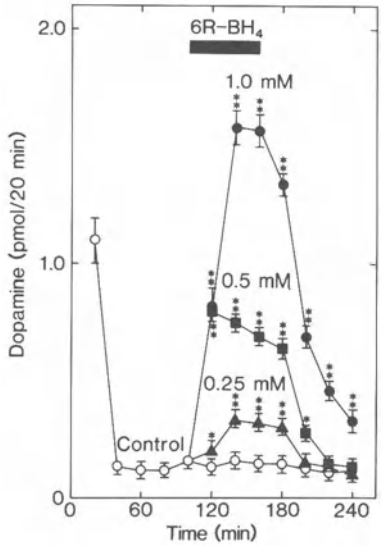

Fig. 1. Effects of 6R-BH_4 on dopamine levels in striatal dialysates.
*, $p < 0.05$; **, $p < 0.01$; significantly different from the value in the corresponding fraction of the control group.

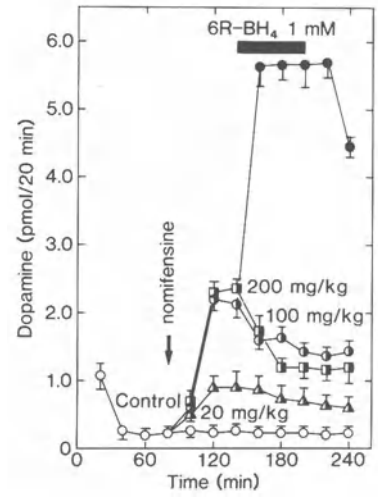

Fig. 2. Effects of pretreatment with nomifensine on 6R-BH_4-induced increases in dopamine levels in striatal dialysates.

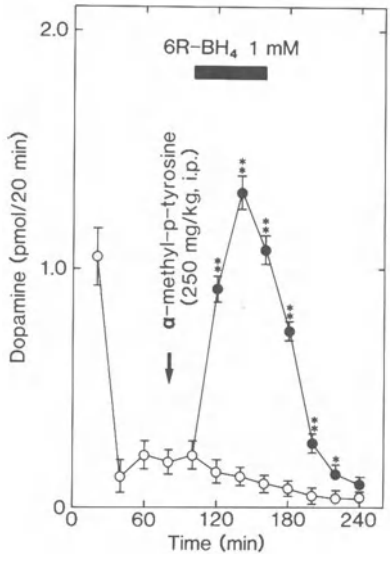

Fig. 3. Effects of pretreatment with α-methyl-p-tyrosine on 6R-BH_4-induced increases in dopamine release. Open circles represent values of the group, which was given α-methyl-p-tyrosine alone.

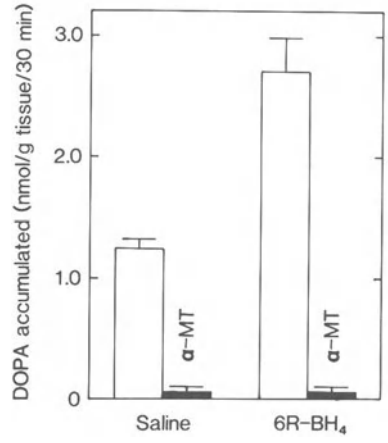

Fig. 4. Effects of 6R-BH_4 on tyrosine hydroxylation in vivo in the absence and presence of pretreatment with α-methyl-p-tyrosine.

pletely inhibited by this dose of α-methyl-p-tyrosine even after administration of 6R-BH_4, the effects of 6R-BH_4 on the rate of tyrosine hydroxylation *in vivo* was examined in the absence or presence of pretreatment with α-methyl-p-tyrosine (Fig. 4). As reported previously (Miwa et al., 1985), tyrosine hydroxylation *in vivo* increased by about 100% after intracerebroventricular injection of 1 mg of 6R-BH_4 but it was completely inhibited by pretreatment with α-methyl-p-tyrosine (250 mg/kg). When rats were pretreated with α-methyl-p-tyrosine, 6R-BH_4 had no effect on tyrosine hydroxylation *in vivo*. These results strongly suggest that most of the 6R-BH_4-induced increase in dopamine release *in vivo* was brought about by a direct stimulating action of 6R-BH_4 on dopamine release.

The concentration of 6R-BH_4 within dopamine nerve terminals is assumed to be approximately 100 nmol/g wet tissue (100 μM) (Levine et al., 1981). In the present study, according to the recovery of 6R-BH_4 (about 3%), when the striatum is dialyzed by the solution containing 1.0 mM 6R-BH_4, the concentration of 6R-BH_4 around the dialysis probe is 30 uM. This value is comparable to the physiological concentration of 6R-BH_4 within dopamine nerve terminals in the rat striatum. Therefore, the present study indicates that 6R-BH_4 physiologically regulates dopamine release *in vivo*.

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PREPARATION OF ANTIGEN AND ANTIBODY TO 3-METHOXY-4-HYDROXYPHENYL GLYCOL(MHPG)

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INTRODUCTION

Norepinephrine is a transmitter not only in the peripheral nervous system but also in the central nervous system. The main metabolite from norepinephrine is MHPG in the brain. The level of MHPG in the brain as well as blood reflects the nervous activity of norepinephrine.

Immunoassay is desirable to measure many samples in a short time. In our previous papers, we have been developing immunoassays of catecholamines(Yoshioka, 1983. Yoshioka et al., 1986a, 1986b), their basic(Shirahata et al., 1980) and acidic metabolites(Yoshioka et al., 1986c). In this paper, we tried to develop their neutral metabolite such as MHPG. Keeton et al. reported a radioimmunoassay of MHPG in 1981. They synthesized the antigen by a reaction of 5-bromopentanoic acid with MHPG, which intermediate was coupled with thyroglobulin, and obtained its specific antibody. We prepared an antigen by one step, in which MHPG was directly conjugated with human serum albumin(HSA) by the Mannich reaction used for the syntheses of the antigens to the acidic metabolites(Yoshioka et al. 1986c). The antibody was produced and applied to enzyme linked immunosorbent assay(EIA).

METHODS

Preparation of antigen

To remove piperazine, 4 ml of 100 mg of MHPG hemipiperazine salt in water was passed through a column(1.5 cm x 6 cm) of SP-Sephadex(H^+ form)

Abbreviations: HVA = Homovanillic acid, VMA = Vanilmandelic acid, DHPG: 3,4-Dihydroxyphenylethyleneglycol, DOPAC: 3,4-dihydroxyphenylacetic acid

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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and eluted with water. Fractions of 1.5 ml each from 7 to 12 were pooled.

The hapten was coupled with a carrier protein by the Mannich reaction described by Yoshioka et al. as shown in Fig. 1. To 3 ml of the pooled solution was added 100 mg of HSA in 1 ml of 0.2 M $NaHCO_3$ and 0.8 ml of 35 % formalin. The solution was adjusted to pH 7.0 with 3 M sodium acetate. After replacing the air with nitrogen gas, the reaction tube was tightly closed. To carry out the Mannich reaction, the mixture was allowed to stand at 17-23°C in the dark for 3 days. The reaction mixture was dialyzed in a Visking tube against 1 liter of water at 4°C 5 times for an hour each. The solution was dialyzed against 0.01 M HCl 1 times and further against water 4 times at 4°C. The dialysate was freeze-dried. The conjugate of MHPG-HSA was obtained as the antigen.

The hapten content in the conjugate was determined by measuring the absorbance of the antigen, hapten and HSA solutions at 280 nm.

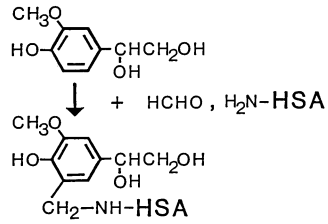

Figure 1. Preparation of antigen by the Mannich reaction

Preparation of antisera

One milliliter of the antigen(2 mg/ml) in 0.9 % NaCl adjusted to pH 7.0 was mixed with an equal volume of complete Freund's adjuvant and stirred vigorously to make a W/O emulsion. For the first immunization, 0.1 ml of the emulsion was intra-peritoneally injected to 10 6-week-old balb/c mice. After several immunizations were done at monthly intervals, using the similar emulsion prepared with incomplete Freund's adjuvant. One week after the immunization, an intravenous blood sample was taken, stood at room temparatrure for 1 h and at 4°C overnight and centrifuged at 3500 g for 5 min. The prepared serum was stored at -80 °C.

Procedure for EIA based on colorimetry

The EIA procedure of M. Yoshioka et al.(1988) was modified so that there was competition between the antigen adsorbed on the surface of the well of the microtiter plate and the hapten in the solution for the antibody binding sites. Fifty microliters of the antigen solution(0.6 μg/ml) in phosphate buffer (PB, 0.1 M KH_2PO_4, pH 7.0) was distributed between the wells. The plate was stood at 37°C for 2 h. Each well was washed with 300 μl of 0.05 % Tween 20 in PB(TPB) 5 times. To the well was added 50 μl of the diluted antiserum solution in TPB. The plate was stood at 37°C for 30 min. The well was washed with 300 μl of TPB 5

times. To the well was added 50 μl of 8 μg/ml goat anti-mouse IgG labeled with alkaline phosphatase solution. The well was stirred at 37 ℃ for 45 min. The well was washed with 300 μl of TPB 5 times. To the well was added 50 μl of 22.4 mM p-nitrophenylphosphate in 100 mM $NaHCO_3$ (pH 9.8) containing 1 mM $MgCl_2$. After 10 min the absorbance of p-nitrophenol produced in the reaction mixture was measured by an EIA reader(Bio-Rad Lab.) at 405 nm.

RESULTS

The antigen of MHPG was obtained by the Mannich reaction as shown in Fig. 1. The conjugation was confirmed by the electrophoresis. The antigen moved against the positive side more than HSA itself. It meant that basic ε-amino group of lysyl residues of HSA was neutralized by the aminomethylation with neutral MHPG. From the increase in the absorbance at 280 nm, it was calculated that 8 mol of MHPG was conjugated with 1 mol of HSA. The attachment site of the protein to the hapten was estimated by ^{13}C-NMR spectroscopy of the antigen, which will be described elsewhere.

Ten antisera obtained after 4 months had high tighter at almost the same extent and higher affinity to the antigen than the carrier, HSA. Cross-reactivities of the analogs were not so much except DHPG which showed less than 10 % at 50 % binding as shown in Fig. 2. From the calibration curve, it was possible to detect more than 20 μM MHPG.

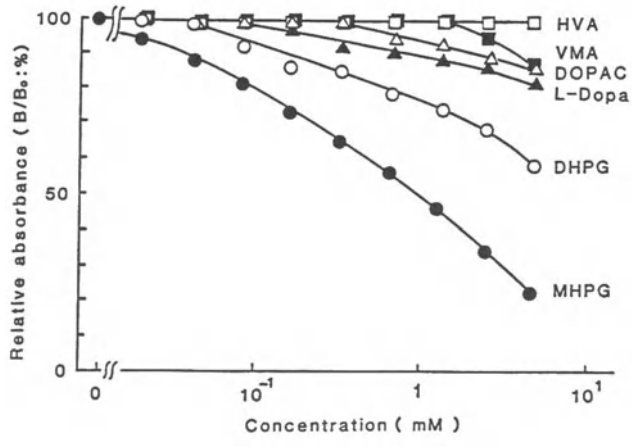

Figure 2. Dose response curves of various haptens by EIA

DISCUSSION

As expected, the antigen was easily prepared and the specific antibody productions were found in all the mice immunized. The probability of the production of the specific antibody to MHPG is higher than those to HVA or VMA. The cross-reactivities to the analogs in our method are less than those described by Keeton et al. in 1981, although the sensitivity of their radioimmunoassay is much higher than those of

our methods. The probabilities of the production of the antibodies to CA are very low, because CA are physiologically active and quickly metabolized as described in our previous paper(Yoshioka et al., 1986a,b). The sensitivity of EIA based on colorimetry corresponds to the urinary level of MHPG, but the one based on fluorimetry(Data not shown) will be to the level of blood and brain. Thus, this method will be useful for the measurements of MHPG in biological materials. The preparation of a monoclonal antibody with the immunized mice is under investigation.

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STUDY OF CENTRAL PUTATIVE NEUROTRANSMITTERS IN RODENT MODELS OF PARKINSONISM

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INTRODUCTION

Considerable insight has been gained into central neurotransmitter mechanisms operating in the extrapyramidal system. Involvement of dopaminergic and cholinergic mechanisms in parkinsonism is now well established. Besides dopaminergic and cholinergic systems, existence of other neurotransmitters has also been reported in the nigrostrial area. Okada (1976) found a high concentration of GABA in substantia nigra (SN) and pallidum. Dray and Straughan (1976) reported the highest concentration of GABA at junction of zona compacta and zona reticulata of SN. The nerve terminals of 5-hydroxytryptamine (5-HT) are present in zona reticulata and in the striatum (Dray and Straughan, 1976). Schwartz et al.(1980) reported the presence of histamine (HA) and its synthesizing enzyme l-histidine decarboxylase in the striatum. However, role of these central putative neurotransmitters - GABA, 5-HT and HA in Parkinson is not clear. Therefore, in the present study the effects of administration of GABA, 5-HT and HA by intracerebroventricular route, have been investigated on experimental models of Parkinsonism in rats.

MATERIAL AND METHOD

The study was conducted on albino rats (wt. 125-150 gm) of either sex. The food and water were allowed ad libitum. The classical signs of Parkinson's disease - tremor, rigidity, hypokinesia and catatonia were produced in rats as follows:

1. Tremor: Oxotremorine (OT) was administered intraperitoneally (ip) to produce tremors. The tremors were scored 5 min after OT according to Coward et al. (1977).

2. Rigidity: Rigidity was induced by reserpine and was assessed 1 hr after of reserpine administration by hind limb pressure method of Goldstein et al.(1975).

3. Hypokinesia: The locomotor activity was counted prior and 2 hr after reserpine administration in a photoactometer for 10 min according to the method described by Dews (1953).

4. Catatonia: Catatonia was scored 4 hr after reserpine injection according to the method of Morpurgo (1962).

Low dose (LD) and maximal dose (MD) of oxotremorine (LD - 0.15 mg/kg ip; MD - 0.5 mg/kg ip) and reserpine (LD - 1 mg/kg ip; MD - 5 mg/kg ip) were used to produce above mentioned responses. GABA, 5-HT and HA were given intra-

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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TABLE 1. Effects of icv GABA, 5-HT and histamine on oxotremorine induced tremors.

Drugs	Dose icv µg	Median tremor score Oxotremorine (mg/kg, i.p.) 0.15	0.5
N. Saline	5 µl	1	3
GABA	50	1	3
	100	1	3
	200	1	3
5-HT	50	1	3
	100	1	3
	200	1	3
Histamine	100	2*	3
	200	2**	3

P * < 0.05; ** < 0.001 significant difference from normal saline control.

TABLE 2. Effects of icv GABA, 5-HT and Histamine on rigidity, hypokinesia and catatonia induced by low dose of reserpine.

Drugs	Dose icv, µg	Reserpine (1 mg/kg, i.p.) Rigidity %	Hypokinesia %	Catatonia Median score
N. Saline	5 µl	30	42.5	1
GABA	50	0	87.2*	0*
	100	10	90.0*	0*
	200	30	94.8*	1
5-HT	50	20	58.4	0*
	100	0	68.9	0*
	200	0	88.8*	0**
Histamine	100	50	53.2	1
	200	70*	50.6	1

P * < 0.05, ** < 0.001 significant difference from saline control.

TABLE 3. Effects of icv GABA, 5-HT and Histamine on rigidity, hypokinesia and catatonia induced by maximal dose of reserpine.

Drugs	Dose icv, µg	Reserpine (5 mg/kg, i.p.) Rigidity %	Hypokinesia %	Catatonia Median score
N. Saline	5 µl	100	88.7	3
GABA	50	20**	95.2	1*
	100	30*	100	1*
	200	90	100	3
5-HT	50	60	96.1	2
	100	20**	94.3	1*
	200	10**	89.8	1*
Histamine	100	100	93.2	3
	200	100	96.0	3

P * < 0.05, ** < 0.001 significant difference from saline control.

- Rigidity has been expressed as %age of rats showing rigidity in a group.
- Hypokinesia has been expressed as %age decrease in locomotor activity counts from pretreatment value after reserpine injection.
- Number of rats in each group was 10.
- Significance of difference between score value was determined by Mann Whitney 'U' test and between % values was determined by Chi square test.

cerebroventricularly (icv) by a polythene cannula implanted according to parameters used by Noble et al. (1962). Location of cannula was confirmed by injecting 0.03 ml of India ink. The volume in icv administration was 5 µl. N. Saline was used as vehicle control. The neurotransmitters were administered 30 min prior to oxotremorine and 15 min after reserpine.

RESULTS

1. Tremors (Table 1): GABA (50-200 µg, icv) and 5-HT (50-200 µg, icv) did not affect the tremors while HA (100 and 200 µg, icv) potentiated the tremors induce by low dose of OT.

2. Rigidity (Table 2 & 3): The rigidity produced by maximal dose of reserpine was significantly inhibited by GABA (50 and 100 µg, icv) as well as by 5-HT (50-200 ug, icv). However, higher dose (200 µg, icv) of GABA did not decrease the rigidity. HA (200 µg, icv) enhanced the rigidity induced by low dose of reserpine.

3. Hypokinesia (Table 2 & 3): Reserpine (low dose)-induced hypokinesia was potentiated by all the doses of GABA and 5-HT. HA did not alter the hypokinesia.

4. Catatonia (Table 2 & 3): GABA (50 and 100 µg, icv) and 5-HT (50-200 µg, significantly antagonized reserpine induced catatonia. GABA in higher dose (200 µg, icv) did not affect the catatonia. HA had no effect on the catatonia.

GABA, 5-HT or HA given in a dose of 200 µg by ip route failed to affect tremor, rigidity, hypokinesia and catatonia.

DISCUSSION

In the present study the effects of central putative neurotransmitters - GABA, 5-HT and HA have been investigated on experimental models of Parkinsonism in rats. Since MPTP does not induce parkinsonian like neurological signs in rats (Langston, 1985), oxotremorine induced tremor (cholinergic hyperfunction model) and reserpine induced rigidity hypokinesia and catatonia (dopaminergic hypofunction models) were employed in this study. These are isomorphic models of parkinsonism. Isomorphic models are those which mimic the signs and symptoms of human disease but differ in etiology (Fisher and Hanin, 1986). These models have been successfully employed in the experimental study of Parkinson's disease as well as for screening anti-parkinsonian drugs (Marsden et al., 1975).

In this study GABA (icv) has been found to suppress rigidity and catatonia. However, this inhibitory effect of GABA was absent with the higher dose (200 µg). Moreover, all the doses of GABA potentiated hypokinesia and did not affect tremor. The lack of uniformity in the pattern of effects of GABA on these responses may be due to the complex neuronal interconnections of GABA in the nigrostriatal area. The nigral cells are inhibited by a descending striatonigral GABAergic tract and this inhibition is blocked by picrotoxin (Pretcht and Yoshida, 1971). The presence of GABAergic interneurons between caudate putamen, globus pallidus and substantia nigra has also been suggested by Okada (1976). GABA inhibits the release of dopamine (DA) as well as of acetylcholine (Ach) (Starke, 1981). Since in the genesis of tremor, rigidity, hypokinesia and catatonia different neuronal pathways are involved and DA and Ach have antagonistic action on these responses (Marsdenet al., 1975), GABA may affect these responses differently because of its complex neuronal connections and influence on the release of both DA and Ach.

In the present study icv 5-HT did not affect tremors. Dogget and O'Farrell (1976) have also obtained similar results in mice. Oelszner et al. (1975) did not find any significant effect of oxotremorine on the level of 5-HT or 5-HIAA in the rat brain. Thus it appears that central 5-HT system has no significant involvement in cholinergic tremors. On reserpine models 5-HT showed inhibitory effect on

rigidity and catatonia. 5-Hydroxytryptophan (5-HTP) precursor of 5-HT, has also been found to antagonize reserpine-induced rigidity (Roos and Steg, 1964; Hornykiewicz, 1966). Orzeck and Barbeau (1970) reported that 5-HTP reduced the content of striatal Ach. Thus anti-rigidity and anti-catatonic effect of 5-HT may be attributed to decrease in the striatal cholinergic activity. However, 5-HT potentiated reserpine-induced hypokinesia. 5-HT is inhibitory to DA neurons in zona compacta of substantia nigra (Dray and Straughan, 1976). The inhibition of nigral DA activity may be a contributory factor for facilitation of hypokinesia by 5-HT.

HA has been proposed as a possible neurotransmitter in the brain (Schwartz et al., 1980) and also implicated in regulation of some brain functions such as behaviour (Nath et al., 1988). In this study HA had a significant potentiating effect on tremor and rigidity. Stern and Igic (1969) have also found enhancement of oxotremorine induced tremors by l-histidine, precursor of HA. It would be pertinent to mention here that use of antihistaminics (H_1 blockers) in parkinsonism is generally attributed to their anticholinergic effects. However, observations of this study indicate that anti-parkinsonian effect of antihistaminics may also involve blockade of HA receptors. HA produces hypokinesia in doses only up to 50 µg, icv, and catatonia in dose of 500 µg icv (Nowak et al., 1977). We have not found any significant effect of HA on reserpine induced hypokinesia and catatonia. This may be due to the use of non-hypokinetic and non-cataleptic doses (100 and 200 ug icv) of HA in this study.

The effects of icv administered GABA, 5-HT and HA discussed above were of central origin since the intraperitoneal administration of these neurotransmitters failed to affect tremor, rigidity, hypokinesia or catatonia.

It may be concluded from this study that central GABA, 5-HT and HA systems play significant modulatory role in parkinsonism. GABA and 5-HT inhibit rigidity and catatonia, and facilitate hypokinesia whereas HA potentiates tremor and rigidity. However, for elucidating the precise mechanisms involved in their functioning, further biochemical studies particularly on the release of neurotransmitters in nigrostrial area are needed.

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POSSIBLE FACTORS RESPONSIBLE FOR ADVERSE EFFECTS OF LONG-TERM L-DOPA THERAPY: SIMULTANEOUS DETERMINATIONS OF DOPAMINE PARAMETERS IN RATS

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INTRODUCTION

After initial benefit, many adverse effects, i.e., "wearing-off", "on-off", or dyskinesia develop following long-term l-dopa therapy. Especially, causes of "wearing-off", which is the most common adverse effect, are proposed to be 1) alterations of l-dopa absorption from the gut, 2) a reduction in l-dopa transport across the blood-brain barrier, 3) changes in dopamine (DA) distribution and metabolism in the CNS, and 4) modification of striatal DA receptor activity.[1]

In order to elucidate the most probable factors for this "wearing-off" phenomenon, we investigated the time course of several parameters for DA metabolism and receptors in rat striatum and serum after single and repeated l-dopa administration.

MATERIALS AND METHODS

Animals and drug treatment

Male Wistar rats (average initial body weight: 280g) were divided into two groups, one given a single administration of l-dopa and the other, repeated l-dopa administration. Each rat received orally either a single dose of 25mg l-dopa (containing 6.25mg benserazide) as a fine suspension in 1 ml of water or the same dose of l-dopa once a day for 28 days. They were sacrificed by cervical dislocation and decapitation 1.5h, 3h, 6h, 12h, 1d, 3d, and 7d after the last l-dopa administration. After decapitation, brains were rapidly removed and paired striata were dissected out and kept frozen at -80 C until analyzed.

Assay methods

Concentration of DOPA and its metabolites in striatum and serum were measured by HPLC-ED (Neurochem, ESA). Tyrosine hydroxylase (T-OH) activities were measured by the radio-chemical method of Hendry and Iversen.[2] D1 and D2 receptor binding were determined with SCH23390 and spiperone, respectively, as ligands.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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RESULTS

DOPA and its metabolites except 3-O-methyl DOPA (3OM-DOPA) in striatum

DOPA. No difference was observed in the peak concentration and the time course between both groups.

DA, DOPAC, HVA. The half-life for DA, DOPAC, and HVA in the striatum of repeatedly administered rats was obviously shorter than that by single administration. However no apparent difference was observed in the peak concentration of either group.

T-OH activity

No detectable change was observed in either group when their values were compared with the control ones throughout the period examined.

Receptor binding assay

Single administration produced an initial increase and a long-lasting increase in the number of D2 binding sites in the striatum. This tendency was even more obvious in the case of D1 binding. On the contrary, repeated administration did not produce any change in the number of either D1 and D2 binding sites. Neither group showed any significant change in the affinity of D1 and D2 receptors throughout the period examined.

3OM-DOPA in the striatum and serum

Compared with other DA metabolites, 3OM-DOPA had a much longer half-life. Seven days were needed for the value to return to the control one both in the serum and in the striatum. The peak concentration was lower and the half-life was shorter in the group of repeated administration than in the single administration group.

DISCUSSION

DOPA uptake into the striatum

Since striatal DOPA levels after repeated l-dopa administration were similar in rats given a single dose, it is unlikely that repeated doses of l-dopa diminish the absorption of l-dopa from the gut and the transport of l-dopa across the blood-brain barrier.

DA synthesis

DA synthesis in the striatum was not influenced by the repeated administration of l-dopa, since the peak concentration of striatal DA after repeated doses was not different from that of single dose. Moreover, the activity of T-OH, one of the enzymes involved in DA synthesis, was not influenced by the l-dopa administration.

DA metabolism

The results presented here indicated DA metabolism in the striatum was accelerated by repeated l-dopa administration. It is suggested, therefore, that the acceleration of DA metabolism may be one of the responsible factors for "wearing-off" after long-term l-dopa therapy.

DA receptor binding

It is noteworthy that the DA receptor binding sites remained at an increased number for a considerably long time after the single dose of l-dopa, in spite of the relatively early return of the normal striatal DA level. This may be one of the causes of the long-lasting beneficial effect at the initial stage of l-dopa therapy in Parkinson's disease. In contrast, neither increase nor decrease of DA binding sites was observed after repeated administration. The loss of supersensitive response may be one of the responsible factors for "wearing-off".

3OM-DOPA

Repeated administration inhibited conversion of DA into 3OM-DOPA. Therefore, it is unlikely that 3OM-DOPA accumulation accounts for adverse effect of long-term l-dopa therapy.

Causes of "wearing-off" phenomenon

Fabbrini and co-workers[3] reported that the "wearing-off" phenomenon is a result of progressive DA neuron degeneration manifested by a reduction in the brain's capacity to synthesize, re-uptake, and store DA synthesized from exogenous l-dopa. Regarding this point, we stated in a previous section that repeated l-dopa administration produces acceleration of DA metabolism, i.e., reduction of DA storage even in the intact rat.

The induction of DA-degrading enzymes such as monoamine oxydase-B (MAO-B) and/or catechol-O-methyltransferase (COMT) is, therefore, suggested as a possible mechanism for the acceleration of DA metabolism. In this respect, brain MAO activity has been shown not to be altered with long-term l-dopa administration.[4] Therefore we mention here a possibility of the induction of COMT activity in brain by long-term l-dopa therapy.

In view of the above, we suggest two factors responsible for the diminished efficacy of l-dopa or "wear-off": 1) acceleration of DA metabolism in the striatum, and 2) loss of supersensitive response of the DA receptor.

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GENERATING AND CONTROLLING MULTIPARAMETER DATA BASES FOR BIOCHEMICAL CORRELATES OF DISORDERS

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INTRODUCTION

Perhaps the most powerful potential use of multiparameter biochemical data bases in studies of degenerative disorders is the unique discrimination of categories. The technology of Coulometric Array Electrode Systems (CEAS) can automatically resolve approximately 400 compounds from biological samples at the picogram level. Among these are tyrosine and tryptophan derived neurotransmitters, precursors, metabolites, conjugates and cofactors, certain purines, pterins and neuropeptides. This capability offers the promise of generating enough relevant data to describe disorders.

The quantity of data obtainable and required for correlative data bases, however, requires very stringent control of the analytical process across long time intervals, as well as automatic data analysis and management. If such data bases are to be used for their eventual intended purpose as a classification or diagnostic aid, in addition to their within study utility for mechanistic inference, they must be tightly controlled. Thus the design of analytical protocols must consider not only such traditional measures as within and among run precision over a 1-2 week study, but also factors which affect the entire pattern of approximately 400 compounds over a year time frame. For example, data bases generated for a cerebrospinal fluid (CSF) composition study in severe facial pain[1] and brain tissue in degenerative disorders in Huntington's disease[2] and Parkinson's and Alzheimer's disease[3] include several hundred samples for 30-40 known compounds and 200-300 unknowns. That is, on the order of 10-30,000 analytical data points, as well as subject data acquired over 1 year.

This paper outlines the procedures being used to generate the data bases cited and considers major quality assurance issues encountered in sample acquisition, storage, preparation, analysis, data reduction and qualification.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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MATERIALS AND METHODS

The primary instruments used for data base generation are 16 channel CEAS instruments equipped with refrigerated autosamplers and gradient liquid chromatography (Neurochemical Analyzer, CEAS 55-0650, ESA, Inc., Bedford, MA). Iron assays are performed on 10-25 µl aliquots of brain tissue extract and CSF to control for blood inclusion using a Ferrochem II (ESA, Inc.). Mobile phases are prepared from Soxhlet extracted (chloroform, propanol, methanol) salts using MilliQ RO water subsequently double distilled. Ion pairing and trace salts are recrystalized from methanol/water. HPLC grade reagents are used for organic modifiers. Batches are then filtered (0.1 u glass fiber). Authentic standards are obtained from various sources. In studies to assess precisison, accuracy, and ease of data management, we have primarily worked with methods for ascorbic acid (ASC); cysteine (CYS); glutathione (GSH); uric acid (URIC); xanthine (XAN); methionine MET); tyrosine (TYR); guanosine (GR); 4-hydroxyphenyllactic acid (4HPLA); 4-hydroxybenzoic acid (4HBAC); 5-hydroxyindoleacetic acid (5HIAA); 4-hydroxyphenylacetic acid (4HPAC); homovanillic acid (HVA); tryptophan (TRP); norephinephrine (NE); vanlilymandelic acid (VMA); 3,4-dihydroxyphenylalanine (LDOPA); epinephrine (E); 3-hydroxykynurenine (3OHKY); homogentisic acid (HGA); 3-methoxy,4-hydroxyphenylglycol (MHPG); homovanillyl alcohol (HVOL); normetanephrine (NMN); 3-hydroxyanthranilic acid (3OHAN); dopamine (DA); 3-methoxytyrosine (3MT); kynurenine (KYN); 5-hydroxytryptophan (5HTP); 5-hydroxytryptophol (5HTOL); 5-hydroxytryptamine (5HT); tryptophol (TPOL); 3,4-dihydroxyphenyl acetic acid (DOPAC); 3OMDOPA (3OMD); and kynurenic acid (KYA).

The time lines, instrumental parameters, and mobile phases for the two methods used in these studies are previously reported.[1,2] In summary, method 1 used for both brain and CSF is a 1 hr gradient profile from 3 to 50% methanol in pH 3.2 phosphate buffer with cation pairing. Method 2 used for brain tissue extracts is a 60 min gradient profile from 0-50% methanol in pH 6.2 phosphate buffer.

CEAS software automatically reduces peak data to digital format, clusters the peaks across detector channels, and determines their ratios of response. Comparison and acceptance of sample data is made automatically by a hierarchy decision based on $\pm$2% retention time and $\pm$20% ratio accuracy.

Essential preliminary studies before beginning data base acquisition are to run repetitive sequences of standard and sample pool with slight variations in the time line of the method - typically changing the injection point $\pm$1 minute in 0.2 min intervals and varying the time that the samples are held at 0 °C. From the studies, a control data base is generated with information on factors affecting sample stability, gradient profile, appropriate concentrations of standards, and appropriate reference peaks. The variation of injection time mimics changes in patterns caused by delay of the gradient or improper delivery of the B mobile phase. The initial standard patterns begin an average standard file for acceptance of mobile phases and column changes. Pattern changes over time at 0 °C define the acceptable time intervals for an automatic run.

Assuming that mobile phase composition is controlled by exact comparison to test patterns, factors affecting the overall stability of a chromatographic pattern can conceptually be separated into fluidic delivery, separations, sensor response, and software preformance.

Fluidic performance is affected primarily by variation in pulse damper volume and B mobile phase delivery. Maintenance and refilling of pulse

dampers is required when the patterns indicate late onset of the gradient. Pressure drift greater than 15 bar delays gradients and requires changes of filters. B mobile phase delivery is evaluated by pressure changes at change over points in the gradient. Greater than 1% drop in pressure from 100% A to 94% A / 6% B indicates a need to change piston seals.

Column factors are controlled by selecting an adequate number for a study from the same lot (assuming 800 assays per column) and by precleaning the columns with 0.1 mM EDTA 1% acetic acid in methanol to eliminate metals which cause degradation of HGA, ASC and 5HIAA.

Sensor response is controlled by using a clean cell function in the timeline and by matching the control standard composition to the sample pool. Peak ratios are typically constant $\pm$40 x the mean value.

Software errors can occur in regions of high complexity which are identified in initial studies. The software flags questionable matches which can then be manually edited. The frequency of manual editing is minimized by slight variations in time lines.

Sample degradation events are controlled by noting peaks that are indicative of oxidation, oversonification, etc. We have used low ASC, HGA, 5HIAA and 5HT as indicators as well as two unknown breakdown products of 5HIAA and MHPG. In brain tissue, decrease in XAN accompanied by increase in guanine indicates warming on sonification.

In these studies we have controlled within instrument among day precision using pool samples as a measure of data validity. The analytical sequence used is standard (3 samples) pool (3 samples) standard. Pools are assayed as samples.

Evaluation of data for rejection is made if its actual vs. calculated value obtained from the regression equation calculated for the compound with all other compounds is outside 3 sigma of all actual values.

RESULTS

The results for control pool precision in the data base for 260 CSF samples [1] and a portion of the putamen brain tissue data base [2] are summarized below.

In the CSF study, manual editing was required on approximately 15% of the values. In the brain tissue study, manual editing was required on approximately 20% of the values and 20% of the coassayed compounds required resolution of differences. Of these, 4 of 230 values were rejected. Forty values that were more than 3 sigma from the mean were tested with regression procedures and none were rejected.

CONCLUSIONS

The analytical validity of large data bases can be controlled using CEAS capabilities to establish and feed back quality control intervals. Pool sample precision provides a more conservative and accurate estimate of data base validity than standard precision which is typically 10-40% lower. Approximately 20% of the time, the complexity and variability of samples causes situations requiring operator intervention and judgement. Data in a large matrix cannot be rejected by single variable criteria.

Table 1

PRECISION OF CONTROL POOLS

	CEREBRAL CSF n = 20 NG/ML		BRAIN TISSUE PCA EXTRACT n = 20 NG/MG PROTEIN	
	Mean	CV%	Mean	CV%
TYR	1537.33	4.1	915.24	3.0
XAN	729.70	3.2	594.58	3.4
KYN	4522.00	7.6	5.89	8.6
GR	21.05	6.6	289.62	4.3
KYA			0.74	12.7
5HIAA	44.41	5.7	13.59	6.8
5HT	0.42	11.2	5.13	6.1
CYS			1028.21	14.1
DA	0.31	18.7	76.18	3.5
DOPAC	1.43	8.3	3.15	4.5
5THOL	0.77	8.9	0.29	26.1
5HTP	1.04	10.2	0.45	
HVA	145.34	6.1	120.46	7.8
TRP	509.07	5.4	231.96	4.7
URIC	4026.00	3.1	81.37	3.2
ASC	653.91	29.1	611.55	18.2
NE	0.31	15.3	0.99	8.6
MET	2479.37	8.1	569.51	10.8
VMA	1.47	20.2	0.11	28.3
LDOPA	0.46	7.2	5.42	7.7
E			0.08	
3OHKY	0.83	10.5	0.74	4.7
HGA	0.17	21.4	0.06	20.6
MHPG	8.61	4.7	0.12	
NMN	1.06	16.1	0.02	
3OHAN	0.08	11.9	0.10	20.4
3OMD	2.81	8.1	23.84	9.4
4HPLA	187.69	4.7	12.32	5.3
3MT			24.28	4.6
HVOL			0.08	
4HBAC	145.35	10.3	0.08	
4HPAC	21.13	4.2	1.91	18.3
TPOL	11.61	9.4	0.90	
GSH	99.24	12.1	2793.24	10.8

1. Note cerebral CSF 5HT is approximately 20 fold higher than lumbar CSF.

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NEUBA®: A MULTICOLUMN, MULTIDETECTOR LIQUID CHROMATOGRAPH WITH ELECTROCHEMICAL DETECTION FOR USE IN THE IDENTIFICATION AND DETERMINATION OF NEUROCHEMICALS AND RELATED SPECIES

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INTRODUCTION

The widespread utilization of liquid chromatography with electrochemical detection to the determination of neurochemicals began to prominently appear in the literature following the initial utilization of a thin-layer electrochemical cell (Refshauge *et al.*, 1974). The initial utilization of rather large diameter particles as the packing material for the liquid chromatograph led to what is now considered poor resolution. At best, the original systems accomplished the separation of only 3-4 compounds in 15-20 minutes. Rapid improvements in the area of liquid chromatography led to the development of columns having packings with particles of only 3 μ in diameter. These systems have provided the separation of up to 18 compounds in $5^1/_2$-$7^1/_2$ minutes (Lin *et al.*, 1984). This represents a substantial advance in the capabilities of neuroscientists to examine the neurochemical mode of action of a variety of behavioral, psychological, and pharmacological events. However, one would certainly like to obtain even further amounts of information concerning such events at the neurochemical level. A hint at a possible route to obtain such further information was provided in a paper we published in 1976 (Blank, 1976). In this paper, we described a dual amperometric electrochemical detector which could be used in a serial arrangement. This detector, employing different potentials at the two electrodes, allowed for some expanded qualitative information concerning eluting components from a liquid chromatograph. This idea has been expanded upon somewhat and, more recently, many investigators have now used multiple electrochemical detectors in both parallel and series configurations to enhance the qualitative information derived from their chromatograms (Roston and Kissinger, 1982). Similar investigations employing multiple coulometric detectors have been reported by Matson and co-workers (Matson *et al.*, 1984; Matson *et al.*, 1987). Employing an initial screen electrode, the coulometric approach relies upon a series arrangement of flow-through carbon detectors which are placed at a variety of potentials. These potentials can be selectively oxidizing followed by oxidation and reduction aimed for particular chemical species, or can be arranged in a straightforward mathematical array to simply provide an on-line hydrodynamic voltammogram for the eluting species. The resultant output from such a multielectrode system is necessarily three-dimensional in character, since it contains information concerning the time, the current, and the applied potential. As expected, the qualitative information contained in such an approach is considerably enhanced over that one can receive from a simple single electrode, single column LCEC setup.

The amperometric approach to multiple electrode detection offers a number of advantages over that provided by coulometric detection. Only a small fraction, typically 2-5%, of the eluting compound is oxidized at a single electrode of conventional size. Thus, the vast majority of the compound is present in its original, electrochemically active identical form at

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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subsequent electrodes located downstream from the first. The electrochemical reversibility upon which selective coulometric detection normally depends is not required for the amperometric detector. The ultimate detection limit which is attainable by an electrochemical detector is intimately associated with the background level or noise current it generates. In fact, analytical chemists frequently describe the detection limit of a device as being that amount of compound which provides a signal which is two or three times this background noise. For electrochemical detectors, both amperometric and coulometric, the background current is almost directly proportional to the surface area. Since coulometric electrodes currently contain surface areas which are typically 100 times larger than their amperometric relatives, they inevitably experience larger background currents. In addition, there exists a lower limit in terms of electrode area which can be achieved by a coulometric detector and yet maintain 100% oxidation of the compound. No such lower limit exists for amperometric detectors. Thus, inevitable size reductions for amperometric detectors will ultimately lead to lower detection limits for these devices. Finally, the small pore size associated with the commonly employed flow-through coulometric detectors used today have been known to experience problems with trapped particles. The resultant electrode clogging has not been observed with amperometric detectors, where the flow through the detector cell is relatively unimpeded.

Of course, one can only achieve a fixed amount of resolution with a single liquid chromatography column in a given unit of time. This parameter, identified as peak capacity by chromatographers, simply says that a given liquid chromatograph only has the capability to resolve a fixed amount of compounds in a fixed amount of time. Since we were concerned that the enhancement in qualitative data provided by multiple electrochemical detectors would not be able to provide sufficient sample throughput of and by itself, we also decided to increase the number of individual liquid chromatography systems within our setup. Our current feeling is that four columns are optimal in this regard. The first three columns are associated with the determination of catecholamines, indoleamines, related compounds, and virtually all directly accessible electrochemically active neurochemically related species. The fourth column has been reserved for the indirect analysis of acetylcholine, choline, and a related internal standard (ethylhomocholine or acetylthiocholine). This fourth column contains a postcolumn enzymatic reactor which converts the species of interest into the electrochemically active hydrogen peroxide.

MATERIALS AND METHODS

The Neurobiological Analyzer (NEUBA®) is primarily composed of four separate, parallel, liquid chromatographic systems. Each system contains its own pump, pressure release valve, bypass line, precolumn, column, and electrochemical detector(s). In addition, each LC system has one flow path in parallel with the bypass line which passes through a dual tandem six-port rotary valve (Rheodyne, Model 7066), the center ports of which are connected to an LC2000 Autoinjector (Dynatech, Lafayette, LA). The tubing connections for the pieces of tubing in the flow path for each system located after the injection port are all constructed of the smallest inside diameter available (0.004-0.005"). This allows maintenance of the dead volume for individual systems in the 10 µL range. The bypass line serves two chromatographic functions. First, it allows flow to proceed in an individual system even though that system is not selected by the six-port rotary tandem valve. Second, it prevents the pressure fluxuations associated with the injection process, thus preserving the maximum possible resolution afforded by the liquid chromatography column. The LC columns are BAS PHASE II®, reverse phase, ODS, 100 x 3 mm, 3 µm. Mobile phases for each of the four liquid chromatography systems are currently isocratic citrate buffers with varying degrees of sodium octadectylsulfate and acetonitrile added to enhance the separation. The only exception to this is the fourth liquid chromatography system, optimized for the separation of acetylcholine and choline, which employs a tris-hydroxymethylaminomethane buffer. Each of the first three liquid chromatography systems has an electrochemical cell containing four individual electrodes. The potential of these electrodes, with respect to a Ag/AgCl reference electrode is shown in the appropriate figures.

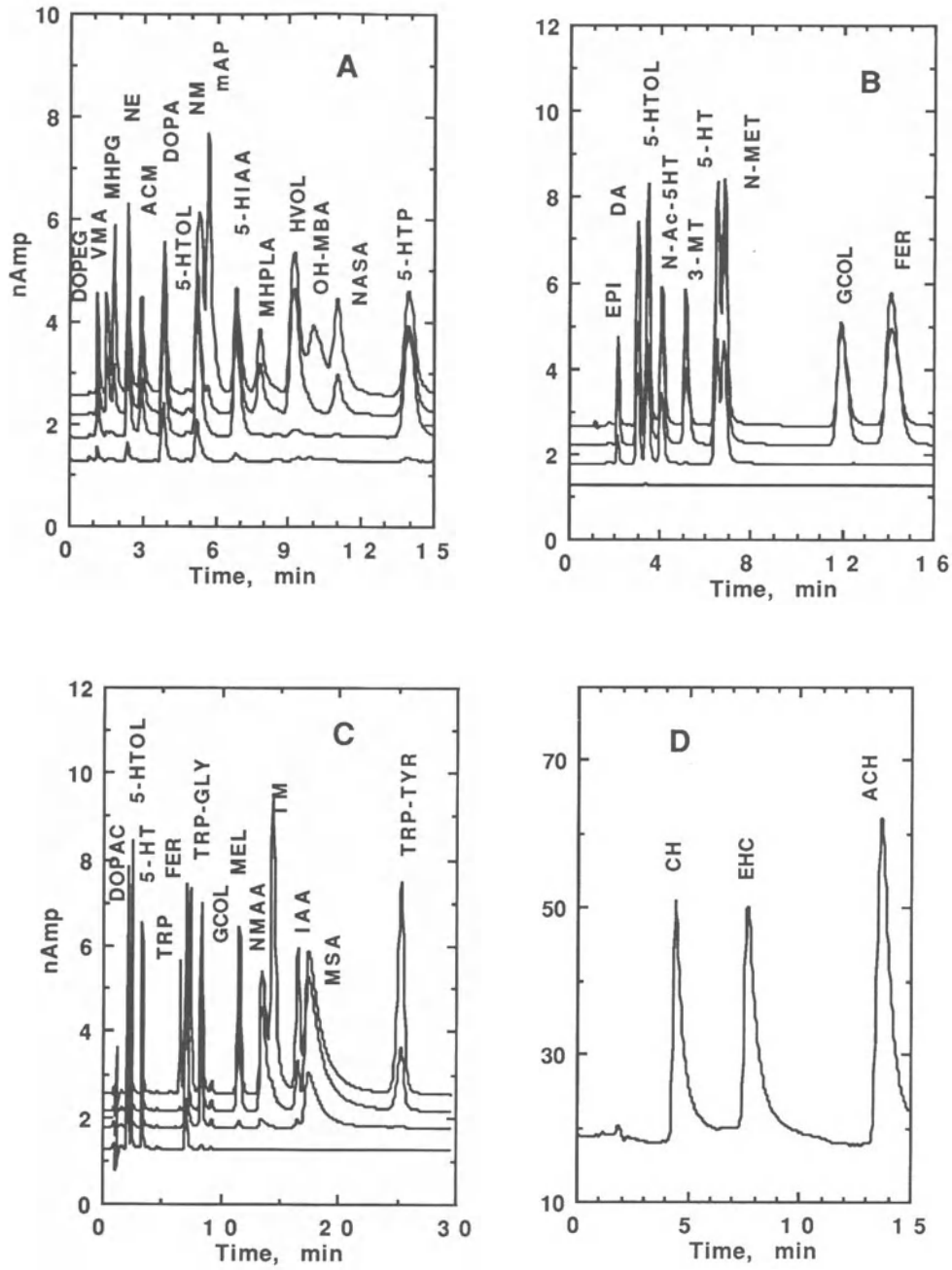

Fig. 1. Typical Chromatograms Obtained Using Standard Mixtures on the Four LC Systems of the Neurobiological Analyzer. The electrode potentials employed were (Volts vs. Ag/AgCl): System 1 **(A)**: 0.50, 0.60, 0.70, and 0.80; System 2 **(B)**: 0.50, 0.60, 0.80, and 0.90; System 3 **(C)**: 0.70, 0.80, 0.90, and 1.0; System 4 **(D)**: 0.55.

The injection volume for each liquid chromatograph is maintained, when possible, at the relatively low value of 5 μL. This small injection volume, in combination with the relatively low dead volume associated with the connecting tubing in each system, allows maintenance of maximum chromatographic performance.

The potentials of each of the thirteen electrochemical detector electrodes contained in the Neurobiological Analyzer are simultaneously controlled by a single Amperometric Controller, constructed by Great Plains Laboratories (Norman, OK). Simultaneously, the currents flowing through each of these electrodes is monitored and amplified by the same controller. The amplified current is subsequently directed to a Macintosh® IIx computer for data collection and subsequent data analysis. The Macintosh® computer also serves to control and direct the injection process/sequence through the issuance of commands to the autoinjector via electronic components contained within the controller unit. Data collection stores values for the currents recorded on all thirteen channels at various times for a typical 20-30 minute run per sample. This data is reduced and also stored for subsequent graphical presentation. A data analysis program is available to identify and quantitate the peaks of interest in the chromatograms. This program also calculates averages, standard deviations, standard errors of the mean, and performs t-tests on groups of samples for each individual compound of concern. The final data presentation in this case is tabular in format.

RESULTS AND DISCUSSION

The Neurobiological Analyzer is readily seen to be applicable to a wide variety of neurochemical, pharmacological, behavioral and psychological investigations. We ultimately hope that it will be applied as well to the chemical categorization of affective and other mental health disorders. A typical chromatogram obtained for standard materials is demonstrated for each of the four liquid chromatography systems in Fig. 1. In this figure, it is readily apparent that peaks which might be difficult to quantitate with a single electrochemical detector become quite easy to separate utilizing the information supplied by the four individual potentials for each of the three primary systems. The total number of compounds which have been determined to be accessible by the Neurobiological Analyzer at this time is ~70. Some of these compounds are listed in Table I. We anticipate that the total number of accessible species will be raised to the 150-200 level in the near term future. Detection limits for reasonably favorable species by the system described are on the order of 10-50 femtomoles.

The broad-ranging and manifold applications of this novel device are just beginning to be explored. We expect to report applications of NEUBA® to tissue, urine, blood, and CSF samples in the near future. Applications to mouse brain tissue samples are contained in two separate reports in this volume (See Satoh *et al.* and Ikarashi *et al.* this volume).

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EXAMINATION OF CENTRAL NERVOUS SYSTEM EFFECTS OF AMPHETAMINE USING NEUBA®

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INTRODUCTION

A brief review of the literature indicates that d-(+)-amphetamine is one of the most heavily investigated CNS stimulants (Kuczenski, 1983). The mode of action of this compound is inevitably connected to, at least, catecholaminergic pathways. The well-known behavioral actions of amphetamine, including locomotor stimulation and stereopathy, can be readily blocked by surgical removal or pharmacological blockade of specific catecholaminergic pathways in the CNS. On the other hand, the involvement of alternative neurotransmitter pathways in the expression of amphetamine psychostimulant effects is relatively less well investigated.

The mode of action of amphetamine as a CNS stimulant certainly incorporates a number of biochemical phenomena. It is a dopaminergic agonist. This agonist action is provided by at least two synergystic actions upon dopamine nerve terminals. First, amphetamine blocks the reuptake of dopamine into these terminals. Secondly, amphetamine stimulates the release of dopamine from these same terminals. The combined action of these two phenomena is a substantially enhanced extracellular concentration of dopamine and, thus, a substantially increased accessibility of this transmitter to its receptor sites on post-synaptic and pre-synaptic neurons. As opposed to other commonly investigated CNS stimulants, amphetamine's releasing activity on dopaminergic neurons appears to be quite unique. The release of dopamine by amphetamine is a calcium independent mechanism which has been labelled as an accelerative exchange diffusion employing reversal of the carrier mediated uptake system (Stein, 1967). And, while the increased extracellular dopamine induced by amphetamine treatment appears to be primarily release associated, the same increase in extracellular norepinephrine levels resulting from such treatment appear to be more connected with its uptake blocking capabilities (Heikkila *et al.,* 1975). Being an α-methylphen-ethylamine, amphetamine additionally blocks monoamine oxidase activity, although most feel that this is not a significant contributor to the mode of action of this drug. Amphetamine treatment leads to increased biosynthesis of dopamine as measured by either the increase in ^{3}H-dopamine from ^{3}H-tyrosine in the striatum or by increased accumulation of L-DOPA in the same region following pretreatment with the dopa decarboxylase inhibitor, NSD-1015. Measurement of the primary dopaminergic metabolites (DOPAC, homovanillic acid, and 3-methoxytyramine) also indicate a substantially increased dopaminergic activity.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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The effects of amphetamine on neuronal activity and other measured phenomena appear to be quite different at high dose levels than they are at low dose levels. For example, the striatal neuronal activity is depressed by low doses of amphetamine, while it is substantially enhanced by higher doses of amphetamine. This phenomenon may indicate an involvement of serotonergic neurons (Rebec *et al.*, 1981).

More recent reports indicate an even more complex picture concerning the neurochemical mode of action of amphetamine. For example, acute amphetamine has been noted to cause an increase in dopamine synthesis in the striatum and olfactory tubercle, while it simultaneously leads to a decrease in dopamine synthesis in nucleus accumbens and prefrontal cortex. Low to moderate doses cause a decrease in the DOPAC levels in striatum and nucleus accumbens, while the same doses produce an increase in the DOPA accumulation measured in striatum. Moderate to high doses (1.0 or 5.0 mg/kg) cause an increase in the release of ascorbic acid as measured by in vivo electrochemical techniques (Rebec *et al.*, 1989). There are indications that p-hydroxyamphetamine is, at least, partly responsible for the dopaminergic and behavioral changes seen with amphetamine pretreatment (Chapman *et al.*, 1989). The motor related activation of central and lateral striatal amphetamine activated striatal cells appears dependent upon an intact cortico-striatal projection (Tschanz *et al.*, 1989). There are even indications that there may exist a genetic predisposition to an individual animal's response to amphetamine treatment. Higher locomotor responses in novel environments, for example, as well as greater sensitivity to stress seem to be highly correlated with the ability of an animal to obtain rapid acquisition of amphetamine self-administration (Deminiere *et al.*, 1989). Acute injections of high doses of amphetamine in combination with iprindole can produce a long-lasting dopaminergic neurotoxic effect (Wichlinski *et al.*, 1989).

Having an ongoing interest in the rather diverse effects of the many established central nervous system stimulants, including amphetamine, we have undertaken a systematic investigation of the neurochemical effects of some of these agents. At the current time, this systematic investigation has been greatly facilitated by the appearance of a multicolumn, multielectrode liquid chromatographic setup with electrochemical detection (Neurobiological Analyzer, NEUBA®). This unit allows the rapid and simultaneous determination of many more catecholamine, indoleamine, and acetylcholine related compounds than was previously available by any alternative approach within a reasonable period of time. The present investigation is focussed on simply the application of this unique analytical methodology to the investigation of the whole brain neurochemical effects of amphetamine in the mouse.

MATERIALS AND METHODS

Mice. Male mice of the ARS HA/ICR Sprague-Dawley strain (Madison, WI, U.S.A.) were employed in these experiments. The animals were maintained on a light/dark cycle with lights on at 07:00 a.m. The temperature was maintained at 22 ± 1°C. The animals were only employed after having been resident in these housing conditions for at least 7 days. The mice were all sacrificed by exposure of the head to 7.5 kW of microwave radiation for 0.25 sec. The microwave irradiation was delivered by a model NJE-2603-10kW unit obtained from New Japan Radio (Tokyo, Japan).

Drug Treatment. The twenty four mice used in this experiment were separated into four individual groups. Each group received a treatment with either isotonic saline or diisopropylfluorophosphate (DFP) 24 hrs prior to treatment with either saline or d-(+)-amphetamine. The DFP treatment consisted of a dose of 6.3 mg/kg, i.p. The dose of amphetamine was 5.0 mg/kg, i.p. The animals were sacrificed 20 min following the final treatment with either AMPH or saline. This treatment schedule resulted in the following four groups:

Group	Treatment
A	Saline/Saline
B	DFP/Saline
C	Saline/AMPH
D	DFP/AMPH

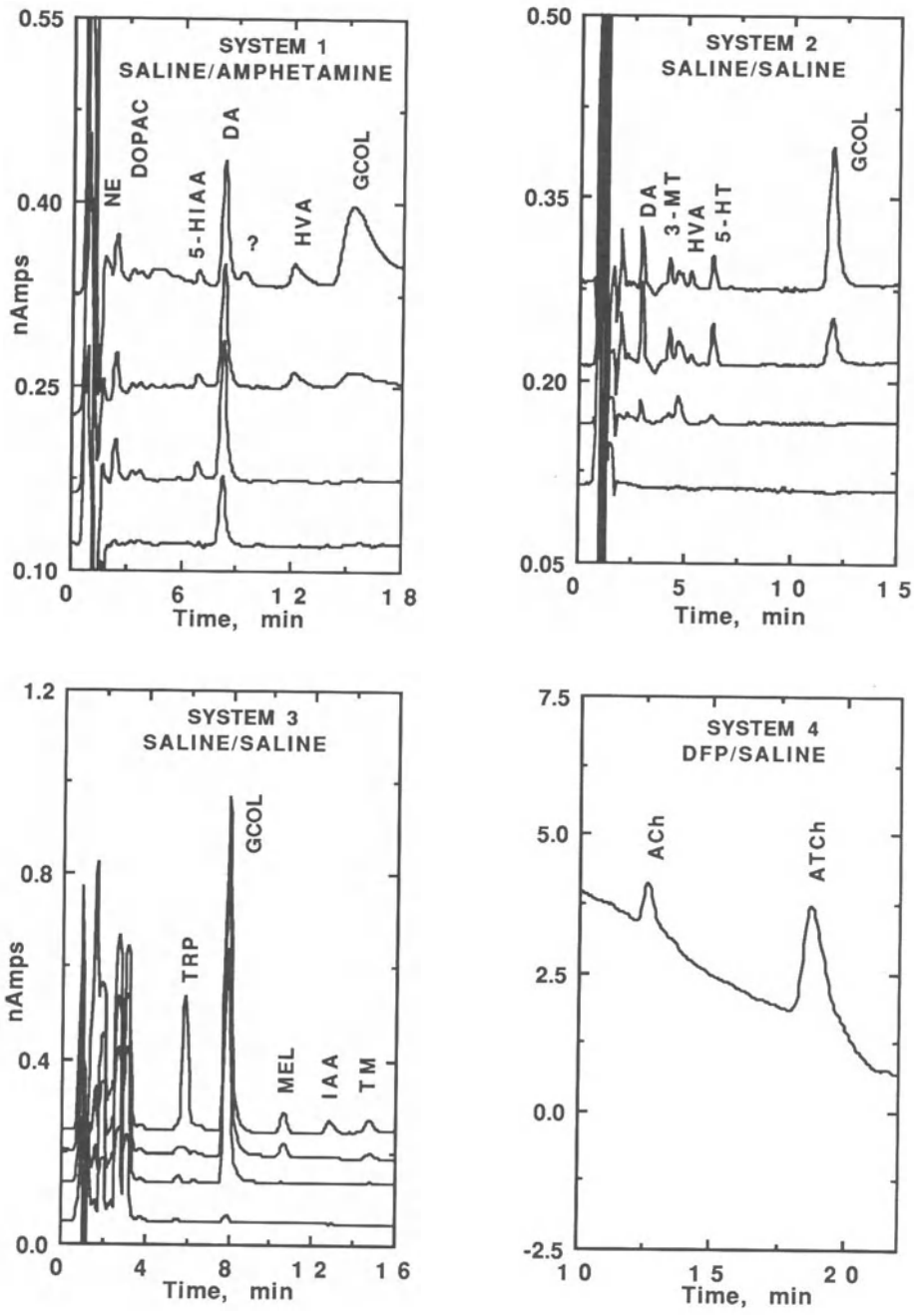

Fig. 1. Typical Chromatograms Observed on the Four LC Systems for Various Drug Treatments.

Tissue Preparation. The meninges and dura/pia matter of each brain was carefully removed. The brains were weighed to the nearest 0.01 mg and stored individually at -80°C. Each brain was homogenized in an acetate/perchlorate buffer system containing 0.1 **M** acetate, pH 4.50, containing 0.4 **M** $NaClO_4$. The homogenization solution also contained internal standard required for each of the four liquid chromatography systems. Each sample was centrifuged at 50,000 x g and 4°C for one hour to remove cellular debris. The super-

natant was further clarified by filtration/centrifugation through 0.45 μ filters. Five μL of the filtrate was then injected into each of the four LC systems in the Neurobiological Analyzer.

Neurobiological Analyzer (NEUBA®). The identification and quantitation of individual chemical species contained in these brain samples was accomplished using NEUBA®. This system contains four parallel liquid chromatographs with multiple electrochemical detectors. Currently capable of quantitating ~70 electrochemically active species, this system offers substantial improvement in the selectivity associated with the derived data in comparison to the usual single LC with a single electrochemical detector. Quantitation of individual peaks was accomplished by the NEUBA® through employment of external standards and consideration of the tissue weights of each sample. All final results are expressed as the mean ± S.E.M. Statistical analyses among groups were performed utilizing Student's t-test.

RESULTS AND DISCUSSION

The primary purpose for utilizing the Neurobiological Analyzer in this investigation was simply to demonstrate that it was applicable to the general, routine analysis of brain tissue samples. This was amply demonstrated. Typical chromatograms contained on each of the four systems for animals from various sample treatment groups are presented in Fig. 1.

As might be anticipated, no radical elevations or decreases occurred in the whole brain levels of any of the neurochemicals investigated. This is consistent with previously reported results. We anticipate that the level differences to be observed will be much more acutely apparent in the brain regional analyses which will follow this study. There was, however, a general increase in DA and a general decrease in the DA metabolite, DOPAC, observed in these whole brain studies following treatment with amphetamine. Likewise, a decrease was noted in the 5-HT level following amphetamine treatment.

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APPLICATION OF MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY (NEUBA®) TO THE DETERMINATION OF NEURONAL CHANGES IN AN ORGANISM

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INTRODUCTION

A very large number of neurochemical investigations incorporate sacrificing as a precursor to the measurement of various neurochemicals following psychological, pharmacological, or behavioral paradigms. The vast majority of such investigations to date have employed either decapitation or cervical dislocation as the primary means of sacrifice. However, such approaches to the examination of CNS mechanisms in mammalian systems suffer quite seriously from a rapid post mortem alteration in the levels of the compounds to be determined. Two separate solutions to this problem have been put forward by various research groups. The first involves rapid freezing of the tissue of concern, thereby inactivating the enzymes associated with the post mortem alteration (Veech *et al.*, 1973). In this approach, one rapidly ejects the CNS tissue from the skull cavity such that it impacts on a plate held at liquid nitrogen temperatures. This rapidly lowers the temperature of the tissue, but, unfortunately, does not allow for easy identification of individual brain regions. Alternatively, one could simply freeze the brain by dropping a decapitated head immediately into liquid nitrogen. This second approach, which allows for reasonable dissection, is not accomplished on as rapid a time scale. In short, both approaches to freezing suffer from some serious deficiencies. Additionally, when frozen tissue is subjected to subsequent homogenization, as is done in almost all procedures, the temporarily inactivated enzymes resume their activity and continue to alter the levels of the compounds which one is attempting to measure. Thus, it appears that the second approach to cessation of rapid post mortem metabolism is more appropriate. In this case, one sacrifices the animal and simultaneously inactivates the enzymes of concern by the utilization of microwave irradiation concentrated on the tissue region of concern. Originally introduced in 1970 (Stavinoha *et al.*, 1970), this technique has rapidly gained favor as the one of choice for halting post mortem decay of neurotransmitters. The microwave radiation leads to direct enzymatic inactivation through protein denaturization processes (Stavinoha, 1983). The major difficulty encountered in the utilization of microwave irradiation is the homogeniety of heat distribution (Ikarashi *et al.*, 1984). The recent introduction of a 10 kilowatt instrument which allows tuning of the magnetron prior to irradiation of each subject and precise location of the animal within the irradiation cavity has led to major advancements in this regard. In particular, mouse brain temperature gradients are found to be less than 4°C when the brain temperature is raised from the nominal 37°C to ~80-85°C. This relatively small temperature differential, compared to previous capabilities, would appear to ensure much more reproducible and reliable results to be obtained from this device.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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The multielectrode, multicolumn liquid chromatograph with electrochemical detectors (Neurobiological Analyzer, NEUBA®) which has been recently constructed offered us the capability to investigate the deleterious effects of decapitation on subsequent neurochemical analyses in somewhat more detail than was previously possible. As such, we have undertaken a systematic investigation which will look at multiple metabolites of the catecholamine, indoleamine, and acetylcholine pathways following sacrifice by either microwave irradiation or decapitation. The current report represents the first phase of this investigation, in which we are comparing the neurochemical levels derived from animals sacrificed in one of these two ways in whole brain tissue samples. Subsequent reports will cover the results for individual brain regions.

MATERIALS AND METHODS

Mice. All animals used were males of the ARS HA/ICR Sprague-Dawley strain (Madison, WI, U.S.A.). The animals were allowed access to food and water ad libitum and kept in a room at 22 ± 1°C with lights on a 07:00 a.m. and lights off at 07:00 p.m. No animals were employed in these experiments until they had been allowed at least one week to become accustomed to the housing conditions following their arrival from the supplier. They typically weighied ~25-30 g at the time of sacrifice.

Microwave Instrument. The microwave irradiation was provided by a 10 kilowatt, 2450 MHz unit supplied by New Japan Radio Corporation (Model NJE-2603). During irradiation the mouse was held in a specially designed applicator chamber to minimize movement within the irradiation field. This chamber was then inserted into the wave guide in an appropriate position to maximize the exposure of the head of the animal to the magnetic component (H-field) of the irradiation. This has previously been shown to provide the most desirable heat distribution upon irradiation.

Sacrifice. The animals were typically sacrificed in the middle of the light portion of the light/dark cycle. Sacrifice by microwave irradiation was achieved by employing a power setting of 7.40 kW and an irradiation time of 250 msec. Decapitated animals were sacrificed by guillotine.

Tissue Preparation. Immediately after sacrifice, the whole brain was removed from the skull and the meninges and dura/pia matter carefully removed. The tissue was placed in a small homogenization tube and weighed. All tissue samples were temporarily stored at -80°C until subsequent homogenization. Homogenization was achieved by ultrasonic cell disruption after addition of 1 mL of homogenizing solution per brain. The homogenizing solution was a 0.1 **M**, pH 4.50, acetate buffer containing 0.4 **M** $NaClO_4$ and containing an appropriate amount of internal standard compounds for the liquid chromatograph. Following homogenization, the samples were centrifuged at 50,000 x g for 1 hour to remove cell debris and large components. The supernate from the first centrifugation was then subjected to centrifugation/filtration through a 0.45 μ filter to provide adequate clarification for injection into the liquid chromatograph. The samples were then stored at -80°C until the day of analysis.

Neurobiological Analyzer (NEUBA®). The Neurobiological Analyzer used for the determination of neurochemicals in this investigation was comprised of four separate liquid chromatography systems, run in parallel fashion. All four of these systems, however, are connected to single automatic injection port such that the sample can be introduced into each of the four systems rapidly and separately. Three of the LC systems possess multiple, glassy carbon electrochemical detectors. Each cell block for these three LC systems contained four individual detectors. Maintenance of separate potentials at each one of the individual electrodes within a given cell block allowed distinctive three-dimensional pictures of eluting components to appear. Thus, many more components than are simply accessible by straighforward chromatographic separation became accessible through this device. A more complete explanation of the Neurobiological Analyzer is supplied in a separate paper in this volume (See article by Turk and Blank).

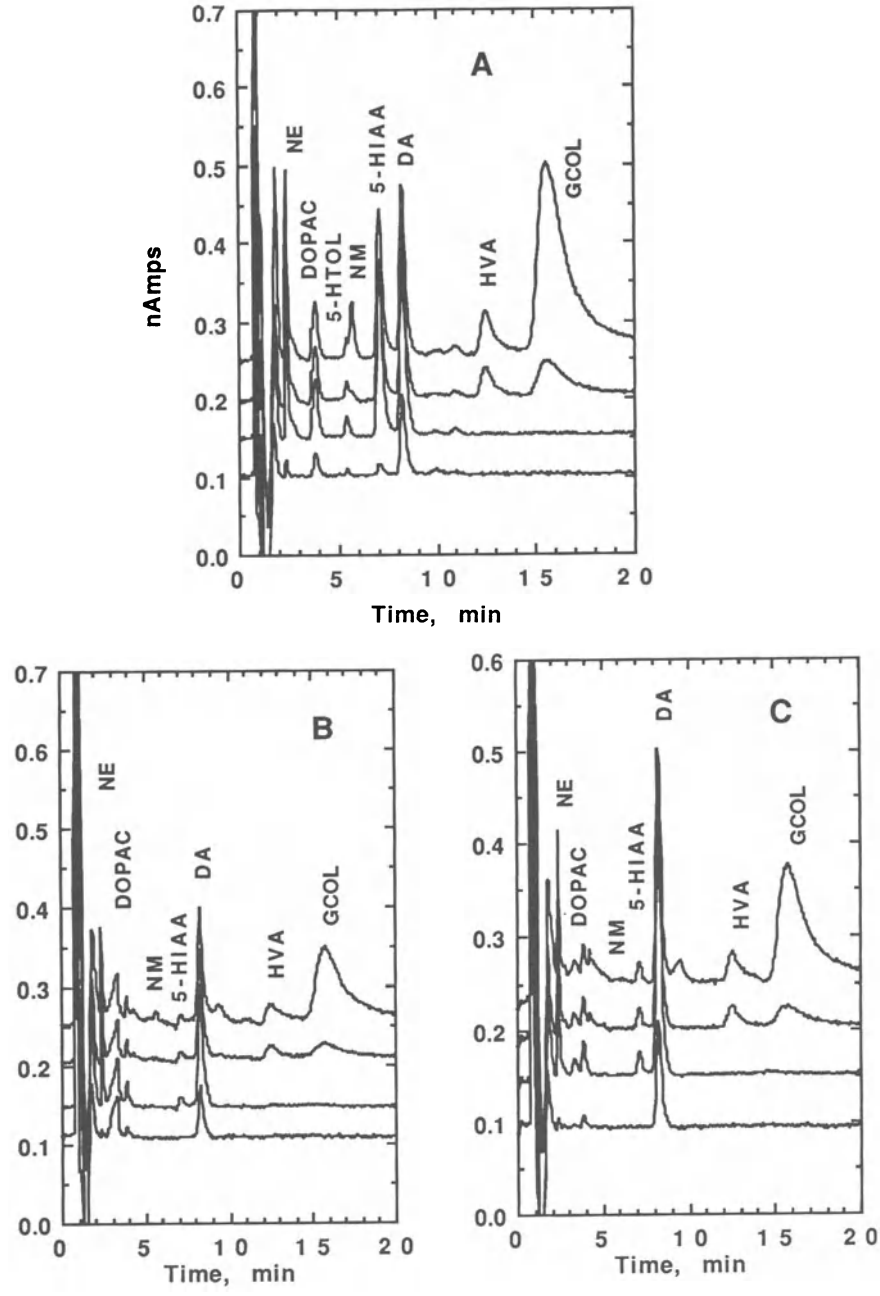

Fig. 1. Chromatograms Obtained from System 1 [of 4 Systems Used] for External Standard **(A)**, Decapitated Brain **(B)**, and Microwaved Brain **(C)**.

RESULTS AND DISCUSSION

Previous investigations (Blank *et al.*, 1979) have indicated that the whole brain levels of neurochemicals are somewhat less than reliable with respect to the individual regional changes that one can expect to ultimately observe. The current investigation, nonetheless,

demonstrates the applicability of the Neurobiological Analyzer to the measurement of these compounds in such whole brain tissue samples. Typical chromatograms obtained on one of the four LC systems monitored by the Neurobiological Analyzer are shown in Fig. 1. The results indicated decreased levels of transmitters (DA, NE, & 5-HT) and increased levels of metabolites (DOPAC, MN, and 3-MT) in the brains of animals which were sacrificed by decapitation. This is, indeed, consistent with the preservation of post mortem neurochemicals through the utilization of microwave irradiation.

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PATTERNS OF TYROSINE AND TRYPTOPHAN METABOLITES IN CONTROLS AND VARIOUS DEGENERATIVE DISORDERS

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INTRODUCTION

Over the last few years, we have been applying techniques for generating and analyzing complex patterns of several hundred small biological molecules to neurodegenerative disorders. The general hypothesis emerging from this work is that patterns of small molecular species are an operational expression of the genome. Under this hypothesis, we would argue that: (1) the underlying genetic makeup of an individual determining their variability in such features as morphology and patterns of enzymes will be reflected through these features in the levels and interrelationships of small molecular species such as transmitters, precursors, metabolites, cofactors; and (2) an individual with a particular degenerative or affective disorder will exhibit a pattern of small molecules that is uniquely different from both normals and other disorders.

The models of small molecule patterns implied by this hypothesis are quite complex since the concentration of species are affected not only, or perhaps not even primarily, by genetic factors. Environmental effects, drug histories, dietary factors, behavioral characteristics, normal aging processes, or sample acquisition variables can be the major correlates of any single value at a given time point. One must also consider the strength of the correlation of genetic factors in a given disorder ranging from Huntington's disease with a clear linkage, to Alzheimer's disease, with a linkage in some cases to Parkinsonism, where environmental effects may trigger a predisposed genome.

The first necessary, but not sufficient, demonstration of the validity of the basic hypothesis is that biochemical parameters can uniquely separate individuals into disorder categories. The boundaries of the size of the study are set by the requirements of having a large enough sample number to include environmental correlates, and a large enough number of relevant analytes to provide correlates of both environmental and genetic factors. As an example, consider what affects the level of an analyte such as homovanillic acid in brain (and what should also be measured to qualify that level): the transport and uptake of parent tyrosine (other amino acids, methionine, tryptophan, etc); the levels of competing pathways (p-hydroxy phenyl acetic acid, norepinephrine and metabolites); the levels of precursors (l-dopa, dopamine, 3-0-methyldopa, etc.); the levels of cofactors (the pyridoxamines, pterins, various metal ions); the levels of precursors

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and metabolites utilizing the same enzymes or cofactors (tryptophan metabolites, purines); as well as post mortem interval, drug and familial history. Any individual compound exists in complex webs or patterns of interlinkages. It is then these patterns which may reflect the genome. Since the number of samples and analytes can only be estimated, we have designed data bases and protocols to study the hypothesis which are dynamic. That is, we are including not only 30-50 known compounds of the approximately 400 resolved, but also approximately 300 unknowns as well as subject data for posthoc identification of chemical species (drug metabolites, endogenous peptides, etc.). Consequently, presenting the entirety of the data for the approximately 800 brain tissue samples that have been tested to date (approximately 50,000 analytical points) is outside the scope of this discussion. (The data base discussed in this paper is available, upon request to the authors, on 5 1/2" floppy disc.)

In this paper, we will outline the protocols of sample handling and analysis; data management and acceptance being used to create data bases for control and degenerative brain tissue; present summary data for an initial 500 samples from various brain regions for 32 components; and describe some of the salient differences and relationships observed in initial data analysis using standard (gaussian assumption) statistical procedures, regression analysis, cluster analysis, use of both known and unknown compounds, and frequency distribution analysis. The use of statistically significant findings in the data base to infer mechanism and guide the design of corroborating experiments will be discussed.

MATERIALS AND METHODS

Age-matched brain tissue samples with post mortem intervals and drug histories for controls (C), Parkinson's (PD), Alzheimer's (AD), and Huntington's (HD) disease are obtained from the brain banks at McLean and Massachusetts General Hospitals. Following previous protocols[1], samples are dissected at -20°C. Duplicate aliquots of approximately 200 mg wet weight are sonified at 0°C with 1 mL 0.1M perchloric acid in 1.5 mL Eppendorf vials. Duplicate aliquots of 10-50 uL of slurry are taken for protein assay. The tissue slurry is centrifuged (12,000 x g, 20 min, 0°C). Aliquots of 100 uL of supernate are placed in 300 uL conical autosampler vials containing 25 uL of a sixfold concentrate of the appropriate A mobile phase prefrozen at -70°C for analysis in separate methods. Residual aliquots are stored for trace element profiles and as spares. Iron is assayed using 20 uL aliquots of extract on an ESA Ferrochem II™ (ESA, Inc., Bedford, MA) to control for blood inclusion in the samples. Iron values greater than 10 ng/mL can affect serotonerigc and kynurenic system levels. Pools of 100 uL of each sample from a tissue type are created and subaliquoted to autosampler vials as controls. All subaliquots and the residual pellet are stored at -70°C. Authentic control standards are made to the approximate levels established by initial assay of pools for 35 compounds.

The samples are analyzed utilizing two sixteen sensor Coulometric Electrode Array Systems (CEAS Model 55-0650, ESA, Inc., Bedford, MA) with gradient HPLC and autosampler options. Separate methods previously reported[2,3] are used, which provided overlap of eight analytes under substantially varied conditions. Method (3) provides complete overlap with an earlier procedure under which 12 controls and 15 HD samples were run.[4]

Samples are compared against standards for inclusion of known compounds and against pools for inclusion of unknown compounds arbitrarily set at a value of 100 in the pool. Data acceptance criteria are ±2% retention time and ±20% ratio accuracy. Assay of the pools provides a measure of the data

base precision (typically ±7% to ±15% CV, depending on analyte). The data files from each assay are converted on the instruments to a format compatible with any standard data base program. In this study, we currently transfer and merge via a Macro program to a Lotus 1-2-3 file and to statistical programs for cluster analysis and multiple regression analysis. Patient data and other results are entered manually.

Analytical data validity is also evaluated from the merger of values from both analytical methods in the data base. Where values among methods and instruments do not agree within the precision of the methods (approximately 10% of analyses), analyses are evaluated manually for faults in the analytical sequence (incorrect baselines, false peak matches) and for degradation of the sample. If there is no clear analytical or sample fault and reanalysis does not resolve the difference, the values are rejected. Within a group of samples, compound values outside 3 sigma of the mean are not rejected. Regression equations are calculated for the compound vs. all others in the data base. Only if the value in question does not agree within 3 sigma of the regression calculated value is it rejected (0 of 40 cases). For data reported as ng/mg protein, merged data from extracted samples from different sources or different times are normalized to each other by the factor of average wet weight/protein for the sample group.

RESULTS

The merged results of a portion of the data from several groups of samples is presented in the following table in condensed form as means and standard errors in ng/mg protein. Because of the different time frames of the putamen studies, several compounds assayed in some groups were not in others. The common abbreviations used in the table have previously been cited.[1,2,3,4]

DISCUSSION

The preliminary evaluation of the data base is focused in two areas: evaluation of various statistical approaches to determining differences among specific compounds and disorder categories and differences among disorder categories in the overall pattern of compounds; and analysis of the data in terms of metabolic pathway relationships and ratios to infer possible mechanisms in a particular disorder and guide the design of various animal model and in vitro studies. We have also used the results of the statistical evaluations to try to assess the nature or structure of the data as a complete entity - whether it is normal, logarithmic, chaotic, etc.

Using procedures that assume gaussian or normal distributions of data to calculate means, standard error, and t values, there are several statistically significant differences ($p<0.01$) among various groups as noted in the tables. Some of these, such as the deficit in guanosine (GR) in AD, the deficit in kynurenic acid (KYA) in HD, and the reduced ratio of tyrosine/xanthine (TYR/XAN) in PD are consistant across various brain regions in striatum and cortex. Some, such as the TYR/XAN ratio in Broadman Area 20, are significant among all groups. The number of significant differences across the range of tissues and disorders investigated is not surprising. Indeed many of the relationships confirm results of prior studies. One surprising and perhaps controversial result, however, has been the lack of linear correlation with post mortem intervals from 1-30 hours, and the lack of correlation with drug histories. As expected, although many compounds are significant among large numbers in a group, there is no single compound that allows the classification of an individual into a particular diagnostic category.

Preliminary total pattern matches for unknown compounds have yielded several promising unknown peaks for classification. Particularly in Broadman A4 cortex between AD and controls, there is a peak at 53.2 min on channel 11 in controls at 100 times AD levels and three peaks on channel 15 at 50.3, 53.4 and 54.6 min in AD at 30 times control level. However, with the relatively limited number of samples for correlation with drug history and the unknown effect of therapeutic history on the region of the chromatograms, the utility of any possible markers will require further expansion of the data base.

Table 1

DISTRIBUTION AMONG DISORDERS IN PUTAMEN

	C(55)	HD(30)	PD(8)	AD(15)
TYR	901(44)	1040(114)	852(175)	758(75)
XAN*	640(25)	607(50)	739(89)	314(19)
KYN*	5(.54)	7.6(1.8)	3.9(.45)	6.9(1.1)
GR*	304(18)	237(18)	441(69)	228(23)
KYA*	.74(.1)	0.51(.15)	.62(.10)	1.3(.24)
5HIAA*	12(.75)	15.9(1.4)	6.4(1.4)	19(3.2)
5HT*	4.1(.3)	6.7(.59)	6.51(2)	4.3(1.2)
CYS*	824(58)	1031(74)	894(275)	1755(152)
DA*	69(5.4)	99.5(15)	2.4(1.2)	92(14.7)
DOPAC*	3.8(.4)	3.22(.28)	.63(.25)	2.4(.38)
4HPLA*	14(1.3)	10.9(1.4)	6.7(.73)	14.2(3)
5THOL	.06(.01)	.06(.01)	.08(.06)	1.6(.87)
5HTP*	.17(.03)	.22(.03)	.70(.14)	1.6(.59)
HVA*	125(6.6)	126(10.8)	54(17)	132(8.4)
TRP*	233(18)	222(39.6)	172(17)	281(30)
URIC	86(6.5)	72(11.8)	67(10.5)	92(15)
ASC	560(95)		612(66)	869(86)
NE*	.97(.12)	1.13(.13)	.26(.08)	1.2(.18)
MET*	547(34)	663(72)	350(78)	567(70)
VMA	.27(.12)		.11(.04)	.19(.04)
LDOPA	4(.49)	3.98(.85)	15.4(11)	6.8(1.2)
E	.05(.02)	.05(.02)	.48(.39)	.06(.01)
3OHKY	.71(.16)	.71(.16)	1.2(.39)	.65(.17)
HGA	.07(.02)		.06(.01)	.12(.02)
MHPG	.29(.09)		.12(.04)	.43(.08)
NMN	.06(.01)		.02(0)	.58(.37)
3OHAN	.10(.03)	.10(.02)	.16(.06)	.04(.01)
3OMD	.49(.12)		23.8(11)	.75(.19)
3MT	20(1.4)	24(2.2)2		
HVOL	.09(.03)	.08(.02)		
4HBAC	.56(.23)		.08(.02)	.84(.13)2
4HPAC	2.9(.54)		1.9(.66)	6.2(.95)
TPOL	1.2(.55)		1.0(.25)	.11(.02)
GSH*	2798(260)	2749(295)	4665(1156)	1074(185)
TRP/SUM*	12.4(1.3)	7.3(.9)	15.2(5.7)	10.3(1.5)
TYR/SUM	4.04(.27)	4.1(.3)	25.7(9.3)	8.89(4.1)

DISTRIBUTION AMONG BRAIN REGIONS OF SOME SELECTED RATIOS & COMPOUNDS

	KYA	GR	TYR/XAN
PUT			
C	.74(.13)	304(18)	1.4(.03)
HD	.51(.15)*	237(18)	1.6(.08)
PD	.62(.10)	441(69)	1.1(.11)*
AD	1.3(.24)	227(23)*	2.3(.15)
A20			
C(40)	.71(.08)	439(19)	1.5(.07)
HD(40)	.22(.03)*	388(15)	.94(.04)
PD			
AD(20)	.21(.06)	206(19)*	1.4(.08)
A21			
C(40)	.64(.10)	449(38)	1.6(.08)
HD(30)	.24(.04)*	396(18)	1.3(.05)
PD			
AD(20)	.46(.09)	240(14)*	1.4(.06)
A9			
C(40)	.43(.06)	391(28)	1.6(.07)
HD(40)	.27(.02)*	338(19)	1.5(.05)
PD			
AD(14)	.43(.06)	163(20)*	1.2(.10)
A4			
C(60)	1.4(.19)	461(30)	1.7(.09)
HD(40)	.31(.06)*	407(13)	1.6(.06)
PD(10)	.92(.09)	242(26)	.62(.80)*
AD(14)	.73(.24)	168(27)*	1.5(.17)

C=control HD=Huntingtons PD=Parkinsons AD=Alzheimers (n)=number
* indicates significance among one or more categories, p<.01
A(n)=Brodmann Area (n)
TRP/SUM = TRP/KYN + OHKY + OHAN + KYA + 5HT + 5HIAA + 5HTP + 5HTOL
TYR/SUM = TYR/HPLA + LDOPA + DA + DOPAC + HVA + NE + 3MT

To separate categories using entire patterns to test the necessary condition of the hypothesis that the genome is reflected in the small molecule patterns, we have initially applied techniques of cluster analysis and linear multiple regression analysis to the putamen data. Using 26 compounds in 30 HD and 55 controls in linear multiple regression with HD scored as 1 and C as 0 yields a regression equation with an R-Sq of 89.2, $p<.0001$. Calculation of the individual case data with the regression coefficients results in 3 HD cases falling within the control group range and 1 control in the HD range. Using the 22 compounds for 105 cases and calculating a regression equation on controls scored as 0, HD as 1, PD as 2 and AD as 3 yields a regression equation with an R-Sq of 72.9, $p<.001$. Calculating individual scores from the regression coefficients results in 17 errors of classification.

The simplest interpretation of these preliminary analyses is that the 22 compounds used separate the four categories with a high degree of probability. A secondary implication is that given a tissue sample of either AD, HD, PD or control, the current data base protocols would classify it with approximately a 20% chance of error.

For cluster analysis, 105 cases and 34 compounds were considered with unassayed values included as blank cells. The resultant Eigen vector significance and dendrite cluster patterns were equivocal. For instance, 55 controls vs. 15 AD showed clustering of 9 AD cases, but when the HD cases were included, the AD's clustered among the controls.

The reason for the equivocal results of cluster analysis and a major possiblity for improvement in regression techniques probably lies in the nature of the data. It can be inferred from the cluster vs. regression results that the patterns of data are chaotic, in a sense that does not allow grouping in a multi-dimensional space, but allows discriminatory solutions to multiple simultaneous equations. It is also evident from preliminary evaluations that many compounds do not have normal gaussian distributions. For instance, KYA in Broadman Area 22 cortex of both HD and control has a log normal distribution. Regression procedures using the appropriate exponents dictated by the actual distributions should yield better categorical separation.

Indeed, as the data base within each category becomes large enough (approximately 100 cases), the frequency distributions themselves should provide a direct route to classification by probability theory without the assumptions of gaussian statistics or linear relationships. Given frequency distributions for category A (for instance AD) and category B (all others) for a series of compounds 1-n and given an unknown sample analyzed for compounds 1-n, we could write: The probability given one value (V1) for the first compound that the sample is in category A (AD) and not B (all others) is:

$$PAB = f(V1)A \,/\, f(V1)A + f(V1)B$$

where f(V1)A is the frequency with which the value V1, occurs in A (AD) and f(V1)B is the frequency with which V1 occurs in B. For n compounds, this is expanded to:

$$PAB = \frac{f(V1)A + f(V2)A \,..\, f(Vn)A}{f(V1)A \times f(V2)A..f(Vn)A + f(V1)B \times f(V2)B..f(Vn)B}$$

This relationship compresses to zero if the sample is not A and is B, and to 1 if it is A and not B. The fundamental, if inelegant approach, relies simply on the ability to generate and control large quantities of data.

One of the utilities of the current data base is to suggest mechanisms and study designs for a disorder. The analysis of metabolic pathways by precursor to metabolite ratios in putamen data implies[1] a significant decrease in turnover of kynurenine (KYN) to KYA in HD which suggests a decrease in KYA's role in exitotoxic protection, and the possibility that it is an expression of primary defect in HD. This finding has guided the design of enzyme assay and lesion studies in the excitotoxic model of HD and is supported by the consistent regional deficit in KYA.

There are several other relationships that are of interest but uncertain significance: (1) the variations among disorders in the ratios of TYR/XAN and the strong correlation between TYR and XAN (r=0.85-0.91 in all tissues) and the variations in GR among disorders (this may infer a role of the purine system); (2) the decrease in HPLA in PD (this may suggest that tyrosine is inhibited in its equilibrium transfer to hydroxyphenylpyruvic acid); (3) the highly consistent ratio of tyrosine to all its metabolites in HD and controls, and the scatter in the ratio in AD and PD (this may suggest differences in the overall kinetics of the entire pathway).

Such observations of variations and relationships should serve as an aid to selection and design of a number of animal model feeding and dialysis studies and in vitro studies of enzyme activity among various disorders.

CONCLUSIONS

The preliminary evaluation of portions of the data base for degenerative disorder in brain tissue indicates that the categories of control, HD, PD and AD are separated with a high probability by 22 components. The current error rate in classifying an individual case can be inferred to be approximately 20%. However, as the number of compounds and cases included has increased, the error rate has dropped. It can be argued that the identification of certain unknown compounds, the inclusion of species (such as the pyrodoximes, dipeptides and additional purines) that can currently be assayed, and further increase in number of cases may eventually allow classification from biochemical data with the same error rate as post mortem examination. Thus, while not demonstrating the necessary unique separation, the preliminary evaluation would indicate that the hypothesis that the genome is reflected in small molecule patterns is at least viable.

The number and nature of correlations and differences among compounds and disorders suggests a model of biochemical patterns with a high degree of interlinkage of all systems. Conceptually, perhaps a web with all points linked by varying forces bounded by conditions of genetics and environment.

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The following is a list of the compounds and their abbreviations as mentioned in this paper.

ASC	Ascorbic Acid
CYS	Cysteine
DA	Dopamine
DOPAC	3,4-Dihydroxyphenyl Acetic Acid
E	Epinephrine
GR	Guanosine
GSH	Glutathione
HGA	Homogentisic Acid
4HBAC	4-Hydroxybenzoic Acid
4HPAC	4-Hydroxyphenylacetic Acid
4HPLA	4-Hydroxyphenyllactic Acid
5HIAA	5-Hydroxyindoleacetic Acid
3OHKY	3-Hydroxykynurenine
5HT	5-Hydroxytryptamine
5HTOL	5-Hydroxytryptophol
5HTP	5-Hydroxytryptophan
HVA	Homovanillic Acid
HVOL	Homovanillyl Alcohol
KYA	Kynurenic Acid
KYN	Kynurenine
LDOPA	3,4-Dihydroxyphenylalanine
MET	Methionine
MHPG	3-Methoxy,4-Hydroxyphenylglycol
3MT	3-Methoxytyramine
NE	Norephinephrine
NMN	Normetanephrine
3OHAN	3-Hydroxyanthranilic Acid
3OMD	3-O-Methyldopa
TPOL	Tryptophol
TRP	Tryptophan
TYR	Tyrosine
URIC	Uric Acid
VMA	Vanillylmandelic Acid
XAN	Xanthine

DEMENTIA IN PARKINSON'S DISEASE

AND CENTRAL CHOLINERGIC FUNCTION

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INTRODUCTION

Dementia occurs in a significant number of patients with Parkinson's disease without substantial pathological changes typical of Alzheimer's disease, i.e. they do not show a higher number of senile plaques and neurofibrillary tangles in the cerebral cortex than those expected by age alone (Candy et al., 1983; Perry et al., 1985). While much is known about the central biochemical changes causing the movement disorders in Parkinsonian subjects (Agid et al., 1989), the neurochemical basis of cognitive impairment and dementia is less clear.

The marked dopaminergic deficiency in basal ganglia nuclei is the major biochemical alteration underlying the motor symptoms in Parkinson's disease (Hornykiewicz, 1982). It has been postulated that deficient dopaminergic transmission in the central nervous system is responsible not only for motor impairment but also for some of the cognitive alterations in Parkinson's disease. This assumption is based on several lines of evidence. Some studies have found an improved performance in neuropsychological tests after the commencement of levodopa treatment (Loranger et al., 1972; Brown et al., 1984). Other studies have shown a positive correlation between the severity of motor symptoms and the severity of cognitive changes in Parkinson's disease (Mortimer et al., 1982), suggesting that both cognitive and motor symptoms result from the same pathological changes in the brain. The significant correlation between the severity of motor impairment and the performance in a neuropsychological test battery could be confirmed in another study (Pillon et al., 1989). However, segregation of the motor symptoms as a function of their response to levodopa leads to a different conclusion. Rigidity and akinesia, both of which respond well to levodopa, were only poorly correlated with performance in the tests. By contrast, symptoms which show little or no response to dopamine replacement such as gait disorder and dysarthria showed strong correlations with neuropsychological test scores. These correlations suggest that cognitive impairment in Parkinson's disease is at least partly related to the dysfunction of non-dopaminergic neuronal systems.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Studies comparing Parkinsonian patients with and without dementia show that cognitive impairment in Parkinson's disease may be associated with altered function in other central neuronal systems using acetylcholine, noradrenalin, serotonin and somatostatin as transmitters (Agid et al., 1989; Scatton et al., 1983).

Alterations of central cholinergic function in Alzheimer-type dementia are well established. The innominato-cortical and septo-hippocampal cholinergic systems degenerate in Alzheimer's disease and decreased choline acetyltransferase (CAT) activity in the cerebral cortex has been related to cognitive impairment (Perry et al., 1978, 1985). In Parkinson's disease the innominato-cortical cholinergic system appears to be damaged, since CAT activity is decreased in the substantia innominata and in several areas of the neocortex (Dubois et al., 1983, 1985; Ruberg et al., 1982) and severe neuronal loss is found in the substantia innominata (Candy et al., 1983; Whitehouse et al., 1983). The septo-hippocampal cholinergic system also seems to degenerate, since CAT activity is reduced in the hippocampus (Ruberg et al., 1982). A loss of cortical CAT activity in Parkinsonian subjects, particularly in the temporal cortex, has been reported to correlate with the severity of dementia (Perry et al., 1985; Ruberg et al., 1982). In the present study alterations of muscarinic cholinergic receptor binding in the temporal cortex (Brodman area 38) and hippocampus in Parkinson's disease were examined.

MATERIALS AND METHODS

Brain tissue was obtained at necropsy from nine patients with neuropathologically confirmed Parkinson's disease and nine matched controls with no evidence of neurological or psychiatric diseases (see table 1). Among Parkinsonian cases dementia had been present in four patients. Demented patients had shown profound progressive disturbances in memory and cognitive impairment. The Parkinsonian patients had all received L-dopa therapy up to the time of death, two patients had also received anticholinergic medication. Control subjects had not received any drugs that are known to affect the central nervous system.

The brain regions examined were the temporal cortex (Brodman area 38) and hippocampus. Using membrane homogenates saturation analysis was performed for the total number of muscarinic receptors with [^{3}H]-quinuclidinyl benzilate (QNB; Amersham International, Amersham, U.K.; specific activity 39 Ci/mmol; concentrations 10 - 300 pM) and for M-1 receptors with [^{3}H]-pirenzepine (New England Nuclear, Boston, Mass., U.S.A.; specific activity 70.1 Ci/mmol; concentrations 0.5 - 64 nM). Specific receptor binding was determined as described by Shimohama et al. (1986). Non-specific binding was defined by 1 μM atropine. The number of binding sites (B_{max}) and the apparent equilibrium constant (K_D) were determined by Eadie-Hofstee analysis. The activity of CAT was determined by a radioenzymatic method (Fonnum, 1975). The protein concentration was measured with the Bio-rad protein assay (Bio-rad laboratories, München, F.R.G.; Bradford, 1976).

Table 1. Patient data (mean ± s.e.m.)

	Controls	Parkinson's disease
Age (years)	74.3 ± 3.9	73.1 ± 2.6
Death to brain removal (h)	19.6 ± 2.7	18.1 ± 3.0

RESULTS

In comparison with controls, large reductions in CAT activity were found in both temporal cortex and hippocampus of demented and non-demented patients with Parkinson's disease (see table 2). In demented Parkinsonian patients CAT activity in the cortex was decreased to a greater extent than in non-demented patients. Demented and non-demented Parkinsonian subjects had increased concentrations of both the total number of muscarinic receptors and M-1 receptors in the cortex and no alterations in hippocampal receptors (see fig. 1). Alterations of KD values were not observed.

DISCUSSION

Even Parkinsonian subjects without cognitive impairment showed markedly decreased cortical CAT activity in this and previous studies (Dubois et al., 1983, 1985; Ruberg et al., 1982) and neuronal loss in the substantia innominata (Nakano and Hirano, 1984) indicating the beginning of degeneration in the innominato-cortical cholinergic system. These observations suggest that degeneration of the subcortico-cortical cholinergic system precedes the clinical appearance of cognitive deterioration. This can be demonstrated pharmacologically by administration of low doses of the anticholinergic drug scopolamine. A subthreshold dose of scopolamine does not induce a deterioration in performance of control subjects in a memory test battery but does reduce performance of Parkinsonian patients without cognitive impairment (Dubois et al., 1987). These results suggest that non-demented Parkinsonian subjects have an alteration of central cholinergic transmission which is involved in memory-related cognitive function.

The increased concentrations of muscarinic cholinergic receptors in the cortex can be induced by anticholinergic treatment prior to death (Westlind et al., 1981). Anticholinergic medication does not, however, appear to be the only reason for the increase of cortical muscarinic receptors in Parkinson's disease, since of nine patients only two had received these drugs and the increase was also seen in patients without anticholinergics. The increase of these receptors and in particular of M-1 receptors, which are thought to be located mainly post-synaptically (Mash et al., 1985), may reflect denervation supersensitivity due to reduced pre-synaptic cholinergic activity. Receptor supersensitivity is not invariably associated with a cortical cholinergic deficit since it is not observed in Alzheimer patients (Lange et al., 1989; Mash et al., 1985; Rinne et al., 1985). Supersensitivity of muscarinic receptors may be an important compensatory mechanism for reduced cholinergic transmission in the cerebral cortex.

Table 2. Choline acetyltransferase activity (nmol/h/mg protein) in cortex and hippocampus (mean ± s.e.m.)

	Controls (n = 9)	Parkinson's disease (n = 9)
Temporal cortex	4.4 ± 0.2	2.4 ± 0.3 *
Hippocampus	13.0 ± 1.1	5.5 ± 1.0 *

In comparison with controls: * $p < 0.05$ (Wilcoxon's rank-sum test)

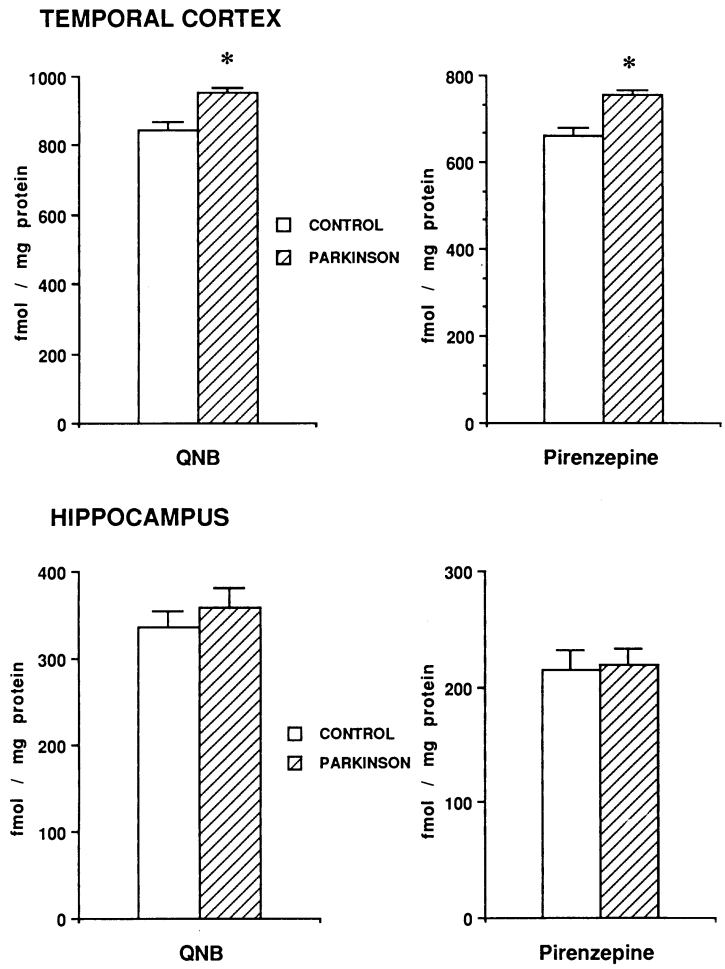

Figure 1. Maximal receptor binding (Bmax, means ± s.e.m.) of [^{3}H]-QNB and [^{3}H]-pirenzepine in the temporal cortex (Brodman area 38) and hippocampus of patients with Parkinson's disease (n = 9) and matched control subjects (n = 9); in comparison with control group: * $p < 0.05$ (Wilcoxon's rank-sum test)

It is very likely that there is a critical threshold for the loss of the cholinergic innervation of the cortex above which there are no clinically overt signs of cognitive impairment due to the compensatory activity of the remaining neurones. In the course of Parkinson's disease, the innominato-cortical cholinergic system may degenerate. In the early stages of altered cholinergic transmission, however, the cortical cholinergic deficit may not be entirely expressed because increased concentrations of cortical muscarinic receptors can maintain normal cognitive function. As degeneration progresses, compensation for the loss of innervation becomes insufficient and cognitive impairment becomes clinically present. At this stage of intellectual impairment, anticholinergic drugs can provoke confusional states (De Smet et al., 1982), probably because pre-synaptic cholinergic denervation is acutely aggravated by blockade of cortical cholinergic receptors.

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SUBTYPES OF NICOTINIC RECEPTORS IN HUMAN CORTEX: SELECTIVE CHANGES IN ALZHEIMER DISEASE

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INTRODUCTION

CHOLINERGIC DEFICITS IN ALZHEIMER DISEASE

Cholinergic deficits in Alzheimer disease (AD) have been well documented. Choline acetyltransferase (ChAt), the synthetic enzyme for acetylcholine (ACh), is consistently reduced by 50-95% in cortex and hippocampus of AD patients compared to age-matched controls (Bowen et al., 1976; Perry et al., 1978; Reisine et al., 1978; Davies, 1979; Zubenko et al., 1989). Reductions are also observed in high affinity choline uptake (HACU) (Rylett et al., 1983; Sims et al., 1983), in <u>in vitro</u> synthesis of ACh and release during depolarization (Blessed et al., 1968; Neary et al., 1986b), in presynaptic muscarinic and nicotinic receptor binding (Mash et al., 1985; Whitehouse, 1987; Kellar et al., 1987; Whitehouse et al., 1988; Giacobini et al., 1988a,b, 1989), in ACh and acetylcholinesterase (AChE) levels in cortex (Richter et al., 1980) and in cerebrospinal fluid (CSF) (Johns et al., 1983; Elble et al., 1987, 1989). The reduction of these presynaptic cholinergic markers is associated with a marked loss of cells in the nucleus basalis of Meynert which project to cortex (Whitehouse et al., 1982). By contrast, postsynaptic muscarinic receptor mechanisms appear to be relatively spared in AD patients (London and Coyle, 1978; Reisine et al., 1978; Davies, 1979; Giacobini et al., 1988a, 1989). The reductions of cortical and CSF cholinergic markers are closely correlated with the extent of neuropathology (senile plaques) and with the severity of cognitive impairment (Bowen et al., 1976; Perry et al., 1978; Fuld et al., 1982; Johns et al., 1983; Francis et al., 1985; Neary et al., 1986a; Elble et al., 1987). We have described changes in ACh and choline (Ch) metabolism in aging animals (Giacobini et al., 1987) and in the CSF of AD patients which may be related to neuronal membrane breakdown and reduced uptake of Ch by cholinergic neurons (Elble et al., 1989).

CORTICAL DEAFFERENTATION IN ALZHEIMER DISEASE

A diagram of the cholinergic systems most affected in AD is shown in Table I. The forebrain nuclei (mainly the basal nucleus), neocortical regions, hippocampus, ventral striatum and amygdala are characteristically affected by the disease.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table I. Cholinergic Pathology of Alzheimer Disease

		Cholinergic Representation	
		Neurons	Projections
1.	Neocortex (front. par. temp)	+	++
2.	Hippocampus	-	+++
3.	Amygdala	-	++
4.	Ventral striatum	++	-
5.	Basal forebrain	+++	-
6.	Locus coeruleus	-	+
7.	Raphe'	-	+

Cortical deficits in AD patients can be interpreted as reflecting denervation phenomena related to major neurotransmitter systems, mainly cholinergic and noradrenergic. This denervation has been related to the severe reduction in number of neurons in the nucleus basalis and in the locus coeruleus (Whitehouse et al., 1982; Bondareff and Mountjoy, 1986; Zweig et al., 1988).

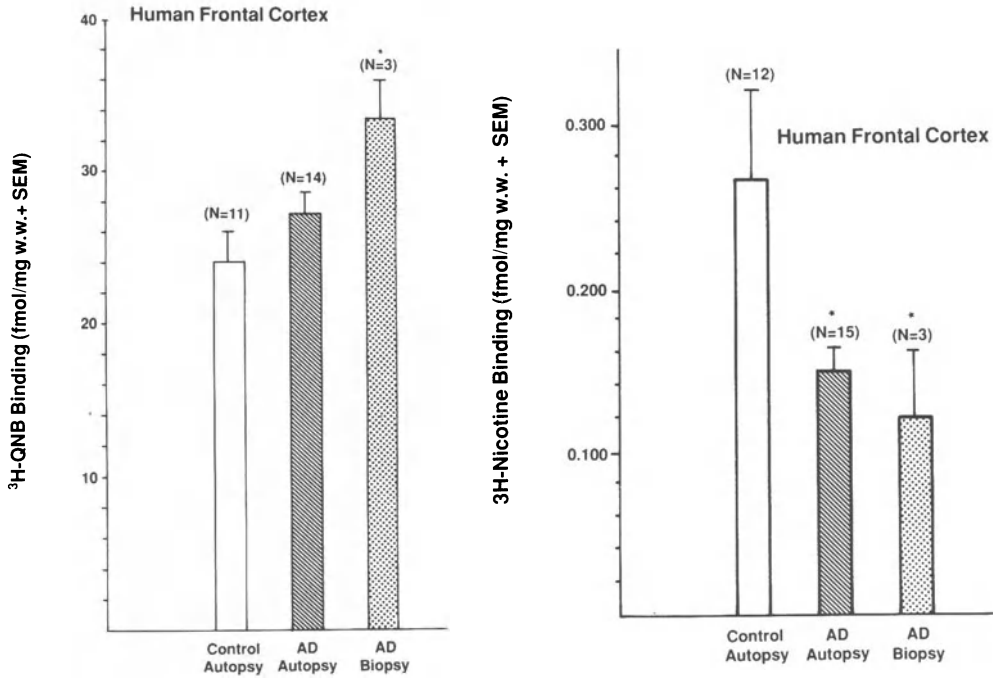

Figure 1. Comparison of ^{3}H-QNB (left) and ^{3}H-nicotine binding (right) (fmol/mg w.w. ± S.E.M.) in samples of human and frontal cortex from normal controls and Alzheimer patients (biopsies and autopsies). Significantly different from controls, $p < 0.05$.

In AD, the degree of cholinergic deafferentation is related to the extent of the cognitive impairment (Perry et al., 1978; Bird et al., 1983; Soininen et al., 1984). Sims et al. (1983) have used neocortical autopsies and biopsies to study presynaptic cholinergic nerve ending function. They showed that ACh synthesis, ChAt activity as well as Ch uptake correlated with the histopathological findings. Other authors studied ^{3}H-ACh release in AD cortical slices of autopsies and its regulation by nicotinic and muscarinic receptors (Nilsson et al., 1987). Receptor changes in AD are probably secondary to neuronal pathology. However, an understanding of receptor alterations is particularly important because it may lead to the development of new therapeutic approaches.

CHANGES IN NICOTINIC RECEPTORS IN ALZHEIMER DISEASE

Surveys, including percent changes in nicotinic receptors in the frontal cortex of AD patients, reveal a striking difference from aging normal controls (Giacobini et al., 1988a, 1989). Nicotinic receptor binding studies are mainly from autopsies using three different ligands [nicotine (NIC), α-BUNGAROTOXIN (α-BTX) and ACh]. Out of eight autopsy studies (1981-1988), including one from our laboratory (DeSarno et al., 1988; Fig. 1), six show decreases from 44-65% and two show no difference. Using ^{3}H-QNB as ligand, we found no differences in specific binding in autopsy material but a significant 39% increase in the biopsies (Fig. 1). Brain muscarinic receptors are differently affected by AD and Parkinson disease (PD). A decrease in M_2-receptor binding is seen in the hippocampus of AD patients while PD patients have unaltered binding (Rinne et al., 1989). Using ^{3}H-NIC as a ligand, we found a 44% decrease in the autopsies and a 55% decrease in the biopsies (Fig. 1). Preservation of the tissue might explain the differences between biopsy and autopsy material. However, in younger subjects it could reflect early changes in AD. These decreases in binding correlate to enzymatic (ChAt and AChE activity) changes (Davies, 1979; Rossor et al., 1982; Bird et al., 1983; Giacobini et al., 1988a,b, 1989) supporting a presynaptic localization of the lesion.

A significant reduction (50-70%) in the evoked release of ACh has been reported by two laboratories (Nordberg et al., 1987; DeSarno et al., 1988; Giacobini et al., 1988a). A reduction in ACh release and in number of nicotinic binding sites in the frontal cortex of AD patients supports the hypothesis of a selective loss of presynaptic nicotinic receptors in AD (Giacobini et al., 1988a).

Most, but not all, nicotinic receptors are insensitive to the snake venom derived α-neurotoxins (reviewed in Chiappinelli, 1986; Egan and North, 1986; de la Garza et al., 1987; Boulter et al., 1987). In contrast, the related snake venom kappa-neurotoxins (Grant et al., 1988) block function at a number of presynaptic and postsynaptic CNS nicotinic receptors, including several subtypes (α_3 and α_4) that have been expressed in frog oocytes (Lipton et al., 1987; Chiappinelli et al., 1988; de la Garza et al., 1989; Calabresi et al., 1989; Vidal and Changeux, 1989; Schulz and Zigmond, 1989).

Kappa-bungarotoxin (K-BTX) or neuronal BTX has been shown to block nicotinic synaptic transmission in a variety of neuronal preparations where α-BTX has no effect. Vidal and Changeux (1989) have demonstrated that the effect of NIC applied by iontophoresis to the prefrontal cortex of the rat is blocked by K-BTX but not by other nicotinic antagonists.

NICOTINIC RECEPTOR SUBTYPES IN HUMAN CORTEX. CHANGES WITH ALZHEIMER DISEASE

Recent molecular genetic and pharmacological studies have revealed the existence of several subtypes of nicotinic ACh receptors in the mammalian and avian brain. At least three discrete nicotinic receptor alpha subunits have been identified in brain (α_2, α_3 and α_4) which are closely related to the single alpha subunit (α_1) found in skeletal muscle (Goldman et al., 1987; Nef et al., 1988; Schoepfer et al., 1988; Wada et al., 1989). In situ hybridization experiments show that each of these alpha subunits has a discrete localization of expression in the brain, suggesting that they are components of three different receptor subtypes (Goldman et al., 1986, 1987; Nef et al., 1988; Schoepfer et al., 1988).

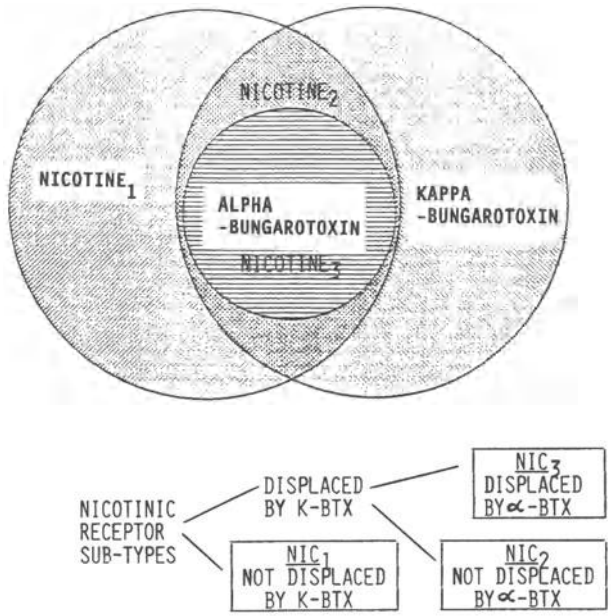

Figure 2. Diagram of subtypes of nicotinic receptors in human frontal cortex demonstrated by using nicotine, α-bungarotoxin and k-bungarotoxin as ligands. See text.

The existence of several subtypes of nicotinic cholinergic receptors in the brain would suggest specific anatomical localizations and functions.

Combining ^{3}H-NIC, ^{125}I-α-BTX and ^{125}I-K-BTX as ligands, the presence of several categories of nicotinic receptors can be postulated in the human brain (Giacobini et al., 1988b). We reported the kinetics, concentration and localization of three subtypes present in the human frontal cortex. Using autoradiography, the localization of these subtypes was described in human cortex. We also described for the first time specific changes related to receptor subtypes in human cortex of AD patients (Giacobini et al., 1988b).

We used autopsy brains from healthy young controls (age 21-57), and healthy elderly controls (age 64-94) as well as from AD patients (age 67-78) from our Regional Alzheimer Center, for homogenate- or slice-in vitro assays of nicotinic binding. The right hemisphere was isolated and stored at -90°C for binding assays, the contralateral was fixed for histological diagnosis.

Table II. Percent Decrease in Number of ^{3}H-(-)-Nicotine, ^{125}I-k- or ^{125}I-alpha-Bungarotoxin Binding Sites In Frontal Cortex of Alzheimer Patients As Compared to Elderly Controls

	High Affinity (% B_{max})	Low Affinity (% B_{max})
^{3}H-(-)-NIC	47	50[a]
^{125}I-k-BTX	0	52[a]
^{125}I-alpha-BTX	0	32[b]

n= 6-8; [a] significant $p < 0.005$; [b] not significant

Specific binding with increasing concentrations of ^{3}H-(-)-NIC, ^{125}I-α- or ^{125}I-K-BTX to membranes of human frontal cortex was saturable. Scatchard plots were curvilinear and Hill coefficients far from unity, indicating the presence of multiple classes of binding sites for these three ligands.

There were significant decreases in the number (B_{max}) of high (47%) and low (50%) affinity binding sites of ^{3}H-(-)-NIC in AD patients as compared to elderly controls (Table II). Alzheimer patients showed a significant decrease of B_{max} in the low affinity binding site of ^{125}I-K-BTX but not in the high affinity site, as compared to young (57%) and elderly (52%) controls. There was only a non-significant decrease in B_{max} in the low affinity binding site of ^{125}I-α-BTX in AD patients, as compared to young and elderly controls (34.0% and 31.6%) (Table II).

Autoradiographic analysis showed that ^{125}I-K-BTX specific binding sites are concentrated mainly in the middle and deep cortical layers. A similar localization has been observed by us in the monkey (Macaca mulatta). With autoradiography, a pronounced decrease in density of ^{125}I-K-BTX binding sites was seen in AD patient vs. elderly controls.

Mapping of nicotinic receptors in rodent brain, using various radiolabelled agonists (Clarke et al., 1985), in situ hybridization (Goldman et al., 1987; Wada et al., 1989) and immunohistochemistry (Swanson et al., 1987) has demonstrated the presence of a high concentration of ACh receptors in the neocortex.

The kinetic characteristics of these three binding sites were studied by means of competition experiments. These demonstrated the presence of two categories of nicotinic binding sites, one which was displaced by K-BTX and one which was not displaced by this toxin (Fig. 2). The binding site not displaced by K-BTX could be subdivided into two subtypes, one which was displaced by α-BTX and one was not.

A functional role of cortical nicotinic receptors can be demonstrated by the effect of BTXs on the evoked fractional release of ACh from rat frontal cortex slices. In these experiments, the first electrical stimulation (S_1, 20 mA, 1 Hz, 5 min) is a pre-drug testing control and the third electrical stimulation (S_3) is a post-drug testing control. The only significant decrease was seen in the S_2/S_1 ratio of ACh release following 1 μM k-BTX but not after 1 μM α-BTX. The S_3/S_1 ratio did not change after the third stimulation. This shows that K-BTX, but not α-BTX, decreases the electrically evoked release of ACh from rat frontal cortex slices and that this effect is reversible.

Our data are in agreement with the molecular biological data on receptor gene families in mammalian species (Heinemann et al., 1988; Lindstrom et al., 1988) suggesting that human cortex has at least three different subtypes of nicotinic receptors, each showing specific kinetics, regional distribution and synaptic localization. These three subtypes are represented in the human frontal cortex. In addition, we have found that parietal, temporal lobe and hippocampus exhibit the same binding sites with a different receptor density and distribution. Hippocampal cortex (CA3) show the highest density in K-BTX binding sites.

The fact that K-BTX, but not α-BTX, decreases the electrically evoked fractional release of ACh in rat brain is a strong indication for a presynaptic localization of K-BTX binding site. This agrees with the finding of Vidal and Changeux (1989) who demonstrated an antagonism of K-BTX (1.4 μM) to stimulation by iontophoretically applied NIC in slices of rat prefrontal cortex.

CONCLUSION

In agreement with our previous data and the data found in the literature (cf. Giacobini et al., 1988a, 1989), with NIC, both high and low affinity binding sites were significantly decreased about 50% in AD patients as compared to elderly controls. With K-BTX as a ligand, only low affinity binding sites were decreased (-52%) while with α-BTX there were no significant changes. A decrease in nicotinic, but not muscarinic, receptors have been correlated to presynaptic changes including ChAt activity (Giacobini et al., 1988a, 1989) and loss of cells in the nucleus basalis of Meynert which project to cortex (Whitehouse et al., 1982; Coleman and Flood, 1989). In human biopsy studies we demonstrated the presence of evoked ACh release which is sensitive to the increase in ACh produced by AChE inhibition (Giacobini et al., 1988a). The effect of K-BTX on ACh release seen by us in rat cortex and the changes in K-BTX binding sites seen in AD cortex suggest the presence of a class of presynaptic nicotinic receptors which modulates ACh release and which is selectively reduced in AD.

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ALTERATIONS IN MUSCARINIC CHOLINERGIC RECEPTORS AND THE SECOND MESSENGER SYSTEM IN THE CEREBRAL CORTEX IN ALZHEIMER-TYPE DEMENTIA

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INTRODUCTION

In Alzheimer-type dementia (ATD), the major finding has been the marked decrease in choline acetyltransferase (CAT), an acetylcholine (ACh) synthesizing enzyme, in the cerebral cortex and hippocampus (1). Thus, decreased neuronal function of the ACh system is believed to play the key role in the pathophysiological mechanisms of ATD.

The ACh system is regarded as the neurotransmitter system that plays the most important role in memory, learning and recognition. This is based on the findings that injections of scopolamine into normal subjects induce similar amnesia to that of demented patients (2), and that the amnesia is improved by the administration of physostigmine (3). The brain has a network of neurons that is supported by the chemical transmission of signals in the synapses. Thus, evaluation of the ACh system function should be based on not only changes in the ACh content and CAT activity, but also on the receptors that recognize ACh and transmit signals to the intracellular second messenger systems. Therefore, analysis of the receptor system is essnetial in considering the pathophysiology and treatment of ATD.

Pharmacologically, muscarinic cholinergic receptors (MCR) has recently been divided into M1-R and M2-R depending on the affinity for pirenzepine. The first reaction following binding of ACh to MCR is believed to be interaction between the MCR and G-protein. There are two or more G-proteins that bind with MCR, and they are linked to several second messenger systems suppression of adenylatecyclase activity (4,5), increased inositol phospholipid turnover (6,7,8) and activation of the K^+ channel (9). Functional determination of the receptors requires clarification of not only the binding site, but also changes in the second messenger systems and the function of coupling to the systems.

Although the MCR associated with ATD was first reported to be normal (10,11), more studies have recently reported on decreases (12,13). Most of the previous studies of MCR employed the radiolabeled receptor assay (RRA), but they did not elucidate changes in the detailed distribution. In the present study, using in vitro autoradiography (ARG) we examined

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the distribution of MCR in the cerebral cortex. In addition, we determined distributional and quantitative changes in forskolin and phorbol ester bindings in the cerebral cortex, as indicators of the second messenger system. Furthermore, we examined the characteristics of binding sites of MCR and coupling activities to G-protein.

MATERIALS AND METHODS

Specimens consisted of frontal cortexes from 3 patients with Alzheimer's disease (AD) and 2 patients with senile dementia of Alzheimer type (SDAT), and age-matched 4 controls.

ARG was conducted by the procedures reported by us (14,15) and others (16,17), and RRA for [^{3}H]QNB was carried out by the previous method (18).

RESULTS

ARG disclosed that the total MCR and M1-R both showed laminal structure in the frontal cortex of normal subjects. In contrast, patients with AD or SDAT showed a higher M1-R content than that of the normal subjects, without laminal structures. Furthermore, MCR were distributed homogenously or in patches, with marked disintegrated laminal structures. In the frontal cortex, [^{3}H]QNB binding activity in AD also showed no decrease in RRA (data not shown).

ARG of the [^{3}H]forskolin binding site revealed differences between AD and SDAT: The AD group showed a marked decrease, but the SDAT group showed a mosaic of areas with markedly decreased binding and insular areas with markedly increased binding. [^{3}H]phorbal ester bindng was decreased in both AD and SDAT.

The substitutive sensitivity to the antagonist atropine for the binding the [^{3}H]QNB was in the order of control>SDAT>AD, and that of the agonist carbachol or pilocarpine was in the order of AD>SDAT>control: Binding of [^{3}H]QNB was easily displaced by the muscarinic agonist in AD (Fig. 1).

Because G-proteins mediate coupling between MCR and the second messenger systems, we evaluated the effect of addition of GTP on agonist-displacement curves in [^{3}H]QNB binding assays. In the control, GTP had little effect with only a 2-fold increase in the IC_{50}. On the other hand, in the AD, the IC_{50} with GTP was 13-fold higher than that without GTP (Fig. 2).

DISCUSSION

In ATD patients, Meynert's nucleus shows marked degenaration, resulting in decreases in ACh and CAT of the cerebral cortex (1,19,20,21,22). Thus, marked pre-synaptic changes in the ACh system exist in ATD patients. Muscarinic receptor binding, an indicator of post-synaptic events, was reported to be normal earlier (10,11), but seems to decrease according to an increasing number of recent reports (12,13). M1-R is said to be decreased (23), or normal (24), in the ATD. The M2-R content remains controversial with respect to the decrease reported earlier (25) and the normal content reported from a more recent study (23). Although it had been believed that M1-R was present in the post-synaptic membrane, and M2-R, in the pre-synaptic membrane, a

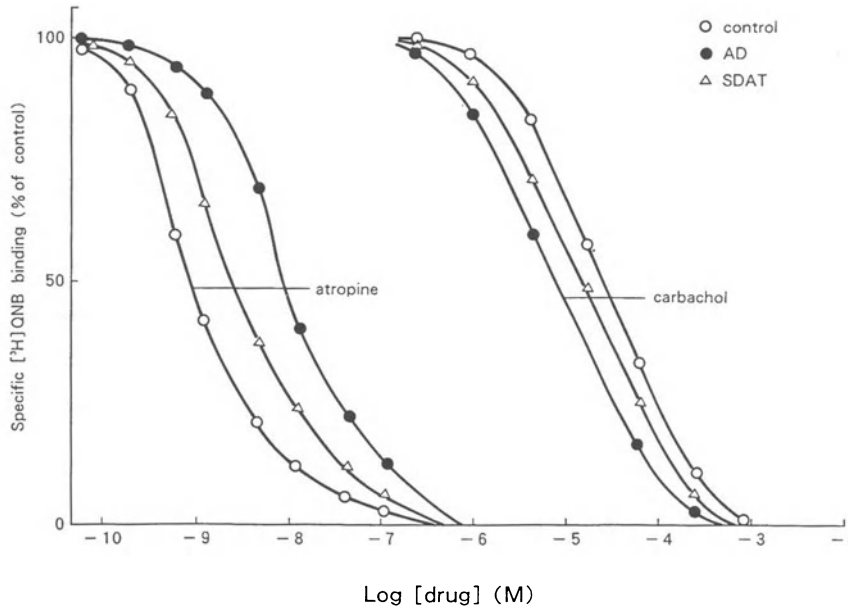

Fig. 1. Displacement curves of atropine (antagonist) and carbachol (agonist) in the [^{3}H]QNB binding to cerebral cortical membranes.

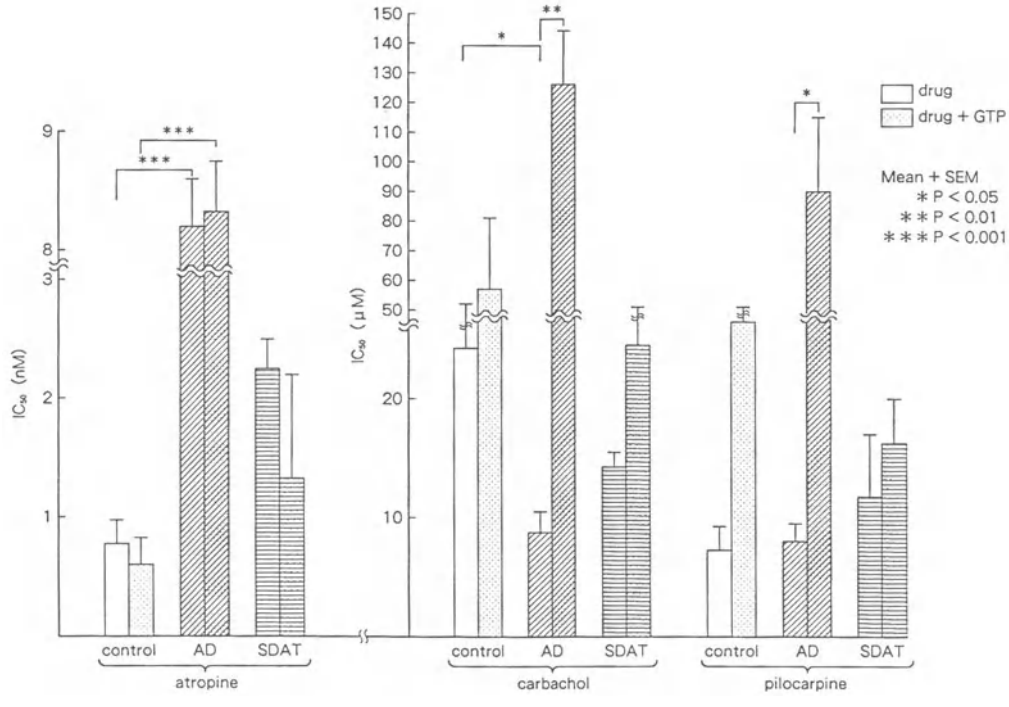

Fig. 2. Effect of GTP (100 μM) on IC_{50} values in displacement by muscarinic antagonist and agonists in competing to [^{3}H]QNB binding.

research group has argued against the proposed presence of receptors on the pre- and post-synaptic membranes (26). Furthermore, the results of detection of M1-R and M2-R may differ depending on the type of radioactive ligand used. Thus, no definite conclusion is currently available.

[^{3}H]QNB used to determine the total MCR content was bound to both pre- and post-synaptic MCR, and that CAT activity decreased in the presence of AD. Therefore, the present ARG results indicate that the neurons of the pre-synaptic ACh system, especially their structures, were markedly disturbed. The increase in M1-R is thought to reflect secondary up-regulation due to degenaration of ACh neurons. Also the loss of the laminal structure of M1-R indicates distruction of post-synaptic structures.

As for the second messenger systems, a report indicates decreased activation of adenylate cyclase due to stimulation with forskolin in the hippocampus of AD patients (27), and in the present study we elucidated not only a quntitative decrease in the binding sites, but also disintegration of the structure. In addition, a decrease in protein kinase C in the microsome fraction of the frontal cortex of the brain of AD patients was recently found (28), which was confirmed by the ARG of the phorbol ester binding sites in the present study.

The previously found normal MCR content in the cerebral cortex of ATD patients has stimulated drug therapy with the ACh precursor choline or lecithin to cause an increase in ACh (29,30) and choline esterase inhibitor such as physostigmine to prevent destruction of ACh (31). However, these drugs did not eliminate symptoms. A recent study reported improvement in the cognitive function of ATD patients by the treatment with tetrahydroaminoacridine (THA), an central inhibitor of ACh esterase (32), which was immediately challenged (33). The administration of arecoline or oxotremoline that directly stimulates MCR was ineffective (29,30,31). The ineffectiveness of these drug therapies is attributable to structural disintegration of MCR, in addition to quantitative decreases in the second messenger systems, as clarified by the present study.

There are two possible directions for the development of drug therapy for ATD. The first is to develop drugs that show a multiplex effect, rather than a single effect on the pre- or post-synaptic ACh system alone. These drugs may be effective in the early stage of ATD. We previously reported that decreased CAT and MCR levels in the 2-year-old senescent rat brain were recovered to normal by the chronic administration of dihydroergotoxine (15). Dihydroergotoxine was also effective for the learning task requiring operant behavior (34). Thus, the brain at senescent age retains the capacity for recovering function and plasticity.

Secondly, the fact that the laminal structure of MCR has been destroyed in the frontal cortex of ATD patients suggests that drug therapy is ineffective in advanced cases. Instead, there is an urgent demand for the development of a technique for diagnosing the disease before progression of structural disintegration and drugs that delay or prevent the progresssion.

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EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTOR mRNA IN THE RAT CEREBRAL CORTEX AFTER LESIONING OF THE NUCLEUS BASALIS MAGNOCELLULARIS

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SUMMARY

We investigated the effect of a unilateral lesion made in the nucleus basalis magnocellularis (nbm) on the expression of nicotinic acetylcholine receptors (nAChR's) in the rat cerebral cortex. Cortical [^{3}H]nicotine binding was not affected by the nbm lesion. Expression of nAChR mRNA in the cerebral cortex was determined by use of cDNA clones coding for nAChR subunits alpha-3, alpha-4, and beta-2. At 1 week after the lesioning, expression of alpha-4 and beta-2 was increased by 82% and 19%, respectively. By 4 weeks afterward, expression levels of these nAChR subunits on the ipsilateral side did not differ from those on the control side. Expression of alpha-3 was not altered. These results suggest that nAChR transcripts are regulated by cell-to-cell interactions and may represent supporting evidence for the occurrence of supersensitivity in deafferentated cholinergic neurons.

INTRODUCTION

Cognitive functions of the brain, such as learning and memory, may be involved in the efficiency of synaptic transmission in neuronal networks, and these functions can be impaired by the reduced availability of synaptic neurotransmitter receptors. In fact, cortical nicotinic acetylcholine receptors (nAChR's) are reduced in number in Alzheimer's disease (Flynn and Mash, 1986; Nordberg and Winbald, 1986; Whitehouse et al., 1985, 1986, 1988). For clarification of the mechanism for this reduction, neuronal regulation of nAChR expression should be precisely analyzed. So we investigated the effect of a lesion made in the nucleus basalis magnocellularis (nbm) on the expression of nAChR's in the rat cerebral cortex. Neurons in the frontoparietal cortex receive the cholinergic projection from the nbm and the lesioned rat could be a model of Alzheimer's disease.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (250-300 g) were anesthetized and placed on a stereotaxic apparatus. Ibotenic acid (0.5 µl, 0.5 µg in 1 µl of 50 mM PBS, pH 7.4) was infused at stereotaxic coordinates: 1.0 mm caudal to bregma, 3.0 mm left from the midline, and 7.6 mm ventral from the skull.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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The rats were sacrificed at 1 week and 4 weeks after the lesioning. The frontoparietal cortex was dissected for the measurement of choline acetyltransferase (ChAT) activity, nAChR content, and expression levels of nAChR mRNA's. Neuronal losses in the nbm were histopathologically confirmed. ChAT activity was measured by the method of Fonnum (1975). The nAChR contents were measured with [^{3}H]L-nicotine as a ligand by the method of Lippiello and Fernandes (1986). RNA was isolated by the guanidium thiocyanate / CsCl method. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography. The rat neuronal nAChR cDNA clones, alpha-3 (Boulter et al., 1986), alpha-4 (Goldman et al., 1987), and beta-2 (Deneris et al., 1987) used in this study were kindly provided by Drs. J. Patrick and S. Heinemann. To minimize cross-hybridization of each probe, we labeled approximately 500 base pairs of 3' end fragments, which have less sequence homology, in the presence of [alpha-^{32}P-dCTP] by the random-primer method. Poly(A)$^+$ RNA was denatured in formaldehyde at 65°C, electrophoresed in 37% formaldehyde-1.0% agarose gels, and transferred to nylon membranes. Prehybridization and hybridization conditions were 50% formamide, 5 x SSPE, 5 x Denhardt's solution, and 0.1% SDS at 42°C. Washing was done in 2 x SSC, 0.2% sodium pyrophosphate, 0.1% SDS at 50°C, followed by 1 x SSC, 0.2% sodium pyrophosphate, 0.1% SDS at 50°C.

RESULTS

ChAT activity

Cortical ChAT activity on the control side was approximately 4.0 μmol/hour/g tissue. ChAT activity on the lesioned side was decreased to an average of 65% and 64% of that on the contralateral control side at 1 week and 4 weeks respectively, after lesioning of the nbm ($p < 0.01$, Student's t test).

nAChR contents

Scatchard analysis of cortical [^{3}H]nicotine binding revealed a single class of high-affinity sites with an average Kd of 23.8 nM and Bmax of 67.2 fmol/mg of protein. Bmax and Kd in the frontoparietal cortex were not affected by the nbm lesion at either 1 week or 4 weeks after the lesioning.

Expression of nAChR mRNA

On Northern blot analysis, the alpha-4 probe detected an approximately 2.4-kilobase (kb) band; alpha-3, a faint 2.0-kb band; beta-2, 3.9- and 5.7-kb bands; and beta actin, a 1.9-kb band. No obvious cross-hybridization was seen between these clones. Therefore, quantification of these mRNA's was performed by densitometer scanning of slot blot films after confirmation that the integral of the scan was proportional to the amount of applied mRNA. At 1 week after the lesioning, alpha-4 expression on the lesioned side had increased by an average of 82% of that on the control side ($n=5$, $p < 0.01$, Student's t test); and beta-2, by an average of 19% ($n=5$, $p < 0.01$, Student's t test). By 4 weeks postlesioning, expression levels of the two mRNA's had dropped, with no significant difference between the lesioned and the control side. Expressions of alpha-3 and beta actin were unaffected by the lesion.

DISCUSSION

We demonstrated that up-regulation of cortical nAChR's with alpha-4 and beta-2 subunits was induced by the nbm lesion in rats. Alpha-4 was

shown to be expressed in the regions receiving cholinergic innervation (Goldman et al., 1987). The co-increase in beta-2 and alpha-4 mRNA's may indicate enhanced synthesis of functional nAChR's, because expression studies using *Xenopus* oocyte have shown that the beta subunit is required for the formation of functional nAChR's in addition to the agonist-binding alpha subunit (Boulter et al., 1987). This increase in nAChR subunit expression may give a supporting evidence for the occurrence of supersensitivity in deafferentated cholinergic neurons. The experimental animals used here could be a model of Alzheimer's disease because the nbm lesion causes a reduction in cortical ChAT activity, deficit in learning and memory (LoConte et al., 1982; Flicker et al., 1983; Salamone et al., 1984; Helper et al., 1985), and histopathological changes including neuritic plaque-like structures, neurofibrillary changes, and neuronal loss (Arendash et al., 1987). Our data provide basic information for interactions of the nicotinic cholinergic neurons in the cerebral cortex, which may correspond to the initial changes in the expression of cortical nAChR's in Alzheimer's disease.

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STUDIES OF CENTRAL NERVOUS SYSTEM CHOLINERGIC RECEPTORS IN ALZHEIMER'S DISEASE (AD)

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ABSTRACT

Much attention has focused on changes in muscarinic acetylcholine receptors (mAChR) in AD. Binding, biochemical and radioautographic studies of human postmortem brain tissue from AD and control (C) patients have been performed. Our data suggest changes occur in mAChR subtypes on both pre- and post-synaptic neurons. Binding assays of [^{3}H](-)quinuclidinyl benzilate ([^{3}H](-)QNB), a highly specific non-subtype selective antagonist, [^{3}H](+)cis-methyldioxolane ([^{3}H](+)CD) which labels the highest affinity mAChR agonist state, [^{3}H]pirenzepine ([^{3}H]PZ), a putative M1 selective mAChR antagonist, [^{3}H]AF-DX 116 ([^{3}H]11-2-[[2-[(diethyl-amino)methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido-(2,3-b)(1,4)benzodiazepine-6-one), a putative M2 antagonist, [^{3}H]hemicholinium-3 ([^{3}H]HC-3), an inhibitor of sodium-dependent high affinity choline uptake (SDHACU) and [^{3}H]N-methylcarbamylcholine ([^{3}H]MCC), a nicotinic (N) ligand, were done as described. No changes in affinity (Kd) values were seen. Among the changes (as %C) in receptor density (B_{max}) were: [^{3}H]AF-DX 116 in temporal cortex (TC)(65); [^{3}H]PZ in hippocampus (H)(67), TC (70); [^{3}H]HC-3 in TC (48), H (70); [^{3}H]MCC in TC (56) and [^{3}H](+)CD in TC. Loss of pre-synaptic input to H and TC and an inability to up-regulate N and/or mAChR subtypes may underlie AD pathology.

INTRODUCTION

Higher than normal densities of plaques and neurofibrillary tangles in cortical regions and marked cell loss in the nucleus basalis of Meynert (nbm) characterize the neuropathology of AD[1]. Most of the cell bodies projecting to the neocortex have been demonstrated to be cholinergic[2]. Adverse effects of anticholinergic drugs on memory and several reports of cholinergic hypofunction in AD eventually lead to the hypothesis that memory dysfunction in AD is related to a primary disturbance of central cholinergic neurotransmission[3]. Diminished activity of the enzyme choline acetyltransferase (ChAT), which acetylates choline to produce acetylcholine (ACh), and thus serves as a specific cholinergic marker has been a consistent finding in numerous laboratories. The severity of dementia has also been correlated with ChAT activity. Thus, while a possible involvement of numerous additional neurotransmitters has been demonstrated the cholinergic hypothesis certainly merits further

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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investigation in order to obtain more precise information regarding the role of CNS cholinergic systems in memory[4]. Here, putative selective ligands were used to examine the distributions and alterations in cholinergic receptors in postmortem brains of AD and control patients.

MATERIALS AND METHODS

Drugs and Reagents

[^{3}H]PZ (82 Ci/mmol), [^{3}H](+)CD (56 Ci/mmol), [^{3}H]AF-DX 116 (81 Ci/mmol), [^{3}H]MCC (87 Ci/mmol), [^{3}H]HC-3 (146 Ci/mmol) and [^{14}C]Acetyl-Coenzyme A (CAT Assay grade, 4.0 mCi/mmol) were purchased from New England Nuclear, Boston, MA. [^{3}H](-)QNB (44 Ci/mmol) was purchased from Amersham Research Products, Arlington Heights, Il. All other chemicals were reagent grade and obtained from commercial sources.

Autopsy Material

Human brains were obtained at autopsy from four AD patients, diagnosed clinically and histopathologically, and from four control subjects without any clinical or morphological evidence of AD. Clinical case histories were obtained and every attempt was made to match the groups for additional parameters such as age and postmortem delay. Causes of death were broadly similar, generally involving cardiorespiratory insufficiency. Brains were dissected immediately following autopsy and the widespread presence of neuronal loss, plaques and neurofibrillary tangles were confirmed in the neocortex and hippocampus of AD cases and noted as absent in the controls. Samples for biochemical analyses were stored at -80°C and later thawed prior to homogenization. Among the CNS regions assayed were the cerebral cortex and hippocampus.

Biochemical Studies

Ligand Binding Assays. Membranes for receptor binding studies were prepared essentially as was described previously[5]. Briefly, homogenates (wet weight/volume) of each CNS tissue were prepared in an appropriate volume (approximately 50 volumes) of ice-cold 0.01 M sodium-potassium phosphate buffer with 0.001 M $MgCl_2$ (pH=7.4) using two 15 sec bursts with a Polytron (Brinkmann, Westbury, NY) homogenizer (setting 5.5) separated by a 30 sec interval on ice. An aliquot of homogenates were used for ChAT activity studies and remaining samples were centrifuged and resuspended in ice-cold buffer for appropriate assays.

With the exception of [^{3}H]HC-3 binding, which was performed in 0.01 M sodium-potassium phosphate buffer with 0.001 M $MgCl_2$ and 0.15 M NaCl, and ChAT activity which was done in phosphate buffer containing NaCl (0.3 M), $MgCl_2$ (0.005 M), disodium EDTA (0.001 M), choline iodide (0.02 M), physostigmine sulfate (0.15 mM) and Triton X-100 (0.5%), all assays were done in 0.01 M sodium-potassium phosphate buffer with 0.001 M $MgCl_2$. Assays were carried out by means of previously described rapid filtration techniques[5-7]. The specific binding of [^{3}H]PZ was determined at 25°C for 60 min with 0.002 mM atropine sulfate to define non-specific binding. [^{3}H](-)QNB, [^{3}H](+)CD and [^{3}H]AF-DX 116 binding were done at 25°C with 0.002 mM atropine for 120 min, 120 min and 60 min, respectively. [^{3}H]MCC binding was done at 0-4°C for 60 min using 0.01 mM nicotine as the displacer and [^{3}H]HC-3 binding was done at 25°C for 30 min with 0.001 mM unlabeled hemicholinium for non-specific binding. All assays were incubated in a 1 ml volume in a shaking water bath until steady state could be reached. Reactions were terminated by rapid filtration of samples under reduced pressure onto glass fiber filters followed by three rapid washes (about 2 ml each) with ice-cold buffer using a Brandel cell

harvester. Filters were presoaked in aqueous polyethylenimine (0.1-0.3%) for 60 min before use to reduce filter binding in each case except for [^{3}H](-)QNB, when distilled water was substituted. After removal, filters were placed into scintillation vials and permitted to dry overnight.

ChAT Activity Assays. ChAT activity in unwashed tissue homogenates was assayed using [^{14}C]Acetyl-Coenzyme A (0.2 mM) essentially as reported by Fonnum (1969) with minor modifications[5,8]. After incubation (15 min, 37°C), tubes were transferred and washed with 3 ml phosphate buffer (pH=7.4, 0-4°C). The [^{14}C]-ACh formed was extracted with tetraphenylboron and quantitated by liquid scintillation spectrophotometry.

Data Analysis

Specific binding for each radioligand was defined as the difference between totals (no inhibitor) and the non-specific (inhibitor) binding. ChAT activity was defined as the net amount of [^{14}C]acetyl-CoA converted in the presence of tissue (tissue-free blank subtracted). For radioligand as well as ChAT activity assays, results were normalized and expressed according to sample protein concentrations as determined by Commassie blue as descibed by Spector (1978)[9]. Values were grouped and statistical analyses were determined using the Student's two-tailed t-test with a minimum sinificance level (P) of 0.05.

RESULTS AND CONCLUSIONS

Histopathology and Effects of Age, Sex and Postmortem Delay

All AD patients showed histological changes consistent with this disorder ($P<0.001$) in contrast to controls. No correlations between age or males and females (data not shown) were seen for any biochemical data obtained. No differences in postmortem delay (data not shown) were seen.

Cholinergic Markers

Reductions seen in postmortem ChAT activity in AD brains were in good agreement with previous data, ranging from 64% to 36% of C[10-15]. The results from binding data of many cholinergic ligands are summarized in Table 1. No significant changes in affinity were observed. K_d values overlapped for AD and C patients in H and TC, ranging from 0.01-0.09 nM for [^{3}H](-)QNB, 3-28 nM for [^{3}H]PZ, 10-99 nM for [^{3}H]AF-DX 116, 2-12 nM for [^{3}H](+)CD, 7-45 nM for [^{3}H]MCC and 1-11 nM for [^{3}H]HC-3.

Table 1. Results of Evaluation of the Status of Several Markers of Cholinergic Synapses in the Hippocampus and Temporal Cortex in AD[a]

Ligand[b]	Hippocampus	Temporal Cortex
[^{3}H](-)QNB	74	79
[^{3}H]PZ	67*	70*
[^{3}H]AF-DX 116	75	65*
[^{3}H](+)CD	77	71*
[^{3}H]MCC	70	56*
[^{3}H]HC-3	48*	70*

[a] Values provided are maximal levels in AD shown as a % of Controls.
[b] Assays were conducted as described in Materials and Methods section.
* Indicates Significance at $P<0.05$ versus Controls by Student's t-test.

Total [^{3}H](-)QNB binding was not significantly altered. However, total binding of numerous selective cholinergic markers was reduced in AD, including SDHACU, N receptors and putative M1 and M2 mAChR subtypes.

DISCUSSION

Discrepancies regarding the status of mAChR's in AD persist[10-17]. Since mAChR's are heterogeneous, changes in subtypes could be obscured by non-subtype selective ligands. A role for N receptors in AD has been similarly postulated and questioned[16-18]. Our data suggest total mAChR densities are not significantly changed in AD but that selective differential alterations occur in mAChR subtypes, as well as in N receptors in discrete areas such as TC. Decreased binding in TC accompanied decreases in HASDCU and ChAT activity. Thus, one may speculate that a loss of presynaptic input to the TC & possibly H, and/or an inability to up-regulate N and/or mAChR subtypes may reflect a pathological process in AD. Moreover, drugs showing selectivity for these sites may be of clinical value.

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CEREBELLAR EXCITATORY AMINO ACID BINDING SITES ARE DIFFERENTIALLY ALTERED IN ALZHEIMER'S DISEASE

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Summary

Excitatory amino acid binding sites in the cerebellum were differentially altered and preserved in Alzheimer's Disease. Quisqualate receptor binding was markedly reduced in the molecular layer while glycine receptor binding was increased in the granule cell layer of Alzheimer cerebellar cortex, compared to age-matched controls. Markers for presynaptic excitatory amino acid terminals, kainate and N-methyl-D-aspartate receptors were unchanged in the same Alzheimer patients. Neuritic plaques were present in the cerebellar cortex in two of the Alzheimer patients. The results suggest that the cerebellum is not spared by the pathophysiological process of Alzheimer's Disease.

Introduction

The pathological hallmarks of Alzheimer's Disease (AD): neuritic plaques and neurofibrillary tangles are most prevalent in the cerebral cortex. Another significant feature of AD is a marked loss of large pyramidal neurones from cerebral cortex (Terry et al. 1981) which are the origins of both cortico-cortical and cortico-fugal projections and which putatively use the excitatory amino acids (EAAs) glutamate and/or aspartate as their neurotransmitter. The actions of glutamate are mediated by three distinct receptor subtypes: quisqualate, kainate and N-methyl-D-aspartate (NMDA) and we have found differential alteration of these receptor subtypes in frontal cortex in AD (Chalmers et al. this volume). In addition, a loss of presynaptic EAA terminals in cerebral cortex in AD has been documented (Cowburn et al. 1988; Simpson et al. 1988). In our study of frontal cortex we found a marked increase in kainate receptor binding which was localised to deep (IV-VI) cortical layers and which was positively correlated with the number of neuritic plaques within those cortical layers. We therefore decided to investigate the cerebellum, a brain region which contains EAA systems but which is usually devoid of neuritic plaques in AD, in order to further examine the relationships between EAA receptors and structural abnormalitites in AD.

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Methods

Brains were obtained at postmortem from six subjects, mean age 84 ± 2 years, who had no known neurological or neuropsychiatric disorders (2 males, 4 females; postmortem delay 11-23 hours), and six patients with neuropathological confirmation of AD, mean age 89 ± 2 years (2 males, 4 females; postmortem delay 3-15 hours). 1cm thick slices of cerebellar cortex, including the dentate nucleus, were dissected out, frozen in isopentane (-40°C) and stored at -80°C. The remainder of the cerebellum was fixed in 10% formalin for neuropathological examination. For receptor autoradiography, blocks of cerebellar cortex were cut into 20μm thick cryostat sections and mounted onto subbed glass slides. Serial sections were used for the determination of ^{3}H-D-aspartate (250nM), ^{3}H-kainate (50nM), ^{3}H-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (^{3}H-AMPA) (130nM), NMDA-sensitive ^{3}H-glutamate (150nM) and strychnine-insensitive ^{3}H-glycine (100nM) binding in both AD and control brains. These ligands bind to the presynaptic sodium-dependent EAA uptake mechanism, postsynaptic kainate, quisqualate, NMDA receptors and the strychnine-insensitive glycine receptor which is linked to the NMDA receptor complex, respectively. Autoradiographic images were quantified by means of computer assisted densitometry with reference to recalibrated ^{3}H-microscales and specific activites of the ligands. Sections approximately 0.5cm medial to those used for receptor autoradiography were examined for neuritic plaques.

Results

^{3}H-AMPA binding to quisqualate receptors was reduced by 70% in the molecular layer of AD cerebella compared to controls while it was unaltered in the granule cell layer (Figure1). In adjacent sections strychnine-insensitive ^{3}H-glycine receptor binding was increased by 40% in the granule cell layer while it was no different in AD cerebella compared to controls in the molecular layer (Figure2). ^{3}H-D-Aspartate binding to presynaptic EAA re-uptake sites, in both molecular and granule cell layer, was similar in AD and control subjects. Moreover, NMDA-sensitive ^{3}H-glutamate and ^{3}H-kainate binding were similarly unaffected in AD.

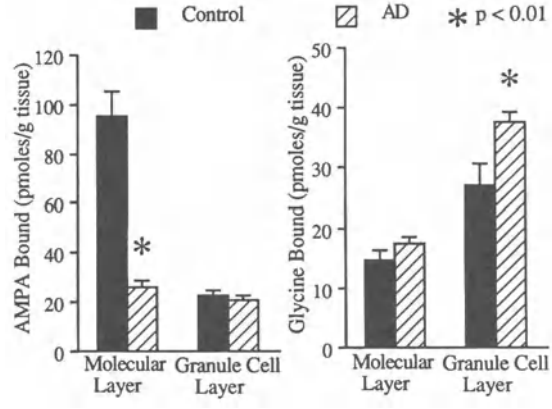

Figure 1

Neuropathological examination of cerebellar sections adjacent to those used for autoradiography revealed that all control and 4 of the 6 AD cerebella were devoid of neuritic plaques. However, one AD subject had 10 and the remaining subject 3 plaques per mm^2 in the cerebellar cortex. There was no evidence of neurofibrillary tangles in either control or AD cerebellar cortices.

Receptor Alterations in Alzheimer Cerebellar Cortex

Ligand	Target Receptor	Molecular Layer	Granule Cell Layer
AMPA	Quisqualate	↓	↔
Glycine	Strychnine-insensitive glycine	↔	↑
D-Aspartate	Presynaptic terminal	↔	↔
Kainate	Kainate	↔	↔
Glutamate	NMDA	↔	↔

Figure 2

Discussion

The cerebral cortex is the major site of pathology in AD with extensive morphological and neurochemical abnormalities. Our finding of a positive correlation between the number of neuritic plaques and the level of kainate receptor binding in frontal cortex in AD prompted the present examination of EAA systems in the cerebellum which has, to date, been regarded as a brain region largely spared by the pathological process of AD.

There was no alteration in the level of presynaptic EAA terminals in cerebellar cortex in AD. This suggests that cerebellar afferent input from climbing fibres and mossy fibre systems as well as granule cell parallel fibre projections to the Purkinje cells remains intact in AD since these systems are believed to contain EAAs. In adjacent sections, from the same patients, there was a marked loss of ^{3}H-AMPA binding to postsynaptic quisqualate receptors in the molecular layer whilst they were unaltered in the granule cell layer. There is evidence from experimental studies in rodents that quisqualate receptors in the molecular layer are localised on the apical dendrites of Purkinje cells. Mice lacking in Purkinje cells exhibit a 70% reduction in the number of quisqualate receptors, while those lacking granule cells have normal levels of this receptor (Olson et al. 1987). The relative distribution of EAA receptor subtypes over molecular and granule cell layers in our study is strikingly similar to that observed in rodents, both kainate and NMDA receptors are enriched in the granule cell layer compared to the molecular layer. Since neither of these two receptors were altered in AD, it is possible that the loss of quisqualate receptors represents a significant loss of Purkinje cells. While there are no quantitative reports of this in AD, Cole et al. (1989) reported an impression that there was "patchy Purkinje cell loss" in AD patients. They also reported that some patients

had cerebellar plaques which were most commonly located in the molecular layer. Two of the six AD patients in the present study also had cerebellar plaques.

The increase in glycine receptor binding confined to granule cell layer found in AD cerebellar cortex is less obviously explicable in terms of possible structural abnormalities. The upregulation of a receptor commonly occurs in response to a loss of presynaptic input (denervation supersensitivity). However, EAA input to the granule cell layer was not reduced in AD. Although ^{3}H-D-aspartate binding provides a measure of EAA presynaptic uptake sites, it is not known whether these terminals are functioning normally, i.e. releasing and/or taking up normal levels of EAAs. Glycine binding sites also may be influenced by other elements of the NMDA receptor complex to which it is linked. Although NMDA receptor binding was unaltered in AD, the functional integrity of the receptor complex may be altered. Abnormalities in the ability of glutamate, acting at the NMDA recognition site, to open the associated ion channel may bring about alterations in the function of the glycine recognition site which acts synergistically with the NMDA receptor in the regulation of channel opening.

In conclusion, we have found significant alterations in both quisqualate and glycine receptor binding in the cerebellum in AD. Whether these changes result from the pathophysiological process of the disease itself or represent functional responses to events initiated in the cerebral cortex is unclear at present. However, the presence of neuritic plaques in some patients and a significant loss of receptors putatively located on Purkinje cells suggests that this brain region may also be susceptible to morphological and neurochemical abnormalities perhaps at a later stage of the disease than cerebral cortex.

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PRE- AND POSTSYNAPTIC CORTICAL GLUTAMATERGIC BINDING SITES IN ALZHEIMER'S DISEASE

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Summary

The distribution and density of Na^+-dependent glutamate uptake sites and glutamate receptor subtypes; kainate, quisqualate and N-methyl-D-aspartate (NMDA), were measured in adjacent sections of frontal and temporal cortex obtained postmortem from six patients with Alzheimer's disease (AD) and six age-matched controls. Binding of [^{3}H]-D-aspartate to Na^+-dependent uptake sites was reduced by approximately 40% throughout AD frontal cortex relative to controls, indicating a general loss of glutamatergic presynaptic terminals. [^{3}H]-Kainate receptor binding was significantly increased in deep layers of AD frontal cortex compared to controls, but unaltered in superficial laminae. There was a positive correlation (r=0.914) between kainate binding and senile plaque numbers in deep cortical layers. NMDA-sensitive [^{3}H]-glutamate binding was slightly reduced (25%) only in superficial layers of AD frontal cortex relative to controls, but was unrelated to senile plaque numbers in these laminae (r=0.104). Quisqualate receptors, as assessed by [^{3}H]-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ([^{3}H]-AMPA) binding, were unaltered in AD frontal cortex compared to controls. There were no significant alterations in pre- or postsynaptic glutamatergic sites in AD temporal cortex relative to control subjects.

These results indicate that alterations in glutamatergic transmission occur in specific regions of the cerebral cortex in AD and that, in the presence of cortical glutamatergic terminal loss, plastic alterations are evident in some glutamate receptor subtypes but not in others.

Introduction

Alzheimer's disease (AD) is characterised neuropathologically by the presence of higher than normal densities of senile plaques and the development of neurofibrillary tangles in neocortical and archicortical regions (Perry,

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1986). In addition, within the cerebral cortex, a loss of pyramidal cells is a prominent feature of AD pathology (Terry et al. 1981). Glutamate is putatively the excitatory transmitter of cortical pyramidal neurons, thus implicating glutamatergic dysfunction as a possible contributory factor in the pathophysiological progression of the disease. A deficit in cortical glutamatergic terminals, assessed by Na^+-dependent [^{3}H]-D-aspartate binding, is a consistent finding of homogenate binding studies in AD brain (Cowburn et al. 1988; Simpson et al. 1988). The integrity of cortical postsynaptic glutamate receptors in AD, however, remains largely unclear. Postsynaptic actions of glutamate are mediated by three receptor subtypes; kainate, quisqualate and N-methyl-D-aspartate, named after the most selective agonist at each receptor. Using quantitative ligand binding autoradiography, we have measured the distribution and density of both Na^+-dependent glutamate uptake sites and the three glutamate receptor subtypes in adjacent sections of frontal and temporal cortex from AD patients and age-matched controls. These neuropharmacological measures could be directly related to the neuropathological severity of the disease by quantification of senile plaque numbers in the same brain region.

Methods

Brains were obtained at postmortem from six subjects, mean age 84 ± 2 years, who had no known neurological or neuropsychiatric disorders (2 males, 4 females; postmortem delay 11-23 hours), and six clinically diagnosed AD patients, mean age 89 ± 2 years (2 males, 4 females; postmortem delay 3-15 hours). At autopsy, brains were cut into 1cm thick coronal slabs and middle frontal gyrus and inferior temporal gyrus were dissected out, frozen in isopentane (-40°C) and stored at -80°C, in preparation for receptor autoradiography. The remaining undissected tissue was fixed in 10% formalin and processed for senile plaque quantification. For receptor autoradiography, blocks of frontal and temporal cortex were cut into 20μm thick cryostat sections and mounted onto subbed glass slides. Serial sections were used for the determination of: [^{3}H]-D-aspartate (250nM + 300mM NaCl); [^{3}H]-kainate (50nM); [^{3}H]-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ([^{3}H]-AMPA) (130nM + 100mM KSCN); and [^{3}H-glutamate (150nM + 5μm quisqualate and 100μm 4-acetamido-4-isothiocyanato-stilbene-2,2'-disulfonic acid) binding in both AD and control brains. Non-specific binding was determined in the presence of 250μM D-aspartate, 50μM kainate, 150μM kainate, and 150μM NMDA. Receptor autoradiograms were quantified using a Quantimet 970 image analysis system. Senile plaque numbers were determined in sections approximately 0.5cm caudal to those used for ligand binding.

Results

Frontal Cortex. In AD frontal cortex, mean ± SEM senile plaque numbers were: 35 ± 6 per mm^2 in superficial layers (I-III) and 21 ± 4 per mm^2 in deep layers (IV-VI). In AD brains, there was a marked reduction in [^{3}H]-D-aspartate binding throughout frontal cortex compared to controls (Fig. 1A), indicating a significant loss of glutamatergic terminals. In adjacent sections from the same patients [^{3}H]-kainate binding in layers IV and V-VI of frontal cortex was

significantly greater than that of control subjects, while [^{3}H]-kainate binding in cortical layers I-III was similar in control and AD brains (Fig. 1B). Furthermore, [^{3}H]-kainate binding was positively correlated with senile plaque numbers in layers IV (r=0.901) and V-VI (r=0.914) of AD frontal cortex, but unrelated to this neuropathological measure in superficial laminae (r=0.089). NMDA-sensitive [^{3}H]-glutamate binding in layers I-II of AD frontal cortex was reduced compared to control brains, although this difference only just achieved statistical significance (p=0.05). [^{3}H]-AMPA binding in AD frontal cortex was not significantly different from controls in any cortical layer.

Temporal Cortex. There were no significant alterations in [^{3}H]-D-aspartate, [^{3}H]-kainate, NMDA-sensitive [^{3}H]-glutamate or [^{3}H]-AMPA binding in AD temporal cortex in comparison to control subjects. The level of local neuropathology, as a function of senile plaque numbers, was, however, similar to that observed in frontal cortex from the same patients: 29 ± 8 per mm^2 in superficial layers; 19 ± 4 per mm^2 in deep layers.

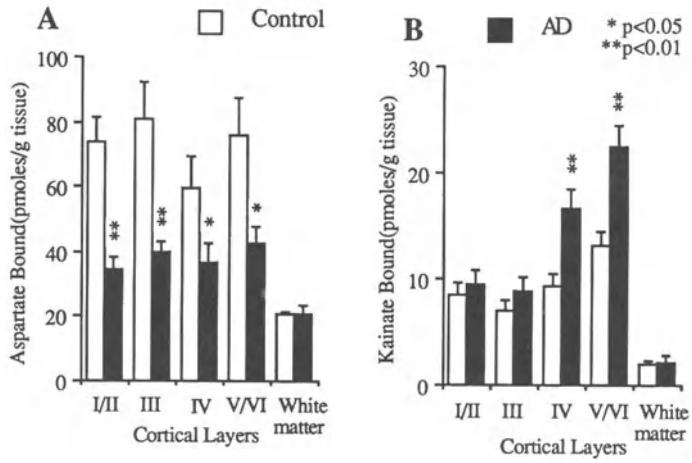

Figure 1

Discussion

In the present study, the marked reduction in [^{3}H]-D-aspartate binding in all layers of frontal cortex in AD subjects indicates a marked loss of glutamatergic terminals in this region. In view of the significant loss of cortical pyramidal neurons in AD (Terry et al. 1981), the cells of origin for intracortical projection fibres, reduced [^{3}H]-D-aspartate binding may reflect the degeneration of excitatory cortico-cortical fibres. Previous homogenate binding experiments have indicated a reduction in [^{3}H]-D-aspartate binding in AD temporal cortex (Cowburn et al. 1988; Simpson et al. 1988). The lack of alteration in [^{3}H]-D-aspartate binding in this region in the present autoradiographic study may have a neuroanatomical basis as homogenate experiments have relied on "pooled" tissue from the temporal lobe rather than examination of a specific gyrus.

Examination of postsynaptic glutamate receptors in AD, to date, have concentrated on NMDA receptors with minimal examination of quisqualate or kainate receptors. In this respect, the most striking finding of the present study has been the substantial increase in kainate receptor binding in deep layers of AD frontal cortex. Autoradiographic saturation analysis of this response indicates that the increase in kainate binding reflects a change in kainate receptor numbers rather than a change in receptor recognition properties.

The loss of a presynaptic glutamatergic marker ([^{3}H]-D-aspartate) together with an increase in kainate receptor numbers may, at a simplistic level, be considered as evidence for denervation supersensitivity in AD frontal cortex. However, the laminar-specific increase in kainate receptors contrasts with the widespread loss of presynaptic glutamatergic terminals from all cortical layers. The neuroanatomical basis for the laminar specificity of the kainate receptor response remains to be established. While this receptor alteration was intimately associated with an index of neuropathological severity of AD (senile plaque numbers), there were no changes in kainate receptors in other cortical layers (I-III), in which similar or more severe neuropathological changes were present. Pyramidal cells in cortical layers V-VI are the source of cortico-fugal projections to the striatum, caudate putamen, thalamus and brainstem. The present binding data may therefore relate to the preservation of cortico-fugal information processing in the presence of intracortical dysfunction.

Alterations in glutamatergic binding sites in frontal, but not temporal cortex, is somewhat surprising in view of the comparative levels of senile plaque numbers in both regions (this study) and similar levels of pyramidal cell loss in both areas (Terry et al. 1981). The functional significance of this anatomically-specific alteration in cortical glutamatergic transmission remains to be established. However, in the present study, it is both the strong association of the kainate receptor response in frontal cortex with local neuropathology and the direction of the response (receptor up-regulation) which indicates the importance of this change in the pathophysiology of AD.

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REDUCED N-METHYL-D-ASPARTATE (NMDA) RECEPTOR-ION CHANNEL COMPLEX IN ALZHEIMER'S DISEASE FRONTAL CORTEX

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INTRODUCTION

In this short report, we will describe the binding characteristics of [^{3}H] N-(1-[2-thienyl] cyclohexyl) 3,4-piperidine ([^{3}H] TCP) and [^{3}H] glutamate to membrane preparations obtained from postmortem human frontal cortical tissues. Under the assay conditions adopted to eliminate the possible effects of endogeneous substances on the binding parameters, we found significant decreases either in [^{3}H] TCP or [^{3}H] glutamate binding capacity in Alzheimer's disease (AD) postmortem brains.

MATERIALS AND METHODS

Autopsy material. Brain tissues were obtained at autopsy from six patients diagnosed clinically and histopathologically as having had AD, and from six subjects with no clinical or morphological evidence of brain pathology. The groups were matched for age and time to autopsy. Immediately after autopsy, brains were halved sagittally and one-half was examined histopathologically. AD was diagnosed histopathologically from the widespread presence of neuronal loss, senile plaques and neurofibrillary tangles in the neocortex and hippocampus. The other half was stored at -80 ^{o}C for biochemical studies. The brain area used in this study was Brodmann area 4 of the frontal cortex.

Membrane preparations. Membranes were prepared according to the method of Ransom and Stec (1988). The samples were homogenized in 10 vol. of 0.32 M sucrose using a Potter-Elvehjem glass homogenizer and the homogenates were centrifuged at 1,000 g for 10 min. The supernatant was collected and centrifuged at 20,000 g for 20 min to obtain a crude mitochondrial pellet. The pellet was resuspended in 5 mM Tris-HCl (pH 7.7) and dispersed with a Polytron homogenizer (PT10) at a setting of 6 for 30 s. The suspension was centrifuged at 8,000 g for 20 min. The supernatant and the buffy layer of the pellet were collected and recentrifuged at 48,000 g for 20 min to obtain a final pellet, which was suspended in 5 mM Tris-HCl and stored at -20 ^{o}C for more than 18 hours.

Binding assays. [^{3}H] TCP binding assays were performed according to the method of Ransom and Stec (1988). The frozen membranes were thawed and centrifuged at 48,000 g for 20 min. The pellet was resuspended in 5 mM

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table 1 Decreased [^{3}H] TCP binding in AD frontal cortex

	Kd (nM)	Bmax (fmol/mg protein)
control (n=6)	42 ± 6	466 ± 35
AD (n=6)	38 ± 7	244 ± 16*

Bidning parameters were obtained from Scatchard analysis of saturation isotherms with [^{3}H] TCP in concentrations of 2-100 nM. * $p < 0.01$

Table 2 Enhancement of [^{3}H] TCP binding by NMDA, L-glutamate and glycine.

(microM)	control (n=5)	AD (n=5)
none	100 (%)	100 (%)
glycine (20)	104 ± 26	99 ± 25
NMDA (50)	164 ± 23	182 ± 26
NMDA (50) + glycine (20)	239 ± 12	263 ± 12
glutamate (50)	199 ± 27	182 ± 26
glutamate (50) + glycine (20)	315 ± 26	331 ± 16

Extensively washed cortical membrane preparations (100 microg protein/assay) from control or AD brains were incubated with 4 nM [^{3}H] TCP in the presence of drugs indicated. Results are expressed as percent of binding values in the absence of drugs, which were 44 ± 12 and 25 ± 6 fmol/mg protein (means ± SEM, n=5) for control and AD membrane preparations, respectively.

Table 3 Decreased NMDA-sensitive [^{3}H] glutamate binding in AD frontal cortex

	[^{3}H] glutamate bound (fmol/mg protein)
control (n=6)	123 ± 12
AD (n=6)	57 ± 8*

NMDA-sensitive [^{3}H] glutamate binding was obtained using 20 nM [^{3}H] glutamate, defined as the difference in the binding capacity between the presence and absence of 100 microM nonradioactive NMDA.
* $p < 0.01$

benzodiazepine receptors ([^{3}H] flunitrazepam binding sites). The massive reduction in [^{3}H] TCP and NMDA-sensitive [^{3}H] glutamate binding sites presented here, thus suggest the specificity of the reduction in NMDA receptor-ion channel complexes in ATD brains, providing a supportive evidence to the hypothesis that excitatory amino acid receptors may play a role in the pathogenesis of ATD; so-called "excitotoxicity theory." (De Boni and MacLachlan, 1985; Choi, 1988).

The role of NMDA receptors in the neuronal damage caused by ischemia/hypoxia now seems to be established (Rothman and Olney, 1987). Activation of NMDA receptors, which leads to increased intracellular

Tris-HCl and incubated at 37 °C for 30 min. After incubation, the suspension was centrifuged at 48,000 g for 20 min, and the pellet was washed 5 times by resuspension in 5 mM Tris-HCl, followed by centrifugation. The final pellet was resuspended in 5 mM Tris-HCl. The plastic tubes, containing [^{3}H] TCP, the membrane preparations (100-200 microg of protein) and 5 mM Tris-HCl to a final volume of 250 microl were incubated at 25 °C for 60 min. The assay was terminated by the addition of 3 ml of ice-cold buffer and filtered under reduced pressure through Whatman GF/C glass-fiber filters which were presoaked in 0.1% polyethyleneimine for at least 2 hours. Filters were washed two times with 3 ml ice-cold buffer, dried, and then counted for radioactivity in 6 ml of scintillation fluid. Nonspecific binding was defined using 100 microM phencyclidine.

[^{3}H] Glutamate binding assays were performed using Triton X-100-treated membrane preparations according to the method modified from Ogita and Yoneda (1988). In brief, the thawed membranes were resuspended in 5 mM Tris-HCl containing 0.08% Triton X-100 and incubated at 37 °C for 30 min. After incubation, the suspension was washed as described above. The final pellet was resuspended in 50 mM Tris-acetate buffer (pH 7.4). The plastic tubes, containing [^{3}H] glutamate, the membrane preparations (100-200 microg of protein), and 50 mM Tris-acetate to a final volume of 250 mcirol were incubated at 2 °C for 10 min. The assay was terminated by the addition of 3 ml of ice-cold buffer and filtered through Whatman GF/C glass-fiber filters. Filters were washed two times with 3 ml ice-cold buffer, dried, and then counted for radioactivity.

Statistical evaluations. Results are given as means ± SEM values from n experiments. Bmax and Kd values of the binding sites were determined from computer assisted linear regression analysis of Scatchard plots.

RESULTS

[^{3}H] TCP binding to human cortical membranes. Schatchard plots of specific [^{3}H] TCP binding to extensively washed cortical membrane preparations were linear, suggesting the presence of a homogenous binding site. Membrane washing resulted in an apparent decrease in the affinity of [^{3}H] TCP for its binding site, with no change in the maximal binding capacity (data not shown). In AD membrane preparations, there was a significant reduction in Bmax values without any change in Kd values (Table 1). In the throughly washed membrane preparations, either L-glutamate or NMDA markedly enhanced [^{3}H] TCP binding. Addition of glycine, which in itself has little effect on [^{3}H] TCP binding, produced a significant increase in L-glutamate- or NMDA-induced enhancement of the binding. The effects of these substances showed no significant change between control and AD membrane preparations (Table 2).

[^{3}H] glutamate binding to human cortical membranes. Schatchard plots of specific [^{3}H] glutamate binding to Triton X-100 treated cortical membrane preparations were linear, regardless of the displacer to be used; l-glutamate, NMDA, quisqualate or kainate (data not shown). In AD membrane preparations, there was a significant reduction in NMDA-sensitive [^{3}H] glutamate binding capacity (Table 3).

DISCUSSION

We have previously reported regional changes in nicotinic and muscarinic cholinergic, α - and β -adrenergic, and benzodiazepine receptors in AD brains (Shimohama et al., 1986a, b, 1987, 1988), and the reduction in number in frontal cortical membrane preparations was recorded only for

calcium concentration, causes neuronal loss which could not be explained by energy failure. The most prominent pathological feature of AD is the massive loss of large cortical neurons and we do not know why these neurons die in this disease. AD is a progressive disease, and, using postmortem brains, we are dealing with the final stage of the disease. If we assume that the neuronal loss in ATD may, at least partly, result from the excitotoxicity caused by the activation of NMDA receptors, one would predict the activated state of these receptors and enhanced calcium metabolism in the initial stage of the disease. We do not have any establised experimental models for AD and the most probable scientific methods to test this hypothesis in the future would be the in vivo imaging of neurotransmitter receptors and cerebral metabolism with positron emission tomography.

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THE INFLUENCE OF PRIMING ON THE BEHAVIORAL EXPRESSION OF DOPAMINE RECEPTOR SUPERSENSITIVITY IN BASAL GANGLIA

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The D-1 agonist SKF 38393 (2 mg/kg s.c.) failed to induce contralateral turning in drug-naive rats, lesioned unilaterally with 6-hydroxydopamine (6-OHDA) from 17 days, while elicited intense contralateral turning 90 days post lesion. Priming with a single administration of a dopaminergic (DA) agonist which elicited contralateral turning by itself, made SKF 38393 very active in producing contralateral turning in rats, lesioned with 6-OHDA from 17 days . The effectiveness of the D-1/D-2 agonist apomorphine as a primer of SKF 39393 induced turning was critically dependent on the interval between the administration of the two agonists. Effectiveness was minimal with an interval of 3 h, increased after 6-12 h, peaking at 72 h and was reduced after 10 days. Priming took place also when the primer and SKF 38393 were administered in different environment, indicating that priming in not dependent from behavioral conditioning. Finally, administration of the N-Methyl-D-Aspartate (NMDA) receptor antagonist (+) MK 801 in conjunction with apomorphine prevented its ability to act as a primer, indicating that the NMDA receptors exert a permissive role on priming.

INTRODUCTION

The model of turning behavior after unilateral 6-OHDA lesion of the nigro-striatal DA projections is extensively used as an animal model of Parkinson's disease (Ungersted, 1971). In this model, systemic administration of DA D-1 or D-2 receptor agonists elicits turning behavior contralateral to the lesion side as a result of DA-receptor denervation supersensitivity (Herrera-Marshitz and Ungerstedt, 1984; Arnt and Hyttel, 1984). Several studies indicated the existence of an interaction between the responses generated by selective stimulation of D-1 and D-2 receptors in intact rats (Gershanik et al., 1983; Barone et al., 1986; Longoni et al., 1987 a,b). We have recently shown that in rats unilaterally lesioned with 6-OHDA, previous stimulation of DA receptors provides a priming of the ability of the D-1 receptor agonist SKF 38393 to induce contralateral turning (Morelli et al., 1987 a,b; Morelli et al., 1989). In this chapter we examine the behavioral and pharmacological characteristics of this phenomenon.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats of 275-300 g of weight were injected in the left medial forebrain bundle with 8 μg/4 μl of 6-OHDA in order to lesion the DA nigro-striatal bundle. Spontaneous

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ipsilateral turning behavior was measured for five days after 6-OHDA lesion; only rats positive to the test for the five days, were used for the subsequent experiments.

For recording of behavior, rats were placed in plastic hemispheres connected with automated rotometers 30 min before administration of the drugs. Rats given distilled water were left in the rotometer bowls for the same time as drug treated rats. The number of contralateral turns (360°) made in 3 min at intervals of 10 min, or the number of total turns was measured.

For place conditioning studies priming was performed in a plastic cylinder of 39 cm diameter and 40 cm height, while challenge with SKF 38393 was performed in a plastic hemisphere (rotameter bowl).

RESULTS

Drug naive rats

Administration of SKF 38393 (2 mg/kg s.c.) to drug-naive rats unilaterally lesioned with 6-OHDA at different time, failed to induce contralateral turning 17 days after lesion. Contralateral turning was instead observed 60 and 90 days after lesion (Fig. 1A). In drug-naive rats, lesioned 17 days earlier, no or slight contralateral turning was observed after 2 and 4 mg/Kg s.c. of SKF 38393 while a low intensity but reproducible contralateral turning was obtained after 6 and 10 mg/kg s.c. of SKF 38393 (Fig 1B).

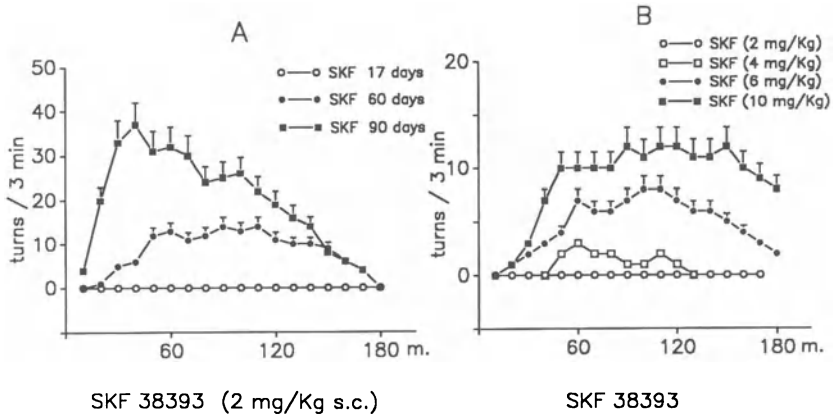

Fig. 1. A: contralateral turning after administration of SKF 38393 (2 mg/Kg s.c.) to drug-naive rats, lesioned with 6-OHDA at different times. $F(2.51) = 40.85$, $P < 0.005$ (two-way ANOVA). B: contralateral turning after administration of different doses of SKF 38393 (2-4-6-10 mg/Kg s.c.) to drug-naive rats, lesioned with 6-OHDA 17 days earlier. $F(3.68) = 56.61$, $P < 0.005$ (two-way ANOVA). The abscissa indicates the time after SKF 38393 administration, the ordinate shows the mean ± SEM of the number of contralateral turns made in 3 min.

Priming after different agonists

Administration of the D-1/D-2 agonist apomorphine (0.1 mg/kg s.c.), to rats unilaterally lesioned with 6-OHDA from 14 days, made the otherwise ineffective dose of 2.0 mg/kg s.c. of SKF 38393, given 3 days later, capable of producing strong and long-lasting contralateral turning. Sensitization (priming) to the contralateral turning induced by SKF 38393 was also obtained by administration of the D-2 agonist LY 171555 (0.2 mg/kg s.c.) or by SKF 38393 (10 mg/kg s.c.) itself (fig 2).

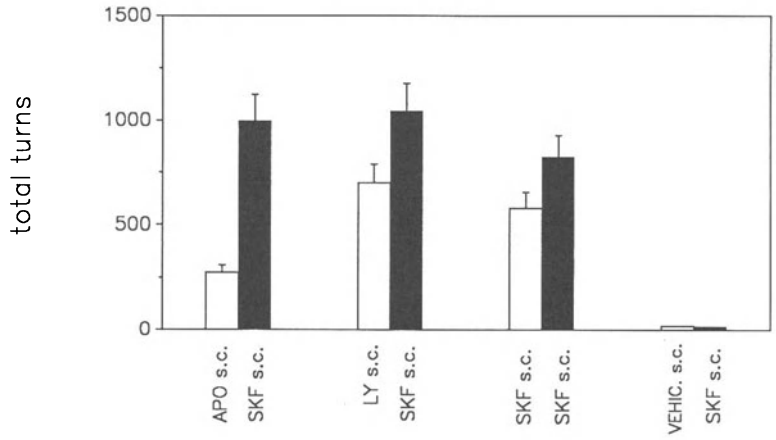

Fig. 2. Contralateral turning in unilaterally 6-OHDA lesioned rats. Fourteen days after lesion rats received apomorphine (0.1 mg/Kg s.c.) or LY 171555 (0.2 mg/Kg s.c.) or SKF 38393 mg/Kgs.c.(10 mg/Kg s.c.) or vehicle, empty columns. Three days later rats received 2 mg/Kg s.c. of SKF 38393, filled columns.

Priming: time relationship

Priming with apomorphine (0.1 mg/kg s.c.) differentially affected the contralateral turning of SKF 38393 depending on the time interval between priming and SKF 38393 challenge. Thus, as shown in fig. 3 while SKF 38393 (2 mg/kg s.c.) elicited sporadic contralateral turning 3 h after apomorphine administration, it was able to induce consistent contralateral turning 6 or 12 h after apomorphine. Intense and long lasting contralateral turning was instead obtained 72 h after apomorphine priming. Further delay of the SKF 38393 challenge (10 days) resulted in a reduction of turning in response to the agonist as compared with the results obtained at 72 h.

On the role of place conditioning

In order to investigate the possible role of environmental conditioning, priming with apomorphine or LY 171555 and challenge with SKF 38393 were performed in different environments. Thus LY 171555 (0.2 mg/kg s.c.) or apomorphine (0.1 mg/kg s.c.) were administered in a plastic cylinder while SKF 38393 (2 mg/kg s.c.) was administered in a rotameter bowl, 3 days later. No significant differences were found in the total number of turns in response to SKF 38393 in rats primed in the cylinder as compared to rats primed in the bowl (data not shown).

Effect of MK 801

To study the role of glutamate receptors of the NMDA-type, three different groups of rats, lesioned unilaterally with 6-OHDA from 14 days, were treated with the isomers (+) MK 801, (-) MK 801 (0.1 mg/kg i.p.) or vehicle and 15 min later administered with 0.1 mg/kg s.c. of apomorphine. Three days later all rats were given a dose of 3 mg/kg s.c. of SKF 38393. Pretreatment with (+) MK 801, the active isomer of MK 801, significantly increased the number of contralateral turns elicited by apomorphine (total turns = 172.4 ± 18) as compared with vehicle + apomorphine treated rats (total turns = 110.5 ± 12, $p<0.05$). In contrast, the inactive isomer (-) MK 801 failed to influence the acute effect of apomorphine (total turns = 121 ± 15) (Fig. 4). Pretreatment with (+) MK 801 however had a striking effect on the subsequent ability of SKF 38393 to induce contralateral turning. In fact, SKF 38393 which elicited vigorous contralateral turning in rats pretreated with vehicle + apomorphine (total turns = 966 ± 98), produced a very weak

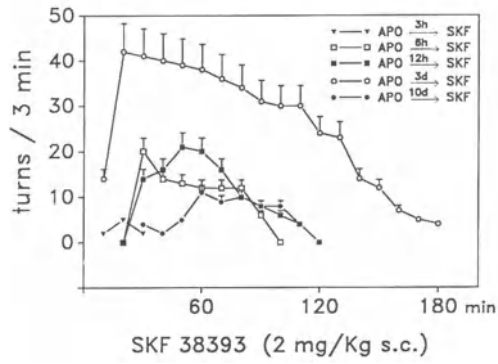

Fig. 3. Contralateral turning after SKF 38393 (2 mg/Kg s.c.) in rats primed with apomorphine 14 days after 6-OHDA lesion. SKF 38393 was administered to 5 different groups of rats 3, 6, 12 hours or 3, 10 days after apomorphine. $F(4.85) = 31.73$, $P < 0.005$ (two-way ANOVA). The abscissa indicates the time after SKF 38393 administration, the ordinate shows the number of contralateral turns made in 3 min. h = hours, d = days.

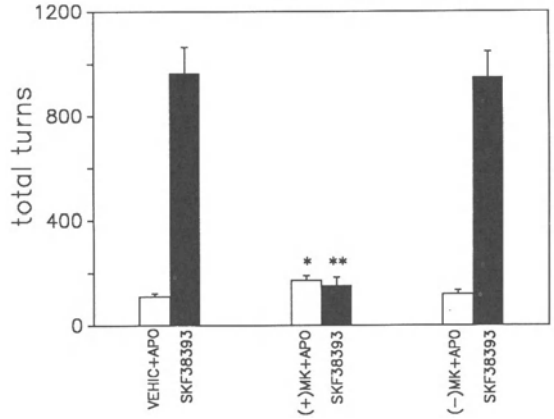

Fig. 4. Contralateral turning in unilaterally 6-OHDA lesioned rats. Fourteen days after lesion 3 different groups of rats received vehicle + apomorphine (0.1 mg/Kg s.c.), (+) or (-) MK 801 (0.1 mg/Kg i.p.) + apomorphine (0.1 mg/Kg s.c.), empty columns. Three days later rats received 3 mg/Kg s.c. of SKF 38393, filled columns.

contralateral turning in rats pretreated with (+) MK 801 + apomorphine (total turns = 154 ± 28, $p<0.005$)(Fig 4). This effect was stereospecific as (-) MK 801 failed to influence the ability of SKF 38393 to induce turning.

DISCUSSION

The present results show that D-1 receptor stimulation by SKF 38393, is poorly effective in eliciting contralateral turning in drug-naive 6-OHDA lesioned rats.

Only starting from doses of 6-10 mg/Kg, SKF 38393 induced consistent, although of a low intensity, contralateral turning. Therefore SKF 38393 can produce contralateral turning in drug-naive rats provided that a sufficiently high dose is given. Doses of 2 mg/kg of SKF 38393, which do not induce contralateral turning in drug-naive rats, lesioned 17 days earlier, become capable of inducing a consistent contralateral turning in rats bearing a 60 day old lesion and even more so in rats, lesioned 90 days earlier. Allowance of a sufficient post-lesion interval, therefore, increases the effectiveness of SKF 38393 in eliciting contralateral turning. Priming with a DA-receptor agonist makes fully effective in producing contralateral turning an otherwise ineffective dose of SKF 38393 (2 mg/Kg s.c.). These results show that priming is not an absolute requirement for the behavioral expression of D-1 dependent supersensitivity in 6-OHDA lesioned rats but strongly facilitates it. Therefore priming appears a facilitatory factor for the expression of DA receptor supersensitivity .

The priming effect of apomorphine was strictly dependent on the interval between priming and the challenge with SKF 38393. Thus, the effectiveness of apomorphine as a primer increased progressively with increasing length of the interval between 3 and 72 h but decreased after a 10 days interval. One could speculate that such a temporal relationship might be due to the time necessary for the synthesis of a molecular substrate of the priming effect. These observations might imply that the mechanisms operative in priming consist of some form of mnemonic process.

A basic issue to be considered in relation to the mechanism of priming is the possible role of conditioning. It has been reported that after previous administration of apomorphine, 6-OHDA lesioned rats show spontaneous contralateral turning when placed in the same environment where turning in response to apomorphine was obtained (Silverman and Ho, 1981). Our results show that priming with apomorphine or LY 171555 was equally effective when performed in an environment different from that where challenge with SKF 38393 took place, thus indicating that environmental conditioning does not play a role in the priming phenomenon.

With regard to the mechanism operative in priming it is interesting to note that when priming was performed in the presence of the NMDA antagonist MK 801 (Wong et al., 1988), the ability of SKF 38393 to induce contralateral turning was practically abolished. Therefore glutamatergic transmission through NMDA receptors, might exert a permissive role on the ability of DA-receptor stimulation to promote priming.

In conclusion, priming is a process of behavioral sensitization promoted by previous stimulation of DA-receptors. This phenomenon shows a distinct time-course which might correspond to the turnover of a molecular substrate of a mnemonic process. Such process might be a form of 'motor memory' and a simplified model of the mechanism by which Basal Ganglia act in motor behavior.

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M1 RECEPTORS STIMULATE DOPAMINE RELEASE VIA PROTEIN KINASE C IN THE STRIATUM OF FREELY MOVING RAT STUDIED BY BRAIN DIALYSIS

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INTRODUCTION

Recent binding and autoradiographic studies have demonstrated that muscarinic receptors in the central and peripheral nervous systems are divided into four subtypes, i.e., M1, M2, M3, and M4. One of various classifications is based on the use of the muscarinic antagonist pirenzepine. The M1-type receptors have a high affinity for pirenzepine, whilst the M2- and M3- types have a low pirenzepine affinity. Muscarinic receptors in the brain may have very important physiological roles. Especially, the receptors in patients with Alzheimer's and Parkinson's diseases may dysfunction. Our recent study indicated that *in vivo* striatal dopamine (DA) release is stimulated by M1 receptors located on the striatal DA terminals.[1]

In the present study, the effects of M1- and M2-receptor agents, which were perfused continuously into rat striatum through dialysis tubing, were investigated. It has been demonstrated that muscarinic receptors are coupled via guanine nucleotide binding proteins to multiple effector systems including adenylate cyclase, phospholipase C, and cardiac potassium channels.[2] In rat striatum, however, it is still not clear whether DA release stimulated by M1 receptors is regulated by second messenger systems. The present study *in vivo* demonstrated the main pathway in cells of striatal DA release evoked by M1 receptors.

MATERIALS AND METHODS

Male Wistar rats (250 - 350g) were used, and a dialysis loop was implanted into the right side of the striatum under sodium pentobarbital anesthesia (50 mg/kg, i.p.). A hollow fiber with an o.d. of 250 mm and 90 % cutt-off of 70-kDa molecular weight was used to prepare a 3 mm length of dialysis tubing.[1] After 2 days for surgical recovery, the dialysis loop was perfused with Ringer's solution (147 mM Na^+, 2.3 mM Ca^{2+} 4 mM K^+, and 155.6 mM Cl^-, pH 6.0) at a speed of 5 ml/min. DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in the

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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dialysates were analyzed by HPLC with electrochemical detection. All drugs were administered into the striatum through the membrane of the dialysis loop.

RESULTS AND DISCUSSION

In our recent study, we found that AF102B, an M1-selective agonist,[3] stimulated DA release in the rat striatum.[1] The stimulatory effect of AF102B was completely inhibited by pirenzepine, an M1-selective antagonist, and was additive to the stimulatory effect of AF-DX116, an M2-selective antagonist. These results suggest that *in vivo* DA release in rat striatum is stimulated by M1-receptors and is inhibited by M2-receptors.

The present study investigated the nature of second messenger system involvement in M1-receptor-mediated DA release in the rat striatum. McN-A-343, an M1-selective agonist, stimulated DA release (Fig. 1). It has been well established that islet-activating protein (IAP, pertussis toxin) decreases the affinity of Gi/Go coupled receptors for agonists.[4] Since pretreatment of the striatum with IAP completely abolished the increase in DA release evoked by McN-A-343 (data not shown), DA release evoked by M1 receptors is coupled with the interaction of Gi and/or Go.

As shown in Fig. 2, forskolin , an activator of adenylate cyclase, treatment increased DA release in the striatum and the release was additively increased by the treatments with forskolin and McN-A-343. These results suggest that *in vivo* striatal DA release evoked by M1 receptors is independent of adenylate cyclase system.

In order to study whether the interaction of M1 receptors with protein kinase-C (PK-C) results in coupling to cell responses, polymyxin B, a rather selective inhibitor of PK-C, was perfused via the dialysis membrane. As shown in Fig. 3, polymyxin B treatment suppressed DA release in a dose-dependent manner. These results confirm the previous data.[5] Furthermore, we found that polymyxin B completely blocked the increasing effect of McN-A-343, which results indicate that *in vivo* striatal DA release mediated by M1 receptors is probably dependent on the activation of PK-C.

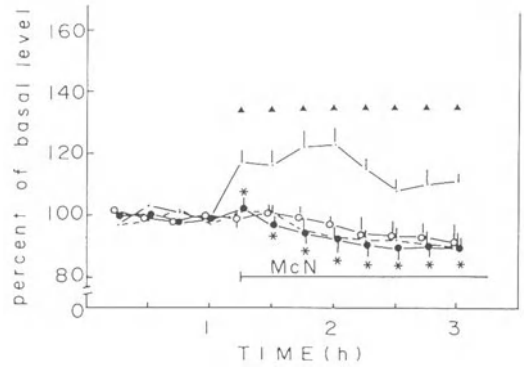

Fig. 1. Effect of McN-A-343 on dopamine release in the striatum of freely moving rats.
---, control; ○—○, IAP-treated; —, McN (10^{-7}M) (▲, $p<0.05$ compared with control); ●—●, IAP + McN(10^{-7}M) (*, $p<0.05$ compared with McN)

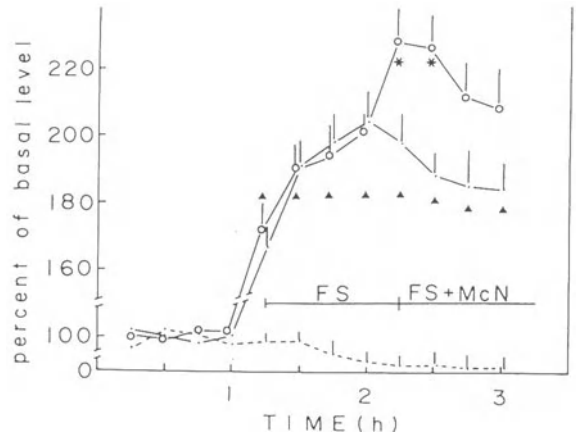

Fig.2. Effects of forskolin on McN-induced and basal dopamine release. Forskolin (FS) at 10^{-5}M was perfused for 2 h. Or, after FS had been perfused for 1 h, McN-A-343 (McN) at10^{-7}M was added to the solution containing 10 M FS and perfusion was continued for 1 h. ---, control; —, FS (▲, $p<0.05$ compared with control); ○—○, FS + McN (*, $p<0.05$ compared with FS).

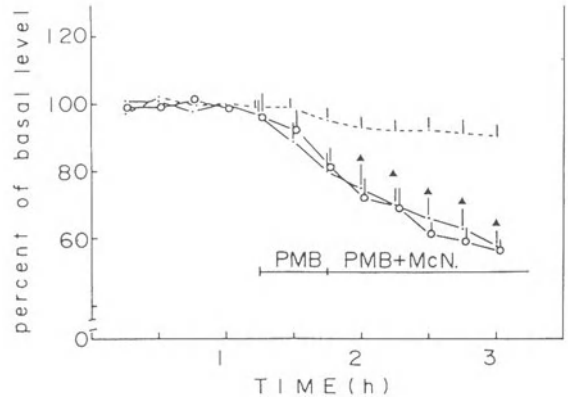

Fig. 3. Effects of polymyxin B on basal and McN-induced dopamine release. Polymyxin B (PMB) at 10^{-4}M was perfused for 2 h. Or, after PMB had been perfused for 30 min, McN (10^{-7}M) was added to the solution containing 10^{-4}M PMB and perfusion was continued for 1.5 h. ---, control; —, PMB (▲, $p<0.05$ compared with control); ○—○, PMB + McN.

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EFFECT OF MPTP ON DOPAMINE D_2 RECEPTORS IN THE AGING MOUSE STRIATUM

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INTRODUCTION

Recently, MPTP (1-methyl, 4-phenyl-1, 2, 3, 6-tetrahydropyridine) present as a contaminant in a synthetic heroin, produced a Parkinson-like disorder following intravenous administration and caused destruction of the nigrostriatal dopamine system in humans (Davis et al. 1979; Langston et al. 1983; Langston and Ballard, 1985). Studies in non-human primates and mice revealed similar degenerative effects of MPTP in the nigrostriatal system (Burns et al. 1983, Heikkila et al. 84, Gupta et al. 1984). Furthermore, it has been shown that aged mice are much more sensitive to MPTP treatment than their younger counterparts treated in a similar manner (Gupta et al. 1986). Degeneration of the dopaminergic nigrostriatal neurons in Parkinson's disease has been shown to increase the number of dopamine D_2 receptors in the striatum (Bokobza et al. 1984; Seeman, 1980). An increase in D_2 receptor density in the medial caudate/putamen has also been reported following dopamine denervation with MPTP treatment in monkeys (Joyce et al. 1986). Sershen et al. (1986) reported that MPTP administration in the young adult mice did not alter ^{3}H-spiperone binding in the mouse striatum despite causing a 63% reduction in ^{3}H-dopamine uptake while Pertouka et al. (1988) reported an increase in ^{3}H-spiperone binding 2 days following MPTP treatment. Since our previous studies have shown that aged mice are more sensitive to MPTP treatment, the present study was undertaken to investigate if dopamine D_2 receptors are altered in aged mice treated with MPTP. Furthermore, our previous studies have shown the presence of high and low agonist affinity states of D_2 receptors; thus, changes in these high and low sites were also investigated in the striatum of aged control and MPTP treated mice.

METHODS

C57BL/6 mice at 21 months of age were given multiple injections of MPTP (total dose 90 mg/kg; i.p. over two days). Ten days after the last injection, control and MPTP treated mice brains were dissected, frozen on dry ice and stored in liquid nitrogen. Cryostat sections 10 microns thick were cut through the striatum and thaw-mounted onto gelatin coated slides. The slide-mounted sections were incubated in ^{3}H-sulpiride (S.A. 72.0 Ci/mMol, Dupont) in a 0.17M Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$ and 0.001% ascorbic acid (Gehlert et al. 1985). High and low affinity binding was determined by incubating sections in the presence of ADTN (2-amino-6, 7-dihydroxy-1, 2, 3, 4 tetrahydronaphthalene) either with or without 10^{-5}M Gpp(NH)p (Guanine-nucleotide). Non-specific binding was determined by incubating sections in the presence of 10^{-6}M haloperidol. Autoradiograms were obtained by apposing the labeled slide-mounted tissue sections to LKB ultrofilm. Quantitation was accomplished through the use of computer-assisted microdensitometry and by using tritium standards.

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RESULTS

The results of these studies show that, at the time period examined, MPTP does not significantly alter the total D_2 binding sites (Fig. 1 and 2) in either the lateral or medial parts of the striatum of the treated animals compared to age-matched controls. The number of binding sites are much higher in the lateral striatum than in the medial striatum in both control and MPTP treated mice. However, in the presence of ADTN and Gpp(NH)p, the number of binding sites were significantly increased in both the lateral and medial compartments of striatum in the MPTP treated group compared to the age-matched controls (Table 1).

DISCUSSION

In non-human species, the dopamine D_2 receptor has been shown to be particularly sensitive to neurotoxic manipulations. A decrease in nigral neurons leads to a decrease in striatal dopamine levels and presumably results in dopamine receptor supersensitivity. The results of this study show that the total D_2 binding, after allowing for non-specific binding, is not altered in the MPTP treated mice although our previous studies have shown decreased dopamine levels in the striatum three weeks after MPTP treatment (Gupta et al. 1985). It is possible that the cells may not be dying although there is a reduction in cell number and dopamine levels, hence membranes may still be present. Sershen et al. (1989) have shown that MPTP does not cause a change in ^{3}H-spiperone binding 12 days after MPTP treatment in the young mice despite a significant reduction in dopamine uptake. Our present findings, therefore, confirm and extend the observation that no change in total D_2 binding is seen in the aged mice ten days after MPTP treatment. However, the data in MPTP treated non-human primates shows a change in dopamine receptors. Although the present studies are very preliminary, incubation of sections in medium containing ADTN in the presence of Gpp(NH)p (which shifts all receptors to the low affinity state), the MPTP treated mice appear to show a significantly increased number of D_2 low affinity binding sites in both the medial and lateral compartments of the striatum compared to the age-matched controls.

The amount of [^{3}H]-sulpiride binding remaining in the presence of ADTN subtracted from specific binding would reflect the amount of high affinity D_2 sites present in the tissue sections. These did not appear to change following MPTP treatment, although there was a trend towards an increased number of high affinity receptors. The amount of specifically bound [^{3}H]-sulpiride remaining in the presence of ADTN reflects the amount of low affinity sites and these also did not change. It was not until we added the guanine nucleotide to the incubation medium that a significant increase in receptor binding was noted. Under these conditions, the high affinity sites should have been shifted to their low affinity conformation and little, if any high affinity binding should remain. It was precisely in this small population of sites that we could measure an increase in binding following exposure to MPTP. This small population of sites was apparently overwhelmed by the large number of high affinity D_2 receptors which are sensitive to guanine nucleotides. These are high affinity sites that appear to be insensitive to guanine nucleotides. Perhaps the receptors are uncoupled from the guanine nucleotide regulatory binding proteins or exist in a stage of development where they are not yet coupled to the regulatory proteins.

Table 1. Showing ^{3}H-Sulpiride Binding (Mean ± SE) in the Striatum (fmol/mg protein)

Groups	Lateral STR	Medial STR
Specific binding (Total - nonspecific)	25.82 ± 2.90	14.56 ± 1.40
Specific - ADTN	23.50 ± 2.97	12.16 ± 1.64
Specific - (ADTN + Gpp) in aged ctrl	0.56 ± 0.38	0.77 ± 0.34
Specific - (ADTN + Gpp) in aged treated	10.54 ± 3.06*	6.23 ± 1.67*

*significantly different from control

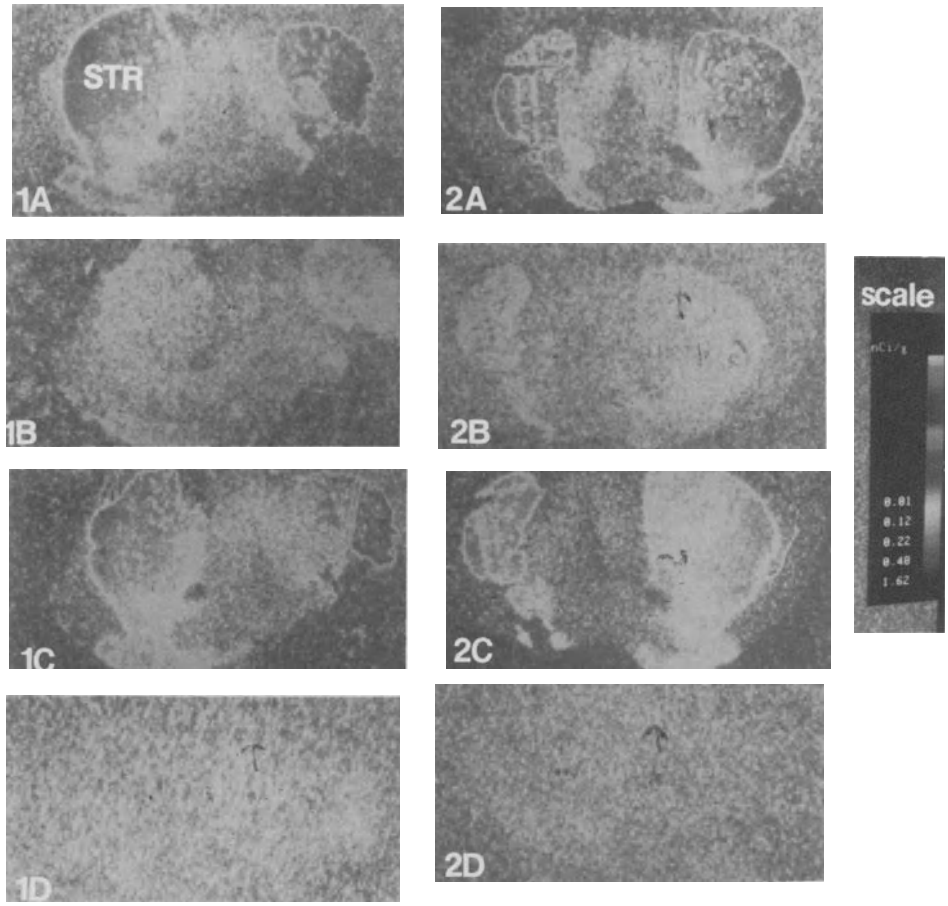

Fig. 1. Autoradiograms of sections through the striatum (STR) of a 21 month old C57BL/6 moused processed for the localization of ^{3}H-sulpiride binding for D2 receptors. Note the high density of grain distribution in the STR (A) representing total binding, binding in the presence of ADTN (B), ADTN + Gpp(NH)p (C), and in the presence of haloperidol (D).

Fig. 2. MPTP-treated mouse for D2 binding in the STR (A-D) as described in Fig. 1.

The latter interpretation seems more plausible and would indicate that at substantially longer time periods after MPTP exposure, we may see significant increases in high affinity D_2 receptor binding. The small increase in the guanine nucleotide insensitive, high-affinity, D_2 receptors we have measured following MPTP treatment would thus be preparative for further increases in the "mature" receptor which occurs at a latter time. In the present study, we have measured these receptors in a stage of transition. Due to a large variability in the normal aging animals, further studies are currently in progress to confirm the present findings and understand the mechanism of receptor alteration using different survival times following MPTP treatment.

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AGE-DEPENDENT EFFECTS OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) IN THE RAT

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INTRODUCTION

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes a Parkinsonian syndrome in humans and monkeys which is accompanied by degeneration of dopamine containing neurones in the substantia nigra pars compacta (SNC) and extensive loss of dopamine in the striatum (for review see Langston, 1987). Rats are much less sensitive to the neurotoxicity of MPTP than primates. Systemic administration of MPTP to rats did not produce neurotoxic effects (Chiueh et al., 1984) and intranigral administration failed to induce lasting damage to the SNC neurones (Chiueh et al., 1984; Bradbury et al., 1986) or lasting behavioural effects (Welzl and Lange, 1986). The neurotoxicity of MPTP seems to be age-dependent. It has been demonstrated that old mice suffer more extensive neuronal damage in the SNC than younger animals in response to MPTP administration (Gupta et al., 1986). The present experiment studied the age-dependent effects of unilateral intranigral administration of MPTP in the rat.

MATERIALS AND METHODS

Eight young adult (aged 4 to 5 months) and eight old (aged 22 to 24 months) male BD IX rats received a unilateral injection of 50 µg/1 µl MPTP (Research Biochemicals, Wayland, U.S.A.) into the SNC through chronic cannulae. Eight young and eight old control rats received a saline solution which was equimolar to the MPTP solution. The stereotaxic coordinates for the SNC injections corresponded to König and Klippel (1963) coordinates: A 2420, V -2.4, L 1.6. Automatic recordings of circling behaviour were made for 30 min on the 1st and 7th day after intranigral MPTP injection and in addition on the 7th day for 30 min after subcutaneous injection of apomorphine (0.5 mg/kg; Woelm Pharma, Eschwege, F.R.G.). Rats were killed 10 days after intranigral MPTP injection. Using washed membrane homogenates of the striatum of the injected hemisphere saturation analysis was performed for dopamine D-2 receptors with [^{3}H]spiperone; nonspecific binding was defined by sulpiride (for details of receptor binding assay see Lange, 1989). The number of binding sites (B_{max}) and the apparent equilibrium constant (K_D) were determined by Eadie-Hofstee analysis.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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RESULTS

Unilateral intranigral injection of MPTP induced ipsiversive circling towards the side of injection in young and old rats on the 1st day after injection (see fig. 1). Seven days after the MPTP injection, ipsiversive circling behaviour was still present in old rats whereas young adult rats did not circle preferentially in either direction. Young and old control rats with saline injections showed no asymmetry in circling behaviour. Old rats with unilateral MPTP injections showed contraversive apomorphine-induced circling 7 days after the injection; young rats did not circle contraversively after administration of apomorphine (see fig. 1).

In old rats the concentration (B_{max}) of D-2 receptors identified by specific [^{3}H]spiperone binding in the striatum of the injected hemisphere was higher after MPTP injection than after saline injection (see table 1). In young rats there were no B_{max} differences between experimental and control animals. K_D changes were not seen.

DISCUSSION

Circling behaviour in the rat can be produced by a lesion of one ascending nigrostriatal dopamine pathway and is thought to reflect an imbalance of dopaminergic activity in the

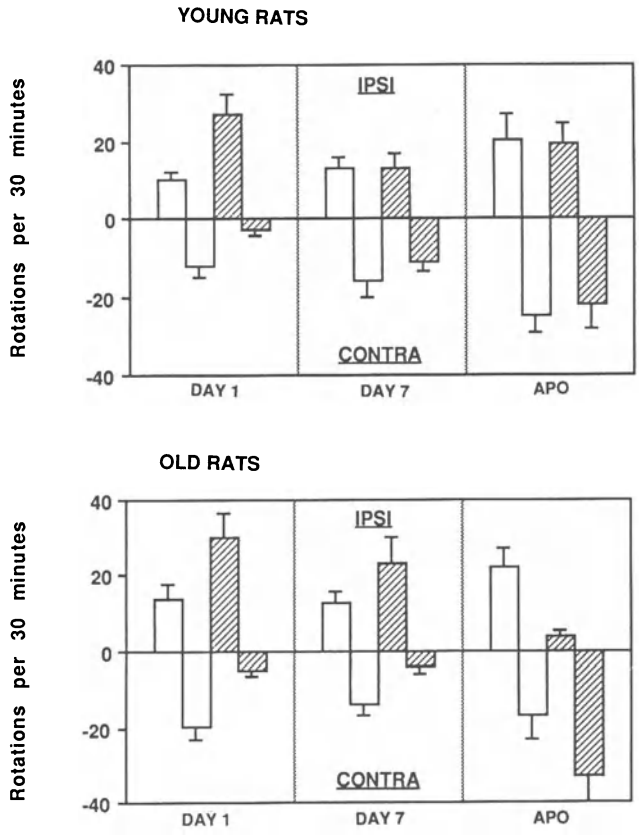

Figure 1. Ipsiversive (IPSI) and contraversive (CONTRA) circling on days 1 and 7 after unilateral intranigral injection of MPTP (hatched bars) and saline (plain bars) in young and old rats and apomorphine-induced (APO) circling on day 7 (means ± s.e.m.)

Table 1. Bmax (pmol/g wet weight of tissue) of dopamine D-2 receptors, identified by specific [^{3}H]spiperone binding, in the striatum of the injected hemisphere on day 10 after unilateral intranigral injection of MPTP or saline in young and old rats (means ± s.e.m.); in comparison with control group: * $p < 0.05$ (Mann-Whitney U test)

Treatment	Bmax in young rats	Bmax in old rats
Saline	23.1 ± 0.6	21.6 ± 0.6
MPTP	22.8 ± 0.7	28.8 ± 0.7 *

striata. The rat circles towards the side of the lesion, i.e. away from the hemisphere of higher striatal dopaminergic activity. After systemic administration of dopamine agonists, circling away from the hemisphere with the destroyed nigrostriatal pathway appears due to supersensitivity of the denervated striatal dopamine receptors (Ungerstedt, 1971; Creese et al., 1977).

The results of the present study suggest that in the rat the neurotoxic effects of MPTP increase with age. MPTP seems to have only a short-lasting depressive effect on nigral dopaminergic neurones in young rats since unilateral injection of MPTP into the SNC induced ipsiversive circling for only a short period. By contrast, in old rats ipsiversive circling was observed for at least one week after MPTP injection; administration of apomorphine induced contraversive circling and the concentration of D-2 receptors was increased in the striatum of the hemisphere with the lesion. These results indicate that MPTP has toxic effects in the nigrostriatal dopaminergic system of old rats and induces receptor supersensitivity in the denervated striatum.

Other studies also demonstrate that the neurotoxicity of MPTP increases with age. Neonatal rats appear to be resistant to the dopamine-depleting effects of MPTP whereas the toxin produces a substantial striatal dopamine depletion in young adult rats (Jarvis and Wagner, 1985). Older mice suffer greater neuronal degeneration in the substantia nigra and greater losses of striatal dopamine than younger mice (Gupta et al., 1986; Ricaurte et al., 1987; Walsh and Wagner, 1989).

The neurotoxic effects of MPTP have been shown to be dependent on its conversion to 1-methyl-4-phenylpyridinium ion (MPP+) by monoamine oxidase subtype B (MAO B; see Langston, 1987). No differences in the striatal dopamine depletion induced by intracerebroventricular administration of MPP+ are observed between older and younger mice (Irwin et al., 1988), suggesting that kinetic factors which increase the concentration of MPP+ at its target site, rather than increased sensitivity of older nigrostriatal neurones to MPP+, account for the age-related effects of MPTP. A possible explanation for the age-dependency of MPTP toxicity is the increase of MAO B activity in the brain with age. In the rat central MAO B activity increases over at least the first two years of life (Benedetti and Keane, 1980). In the mouse MAO B activity in the brain increases with age (Walsh and Wagner, 1989), striatal MPP+ concentrations increase directly with the age of the animals injected with MPTP (Langston et al., 1987) and a significant correlation between MAO B activity and the degree of lesion induced by MPTP is observed (Walsh and Wagner, 1989).

CONCLUSION

The present study suggests that the neurotoxic effects of MPTP are age-dependent in the rat. In old rats MPTP destroys dopaminergic neurones in the substantia nigra and induces receptor supersensitivity in the striatum, whereas in young rats it has only a short-lasting depressive effect on nigral dopaminergic neurones.

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MORE RAPID DEVELOPMENT OF SUPERSENSITIVITY OF D-2 RECEPTORS THAN D-1 RECEPTORS AS EXAMINED BY ROTATIONAL BEHAVIOR

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INTRODUCTION

Injection of a dopamine agonist such as apomorphine induces rotation of the rat given 6-hydroxydopamine (6-OHDA) in the nigrostriatal pathway, because destruction of dopaminergic pathway causes supersensitivity of the dopaminergic receptors innervated by these fibers (Ungerstedt, 1971; Creese et al., 1977). This rotation is reported to be induced by both DA D-1 and D-2 agonists. However, whether the rotation induced by DA agonists is due to the distinct action on D-1 and D-2 receptors or synergic interaction between D-1 and D-2 receptors is unknown (Herrera-Marschitz and Ungerstedt, 1984, 1985: Arnt and Hyttel, 1984, 1985; Arnt and Perregaard, 1987; Morelli et al., 1987; Karlsson et al., 1988; Casas et al., 1989). In addition, a little is known regarding the time course of development of supersensitivity of distinct D-1 and D-2 receptors after lesion of the dopaminergic pathway. We examined the role of D-1 and D-2 receptors in rotational behavior and the time course of appearance of supersensitivity of D-1 and D-2 receptors using selective D-1 and D-2 agonists as well as antagonists.

METHODS

Male Wistar rats (150-200 g) were used. Under anesthesia with chloral hydrate (300 mg/kg, i.p.), 8 μg of 6-OHDA (calculated as the base) dissolved in physiological saline containing 0.3% ascorbic acid, was injected into the pars compacta of the left substantia nigra (A: -4.4 from bregma; L: 1.2; H: -7.8 mm from the brain surface) through the cannula at the rate of 1 μl/min and the cannula was kept there for a further 2 min after termination of the injection. One week later following 6-OHDA treatment, rotation contralateral to the injection site was induced by DA agonists such as apomorphine (0.1 mg/kg, s.c.), SKF 38393 (2 mg/kg, s.c.) and quinpirole (0.1 mg/kg, s.c.). The number of 360 degrees turns was recorded using an automated rotometer for every 5 min. SCH 23390 (0.5 mg/kg, s.c.) was injected 30 min before the application of apomorphine. After all studies were completed, the effects of apomorphine were reexamined and the data were obtained from the animals which showed a more than 400 rotation for 90 min and two-peak pattern of rotation.

Catecholamine fluorescence in the nigrostriatal pathway was examined in some animals after completion of the experiment. The animal's heart was

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perfused with FAGLUPAGAS SOLUTION (Imai et al., 1982; 4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% picric acid, 2% glyoxylic acid), and then the brain removed was placed in FAGLUPAGAS SOLUTION containing 15% sucrose for 24 hr. Then, 30-μ thick frontal sections were made to observe catecholamine fluorescence under a fluorescence microscope.

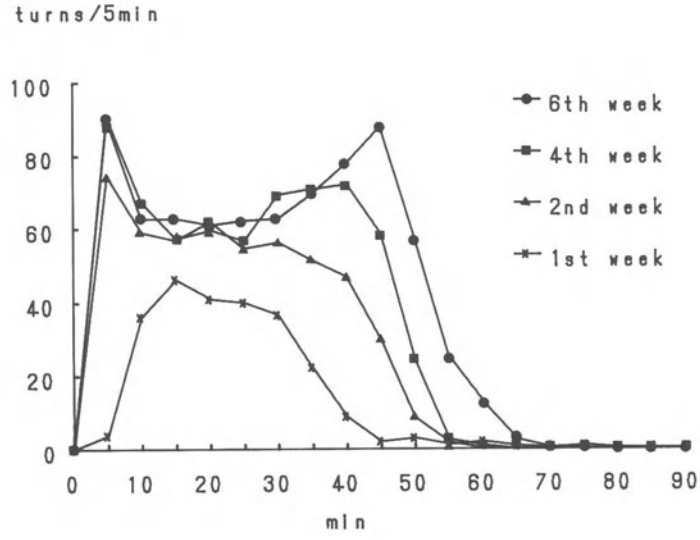

Fig. 1. Number of rotation for 5 min induced by apomorphine (0.1 mg/kg s.c.) 1, 2, 4 and 6 weeks after unilateral lesion of the substantia nigra.

RESULTS

Effects of apomorphine and D-1 antagonist

When apomorphine was injected one week after lesion of the substantia nigra, one peak of the rotation was seen 10-20 min after the injection. Two weeks after lesioning, the peak shifted 5 min after the injection. Three to 4 weeks later, a two-peak pattern with apomorphine was observed with the first peak at 5 min and the second between 30 and 60 min (Fig. 1). The total number of rotations gradually increased until 3 weeks after lesioning and thereafter reached a plateau (Table 1).

Injection of SCH 23390 30 min prior to the injection of apomorphine inhibited the appearance of the second peak of the rotation induced by apomorphine, although the first peak was still obtained. The duration of the rotation with apomorphine was not affected by pretreatment with SCH 23390.

Table 1. Total Number of Rotation (3 hr) induced by Apomorphine, SKF 38393 and Quinpirole 1, 2, 3, 4 and 6 Weeks after Unilateral Lesion of the Substantia Nigra

Drugs	Doses (mg/kg s.c.)	Weeks after Lesioning				
		1	2	3	4	6
Apomorphine	0.1	245	501	637	630	743
SKF 38393	2	153	985	1952	2370	2136
Quinpirole	0.1	370	1166	1378	1343	1214

Effects of SKF 38393 and quinpirole

Injection of SKF 38393 did not induce the rotation one week after lesioning, and did produce a long-lasting rotation 2-6 weeks later: the rotation lasted for 3 hr without the obvious peak. By contrast, quinpirole induced rotation one week after lesioning and more long-lasting rotation for over 3 hr without the peak 2 weeks later on (Table 1).

Catecholamine fluorescence in the substantia nigra

When catecholamine fluorescence was examined 4-6 weeks after lesioning with 6-OHDA, the number of neurons with the fluorescence in the pars compacta of substantia nigra given 6-OHDA was reduced to less than 10% of those in the untreated side.

DISCUSSION

In accordance with the results obtained by Herrera-Marschitz and Ungerstedt (1984, 1985), the two-peak pattern of rotation was observed with apomorphine 2 weeks after lesion of the nigrostriatal pathway. Furthermore, SCH 23390, a D-1 antagonist, inhibited the appearance of the second peak, but not the first peak, of the rotation induced by apomorphine, being in agreement with the findings by Herrera-Marschitz and Ungerstedt (1985). SKF 38393, a D-1 agonist, induced the long-lasting rotation, although the obvious second peak was not seen with this drug, coinciding with the findings obtained by Arnt and Hyttel (1984, 1985). Therefore, the second peak of the rotation seen between 30 and 60 min after injection of apomorphine is considered to be induced by activation of D-1 receptors, as described by Herrera-Marschitz and Ungerstedt (1985) and Arnt and Hyttel (1985).

The present study also confirmed the findings by Arnt and Hyttel (1985) that quinpirole, a D-2 agonist, induced rotation and the first peak with apomorphine was modified by spiroperidol, a D-2 antagonist, but not by SCH 23390, a D-1 antagonist. Therefore, the first peak of the rotation induced by apomorphine at 5 min is considered to be due to activation of D-2 receptors, although activation of D-1 receptors appears to be required for full expression of the first peak.

The time course of the effects of apomorphine was examined every week for 6 weeks after lesioning. The drug was found to induce one peak

of rotation 10-20 min after injection one week after lesioning and then two peaks at 5 min and between 30 and 60 min 2 to 6 weeks later. Similarly, quinpirole induced rotation one week after lesioning, but SKF 38393 did so two weeks later. Therefore, supersensitivity of D-2 receptors is suggested to develop more rapidly than D-1 receptors.

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FEATURES OF THE DOPAMINERGIC NEUROTOXICITY OF METHAMPHETAMINE: A COMPARISON WITH MPTP

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The amphetamines and cocaine are powerful CNS stimulants and common drugs of abuse. It is widely accepted that both the amphetamines and cocaine owe their stimulant properties to an interaction with brain dopaminergic systems. As an example, the administration to mice of d-amphetamine or of cocaine results in a large increase in locomotor activity of a relatively short duration which can be blocked by a number of dopamine receptor antagonists. Moreover, both cocaine and d-amphetamine cause an ipsilateral rotation in rats with a unilateral lesion of the nigrostriatal pathway. This rotational behavior also can be blocked by pretreatment of the rats with dopamine receptor antagonists. These data might suggest that these behavioral responses to d-amphetamine and cocaine are due to a common pharmacological mechanism. However this is not the case. It is generally accepted that d-amphetamine and several of its structural analogs are CNS stimulants because they facilitate the release of dopamine. In contrast, cocaine and several structural analogs are thought to be stimulants because they block the reuptake (uptake) of synaptically released dopamine. The differences in their mode of action can be demonstrated by the observations that treatment of mice with the tyrosine hydroxylase inhibitor α-methyl-para-tyrosine prevents the increased locomotor activity caused by the amphetamines but has no effect on that caused by cocaine. In contrast, treatment of mice with reserpine prevents the increased activity caused by cocaine and other dopamine uptake inhibitors but has no effect on that caused by the amphetamines (Ross, 1978). These data suggest that the behavioral effects of d-amphetamine and related compounds are due to their release of newly synthesized dopamine while the behavioral effects of cocaine and other dopamine uptake inhibitors are due to their blockade of uptake of dopamine which is derived mainly from the reserpine-sensitive storage pool.

In addition to being powerful CNS stimulants, the amphetamines are also potent dopaminergic neurotoxins. In contrast, cocaine is not known to possess dopaminergic neurotoxicity. Treatment of experimental animals including rats and mice with d-amphetamine or methamphetamine, at doses somewhat higher than those which elicit increases in locomotor activity, causes long-lasting and severe effects on the dopaminergic nigrostriatal pathway (Hotchkiss and Gibb, 1980; Fuller and Hemrick-Luecke, 1984; Preston et al., 1985). For example, mice treated with methamphetamine exhibit a large and long-lasting decrement in the neostriatal content of dopamine and its metabolites and a parallel decrease in neostriatal tyrosine hydroxylase activity and in the capacity of neostriatal synaptosomes to accumulate ^{3}H-dopamine (Steranka and Sanders-Bush, 1980; Sonsalla and Heikkila, 1988;

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Sonsalla et al., 1989). Rats treated with methamphetamine also exhibit a decrement in their neostriatal content of serotonin and in neostriatal tryptophan hydroxylase activity (Hotchkiss and Gibb, 1980).

In the present report we will summarize some of our observations on the dopaminergic neurotoxicity of methamphetamine in mice. We will compare these observations to those obtained with another widely used and better-known dopaminergic neurotoxin, namely 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The results will clearly demonstrate that while these two dopaminergic neurotoxins have some features in common, their mechanisms of action are more dissimilar than they are similar.

In the last several years, we have carried out research with a number of strains of mice, including C57 black mice and Swiss-Webster mice obtained from a number of different suppliers. In these studies we observed that MPTP was considerably more potent as a dopaminergic neurotoxin in C57 black mice than it was in several other strains of mice tested. For example, C57 black mice treated with 4 i.p. injections of MPTP at 20 mg/kg per injection at 2 hr intervals exhibited severe dopaminergic deficits including a loss of neostriatal dopamine of approximately 90% (Sonsalla and Heikkila, 1986). Under identical conditions, MPTP caused approximately a 60% decrement in neostriatal dopamine content in CF-W mice. We speculated that C57 black mice might also be particularly sensitive to methamphetamine. It became apparent that our speculation was without merit when we found that C57 black mice were considerably less sensitive than CF-W mice to the dopaminergic neurotoxicity of methamphetamine (Sonsalla and Heikkila, 1988). In retrospect, we should perhaps not have been surprised by these results. A glance at the summary of some observations obtained in mice treated with MPTP and with methamphetamine, which are presented in Table 1, indicates that their mechanisms of action are completely different. Perhaps the major similarity is that the dopaminergic neurotoxicity of both MPTP (Javitch et al., 1985) and of methamphetamine (Schmidt and Gibb, 1985) can be prevented by pretreatment of the experimental animals with dopamine uptake inhibitors. This most likely is due to the fact that the uptake inhibitors prevent the uptake and concentration by the dopaminergic neuron of the major metabolite of MPTP, namely the 1-methyl-4-phenylpyridinium species (MPP^+), or of methamphetamine itself.

A major point of difference between the two is that inhibitors of monoamine oxidase-B (MAO-B) are protective against MPTP-induced but not against methamphetamine-induced dopaminergic neurotoxicity. For example, mice treated with deprenyl prior to MPTP administration do not exhibit the dopaminergic deficits normally caused by MPTP. In parallel, deprenyl and other MAO-B inhibitors markedly lower the formation of MPP^+ from MPTP. It follows that this protection against MPTP-induced dopaminergic neurotoxicity by MAO-B inhibitors is due to their attenuation of the formation of MPP^+, which is an extremely potent cytotoxin and which is believed to be the actual neurotoxic species (Markey et al., 1984; Heikkila et al, 1984). It has been suggested that inhibitors of MAO, particularly of MAO-A, may in fact potentiate the neurotoxicity of the amphetamines by raising cellular levels of dopamine which are available for release.

Administration of α-methyl-para-tyrosine to mice not only prevents methamphetamine-induced increases in locomotor activity but also prevents methamphetamine-induced dopaminergic neurotoxicity (Schmidt et al., 1985). In contrast, treatment with α-methyl-para-tyrosine has no such protective effect on MPTP-induced dopaminergic neurotoxicity (Fuller and Hemrick-Luecke, 1985). These data suggest that newly synthesized dopamine is

Table 1. A Summary of the actions of MPTP and methamphetamine (METH) in mice.

	MPTP	METH
Toxic to Nigrostriatal Dopaminergic Neurons	Yes	Yes
Actual Toxic Species	MPP^+	?
Possible Mechanism of Toxicity	Inhibition of Mitochondrial Respiration?	?
Protection Against Neurotoxicity by:		
MAO-A Inhibitors	No	No
MAO-B Inhibitors	Yes	No
DA Uptake Inhibitors	Yes	Yes
Inhibitors of DA Synthesis	No	Yes
DA Receptor Antagonists	No	Yes
NMDA Receptor Antagonists	No	Yes

required for methamphetamine but not for MPTP to exert its dopaminergic neurotoxicity. Administration of dopamine receptor antagonists to mice also results in protection against the dopaminergic neurotoxicity of methamphetamine but not of MPTP (Buening and Gibb, 1974; Fuller and Hemrick-Luecke, 1985). This protection by dopamine receptor antagonists indicates that the interaction of dopamine with its receptor is required for the neurotoxic actions of methamphetamine. What happens after methamphetamine causes the release of dopamine and dopamine interacts with its receptor has been a matter of considerable speculation. We previously reported that non-competitive antagonists of the N-methyl-D-aspartate (NMDA) receptor including MK-801, phencyclidine and ketamine could protect against methamphetamine but not MPTP-induced dopaminergic neurotoxicity in mice (Sonsalla et al., 1989). In more recent studies, we have found that competitive antagonists of the NMDA receptor also provide protection. In other experiments, NMDA administration potentiated the neurotoxic actions of methamphetamine. All of these data indicate that stimulation of the NMDA receptor plays an important role in the neurotoxic process. Interestingly, competitive and non-competitive antagonists of the NMDA receptor have recently been found to be protective against several types of neurodegeneration.

Exactly how stimulation of the DA receptor and stimulation of the NMDA receptor are important features in the neurotoxicity caused by methamphetamine and related compounds is unclear. Perhaps there is a role for free radicals and oxidative stress as has been suggested by some. It is possible that there is a role for intracellular calcium. Perhaps the release of dopamine by methamphetamine leads to an alteration in the amount of glutamic acid which is released, which in turn controls some as of yet undefined process. We and others are currently trying to determine the exact cellular

events which lead from the enhanced release of dopamine to the neurotoxic event. If a facilitated release of dopamine caused by methamphetamine can lead to neurotoxicity, it is not unreasonable to speculate that an enhanced dopamine release caused by some undefined mechanism might play a critical role in some naturally occurring neurodegenerative process.

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ALTERATION OF [^{3}H]Ins(1,4,5)P$_3$ AND [^{3}H]PDBu BINDING SITES IN BRAINS OF PATIENTS WITH PARKINSON'S DISEASE AND MULTIPLE SYSTEM ATROPHY

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INTRODUCTION

The roles of membrane inositol phospholipid turnover in intracellular signal transduction have been vigorously researched. Stimulation of several receptors by neurotransmitters, hormones, and growth factors leads to accelerated turnover of polyphosphoinositides. Hydrolysis of phosphatidylinositol 4,5-bisphosphate by activated phospholipase C generates inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) and 1,2-diacylglycerol, both of which act as second messengers. The former increases intracellular Ca^{2+} and the latter activates protein kinase C (PKC) (Nishizuka, 1986).

Tumor-promoting phorbol esters specifically bind to and activate PKC and their binding sites are co-purified with PKC. Thus one can localize PKC by monitoring the binding of phorbol esters. Intracellular mobilization of Ca^{2+} by Ins(1,4,5)P$_3$ is thought to be mediated through specific receptors. Direct ligand-binding studies using radio-labeled Ins(1,4,5)P$_3$ revealed high-affinity and selective binding sites for Ins(1,4,5)P$_3$. Ins(1,4,5)P$_3$ and phorbol ester binding sites abound in the brain (Worley et al., 1987).

Parkinson's disease (PD) is a common neurodegenerative disease and histopathologically characterized by degeneration of nigrostriatal dopamine neurons and the presence of Lewy bodies. Multiple system atrophy (MSA) is characterized by neuronal cell loss and gliosis occuring in the olivopontocerebellar, striatonigral (putamen-substantia nigra), and autonomic nervous systems. Here we have biochemically examined possible alterations of second-messenger systems coupled with the phosphoinositide cycle in the basal ganglia of autopsied brains from patients with PD or MSA, by monitoring the binding sites of [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]4-β-phorbol 12,13-dibutyrate ([^{3}H]PDBu), the latter of which is thought to label PKC.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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MATERIALS AND METHODS

Patients

Brains were obtained at autopsy from 20 patients with PD (11 men; 9 women; age, 69 ± 1.2 years), from 10 patients with MSA (4 men; 6 women; age, 65 ± 1.7 years), and from 32 control subjects (22 men; 10 women; age, 62 ± 2.5 years) with no known neuropsychiatric disease. When comparing the binding activities in these three groups, we used matched controls with respect to age and post-mortem delay.

Binding Assay

[^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding assays were performed as previously described (Kitamura et al., 1989; Nishino et al., 1989). For the [^{3}H]PDBu binding assay, when the tissue was homogenized in the absence of the chelators, the majority of [^{3}H]PDBu binding activity was retained in the crude membranes (Nishino et al., 1989). Therefore, we used crude membranes prepared with Tris-HCl buffer. Protein assay was made by the method of Lowry et al. (1951).

Calculation and Statistics

Each sample was assayed in triplicate, and the significance of the difference between groups was analyzed by the two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Multiple System Atrophy

[^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu bindings were significantly decreased in the putamen of MSA patients as compared with their levels in the controls, the binding activity being 44 % and 29 %, respectively, of the latter ($P < 0.01$, $P < 0.001$). In the caudate nucleus of patients, there was no significant change in either [^{3}H]Ins(1,4,5)P$_3$ or [^{3}H]PDBu binding as compared with that of controls (Table 1). We confirmed histopathologically that severe neuronal cell loss and gliosis were present in the putamen of our patients, but not in the caudate nucleus (submitted for publication). Loss of both binding sites in the putamen of the patients with MSA suggests that [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding sites are predominantly localized on neurons in the basal ganglia.

Parkinson's Disease

As previously described (Kitamura et al., 1989; Nishino et al., 1989) [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu bindings were significantly decreased in the caudate nucleus of these patients as compared with those in controls, the binding activity being 62 % and 68 % of controls, respectively ($P < 0.05$). No significant difference in either binding activity, however, was found between controls and 5 patients clinically diagnosed as stage III - IV PD according to Hoehen and Yahr. In patients diagnosed as stage V, [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding activities were much more reduced, by 51 % and 47 %, respectively ($P < 0.01$), as seen in Table 2. It is generally accepted that loss of at least 80 % of

Table 1. [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu Binding in the Caudate Nucleus (CN) and Putamen (PUT) of Patients with MSA

		Controls (10)	Patients (10)
[^{3}H]Ins(1,4,5)P$_3$ bound (fmol/mg protein)	CN	116±13	122±19
	PUT	103±13	45±9**
[^{3}H]PDBu bound (pmol/mg protein)	CN	12.7±2.3	9.8±1.4
	PUT	10.1±1.2	3.0±0.5***

Values expressed as mean±SE. Numbers in parentheses are numbers of samples. Ligand concentrations in incubation media were 2nM [^{3}H]Ins(1,4,5)P$_3$ and 10nM [^{3}H]PDBu. Statistical significance of difference between 2 groups: $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t* test, two-tailed).

Table 2. [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu Binding in the Caudate Nucleus of Patients with Parkinson's Disease (PD)

[^{3}H]Ins(1,4,5)P$_3$ (fmol/mg protein)	Control (N=20)	All PD (N=16)	Yahr III,IV (N=5)	Yahr V (N=11)
	127±14	80±14*	117±8	63±17**
[^{3}H]PDBu (pmol/mg protein)	Control (N=23)	All PD (N=18)	Yahr III,IV (N=5)	Yahr V (N=13)
	2.2±0.2	1.5±0.2*	2.4±0.2	1.2±0.2**

Values expressed as mean±SE. Numbers in parentheses are numbers of samples. Ligand concentrations in incubation media were 2nM [^{3}H]Ins(1,4,5)P$_3$ and 5nM [^{3}H]PDBu. Statistical significant difference between 2 groups: $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t* test, two-tailed).

the nigral neurons along with striatal dopamine content is necessary for frank symtoms of PD to appear. Since no changes in each binding site were observed in the caudate nucleus of patients of stage III - IV, [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding sites may not be solely localized on nigrostriatal dopamine neurons. Reportedly, lesion studies in rats suggest that [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding sites occur in presynaptic terminals in the striatonigral projection (Worley et al., 1987). Furthermore, we found about 70 % decrease in both binding sites in the caudate nucleus of patients with Huntington's disease, a disease characterized by degeneration of striatonigral neurons, namely, GABA-containing neurons (in preparation). Changes in

striatonigral and -pallidal neurons such as GABA-, substance P-, and enkephalin-containing neurons have been reported in cases of PD (Agid et al., 1987). Changes in these neural systems other than dopamine neurons may possibly be related to the decrease in these two binding sites in patients of stage V . We cannot, however, exclude the possibility that the decrease of [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding sites may reflect the loss of or pathologic changes in striatal intrinsic neurons, since our patients showed low choline acetyltransferase activities (Nishino et al., 1988).

CONCLUSIONS

1) [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding activities were markedly decreased in the putamen of patients with MSA compared with those in age-matched controls, while there was no change in either binding in the caudate nucleus. These findings were consistent with the histopathological findings of MSA that the putamen is much more affected by neuronal cell loss than the caudate nucleus.

2) [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding activities were significantly decreased in the caudate nucleus of patients with PD clinically diagnosed as stage V according to Hoehn and Yahr, but not in patients diagnosed as stage III - IV. Our findings suggest that [^{3}H]Ins(1,4,5)P$_3$ and [^{3}H]PDBu binding sites are not solely localized on nigrostriatal dopamine neurons and that loss or pathologic changes of striatonigral and -pallidal neurons including striatal intrinsic neurons may be related to the decrease in number of these two binding sites in patients of stage V.

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THE MECHANISM OF ACTION OF NERVE GROWTH FACTOR

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Nerve growth factor (NGF) is a trophic peptide, discovered by Levi-Montalcini and Hamburger some 40 years ago[1,2]. The complete structure of NGF is known[3], as is the general nature of its receptor(s)[4], and the character of the gene encoding it[5]. It is also clear that NGF has a large number of effects on its various target cells, some of which depend on the cell type itself, and some of which depend on the conditions under which it is applied. Generally speaking, there are two types of actions observed after NGF interacts with its cell membrane receptor. Some of its actions occur at the membrane, are relatively rapid, and do not require the synthesis of RNA. Typical of these are alterations in cell membrane structure[6], stimulation of the transport of small molecules[7], and increases in the levels of cyclic nucleotides[8]. A second type of action occurs at the nucleus and involves changes in the transcription of specific genes. Typical of these are increases in the synthesis of neurotransmitter-metabolizing enzymes[9], and the generation of neurites[10]. The best evidence[11] indicates that although NGF influences the transcription of specific genes, NGF itself does not act directly on the nucleus; it is generally believed that the signal initiated by the combination of NGF with its receptor at the membrane is carried to the nucleus by a series of second messengers, the nature of which are as yet not completely clear.

There are two types of NGF receptors on most NGF-responsive cells[4], the high-affinity and the low-affinity. These are also called the 'slow' and the 'fast', respectively, to indicate that the difference in the binding is due largely to the off-time, the speed with which bound NGF dissociates from the receptor. Most measurements indicate that NGF is bound to the slow receptor with an affinity 100x that of the fast receptor. The low-afffinty receptors comprise about 90% of the total receptors on most responsive cells, but it is the high-affinity receptors that appear to be responsible for the action of NGF on its target cells. Cells which have only low-affinity receptors do not respond physiologically to NGF[12,13]. The exact relationship between the two classes of receptors is not known, but it is known that they both contain the same core protein[14]. It is generally found that the molecular weights of the two classes of receptors are different, the high-affinity having a higher molecular weight[15]. There is some support for the suggestion that the high-affinity of the high-affinity receptor is obtained by the recruitment of an accessory protein.

The combination of NGF with its receptor leads to changes in the levels of a number of second messengers. Among these are cAMP[8,16], intracellular calcium[17,18], phosphoinositide[19], arachidonic acid[20], and inositol phosphate glycan[21]. In spite of the now-abundant evidence that the metabolism of a number of second messengers is altered by NGF, it is not yet clear which of these messengers, if any, are the mediators of NGF action in the cell. It can be inferred, however, from the changes in cAMP levels, intracellular

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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calcium concentrations, and phosphoinositide turnover, that the activities of several protein kinases are affected.

Indeed, it has been shown directly that NGF treatment changes the phosphorylation of a number of proteins and the activity of a number of protein kinases in the cell[22,23]. Work from several laboratories has indicated that there are changes in the phosphorylation of proteins in the cytoplasm, the cytoskeleton, the ribosomes, and the nucleus. In many cases these changes have been studied in detail and the specific kinases responsible for the changes in phosphorylation have been identified[24,25]

Studies in this laboratory have focused on three different systems. The first involves the phosphorylation of a nuclear protein, called by us SMP[22]. SMP is a basic protein with a molecular weight of some 30,000 that is a member of the low-mobility class of non-histone proteins. The NGF-induced increase in the phosphorylation of SMP is rapid, long-lasting, and occurs exclusively on serine residues. A cell-free system reflecting this increase has been prepared[26]; isolated nuclei from NGF-treated PC12 cells, incubated with radioactive ATP, phosphorylate SMP more vigorously than do comparable nuclei from control cells. Unfortunately, all further attempts to fractionate these nuclei and to isolate and study the kinase involved have been unsuccessful. A study of the details of this NGF-induced increase in phosphorylation could be particularly rewarding because there is a possibility that SMP is a transcription factor. In any case, the study should shed light on how the NGF signal is transmitted to the nucleus and perhaps on how NGF regulates gene transcription.

A second, more informative system involves the phosphorylation of a cytoplasmic protein with a molecular weight of 100,000. The phosphorylation of this protein, called by us Nsp100, decreases in cells treated with NGF. The decrease is rapid, transient, and occurs exclusively on threonine residues[27]. Nsp100 kinase has been purified and its characteristics detailed[28]. It is now known[29] that Nsp100 is elongation factor 2, a component of the protein synthesis machinery. It is equally clear that the kinase phosphorylating EF-2 is the kinase described by Nairn, Bhagat, and Palfrey[30] and originally called calcium/calmodulin kinase III. The mechanism by which the activity of EF-2 kinase is decreased is clear; EF-2 kinase is itself phosphorylated. Evidence from this laboratory[31] indicates that EF-2 kinase is phosphorylated by kinase C, while studies from another group[32] implicate cAMP-dependent kinase in this regard.

A third system that we have used concerns the phosphorylation of the ribosomal protein S6. The phosphorylation of S6 in PC12 cells is increased by treatment of the cells with NGF[23]. The increase is rapid and serine-specific. The S6 kinase stimulated by NGF has been purified[32] and its action studied. The mechanism by which the activity of this S6 kinase is increased has been explored[32] and it seems clear that this kinase, also, is subject to phosphorylation. The best evidence presently available indicates that the NGF-sensitive S6 kinase from PC12 cells is stimulated by phosphorylation by a cAMP-dependent kinase[32].

Even a partial listing of the proteins whose phosphorylation is known to be altered by treatment of various target cells with NGF is now quite extensive. It is known that the phosphorylations of tyrosine hydroxylase, synapsin, vinculin, microtubule-associated proteins, and neurofilament proteins are also altered by NGF treatment[24,25]. Indeed, it is possible, even likely, that the phenotypic changes produced by nerve growth factor treatment are a summation of all the changes in phosphorylation, and consequently, in function, of these different cellular proteins.

Based on the evidence from this and other laboratories it can be suggested that these various phosphorylative changes are due to the activation, by NGF, of a series of parallel phosphorylative cascades in the cell. One of these, for example, might involve the activation of kinase C, the subsequent phosphorylation of EF-2 kinase, and the phosphorylation of EF-2. A second could involve the activation of cAMP-dependent kinase, the phosphorylation of S6 kinase, and, finally, of S6. But although there is good evidence for the activation of these various phosphorylative cascades, there is no information as to how they are initiated at the NGF receptor. This is a particularly intriguing question since the NGF receptor, unlike the receptors for many other ligands, is clearly not, itself, a

kinase. Indeed, the now-known sequence of the receptor does not contain any kinase-like domain or any ATP-binding site[33].

There is presently no answer to this puzzle, but a newly-developed tool may eventually lead to some information on this score. This tool is the kinase inhibitor K-252a. K-252a is an alkaloid-like molecule first isolated from the culture medium of Nocardiopsis sp. The first data on this compound indicated that it was a strong inhibitor of protein kinase C[34], although subsequent studies suggest that it is not specific for this kinase[35,36]. The material does have the interesting property of inhibiting all the actions of NGF on PC12 cells, but not the comparable actions of other ligands[37,38]. That is, at 200 nM, K-252a will prevent NGF-induced neurite outgrowth, but not the outgrowth produced by fibroblast growth factor. It will inhibit ornithine decarboxylase induction by NGF in PC12 cells, but not ornithine decarboxylase induction by epidermal growth factor (EGF) in these same cells. In short, every action of NGF on PC12 cells is selectively blocked by K-252a. It has also been shown that K-252a blocks NGF actions in normal cells as well[39,40]. Since K-252a is clearly an inhibitor of kinases, and since its action must be very close to the receptor itself, it is reasonable to postulate that K-252a inhibits a unique kinase involved specifically in the actions of NGF.

In spite of the accumulating information about the intracellular events mediating NGF action, it is still not at all clear what sequences of steps are involved in the more complex phenotypic changes wrought by NGF. For example, we do not know, in PC12 cells, the molecular events in neurite outgrowth, or in synapse formation, or in the development of electrical excitability. But work from this laboratory may provide some insight into how NGF instructs the PC12 cells to stop dividing. These cells have the interesting property of responding to both NGF and EGF. NGF is, of course, a differentiating agent in this system, and EGF, a powerful mitogen for many cells, is a mild mitogen for PC12[41]. Since EGF encourages the cells to divide and NGF stops them from dividing, we asked what happens when the cell is exposed to both. The answer is that NGF-treated cells become unresponsive to EGF. The reason they become unresponsive is that NGF causes a down-regulation of the EGF receptor[41,42]. Although the molecular mechanism by which this down-regulation takes place is as yet unknown, the interpretation of the experiment seems clear. At least part of the mechanism by which NGF causes the cells to stop dividing could be that it blinds them to the mitogen EGF. In more general terms, it may be that one way in which NGF instructs its target cells to stop dividing and differentiate is by preventing their interaction with the mitogens that normally control their growth.

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NERVE GROWTH FACTOR PROMOTES SURVIVAL OF CULTURED CHOLINERGIC NEURONS FROM NUCLEUS BASALIS OF MEYNERT OF 2-WEEK-OLD RATS

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INTRODUCTION

One of the possible causes of Alzheimer's disease is thought to be a lack of nerve growth factor (NGF), a molecule that plays an important role in the neuronal differentiation and cell survival of basal forebrain cholinergic neurons.[1] These neurons are scattered over the following three areas: medial septum nucleus, diagonal band of Broca (vertical and horizontal limbs), and nucleus basalis of Meynert. The action of NGF on cells in the former two regions has already been investigated in detail.[2-6] However, its action on the cholinergic neurons in the nucleus basalis of Meynert has not been investigated extensively. The reason for this sparse research might be that, in the rat and many other experimental animals, detection and isolation of the cholinergic neurons of this nucleus are difficult. In this communication, we measured the number of viable cholinergic neurons in cultures of the nucleus with or without NGF.[7,8] This is the first report on the effects of NGF on the functions of cultured cholinergic neurons of the nucleus basalis of Meynert from postnatal 2-week-old rats.

RESULTS AND DISCUSSION

NGF has been detected at high levels in the brain regions of the basal forebrain cholinergic pathway. Fig.1 illustrates the mode of NGF action in the pathway. The target areas, hippocampus and neocortex, contain high level of NGF mRNA. The cholinergic neurons in the basal forebrain including nucleus basalis of Meynert have specific NGF receptors, which can be detected autoradiographically by high affinity ^{125}I-NGF binding, and also by immunocytochemistry with a specific anti-NGF receptor monoclonal antibody, MC192.[7]

We have already demonstrated that NGF prevents the time-dependent decline of choline acetyltransferase (ChAT) activity seen in cultures without NGF.[3,4] Now we examined the direct

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effect of NGF on the cholinergic functions of cultured neurons from the nucleus basalis of Meynert. The area definitely containing the nucleus was dissected out from 400 μm-thick brain slices prepared from the forebrain of 2-week-old Wistar rats. The dissected portions of the slices were digested by papain as described previously. After 5 days in culture, without or with NGF at the final concentration of 100 ng/ml, total ChAT activity and the number of viable acetylcholinesterase (AChE)-positive neurons were determined. As shown in Fig. 2, the number of viable AChE-positive neurons of NGF-treated cultures was nearly two fold higher than that of NGF-untreated cultures. Cellular ChAT activity was calculated by dividing for each well total ChAT activity by AChE-positive neuron number. Cellular ChAT activities without and with NGF were 241 and 298 fmol/min/cell, respectively. More extensive and denser cholinergic neurites were observed after the AChE-staining of nucleus basalis neurons cultured with NGF than without it.

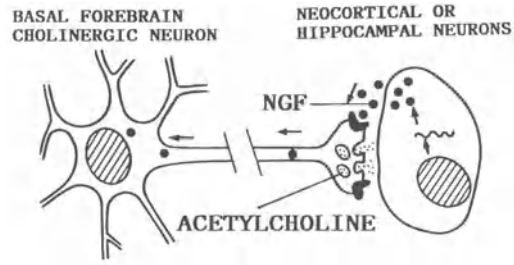

Fig. 1. Possible role of NGF as target-derived neurotrophic factor in basal forebrain cholinergic pathway.

In summary, the present results showed that (1)we were successful in establishing a new culture method for cholinergic neurons from 2-week-old rat nucleus basalis of Meynert; (2)NGF can promote survival of cultured AChE-positive magnocellular neurons of the nucleus from 2-week-old rats; and (3)NGF can enhance extension of AChE-positive neurites from cultured neurons.

So far it is not known whether the neurons in the nucleus basalis of Meynert in the rat are responsive to NGF. In the present work, we demonstrated that NGF promoted cell survival and extension of neurites of cultured AChE-positive neurons from the nucleus basalis. It is, however, difficult to examine the effects of NGF on cholinergic neurons in the nucleus from the embryonic rat brain, because an exact position of the nucleus in the brain is thought to be impossible to be found out.[2]

From the present results showing the NGF-elicited prevention of loss of nucleus basalis cholinergic neurons, we consider NGF to be a possible candidate for the medical treatment of Alzheimer's disease.

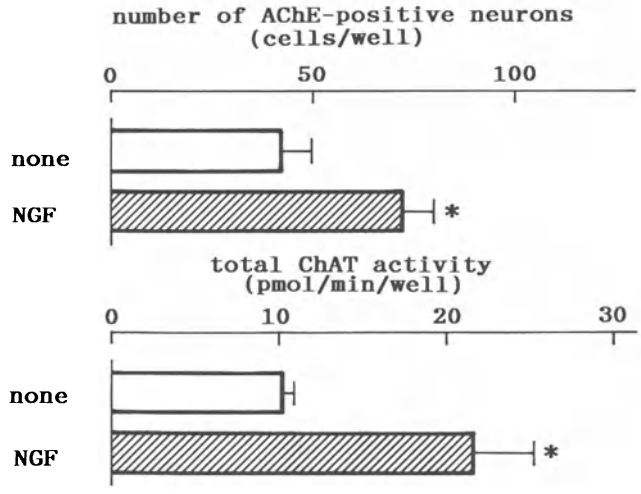

Fig. 2. NGF-mediated cell survival of cholinergic neurons in nucleus basalis of Meynert cultured from 2-week-old rats.

The brains of 2-week-old rats were attached to a sterile gelatin block with adhesive resin. From the brain slices obtained by using a vibratome (Microslicer DTK-3000W), tissue fragments containing the nucleus basalis of Meynert were excised under a dissecting Nikon microscope with a fiber optic light source. The fragments were collected in cold oxygen-bubbled L-15 medium and then digested twice at 37℃ for 15 min in freshly prepared PBS containing 0.05% papain (Cooper), 0.01% DNase I (Sigma), 0.2% cysteine and 5% glucose. After the digestion, a dissociated cell suspension was obtained by trituration in serum-containing DF medium with 1μ M cytosine arabinoside. The number of viable cells obtained was 4×10^5 cells/rat. The cells were seeded at a density of 1.6×10^5 cells/well on a feeder layer of astroglial cells in 48-well plates. NGF was added immediately at a concentration of 100 ng/ml to some cultures, and others received no growth factor. After 3 days in culture, the medium was changed for a cytosine arabinoside-free medium. Determination of ChAT activities and AChE-staining of cultured cells were described previously.[2-4]

Values represent means± S.D.(n=4). * , $P< 0.001$.

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ALTERATIONS IN NERVE GROWTH FACTOR RECEPTOR CONTAINING NEURONS IN PARKINSON'S DISEASE

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INTRODUCTION

At the turn of the century, neuronal loss associated with the nucleus basalis of Meynert was first observed in patients with Parkinson's disease (PD) by Lewy[1]. Neuronal loss in this region has been based primarily on material stained for Nissl substance using perikaryal diameter as the criterion for including a neuron as part of the nucleus basalis[2,3]. The caveats associated with such investigations have been discussed in detail, elsewhere[4]. The magnocellular neurons of the nucleus basalis (Ch4) have been shown to contain the specific cholinergic marker choline acetyltransferase (ChAT)[5,6,7], and that these cell bodies provide the major cholinergic innervation of the neocortex and amygdala[5,8]. Degeneration of these neurons may underlie deficits in cognition often seen in Alzheimer's and Parkinson's disease[9,10] and that these cell bodies also are under the influence of the trophic substance nerve growth factor (NGF)[11]. We have shown using antibodies for the NGF receptor (NGFR) an almost complete colocalization between neurons expressing NGFR and the cholinergic marker ChAT within the human nucleus basalis[7]. Thus, the characterization of an antibody which visualizes the primate NGFR[6,7] provides a unique probe for assessing the integrity of nucleus basalis cholinergic neurons in human disease. In this chapter, we present evidence of basal forebrain neuronal dysfunction in neurons which localize the NGFR without AD-type pathology in patients with PD.

METHODS AND RESULTS

The present results are based on brain tissue from five neurologically and psychologically normal aged (mean age 74 yr; range 68 - 78 yr) and four PD (mean age 79 yr; range 73 - 88 yr) patients obtained at autopsy. Brain stems containing the substantia nigra as well as 1 cm thick coronal slabs including the nucleus basalis were fixed and sectioned as previously described[4,7,12,13]. Clinical diagnosis of PD was confirmed postmortem by an extensive loss of melanin containing neurons and Lewy bodies in the substantia nigra pars compacta using hematoxylin and eosin staining. Evaluation of subcortical and cortical neuritic plaques (NPs) and

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neurofibrillary tangles (NFTs) was performed using Thioflavin-S[4,12,13]. Alterations in NGFR expressing neurons in the nucleus basalis were analyzed using NGFR immunohistochemistry and NGFR mRNA in situ hybridization methods combined with counts of NGFR containing perikarya according to procedures previously described[4,7,12,14]. Information related to mental state of PD patients was obtained by a retrospective analysis of hospital charts and discussions with next of kin. This evaluation revealed no evidence of associated dementia in the PD cases examined.

Tissue reacted immunocytochemically for the NGFR clearly delineated the Ch4 subfields. Although the staining pattern was heavy in certain regions, particularly in control patients, individual neurons were visible. Qualitative evaluation revealed substantial reductions in NGFR immunoreactive perikarya within the anterior, intermediate and posterior subdivisions of Ch4 in the PD cases (Fig. 1). The pattern of cell loss varied between patients with regard to the Ch subgroup most severely affected. Counts of NGFR immunoreactive cell bodies revealed the greatest reduction in immunoreactive neurons in the posterior subfield of Ch4. The overall mean loss of NGFR containing neurons within the Ch4 complex was 68% (range 37% - 87%) in the PD cases as compared to age-matched controls. Preliminary in situ hybridization experiements, performed on sections adjacent to those processed for NGFR immunohistochemistry, revealed decreased NGFR mRNA positive neurons in Ch4 as compared to normal aged patients. In order to determine the impact of Ch4 neuronal loss on cortical cholinergic fiber innervation, sections from temporal cortex (Brodmann areas 20, 21, 22) were reacted for the indirect cholinergic marker AChE[15]. In contrast to the extensive AChE fiber plexus seen in controls, there was an almost total absence of this staining pattern in PD patients (Fig. 2). Sections stained or counterstained with Thioflavin-S revealed occasional intra or extracellular NFT or NP similar in density to those seen in age-matched controls.

DISCUSSION

The present findings demonstrate that there is an extensive reduction in NGFR gene expressing neurons in all subregions of the nucleus basalis (Ch4) in non-demented patients with PD. Cortical sections reacted for AChE revealed an almost complete loss of cortical cholinergic fibers associated with the Ch4 neuronal loss in these patients. However, virtually no Alzheimer's type pathology was seen in cortex or in nucleus basalis. We recently demonstrated that NGFR gene expressing neurons of the nucleus basalis are severely depleted in AD and that these patients exhibit extensive multifocal cortical neuritic plaque formation[4]. The fact that AD-type pathology was not evident in our PD cases, despite loss of NGFR containing perikarya within the nucleus basalis, is of particular interest since it has been suggested that basal forebrain cholinergic cell loss contributes to the formation of cortical neuritic plaques[16]. However, our results suggest that the loss of Ch4 neurons, which provide the major cholinergic innervation to the entire cortical surface, is not a sufficient prerequisite for the formation of neuritic plaques in the cortex of PD patients.

Based on the evaluation of AD and PD patients with dementia who exhibit extensive cell loss in the Ch4 system, it has been postulated that this region is intimately involved in normal mental function[3]. Although the present study relies on a retrospective analysis of the cognitive state of the PD patients, the results do not support the view that such lesions alone cause dementia. In fact, de la Monte and coworkers[17] suggest that Parkinsonian dementia is primarily due to multifocal degeneration of numerous subcortical structures. The fact that no significant cortical AD pathology was seen in our PD cases, despite

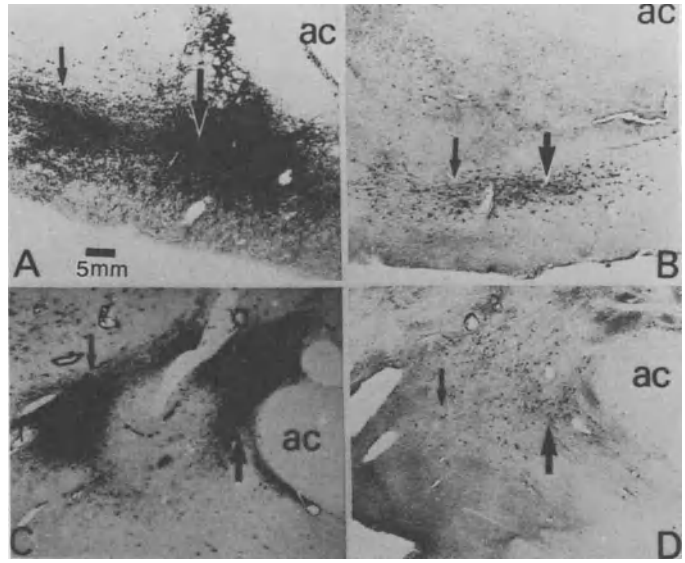

Fig. 1 NGFR immunoreactivity in (A) Ch4am (small arrow), Ch4al (large arrow) and (C) Ch4iv (small arrow), Ch4id (large arrow) of 78 yr old normal female. Compare this with the loss of NGFR staining in (B) Ch4am (small arrow), Ch4al (large arrow) and (D) Ch4iv (small arrow), Ch4id (large arrow) of 82 yr old PD patient.

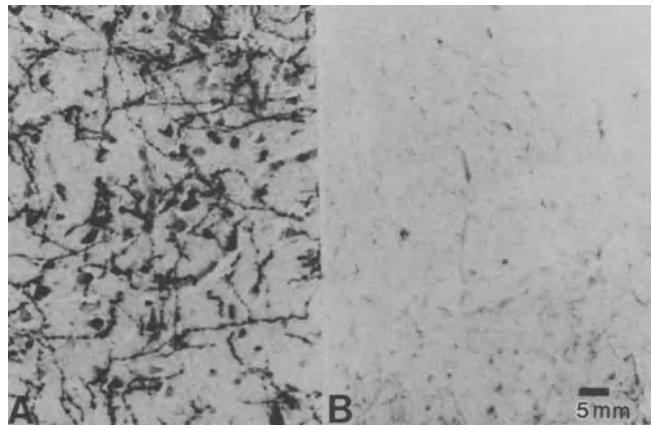

Fig. 2 Comparison of AChE containing fibers in the temporal cortex of a normal aged (A) and Parkinson's (B) patient.

extensive Ch4 neuronal loss, suggests that an additional process may be superimposed upon this subcortical damage which leads to plaque formation.

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A STRIATAL-DERIVED DA NEURON GROWTH FACTOR: IMPLICATION IN PARKINSON'S DISEASE

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INTRODUCTION

The anti-Parkinsonian efficacy of adrenal medulla-to-brain transplantation was initially thought to result from increased dopamine (DA) secretion from the implanted chromaffin cells. However, examination of the CSF from transplant recipients, failed to demonstrate increased levels of DA despite clinical improvement.[1] This would suggest that mechanisms other than increased DA secretion may be responsible for the therapeutic benefits resulting from medulla transplantation. Any alternative hypothesis advanced must consider recent observations suggesting that: 1- endogenous DA tone appears increased by medulla transplantation,[3] 2- transplantation leads to enhanced sprouting of tyrosine hydroxylase positive fibers,[6] and 3- transplantation results in bilateral clinical improvement.[5] The release of a neuro-trophic-factor (NTF) capable of diffusing through CSF is consistent with these observations.

Various investigators have described a DA-NTF present in the target structures of DA neurons.[6,8,10] We have recently observed that striatal tissue taken from animals chronically treated with the DA antagonist haloperidol, produced enhanced DA neuron growth relative to normal saline treated controls when homogenates of the striatum, were added to rostral mesencephalic tegmental (RMT) cultures. This growth promoting effect was dose-dependent and boiled homogenates failed to stimulate cell growth. These observations are consistent with the notion that striatal tissue contains a soluble and inducible DA-NTF capable of stimulating DA neuron growth. They further suggest that presynaptic DA tone can influence the release of this NTF. Since chronic treatment with a DA antagonist resulted in an enhanced growth promoting effect, we decided to examine the effects of chronic treatment with the indirect DA agonist, amphetamine, using a similar experimental paradigm.

METHODS AND MATERIALS

Sprague Dawley rats (N=32), were treated for two months with d-amphetamine sulfate (AMPH; 2.5 mg/kg; (n=8) or 10.0 mg/kg, (n=8)), normal saline (NS; n=8), or haloperidol (HAL; 1.25 mg/kg; n=8). Six days

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following their last treatment, the animals were sacrificed and their brains were removed. The striata, and an equivalent size section of the cerebellum, were dissected out on ice, weighed, and immediately homogenized in Hank's Balanced Salt solution (HBS; 40 mg wet weight/ml of HBS). The samples were spun down, the supernatants were removed, pooled by treatment group, and assessed for total protein. All homogenates were diluted to yield an equivalent protein content. (RMT) cultures were established from E-13 rat embroyos using the methodology of Tomozawa and Appel.[10] Cells were plated at 250,000 cells per well in 500 ul of culture media (DMEM and Hamm's F-12; 50:50) and 10% fetal calf serum. The cultures were allowed to grow for 1-3 days prior to homogenate addition. 200 ul of the various supernatants were added to each well. 800 ul of media, without serum, was then added to yield a final volume of 1.0 ml. Control cultures were incubated with 800 ul media and 200 ul of HBS supplemented with an equivalent quantity of bovine serum albumin. The cultures were then incubated for 6 days at 37C in humidified air/5% CO_2.

RESULTS

Figure 1 depicts the typical effect of incubating the RMT cultures with the supernatants from the various striatal homogenates. The supernatants from striatal homogenates taken from NS treated animals were capable of supporting growth in the absence of additional growth promoting factors (as would be found in fetal calf serum). The HAL supernatants induced a more pronounced growth promoting effect. More cells were present in these cultures and the processes on these cells were longer, more branched, and generally thicker than those present in NS cultures. In contrast, the supernatants of homogenates taken from animals treated chronically with AMPH did not appear to support cell growth. There were significantly fewer cells and many cells were devoid of processes. When processes were present, they were shorter and less branched than those observed in NS treated cultures. This was especially evident in the cultures incubated with 10 mg/kg AMPH. The cultures incubated with the supernatants taken from the animals treated with 10 mg/kg AMPH were similar to those observed in control cultures (not shown). There were no overt differences among the cultures which were incubated with the various cerebellar supernatants. Cerebellar supernatants were capable of supporting growth although the cell density, process extension, and process branching observed in these cultures was not as pronounced as that seen in the NS striatal cultures.

DISCUSSION

The results presented here demonstrate that striatal homogenates taken from animals chronically treated with the DA agonist, amphetamine, had less growth promoting effect on RMT cultures than striatal homogenates from NS treated animals. This effect was dependent upon the AMPH dose delivered chronically. In contrast, striatal homogenates taken from animals chronically treated with HAL had a growth enhancing effect relative to NS. This effect was probably not the result of residual drug carried over to the cultures in the homogenates because of the six day drug-free interval and the fact that cerebellar homogenates from the various treatment groups were equivalent in their ability to support culture growth. Therefore, it seems likely that the growth regulating effect of the striatal homogenates was a consequence of the effects of chronic drug treatment on striatal DA function. Since HAL treatment led to enhanced culture growth while AMPH treatment led to reduced culture growth, the effect appeared to be inversely proportional to DA tone.

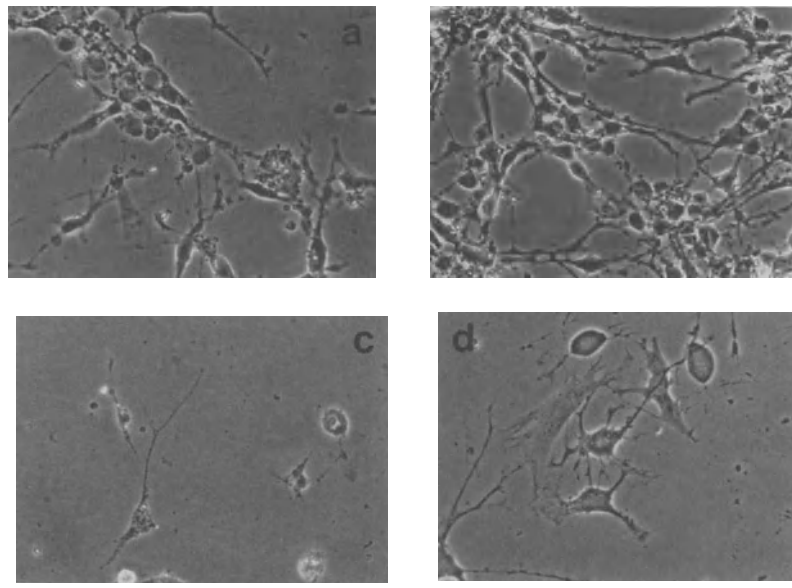

Fig. 1. This figure depicts the effect on cell growth resulting from incubation of mesencephalic cultures with homogenates of striatal tissue taken from animals chronically treated with normal saline (a), haloperidol (b), high dose (10 mg/kg) amphetamine (c), and a low dose (2.5 mg/kg) of amphetamine (d).

A variety of factors found in the striatal homogenates, growth promoting as well as inhibiting, could have contributed to this effect. If the effect was a result of alterations in a NTF secondary to chronic drug treatment, it would not likely be Nerve Growth Factor (NGF) since NGF does not support RMT culture growth.[10] Thus far, only epidermal-derived growth factor and insulin-like growth promoting factor have been shown to support growth in this culture system.[7] Establishing the existence of this hypothesized DA-NTF would have significant implications in our understanding of the DA neuron system.

The fact that homogenates from NS treated animals supported culture growth suggests that it is present in normal tissue and raises the possibility that this hypothesized DA-NTF participates in the maintenence of circuitry involving DA neurons. Prochiantz et al.,[8] previously demonstrated that striatal tissue taken from rats with lesions of the substantia nigra resulted in culture growth promoting effects similar to those produced here by chronic HAL. This might suggest that the striatum responds to a loss in DA tone by increasing the production of this DA-NTF. Similar increases in DA-NTF production may occur in Parkinson's disease (PD) leading to increased sprouting of remaining DA neurons to compensate for cell loss. Since chronic AMPH treatment apparently reduced the production of the DA-NTF, it is possible that other indirect acting DA agonists, like levodopa, would have similar effects. Thus, chronic treatment with levodopa may reduce DA-NTF production and therefore reverse the striatal compensation involving the DA-NTF resulting from DA neuron loss. The existence of this hypothesized DA-NTF would also be expected to participate in transplantation (PD). It is possible that adrenal

medulla transplantation stimulates the release of the DA-NTF resulting in enhanced sprouting of remaining DA neurons with subsequent mild clinical improvement. Alternatively, transplantation of fetal DA neurons into striatal tissue which has been chronically treated with levodopa, may have low growth potential due to decreased DA-NTF production. This may explain the recent observation that chronic levodopa treatment reduced the viability of implanted fetal DA cells.[9] The isolation of this hypothesized DA-NTF and the development of an efficacious delivery system would therefore be expected to have significant therapeutic implications in the treatment of PD.

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CO-LOCALIZATION OF CHOLINERGIC AND GABAERGIC TRAITS IN IN VITRO SEPTOHIPPOCAMPAL NEURONS FROM DEVELOPING RATS

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INTRODUCTION

It is well known that basal forebrain neurons are involved in Alzheimer's disease. Massive neuronal loss occurs in the basal forebrain as well as in the cortical areas that are interconnected with this region. In rodents, nerve growth factor (NGF) acts on cholinergic neurons in the basal forebrain to induce choline acetyltransferase activity.[1] It was suggested that survival of the basal forebrain neurons is also dependent on NGF during development[2] and in adult life.[3] We have offered direct in vitro evidence that NGF in fact promotes the survival of hippocampus-projecting neurons in the medial septum and vertical limb of the diagonal band of developing rats.[4] The septohippocampal neurons were identified by retrograde-labeling with fluorescent latex microspheres that had been injected into the hippocampus in vivo. The number of microsphere-labeled projection neurons in the cultures supplemented with 100 ng/ml NGF was much greater than that without NGF.

The septohippocampal system is known to include cholinergic and GABAergic projection neurons.[5] Recently, we also found that most of the retrogradely-identified developing septohippocampal neurons expressed NGF receptor-immunoreactivity (IR) in culture and that the identified neurons included GABA-immunoreactive as well as AChE-positive ones.[6] In the present study, we examined possible co-localization of the cholinergic and GABAergic traits in cultured developing septohippocampal neurons by a double labeling experiment combining AChE-histochemistry and GABA-immunohistochemistry.

MATERIALS AND METHODS

Four-day-old rats of Wistar stain were used. Fluorescent latex microspheres were injected bilaterally into the hippocampus. After 20 to 24 hrs from the injection, the rats were killed and cells from the medial septum and vertical limb of the diagonal band were dissociated with papain and plated on an astroglial feeder layer prepared in wells of tissue culture chamber/slides (Lab-Tek, #4818; 0.79 cm^2/well; $3x10^5$/well). The cells were cultured for 3 days with supplementation of 100 ng/ml NGF. They were then fixed with a solution containing 0.2 % picric acid, 4 % paraformaldehyde, and 0.05 % glutaraldehyde in 0.1 M sodium phosphate

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buffer (pH 7.4) for 2 min and then with 4 % paraformaldehyde in the phosphate buffer for additional 30 min. To identify GABA-immunoreactive neurons, we washed the cells with phosphate-buffered saline (PBS, pH 7.4) and treated them sequentially with 5 % normal goat serum in PBS containing 0.02 % Triton X-100 (5NGS/T; 20 min), rabbit anti-GABA antiserum (Immunotech, 1/2000 dilution in 5 NGS/T; 2 hrs) and FITC-conjugated goat anti-rabbit IgG (Cappel, 1/50 dilution in 5NGS/T; 1 hr). The slides were coverslipped with glycerol/PBS (1:1). The neurons labeled with fluorescent latex microspheres were identified under a fluorescence microscope (Olympus BH2) with a filter set for rhodamine and the identified cells were then photographed through another filter set for FITC for GABA-IR. The coverslips were then removed and the cells were stained for AChE activity as described elsewhere.[7] The fluorescent latex microsphere-labeled neurons were photographed again for AChE activity under bright field illumination. Detailed methods for retrograde cell labeling and cell culture were described previously.[4,6]

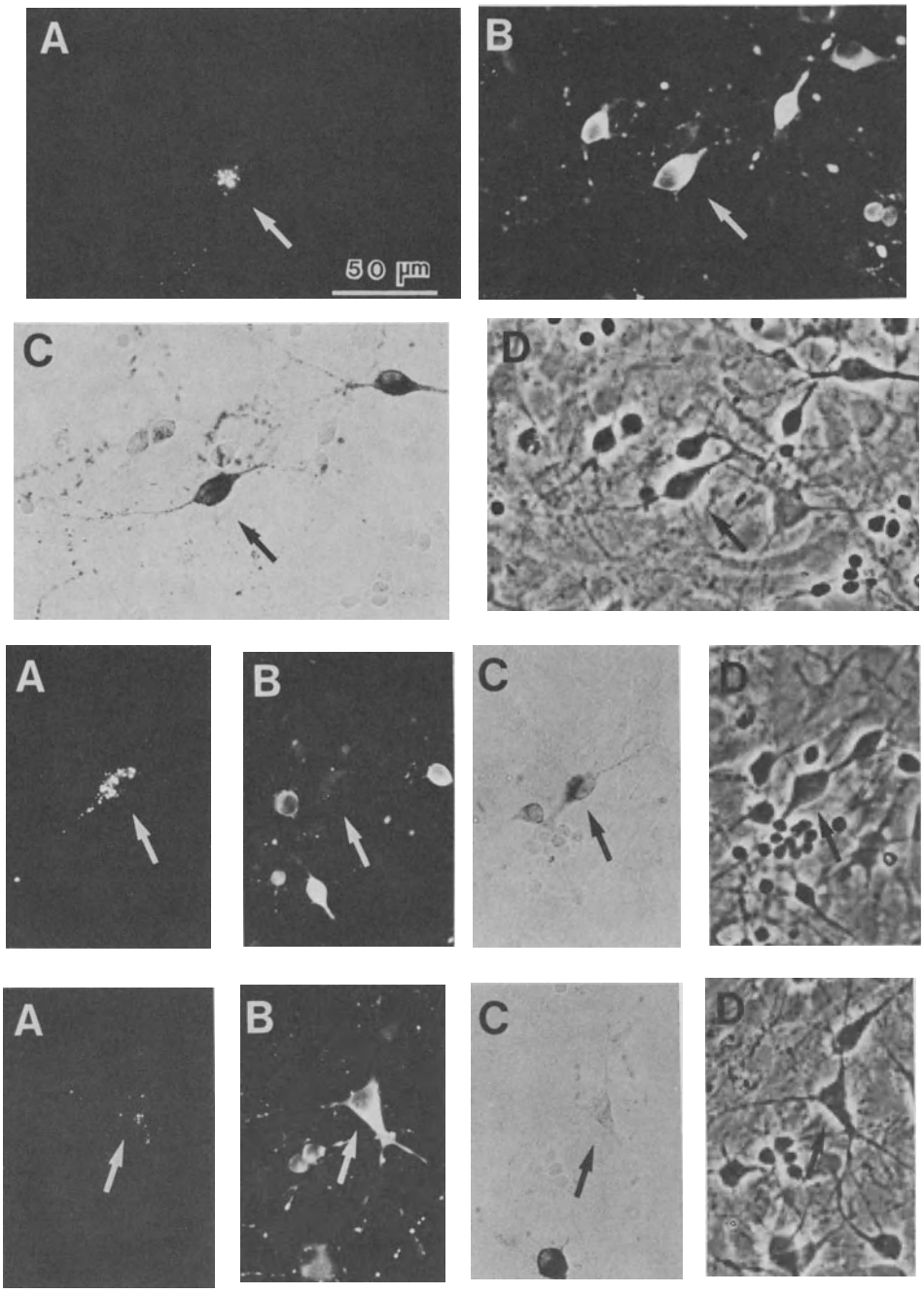
In an additional experiment, septal cells from 5-day-old rats were cultured for 7 days to examine the co-localization of AChE activity and GABA-IR within NGF receptor-immunoreactive neurons. The cells were fixed as above and treated sequentially with 1) 5NGS/T, 2) anti-NGF receptor monoclonal antibody MC192 (2 hrs), 3) rhodamine-conjugated anti-mouse IgG (Cappel, 1/50 dilution; 1 hr), 4) rabbit anti-GABA antiserum (1/2000 dilution in 5NGS/T; 1 hr), and 5) FITC-conjugated goat anti-rabbit IgG (1/50 dilution in 5NGS/T; 1 hr). The cells were photographed for NGF receptor- and GABA-immunofluorescence and were then stained and photographed for AChE activity as above.

RESULTS

Since treatment of the fixed cells with Triton X-100 was necessary for better visualization of intracellular GABA-IR, we examined the effects of various concentrations of Triton X-100 on the intensity of AChE staining before conducting the double labeling experiments combining GABA-immunohistochemistry and AChE-histochemistry. The addition of 0.1 % Triton X-100 to every working solution for immunostaining for GABA completely abolished AChE staining. In contrast, staining intensities of cells treated with 0.02 % Triton X-100 were similar to those not treated with Triton X-100. The staining intensities of cells treated with 0.05 % Triton X-100 were partially reduced. Thus, in the following double labeling experiments, we used 0.02 % Triton X-100-containing solutions for GABA-immunostaining.

As shown in Fig. 1, many fluorescent latex microsphere-labeled neurons were positive both for AChE activity and GABA-IR. There were also neurons that were positive for GABA-IR but not for AChE (Fig. 2), those positive for AChE but not for GABA-IR (Fig. 3), and those which were never stained for either(not shown). Among 68 microsphere-labeled neurons counted, more than half of the neurons (59 %) were AChE-positive/GABA-positive and 12 to 16 % of cells were either AChE-positive/GABA-negative, AChE-negative/GABA-positive or AChE-negative/GABA-negative (Table 1).

We also examined the co-localization of AChE activity and GABA-IR in NGF receptor-immunoreactive cells since previous studies had shown that cultured developing septal cells include neurons containing both NGF receptor-IR and AChE activity,[8] and also those containing both NGF receptor- and GABA-IR's.[6] As shown in Table 2, more than half (57 %) of 35 NGF receptor-immunoreactive cells were positive for both AChE activity and GABA-IR at 7 days *in vitro*. Control cultures treated with anti-NGF receptor antibody but not with anti-GABA antiserum, and those treated with anti-GABA antiserum but not anti-NGF receptor antibody, showed no specific FITC- and rhodamine-fluorescence, respectively.



Figs. 1-3. Double staining of fluorescent latex microsphere-labeled septohippocampal neurons for AChE activity and GABA-IR. AChE+/GABA+, AChE+/GABA-, and AChE-/GABA+ neurons are seen in Figs. 1, 2 and 3, respectively (arrows). A, rhodamine-fluorescence of fluorescent latex microspheres; B, FITC-immunofluorescence for GABA; C, AChE staining; D, phase-contrast view. The FITC-fluorescence in the microsphere-labeled neuron in Fig. 2 was as weak as that seen in control cultures not treated with anti-GABA antiserum (not shown).

Table 1. Co-localization of AChE activity and GABA-IR in fluorescent latex microsphere-labeled septohippocampal neurons in vitro

AChE	+	+	-	-
GABA	+	-	+	-
Cell number	40	9	11	8
(%)	(59)	(13)	(16)	(12)

Table 2. Co-localization of AChE activity and GABA-IR in NGF receptor-immunoreactive septal neurons in vitro

AChE	+	+	-	-
GABA	+	-	+	-
Cell number	20	11	3	1
(%)	(57)	(31)	(9)	(3)

DISCUSSION

The GABAergic projection neurons have been suggested to play an important role in the basal forebrain-cortical systems of the mammalian brain. Certain GABA-containing neurons in the septum have been shown to innervate most of the GABA-containing interneurons in the hippocampus and seem to be involved in the reduction of local hippocampal inhibition.[9] We showed in our previous studies that NGF promotes survival of GABA-immunoreactive septohippocampal neurons[6] and that certain septal neurons with NGF receptor-IR express [^{3}H]GABA uptake activity[10] and/or GABA-IR.[6] The present results that more than half of the cultured septohippocampal neurons and those of NGF receptor-immunoreactive neurons contained both GABA-IR and AChE activity strongly support the assumption that the GABAergic and cholinergic systems are developmentally and functionally correlated and may originate from a common source.[11] This is consistent with a prediction that GABAergic septohippocampal neurons might also be involved in Alzheimer's disease.

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A HIPPOCAMPAL CELL LINE EXPRESSES A MEMBRANE-ASSOCIATED CHOLINERGIC TROPHIC ACTIVITY NOT ATTRIBUTABLE TO NERVE GROWTH FACTOR

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INTRODUCTION

Basal forebrain cholinergic cells are prominently affected in Alzheimer's disease (AD) (Hefti and Weiner, 1986). Choline acetyltransferase (ChAT) is decreased in cholinergic target areas such as the hippocampus and neocortex, and there is a loss of ChAT-positive cells in the basal forebrain, including the septal region. One of the postulated pathogenetic alterations in AD is a deficiency in the normal trophic interactions through which target neocortical and hippocampal cells promote the survival and function of projecting cholinergic neurons (Appel, 1981). Accordingly, trophic factor therapy has been proposed as a potential future approach to the management of AD. Even if abnormalities in trophic interactions do not play a specific pathogenetic role in AD, trophic agents might be expected to help maintain cholinergic innervation of cortex and thus possibly influence the clinical course of AD.

It is clear that hippocampal cells can promote septal cholinergic neuron survival and function (Gahwiler and Hefti, 1984; Rimvall et al., 1985; Hsiang et al., 1987). Recent evidence indicates that target-derived nerve growth factor (NGF) is one of the molecules mediating hippocampal influences on cholinergic cells (Hefti and Weiner, 1986; Whittemore and Seiger, 1987). There is also evidence that other, non-NGF, hippocampal-derived molecules play a role (Crutcher and Collins, 1982; Heacock et al., 1986; Bostwick et al., 1987; Emerit et al., 1989). However, relatively little is known about the agent(s) mediating these non-NGF effects on cholinergic neurons. In order to study these molecules, we developed a strategy for their isolation and identification. Since the purification of trophic factors directly from brain tissue is complicated by cellular heterogeneity and the likelihood that such factors are present in very low concentration, we chose the alternative approach of using clonal cell lines as a source of material for purification. Accordingly, we developed a technique for deriving permanent neural cell lines from particular regions of brain which express differentiated characteristics typical of the cells of origin (Hammond et al., 1986). We then used this method to derive cell lines from hippocampus, and have examined these lines for the expression of cholinergic trophic activity not mediated by NGF.

METHODS

In order to develop hippocampal cell lines, we dissected and dissociated embryonic hippocampi, and fused the cells with hypoxanthine phosphoribosyltransferase (HPRT)-deficient N18TG2 cells, using polyethylene glycol. The fusion products were plated in medium with aminopterin to select for the growth of hybrid cells, and against the growth of unfused HPRT-deficient N18TG2 cells.

Medium conditioned by hippocampal cell lines and by N18TG2 cells was prepared as follows. Cells were grown in Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal calf serum (FCS), rinsed five times with serum-free DMEM, and subsequently incubated for 24-36 hours in serum-free DMEM. The conditioned medium was collected, and centrifuged at 100,000 x g. The 100,000 x g supernatant was then partially filtered through an Amicon YM5 filter (5,000 Dalton cutoff) yielding a concentrated retentate, which was tested.

We also established a bioassay to screen for effects of hippocampal cell line preparations on primary septal cells in culture. Embryonic septa were dissected and dissociated, and resuspended in serum-free N2 medium modified after Bottenstein (1985). The cell suspension was then plated in microtiter wells which had been poly-

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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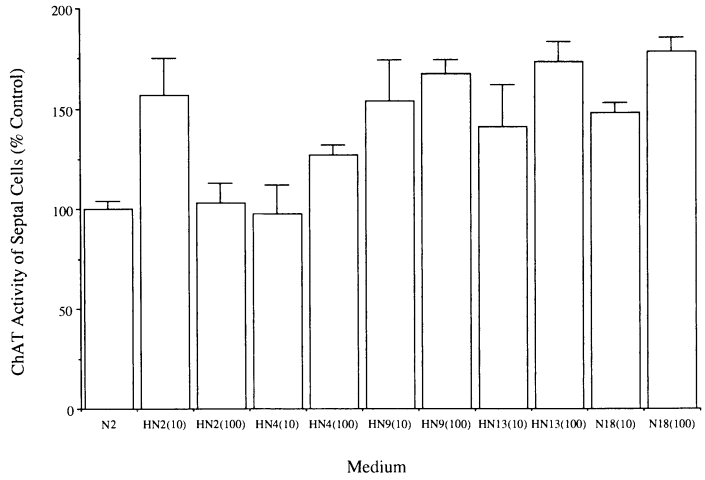

Fig. 1. Septal cells were cultured in N2 medium alone (N2), or in N2 supplemented with medium conditioned by hippocampal cell lines (HN lines) or by N18TG2 cells (N18), with a final concentration in the well of either 10 µg/ml (10), or 100 µg/ml (100) conditioned medium protein. Media conditioned by HN2, HN4, HN9, HN13, and N18TG2 increased septal ChAT activity by 0-75% over controls. The mean ± the standard error of the mean of 3 cultures is displayed.

lysine coated. Four to eight hours later, the agent to be tested was added, and the cells were then maintained in culture for a total of seven days. As an outcome measure, septal cell ChAT activity was quantitated as modified after the method of Fonnum (1975).

In order to extract HN10 activity from a membrane preparation, HN10 cells were washed thoroughly and lysed in 10 mM phosphate buffer, containing phenylmethanesulfonyl fluoride (PMSF), leupeptin, and pepstatin. The lysate was then centrifuged at 600 x g and the supernatant subsequently centrifuged at 100,000 x g. The 100,000 x g pellet was resuspended in phosphate-buffered saline containing protease inhibitors, and incubated for 1 hour. This suspension was then centrifuged at 100,000 x g, and the supernatant was tested.

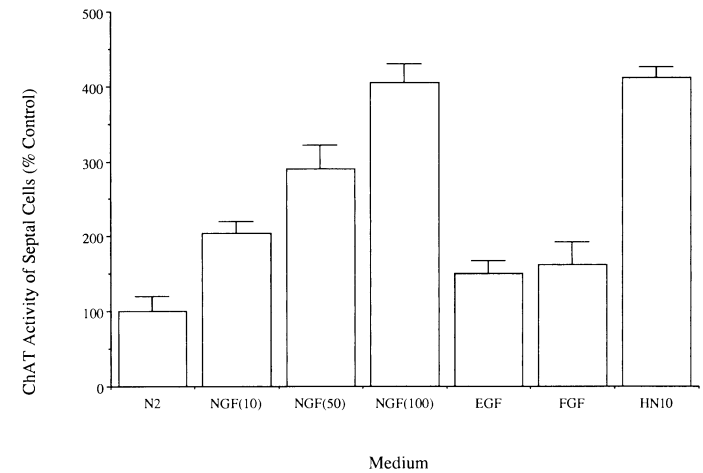

Fig. 2. Septal cells were cultured in N2 medium alone, and in N2 with NGF at 10 ng/ml (NGF (10)), at 50 ng/ml (NGF (50)), or at 100 ng/ml (NGF (100)). Cells were also cultured in N2 with EGF, N2 with basic FGF, or N2 with HN10 conditioned medium at a final protein concentration of 100 µg/ml. HN10 conditioned medium increased septal cell ChAT activity to approximately 400% of control values, similar to the effect of 100 ng/ml purified, exogenous NGF.

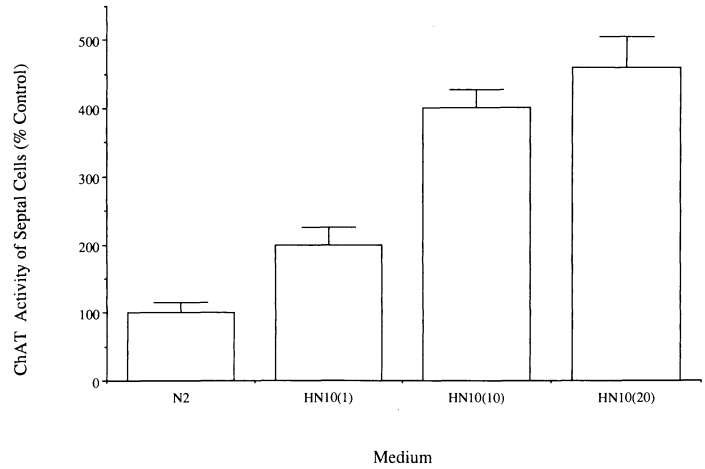

Fig. 3. Septal cells were cultured in N2 medium alone, and in N2 with the HN10 membrane extract at final protein concentrations of 1, 10, and 20 μg/ml. Treatment with the membrane extract increased septal ChAT activity in a dose-dependent fashion.

RESULTS AND DISCUSSION

A series of 19 cell lines derived from hippocampus ("HN" cell lines), as well as the neurotumor parent, N18TG2, were screened for effects on primary septal cells in culture. Conditioned media were added to primary septal cell cultures at final protein concentrations of 10 μg/ml or 100 μg/ml. After 7 days, the ChAT activity of each culture was quantitated. Media conditioned by 18 of the hippocampal cell lines, and by N18TG2 cells, increased septal cell ChAT activity by less than 75% over control wells treated with N2 medium alone. The results of a typical screening assay are shown in Fig. 1.

However, when media conditioned by the hippocampal cell line HN10 was added to primary septal cells in culture at a final protein concentration of 100 μg/ml, there was an approximately 4-fold increase in ChAT activity compared to controls (Fig. 2). In an initial attempt to ascertain whether this activity might be mediated by NGF in the HN10 conditioned medium, we determined the concentration of purified, exogenous NGF required

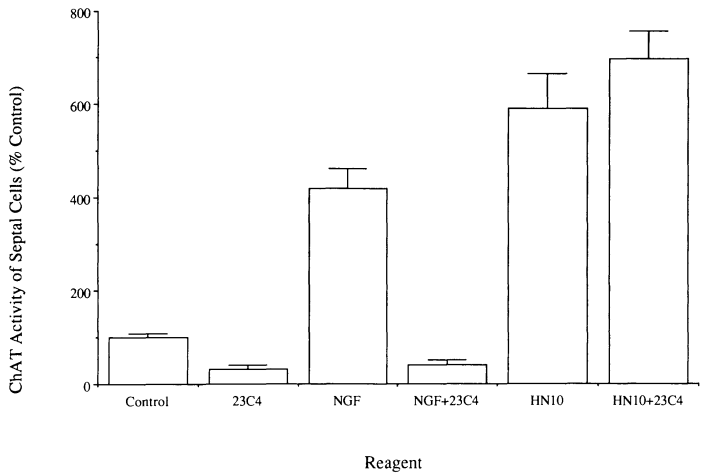

Fig. 4. Septal cells were cultured in N2 medium alone (control), and in N2 with anti-NGF antibody 23C4, N2 with NGF, N2 with NGF plus 23C4, N2 with HN10 membrane extract, and N2 with HN10 membrane extract plus 23C4. The addition of the anti-NGF monoclonal antibody blocked the effect of exogenous NGF on septal cell ChAT activity, but did not decrease the effect of HN10 membrane extract.

to achieve a similar effect on septal cell ChAT activity. (NGF was graciously provided by W.C. Mobley.) As shown in Fig. 2, the increase in ChAT with HN10 was approximately equivalent to the increase seen with NGF at a concentration of 100 ng/ml. We then quantitated the NGF in HN10 conditioned medium using a two-site enzyme-linked immunosorbent assay modified after the method of Otten and colleagues (Weskamp and Otten, 1987). Direct quantitation of NGF in the HN10 conditioned medium, performed before the conditioned medium was added to the septal cultures, revealed that the final concentration of HN10-derived NGF in the wells was less than 1 pg/ml, over 10,000-fold less than the 100 ng/ml of purified, exogenous NGF required to stimulate the same level of ChAT activity.

We also performed experiments to determine, preliminarily, whether the HN10 activity might be mediated by epidermal growth factor (EGF) or fibroblast growth factor (FGF), two polypeptides which have been reported to influence developing neurons from the central nervous system (Walicke, 1989). When EGF or FGF were added at concentrations reported to result in maximal effects in other systems (1 nM), ChAT activity was increased by only 50% and 60%, respectively, over controls, suggesting that HN10 activity is not mediated by EGF or FGF (Fig. 2).

HN10 activity could also be extracted in soluble form from a crude membrane preparation. When the extract was added to primary septal cells in culture, there was a dose-dependent increase in ChAT activity (Fig. 3). HN10 extract at a final protein concentration of only 20 µg/ml resulted in a 4 1/2-fold increase in ChAT activity (Fig. 3). The HN10 extract thus demonstrated an approximately 5 1/2-fold increase in specific activity compared to the HN10 conditioned medium. Using the soluble extract of the crude HN10 membrane preparation, we then sought to determine more definitively whether HN10 activity was mediated by NGF. An antibody blocking experiment was performed in which primary septal cells were incubated with NGF or HN10 extract in the presence or absence of the anti-NGF monoclonal antibody 23C4 (kindly provided by U. Otten). We found that the addition of 23C4 blocked the effect of NGF, but not the effect of HN10 extract, on primary septal cell ChAT activity (Fig. 4). These data provided further evidence that the HN10 activity is not mediated by NGF.

More recently, we began to investigate the physicochemical nature of the molecule(s) mediating the HN10 effect on septal cell ChAT activity. When HN10 membrane extract, prepared as described above, was heated to 90°C for 20 minutes, there was loss of virtually all activity (data not shown). Activity was also lost when HN10 membrane extract was prepared in the absence of protease inhibitors, indicating that the activity was sensitive to endogenous proteases (data not shown). Heat and protease sensitivities are consistent with the possibility that intact protein(s) are required for the HN10 activity.

In summary, HN10, expresses an activity which increases ChAT activity of septal cells in culture, and which appears to be membrane-associated. This is of interest in view of recent reports suggesting a role for membrane-related molecules in the regulation of neuronal phenotypic expression (Wong and Kessler, 1987; Adler et al., 1989; Emerit et al., 1989). The HN10 activity does not appear to be mediated by EGF or by FGF, since under these culture conditions purified preparations of these two growth factors do not exert comparable effects on septal cells. Furthermore, HN10 activity does not appear to be mediated by NGF since direct quantitation reveals that the concentration of NGF in HN10 preparations is many fold less than the concentration of purified, exogenous NGF required to achieve a similar effect. Additionally, a monclonal antibody which blocks the bioactivity of purified NGF does not block HN10 activity. Taken together, these studies indicate that a membrane-associated factor influences central cholinergic neurons in culture, and, furthermore, suggest that the activity is not mediated by NGF, EGF, or FGF.

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INTERLEUKIN-6 AS A NEUROTROPHIC FACTOR FOR PROMOTING SURVIVAL OF SEPTAL CHOLINERGIC NEURONS AND MESENCEPHALIC CATECHOLAMINERGIC NEURONS FROM POSTNATAL RATS

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INTRODUCTION

Neuronal differentiation and survival are supported by several kinds of neurotrophic factors in the central nervous system (CNS) as in the peripheral nervous system (PNS). The well-known nerve frowth factor (NGF), target derived growth factor, is synthesized[1-4] and acts on cholinergic neurons[5-12] in the CNS; however the action of NGF on other neurons is limited. We have been studying such factors that act on neuronal survival by using primary cultures of postnatal rat brain.

Interleukin 6 (IL-6) is a lymphokine involved in the final maturation of B-cells to antibody-forming cells.[13,14] Recently it was reported that IL-6 acts on a variety of cells aside from B-cells. Additionally, it is reported that IL-6 is produced by astroglia and microglia after a virus infection[15] and its mRNA is detected in astrocytoma and glioblastoma cell lines after IL-1 stimulation.[16] These findings suggest that IL-6 is produced universally in CNS after virus infection or injury. Therefore we decided to investigate the effect of IL-6 on neuronal survival.

MATERIALS AND METHODS

Recombinant IL-6, which was prepared by expression of a cDNA for IL-6[17] was a kind gift from Drs. T. Hirano and T. Kishimoto. NGF was purified from male mouse submaxillary glands by the method of Bocchini and Angeletti[18] with the modification of Suda et al.[19] Primary culture from postnatal (P10-P15) rat brain were performed as described in Hatanaka et al.[8] Basal forebrain for septal cholinergic neurons and the bottom part of the midbrain for catecholamine neurons were dissected out from postnatal (P10-P15) rat brain and digested with papain and 0.01% DNase I.

Choline acetyltransferase (ChAT) activity was measured by the method of Fonnum[20] with modifications by Hatanaka and Tsukui.[6] Cultured neurons from midbrain were stained by a monoclonal anti-TH antibody. Cells were fixed with 4% paraformaldehyde (PFA), and TH-positive neurons were visualized by peroxidase and diaminobentidine.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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The demonstration of strong AChE staining in the cultured cells was carried out by the modified method of Hefti et al.[9] Briefly, after fixation and washing with PBS, cultures were incubated for several days at 4°C in a solution containing acetylthiocholine as substrate, gelatin for preventation of the diffusion of the reaction products, and tetraisopropylpyrophosphoramide as the pseudocholinesterase inhibitor.

RESULTS AND DISCUSSION

In primary cultures of septal neurons from 10-day-old rats, choline acethyltransferase (ChAT) activity decreased during the culture period, and this decrease paralleled that in the number of cholinergic neurons identified by acetylcholine esterase (AChE) staining. As nerve growth factor (NGF) acts on septal cholinergic neurons, NGF prevents this decrease in ChAT activity and in the the number of AChE positive neurons. In this system remaining ChAT activity indicates the activity supporting neuronal survival. As shown in Fig. 1 remaining ChAT activity in cultures from the septal area of P10 rat brain treated for 6 days with IL-6 was higher than that in control cultures (without IL-6). The effect of IL-6 was observed significantly at 5 ng/ml, and the maximal effect was obtained at 50 ng/ml. Moreover, cells cultured with IL-6 at 50 ng/ml and NGF at a maximal dose of 100 ng/ml showed a synergistic effect of the agents. This effect of IL-6 on remaining ChAT activity indicates the survival effect of IL-6, and it was observed in serum-free, TIP/DF medium. Then we examined the effect of IL-6 on the number of AChE-positive neurons. After 6 days with IL-6 (50 ng/ml), cultures from P13 rat brain septal area had a number of AChE-positive neurons significantly greater than cultures without IL-6 (Fig. 2); and, also, cultures with IL-6 (50 ng/ml) and NGF (100 ng/ml) showed a synergistic effect.

These data indicate clearly that IL-6 acts to support the neuronal survival of cultured septal cholinergic neurons from the postnatal (P10-P15) rat. However, IL-6 had no effect on the differentiation of cultured septal cholinergic neurons from embryonic rats, though NGF induces ChAT activity in cultured embryonic cholinergic neurons.[21] This suggests that the survival effect of IL-6 is not mediated by NGF secretion.

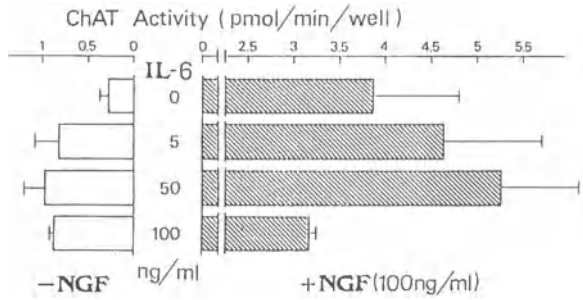

Fig. 1. Effect of IL-6 on remaining ChAT activity in cultured postnatal septal cholinergic neurons. Cells from P10 rat septum were cultured in serum-containing medium on polyethyleneimine-coated, 48-well plates. In the presence of various amounts of IL-6 with or without NGF at a concentration of 100 ng/ml, cells were cultured for 6 days. Medium was changed every 3 days. ChAT activity was determined as described in the text. Values are presented as pmol/min/well. Each value shows the mean ±S.D. (n=4). *P<0.05 compared with the effect of NGF alone. **P<0.01, ***P<0.001 compared with control (no treatment).

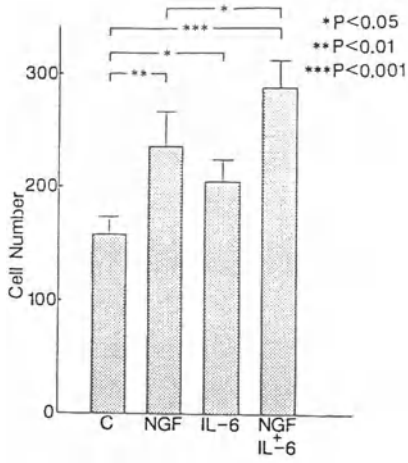

Fig. 2. Effect of IL-6 on the number of septal cholinergic neurons. Cells from P13 rat septum were cultured on an astroglial feeder layer with IL-6 (50 ng/ml) or NGF (100 ng/ml) or IL-6 (50 ng/ml) and NGF (100 ng/ml) in serum-containing medium for 6 days. Medium was changed every 3 days. AChE staining was done as described in the text and AChE-positive neurons were counted. Each value is the mean ±S.D. n=5.

We also examined the effect of IL-6 on cultured catecholamine neurons from P10-P15 rat midbrain, and found that IL-6 also promotes the survival of cultured catecholamine neurons from P10-P15 rat midbrain in both serum-containing and serum-free medium. The maximal effective dose was 50 ng/ml. And also the effect of IL-6 on cultured catecholamine neurons from embryonic rat brain was different from that on mature neurons (manuscript in preparation).

From all our results, it is clear that IL-6 acts as a survival factor for cultured septal cholinergic neurons and midbrain catecholamine neurons from P10-P15 rat; however, the effects of IL-6 on embryonic neurons are different from those on cultured neurons from postnatal rat brain. It is known that the basal forebrain cholinergic neurons play a crucial role in senile dementia and that nigral dopaminergic neurons are damaged in Parkinson's disease. From our results, IL-6 may be able to help repair some of these damaged neurons in situ. Thus it is important to study the role and mechanism of IL-6 action in the adult CNS.

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DISTRIBUTION OF NGF RECEPTORS IN CONTROL AND ALZHEIMER'S DISEASE BASAL FOREBRAIN

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INTRODUCTION

Alzheimer's disease (AD) is characterised by a loss of memory and confusion. Neuropathological changes indicate neuronal loss and degeneration in the neocortex and the cholinergic basal forebrain. The cholinergic system is involved in some aspects of learning and memory and it has been suggested that some of the memory defects observed in AD are caused by this degeneration.

β-Nerve growth factor (NGF) has recently been identified as a trophic factor for basal forebrain cholinergic neurones. The internalisation and retrograde transport of radiolabelled NGF in these neurones indicates the presence of receptors for NGF on these neurones (Seiler and Schwab 1984). This localisation has been confirmed by receptor autoradiography studies (Richardson et al., 1986; Raivich and Kreutzberg, 1987) and by immunocytochemistry in the rat basal forebrain (Dawbarn et al., 1988a; Gomez-Pinilla et al., 1987) and in human brain (Hefti et al., 1986). A high degree of coexistence has been shown between NGF receptor bearing and choline acetyltransferase (ChAT) immunoreactive neurones (Dawbarn et al., 1988b). The site of synthesis of the NGF receptor (in the human forebrain) has been localised to the magnocellular neurones by in situ hybridisation with a labelled probe to the human NGF receptor (Allen et al., 1989).

NGF administration apears to have a stimulatory effect on ChAT activity in vivo (e.g. Mobley et al. 1986) and in vitro (e.g. Hefti et al. 1985) and prevents basal forebrain neuronal degeneration associated with lesioning of the septo-hippocampal pathway. Furthermore, infusion of NGF has been shown to reverse behavioural impairment in a subpopulation of aged rats showing a deficit in spatial memory tasks (Fischer et al. 1987). It has been speculated that administration of NGF to AD patients may result in the amelioration of symptoms. Recently we have examined immunohistochemically the distribution of neurones in the human forebrain (Allen et al., 1989) as a basis for comparison in AD brain.

The present study compares the number and size distribution of NGF receptor positive neurones in the control and AD forebrain.

METHODS

Serial sections of basal forebrain were used from tissue obtained at autopsy from five intellectually unimpaired patients (mean age 81 years, mean post mortem delay 27 hours) and from five AD patients (mean age 89 years, mean post mortem delay 37 hours). All patients were prospectively assessed before death for evidence of dementia. The cerebral hemispheres were divided sagitally at the time of autopsy and the right hemisphere and hindbrain were fixed in 20% formaldehyde for routine histological examination. The occipital cortex was dissected from the unfixed left cerebral hemisphere and assayed for ChAT activity (Fonnum, 1975). The left

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

hemisphere was sliced and immersed in 4% paraformaldehyde (pH7.4), at 4°C for four days and then transferred to 30% buffered sucrose at 4°C.

Sections 30μm thick were cut on a freezing microtome and collected in triplicate, free-floating, every 300 or 600μm and processed by the peroxidase anti-peroxidase technique using a monoclonal antibody to the human NGF receptor (ATCC, Rockville, USA; HB8737, clone 200-G-64). Adjacent sections were stained for myelin and for Nissl substance. A third series of sections were processed omitting the primary antibody to check for non-specific staining. One section from each region, stained for β-NGF receptor was costained with cresyl violet and the percentage of hyperchromic magnocellular neurones which stained for β-NGF was assessed.

The magnocellular nuclei of the basal forebrain were subdivided into the diagonal band of Broca (dbB) and anterior, intermediate and posterior regions of the nucleus basalis of Meynert (nbM). Two sections from each of the four regions of the basal forebrain were examined for number and size of neurones expressing NGF receptors by semi-quantitative image analysis (Dell200/Imagan2, Kompira ltd). A further two sections were then examined from each region for number of neurones.

The control and AD populations were compared using nested analysis of variance (Genstat statistics, Rothampsted):

$$(Y_{ijk})^{1/2} = \mu + A_i + B_{ij} + E_{ijk}$$

where μ is the overall mean, A_i is the mean difference between AD and control neuronal cross-sectional areas, B_{ij} is a random effect of variation of data for each patient and E_{ijk} is the random error between all observations. This model was used to allow for the variation amongst patients. Frequency distribution of data was also evaluated by chisquare analysis.

RESULTS

β-NGF receptor positive neurones were present in all of the nuclei of the basal forebrain examined in all control and AD subjects. Staining was punctate in appearance in both perikarya and dendrites and perinuclear staining was distinct. Untransformed values of neuronal cross-sectional area were found to be normally distributed in both control and AD. Different nuclei were differentially affected by neuronal loss (Table 1). There was a significant reduction in neuronal number in NGF receptor bearing neurones in the posterior region (34% $p<0.05$) with no significant loss in the ndbB, anterior or intermediate areas of the nbM. Analysis of variance of data indicated a significant ($p<0.001$) difference in distribution in the intermediate and posterior regions of the nbM in AD compared with control, although not in the anterior nbM and ndbM.Chi square analysis revealed a significant difference in frequency distribution in all regions of nbM in AD basal forebrain compared with control with no change in ndbB. There was no change in mean or median neuronal size in any region. The percentage of β-NGF receptor positive neurones did not appear to change in any region examined in AD brain (Table 2).

Table 1. Mean number of β-NGF positive neurones and s.e.m. counted in two sections in the ndbB and the anterior (nbMa), intermediate (nbMi) and posterior (nbMp) regions of the nbM in five normal and five AD brains. Two sections with the greatest number of neurones were compared to eliminate bias. (* significantly different $p<0.05$).

	ndbB	nbMa	nbMi	nbMp
Control	679 ± 117	1016 ± 151	1971 ± 355	1098 ± 86
AD	525 ± 67	791 ± 131	1510 ± 296	728 ± 140*

Table 2. Mean percentage of cresyl violet stained neurones which express β-NGF receptors counted in one section in the ndbB, the anterior (nbMa), intermediate (nbMi) and posterior (nbMp) regions of the nbM in five normal and five AD brains.

	ndbB	nbMa	nbMi	nbMp
Control	60	72	78	88
AD	50	61	76	84

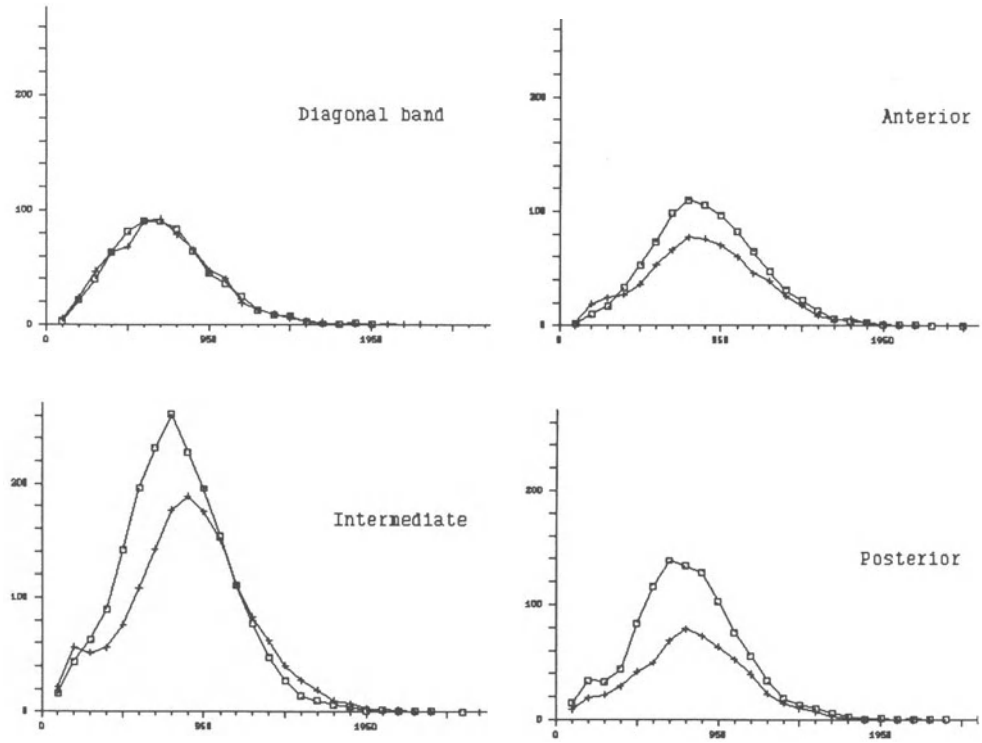

Figure 1. Size distribution of NGF receptor immunoreactive neurones in control and AD basal forebrain. Results are shown as mean number of neurones per subject. The x axis represents neuronal area in μm^2 and the y axis is the number of neurones counted per subject (squares represent control values and crosses represent AD).

DISCUSSION

We report a comprehensive assessment of number and size distribution of NGF receptor positive neurones in basal forebrain of aged controls and AD patients. In agreement with reports of Nissl stained cells, we find a reduction of 34% of large cells (greater than 350 μm, equivalent maximum diameter of 30 μm) in the posterior region of the nbM in AD. No significant change was observed in any of the other regions examined.

It has been suggested that AD may be a result of a lack of a specific neurotrophic factor such as NGF. Since NGF has a stimulatory effect on ChAT activity in cholinergic neurones and promotes cell survival in vivo, the finding that there are many neurones present in the ndbB and the nbM of AD brain which express NGF receptors makes it likely that NGF will be of therapeutic value in AD

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TROPHIC EFFECTS OF HIPPOCAMPUS CELL MEMBRANE FRAGMENTS ON CULTURED MEDIAL SEPTAL CHOLINERGIC NEURONS

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INTRODUCTION

In the developmental stage of the nervous system, synaptogenesis plays an important role in the survival of pre-and postsynaptic neurons. Presynaptic neurons die if they do not have synaptic contact with an optimal number of postsynaptic target neurons.[1] Conversely, neurite outgrowth and neurotransmitter synthesis in presynaptic neurons are promoted by co-culture with postsynaptic neurons.[2] The mechanisms of these phenomena are not yet clear; however it has been postulated that the presynaptic growth cones may recognize a specific molecule of the postsynaptic target neurons.[3]

We report here evidence that hippocampus cell membrane fragments have trophic effects on transmitter synthesis of cultured cholinergic septal neurons.

MATERIALS AND METHODS

Explant cultures

Medial septal explants from 16-day-old rat fetuses(Sprague-Dawley strain, Charles River Co.) were cultured on polylysine-coated plastic dishes with Dulbecco's modified Eagle's medium supplemented with transferrin, insulin, progesterone, putrescine, and selenium.[4] Hippocampus membrane fragments or extracts were added to the cultures on the 3rd and 6th days.

Preparaton of hippocampus membrane fragments and their extracts

The hippocampus was homogenized in 4 volumes of phosphate-buffered saline(PBS), pH7.2, with a Teflon homogenizer. The homogenate was spun at 100,000g for 90 min, and the pellets were washed once with cold PBS. For subcellular fractionation of the pellets, the tissue was prepared by the method of Gray and Wittaker.[5] Membrane fragments from the hippocampus, cerebral cortex other than the hippocampus, cerebellum or liver

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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were homogenized in 10 volumes of 0.1% sodium deoxycholate in PBS at pH7.2. The homogenate was spun at 100,000g for 90 min. The supernatants containing the detergent were dialyzed against PBS for 48 hr at 4 ℃. The hippocampus pellets or their deoxycholate extracts were treated with trypsin, neuraminidase, N-acetyl hexosaminidase, β-galactosidase, non-specific protease, or phospholipase C under optimal conditions.

Assay of acetylcholine(ACh) synthesis

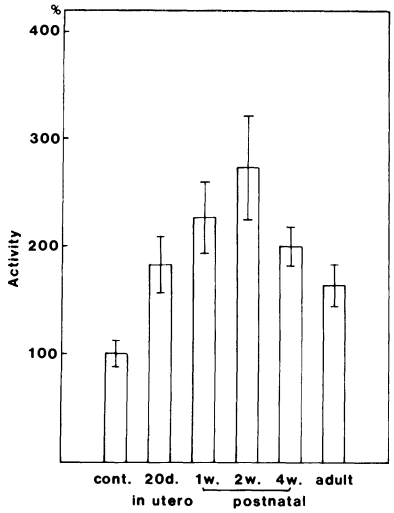
On the 9th day of culture the cells were incubated with ^{3}H-choline, and the newly synthesized ^{3}H-ACh in the cells was measured.[6,7] The amount of ^{3}H-ACh was expressed as the number of fmoles of ^{3}H-ACh per specimen, and was referred to as the cholinergic activity.

RESULTS

The amount of synthesized ^{3}H-ACh was 5 to 10 fmoles per piece of hippocampus. This value differed from culture to culture, so the cholinergic activity was expressed as the relative percentage compared with the control value.

The septal explants were cultured with the hippocampus membrane fragments taken from 20-day-old fetuses, 1-, 2-, 4-week-old neonates, or adult rats. Fig. 1 shows the cholinergic activity of these membrane fragments. The fragments from 2-week-old neonates had the highest activity.

The fragments from the 2-week-old neonates were prepared for subcelluar fractionation. Fig. 2 shows the cholinergic activity in the myelin, synaptic membrane, mitochondrial, and microsomal fractions. High activity was found in the synaptic membrane and microsome fractions. The activity in the myelin fraction differed from rat to rat. Because of poor myelination during the early neonatal period and low recovery of the myelin in the fractionation, we concluded that the activity in the myelin fraction was derived from synaptic membrane and microsome cross-contamination.



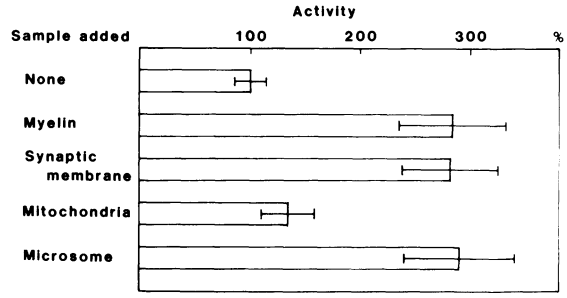


Fig. 2
Activity of subcellular fractions.
Protein conc., 10 μg/ml.

Fig. 1 Cholinergic activity of the hippocampus membrane fragments. Each column shows average and standard error(n=4).

Table 1. Activity of the membrane extracts.

Sample	Cholinergic activity(%) (n =4)
Control	100 ± 18
Hippocampus	555 ± 61
Cerebral cortex	365 ± 40
Cerebellum	287 ± 27
Liver	329 ± 39

In the next experiment we ascertained whether the factors are extractable with sodium deoxycholate. Table shows the activity of the extracts from four sources: the hippocampus, cerebral cortex, cerebellum and liver. As shown in the Table, the hippocampus membrane fragments extracts had the highest activity.

The hippocampus membrane fragments were treated with different kinds of enzyme. The activity was resistant to trypsin, neuraminidase, N-acetyl hexosaminidase, and β-galactosidase. Then the deoxycholate extracts of the hippocampus were treated with protease or phospholipase C. The activity was completely inactivated by the phospholipase C.

DISCUSSION

In this paper we have presented evidence that hippocampus cell membrane fragments and their detergent extract can promote cholinergic activity in cultured rat septal cholinergic neurons. The highest activity was found in the fragments derived from 2 week-old neonatal hippocampi. This finding is compatible with the evidence that active synaptogenesis in the hippocampus takes place during the neonatal period.[8]

Subcellular fractions such as synaptic membranes and microsomes contain presynaptic nerve terminals, postsynaptic membrane fragments, and fragmented plasma membranes.[5] Since these fractions had higher activity in our study, the cholinergic neuronal growth cone may interact with these subcellular components.

The activity was extractable with detergent. It was resistant to proteolyic enzyme, hexosaminidase, galactosidase, and neuraminidase. However, it was susceptible to phospholipase C. At least in part, therefore, phospholipids in membrane fragments are responsible for cholinergic-promoting activity.

Prochiantz et al.[9] have reported that striatal membrane fragments stimulate dopamine uptake in substantia nigra cultures. The active substance was likely a protein. Our active substance differs from theirs, however, because it is resistant to proteolytic enzyme.

Phospholipids are constituents of plasma membrane and organella. The variation of phospholipid components in various types of membrane is small, although sphingomyelin are relatively rich in myelin, and phosphatidyl ethanolamine, in mitochondria.[10] The regional distribution of phospholipid components in the developing neonatal brain is poorly documented.

What is the role of phospholipids in the cholinergic-promoting effect? It is presumed that phospholipids alter membrane components of the choline uptake channel as they do gamma-amino butyric acid binding in the striatum.[11] Further study is required to clarify the nature of this factor.

CONCLUSIONS

Rat fetus medial septum explants were cultured with hippocampus membrane fragments or their extracts. The amount of newly synthesized ACh in the cultured neurons was referred to as the cholinergic activity. The hippocampus cell membrane fragments enhanced the cholinergic activity. The activity was higher than that of similar fragments from cerebellum, cerebral cortex, or liver. The hippocampus fragments from 2 week-old neonates had the highest activity. Higher activity was found in the synaptic membrane and microsomal fractions. Detergent extracts of the fragments also promoted the cholinergic activity. The active factor was resistant to proteolytic enzyme. However it was susceptible to phospholipase C. These results suggest that some membrane-associated phospholipid(s) in the hippocampus cells of neonatal rats has specific trophic effects on presynaptic septal cholinergic neurons.

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CHANGES IN BASAL FOREBRAIN CHOLINERGIC SYSTEMS FOLLOWING EXCITOTOXIC CELL DEATH IN THE HIPPOCAMPUS AND CEREBRAL NEOCORTEX

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INTRODUCTION

Many areas of the brain that exhibit neurodegenerative changes in Alzheimer-type dementias (DAT) appear to be interconnected via neuronal projections. The severity of pathology in the neocortex correlates statistically with known patterns of intracortical connections (Pearson et al., 1985), and the major affected subcortical regions, such as the nucleus basalis, locus coeruleus and dorsal raphe project prominently to the allo- or neocortex (Saper et al., 1987). Observations such as these have led to suggestions that the degeneration in some regions of the brain might be secondary to loss of neurons in other areas, resulting from a loss of target-derived trophic support (Appel, 1981; Hefti, 1983). The evidence that this might be the case is particularly suggestive for cholinergic neurons of the basal forebrain.

Basal forebrain cholinergic neurons can be divided into several subgroups, those in the medial septum, diagonal band and basal nucleus, and these subgroups project in a topographically organized manner to different portions of allo- and neocortex (Pearson et al., 1983a; Cuello and Sofroniew, 1984). In DAT, the severity of pathology noted in these subgroups correlates positively with the degree of pathology in the particular tartet cortex (Arendt et al., 1985). Work in experimental animals has shown that basal forebrain cholinergic neurons bear nerve growth factor receptors (NGFr) and retrogradely transport radioactively labelled nerve growth factor (NGF) injected into cortical areas where target neurons normally make NGF and its mRNA (Korsching, 1986; Ayer-Lelievre et al., 1988; Dawbarn et al., 1988; Seiler and Schwab, 1978). In addition, NGF has demonstrable effects on these cells. In tissue culture, NGF effects both the production of choline acetyltransferase (ChAT) and degree of neurite outgrowth (Hefti et al, 1985, Gahwiler et al., 1987). In vivo, the majority of septal cholinergic neurons are lost after axotomy in the septo-hippocampal projection (Gage et al., 1986; Sofroniew et al., 1987), and this loss can be prevented pharmacologically by administration of exogenous NGF (Hefti, 1986; Williams et al., 1986; Kromer, 1987). Similarly, pharmacologic doses of NGF can reverse the atrophy of cholinergic neurons in the basal nucleus in aged rats (Fischer et al., 1987). Nevertheless, the precise effects normally exerted upon these neurons by NGF or other target-derived factors are poorly understood, and the consequences of withdrawing target-derived trophic support in the adult rat are not known. We have therefore examined the effects upon basal forebrain cholinergic neurons and their terminal networks of removing their target neurons.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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EFFECTS OF TARGET NEURON LOSS ON LOCAL CHOLINERGIC TERMINAL NETWORKS

To ablate target neurons without transecting the afferent fibers of basal forebrain cholinergic neurons, excitotoxic amino acids were either applied unilaterally to the dura overlying the cerebral cortex (Sofroniew and Pearson, 1985; Sofroniew et al., 1987; Isacson and Sofroniew, 1990) or stereotaxically injected unilaterally into multiple sites in the hippocampus in adult rats ((Sofroniew et al., 1989). After various survival times the rats were sacrificed.

Histological analysis of the cerebral neocortex (Isacson and Sofroniew, 1990) showed initial gliosis followed by gradual shrinkage of the tissue. There was also a gradual reduction in the overall network of AChE-positive cholinergic fibers which lagged behind the shrinkage of the neocortex such that the density of cholinergic fibers appeared slightly elevated during the phase of active shrinkage (1 to 4 weeks). After survival times of several months to over one year, the density of cholinergic fibers in the cortical remnant was similar to that in normal cortical tissue. Tracing experiments conducted after one year showed that cholinergic neurons in the basal nucleus still project to the cortical remnant (Isacson and Sofroniew, 1990; Sofroniew et al., 1990). Similarly, measurements of the biochemical marker for afferent cholinergic fibers, ChAT, showed a significant increase in ChAT concentration during the phase of active cortical shrinkage after loss of intrinsic neurons, and a return to concentrations similar to that found in unlesioned tissue after shrinkage had more or less stopped (Isacson et al., 1988; Isacson and Sofroniew, 1990; Sofroniew et al., 1990). At no time were concentrations lower than normal observed. These findings are in marked contrast to the changes in cholinergic markers seen in DAT cortical tissue, where there is a profound drop in the concentration of ChAT and in the density of cholinergic axons. Our results therefore strongly suggest that the loss of cholinergic markers from the cortex in DAT cannot be due solely to a loss of intrinsic cortical neurons.

Histological analysis of hippocampal tissue (Sofroniew et al., 1989) showed the absence of virtually all intrinsic neurons, pronounced gliosis and little change in cholinergic fiber density as revealed by AChE histochemistry after 7 and 28 days. By 90 to 120 days gliosis had largely subsided and massive shrinkage had occured, such that less than 10% of hippocampal tissue remained. Many AChE positive fibers were present but their density was somewhat lower than normal, indicating that a substantial reduction in the total extent of the original network of cholinergic axons had occured.

EFFECTS OF TARGET NEURON LOSS ON BASAL FOREBRAIN CHOLINERGIC NEURONS

Following extensive excitotoxic lesions of the cerebral neocortex, afferent cholinergic neurons atrophy in the form of shrinkage, but are not reduced in number (Sofroniew and Pearson, 1985; Sofroniew et al., 1987). Foetal grafts containing cortical neurons are able to sustain afferent cortical fiber networks and prevent the atrophy of cholinergic neurons in the basal nucleus (Sofroniew et al., 1986; Isacson and Sofroniew,1990). These findings suggest that target neurons may regulate the extent of cholinergic terminal networks and the size of basal forebrain cholinergic neurons, but may not be essential for their survival. In this system, however, axotomy also does not lead to retrograde cell death and causes a reversible decline in ChAT activity (Sofroniew et al., 1983; Stephens et al., 1985; Sofroniew et al., 1987), so that it may be argued that cholinergic neurons are able to derive enough trophic support from other target neurons through collateral branches. For this reason we have also studied the septo-hippocampal system, where septal cholinergic neurons die after axotomy of their fibers projecting to the hippocampus through the fimbria-fornix (Tuszynski et al., 1990; O'Brien et al.,1990).

Following the near total ablation of hippocampal neurons by excitotoxic amino acids, there is no loss of septal cholinergic neurons for up to 120 days (Sofroniew et al., 1989), indicating that septal cholinergic neurons are not directly dependent upon target neurons for survival. There is, however, some atrophy of the cholinergic neurons in the form of significant cell shrinkage and a decline in optical density indicative of a mild loss of intracellular ChAT. The persistance of septal cholinergic neurons after ablation of their

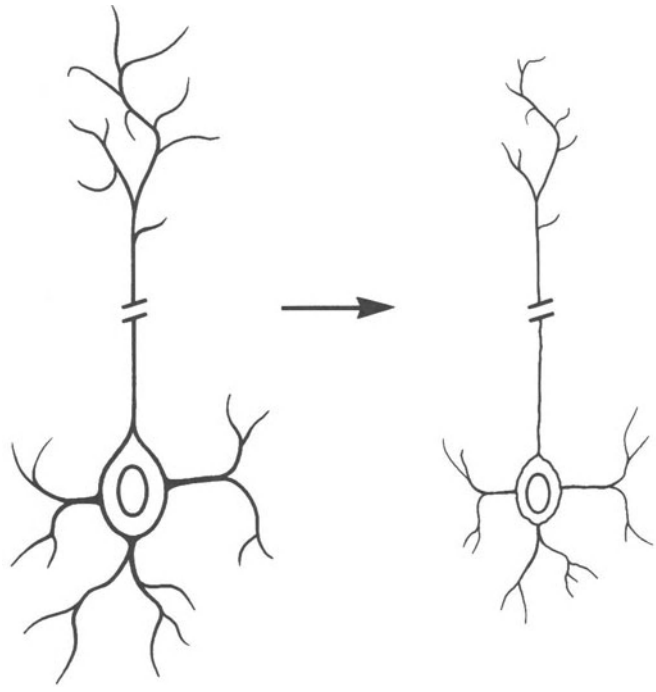

Fig. 1. Schematic drawing illustrating the effects of loss of target neurons on basal forebrain cholinergic neurons. There is a reduction in the extent of the terminal axonal network, accompanied by shrinkage of the cell body and dendrites, but cell death does not occur.

target neurons is in striking contrast to the massive cell death seen 14 to 28 days after axotomy. Interestingly, the loss of septal cholinergic neurons is equally severe following axotomy after excitotoxic hippocampal lesions, as in unlesioned animals. This result demonstrates that the majority, if not all, axons from septal cholinergic neurons are still present in the remnant of the fimbria-fornix 120 days after the loss of all hippocampal neurons. Tracer studies using different fluorescent tracers injected into the hippocampus and other reported potential target regions showed that fewer than 18% of neurons in the medial septum project to sites other than the ipsilateral hippocampus, and less than 5% project both to the hippocampus and another site (Sofroniew et al., 1989). Thus, the survival of septal cholinergic neurons after hippocampal NMDA lesions is not due to the presence of axon collaterals to other targets.

These findings show that uninjured basal forebrain cholinergic neurons in adult rats do not die in the absence of target neurons and are either not dependent on neurotrophic factors for survival or can obtain trophic support from other sources for at least 120 days after target neurons are lost. Loss of target neurons will, however, result in shrinkage of the cell body and dendrites, and a reduction of the cholinergic terminal network (Fig. 1).

LOSS OF TARGET NEURONS, TROPHIC SUPPORT AND CHANGES IN BASAL FOREBRAIN CHOLINERGIC SYSTEMS IN DAT

Our findings thus far indicate that the loss of intrinsic neurons from the cerebral cortex in experimental animals does not lead to a decline in the concentration of biochemical cholinergic markers or in the density of cholinergic fibers in cortical tissue. This contrasts greatly with the pronounced loss of such markers seen in DAT. Moreover, the loss of cortical neurons does not lead to cholinergic cell death, whereas cholinergic cell loss appears to be characteristic of severe or advanced DAT (Whitehouse et al., 1982;

Arendt et al., 1985). These observations suggest that the loss of intrinsic cortical neurons would not alone lead to the changes noted in cholinergic systems in DAT, even though these neurons are a main site of NGF production in normal animals (Ayer-LeLievre et al., 1988, Whitmore et al., 1988), and this NGF appears to be directed towards cholinergic neurons (Seiler and Schwab, 1978; Korsching, 1986; Dawbarn et al., 1988).

Loss of neurotrophic support has been proposed as a possible cause of changes in cholinergic systems in DAT (Appel, 1981; Hefti, 1983). However, the concentration of mRNA for NGF is not reduced in the DAT cortex (Goedert et al., 1986), and extract of DAT cortex contains more survival-promoting activity for neurons maintained *in vitro* than extract from normal cortex (Uchida et al., 1988). Moreover, our findings suggest that a failure of the supply of factors by target neurons will not lead directly to the death of afferent cholinergic neurons, as generally seen in DAT, but will result in the retraction of terminals and shrinkage of the cell body and dendrites. These changes are similar to the shrinkage, without loss, of cholinergic neurons noted in some cases of DAT (Perry et al., 1982; Pearson et al., 1983b; Etienne et al. 1986; Zweig et al., 1989), and a loss of trophic support might underlie atrophy of this kind. Whether these cases represent early stages or different and less severe forms of DAT is not known. It is possible that the additional loss of trophic support from other sources, or perhaps a failure of uptake, transport or intrinsic ability of the cholinergic neurons to respond to trophic factors, might be responsible for the more severe changes noted in cholinergic systems in advanced DAT, but there is no evidence to support this so far. Experiments to test some of these possibilities are currently in progress. Nevertheless, our observations do not preclude the possiblity that neurotrophic factors such as NGF might be of pharmacological benefit to cholinergic neurons in DAT, either by promoting survival or upregulating the function of surviving neurons (Gage et al., 1988; Hefti et al., 1989), but this will require precise evaluation.

In conclusion, our evidence suggests that the degenerative changes in cholinergic systems seen in DAT are not merely a retrograde reaction to the loss of target cortical neurons or factors produced by them. Since the basal forebrain cholinergic system is one of many regions affected in DAT that appear to be interconnected by neuronal projections (Pearson et al., 1985, Saper et al. 1987, Talamo et al., 1989), it will be interesting to see if evidence can be obtained for retrograde degeneration in other systems, or for the spread of a specific disease process by way of neuronal projections.

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INTERLEUKIN-3 PROMOTES NEURITE OUTGROWTH AND ELEVATES CHOLINE ACETYLTRANSFERASE ACTIVITY

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INTRODUCTION

Alzheimer's disease (AD) is associated with the degeneration of cholinergic neurons in the hippocampus and basal forebrain. Since nerve growth factor (NGF) was found to enhance choline acetyltransferase (ChAT) activity, and the infusion of NGF into the rat cerebral ventricle prevented death of lesioned septal cholinergic neurons and improved cognitive functions in old animals, NGF has been proposed as a therapeutic agent for AD. As basic fibroblast growth factor has a similar effect in vivo, we looked for other trophic factors for central cholinergic neurons. In this study we demonstrate that interleukin-3 (IL-3), which plays a role in the growth and differentiation of hematopoietic lineage cells, also acts as a novel trophic factor for central cholinergic neurons in primary cultures of mouse septal neurons and in cholinergic hybridoma line cell SN6.10.2.2.

MATERIALS AND METHODS

Neurons obtained from the septal region of BALB/c mice on embryonic day 15 were dissociated and cultured in dishes coated with 10 μg/ml poly-L-lysine and containing the serum-free medium defined below. Cells were seeded at 6×10^5 cells/ml for ChAT assay or 1×10^5 cells/ml for the study of neurite outgrowth. Serum-free medium was composed of equal volumes of Dulbecco's modified Eagle medium and Ham's F-12 medium supplemented with 100 μg/ml human transferrin, 25 μg/ml bovine crystalline insulin, 30 nM sodium selenate, 15 mM Hepes buffer, 20 nM progesterone, 20 nM hydrocortisone-21-phosphate, 10 μM L-carnitine, 30 nM 3,3',5-triiodo-L-thyronine, 7 ng/ml tocopherol, 7 ng/ml retinol, 1 μM thioctic acid, and 1 μl/ml mineral mixture as formulated by Hutchings and Sato[1]. Cholinergic hybridoma line cell SN6.10.2.2 was a gift from Dr. B. Wainer (Univ. Chicago) and cultured at a cell density of 2×10^5/ml in the serum-free defined medium for the study of ChAT activity. For ChAT assays Fonnum's method[2] was used with partial modification, and the activity was assessed on day 5 of primary cultures or day 2 for the line cell. Protein content was measured by Lowry's method. To estimate the neurite outgrowth, we took pictures of random fields under a phase contrast microscope after 0, 20, 40, 50 hr and neurite-bearing cells were counted on printed pictures. Human recombinant IL-3 (hIL-3), 10, 50, 100 U/ml; highly purified mouse IL-3 (mIL-3), 10, 50, 100 U/ml; human recombinant interleukin-1α (IL-1α),

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Table 1. Effect of IL-3 on ChAT Activity in Cultured Septal Neurons

	n	Specific activity (pmoles/mg/min)	Total protein (μg/well)	Total ChAT activity (pmoles/hr/well)	Relative activity (%)
Control	3	12.3±2.0	120.8±29.5	89.2±27.7	100
β-NGF	3	18.1±3.4	158.6±39.1	172.2±37.2	193±35
Control	3	11.4±2.4	70.6±33.1	48.3±12.2	100
mIL-3	4	20.9±5.3	91.4±35.5	114.6±35.8	237±62
Control	3	14.6±4.6	68.9±18.7	60.4±35.9	100
hIL-3	4	32.2±8.7	111.7±21.0	215.8±49.8	357±25
Control	2	11.5	193.2	133.3	100
hIL-3	2	44.9	140.3	378.0	284
hIL-3+α-IL-3	2	15.4	184.3	170.3	128
α-IL-3	2	13.2	169.4	134.2	101

26.4 U/ml; human recombinant interleukin-1β (IL-1β), 18.8 U/ml; human recombinant interleukin-2 (IL-2), 20 U/ml; human recombinant interleukin-6 (IL-6), 90 ng/ml; human recombinant tumor necrosis factorα (TNFα), 20 ng/ml; mouse recombinant granulocyte-macrophage colony stimulating factor (GM-CSF), 10 U/ml; βNGF, 100 ng/ml; and monoclonal antibody against hIL-3, 13.3 μg/ml were each added to separate cultures at the time the cultures were started, and the medium was changed on day 2 or 3. To characterize the primary culture cells, we stained them with polyclonal anti-chicken neurofilament protein (NFP, 70KD) antibodies, polyclonal anti-porcine glial fibrillary acidic protein (GFAP) antibodies, and reagent for the staining of non-specific esterase (NSE).

RESULTS

Most cells in primary culture were stained with NFP antibodies but not with GFAP ones or NSE. Among the cytokines and factors tested, only IL-3 promoted neurite outgrowth (50%) in treated cultures, compared with controls (25%), after 20 hrs. IL-3 also enhanced ChAT activity (Table 1); relative activities (RA) of ChAT (based on control values) for mIL-3 and hIL-3 were 240% and 360%, respectively. These effects were completely blocked by α-IL-3. In Table 2, the effect of mIL-3 on SN6.10.2.2 cells is shown. This cytokine had an RA of 180% toward these cells.

DISCUSSION

IL-3 is one of the colony-stimulating factors, that is produced by antigen- or mitogen- activated T cells and is involved in regulating the growth and differentiation of a wide range of hematopoietic lineage cells. Although mIL-3 has only 40% homology to hIL-3 including conserved amino acid residues, a single disulfide bridge between cysteins residues 17 and 80 is conserved for biological activity in both human and mouse IL-3.

Table 2. Effect of IL-3 on ChAT Activity in Cholinergic Line Cells

	n	Specific activity (pmoles/mg/min)	Totel protein (μg/well)	Total ChAT activity (pmoles/hr/well)	Relative activity (%)
Control	2	14.7	369.8	326.2	100
mIL-3	2	19.7	506.4	598.6	184

Recently the interaction of the immune and nervous systems in AD has generated great interest in investigators. Neuroleukin, which is produced by T cells and induces B cell differentiation, has a trophic effect on spinal cord neurons. Interleukin-6, essentialy a cytokine for B cell differentiation, also acted on rat's pheochromocytoma line cell PC12.

Frei and colleagues[3] reported that astrocytes produced IL-3-like factors that enhanced proliferation and growth of microglial cells in the mouse culture system. Farrar et al.[4] demonstrated a large amount of IL-3 mRNA expressed in mouse brain by in situ hybridization and Northern blot analysis. But the function of IL-3 has remained unclear. Here we indicate IL-3 promotes neurite outgrowth and induces elevated ChAT activity in primary cultures of septal neurons. Since these effects were also found in cholinergic line cell SN6.10.2.2 and were blocked by α-IL-3, IL-3 appears to have a direct effect on cholinergic neurons. So we conclude[5,6] IL-3 is a novel trophic factor for central cholinergic neurons at least in vitro. Although there are many reports about IL-3 acting on hematopoietic lineage cells, little is known of its action in the central nervous system. Our results indicate that IL-3 should be added to the list of therapeutic agents for AD. Further studies to examine the status of IL-3 in AD patients are proposed.

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MEMORY IMPAIRMENT IN RATS INDUCED BY AN ACTIVE FRAGMENT OF NERVE GROWTH FACTOR ANTIBODY

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INTRODUCTION

Nerve growth factor (NGF) is a protein of known importance for the development and maintenance of peripheral sympathetic neurons (Levi-Montalcini and Angeletti, 1968; Yu et al., 1978). Recent anatomical, behavioral, and biochemical studies have been interpreted as suggesting a possible role for NGF in the central nervous system (Honegger and Lenoir, 1982; Gnahn et al., 1983; Fischer et al., 1987). However, no direct evidence exists implicating altered levels of NGF as causative in either normal aging or in accelerating neuro-degenerative processes such as Alzheimer's disease. Furthermore, intra-ventricular and intracortical injection of anti-NGF-antibody failed to produce anadverse effect on forebrain cholinergic neurons (Gnahn et al.,1983). It is so far not known whether a deficiency of NGF is responsible for impairments in learning and memory. We report here that continuous intracerebral infusion of a specific Fab' fragment of antiserum to NGF (anti-NGF) over a period of four weeks impairs learning and memory retention of the water maze and habituation tasks in rats.

MATERIALS AND METHODS

Male KBL Wistar rats weighing about 250 g were used. Their ages were 50-56 days at the start of the study. Cannulae attached to modified mini-osmotic pumps were implanted into the lateral ventricles of all the rats used in the experiments. The rats were divided into 7 groups. For the control rats, the pump was filled with artificial cerebrospinal fluid (CSF) containing bovine serum albumin (5 mg/ml) and non-specific Fab' fragment of IGg. For the experimental rats, the pump was filled with CSF containing anti-NGF Fab' fragment (30, 100, 300, 500, 1000, or 2000 μg/ml) and bovine serum albumin. The estimated cumulative anti-NGF doses were 12, 40, 120, 200, 400 or 800 μg per 4 weeks. The pumps were replaced with new ones after 15 days.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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The rats were put through the test once a day (one training session a day) in a water maze during the anti-NGF infusion period, between day 8 and day 14 (2nd week), and between day 22 and day 28 (4th week) after the start of infusion. In the 4th week, the cues for navigation were rearranged. The behavioral trace, the distance of swimming, and goal latency in the water-maze task were monitored by TV camera and analyzed by computer. Each rat was put into a plastic cage and the locomotor activity of the rat was recorded by automex for 10 m in on 25th and 27th days after the start of infusion. Choline acetyltransferase (CAT) activity was assayed immediately after the last training session. Data were analyzed by an analysis of variance, Mann-Whitney'U-test, and Student't-test.

RESULTS

Both the distance of swimming and the goal latency in the control rats rapidly shortened day by day compared those of the anti-NGF-treated rats, when rats were trained continuously in the water-maze task. By the end of training, the distance of swimming was down to approximately 173 cm/training session for the control rats, but approximately 389 cm/training session for the anti-NGF (400 μg/4 weeks)-treated ones. The goal latency was down to approximately 17.7 s/training session for the control rats, but approximately 31.6 s/ training session for the anti-NGF (400μg/4 weeks)-treated animals. By watching the swimming path of the anti-NGF-treated rats, we noted that they appeared to approach the platform by swimmimg in a fixed direction around the sides of the pool. In the statistical analysis for the 2nd and 4th weeks, ANOVA showed differences between the groups in both areas of evaluation for the whole test week (Fig.1, the distance of swimming; 2nd week, $F(6,392)=9.60$, $p<0.01$. 4th week, $F(6,294)=4.14$, $p<0.01$: the goal latency; 2nd week, $F(6,392)=7.57$, $p<0.01$. 4th week, $F(6,294)=3.85$, $p<0.01$.).

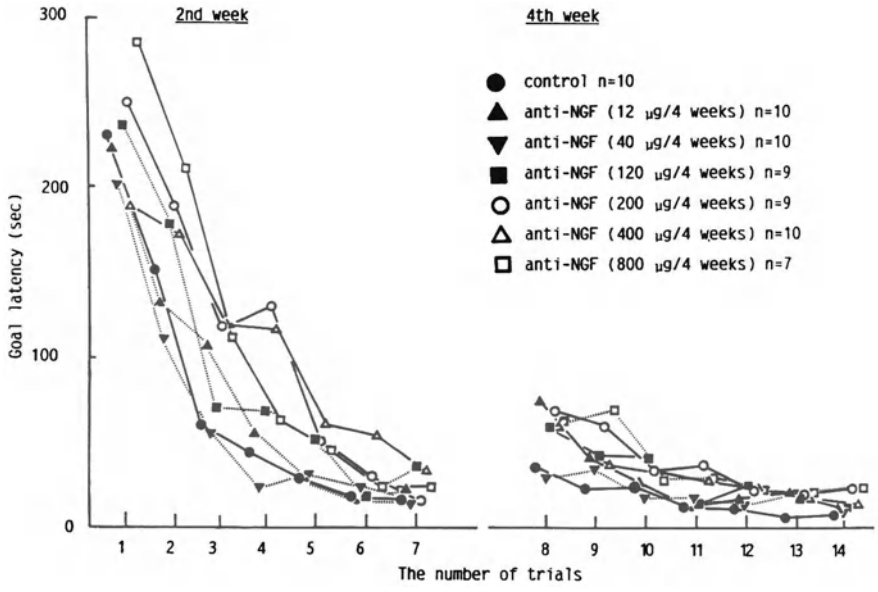

Fig.1 Effect of chronic infusion of an active Fab' fragment of anti-NGF (anti-NGF: 12-800 μg/4 weeks/rat, i.c.v.) on the learning and memory of rats in the water-maze task.

In the habituation task, when rats were placed in unfamiliar circumstances, their locomotion increased as they examined the new environment. Although the movement counts for the rats were different for the control and anti-NGF-treated groups for the first exposure (25th day after the start of infusion; 200 and 800 μg/4 weeks, P<0.05), the movement counts for the control rats sig- nificantly decreased when the animals were exposed again to the same environment on the 27th day after the start of infusion, since they remenbered the circumstances. However, the degree of reduction of the movement counts for the anti-NGF-treated rats was significantly smaller than that of the control rats. (Fig.2, 400 μg/4 weeks; p<0.05).

Administration of anti-NGF had no effect on the striatum, the cortex or the hippocampus CAT activity.

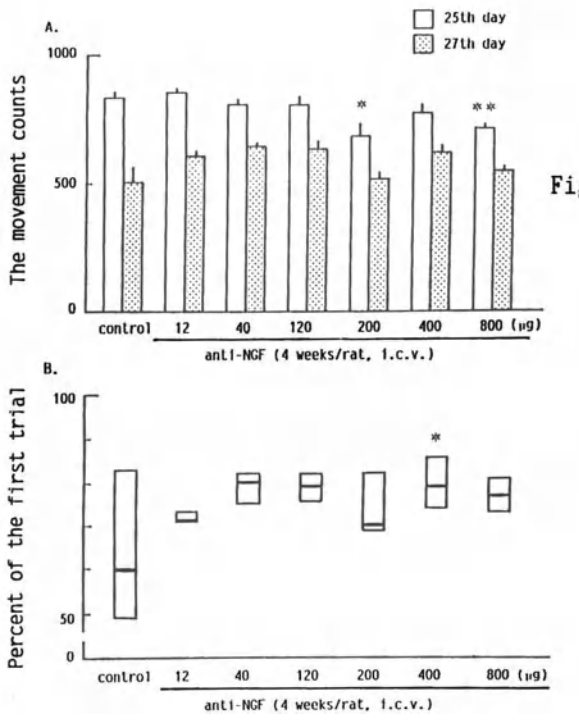

Fig.2 Effects of chronic infusion of an active Fab' fragment of anti-NGF (anti-NGF) on the habituation of rats. All rats were infused with anti-NGF (12-800 μg/ 4 weeks/rat, i.c.v.) by osmotic minipump and habituation task was carried out on 25th and 27th days after the start of infusion. * p<0.05 vs. the control group.

DISCUSSION

Our results showed that chronic anti-NGF-infusion produced impairment of learning and memory. Such, chronic infusion of the anti-NGF Fab' fragment into the cerebral ventricle may decrease the NGF level in the cholinergic neurons. We suggest that the infused anti-NGF exerts its effects mainly through local diffusion into the brain tissue, including the part of the striatum close to the infusion site, since CAT activity was not changed by anti-NGF treatment. Perhaps this ermits the infused anti-NGF to be taken up into the striatal cholinergic interneurons and by the axons of the basalo-cortical cholinergicneurons.

Viewing the question from a different angle, it has been reported that intraventricular injection or infusions of NGF in young adult rats prevents retrograde neuronal cell death (Hefti, 1986; Williams et

al., 1986; Kromer, 1987) and promotes behavioral recovery after damage to the septo-hippocampal connections (Will and Hefti, 1985). In addition, continuous intracerebral infusion of NGF can partly reverse cholinergic cell body atrophy and improve retention of a spatial memory task in aged rats (Fischer et al., 1987). The above results suggest that the ability of NGF to counteract retrograde degeneration of the cholinergic neurons after axotomy may be due to a trophic, protective action on the damaged cholinergic neurons.

It seems possible that the impairment of memory by means of anti-NGF infusion in the rats could be due to the degenerative effect of anti-NGF on the forebrain cholinergic neurons through deprivation of NGF. The present results suggest the possibility that the age-dependent structural and functional deterioration of the forebrain cholinergic neurons, implicated in Alzheimer's disease, may be due to either the loss or dysfunctional utilization of NGF induced by auto-immunization. The experiments reported here represented a model in the rat to study physiological changes due to a deficiency of NGF in the CNS. However, we could not determine any change in CAT activity in discrete brain areas after anti-NGF treatment. We should employ more a sophisticated method of analysis to detect CAT activity in small, discrete brain areas.

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BRAIN'S CALCULATING PRINCIPLE

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INTRODUCTION

The testing of psychotropic drugs on humans requires that consciousness itself be evaluated. With laboratory animals, conscious activity cannot be diagnosed. How can this handicap be overcome?

This is possible if we postulate a pair of potentials--sleep potential (SP) and wakefulness potential (WP)--that are constantly changing at high speeds and combining to form the self-conscious mind once over every unit time. It is also necessary to attribute energy to the pair of potentials--energy that is produced while the nervous system computes probability amplitude to perceive information. As for the reason that SP and WP occur as a pair, it would be logical to surmise that, for some reason, an organism needs to recognize both incoming (sensory) information and inherent (DNA and memory) information simultaneously.

BACKGROUND CONCEPT 1

Science has long sought an answer to the question: Why do we sleep? It does not seem to be possible to reach an understanding of the functions of sleep by studying only sleep itself. The following viewpoint about sleep appears to be necessary: Two phenomena occur simultaneously and in opposite directions. One is a potentialization toward sleep, and the other is a potentialization toward wakefulness. These potentials are constantly changing independently and at high speeds, and the combined output of both potentials determines whether an organism is in a state of sleep or wakefulness.

These antagonistic potentializations generated in all cell membranes go through an integration process in which consciousness and the self-conscious mind are created and, thereby, recognition of information is made possible. This process can be replaced with the operating system (OS) of the brain for handling information. While the sleep and wakefulness potentializations are integrated, an operating system is created, memory is accessed, and the OS is then modified.

More specifically, each bit of information that the brain takes in is distributed to localized neural circuits and a new address is established for storage. The information is compared with and integrated into a past fixed memory, the entire system of information is reorganized, and the add-

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ress of the neural circuit where the information is stored is renewed. From this viewpoint, wakefulness can be regarded as a state in which the brain concentrates more on taking in information, such that the recognition of information cannot catch up. Sleep is a state in which the brain has given up taking in information and concentrates on the reorganization of information.

It may be possible that this hypothesis can be verified partly through experiments conducted through the use of reverse diagnosis, a method in which very brief moments of sleep in mice are regarded as wakefulness periods and vice versa.

BACKGROUND CONCEPT 2

One example involves an imaginary stopwatch hand and the fundamental concepts of quantum mechanics. The other is related to the elegant geometric curve known as the cycloid.

Richard P. Feynman asserts that the fundamental concepts of quantum mechanics are understandable for anyone who knows how to read a clock (Feynman's book: The Strange Theory of Light and Matter, Princeton University Press, 1988). The physicist sets up a light source and a detector to discuss the path in which a photon travels and the length of time it takes a photon to reach the detector. The next step is to consider the clock hand as a unit vector and add all the resulting clock hands obtained. The magnitude of this resultant vector provides the probability amplitude, and the square of the magnitude represents the probability with which a photon travels from the light source to the detector.

Feynman asserts that this simple model can explain all phenomena concerning light and electrons as well as the diversity of nature and thus allows prediction of the future. This statement has the effect that one beam of light does not suffice to detect light but that a bundle of beams is necessary. This aspect of the behavior of nature should be true of vibration and time, and it should apply to the sensation and perception mechanism of an organism.

A cycloid is also called brachistochrone or tautochrone. Analytical dynamics can be said to be the mechanics version of Fermat's principle in optics. The Hamiltonian function (H) represents the total energy that the object (a system) has. When H satisfies the canonical equation, the motion of an object appears to be constant "regardless of time". If we refer to a length of time within which "time cannot be resolved", then the size of a cycloid can be thought of as a unit time, and at least two consecutive cycloids are necessary for recognition of a change in time or a point in time. Attention should be paid to whether it is numerically countable. The smallest possible cycloid can also be regarded as "the quantum of time".

Imagine a series of small cycloids laid one next to another all of which are contained in a larger cycloid. In this situation, the subject with a shorter unit time, which is located on a lower level in the hierarchy, is able to resolve a certain length of time that may appear isochronal to the subject with a longer unit time, located on a higher level in the hierarchy. A cycloid can be regarded as the unit of proper time because time cannot be resolved.

We can hypothesize that an impulse processing system has a hierarchy of proper times in which the unit time of each level lengthens in ascending order, or in the order in which an impulse is transmitted from the receptors up to the brain for perception. Under such a hypothesis, probability calculation is the only computation principle that can be used to explain the

information processing system of a biological system. This concept should help us to understand why there is uncertainty in localizing consciousness.

CONCLUSION

We assume that without comunication between the self-conscious mind and the neural networks there would be no learning and memory. Unlearning is a process in which an address for storing information or memory is changed. Neither information nor memory is lost.

Hypothesis 1

The self-conscious mind is a combination of the three integrated potentials (the sleep, wakefulness, and conscious activity potentials), each activating the address potential on the same level as itself. In other words, the self-conscious mind chooses three functional modules out of several million modules, all lined up on a scale, and specifies their addresses to activate them. This allows the memory stored in those particular functional modules to occur to the mind. In this process, two types of functional combinations--sleep type (C-type) and the wakefulness type (E-type)--occur. We assume that the recognition memory is induced by the E-type functional combination and the neural reconstruction is performed by the C-type functional combination. Both have the direction from the higher address potential to the lower address potentials. In this case, the initial value and the object (final) value for accessing must be determined at every moment. In comparison with computers, E-type and C-type correspond to read/write and delete/rewrite instructions, respectively. Fig. 1 simplifies this rather complicated dynamics into a model where only ten modules are shown lined up on the scale.

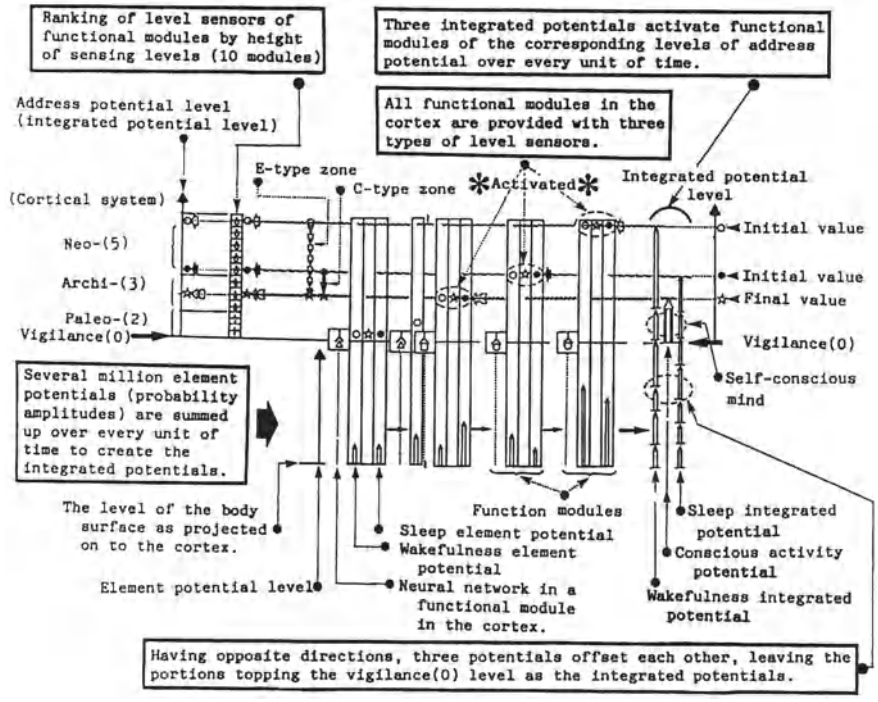

Fig. 1. Functional combination of three types of address potentials inherent in columns (functional modules) of the cortex and three types of integrated potentials (the self-conscious mind).

Hypothesis 2

Quantum mechanics leads us to the prediction that the upper (larger) cycloid recognizes the energy flow pattern--or the meaning of a signal or phenomenon--by adding the probability amplitudes. The brain replaces the energy flow pattern derived from each of its function systems with an probability amplitude and then adds or multiplies the amplitudes to calculate the probability of a phenomenon that has just occurred in the outside/inside world. Fig. 2 summarizes these relation into a simple model.

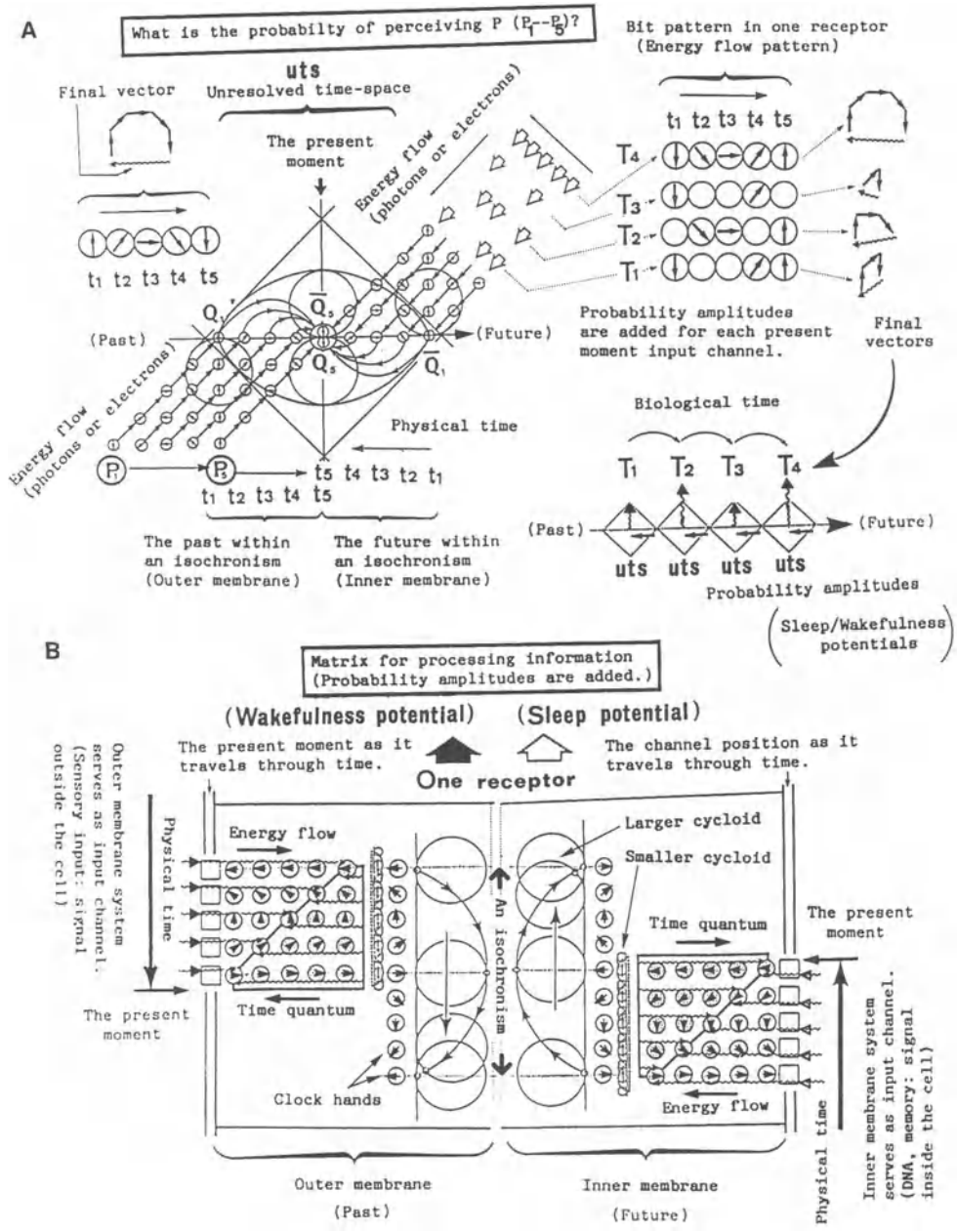

Fig. 2. A mechanism for perception of information using time quantum as a parameter. (A) Minkowski space-time. (B) Calculating membrane model.

SCREENING FOR PSYCHOTHERAPEUTIC CHEMICALS BY ANALYSIS OF CONSCIOUS ACTIVITY IN THE MOUSE

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INTRODUCTION

The notions and methods discussed below will at least allow the screening of new types of drugs. New types of drugs in the screening stage will not only improve the synaptic mechanism for excitation and inhibition, as conventional drugs do, but will also improve the performance of the operating system of the brain. This suggests the existence of chemical compounds that cannot be inferred through limited (localized), sharp and realistic experimental analysis of biochemistry and neurophysiology, since the variables for the integration of varid phenomena in the brain must also be analyzed.

NOTION

Eccles' liaison brain hypothesis[1] argues that the self-conscious mind does not originate in the cerebrum and yet is very likely to be produced through some sort of material structure. It also argues that perception is made possible when the self-conscious mind "scans" and "comunicates" with function modules (column structure). In addition, memory is of two clearly distinguishable types: brain storage memory and recognition memory. Brain storage memory is stored in function modules that correspond to individual types of information, whereas recognition memory occurs to the mind when communication between the self-conscious mind and the individual function modules has been established. The liaison brain hypothesis focuses on the brain during wakefulness periods and regards a sleep period as a disordered state in which the self-conscious mind cannot communicate with the function modules.

The reverse learning (unlearning) hypothesis[2] proposed by Crick and Mitchison (1983) stresses the positive function of sleep phenomena. Dreaming is regarded as a process in which abnormal information that is undesirable for the neural networks of the cerebral cortex is eliminated. Dreaming thus plays a role of "tuning the brain".

The above hypotheses concerning consciousness and sleep phenomena can be understood in a unified way by the introduction of some new viewpoints[3] corresponding to each of the hypotheses. Neither Eccles' nor Crick's hypothesis provide an approach for verification. A method using a model experi-

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ment to verify the hypotheses will be described below. This limited method was developed by observation of the characteristic frequent alternation of sleep and wakefulness in mice.

METHODS

The duration in seconds of sleep and wakefulness in mice can be diagnosed by the dynamic behavioral stage analysis method (DBSAM).[4] The top rhythm in Fig.1,A shows an example of a series of vigilance levels per sec, derived from a diagnosis using DBSAM. As the first step, after the standard levels for X and Y have been specified, the bits of data are classified into three stages: the moving stage (MV), the deep sleep stage (EP), and the transient "other" stage (OT). The output of this process is represented by LINE 1. In the next step, bits in the OT stages are classified, by use of one of the DBSAM algorithms, into the entering sleep stage (ET), the awakening stage (AW), the light sleep stage (LS), or the quiet stage (QU). LINE 1 and LINE 2 are diagnostic results of DBSAM. The process by which LINE 3 is obtained from LINE 2 is as follows: The leftmost EP in LINE 2 is selected as an example. First, EP is replaced with QU, and then the two LS's before and after it are also replaced with QU's. Since a QU is a wakefulness stage, the 3-second duration, which was occupied by EP and LS's before, has now been converted into a wakefulness stage. As a result, the two wakefulness stages (MV) that were separate before the conversion have now been connected by the newly-formed three-second wakefulness stage, which together form a longer wakefulness stage than either of the previous wakefulness stages. This is the output of the rediagnosis. This type of diagnostic output is defined as rediagnostic output of MSLP=1 (sec). Thus, one can create "apparently continuous wakefulness stages" by first declaring MSLP=t (sec) and then changing the value of t until no stages other than wakefulness stages are left in a given area. Similarly, if we use the duration of the MV stage (t seconds) as the index and declare MACT=t, so that all stages are changed into LS stages, we can obtain "apparent sleep stages" as the output.

What such processing actually does is to artificially change the vigilance(0) level and to rediagnose "the apparent wakefulness or sleep stage", derived from the results of diagnosis using DBSAM with the vigilance(0) standard. When depth of sleep and intensity of activity have been diagnosed in detail, it is more appropriate to regard it as the shifting of the diagnostic standard on the vigilance scale upward and downward. Although it is the lengths of sleep and wakefulness states, shown on the horizontal axis, that are taken into consideration for reverse diagnosis, the aim of this processing is to vertically shift the evaluation standard for depth of sleep and height of wakefulness upward or downward along the vigilance scale, i.e. the vertical axis. This adjustment enables one to review, in relative terms, the original diagnostic results from different diagnostic standards. This process can be easily understood by the use of three specific vectors as shown in the right side of Fig. 1,B.

By using the magnitude of "the apparent conscious activity vector" (AVC) as the probe, we can hypothesize the levels of the integrated sleep potential and the integrated wakefulness potential. We should note that in this paper the conscious activity potential obtained from diagnosis with the vigilance(0) level serves as the conventional concept of 'depth of sleep' or 'height of wakefulness'. This hypothesis postulates that the integrated sleep potential and the integrated wakefulness potential, both currently immeasurable, occur once over every unit of time and, as a pair, activate--or functionally combine with--the neural circuits lying on the same levels (address potentials) as the potentials themselves.

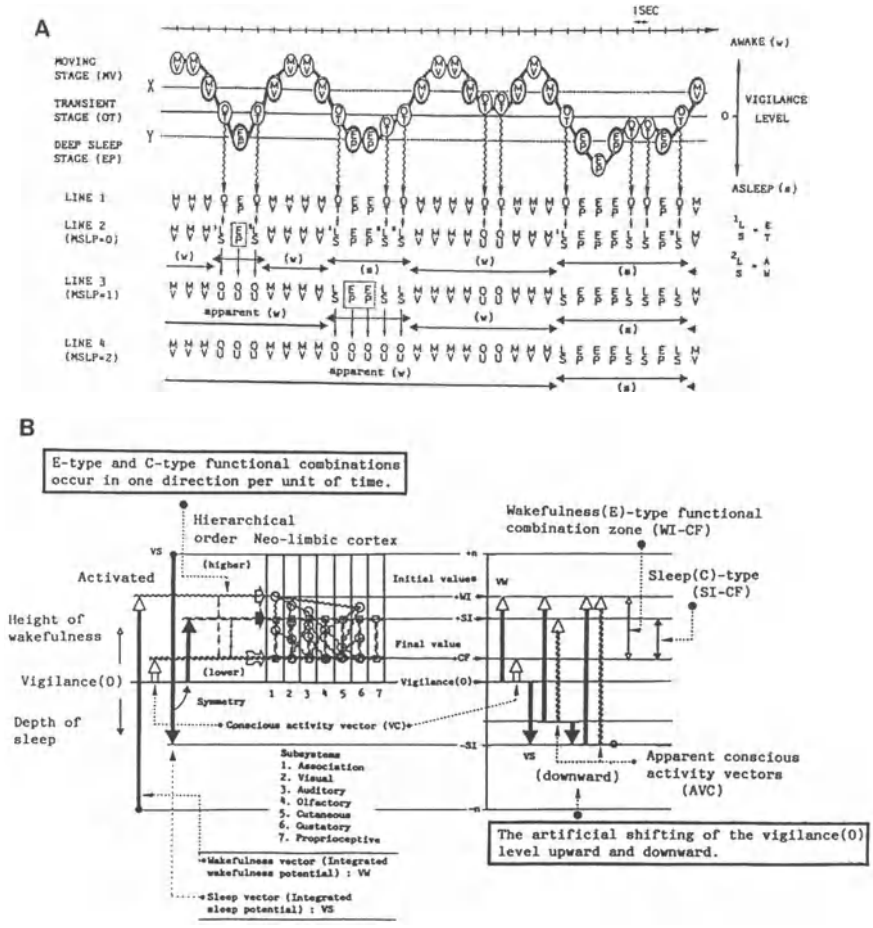

Fig.1. (A) Rediagnosis by reverse-processing of sleep as wakefulness. (B) A generalized drawing showing the correlation between sleep/wakefulness/conscious activity vectors and functionally combined (activated) zones of functional modules.

Generally, where there are three unknown figures, the measurement of one of them does not suffice for the determination of the other two. Our intention is to apply reverse diagnosis to sufficiently lengthy, continuous data with a wide range of variables, to imagine a number of operational equations of the brain, and to guess the tendency of changes in two unknown figures from the equations.

RESULTS

Fig. 2 shows an example of rediagnosis in which the mouse was administered 0.01 mg atropine sulfate (1/2AP) at 1900 and 0.02 mg (AP) at 0720. The cumulative curve with the variable 0 is the original result of diagnosis with the vigilance(0) standard; reverse diagnosis has not been applied. The baseline and the cumulative curve respectively show continuation of sleep state and continuation of wakefulness state. All of the other cumulative curves have been derived from the application of reverse diagnosis to the original result and show continuation of the apparent wakefulness state. This type of diagnostic output is defined as rediagnostic output of MSLP=5, 10, 20, 40, 60, 120, and 180 sec. In this case, the standards for rediagnoses are at a minus level of vigilance(0). In the light period, notable

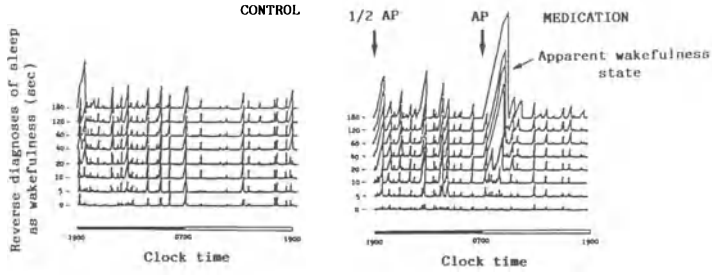

Fig.2. Examples of reverse-processing of continuous data.

characteristics can be observed in the rows of the cumulative curves in which parameter=40-180 sec, which are not seen in rows with conditions smaller than parameter=20 sec. Notable differences can also be observed in the rows in which parameter=420-600 sec, which are omitted in this figure.

If too large a value is selected for t, or the parameter for reverse diagnosis, then the cumulative curve for the control enlarges, which makes the difference between the control data and the data with drug administration unclear. In other words, only within a certain range of parameter t is this difference shown most clearly and the sensitivity of evaluation of the pharmacological effects increased. This tendency seen with atropine can be seen with other psychotherapeutic drugs such as chlorpromazine.

CONCLUSION

The conventional notions of depth of sleep or height of wakefulness have been somewhat ambiguous. This hypothesis defines the formation mechanism for depth of sleep and height of wakefulness. In this hypothesis, the difference in the curves from the control data, which were caused by the drug, can be thought of as changes in the number of functional combinations of neural networks that occur per unit time of diagnosis, in a specific zone on the vigilance scale. The unit of time for the regulation by the brain is thought to be much shorter than 1 sec, which is the unit of time used in this experimental system. The results in this experiment, therefore, signify an averaging of the number of occurrences of functional combinations per unit of diagnosis.

If we assume that these drugs induce functional combinations of particular neural networks in a highly selective manner, then the sensitivity of the detection of their changes induced by chemicals ought to be higher when one compares only the functionally combined zone of a neural circuit with the control, rather than when other zones not directly affected by the functional combination are compared. This is because E-type and C-type functional combinations have competing properties and because there is a ranking of neural circuits by address potential.[3]

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REGULATION OF NEOCORTICAL ELECTRICAL ACTIVITY BY CHOLINERGIC AND NORADRENERGIC SYSTEMS

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INTRODUCTION

Cognitive impairment associated with aging and an early stage of Alzheimer's disease (AD) may be related to changes in the cholinergic projections from the basal forebrain to the neocortex and hippocampus (2,4). The maintenance of vigilance, the capacity to allocate attention and sharpness of memory are adversely affected during aging.

Selective lesioning of the nucleus basalis magnocellularis (nbm) increases the amount of slow wales (delta power) and the number of high voltage spindles (HVS) in the neocortical recordings (3,6). The increase in delta power is due to destructions of nbm neurons which directly activate the neocortex. The generation of rhythmic spindling activity in the reticulothalamo-thalamo-cortical cicuitry is released by lesions within the cholinergic nbm which result in an increased incidence of HVSs.

An increased amount of delta power has also been found in some patients during progression of AD (9,10). The importance of the cholinergic deficit in the EEG pathophysiology observed in AD patients is supported by results showing correlations between the decreased activity of the cerebrospinal cholinergic marker enzyme, acetylcholinesterase (AChE), and increased slow wave activity (7). Furthermore, patients with rapidly deterioriating EEG also had significantly decreased choline acetyltransferase (ChAT) and AChE activities in the neocortex as assessed by post-morten examination of the brain. Moreover, patients with fairly normal EEG (no change in delta power) had ChAT and AChE activities within the control range (7) In the present study we wanted to further examine the role of the cholinergic system in the regulation of cortical EEG. Specifically, we assessed the role of the nbm-cortical projection and of the interactions

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between muscarinic and nicotinic receptors in cortical activation. Another major aim was to study the effects of tacrine (THA, anticholinesterase) on both scopolamine and nbm-lesion induced EEG change.

In addition to cholinergic pathology, noradrenergic neurons are also adversely affected in Alzheimer's disease. Furthermore, NA-system may interact with the cholinergic system in the neocortex, thalamus and basal forebrain (8,11). Thus, one major aim of this study was to investigate the role of the noradrenergic system in modulating neocortical electrical activity. We investigated the effects of noradrenergic drugs (alpha-2 agonists and antagonists) on the cortical EEG. Moreover, the effects of noradrenergic drugs on scopolamine and nbm-lesion induced EEG slowing were studied to reveal interactions between these systems.

MATERIALS AND METHODS

Animals

Male Wistar rats aged sixteen weeks were used in these studies. The rat were singly housed in a controlled environment with food and water freely available.

Lesions

For the lesioning of the nbm, the animals were anesthetised with chloral hydrate (350 mg/kg) and placed in a stereotaxic frame. Ibotenic acid (0.7 ug/0.7 ul) was infused into the nbm (AP:-0.8 mm, DV: -7.7mm and ML: +/-2.6mm relative to the bregma). Sham-operated controls received saline.

Administration of drugs

Scopolamine (muscarinic antagonist), mecamylamine (nicotinic antagonist), tacrine (anticholinesterase, THA), quanfacine (alpha2-agonist) and clonidine (alpha2-agonist) were all injected intraperitoneally 30 minutes before testing.

Atipamezole (alpha2-antagonist, Farmos Ltd., Finland) was injected subcutaneously 30 minutes before recordings.

Recording and analysis of EEG

The active recording electrodes (small stainless steel screws) were symmetrically implanted on both sides of the skull in the frontal and occipital pole. The reference electrode was located in the midline above the cerebellum.

Two behavioural stages were selected for spectral power components: waking-immobility and movement. Four 8-second,

artefact-free epochs were selected and digitized using a sampling rate of 250 Hz or 125 Hz. The fast Fourier transformation was used for analysis of the EEG. The EEG spectrum was compressed to 1.5-3 Hz, 3-5 Hz, 5-10 Hz, 10-20 Hz, 20-30 Hz and 30-60 Hz (125 Hz sampling frequency) or 1-4 Hz, 4-8 Hz, 8-12 Hz, 12-20 Hz, 20-30 Hz, 30-60 Hz (250 Hz sampling frequency).

Histology

After decapitation of the animal, a coronal slice was cut three millimeters anterior and posterior to the needle tract and put in 4% formalin. Lesions were located using hematoxylin-eosin and acetylcholinesterase stainings.

Biochemistry

Choline acetyltransferase activities were measured using a radioenzymatic method. High pressure liquid chromatography with electrochemical detection was applied to the measurements of catecholamines and their metabolites.

Statistical analysis

An SPSS program was used for statistical analysis (Mann Whitney U-test, Wilcoxon test).

RESULTS

The effect of cholinergic manipulation on neocortical electrical activity

The ChAT activity of nbm-lesioned rats was significantly decreased in the frontal cortex, but remained unchanged in the occipital cortex.

Nbm-lesioned rats had an increased level of delta power and a decreased amount of alpha power in the immobility-related EEG of the frontal cortex. In the mobility-related EEG of the frontal cortex, the amount of delta power was higher, but theta and beta powers remained unchanged in the nbm-lesioned rats. Nbm-lesioned rats also had an increased number of high voltage spindles in the frontal cortex (5/25 minutes of waking-immobility, nbm-lesioned vs. 1/25 minutes of waking-immobility, controls) . In the occipital cortex, none of the parameters of spectral EEG differed between nbm-lesioned and sham-operated controls.

In the frontal cortex, ChAT activity correlated significantly with the delta power both in the group of nbm-lesioned rats ($r=0.88$, $p<0.05$, $n=7$) and in the combined group of sham-operated and nbm-lesioned rats ($r=0.78$, $p<0.05$, $n=14$).

A combination of subthreshold doses of mecamylamine (5

Table 1. The effects of cholinergic antagonists on the spectral EEG in the frontal cortex of normal rats (controls), nbm-lesioned and sham-operated rats during waking immobility

Waking-immobility

	DELTA (1-4Hz)	THETA (4-8Hz)	ALPHA (8-12Hz)	BETA (12-20Hz)
CONTROLS	51±8	76±14	41±9	52±12
S	52±10	73±11	40±11	49±16
M-L	55±9	70±13	42±7	53±9
S+M-L	79±13*	101±11*	67±13*	74±9*
M-H	55±14	73±15	46±11	50±9
SH+M-H	85±13*	100±12*	75±17*	80±16*
SH	89±15*	105±17*	80±13*	81±17*
SH+SH	87±17*	109±12*	77±15*	79±13*
CONTROLS	53±9	73±13	45±8	50±10
S-M	79±9#	104±10#	67±10#	70±10#
NBL	71±10#	109±13#	65±7	50±9#
S-M:S+M-L	80±13#	100±11#	65±9#	72±13#
NBL:S+M-L	80±10#	107±13#	61±9#	55±7#

The amplitude (μV) of different frequency bands are expresed as means ±SD.
Abbreviations: CONTROLS (saline treated rats); S (scopolamine, 0.1 mg/kg i.p.); M-L (mecamylamine, 7.5 mg/kg i.p.); M-H (mecamylamine, 20 mg/kg i.p.); S-H (scopolamine, 2 mg/kg i.p.); S-O (sham-operated); NB-L (nbm-lesioned);
* $p < 0.05$ as compared to controls using Wilcoxon-test
# $p < 0.05$ as compared to sham-operated rats using Wilcoxon-test or Mann Whitney-test
The number of rats in S-O and NBL groups were 10, in other groups 14

mg/kg) and scopolamine (0.1 mg/kg) increased the power of all spectral components during waking-immobility (Table 1), but during movement only the alpha power was increased. A high dose of scopolamine (2.0 mg/kg) increased all spectral components, but no further changes were seen with a higher scopolamine dose (4 mg/kg) or with a combination of scopolamine 2.0 mg/kg and mecamylamine 20 mg/kg (Table 1).

In the nbm-lesioned rats, pre-lesion recordings confirmed interaction between the muscarinic and nicotinc systems in the regulation of cortical EEG. (Table 1). In post-lesion EEG recordings of the nbm-lesioned rats, no significant changes were seen with the same combination of nicotinic and muscarinic antagonists which had induced an EEG slowing in non-lesioned rats (Table 1).

Administration of THA (7.5 mg/kg, i.p.) decreased the amount of delta power, but did not reverse the beta power decrease in nbm-lesioned rats (Table 2). Administration of THA 7.5 mg/kg also reversed the scopolamine (0.8 mg/kg) induced increase in the spectral powers. THA 2.5 mg/kg did not reverse either the nbm-lesion or scopolamine induced EEG alterations (Table 2). THA 7.5 mg/kg but not 3 mg/kg could reverse scopolamine induced EEG slowing (Table 3).

The effect of noradrenergic manipulation on neocortical electrical activity

Atipamezole (3 and 10 mg/kg) increased alpha and beta activity during waking-immobility and movement periods, but no changes were seen in the delta or theta bands (Table 4). Yohimbine produced an EEG profile similar to that of atipamezole (Table 4). Clonidine (1 mg/kg) increased the power of all the spectral components during waking-immobility. Movement recordings indicated that theta, alpha and beta powers were increased by clonidine (1 mg/kg).

None of the alpha2-antagonist compounds reversed scopolamine (0.8 mg/kg) induced EEG slowing (Table 4). In waking-immobility related EEG recordings, a combination of clonidine (1 mg/kg) and scopolamine (0.8 mg/kg) induced a more severe EEG slowing than did administration of either of the drugs alone (Table 4). During movement, no additive interaction was seen between clonidine and scopolamine in their effects on EEG parameters (Table 4).

In nbm-lesioned rats, the increase in slow activity, but not the decrease in fast activity was partly reversed by atipamezole (10 mg/kg) (Table 5). Yohimbine (1 mg/kg) did not reverse theta amplitude increase induced by nbm lesions. Clonidine (1 mg/kg) produced a further synchronisation of the EEG in nbm-lesioned rats as the amplitudes of all spectral components were increased (Table 5).

The results of the HVS analysis are shown in Table 6. Administration of the noradrenergic alpha-2 agonist, quanfacine (0.004-0.02-0.1 mg/kg), increased the number of high

Table 2. The effect of cholinergic drugs, THA and pilocarpine, on the spectral EEG in the frontal cortex of nbm-lesioned rats during waking-immobility and movement

	DELTA (1.5-3Hz)	THETA (3-5Hz)	ALPHA 5-10Hz)	BETA (10-20Hz)
Waking-immobility				
Sa	98±20	72±25	108±30	82±21
TL	92±27	67±20	107±22	78±30
TH	53±17*	47±17*	100±27	78±21
PILO	51±20*	50±21*	101±17	87±22
Movement				
Sa	55±12	42±13	100±21	68±17
TL	50±10	44±14	107±21	66±21
TH	40±13*	45±14	102±29	68±30
PILO	37±15*	36±18	104±31	73±17

The amplitude (μV) of different frequency bands are expressed as means ±SD.
Abbreviations: Sa (saline treated); TL (THA 2.5 mg/kg i.p.); TH (THA 7.5 mg/kg i.p.); PILO (pilocarpine 10 mg/kg i.p.). Drugs were injected 30 minutes before recordings.
* $p < 0.05$ as compared to saline treated (Sa) nbm-lesioned using Wilcoxon-test
The number of rats is 12 in each group

Table 3. The effect of cholinergic drug, THA and pilocarpine on the spectral EEG in the frontal cortex of saline and scopolamine treated rats during waking-immobility and movement

	DELTA (1.5-3Hz)	THEA (3-5Hz)	ALPHA (5-10Hz)	BETA (10-20Hz)
Waking immobility				
CONTROLS	41±8*	47±8*	107±12*	116±19*
TL	42±9*	50±7*	102±9*	112±15*
S	54±7	62±6	125±10	141±13
S+TL	52±8	61±7	130±17	131±20
S+TH	43±7*	51±7*	107±7*	119±17*
Movement				
CONTROLS	37±8	38±10	100±16*	90±12
TL	32±7	37±16	82±17·*	66±10·
S	38±18	40±17	128±19	93±14
S+TL	35±5	40±8	121±25	89±18
S+TH	32±7	37±6	100±9*	87±17

The amplitude (μV) of different frequency bands are expressed as mean ±SD.
Abbreviations: CONTROLS (saline treated); S (scopolamine, 0.8 mg/kg i.p.); TL (THA, 2.5 mg/kg i.p.); TH (THA, 7.5 mg/kg i.p.)
* $p < 0.05$ as compared scopolamine treated rats using Wilcoxon-test
· $p < 0.05$ as compared to controls recordings
The number of rats is 16 in each group

Table 4. The effect of alpha-2 adrenergic drugs on the spectral EEG in the frontal cortex of control and scopolamine (0.8 mg/kg) treated rats during waking-immobility and movement.

	DELTA (1.5-3Hz)	THETA (3-5Hz)	ALPHA (5-10Hz)	BETA (10-20Hz)
Waking-immobility				
CONTROLS	41±8	47±8	107±12	118±19
ATI-3	42±5	52±7	132±14*	136±12*
ATI-10	45±7	54±8	137±18*	135±17*
YOH	43±2	50±9	130±17*	121±12
CLO	58±11*	68±10*	127±14*	135±12*
SCOP	54±7	62±6	125±10	141±13
SCOP+ATI-10	50±12	60±2	115±19	121±26
SC+YOH	51±13	61±17	120±21	130±17
SC+CLO	70±17	80±17#	135±21	142±17
Movement				
CONTROLS	37±8	38±10	100±16	90±12
ATI-3	39±7	37±10	127±12*	117±14*
ATI-10	41±8	39±9	137±19*	128±27*
YOH	40±7	41±8	126±10*	109±7*
CLO	49±8	58±10*	130±11*	124±13*
SCOP	38±18	40±17	128±19	93±14
SCOP+ATI-10	40±7	37±10	90±15	87±11
SCOP+YOH	41±9	42±7	87±19	86±12
SC+CLO	39±12	45±16	135±25	104±24

The amplitudes (μV) of different frequency bands are expressed as means ±SD.
Abbreviations: CONTROLS (saline treated); ATI-3 (atipamezole, 3 mg/kg i.p.); ATI-10 (atipamezole, 10 mg/kg i.p.); YOH (yohimbine, 3 mg/kg i.p.); CLO (clonidine, 1 mg/kg i.p.); SCOP (scopolamine, 0.8 mg/kg i.p.)
* $p < 0.05$ as compared to controls using Wilcoxon-test
# $p < 0.05$ as compared to scopolamine treated using Wilcoxon-test
The number of rats is 16.

voltage spindles in the neocortex. Interestingly, the largest dose produced the smallest increase in HVSs. The average duration of the HVSs was not increased by quanfacine in young rats. Atipamezole (1-10 mg/kg) blocked the appearance of spindles in young rats, and pilocarpine (10 mg/kg) blocked the generation of HVSs.

In young rats, the number of quanfacine (0.1 mg/kg) induced spindles were decreased by atipamezole (1 mg/kg) and pilocarpine (10 mg/kg). A combination of these drugs blocked the appearance of the quanfacine induced spindles more effectively than either of these drugs alone.

In aged rats, the incidence of HVSs was higher than in young rats. No differences were observed between the effects of different doses of quanfacine on the incidence of HVSs. The mean duration of HVSs was in a dose-dependent manner increased by quanfacine. Atipamezole administration markedly reduced the incidence and the duration of quanfacine-induced HVSs. Pilocarpine (10 mg/kg) decreased the number of HVS periods. The combination of pilocarpine (10 mg/kg) and atipamezole (10 mg/kg) completely blocked the appearance of HVSs in aged rats.

DISCUSSION

Our findings concerning the effects of nbm lesion on the neocortical electrical activity are consistent with the results of previous reports (3, 6). The present experimental results showing a marked correlation between ChAT activity and delta power as well as a decrease in delta power due to THA treatment in nbm-lesioned rats support our clinical findings that the level of delta power is related to the degree of cholinergic deficit in the basal forebrain (7). Furthermore, our results demonstrate the important role of both nicotinic and muscarinic receptors in the nbm-cortical ascending cholinergic activating input.

Nbm-lesioned rats provide an interesting parallel to the cholinergic deficit occurring in normal aging and AD. In experimental animals, cognitive impairments and EEG alterations are produced by nbm lesions (3,6). If the cholinergic system is involved in normal EEG regulation and cognitive processing, it would be logical that the degree of cholinergic deficit would be related to the severity of the EEG pathophysiology and cognitive decline. Indeed, our recent CSF and autopsy findings have tentatively demonstrated that the loss of cholinergic markers is correlated with an EEG slowing (7). Moreover, previously it has been shown that a decrease in neocortical ChAT activity is related to the degree of dementia (2). Thus, behavioral and neurophysiological studies with nbm-lesioned rats are valuable models for developing cognitive enhancers with the aim of alleviating age- and AD-related cognitive deficits.

The present result indicate that atipamezole, alpha-2 antagonist which increases the turnover of noradrenaline in

Table 5.

	DELTA (1.5-3Hz)	THETA (3-5Hz)	ALPHA (5-10Hz)	BETA (10-20Hz)
Waking-immobility				
NBL	98±20	72±25	108±30	82±21
ATI	60±20*	50±17	117±20	93±26
JOH	70±20*	60±27	109±21	84±17
CLO	129±17*	109±21*	127±30*	107±17*
Movement				
NBL	55±12	42±13	100±21	68±17
ATI	43±17*	45±11	112±21	73±12
JOH	50±12	44±13	105±19	66±14
CLO	67±24	52±19	110±27	75±12

The amplitude (μV) of different frequency bands are expressed as means ±SD.
Abbreviation: NBL (nucleus basalis lesioned); ATI (NBL+ atipamezole, 10 mg/kg); JOH (NBL+yohimbine, 3 mg/kg); CLO (NBL+clonidine, 1 mg/kg)
* $p < 0.05$ as compared to NBL recordings

Table 6. The number of high voltage spindles (HVS) in cortical electroencephalogram of young and aged rats under different drug treatments

	Aged		Young	
	Number	Duration	Number	Duration
Baseline	54	5.3±4	8	5.5±52
ATI-1	21*	2.0±1.8*	0	0
ATI-10	3 *	0.5±0.2 *	0	0
QF-0.004	106*	9±8	209 *	5±5
QF-0.02	111*	12±3*	230*	3.5±3
QF-0.1	102*	18±7*	120*	5±4
PILOH	5	2.0±1*	0	0
PILOL+ATI-1	1	1.1	0	0
PILOL	20*	4.0±2	0	0

The number of duration(s) of HVSs are expressed as mean ±SD in the recordings lasting for 30 minutes.
Abbreviations: Baseline (saline treated); ATI-1 and 10 (atipamezole 1 mg/kg and 10 mg/kg i.p.); QF-0.004, -0.02 and -0.1 (quanfacine 0.004 mg/kg, 0.02 mg/kg and 0.1 mg/kg i.p.); PILOH (pilocarpine 10 mg/kg i.p.) and PILOL (pilocarpine 3 mg/kg i.p.).
* $p < 0.05$ compared to baseline using Wilcoxon-test

the neocortex, decreased the level of delta power in nbm-lesioned rats. On the other hand, quanfacine and clonidine, alpha-2 agonists had a deleterious effect on the neocortical electrical activity, i.e. an increase in the number of HVSs and amount of slow waves. Moreover, scopolamine and nbm lesion induced EEG slowing was augmented by quanfacine. Interestingly, it was also observed that HVS generation is jointly regulated by the cholinergic and noradrenergic system. Atipamezole and pilocarpine decreased the incidence of HVSs in young and aged rats, and a combination of these drugs proved to be more effective than either of the drugs alone. Furthermore, alpha2-agonist induced spindles were blocked not only by atipamezole, but also by pilocarpine. Therefore, our results suggest that both the direct cortical and thalamo-cortical activation of EEG are jointly modulated by cholinergic and noradrenergic systems.

In conclusion, our findings highlight the important role of cholinergic and noradrenergic interaction in the cortical activation. Our results in young (lesion, receptor modulation) or old rats demostrating partial EEG recovery by the alpha2-antagonists and cholinomimetics give additional empirical support for the use of these compounds in clinical trials of treatment strategies for Alzheimer's disease. In addition, these findings underscores the importance of pharmaco-EEG studies as an indicator of the activity of drugs stimulating either the noradrenergic or cholinergic system.

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ASCENDING CHOLINERGIC AND MONOAMINERGIC SYSTEMS IN THE BRAINSTEM: DO THEY CONSTITUTE A RETICULAR ACTIVATING SYSTEM?

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INTRODUCTION

The concept of the "ascending reticular activating system" (ARAS), promulgated by Moruzzi and Magoun (1949) as the essential element in the neural mechanisms underlying wakefulness, suffers significantly from a lack of specificity as to the neurons which might actually comprise the ARAS. With the new techniques of neuroanatomy several clearly defined structures with ascending projections have been discerned in or near the putative ARAS: the noradrenergic projection from the locus coeruleus (LC), serotonergic projection from the dorsal raphe (DR), and the cholinergic projection originating in the laterodorsal and pedunculopontine tegmental nuclei (LDT and PPT). These projection systems all have anatomical features appropriate for the ARAS such that they send network-like axons with many varicosities directly to diffuse areas of the forebrain, except that the cholinergic influence on most cortical areas is indirect via thalamus, hypothalamus or basal forebrain nuclei (Fig. 1). The obvious question is whether one or more of these newly specified entities is, or constitute a major component of, the ARAS.

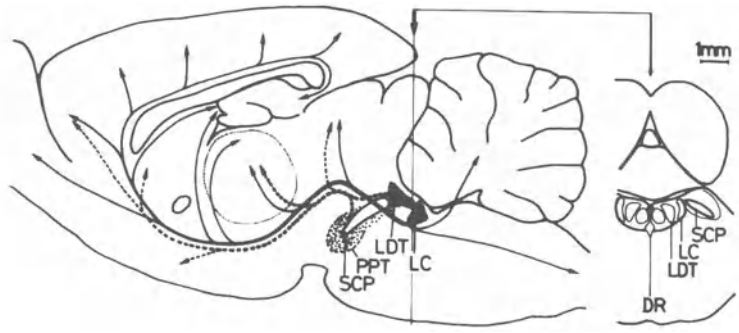

Fig. 1. Left, schematic illustrations of noradrenergic (solid line) and cholinergic (broken line) projections (serotonergic projection is omitted to avoid complexity) from the brainstem of the rat. Right, coronal section at the plane shown by the thin line with arrow. SCP, superior cerebellar peduncle.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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This question will be discussed in this paper, based mainly upon two kinds of our studies: 1) the recording of neuronal activity in these brainstem nuclei (LC, DR and LDT), and 2) the effects of stimulating each of these nuclei on activity of the forebrain. In the latter experiments single neuronal activity was recorded in the dorsal lateral geniculate nucleus (LGNd). (For methodological details, see Kayama et al., 1982; 1986; 1987; 1989). All of our studies were done in rats (anesthetized with urethane), since in this animal the noradrenergic neurons in the LC and the cholinergic neurons in the LDT are tightly grouped and segregated clearly from each other (Fig. 1, right), in contrast to the cat where noradrenergic and cholinergic neurons are intermingled in the parabrachial region (De Lima and Singer, 1987).

I propose that the ARAS is not a single system, but is composed of two systems arising from the brainstem: the noradrenergic projection conveying a rather tonic control, and the cholinergic projection responsible for the phasic aspects of the activating system. (Similarly to the former, serotonergic influence is tonic, but inhibitory in nature.)

PHYSIOLOGICAL PROPERTIES OF INDIVIDUAL BRAINSTEM NEURONS

We recorded from neurons in the central gray of the midbrain and pons whose positions marked with pontamine sky blue. Spikes recorded extracellularly with a low-cut filter of 80 Hz were clearly divided into two groups; broad-spike neurons and brief-spike neurons. While the latter were similar to those recorded commonly in the cortex or thalamus, the former were characteristic of brainstem neurons. The LC was occupied exclusively by the broad-spike neurons, while in the DR and LDT this sort of neuron was the major component, but 20-30% of the total population were brief-spike neurons. The broad-spike neurons in the three nuclei were very similar in many electrophysiological characteristics. Many of them had widely branching collateral axons as evidenced by antidromic responses to stimulation of multiple forebrain sites. Their conduction velocities were in the range of unmyelinated fibers (Kayama and Ogawa, 1987).

The broad-spike LC and DR neurons are very probably noradrenergic and serotonergic, respectively (Aghajanian and Vandermaelen, 1982). The broad-spike LDT neurons are presumed to be cholinergic, as suggested by the fact that (1) 86% of LDT neurons projecting to the forebrain (or 93% of those projecting to the LGNd) had broad-spikes (Kayama and Ogawa, 1987), and (2) in an anatomical study about 90% of retrogradely labeled LDT neurons after HRP injection in the thalamus were cholinergic (Sofroniew et al., 1985), although this needs to be confirmed with intracellular techniques.

The broad-spike neurons of the LC, DR and LDT resembled each other also in that, under urethane anesthesia, they fired rather regularly with a low rate (0.5-5 Hz), except that there were a considerable number of LDT neurons not spontaneously active (Kayama and Ogawa, 1987). However, responses of LC, DR and LDT neurons to sensory stimulation such as tail pinch were very different from each other. The majority of LC neurons responded with a strong phasic excitation followed by a mild tonic firing, and the firing was transiently suppressed after the cessation of stimulation (Fig. 2A). This response pattern of LC neurons was stereotyped; under deeper anesthesia some LC neurons seemed to lose the tonic component of the response, but other types of response such as pure inhibition during stimulation were not observed. In contrast, responses of DR and LDT broad-spike neurons differed from one to another. While responses of DR neurons were most frequently inhibitory (Fig. 2B), those of LDT neurons were predominantly excitatory. In about 2/3 of the excited LDT neurons the response was only phasic, as shown in Fig. 2C; others had tonic components.

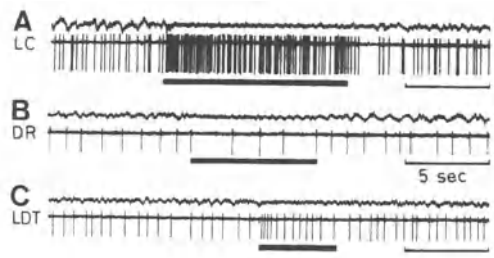

Fig. 2. Effects of tail pinch on activity of an LC neuron (A), a DR neuron (B) and an LDT neuron (C), recorded simultaneously with cortical EEG. The tail was pinched with a forceps for a period shown by the thick bar under each record. The largest EEG slow waves are about 0.4 mV.

When the cortical EEG was recorded simultaneously with neuronal activity, the duration of the tonic excitation of LC neurons induced by tail pinch was essentially identical with that of EEG desynchronization (Fig. 2A). In contrast to this, the phasic excitation induced in LDT neurons did not coincide with EEG desynchronization in its duration (Fig. 2C). Another difference was noticeable in the excitations of LC and LDT neurons induced by sensory stimulation (Kayama and Ogawa, 1987). When the stimulation was repeated, LC neurons responded each time if EEG desynchronization was induced by the stimulation, while most LDT neurons responded only at the first stimulation and failed to respond to subsequent stimulations.

EFFECTS OF BRAINSTEM STIMULATION ON THALAMIC NEURONAL ACTIVITY

After Moruzzi and Magoun (1949), stimulation of the mesencephalic reticular formation have been used to study ascending activating system. To avoid an ambiguity as to what was stimulated (axons or somas?), we introduced stimulating electrode to a nucleus (LC, DR or LDT) in which neurons having an identified transmitter were packed tightly. Effects of repetitive stimulation (<6 V, 0.05 ms, 200 Hz, several to 10 s) of the nucleus were examined on LGNd relay neurons, since this neuron species is easily identified electrophysiologically, and its function is evident (final output of a neuronal circuit). The transmitter mediating the effects was checked by blockers applied ionophoretically. (Other details of the experiments, see Kayama et al., 1982; 1986; 1989).

LC stimulation Upon repetitive LC stimulation, the great majority of LGNd relay neurons gradually increased their firing (Fig. 3A). The firing remained elevated during LC stimulation, and decreased gradually after cessation of the stimulation, taking more than several seconds (in the longest case, about 1 min). Increase in spontaneous firing induced by LC stimulation was accompanied by increased responsiveness to optic nerve inputs. The excitatory effects of LC stimulation were antagonized by an alpha-adrenergic blocker, but not by a beta-adrenergic blocker or cholinergic blockers (Kayama et al., 1982).

DR stimulation No consistent change in firing of LGNd relay neurons was observed during repetitive stimulation of the DR; firing increased in some neurons, decreased in others, and did not change in still others. In some neurons it changed from time to time even in a given neuron. However,

a long-lasting suppression of firing was induced consistently after the cessation of the repetitive DR stimulation (Fig. 3B). The suppression became manifest 4 to 20 seconds after the cessation of DR stimulation and lasted for 10-60 seconds. The serotonergic nature of the long-lasting suppression was confirmed by pharmacological experiments (Kayama et al.,1989).

LDT stimulation As in the case of LC stimulation, upon repetitive stimulation of the LDT for several seconds the great majority of LGNd relay neurons increased their firing (Fig. 3C). Conspicuous differences of the LDT-induced excitation from that induced via the LC were that the increase in firing usually ended soon after the cessation of LDT stimulation (Fig. 3C, left), and that, when LDT stimulation was maintained longer than several seconds, the increased firing nevertheless started to decline (Fig. 3C, right). In some neurons with no spontaneous activity LDT stimulation might induce only several spikes at first without any activity thereafter during the stimulation. The habituation of excitation during LDT stimulation was not due to a postsynaptic mechanism, because ionophoretically applied acetylcholine induced an increase in firing as long as the application continued. The LDT-induced excitation was proved to be cholinergically mediated via muscarinic receptors (Kayama et al., 1986).

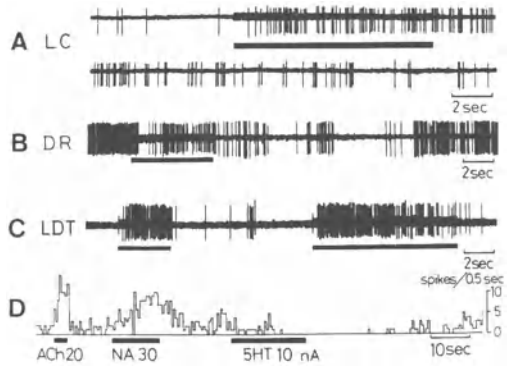

Fig. 3. Effects of repetitive stimulation of LC (A), DR (B) and LDT (C), and effects of ionophoretically applied transmitters (D), on activity of LGNd relay neurons. D, shown by output of a ratemeter. Duration of stimulation or application is shown by the thick bar under each record.

Ionophoresis of transmitters As in the experiments with LC, DR or LDT stimulation, ionophoretic application of noradrenaline (NA) increased, that of serotonin (5HT) decreased, and that of acetylcholine (ACh) increased firing of the majority of relay neurons of the LGNd (Fig. 3D). A clear difference was noted in the time course of appearance and disappearance of the effects. The effect of acetylcholine appeared within 1 sec and reached its maximum very quickly. It also disappeared very quickly after stopping the application. In contrast, increase in firing by noradrenaline started after a delay of several to 20 sec, and gradually became strong. It also decreased gradually to the control level after the cessation of application. Appearance and disappearance of the effects of serotonin took a course similar to, or longer than, that of noradrenaline.

CONCLUDING REMARKS

As described above, the noradrenergic and cholinergic systems arising from the brainstem have somewhat similar properties: both noradrenergic and (possibly-)cholinergic neurons, which send axons to diffuse areas of the forebrain, respond to sensory stimulation primarily with excitation, and both noradrenergic and cholinergic projections induce excitation of thalamic relay neurons. Thus, the properties of the noradrenergic and cholinergic systems are concordant with those expected for the ascending reticular activating system. (The serotonergic system is also a member of the brainstem systems controlling activity of the forebrain, but not the "activating" system.)

A more detailed consideration of above results, however, reveals some significant differences between the noradrenergic and cholinergic systems, both in response to sensory stimulation and in their action on thalamic relay neurons. The noradrenergic system is considerably more tonic in both its action and sensory response than is the more phasic cholinergic system. These observations prompt us to hypothesize that the ascending reticular activating system is not a single system, but rather is composed of two systems, i.e., the predominantly tonic, noradrenergic system and the rather phasic, cholinergic system. It may be that the former is concerned more strongly in induction and maintenance of such tonic activation phenomena as wakefulness, and the latter in induction of phasic activation phenomena such as attention.

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ROLE OF THE MEDIAL LIMBIC SYSTEM IN THE MEMORY PROCESS: A HYPOTHESIS BASED ON A RADIOACTIVE 2-DEOXYGLUCOSE STUDY

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INTRODUCTION

Alzheimer or Parkinson patients suffer from some memory deficits in the later course of their illness. It is also reported that MPTP-treated monkeys showed cognitive disturbances without Parkinsonian motor deficits.[1] Therefore, it would be very helpful to determine the activity of memory-related structures in the normal brain for the development of more appropriate therapeutic or clinical treatments. In the present study, the 2-deoxy-d-glucose (2-DG) method[2] was employed to reveal active sites in the brain while the monkey was performing a delayed response. This paradigm is known to test for short-term memory. The results have been already published in final form.[3,4]

METHODS

Details of the methods were given in a previous paper.[3] In this report, a brief description should suffice. Nine monkeys were used in the present experiment. Five of them were trained to perform a delayed-response task. Another two monkeys were accustomed to sit quietly in a monkey chair (Quietly sitting monkeys). The other two were anesthetized with sodium pentobarbital (Anesthetized monkeys).

In the delayed-response paradigm, the monkey was trained to move a handle from a central start zone to a predetermined target zone following visual instruction controlled with a mini-computer (DEC, PDP 11). The target zone appeared at the left or right in a pseudorandom order. When the monkey set the handle in the start zone, a central LED (light-emitting diode) and a target LED turned red. After 1 second, the target LED turned off, and a delayed period of 5 seconds began. When the 5 seconds were over, the central LED changed to green, signifying GO. Then the monkey had to move the handle into the

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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target zone in less than 2 seconds in order to get a reward (a drop of water or juice).

Surgical operation was conducted when the monkey had mastered the task at the criterion level of 95 %. Under anesthesia, an L-shaped metal piece was attached to the skull with the aid of acrylic resin, screws, and bolts. The metal piece was connected to a head holder during an experimental run. One end of a Teflon tubing (5Fr. i.d.) was inserted into the jugular vein to inject isotope solution and anesthetics in an isotope experiment. The other end was subcutaneously guided to the acrylic bed previously prepared on the skull. The monkey was carefully managed after the operation.

Isotope experiments were conducted after complete recovery of the monkey from the surgery. On the day of the experiment, monkey was sat on a monkey chair and radioactive 2-deoxy-d-glucose (100 μCi/kg) was injected via the Teflon tubing into the vein. Then the monkey was allowed to continue the delayed-response task for 45 minutes. After that time, an overdose of pentobarbital (50mg/kg) was injected into the vein through the tubing to kill the monkey. After the death of the monkey, the brain was removed as quickly as possible, and cut into a few blocks for more rapid freezing in cooled isopentane (-35 to -40 °C).

The blocks of brain were sliced (30 μm thick), mounted on slide glasses, and dried at 70 °C. Then they were arrayed in a light-proof cassette after having been covered with radiosensitive film for autoradiography. After one week the film was processed and the density of the autoradiographs was measured with a microdensitometer (PDS 15, Konishiroku Photo Co.) in several brain structures that were interesting in terms of memory. The density in each structure was converted into radioactivity in reference to the polymer standards (Amersham). The densities were also normalized to those in the corpus callosum.

RESULTS

The brain areas and radioactivities measured in the present experiments were as follows (values in parentheses are in μCi/g tissue/45 min for 5 experimental versus 2 quietly sitting control monkeys): Prefrontal cortex (638±100 vs. 530±21), motor cortex (933±134 vs. 645±7), somatosensory cortex (983±159 vs. 640±28), auditory cortex (1053±81 vs. 718±96), visual cortex (915±109 vs. 620; only one sitting monkey was available for this analysis), retrosplenial cortex (835±248 vs. 568±25), area 24a of the anterior cingulate cortex (372±74 vs. 333±11), anterior nucleus (750±143 vs. 575±35), dorsomedial nucleus (666±117 vs. 505±64), ventrolateral nucleus (585±103 vs. 450±70), caudate nucleus (634±119 vs. 560±14), putamen (697±155 vs. 570±28), globus pallidus (314±37 vs. 275±35), amygdala (442±73 vs. 345±35), hippocampus (454±55 vs. 345±35), corpus callosum (137±12 vs. 134±7).

Significant p values were obtained for motor ($p<0.01$), somatosensory ($p<0.05$), and auditory cortex ($p<0.05$). In other areas, p values were $0.1<p<0.2$ (anterior nucleus, dorsomedial

nucleus and hippocampus), or $0.2<p<0.3$ (retrosplenial cortex, prefrontal cortex, and amygdala).

HYPOTHETICAL CONSIDERATION

The Papez circuit is considered to form a neurologic basis of the memory system, though it was originally proposed to explain emotion.[5] The cingulate cortex, anterior nucleus (AN), and hippocampus are the main members of this circuit. In the present experiments, the AN and area 29 of the posterior cingulate (usually called the retrosplenial [RS] cortex), both of which are closely interconnected with each other, incorporated a considerable amount of 2-DG. Also other memory-related structures like the hippocampus, amygdala, prefrontal cortex, and dorsomedial nucleus incorporated 2-DG in a similar mode of increase, though their respective absolute values were different from one another. Although the functional role of these structures is fairly well understood to be possibly related to memory, the function of the RS has not been determined. However, a recent clinical study reported that lesions in the human retrosplenial cortex caused amnesia (retrosplenial amnesia).[6] Also it was well documented in the rodent that area 29 of the posterior cingulate, an area homologous to the primate RS cortex, is intimately concerned with conditioned avoidance learning.[7] Neuronal activities were differentially related to CS(+) and CS(-). Therefore, the present findings of the remarkable 2-DG uptake into the primate RS cortex reflect some aspect of memory or learning processes required to perform the delayed response.

It has been elucidated ontogenetically and histologically[8,9] that the RS cortex and other cingulate and septal areas form the medial limbic system while the hippocampal areas, piriform, and a part of the orbitofrontal cortex form the lateral limbic system. Because almost all these neuronal structures have essential roles in memory or learning, we suppose that two main information flows exist in the monkey limbic system in relation to memory, e.g., medial and latelal limbic flows. And these two limbic flows would be equally related to memory or learning processes of animal behavior.

It has been pointed out that the part of the neocortex anterior to the central sulcus is mostly related to motor functions, while the posterior part is related to perceptual information processing. In a similar way, the anterior part of the limbic system is probably related to the motor functions in a broad sense and the posterior limbic system probably has perceptual functions. Therefore, the RS cortex, together with the posterior cingulate cortex (area 23), would be more related to cognitive memory, while the anterior medial limbic (area 24) would have a nature to process "motor memory", provided the medial limbic system is admitted to process some kinds of memory.[3,4]

Furthermore, the anterior cingulate (area 24) projects fibers to the supplementary, premotor, and prefrontal cortex. These three structures are interconnected with one another, cortico-cortically or through subcortical routes.[10] Although these three areas did not show a strict hierarchical

organization, we can still conceive of the existence of some hierarchical order composed of "motor association cortices". Therefore, these three structures could have the ability to store "motor memory". Likewise, the anterior part of the basal ganglia, largely composed of the head of the caudate and putamen, could also store "motor memory", although the type of "motor memory" would be a little different from that in the "motor neocortical association areas".

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EFFECT OF MAGNETISM ON LEARNING FUNCTION

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INTRODUCTION

Magnetism has been used in medical and industrial fields to a considerable extent. As magnetism is not detected by sensory system or visual system, the effect of it on humans is not clear. The technique of pulsed magnetic stimulation of the human brain has recently been reported. [1-5] The magnetism used in this stimulation is strong but of very short duration. The magnetic stimulation with short duration is able to excite the nerves, which enables us to study nerve conduction in the central nervous system. Unlike electrical stimulation, magnetic stimulation excites the motor cortex without discomfort to the subject. Thus this method might be useful as a new clinical test to study central motor pathways. Although no deleterious effects have been observed thus far, the safety of this technique [6] has not fully been established. Our recent study showed decreases in dopamine and serotonin 60 minutes after 50 pulses of magnetic stimulation. These results led us to investigate, by use of the passive avoidance method, the effect of pulsed magnetic stimulation of the brain on learning function.

MATERIALS AND METHODS

A pulsed magnetic discharge system, consisting of a high-voltage capacitor bank and a flat circular coil of insulated copper wire, was used for brain stimulation. The high-voltage capacitor bank was discharged to a maximum current flow of 8,000 amperes and had a capicitance of 1,637 μF. The flat coil consisted of 12 turns of insulated copper wire having an external diameter of 88 mm and internal diameter of 15 mm. Its magnetic field was 2.34 Tesla with an inductance of 7 μH and a peak time of 137 μs. Mice were placed in long, narrow cylindrical chamber with a mesh covering around their head, and received the pulsed magnetic stimulation (MS), 50 times or 10 times at 0.1 Hz via the flat circular coil, or electroconvulsive shocks (ECS).

Two hundred twenty-six normal slc : ddY male mice, each weighing 27-29 g, were used in this study. The study was planned in 4 sessions with different mice in each. The mice were separated into three groups in each session.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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In the first session the mice in the MS group were stimulated 10 times, one hour before the learning by the passive avoidance method. Mice in the ECS group also received their stimulation one hour before the learning. In the second session, animals in the MS group received MS 10 times one hour after the learning, and those in the ECS group received ECS one hour after the learning. In the third session, mice in the MS group received MS 50 times one hour before the learning. Mice in the ECS group received ECS also one hour before the learning. In the fourth session, the MS group received MS 50 times one hour after the learning, and the ECS group, ECS one hour after the learning. In each session, mice in the control group received neither MS nor ECS before or after learning.

Apparatus for passive avoidance learning

The passive avoidance equipment [7-9] consisted of two compartments (light and dark chambers). The light chamber was constructed entirely of clear plastic and had inside dimensions of 7 ×9 ×14 cm. It was illuminated by a 15 W incandescent light. The dark chamber had a wooden roof and sides and the interior was painted black (inside dimensions : 14×14×14 cm). The floor consisted of stainless steel bars 3 mm in diameter and placed 7 mm apart. A foot shock of constant current DC pulses could be delivered to the bars by an shock generator and scrambler (type 11-13, BRS/LVE company). The light and dark compartments were connected by a 3×3 cm hole that could be covered by a sliding metal door.

Procedure for passive avoidance learning [9]

The standard day 1 (learning) procedure was to place the mouse in the light chamber so that it faced away from the hole leading into the dark chamber. The animal's latency to step through into the dark chamber after stimulation was then measured with a stop-watch (latency of learning). The endpoint taken was when the animal had all four paws on the grid floor of the dark chamber.

The mice were tested 24 hours later (day 2) by the same procedure except that no stimulus was delivered (latency of test). If the mice did not step through into the dark compartment within 300 sec, the trial was terminated and the latency for step-through was recorded as 300 sec. [9]

RESULTS

Mice exhibited no significant difference among the three groups in latencies to step-through to the dark chamber of the passive avoidance equipment during learning in each session.

Prelearning treatment or postlearning treatment of mice with MS 10 or 50 times did not impair the passive avoidance response significantly, whereas prelearning treatment or postlearning treatment of mice with ECS significantly impaired passive avoidance response in every session, as there was a significant reduction in day 2 test latency to step-through (Figure 1). There was no significant difference in latencies of test between prelearning treatment group and postlearning treatment group. And there was no significant difference in latency of test between 10 pulses of MS and 50 (Figure 2).

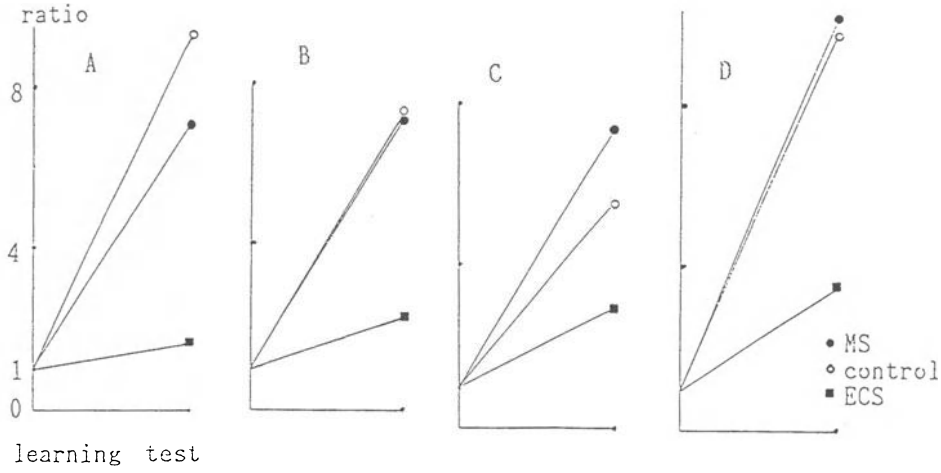

Figure 1 . The ratio of latency of test to latency of learning by the passive avoidance method. See Materials and Methods for details. A ; before Learning, MS ×10. B ; after learning, MS×10. C ; before learning, MS×50. D ; after learning, MS×50. In all 4 sessions, the ECS group showed a significantly decreased ratio (Kruskal-Wallis H-test, χ^2 -test ; $p<0.01$).

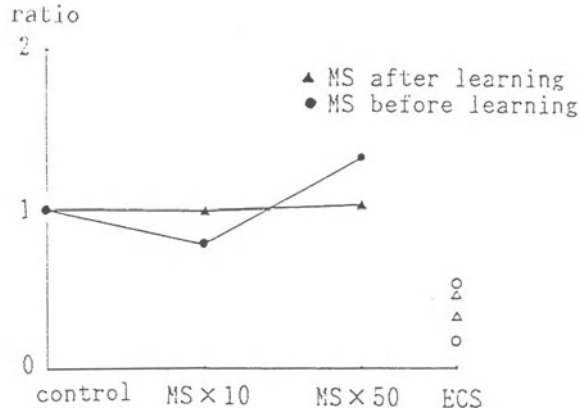

Figure 2 . The ratio of (latency of test/latency of learning) in MS group to (latency of test/latency of learning) in control group. MS×10 or 50 : MS, 10 times or 50 times. MS after or before learning : MS one hour after or before learning by passive avoidance method. These ratios did not show any significance statistically (Mann-Whitney U-test).

DISCUSSION

In the present data from the passive avoidance method, the pulsed magnetic stimulation is considered to have no notable effect on the learning process of the passive avoidance, whereas ECS appears to affect the learning process. In our recent report [6] of a kinesiological, neurochemical and neuropathological study on the effect of pulsed magnetic stimulation, we stated that dopamine and serotonin metabolism were changed significantly, but transiently, after pulsed magnetic stimulation of rat brain, although there was no significant change in movement or neuropathology. Each pulsed magnetic stimulation excites the neurons, with consequent neurotransmitter release from the synapse, thus accounting for the observed changes in neurotransmitter metabolism. The muscles of the rat's body were contracted at every magnetic stimulation. However, after the cessation of stimulation, no special behavior or movement was observed at all. These observation suggests that transient changes in neurotransmitter level by magnetic stimulation do not interfere with the memory, behavior or muscle contraction, but further studies on the effect of MS on long-term memory, and on longer and stronger exposure to magnetism on short-term memory are needed. [10,11]

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MORPHOLOGICAL CHANGES OF THE BRAIN IN SENESCENCE ACCELERATED MOUSE (SAM)-P/8, A NEWLY DEVELOPED MEMORY-DEFICIENT STRAIN

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INTRODUCTION

Senescence Accelerated Mouse (SAM) shows an earlier onset and irreversible advancement of senescence manifested by the following signs and lesions: a loss of activity, skin coarseness, a shortened life span, systemic senile amyloidosis, senile cataract ,senile osteoporosis[1-3] etc. Further, it was reported that SAM-P/8, a substrain of accelerated senescence prone series with a low incidence of senile amyloidosis, showed the following age-related deterioration in learning ability: SAM-P/8 as well as SAM-P/8/Ta, which is SAM-P/8 bred under specific pathogen-free conditions, exhibited remarkable age-related deterioration in memory and learning in passive avoidance response and hyperactivity in the open field, compared with SAM-R/1 control, a substrain of accelerated senescence resistant series[4-5]. In searching for the structural basis of such behavioral changes and accelerated senescence, we found age-related pathomorphological changes in the SAM-P/8 brain[3]. Several changes related to the learning and memory deficits are described herein.

METHODS AND RESULTS

Age-dependent pathomorphological changes observed in the SAM-P/8 brains can be summarized as follows: cell loss in the locus coeruleus and dorsolateral tegmental nucleus, cortical atrophy in the pyriformis, lipopigmentation, thalamic neuronal inclusion, astrocytosis, PAS-positive intracellular granular structures (PGS), spongy degeneration, reduction of dendritic spines of hippocampal pyramidal neurons, and spheroid in gracile nuclei (Table 1).

1. PAS-positive granular structures (PGS)

SAM-P/8, SAM-R/1 and DDD mice were used in this study. Abnormal granular structures positive for periodic acid-Schiff were found in the hippocampus, piriform cortex, olfactory tubercle, nucleus of trapezoid body and cerebellar cortex. PGS were small, round to ovoid homogeneous structures measuring up to 5 μm in diameter and usually grouped in clusters with a close anatomical relationship to GFAP-positive astrocytic processes[6] . PGS were most frequently found in the hippocampus, especially in CA1, CA2 and CA3. Although they were disseminated in all of the hippocampal layers except the alveus, the most prominent distribution was observed in the

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stratum radiatum. For a quantitative analysis, six level-matched sections were selected and the number of clusters of PGS in the bilateral hippocampal regions was counted. Figure 1 shows the age-dependent increase of PGS in the brains of these mice. In SAM-P/8, PGS appeared in the hippocampus at age 3 months, and the number increased rapidly with aging from age 6 months. PGS were also observed in the control mice, SAM-R/1 and DDD, into old age. Even when the difference of mean life span was taken into consideration, the occurrence of PGS in the control mice was much later and the increment with aging was much less than in SAM-P/8. Ultrastructurally, the PGS showed a morphology similar to "Polygluconsan bodies" (Fig. 2). The histochemical nature and the distribution pattern of PGS, however, differs from corpora amylacea and other structures noted previously. PGS consist of electron dense granular or filamentous substances. It is not clear whether PGS are present in the dendrite or astrocytic process, however, the former possibility is most likely.

2. Spongiform degeneration in the brain stem

Vacuoles of various sizes in neuropils were observed in the brain stem reticular formation (RF) of the SAM-P/8 and SAM-P/8/Ta brains[7]. They could be seen at the age of 1 month and reached a maximum in number and size at the age from 4 to 8 months (Table 2). They were dispersed and were also evident in other areas at the age of 11 months, although the total number decreased. Inflammatory reactions such as cellular infiltration and vascular changes were not evident. Neuronal alterations and loss were not apparent. Ultrastructurally, mild dendritic swelling occurred at one month of age. At two months of age, moderate postsynaptic swelling and widening of intracellular membrane structure were observed, and at age five months there were large vacuoles circumscribed by membranous lamellae, identifiable as myelin (Table 3, Fig. 3).

3. Reduction of dendritic spines of hippocampal pyramidal neurons

Using the Golgi method, dendritic spines in the hippocampal CA1 pyramidal cells of 8 month-old SAM-P/8/Ta and R/1/Ta brains were quantitatively studied[8]. Spines on the basal dendrites were counted per 30 μ segment up to 120 μ. (Thirty well-impregnated pyramidal cells for each subject were randomly chosen.) In SAM-P/8/Ta, as compared to SAM-R/1/Ta, spines decreased significantly on the 30 $\sim$60 μ segment in males ($P<0.01$), and in females on the 0 $\sim$30 μ and 30 $\sim$60 μ segments ($p<0.001$). In each segment, the females had fewer spines than did the males.

4. Astrocytosis

Proliferation of GFAP-positive astrocytes was apparent in the spongy areas of the brain stem, but was also noted in other regions without spongy changes, e.g. hippocampal cortex, piriform cortex, neocortex, and various nuclei in the brain stem. The astrocytosis became more prominent with advancing age.

DISCUSSION

Age-related massive occurrence of PGS, especially in the hippocampus and the spongiform degeneration in the brain stem RF, are peculiar to the SAM-P/8 brain. On the basis of the histochemical nature of PGS, they are suspected to contain certain amounts of glucose polymers as a part of the polysaccharide molecules. Other histochemical results show that PGS do not contain much protein or lipid. In addition, the staining properties of PGS do not resemble those of lipofuscin, amyloid or acid mucopolysaccharides. PGS were found only in some regions of the brain, and the distribution pattern was unique. They were localized in the allocortices such as the hippocampus, piriform cortices and olfactory tubercles, and were not present in the neocortices. The selective anatomical distributions of PGS may be related to the cause of the formation of these structures. Our results also revealed an age-associated increase of PGS in the brain of both SAM-P/8 and

Table 1

Mophological changes in SAM-P/8 brains

1)	Brain weight	P/8 ≒ R/1
2)	Cell loss (TLD*, LC*)	P/8 ≧ R/1
3)	Cortical atrophy (pyriformis)	P/8 > R/1
4)	Spongy degeneration	P/8 ≫ R/1
5)	Astrocytosis	
	a) regions with spongiosis	P/8 ≫ R/1
	b) other regions (cortex, hippocampus,etc)	P/8 > R/1
6)	Lipofuscin (Purkinje cell,etc)	P/8 ≧ R/1
7)	PGS	P/8 ≫ R/1
8)	Thalamic neuronal inclusion	P/8 ≧ R/1
9)	Alteration in hippocampal dendritic spines	P/8 > R/1
10)	Spheroid in gracile nuclei	P/8 > R/1

*TLD: dorsolateral tegmental nucleus
*LC : locus coeruleus

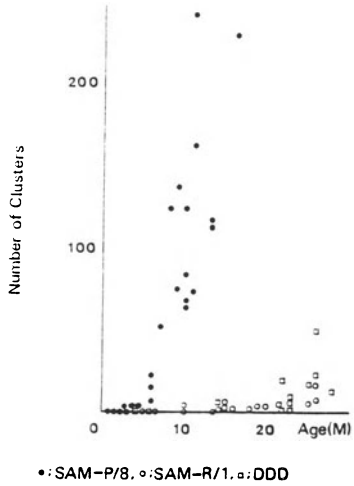

Fig. 1 Number of clusters of PGS in the hippocampus of six level-matched 6-μm-thick sections

Table 2

Vacuolation in brain stem and Spinal Cord in SAM-P/8

	1 W	1 M	2 M	4 M	8 M	11 M
R	O	O	+	++	++	+
PnO	O	O	+	++	++	+
PnC	O	O	+	+++	+++	++
Gi	O	+	+++	+++	+++	++
Spi	O	+	++	++	+	+

Legend: O, no vacuoles; +, some vacuoles;
++, dozens of vacuoles;
+++, a moderate number of vacuolation;
+++, severe vacuolation
Abbreviation: R, red nucleus;
PnO, pontine reticular nucleus, oral;
PnC, pontine reticular nucleus, caudal;
Gi, gigantocellular reticular nucleus;
Spi, spinal cord

Table 3

Ultrastructural changes of vacuolation with advancing age in SAM-P/8 brains

	1 M	2 M	5 M	8 M
Vacuolation of* Postsynapse	+	++	+++	++
Vacuolation** of Myelin	O	O	+++	++
Reaction of Astrocyte	+	+	++	+++
Remyelination	O	O	+	++

Legend: O, no changes; +, mild;
++, moderate; +++, severe
*, fragility of neuronal process membrane ?
**, dying-back gliopathy ?

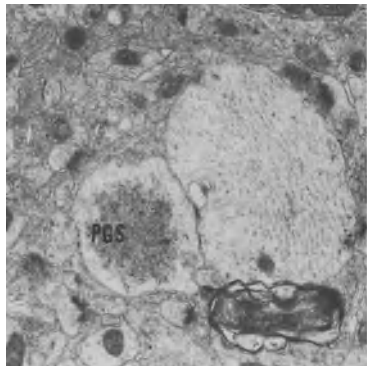

Fig. 2 Electron micrograph of PGS (SAM-P/8, hippocampus, X 20,000)

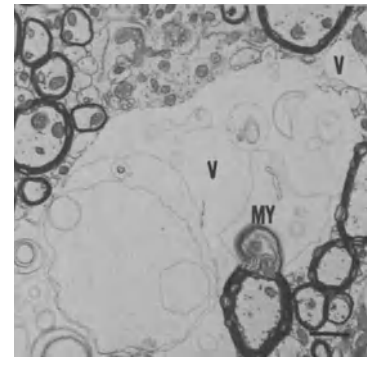

Fig. 3 Electron micrograph of vacuoles in RF (SAM-P/8, 5 M, X 22,500)

the control mice, SAM-R/1 and DDD. It should be emphasized that PGS are considered to be a new morphological manifestation of senescence, and that they appeared more numerously in the brains of SAM-P/8, a strain with learning and memory deficits.

The earliest pathomorphological finding observed in SAM-P/8 brain was spongy degeneration in RF, which appear 1 ~2 month-old mouse and precede or simultaneously appear with learning disabilities. The role of RF is considered to modulate learning and memory process as well as alertness, attention and sleep. So, it can be assumed that there is a possible pathophysiological relationship between the spongy state and learning and memory deficits. Genetic disorders linked to spongy degeneration have been noted in various mice, however, pathogenesis of spongy degeneration in SAM-P/8 brain is not clear. Vacuoles in SAM-P/8 proved to be both swollen neuronal processes and oligodendroglial processes. It may be assumed, however, a major part of vacuoles in this model is related to oligodendroglial processes and membranous myelin structures. In general, myelination in the CNS ends early in the postnatal period (1 month in mice), however, completion of myelination in RF is late as well in association areas. From this characteristic myelination of RF, spongy degeneration, which is based on the mechanism interaction between vacuolation and repair, seems to progress because of a relatively more potent vacuolation and/or weaker repair up to 4 to 8 months of age. It may tend to diminish because of a relatively weaker vacuolation and/or more potent repair over 8 months of age. These SAM-P/8 and SAM-P/8/Ta strains of mice are newly developed memory-deficient strains with spontaneous spongy degeneration associated with aging.

Morphometric analysis of Golgi-impregnated sections indicated a significant reduction of spine density in the proximal to middle portions of basal dendrites of hippocampal pyramidal neurons in SAM-P/8 brain. This post-synaptic alteration of hippocampal neurons may also be related to learning and memory disabilities. The age-related increase of astrocytosis, thalamic inclusion, and lipopigmentation, generally regarded as senescence-associated changes, are also prominent in the SAM-P/8 brain.

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AGE-RELATED CHANGES IN BLOOD-BRAIN BARRIER (BBB)

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INTRODUCTION

The BBB is a major modulator of nutrient delivery to the central nervous system (CNS) and a major keeper from environmental toxins. Any age-related deteriorations in the BBB may lead to brain damage and consequently to progressive deterioration and loss of memory. Some data have suggested that the BBB is broken down in Alzheimer's disease,[12] but no evidence supporting this view has been obtained by positron emission tomography (PET).[9] Most of the studies reported so far have failed to show a significant age-related alteration in BBB permeability to water-soluble substances and high molecular weight solutes in the absence of neurological disease.[6,8] We evaluated the brain uptake of serum albumin, the most common circulating macromolecule, in SAM-P/8 and SAM-R/1, as SAM-P/8 but not SAM-R/1 showed a marked age-related deterioration in the ability of memory and learning in passive and active avoidance responses.[5,10,11]

MATERIALS AND METHODS

Animals

Three, 6, and 12-month-old (n=18) SAM-P/8 and 3, 12, and 21-month-old (n=15) SAM-R/1 were maintained under conventional conditions and fed a commercial diet (CE-2, Nihon CLEA) and tap water ad libitum.

Experimental procedure

Brain uptake of human serum albumin was measured by the double-isotope method of Leibowitz and Kennedy with some modification.[3] Iodination of HSA by carrier- free $Na^{125}I$ (IMS-30, Amersham Corp.) or carrier-free $Na^{131}I$ (IBS-3, Amersham Corp.) was performed by the ICl method.[4] Unbound iodine was removed by an anion-exchange resin column (column size, 0.5ml) and overnight dialysis against phosphate-buffered saline (PBS), pH 7.2, at 4° C. Specific activities ranged from 1.6-2.5 x 10^6 (cpm / μg protein) for ^{125}I-HSA and 1.8-3.6 x 10^6 (cpm / μg protein) for ^{131}I-HSA. The ratio of trichloroacetic acid precipitable to total counts always exceeded 99%. Mice were infused via a tail vein with 0.5 μg protein of ^{125}I-HSA per gram of

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body weight. At 5 minutes and 1, 6, and 12 hours after injection, blood samples were taken from a tail vein and put into capillary tubes. Exactly 10 μl of serum was obtained from each sample, and the radioactivity was counted in an Aloka Auto Well Gamma System (ARC-300). Five minutes to 24 hours later, these same mice were infused from a tail vein with 0.1 μg protein of ^{131}I-HSA per gram of body weight and then were given i. p. an overdose of pentobarbital sodium. A sample of 500 μl of blood was taken from the heart exactly 5 minutes after the injection of ^{131}I-HSA and the animals were then immediately decapitated. The brain was removed and the dura and the subarachnoidal vessels were carefully excised and discarded. The brain surface was washed clean with phosphate-buffered saline and hemisectioned at the midline and quickly dissected into several regions (olfactory bulb, frontal cortex, parietal cortex, caudate putamen, hippocampus, pons, and cerebellum) in a room kept at 4°C. The radioactivity in each brain region was measured. The ^{125}I activity was corrected for counts due to ^{131}I "breakthrough" into the ^{125}I channel.

Calculations

To evaluate the brain uptake, we calculated the ratio (accumulation index, AI) of the accumulation of radioactivity per unit wet weight in the brain region, from which intravascular radioactovity was subtracted, to the plasma concentration integral.

$$AI = ({}^{125}Br - {}^{125}Va) / Wbr \times \int_0^T Cp(T)dT \quad (1)$$

where ^{125}Br is the measured activity of ^{125}I-HSA in the brain at time T, ^{125}Va is the activity in the vascular compartment, Cp(T) is the plasma concentration at time T, Wbr is its weight, and T is the experimental time. ^{125}Va was calculated from the plasma radioactivity of ^{125}I-HSA at decapitation time and the regional blood volume, which is defined as ^{131}Br/^{131}WB, where ^{131}Br is the measured activity of ^{131}I-HSA in the brain at time T and ^{131}WB is the activity of ^{131}I-HSA in the whole blood per unit volume at time T.

Data Analysis

All values in the text are expressed as means ± SEM. Comparisons were made by two-way analysis of variance (ANOVA).

RESULTS

All the accumulation indexes in both mouse strains except those for caudate putamen and pons in SAM-R/1 changed with age in the same fashion. That is, they declined with age at first and thereafter rose. The mean accumulation index in SAM-P/8 was 5.67±0.60, 4.65±0.42, and 7.30±0.76 (x 10^{-5} μl/mg/h) at age 3, 6, and 12 months, respectively. The index was significantly higher in 12-month-old SAM-P/8 than in 3 or 6-month-old ones ($p<0.05$). The mean accumulation index in SAM-R/1 was 5.22±0.60, 4.62±0.39, and 7.16±0.81 (x 10^{-5} μl/mg/h)at age 3, 12 and 21 months, respectively. The index was significantly higher in 21-month-old SAM-R/1 than in 3 or 12-month-old animals ($p<0.05$). The index was 1.58 times higher in 12-month-old SAM-P/8 than in 12-month-old SAM-R/1 ($p<0.05$). The age was younger in SAM-P/8 than in SAM-R/1 when the index began to rise. In addition, the accumulation index was significantly higher in the olfactory bulb than in other brain regions regardless of age and strain ($p<0.01$), and it was 2.6 times higher in the olfactory bulb than in the cerebellum.

DISCUSSION

Our findings indicate that the barrier function in the brain of SAM-P/8 and -R/1 weakens in the latter period of life and the breakdown of the BBB occurs in SAM-P/8 earlier and more severely than in SAM-R/1. Therefore, aging may be related to breakdown of the BBB. In addition, we obtained evidence for the ready penetration of intravascular circulating macromolecules into the olfactory bulb in mice. This would suggest that the olfactory bulb is loosely barriered against blood circulation.

Rhinencephalo-limbic structures are known to be easily affected in patients with Alzheimer's disease.[2,7] Our findings should contribute to the elucidation of the pathogenesis of Alzheimer's disease. A PET study using a protein as a tracer should be carried out, if possible. We have found several age-related pathomorphological changes in the SAM-P/8 brain.[1] Our data suggest that SAM-P/8 have senescence-accelerated changes also in the BBB, and thus these animals provide a convenient experimental model to examine further these changes.

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EFFECT OF AGING ON NADPH-DIAPHORASE NEURONS IN LATERODORSAL TEGMENTAL NUCLEUS AND STRIATUM OF SENESCENCE ACCELERATED MOUSE (SAM)

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INTRODUCTION

Age-related reduction of neuronal number and/or size has been reported in some brain regions of human and experimental animals. Of particular interest are the age-related alterations of cholinergic neurons in the basal forebrain. This cholinergic cell group sends ascending projections into the neocortex, amygdala and hippocampus, and may be associated with memory and cognitive function, both of which are affected in older experimental animals. In fact, neuronal size is reduced in this cholinergic cell group in aged rodents [1, 2]. In addition to the basal forebrain cholinergic neurons, a conspicuous cell group in the pedunculopontine tegmental nucleus (TPP) and laterodorsal tegmental nucleus (TLD) of several species has been described to be cholinergic [3]. This cholinergic cell group in the hindbrain also gives rise to widespread ascending or descending projections to various regions.

Previous studies have demonstrated that NADPH-d histochemistry selectively stains these cholinergic neurons in a Golgi-like manner [4], as well as stains a class of striatal neurons containing both somatostatin and neuropeptide Y in the rat [5]. In the present study, NADPH-d histochemistry was used for the identification and morphometric analysis of the cholinergic neurons in the TLD of normal mice and senescence-accelerated mouse (SAM) [6], in which the substrain SAM-P/8, but not the substrain SAM-R/1, shows an age-related deteriolation in its ability to acquire memory and learning [7]. NADPH-d positive cells in the striatum were also analyzed and compared with those in the TLD.

MATERIALS AND METHODS

Animals

Twelve male DDD mice (six 2 months old and six 25-30 months old) and 20 male SAM mice (five 2 months old and five 14-17 months old of each substrain) were obtained from the colonies of the Division of Senescence Biology, Chest Disease Research Institute, Kyoto University, Japan. In this laboratory and for these three strains, the ages selected represent an almost 99% survival rate for the younger group and less than a 1% survival rate for the older group.

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Histochemical Methods

The mice were deeply anesthesized with an intraperitoneal injection of sodium pentobarbital and perfused transcardially with ice-cold phosphate-buffered saline (PBS, pH 7.4), followed by an initial fixative of 0.35% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), and a second fixative of 200ml of 1% paraformaldehyde in 0.1M PB. The brains were removed immediately and soaked in 20% sucrose in 0.1M PB overnight at 4°C. Coronal blocks from midbrain to pons were cut serially into 30-μm thick sections with a cryostat. Forty-μm thick sections of the caudate-putamen at the level of the anterior commissure were cut in a similar way. NADPH-d histochemistry was performed according to the method by Scherer-Singler et al. [8] with some modifications. In order to examine the relationship between NADPH-d activity and choline acetyltransferase (ChAT) immunoreactivity in the ponto-mesencephalic tegmentum of mice, a well-characterized monoclonal antibody to ChAT was used.

Morphometric Methods

A morphometric analysis was performed on the positive cells located in the TLD on the left side of the sections, but all the NADPH-d positive cells in the TLD were counted. The cell size was measured with a Quantimet 720 image-analyzing computer (Cambridge Instrument, U.S.A.). Since, in the TLD, some of the positive cells and neurites were closely aggregated and overlapped, a manual editing method was employed in order to eliminate neurites from the morphometric procedure, and also to separate overlapped positive cells from each other. The whole area was examined precisely with neither overlap nor gaps in the TLD. The neuronal counts were corrected by Abercrombie's formula to avoid double counting of split cells. In the present study, the morphometry of the caudate-putamen was limited to positive cells found in 25 randomly selected microscopic fields from 2 to 4 sections at the level of anterior commissure from each animal. The output data from the Quantimet 720 were processed with a PDP11 computer (Digital Equipment Corp., U.S.A.). The cross-sectional area and diameter of all the individual cells in the TLD were calculated. The numerical density, indicated by cell number per microscopic field, and cross-sectional area of positive cells in the striatum were also computed. The individual differences between the two age groups and between the three strains were evaluated by use of a two-sided Mann-Whitney's U test.

RESULTS

NADPH-d reactive material was observed as a diffuse intense blue reaction product within neuronal somata, dendrites, and varicose fibers. No glial cells were stained. Large bipolar, triangular, or multipolar neurons were found in the TLD and TPP, and medium-sized cells with a fewer dendrites were stained in the striatum. The topographic distribution of positive cells in the striatum and brainstem appeared similar to that previously reported in the rat. Although the morphologies of positive somata were similar in both age groups, the number of positive neurites appeared to be less in the old mice than in the young ones. Figure 1 is a histogram of the positive neurons in the TLD and caudate-putamen of the young and aged mice, and indicates a decrease in number of NADPH-d positive neurons in the TLD of the SAM strain: the reduction from an early age in SAM-P/8 and with aging in SAM-R/1. The mean numbers of TLD neurons in aged mice were approximately 3%, 13%, and 1% less than those in the respective young DDD, SAM-R/1, and SAM-P/8 mice, although these differences were not statistically significant except for the SAM-R/1 strain. When, however, NADPH-d positive neurons in the TLD were divided into two groups based on their sizes, the mean number of large positive cells, whose diameter was greater than 17um, was 34%, 3%, and 18% less in the aged than in the young DDD, SAM-R/1, and SAM-P/8 mice, respectively. In the TLD, the mean cross-sectional area of positive cells in the old group was 3.0%, and 12.8% less than that in the respective young group of DDD, SAM-R/1, and SAM-P/8 strains (Fig. 2).

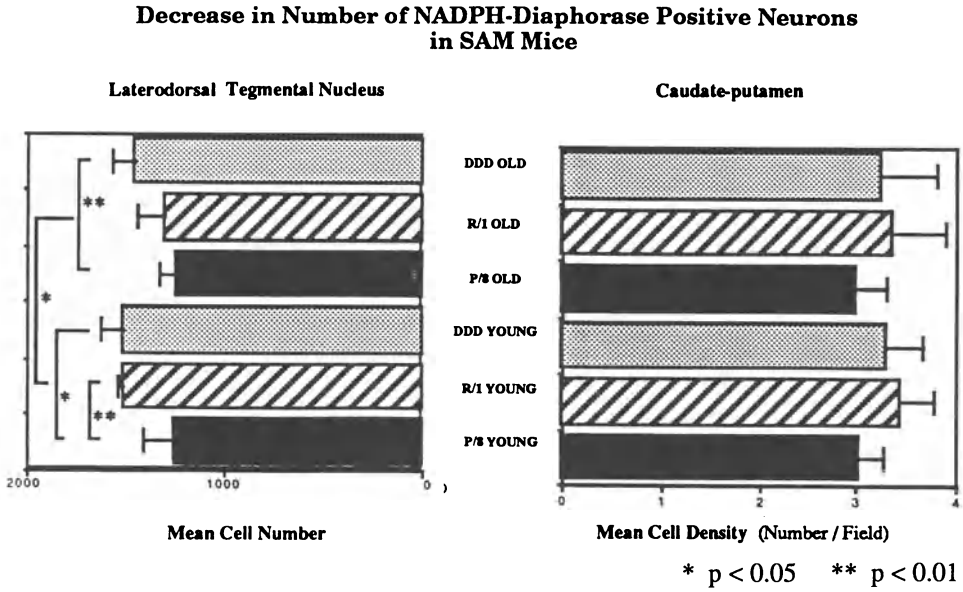

Fig. 1. Data are shown as mean ± standard deviation.

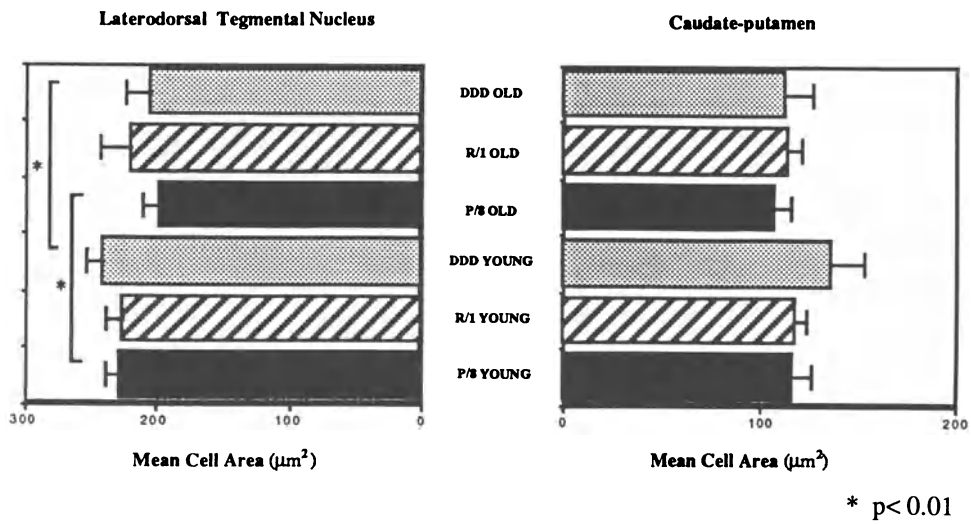

Fig. 2. Data are shown as mean ± standard deviation.

DISCUSSION

The present study has revealed that NADPH-d containing neurons undergo shrinkage with increasing age in the TLD, but not in the caudate-putamen of DDD and SAM-P/8 mice, while the numbers of these neurons are preserved with age in both regions of DDD mice but not in the TLD of SAM mice. NADPH-d containing neurons in the TLD of mice correspond in this regard to cholinergic cells in this nucleus of rats. Thus, cholinergic neurons in the TLD appear to undergo age-related shrinkage in all three strains and also show cell loss in the two strains of SAM mice. Since NADPH-d neurons in the caudate-putamen are non-cholinergic [5], this age-related shrinkage appears to be more prominent in cholinergic NADPH-d neurons in the TLD than in non-cholinergic NADPH-d striatal neurons. In agreement with the present results, it has been suggested that cholinergic neurons are the most susceptible to aging among the neurons in the central nervous system [9].

Similar age-related shrinkage has been reported in cholinergic neurons of the basal forebrain in mice, where neuronal numbers were preserved [1, 2]. It appears therefore that cholinergic neurons in the TLD undergo aging changes similar to those in the basal forebrain. These cholinergic TLD neurons have been shown to send wide ascending projections into the medial prefrontal cortex, basal forebrain, striatum, and thalamus. Although the functional role of cholinergic neurons in the hind brain is yet to be fully understood, severe neuronal loss in the TPP was recently described in patients with progressive supranuclear palsy, and abnormality in this cholinergic nucleus might underlie the clinical symptoms such as parkinsonian features and dementia characteristic of the disease [10]. And also neurofibrillary tangles in NADPH-d neurons and neuritic plaque formations were found in the TLD and TPP of postmortem human brainstems of Alzheimer's disease patients [11]. Thus, the age-related shrinkage of cholinergic neurons in the TLD may result in the reduction of cholinergic afferents to the forebrain of senescent mice and may cause motor or cognitive dysfunction in aged animals. In addition to such neuronal shrinkage, the smaller number of neurons in the TLD may be also related to the age-related disability of acquisition of memory and learning that occurs in SAM-P/8 mice.

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SENESCENCE-ACCELERATED MOUSE SAM-P/8 SHOWS SPONTANEOUS AGE-RELATED IMPAIRMENT OF ABILITY OF ACQUISITION OF LEARNING AND MEMORY: AN ANIMAL MODEL OF DISTURBANCES IN RECENT MEMORY WITH AGING

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INTRODUCTION

Several pairs of AKR strain mice were donated to our laboratory by the Jackson Laboratory(Bar Harbor) in 1968. We continued the sister-brother mating of these mice and became aware of some litters which showed a moderate to severe loss of action, hair loss, skin coarseness, and a shortened life span. We selected and maintained 6 substrains with severe exhaustion as accelerated senescence prone(SAM-P/1,-P/2,-P/3,-P/4,-P/6,-P/8) and 3 substrains with normal aging process as accelerated senescence resistant(SAM-R/1,-R/2, -R/3).[1] Judging from findings in the survivors and for Gompertzian function of the growth pattern, the aging pattern in this SAM model seems to relate to an accelerated senescence rather than to premature aging. Recently, SAM-P/8 was reported to show age-related learning and memory deficits in passive avoidance performance under specific pathogen-free conditions.[2] In this present study, we examined age-related changes in memory of SAM-P/8 under conventional conditions and found that SAM-P/8 is a pertinent model for researching disturbances of recent memory with aging in animals and humans.

MATERIALS AND METHOD

Male SAM-P/8 mice and SAM-R/1 controls bred under conventional conditions were used in the experiment.

Passive Avoidance Test

Single-trial passive avoidance tests were carried out by use of a step-through-type apparatus with light and dark chambers and by application of a 0.5mA-3s foot shock to the mice.[3] The latency to enter the dark chamber was measured 24 h after foot shock. Acquisition tests were conducted by repetition of the foot shocks till the mice reached the criterion of latency(300s), and the number of the shocks was recorded. Retention tests were carried out one month after the mice had achieved the acquisition criterion.

Maze Test

T-maze avoidance tests were carried out in a gray plastic T-maze with a grip floor, and 0.5mA foot shocks were applied to the mice under stay condi-

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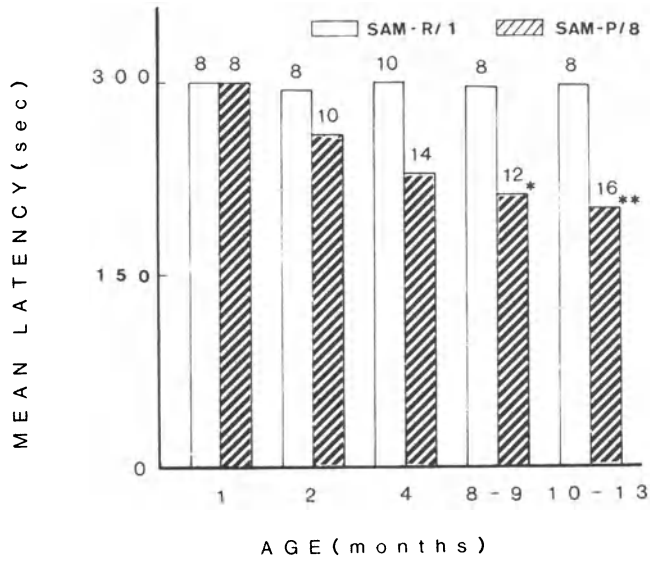

Fig.1. Age-related changes in single-trial passive avoidance response in SAM-P/8 and SAM-R/1. Numbers in parentheses are the number of mice tested. *P<0.05,**P<0.01 vs. SAM-R/1 controls.

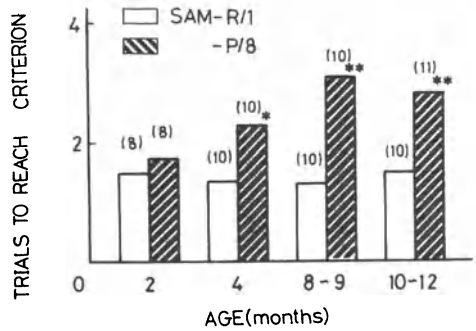

Fig.2. Age-related changes in ability of acquisition in SAM-P/8 and SAM-R/1. The ability of acquisition is represented as the number of trials needed to achieve the criterion(300s).[3]

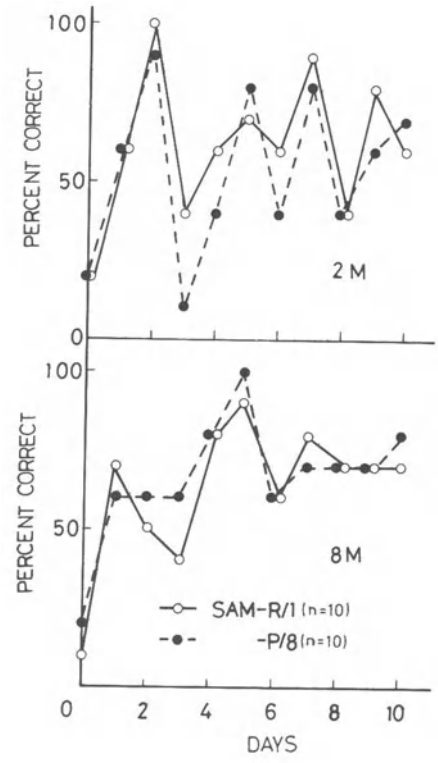

Fig.3. T-maze avoidance performances in SAM-P/8 and SAM-R/1. The test was carried out once daily for 10 consecutive days.[3]

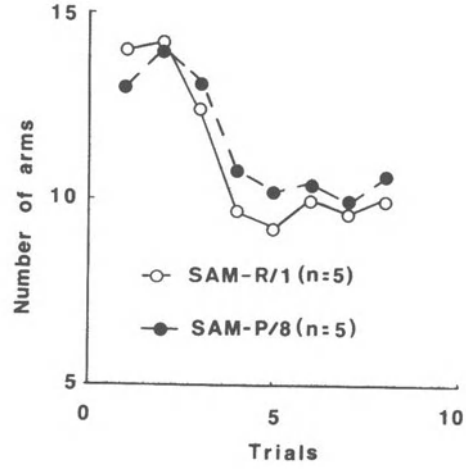

Fig.4. Radial maze performances in SAM-P/8 and SAM-R/1 at age of 8 months.

tions.[3] Radial maze tests were conducted in an eight-arm radial maze after reduction of the body weight of the mice to 80% of their ad lib.weight. The total number of arms that the mice entered to eat all pellets placed at the end of each arm was recorded.

RESULTS

Passive Avoidance Test

In the single-trial passive avoidance test, the mean latency of SAM-P/8 decreased with aging, while that of SAM-R/1 controls was almost 300s of the criterion at all ages tested(Fig.1). Thus, SAM-P/8 showed age-related deterioration in single-trial passive avoidance performance. In the acquisition test, the number of foot shocks that the mice received to achieve the criterion was almost constant in SAM-R/1 controls, while it increased with advancing age in SAM-P/8; SAM-P/8 showed marked impairment of acquisition ability(Fig.2). In the retention test, there was no significant difference between the two groups.

Maze Test

In the T-maze avoidance test, the percentage(percent correct) at which the mice chose a correct arm under stay condition finally reached a constant value of 70 in both SAM-P/8 and SAM-R/1(Fig.3). In the radial maze test, the total number of arms entered decreased with increasing trials in both groups (Fig.4). Thus, there was no significant difference in T-maze avoidance and radial maze performances between the two groups.

Discussion

SAM-P/8 mice under conventional conditions showed age-related deficits in learning and memory in the single-trial passive avoidance response. The results of the acquisition and retention tests revealed that the memory deficit in SAM-P/8 was mainly due to a deterioration of the ability of acquisition and not due to a deficit in the ability of retention. On the other hand, there was no significant difference in the results of the maze test between SAM-P/8 and SAM-R/1. Maze tests reportedly reflect working memory; therefore, the results of the maze test suggest that the working memory of SAM-P/8 is fairly intact despite the memory impairment in the passive avoidance. Different memory-related mechanisms may operate between the passive avoidance(reflecting sensory memory) and maze tests(reflecting working memory). Thus, SAM-P/8 is a good model to show an age-related deficit of memory, especially sensory memory, and may be a pertinent model to study the impairment in the ability of acquisition in memory seen in senile humans and in patients with Alzheimer's disease.

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AN EXPERIMENTAL ANIMAL MODEL FOR THE STUDY OF CHOLINERGIC AND SEROTONERGIC NEURONAL ACTIVITY IN AGING BRAIN

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INTRODUCTION

Physiological aging of neurons associated with cholinergic as well as serotonergic activities in experimental animals may well be compatible with senile deterioration of selective neuronal activity in demented brain. To date, cumulative information has been reported on decreased synaptic activity of cholinergic neurons in aging rodents, with most studies done with Fischer rats. However, serotonergic involvement, which is persistent in human aging brain, remains unchanged in aging rats as well.

Suncus murinus(house musk shrew), a primitive mammalian insectivore situates at the stem of the mammalian phylogenic tree, has been proposed to retain common ontogenic and gerontogenic similarities to the primates and to have a nervous system that operates according to the basic biological processes of the human nervous system.

Earlier we reported a difference in properties between aging Suncus and rats in terms of the binding capacity of serotonergic ligands to the membrane fraction of aging cerebral cortex[1], and the responsiveness neurite outgrowth of their ganglion cells in culture to nerve growth factor[2].

In an attempt to elucidate molecular mechanisms that may account for the decreased activity in calcium uptake by aging synaptosomes, we investigated and the effect of some modulator molecules that have been established to promote differentiation of developing neurons, i.e., some gangliosides[3] and certain types of protease inhibitors[4].

EXPERIMENTAL METHODS

Experimental animals

Suncus and Fischer rats were commercially supplied, and Suncus were maintained on high protein pellets from Kurea

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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Laboratory until the late period of their life spans, which rarely exceeds 2 years in the case of Suncus. The average life span of Suncus is around 20 months, which is almost two thirds of that of rats.

Acetylcholine content

Homogenates of different brain regions were prepared in the presence of neostigmine, an inhibitor of choline esterase, to prevent degradation of acetylcholine during the preparation. The deproteined supernatant by trichloroacetic acid was applied to an HPLC/ECD system connected to an enzyme-immobilized column.

DPAT binding assay

^{3}H-8-Hydroxy-2-(Di-n-Propylamino)Tetralin(^{3}H-OH-DPAT) was obtained from NEN Research Laboratory and incubated with the membrane fraction of cerebral cortex from aging Suncus as described elsewhere.[5]

Phosphorylation study on synaptic membrane

Synaptosomal membrane fractions were prepared by the conventional differential centrifugation technique[6] and incubated with γ-^{32}P-DAPT in the presence of $CaCl_2$ or EGTA at 25°C for 5 minutes. Following SDS-PAGE of the reaction mixture, phosphorilated synaptic membrane proteins were stained by Coomasie Brilliant Blue; then dried gel was exposed on a X-ray film. The phosphorylated bands were excised from the dried gel and counted for radioactivity in scintillation vials

RESULTS

As has been reported, calcium uptake by aging synaptosomes is markedly decreased in the senile brains of experimental animals.[1] Acetylcholine content in the cerebrum and hippocampus of aged animals was extremely decreased, as is shown in Figure 1. On the contrary, the binding capacity of serotonergic ligands such as imipramine and 5-hydroxytryptamine to the membrane fraction of aging animals was not decreased in rats but was in Suncus[1]. DPAT is considered to be a specific lingand of the $5HT_{1A}$ receptor. As shown in Table 1, the binding capacity of the ligand in aging Suncus 22 months old was decreased to about 30% of that of the adult animal. Diaminopyridine(DAP) has been reported to modulate decreased uptake of calcium by aging synaptosomes,[7] whereas DPAT binding was inhibited by DAP at higher rate with aging.

In the biological transduction of signals through the membrane, phosphorylation and dephosphorylation of membrane proteins regulate molecular conformations and serve to couple signals to cellular functions. Therefore, it may be interesting to disclose the change in autophosphorylation activity of the synaptic membrane of aging Suncus. Among major phosphoproteins in the synaptic membrane, those that were phosphorylated by calcium-dependent and -independent protein kinases were moderately decreased in aging

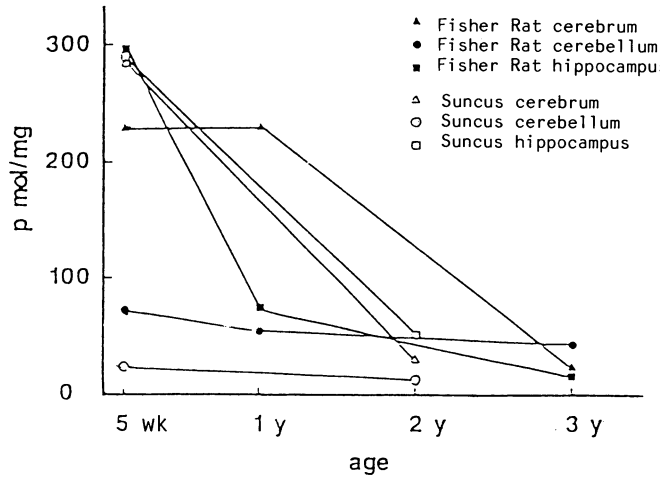

Figure 1. Acetylcholine content in aging brain.

TABLE 1 Effect of DPAT Binding in Cortical Membrane in Aging Suncus.

Age M	Control (f moles / mg protein)	DAP	% of control
4	47±5	40±4	84.6
6-8	42±4	37±5	88.1
10-12	46±7	34±4	73.9
17	36±6	11±4	30.5
22*	16	13	

Mean of two experiments±s.d.
* Single experiment in triplicate.

TABLE 2 Effect of Modulators on Phosphorylation of Synaptosomal Membrane Proteins.

	BBG		GM1		Aprotinin	
	5W	25M	5W	25M	5W	25M
88K	→	↑	→	↑	↑	↑
68K	→	→	↑	→	→	↑
51K	→	→	→	→	→	↑
20K	→	↑	→	↑	↑	↑

synaptosomes. Unlike calcium uptake by the synaptosomes or DPAT binding to the membrane fraction in aged animals, autophosphorylation activities were retained fairly well by the aged synaptosomes(data not shown). Bovine brain gangliosides(BBG), one of its component monosialoganglioside(GM_1), and aprotinin, serine protease inhibitor, all of which have been reported to promote synaptogenesis [3,4] showed some modulatory effect in phosphorylation by certain types of protein kinases, as is shown in Table 2. Among the three, aprotinin seemed to enhance overall autophosphorylation of aging synaptic membrane proteins, while gangliosides, but not asialoganglioside, inhibited it.

DISCUSSION

The pathogenesis of senile dementia(SDAT) and of Alzheimer's disease have yet to be elucidated, perhaps owing to the difficulty in establishment of animal models for basic research. However, the involvement of cholinergic neurons has been well established in medioseptal and hippocampal neurons in demented brains.

The new experimental animal Suncus Murinus has been recently domesticated because of its biological similarity to the primate, for it is more closely related to them than to rodents in some respects, including the cerebral cortex. But, as a matter of fact, it is a more primitive mammalian than rodents. The life span is also shorter than rodents, which may be favourable for the study of aging. As was demonstrated in the present study on aging brain in experimental animals and has been shown previously,[1] cholinergic deterioration in Suncus is comparable to that in the rat. And yet, serotonergic activity was also decreased in aging Suncus, but was not decreased in aging rat brain. Senile changes in cholinergic as well as serotonergic neuronal activities, therefore, it is useful to introduce such an experimental animal like suncus, which displays for examination of proposed modulator molecules that affect neuronal activity and might be effective in the treatment of deteriorated neuronal activity found in senility.

Gangliosides play diverse roles in cell growth[3] or modulation of phosphorylation of some of the synaptic membrane proteins;[6] however, asialogangliosides are not effective in either activation or inhibition of the autophosphorylation of synaptic membrane proteins.

Protease inhibitors protect proteins from degradation; however, aprotinin has been reported to have some homology to amyloid precursor protein[4] and promotes neurite outgrowth of neuronal cells in culture. In the present study, aprotinin increased norepinephrine in brain cells in culture and modulated autophosphorylation of the aged synaptic membrane proteins. Nevertheless, the activation mechanism of aprotinin to promote protein kinases is yet to be disclose.

Diaminopyridine increased calcium uptake by aged synaptosomes[7], but inhibits serotonergic receptor binding. Pharmacological effect of diaminopyridine is apparent, yet

it is difficult to understand its overall modulatory effect on neuronal activity in the CNS.

ACKNOWLEDGEMENTS

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AGING EFFECTS ON SCHEDULE-CONTROL DISCRIMINATION LEARNING TEST IN AGED RATS AS A MODEL OF ALZHEIMER'S DISEASE

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INTRODUCTION

A gradual deterioration of learning ability is one of the common symptoms of advanced age.[1] Recent interest has been focused on neural mechanisms and pharmacological treatments of such cognitive deficits in the aged.[2] Although appropriate animal models of cognitive aging are necessary to evaluate pharmacological treatment, there is a wide variety of inconsistencies in the literature on learning ability in aged animals. Many factors such as task difficulty, genotype, and early experience are involved in these discrepancies.[3] Concerning discrimination learning, diverse findings have also been reported. Several investigators found clear age differences in visual and place discrimination tasks in primates and rodents.[3,4] In contrast, a number of other reports have shown no age-dependent deficit in similar kinds of discrimination tasks.[5,6]

Most previous studies with rats used an aversively-motivated discrimination or simple appetitive place discrimination problem in a Y-maze or T-maze. Aversively-motivated tasks have disadvantages for psychopharmacological assessments because the effects of stress cannot be excluded from the results. Especially, aged rats have shown many differences in threshold and in moving response to aversive stimulus like an electric shock.[7] On the other hand, experiments using simple appetitive tasks such as a Y-maze or a T-maze failed to find performance deficits in the aged animals. In more than 14 choice maze tasks, aged rats showed some deficiency.[8]

In this experiment, we examined the acquisition of a brightness discrimination task in aged rats by using an operant-type experimental setting, that would be more expected to require one of the highest abilities of the central nervous system, because a positive reinforcement schedule of discrimination has been considered to be a more physiological state of conditioning,[9] and the results might be more meaningful for real learning ability. In our task it was necessary for the rat to obtain a rate of more than 85 percent correct response ratio as a criterion[10,11] in over more than 20 consecutive daily sessions. Although the task was difficult and timeconsuming, it was effective to distinguish a difference between aged and young. This experiment utilized an appetitive brightness discrimination task in an operant paradigm has provided further information about the discrimination ability of aged rats.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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MATERIALS AND METHODS

Subjects

Twenty male Fischer-344 rats, aged 3 months (n=10) and 24 months (n=10), were used. Body weight was 350 to 400 g. They were housed in groups of two or three with food (Oriental Co. Japan) and water ad libitum under 12-h/12-h light dark schedule (light on between 7:00-19:00) in a climate-controlled room that was kept at 22 + 2 C. The rats were then individually housed in stainless steel cages for at least 1 week prior to the start of the experiment. Access to food was controlled so as to maintain the animals at approximately 85% ad libitum body weight.

Apparatus

The apparatus for the discrimination task was a Skinner box (Ralph Gerbrands Company, G7010). It contained a recessed food magazine on its front wall and a lever (3x4 cm) near the lower right-hand corner of the small wall. The rear and front walls of the chamber were made of clear Plexiglass. The chamber was enclosed within a sound- and light-attenuating shell. A light screen (4 cm in diameter) for presenting a positive or a negative stimulus (S+ or S-) was located on the front wall 10 cm above the floor of the chamber. The brightness of this screen was 5 x 10^4 foot Lambert (fl) as a S+ stimulus and 1/1000 brightness of S+ as an S- stimulus. The latter was produced by inserting a Kodak filter (ND 4.0).

In a control room adjacent to the testing room, experimental events were controlled and recorded automatically by a microcomputer, which all trials were continuously monitored on a video monitor. A white noise stimulus of 60 dB was presented through a speaker.

Procedure

The rats underwent shaping in a Skinner box according to a continuous reinforcement schedule (CRF) with food pellets (40 mg) during which the brighter light (5.0 x 10^4 fl.) was shown on the front screen of the box. After shaping, a variable interval (VI schedule) training was interjected. The interval of pellet reinforcement was gradually increased from a VI-5 sec through VI-10 to a VI-15 sec extinction schedule. When the brighter light of 5 x 10^4 fl. was given on the screen as a positive stimulus (S+) for 20 sec, the lever pressing responses were reinforced under the VI-15 sec. schedule. When the darker light (S-) (i.e., 1/1000 of S+) was presented, no pellet was given in response to the lever pressing. The stimulus was provided for 20 sec with a 5-sec black-out interval. The presentations were randomized according to a Gellermann sequence with a single session consisting of twenty each of S+ and S-. Sessions were run daily. A correct response ratio was calculated from the number of correct responses (R+) during S+ presentation and incorrect responses (R-) during S- presentation for a session.

RESULTS

The mean duration to complete the CRF acquisition schedule, 83.1 + 6.6 min (mean + SEM), in young rats was significantly less than the 302.6 + 37.2 min recorded for aged rats ($t=5.81$, p 0.01). The responses of young and aged group on the MULT VI-15 sec extinction schedule are shown in Fig.1 A and Fig.2 A. Two-way repeated measures analysis of variance (group x session) revealed that the total number of responses (R+ + R-)

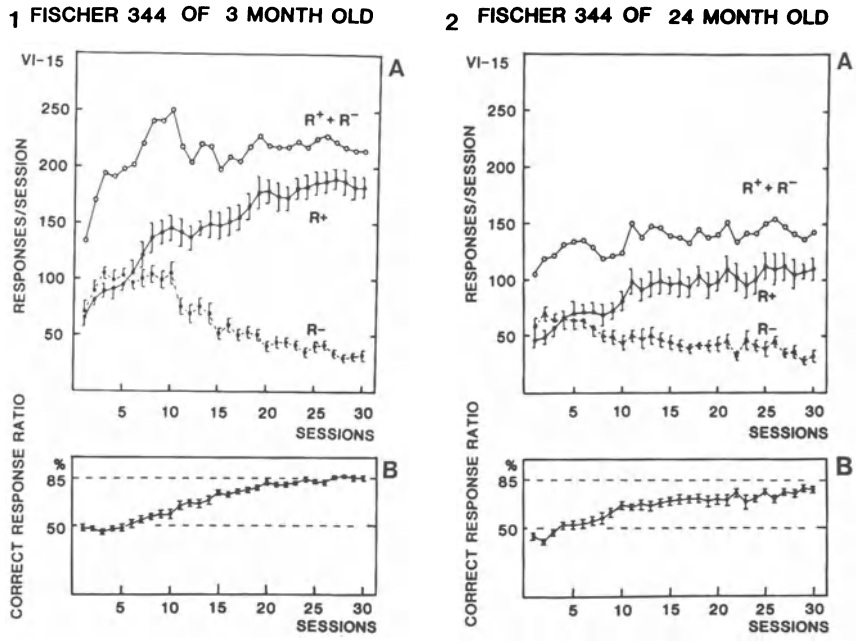

Fig. 1 A, B. Responses and correct response ratios in brightness discrimination learning test in 3-month-old Fischer 344 rats.

Fig. 2 A, B. Responses and ratios in 24-month-old Fischer 344 rats.

in the aged group was markedly lower than that in the young group ($F(1,540)= 489.65$, $p < 0.01$). The number of R+ gradually increased and the number of R- decreased in both groups throughout the 30 sessions. These results suggest that both young and aged animals discriminate between S+ and S-. The correct response ratio of the young rats increased session by session, and after approximately the 27th session, it increased to more than 87%, as shown in Fig.1 B. In contrast, the correct response ratio of aged rats did not reach the 80% level until the 30th session (Fig.2 B).

Two-way repeated measures analysis of variance (group x session) revealed that the correct response ratios of young vs. aged groups were significantly different ($F(1,540)=46.7$, $p < 0.01$).

DISCUSSION

This experiment clearly demonstrated an impairment in the acquisition of a brightness discrimination task of the operant type in aged Fischer-344 male rats. More specially, aged rats engaged in less lever-pressing responses than young controls, which reflects a general activity decline with increasing age. Furthermore, aged rats required more CRF acquisition trials to learn the discrimination task. Concerning the discrimination performance per se, the correct response ratio of aged rats was markedly lower than that of young rats. These results are consistent with previous studies that successfully demonstrated deficits in discrimination tasks in aged animals.[12]

There were many differences between young and old rats in this kind of learning examination. The motivation differences between young and aged rats were remarkable. As shown of appetitive deprivation procedure,[3] aged rats should be deprived to 80 % or less of their ad libitum body

weight; and young, to 85 %.[3] The attention also differed between aged and young rats. The activity difference was remarkably low in aged rat. The decrement activity of aged rats to learn this type of behavior might be estimated higher than that of negative learning paradigms such as an escape or avoidance behavior to an electric shock.[9,13] Even though a Y- or T-maze was used, the aged rat could not run so quickly as the young rat. So behavioral examination requiring the use of motor activity is not an excellent method for use in aging experiment, for in many cases overestimation may occur.

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EFFECT OF THE BASAL FOREBRAIN LESIONS ON MEMORY AND LEARNING PERFORMANCE IN MICE

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INTRODUCTION

The loss of cholinergic cells in the basal forebrain (BF), which correlated with the degree of dementia(1), has been found in Alzheimer's disease patients. The BF cholinergic system has been demonstrated to play an important role in memory function, and many investigation using rats and monkeys indicate that severe deficits in many kinds of learning tasks result from experimental lesioning of this region(2,3).

Basic fibroblast growth factor (bFGF) is one of the mitogenic proteins which promote proliferation of astroblasts(4). Recently bFGF has been demonstrated to promote survival and neuritic growth of several central nervous system neurons in vitro(5) and to rescue medial septal neurons after fimbria fornix transection(6).

Recently, learning impairment caused by BF lesion was demonstrated in mice in our laboratory. In the current study, the effects of bFGF on memory and learning performance abilities were studied in BF lesioned mice.

MATERIALS AND METHODS

Under anesthesia with ketamine and xylasine mixture, male ddY mice (35-39g, 8 weeks old) were placed on a stereotaxic apparatus and received bilateral BF lesion by delivery of radiofrequency current. The coordinates of the electrode placements were; 0.8mm posterior to Bregma, ± 2.8mm lateral to the sagittal suture, and 4.3mm below dura (incisor bar: 2.2mm ventral to the horizontal plane). The stainless steel electrode (0.25mm diameter) was inserted and the tip of it was heated at 70°C, 60 sec. For the sham-operated mice (SHAM, n=15), the inserted electrode was not heated.

bFGF (Recombinant human basic fibroblast growth factor, mutein CS23, kind gift from TAKEDA CHEMICAL INDUSTRIES, LTD., Osaka, Japan) were injected bilaterally in the lesioned position soon after lesion. bFGF (5 and 50ng/1µl of 0.01 % BSA containing PBS/side; FGF 5, FGF 50, respectively, n=15), was applied at the speed of 0.5µl/min and then the cannula was left for 5 min at the injection point. Vehicle was injected to SHAM and lesioned control group (LESION, n=15). Only anesthetized mice were used as control group (CONT, n=15). In case of lesioned mice, milk was given twice a day until 15 days after surgery.

After surgery, when the body weight of the lesioned mice were recovered, step through and step down tests were performed according to Segawa's method(7) for 11 days sequentially after measuring motor activiteis with tilting-type ambulometer for 30min.

After the behavioral tests, 31 days after lesion, choline acetyltransferase (ChAT) activities of anterior and posterior cerebral cortices were measured by the method of Fonnum(8). The subcortical region was immersed in Bouin's fixative, eight µm paraffin sections were made and stained with H.E. The location and histological alterations of the lesioned site were assessed by microscopic observation.

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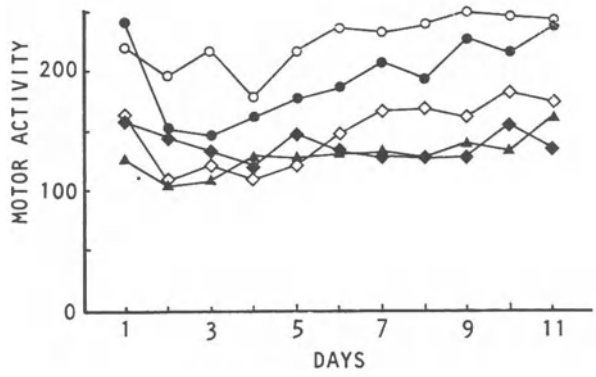

Fig. 1. Motor activity during behavioral testing. o: CONT, ●: SHAM, ▲: LESION, ◇: FGF 5, ◆: FGF 50. LESION, FGF 5 and FGF 50 exhibited significantly lower motor activity than CONT and SHAM on almost all days during testing period (DUNCAN's multiple range test).

RESULTS AND DISCUSSION

Lesioned mice lost their body weight from soon after surgery to about 1 week, as it is often seen in BF lesioned animals. Many mice recovered during 15 days, but 2, 2 and 5 animals were dead in LESION, FGF 5, and FGF 50, respectively, because they did not regain their body weight.

By microscopic examination, lesions were located in the ventromedial region of globus pallidus, which correspond to rodent's BF.

Fig. 1. shows that LESION, FGF 5 and FGF 50 exhibited significantly decreased motor activity compared to CONT and SHAM. However, there was no significant change between FGF 5, FGF 50 and LESION.

In step through test, mean latency to entering the dark chamber on learning trial was not different in each group, while on the first retention test, the latency in LESION was significantly shorter than that of SHAM and CONT (Fig. 2(a)). On the other hand, FGF 50 exhibited significantly longer latency. Percentage of animals that did not enter the dark compartment, which significantly decreased in LESION, was also increased slightly in bFGF treated groups, though the difference was not significant (Fig. 2(b)). These groups also tended to elongate the mean days until the mice made error (memory retention) which significantly shortened in LESION (Table 1). bFGF treated groups tended to improve the total number of errors during the 10 days retention tests(Table 1) increased in LESION.

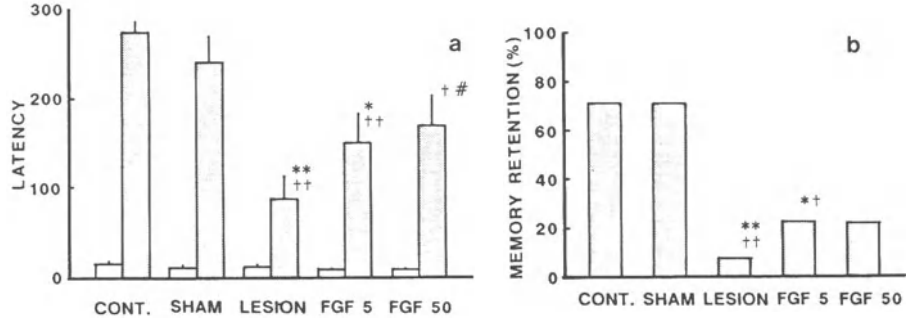

Fig. 2. (a) Mean latency to enter the dark chamber during acquisition (open column) and the first retention trial (dot column) in step through test. The bars represent S.E. *: $p<0.05$, **: $p<0.01$ vs SHAM, †: $p<0.05$, ††: $p<0.01$ vs CONT, #: $p<0.05$ vs LESION, Mann-Whitney's U-test. (b) Percentage of mice which did not enter the dark compartment in the first retention trial. *: $p<0.05$, **: $p<0.01$ vs SHAM, †: $p<0.05$, ††: $p<0.01$ vs CONT, chi square-test.

Table 1. Memory Retention and Number of Total Errors in Step Through and Step Down Tests

		Memory Retention [days]	No. of Total Errors
ST	CONT	3.6±0.8	2.3±0.3
	SHAM	3.4±0.8	2.6±0.7
	LESION	1.1±0.1 **††	6.2±0.7 **††
	FGF 5	1.4±0.2 *†	4.5±0.6 *†
	FGF 50	1.3±0.2 *†	5.4±0.8 *††
SD	CONT	4.4±0.7	2.3±0.4
	SHAM	3.1±0.8	3.3±0.7
	LESION	2.0±0.3 ††	4.5±0.6 †
	FGF 5	3.5±0.9	3.5±0.7
	FGF 50	4.8±1.2	2.9±0.8

Results are given as means±S.E. ST: step through test, SD: step down test. *: $p<0.05$, **: $p<0.01$ vs SHAM, †: $p<0.05$, ††: $p<0.01$ vs CONT, Mann-Whitney's U-test.

On the contrary to step through test, BF lesion resulted in only slight memory impairment in step down task (Fig. 3, Table 1). In the learning trial, no significant difference was seen in mean number of descents in each group. However, on the first retention test, this value in LESION significantly increased compared with that of CONT, while the difference was not significant in comparison to SHAM. Same tendency also can be seen in percentage of animals which did not failure, total number of errors and memory retention days. bFGF tends to ameliorate these indexes.

It is known that decrease of motor activity often causes the apparent improvement in the passive avoidance tasks. However, in the present data, LESION exhibited both decrease in motor activity and leaning impairment. So this learning deficit may not be due to the change of motor activity, but due to the memory deterioration per se. However the cause of the motor activity decrease is still unknown.

Table 2. summarized the ChAT activity in anterior and posterior cerebral cortices. In anterior cortex, ChAT activity significantly (ca.20%) decreased in LESION, while this decrease was much less in posterior cortex. These data indicate that cholinergic neurons which were lesioned in this experiment project to anterior cortex when compared to posterior cortex. bFGF treatment did not recover these reduction both in both part of cortices. On the contrary, high dose of bFGF deteriorated the decrease of ChAT activity in posterior cortex.

The significant decrease of ChAT activity in cortex of LESION suggests that the learning deficit exhibited in this group correlated to the decrease of cholinergic projection from BF to cortex. However, no improvement of ChAT activity was seen in bFGF treated group in spite of partial amelioration in behavioral tasks, suggesting that bFGF improves lesion-induced memory deficits not by effecting on the cortical ChAT activity.

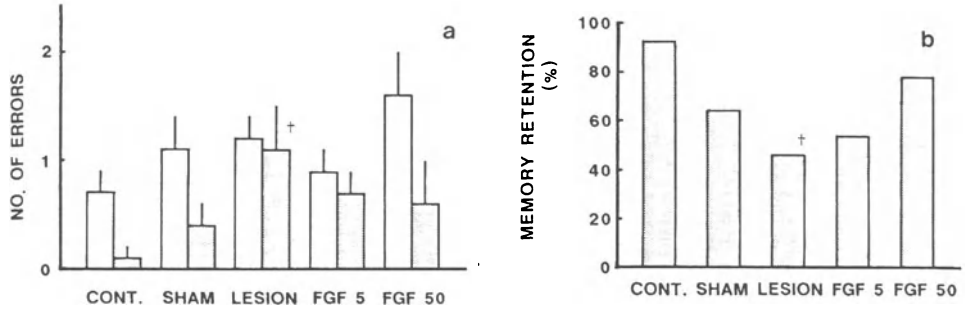

Fig. 3. (a) Mean number of descents during acquisition (open column) and the first retention trial (dot column) in step down test. The bars represent S.E. †: $p<0.05$ vs CONT, Mann-Whitney's U-test. (b) Percentage of mice which did not descend in the first retention trial. †: $p<0.05$ vs CONT, chi square-test.

Table 2. Change in Cerebral Cortices Choline Acetyltransferase Activity Measured at 31 Days After Surgery

	Specific Activity	
	Anterior Cortex	Posterior Cortex
CONT	0.265±0.015	0.286±0.008
SHAM	0.268±0.011	0.292±0.007
LESION	0.218±0.012 **†	0.263±0.009 †
FGF 5	0.219±0.008 **††	0.268±0.009
FGF 50	0.215±0.014 *†	0.246±0.009 **††

Results are given as means±S.E. (pmol/μg protein/min)
*: p<0.05, **: p<0.01 vs SHAM, †: p<0.05, ††: p<0.01 vs CONT, DUNCAN's multiple range test.

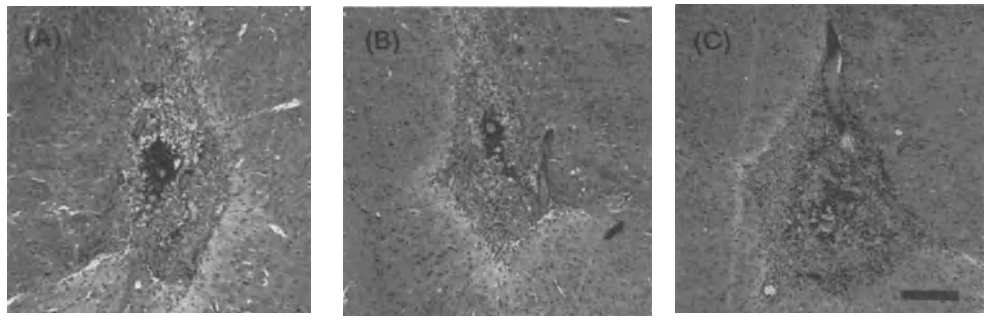

Fig. 4. Histological visualization of lesioned area. Sections were stained with H.E. (A), (B) and (C); 8μm coronal sections of LESION, FGF 5, and FGF 50, respectively. Bar in (C)=200μm. No specific histological change can be seen in each group.

By the microscopic examination, gliosis were observed in lesioned area, but there seemed to be no distinct pathological difference between LESION, FGF 5 and FGF 50 (Fig. 4). The lesioning method used here was not specific for cholinergic neurons, so the effect of bFGF on the other transmitter system should also be considered.

In conclusion, cholinergic projection from BF to cerebral cortex was successfully lesioned in mice, and bFGF injected directly into the lesioned area partially improved the learning deficits resulted from BF lesions.

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EFFECTS OF DESTRUCTION OF NUCLEUS BASALIS MEYNERT ON OPERANT STUDY AND NEUROTRANSMITTERS IN RATS

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INTRODUCTION

In senile dementia of the Alzheimer type (SDAT), it has already been reported that the activity of neurotransmitters[1-3] and number of cells in the nucleus basalis Meynert (nbM)[4] are decreased. The purpose of our study was to evaluate changes in and correlation between the activity of neurotransmitters and response of ibotenic acid-lesioned WKY rats (a model of Alzheimer's disease) in an operant study.

MATERIALS AND METHODS

(1) Alzheimer model (WKY, 4 months old)

According to the procedure of Miyamoto[5], we injected ibotenic acid (10mg/ml, 05ul) to the bilateral nbM (A:6.4, L:2.8, H:1.8) by using a microinfusion pump. H.E. stain was done to check the lesion.

(2) Operant study

WKY (4 months old) rats (control, n=10; Alzheimer model, n=8) were used for this study. Body weight was decreased to 85 percent of the ad libitum valve and kept there during the study. Room temperature was maintained at 22+2°C, and the room was illuminated from 7:00 to 19:00.

During the S^+ time (room light blight), the rats could get the food by tapping the bar at a variable interval up to 15 seconds (VI-15). During the S^- time (room light dark), they could get no food even if they tapped the bar. One session, having 20 S^+ and S^- in a Gellermann roll, was done each day. A total of 30 sessions were carried out continuously. Correct response (R^+): number of bar taps during S^+; incorrect response (R^-): number of bar taps during S^-. Correct response ratio was expressed as $[R^+/(R^+ + R^-)]x100$. We conclude that rats completed this study when an 85 percent correct response ratio was maintained for 3 consecutive days.

(3) Methods of mesurement of the neurotransmitter

Monoamines were detected by HPLC-ECD; and ChAT (Choline Acetyltransferase) and AchE (Acetylcholine Esterase) were measured by the methods of Rossive and Ellmans respectively.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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RESULTS

As shown in Fig.1, R^+ (correct responses) of the WKY control group was 82 for the first session, 188 for the 10th, 210 for the 20th, and 250-260 for later ones. R^- (incorrect responses) was 88 for the first session, increased to 120 at the 10th session, and gradually decreased to 60 thereafter. Total response (R^++R^-) gradually increased to 300 by the 10th session and remained above that number in following sessions. The correct response ratio was 85% on the 20th session and kept this value through the 30th session.

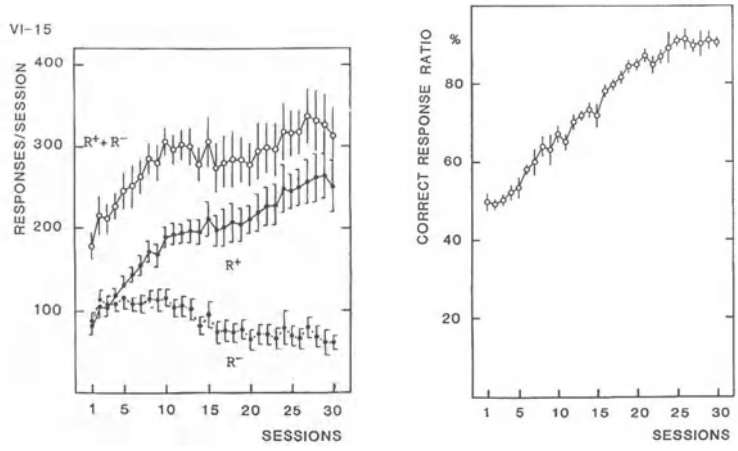

Fig-1. WKY Control Group

In the Alzheimer model group, R^+ was 53 for the first session, 125 for the 10th, 148 for the 20th, and 140-170 thereafter. On the other hand, R^- showed a value of 59 for the first session, 71 for the 10th, and 77 for the 20th session; and it did not decrease during following sessions. R^++R^- gradually increased to 192 at the 5th session but did not increase much further thereafter. The correct response ratio of the Alzheimer model group was 60% on the 6th session and did not improve after this session. (Fig-2)

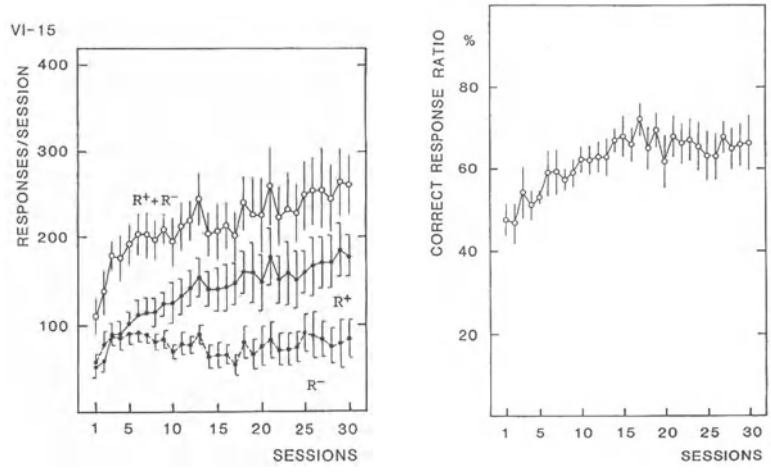

Fig-2. Alzheimer Model Group

Brain wet weight decreased in the left striatum and cerebellum in the Alzheimer model compared with the corresponding weights in the control group (Table 1).

Table 1. Brain Wet Weight

M:Medulla oblongata, Ce:Cerebellum, Str:Striatum, AC:anterior cortex
Hi:Hippocampus, PC:posterior cortex, Br-stem:brainstem

	M	Ce	Str(R)	Str(L)	AC(R)	AC(L)	Hi(R)	Hi(L)	PC(R)	PC(L)	Br-stem
control n=7	0.235 ±0.025	0.275 ±0.015	0.042 ±0.012	0.051 ±0.011	0.148 ±0.022	0.159 ±0.023	0.072 ±0.008	0.072 ±0.004	0.236 ±0.026	0.233 ±0.021	0.297 ±0.018
ALZHEIMER MODEL n=8	0.232 ±0.032	0.240 ±0.025 **	0.036 ±0.012	0.038 ±0.011 *	0.136 ±0.019	0.135 ±0.024	0.073 ±0.010	0.073 ±0.011	0.215 ±0.031	0.203 ±0.035	0.286 ±0.033

(gr.)

* P<0.05
** P<0.01

As shown in Table 2, choline acetyltransferase (ChAT) was decreased in the left anterior cortex ($p<0.05$), left hippocampus ($p<0.05$), and right posterior cortex ($p<0.05$) in the Alzheimer model.

Whereas acetylcholine esterase was decreased in the left anterior cortex ($p<0.05$) only (Table 3).

Table 2. ChAT Content (nmol/mg)

	M	Ce	Str(R)	Str(L)	AC(R)	AC(L)	Hi(R)	Hi(L)	PC(R)	PC(L)	Br-stem
control n=4	30.42 ±18.08	4.77 ±0.24	80.71 ±10.52	72.35 ±22.06	47.48 ±24.23	32.30 ±21.21	34.54 ±21.21	96.37 ±23.38	28.86 ±9.91	39.76 ±25.01	19.52 ±15.54
ALZHEIMER MODEL n=5	14.14 ±7.78	3.33 ±1.77	41.35 ±33.08	49.44 ±23.70	22.45 ±9,23	21.11 ±8.28 *	49.50 ±32.37	41.06 ±21.69 *	14.84 ±7.13 *	20.88 ±14.25	8.19 ±2.34

* P<0.05

Table 3. Ach. E Content (nmoles/mg)

	M	Ce	Str(R)	Str(L)	AC(R)	AC(L)	Hi(R)	Hi(L)	PC(R)	PC(L)	Br-stem
control n=4	6.6 ±4.2	7.0 ±7.2	32.1 ±3.9	30.0 ±4.8	13.9 ±5.6	14.5 ±3.5	12.8 ±11.6	22.7 ±7.4	13.6 ±14.9	11.9 ±5.7	4.8 ±3.5
ALZHEIMER MODEL n=5	3.2 ±2.2	2.9 ±2.3	47.0 ±24.2	37.8 ±10.3	15.1 ±6.6	7.8 ±3.1 *	11.8 ±8.4	14.5 ±11.9	6.6 ±3.0	7.9 ±4.6	2.8 ±0.9

* P<0.05

Table 4. Contents of Monoammine (ng/mg protein) in the Left Striatum

	NE	DOPAC	DA	HVA	3MT	5HT	5HIAA
control n=5	2.0 ±1.1	4.5 ±2.2	15.8 ±3.1	5.1 ±0.8	2.0 ±0.5	0.9 ±0.4	51.4 ±17.8
ALZHEIMER MODEL n=5	3.9 ±3.2	4.4 ±3.1	36.3 ±4.4 **	7.9 ±2.0 *	3.6 ±1.0 *	1.2 ±0.7	76.5 ±14.4

* P<0.05
** P<0.01

Table 5. Contents of Monoamines (ng/mg-protein) in the Right Striatum

	NE	DOPAC	DA	HVA	3MT	5HT	5HIAA
control n=5	2.1 ±0.7	5.1 ±2.1	18.4 ±5.9	4.6 ±0.3	4.5 ±1.8	1.8 ±0.9	46.0 ±17.8
ALZHEIMER MODEL n=5	3.5 ±1.0	3.4 ±3.0	35.4 ±12.8 *	7.8 ±2.2	5.4 ±2.1	3.0 ±1.7	73.0 ±20.1

* P<0.05

In the striatum, dopamine ($p<0.01$), HVA ($p<0.05$), and 3MT ($p<0.05$) were increased on left side and dopamine was increased on the right side (Tables 4,5).

DISCUSSION

There have been many reports on experimentl studies of dementia, but we have never seen any paper on an operant study using the nbM-lesioned rat. We made the Alzheimer model rat according Miyamoto. The alzheimer model showed a remarkable decrease in the activity of movements ($p<0.001$), correct responses ($p<0.001$), and correct response ratio ($p<0.001$). The operant study results were very similar to the clinical findings made in Alzheimer disease patients.

Neurotransmitters, especially ChAT and AChE, were decreased in this Alzheimer model. ChAT was decreased in the left anterior cortex, hippocampus, and right posterior cortex, but it did not decrease in the entire brain as in Alzheimer patients. AChE was also decreased in the left anterior cortex.

Our study has thus shown that (1) deterioration of cholinergic neurons occurred in the Alzheimer model, but not in the entire brain, (2) monoamines did not decrease in the model but rather increased, although reports on autopsied Alzheimer brain showed them to decrease, (3) the operant study gave results very similar to the clinical features of the Alzheimer patient.

CONCLUSIONS

The disruption of the nbM caused a decrease in activity of movements and memory disturbance, which is very highly related to the decrease in the number of cholinergic neurons known to occur in Alzheimer's disease. We think this model is very useful to study Alzheimer's disease.

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CHARACTERISTIC OF LEARNING DEFICIT INDUCED BY IBOTENIC ACID LESION OF THE FRONTAL CORTEX RELATED WITH THE NUCLEUS BASALIS OF MEYNERT IN RATS

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INTRODUCTION

The final goal of this study is to develop an animal model of Alzheimer's disease (AD) for preclinical evaluation of therapeutic drugs. AD has classically been defined as a progressive dementia accompanied by characteristic neuropathological changes such as senile plaques and neurofibrillary tangles, both of which are hallmarks of AD. However, no animals having these neuropathological characteristics exist. On the other hand, the memory dysfunction of AD has been associated with a cortical cholinergic deficiency (DeFeudis, 1988). Therefore, memory dysfunction based on nerve cell loss of the cortex in animals may be adequate as an animal model of AD.

The cholinergic component of AD can be modeled in the rat by ibotenic acid (IB) lesioning of the nucleus basalis of Meynert (NBM). IB lesions in the NBM of the rat reduce choline acetyltransferase (ChAT) activity in the dorsolateral frontal cortex (DFC), medial prefrontal cortex (MPC), and parieto-temporal cortex (Mayo et al., 1984). Although the NBM lesion has been reported to induce learning impairment in animals, the relationship between memory function and these cortex regions having cholinergic input from the NBM is not yet clear.

Our previous study indicated that IB lesions of the DFC and MPC impaired retention of discrimination avoidance learning (DAL) in rats similar to that of the NBM lesion (Hara et al., 1989). In addition, the DFC and MPC lesions reduced glutamic acid decarboxylase (GAD) activity in the cortex, but the NBM lesion did not. In contrast, the NBM lesion reduced ChAT activity in the cortex, but the DFC and MPC lesions did not. Therefore, these results suggest that memory deficit resulting from nerve cell loss of the frontal cortex induced by IB may be based on the GABAergic neural system.

The present study investigated effects of DM9384, a new pyrrolidone derivative that activates ChAT activity (Kawajiri et al., 1988) and improves learning impairment induced by GABA antagonists (Nabeshima et al., 1988), and tetrahydroaminoacridine (THA), a centrally acting cholinesterase inhibitor (Heibronn, 1961), on retention impairment of DAL induced by IB lesioning of the frontal cortex having cholinergic input from the NBM versus those on the impairment induced by IB lesioning of the NBM.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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MATERIALS AND METHODS

Male Wistar strain rats (8 weeks old) were used. The animals were daily trained DAL in a two-compartment shuttle box to discriminate the positive conditioning stimulus (PCS) from the negative one (NCS) to avoid foot shock. The conditioning stimuli used were two pure tones with different frequencies. The avoidance response, that the animals moved into the opposite compartment during the PCS prior to foot shock, was recorded as the correct response (CR). The transfer to the opposite compartment during the NCS was regarded as the false response (FR). The NCS was not followed by foot shock. Transfer between compartments in the shuttle box during the intertrial period was recorded as a spontaneous response (SR). Following the daily training of DAL consisting of 20 trials of PCS and 20 trials of NCS, only the rats that showed both over 80% CR and under 20% FR were subjected to the brain lesioning by IB. IB (7.5 μg/0.75μl for 3 min) was injected bilaterally into the DFC or MPC, or unilaterally into the NBM according to the brain atlas of Paxinos and Watson (1986). Animals in the sham-operated group were injected with saline. On Day-7 and 14 after the surgery, the retention test with the 40 trials was loaded. From Day-14 after surgery, only the rats with the retention impairment of DAL were administered DM9384 (Daiichi Seiyaku, Japan, 10 mg/kg, p.o.) or THA (0.5 mg/kg, i.p.) daily for 1 week. DM9384 was suspended with 0.5% CMC, and THA was dissolved in saline. On the test day, DM9384 and THA were treated 1 hr and 30 min before the retention test, respectively. One week after the final drug dose, the retention was re-tested. After termination of the experiment, the lesioned site was verified histologically. The statistical evaluation was performed by ANOVA followed by Scheffe's test.

RESULTS

Sham-operated groups clearly showed the retention of DAL for 4 weeks after the surgery (Figs. 1-3). The DFC-lesioned rats showed retention impairment within 2 weeks after the surgery, as seen from the reduced CR's and increased FR's (Fig. 1) similar to the NBM-lesioned rats (Fig. 3). The MPC-lesioned rats showed the retention impairment accompanied by increased FR's without a marked change in CR's within 2 weeks after the surgery (Fig. 2). DM9384 and THA improved the retention impairments induced by the DFC, MPC, and NBM lesions (Figs. 1-3). The improvement by DM3997 was retained even 1 week after cessation of drug treatment. That by THA was also retained, except in case of the MPC lesion.

DISCUSSION

In the present study, the memory deficit based on nerve cell loss of the frontal cortex regions having cholinergic input from the NBM was improved by DM9384 and THA. IB lesioning of the frontal cortex induces reduction in GAD activity, but not in ChAT activity, in the cortex (Hara et al., 1989) similar to the kainic acid lesion (Lehmann et al., 1980). On the other hand, THA enhances central cholinergic functions by its cholinesterase-inhibiting action (Heibronn, 1961). DM9384 not only activates ChAT activity (Kawajiri et al., 1988), but also GAD activity in the cortex (Watanabe et al., 1989). In addition, DM9384 increases the turnover of GABA and acetylcholine in the cortex (Watanabe et al., 1989). These effects of DM9384 also have been reported in this conference. Therefore, the results of the present study suggest that memory dysfunction based on nerve cell loss in the cortex implicates the GABAergic, as well as cholinergic, neural system, and, in addition, that DM9384 may be applicable for therapy of AD.

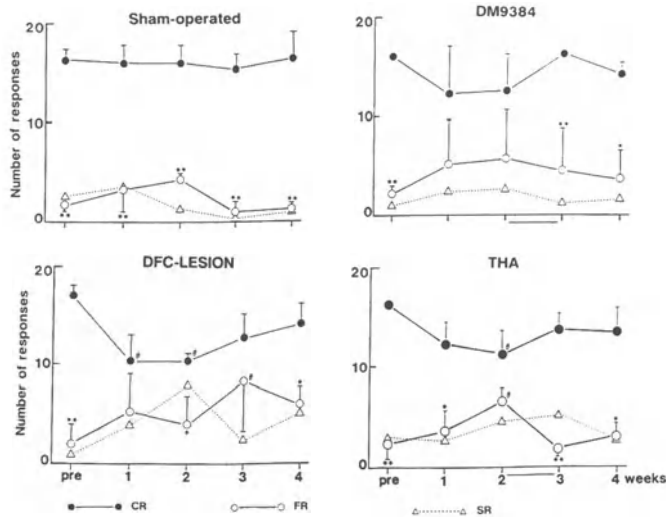

Fig. 1. Effects of DM9384 and THA on retention impairment of discrimination avoidance learning (DAL) induced by ibotenic acid (IB) lesioning of the dorsolateral frontal cortex (DFC) in rats. DM9384 (10 mg/kg, p.o.) and THA (0.5 mg/kg, i.p.) were administered daily for 7 days from 2 weeks after the surgery. Each group consists of 4 animals. *p<0.05,**p<0.01: statistical difference from correct response (Scheffe's test). θp<0.05: statistical difference from sham-operated group (Scheffe's test).

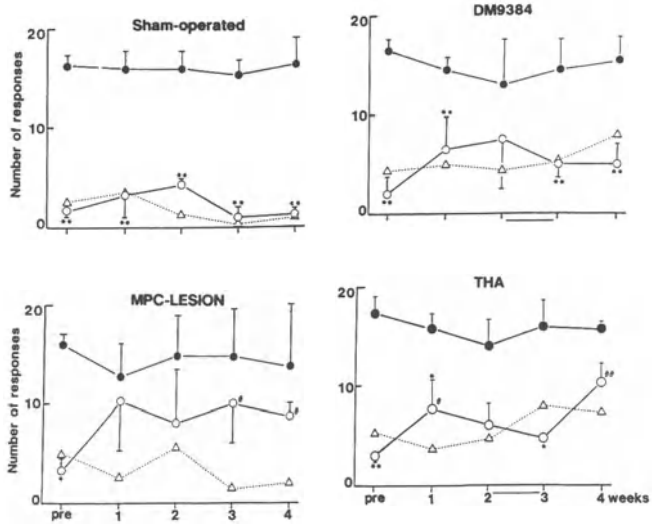

Fig. 2. Effects of DM9384 and THA on retention impairment of DAL induced by IB lesioning of the medial prefrontal cortex (MPC). Each group consists of 4 animals. Abbreviations are as in Fig. 1. *p<0.05,**p<0.01: statistical difference from corect response (Scheffe's test). θp<0.05, θθp<0.01: statistical difference from sham-operated group (Scheffe's test).

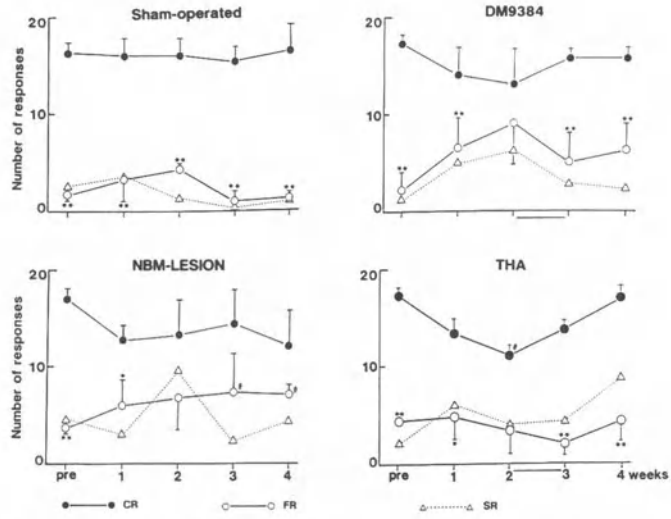

Fig. 3. Effects of DM9384 and THA on retention impairment of DAL induced by IB lesioning of the unilateral nucleus basalis of Meynert (NBM). Each group consists of 4 animals. Abbreviations are as in Fig. 1. *p<0.05,**p<0.01: statistical difference from correct response (Scheffe's test). θp<0.05: statistical difference from sham-operated group (Scheffe's test).

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A NEW ANIMAL MODEL OF ALZHEIMER'S DISEASE BY SELECTIVE DESTRUCTION OF THE CHOLINERGIC NEURONS IN THE BASAL FOREBRAIN

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INTRODUCTION

In Alzheimer's disease (AD), several neurochemical and pathological changes have been reported. Among them, the decrease of acetylcholine in the cerebral cortex and selective degeneration of the basal magnocellular nucleus of Meynert(BMN), sending cholinergic projections to the cortex, were the most conspicuous findings (Davies and Maloney, 1976; Whitehouse et al., 1982; Friedman et al., 1983; Beatty et al., 1986; Mesulam, 1986). In order to analyze the pathogenic mechanism(s) of AD, various attempts to produce an animal model of AD have been made (Coyle and Schwarcz, 1983; Smith, 1988). The neurons in the BMN have been destroyed by electrical means and by injection of axon-sparing neurotoxins such as kainic acid and ibotenic acid. However, not only cholinergic neurons but also noncholinergic neurons in the BMN are destroyed by these methods. In addition, by the electric destruction a number of cholinergic or non-cholinergic passing fibers are also destroyed. Quinolic acid, an endogenous neurotoxin, has the same drawback as the other neurotoxins. Sofroniew and Pearson(1985) reported the transneural retrograde degeneration of BMN neurons by topical application of kainic acid or N-methyl-D-aspartic acid on the cortex. The events were specific neither to the cholinergic system nor to kainic acid. On the other hand, injection of ethylcholine mustard aziridinium ion (AF 64A), a specific cholinotoxin, was made in order to produce an animal model of AD (Walh et al., 1984). However, because AF64A affects all the cholinergic neurons, it could also affect passing cholinergic fibers in the BMN when applied. Furthermore, the possibility that AF64A has an affect on noncholinergic systems should be considered. Accordingly, an attempt to develop a method that selectively destroys the cholinergic neuron in the BMN is needed.

Recent investigations have shown the presence of nerve growth factor (NGF) in the cerebral cortex (Korsching et al., 1985; Whitemore et al., 1986) and NGF receptors in the BMN (Raivich and Kreutzberg, 1987; Woolf et al., 1989). The NGF is suggested to be incorporated into NGF receptor-bearing axon terminals and then retrogradelly transported to cholinergic neurons in the BMN . In fact, isotope-labeled NGF exogenously applied into the cerebral cortex is specifically accumulated in the cholinergic soma of the basal forebrain (Stoeckel and Thoenen, 1975; Seiler and Schwab, 1984). This finding allows us to selectively kill the cholinergic neurons in basal forebrain if NGF is conjugated to some toxic substance whose effect begins when it enters the cytoplasm. In the present study, based upon this

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strategy, we attempted to make an animal model of AD in which the basal forebrain cholinergic neurons is selectively destroyed.

PREPARATION OF NGF AND DIPHTHERIA TOXIN CONJUGATE(NGDT)

Fifteen microliters of 2.5S NGF dissolved at lmg/ml in phosphate-buffered saline (PBS) (Collaborative Res., U.S.A. or Seikagaku Kogyo Co., Japan) was conjugated to 40 µl of diphtheria toxin PBS solution (1 mg/ml) with the use of 15 µl of 1 ethyl-3-(3-L-dimethylaminopropyl) carbodiimide-HCl(140 mg/ml). The conjugate was dialyzed against PBS. NGDT was checked with SDS-polyacrylamide gel electrophoresis and silver staining (Nakarai Chemical Co., Japan). Protein concentration was measured with a protein assay kit from Bio-Rad (U.S.A.)

INJECTION OF NGDT INTO CEREBRAL CORTEX OF THE RAT OR MOUSE

A volume of 0.5µl of NGDT (0.5 µg) solution was stereotaxically injected into 1-3 different parts of the parietal cerebral cortex (0.5-1.0 mm depth) with a Hamilton syringe under pentobarbital anesthesia. In other animals, approximately the same amount of diphtheria toxin, NGF, or cytochrome C,the latter of which has almost the same molecular weight as NGF was injected as the control. One, 3, and 7 days later, the animals were perfused transcardially with saline followed by Zamboni's fixative. The brains were removed and examined immunocytochemically for the cholinergic marker choline acetyltransferase (ChAT). In addition, in order to test the effect of NGDT on catecholamine neurons and peptidergic neurons, tyrosine hydroxylase immunoreactivity, a catecholaminergic marker, in the locus coeruleus and somatostatin and a neuropeptide Y immunoreactivities in the cerebral cortex were checked. NGF and cytochrome C injection resulted in no decrease in ChAT immunoreactivity in the BMN. Diphtheria toxin was highly toxic and almost all of the cells on the injected side of the cerebral cortex degenerated.

In the animals that were killed one day after the injections of NGDT into the cerebral cortex, no significant changes in ChAT-like immunoreactive neurons were identified in the brain. Three and 7 days after the injections, a remarkable decrease in the number of ChAT-like immunoreactive neurons were found in the BMN and in the horizontal limb of the nucleus of diagonal band (DB)(Fig. 1A,B). However, no decrease in the number of ChAT-like immunoreactive neurons was detected in the septal region and vertical limb of the DB which contains NGF receptors but does not project to the parietal region of the cortex. Also no decrease was detected in the number of ChAT-like immunoreactive neurons in the laterodorsal tegmental nucleus, which projects to the cerebral cortex, and in the intrinsic cortical neurons and other cholinergic neurons (Kudo et al., 1989).

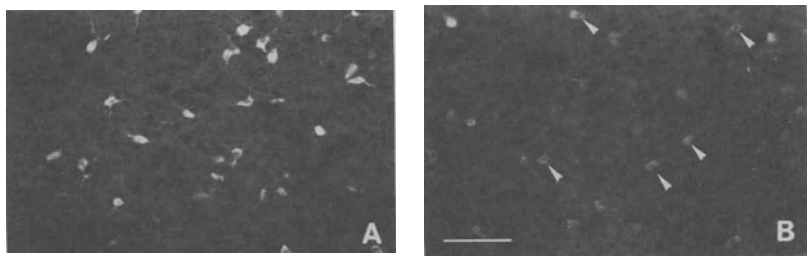

Fig. 1 Fluorescence micrographs showing the change in ChATi cells in the horizontal limb of the diagonal band after injection of NGF-diphtheria toxin conjugate into the unilateral cerebral cortex of the rat. Control(A) and Injection side(B). Bar=100 µm. Arrowheads show faintly labeled ChATi neurons.

CHOLINE ACETYLTRANSFERASE(CHAT) ACTIVITY IN THE NGDT-INJECTED ANIMAL

ChAT activity was determined according to Fonnum (1975) and compared between bilaterally NGDT-injected and saline-injected groups. ChAT activity in the cerebral cortex was approximately 10 % decreased ($P<0.05$) in the NGDT-injected group compared with the saline-injected one (data not shown).

PASSIVE AVOIDANCE LEARNING TEST

Experiment 1

DDY mice bilaterally injected with NGDT were used in the experiments. The testing procedure was described elsewhere (Friedman et al., 1983; Beatty et al., 1986). Briefly, NGDT- and saline (control)- injected mice were habituated to a two-chamber test apparatus so that upon being placed in the light compartment they would promptly enter the dark compartment. A three-sec., 0.5 mA electric shock(AC) was then delivered via a metal grid floor when the animal entered the dark chamber. Normal mice are inhibited from re-entry into the dark compartment during subsequent placement in the bright compartment. Latency to re-enter the dark in immediately subsequent tests provides a measure of task acquisition; and when measured 8 days later it provides a measure of retention of the inhibitory avoidance. The means of the latency before animals entered the dark during the retention tests were compared between NGDT- and saline-injected groups by use of Mann-Whiteney's U-test (two tails). For the analysis of latency periods of longer than 300 sec., Fischer's exact probability test was employed.

A predetermined latency of 120 sec. was established as the criterion for acquisition. The normal control group learned the avoidance after 1.28 ± 0.084 trials (mean ± S.E.M.), whereas more trials (1.45 ± 0.106) were required for the bilaterally NGDT-injected group. Retention was tested 8 days later. When entrance of the upper body into the dark (upper body criterion) was used as the criterion, 48.3% of the control mice did not re-enter for more than 300 sec., whereas the value for the NGDT-injected mice was only 17.2%($P<0.05$). When entrance of whole body was used as the criterion (whole body criterion), the percentages of entrance to the dark were 82.8% for the control and 65.5% for the NGDT-injected group. Upper body latency (the latency by upper body criterion) and whole body latency (the latency by whole body criterion) were 231.3 ± 14.4 sec. (N=29) and 285.0 ± 7.9 sec. (N=29),respectively, in the control animal, while they were 151.9 ± 16.9 sec. (N=27) and 256.2 ± 14.5 (N=27),respectively, in the treated animals. The upper body latency was significantly lower in the experimental group ($P<0.001$).

Experiment 2

In this experiment, NGDT was administered immediately after the acquisition trial, and 8 days later retention was tested in the same manner as in Experiment 1. Upper body and whole body latencies of the control were 179.2± 22.7 sec. (N=26) and 252.3 ± 15.3 sec. (N=26), respectively, while the corresponding values of 105.0 ± 20.7 sec.(N=26) and 192.1 ± 19.8 (N=26) were found for the NGDT-injected group. In both criteria, the NGDT-injected group showed significantly shorter latency than the control group ($P<0.01$, $P<0.05$, respectively).

In conclusion, our present study showed that cholinergic neurons in the basal forebrain which project to cerebral cortex are closely involved with acquisition and retention of memory. Furthermore, this animal model outlined here was shown to be very useful for analysis of the pathogenesis of Alzheimer's disease.

ACKNOWLEGMENT

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IMPAIRMENT OF AVOIDANCE BEHAVIOR PRODUCED BY DESTRUCTION OF THE CHOLINERGIC PROJECTION FROM THE PEDUNCULOPONTINE NUCLEUS TO THE MEDIAL THALAMUS

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INTRODUCTION

The pedunculopontine nucleus (PPN) is located bilaterally in the lower midbrain and pons. The larger PPN neurons utilize acetylcholine as a neurotransmitter (Kimura et al., 1981). The major ascending efferent fibers of the PPN project to the medial thalamus (Med Thal), substantia nigra, subthalamic nucleus, and almost all parts of the diencephalon and the limbic system, while other efferent fibers descend to the brainstem reticular formation and spinal cord (Niijima and Yoshida, 1988). Although the function of the PPN is not yet clearly understood, it is thought to modulate the sleep cycle and consciousness level by its cholinergic excitatory action. The PPN might also activate the midbrain dopamine neurons in the pars compacta of the substantia nigra and ventral tegmental area. Also, the nucleus is thought to be one of the locomotor centers (Fujimoto et al., 1989).

In Alzheimer's disease, the well-known cholinergic neurons of the basal forebrain undergo selective degeneration, however, severe pathological changes are also seen in the PPN (Mufson et al., 1988). It may point to a possibility that PPN is involved in memory mechanisms which are impaired in Alzheimer's disease.

We lesioned the PPN by local injection of ibotenic acid. We also lesioned the Med Thal, which receives cholinergic fibers from the PPN, by local injection of AF64A. We examined behaviors in these rats and assayed choline acetyltransferase (ChAT) activity in various parts of the brain.

MATERIALS AND METHODS

Forty-nine adult male Wistar rats were used for passive and active avoidance tests, and 20 adult male Wistar-Imamichi rats were used for a radial maze test. Under anesthesia with sodium pentobarbital, bilateral lesions of the PPN were made by stereotaxic injections of ibotenic acid (IBO) (8 nmol/0.5µl/side). Control rats underwent similar surgical procedures except that saline was injected instead of IBO. The solution of AF64A (1 nmol/0.5µl/side) was injected into the bilateral Med Thal. Control rats were injected identically with saline.

Fourteen days after the injection, each rat was tested for passive avoidance responses by a two-compartment step-through passive avoidance

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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apparatus. For the initial training, each rat was placed in the illuminated chamber and the door was opened, thus allowing access to the dark chamber. The latency between the door opening and the entrance into the dark chamber was measured. As soon as the rat entered the dark chamber, an electric shock was delivered to the floor for 2 sec. The training was terminated when the rat went back into the illuminated chamber. Twenty-four hours after the initial training, each rat was tested for retention of memory of the adverse experience. The rat was placed again in the illuminated chamber and the door was opened and the latency period was recorded. When the rat remained in the illuminated chamber for more than 300 sec, the test was terminated.

Eighteen days following the injection, each rat was tested for active avoidance responses by a two-way shuttle box. A buzzer and light attached to the ceiling were used to present the conditioned stimuli. The unconditioned stimulus was an electric foot shock (maximum 3 sec). If the rat ran to the opposite compartment during application of the conditioned stimuli (for 5 sec), the rat thus avoided the electric shock, and such behavior was recorded as an avoidance response. One hundred and twenty trials were given daily at an interval of 25 sec for 5 days.

Only the PPN-lesioned rats and their controls were tested for spatial memory performance by an 8-arm radial maze test. Food deprivation was begun one week prior to testing. At the start of each session, food was placed in the end of each arm. Each rat was placed on the central platform and all doors were raised allowing access to any of the arms. When a rat entered an arm which it had not yet entered before and ate the food, this was defined as a correct choice. However, if the rat reentered an arm which it had already selected, this was defined as an error choice. When the rat had eaten all the food or 10 min had elapsed, the session was terminated. Training was performed at the rate of one session per day.

On the 23rd day after injection, 9 PPN-lesioned, 10 Med Thal-lesioned, and 10 sham-operated Wistar rats were selected randomly, anesthetized with ether, and sacrificed by decapitation. The brains were frozen rapidly and samples were dissected out with a razor blade. After determination of wet weight, the samples were homogenized and the ChAT activities were assayed by a radiochemical method.

The location and size of the PPN lesions were stained by the Klüver-Barrera method and histologically examined.

RESULTS

IBO-induced lesions were located within the PPN in all rats histologically examined, as shown in Fig. 1.

In the initial trial of passive avoidance, the sham and the PPN-lesioned rats placed in the illuminated chamber entered almost immediately into the dark chamber (Table 1-A). No statistical difference between the 2 groups was observed. After 24 h the rats were tested again. In the sham-operated rats the mean latency before entering the dark chamber was 300 sec or more; whereas in the PPN-lesioned rats, it was significantly shorter, being 198±116 sec. The Med Thal-lesioned rats were also tested, and the results (Table 1-B) were quite similar to those of the PPN-lesioned rats.

The results of active avoidance tests are illustrated in Fig. 2 and 3. The avoidance rate indicates number of avoidance response/20 trials. The points in Fig.2 and 3 represent the mean avoidance rates obtained for each of six 20-trial sequences. In both groups of sham-operated rats, the avoidance rate in the 1st 20-trial sequence was lower than 20% on the 1st day and increased day by day, reaching 60% on the 5th day and finally higher than 80% in the last 20-trial sequence of the 5th day.

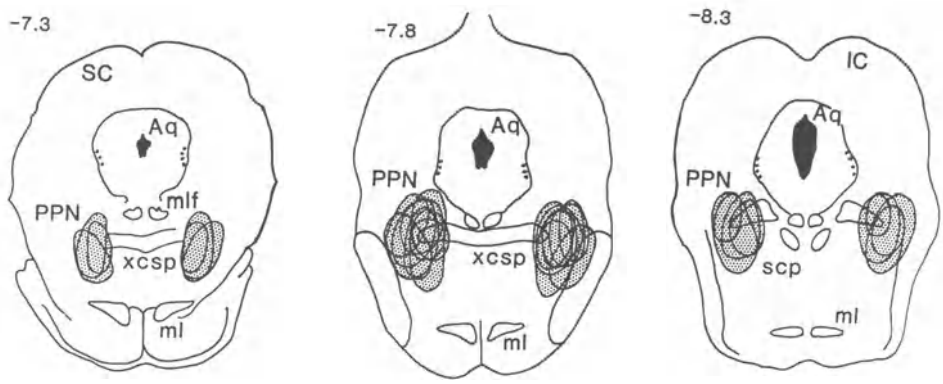

Fig. 1. Brainstem regions where cell loss or gliosis due to toxic effect of ibotenic acid are enclosed and shaded. Number of each section indicates distance in mm posterior to bregma. Aq, aqueduct; IC, inferior colliculus; ml, median lemniscus; mlf, medial longitudinal fasciculus; PPN, pedunculopontine nucleus; SC, superior colliculus; scp, superior cerebellar peduncle; xcsp, decussation of superior cerebellar peduncle.

Table 1. Passive Avoidance of PPN- and Med Thal-lesioned rats

A) PPN-lesioned Rats					B) Med Thal-lesioned Rats			
Initial Trial		Test Trial			Initial Trial		Test Trial	
Sham	Lesion	Sham	Lesion		Sham	Lesion	Sham	Lesion
18±10	18±8	300	198±116	Latency to Enter Dark Chamber(sec)	23±14	28±22	296±15	182±100
ns		**			ns		**	

Latencies for sham and lesioned rats to enter the dark chamber in passive avoidance task. Maximum latency was set at 300 sec. mean±SD ns: not significant **: $p<0.01$, Student's t-test.

In contrast, in both the PPN-lesioned and the Med Thal-lesioned rats, the avoidance rate always remained below 40% for all 20-trial sequences in the 1st as well as all of the subsequent experimental days. There were statistically significant differences (ANOVA, $P<0.01$) between the lesioned and sham-operated rats in the 1st and every consecutive day.

In the radial maze test, on the first experimental day, the mean number of correct choices out of the first 8 choices was about 2.6 in both sham and PPN-lesioned rats. They began to choose correctly day by day. It took 7.7±5.6 days for sham-operated rats and 7.1±2.9 days for PPN-lesioned rats respectively to achieve the criterion, which was to chose 7 different arms in the first 8 choices, 5 days in succession. There was no statistical differences between sham and lesioned rats, in both the acquisition curve (Two-way ANOVA) and the days to achieve the criterion (Student's t-test).

The results of ChAT activities are shown in Table 2. In the PPN-lesioned rats, the ChAT activity of the Med Thal decreased about 20% compared with that of the sham-operated rats ($p<0.01$, Student's t-test), while the activities in the lateral thalamus, hippocampus, and cortex showed no significant differences between the two groups. In the Med

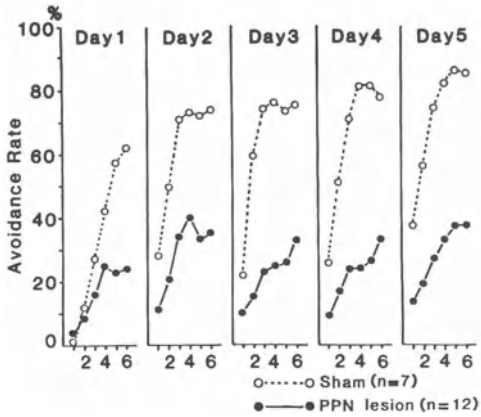

Fig. 2. Acquisition of Active Avoidance in PPN-lesioned rats.

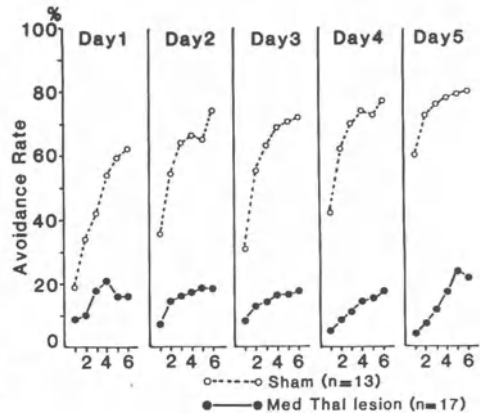

Fig. 3. Acquisition of Active Avoidance in Med Thal-lesioned rats.

Table 2. Choline Acetyltransferase Activity (μmol/g/h)

	Sham	PPN-lesion	Med Thal-lesion
Lateral Thalamus	4.71±0.62	4.15±1.00	3.78±0.30*
Medial Thalamus	16.24±1.97	12.48±2.40**	6.53±1.40**
Hippocampus	6.62±1.80	6.26±1.90	5.88±1.56
Cortex	1.90±0.18	1.91±0.48	1.96±0.26

ChAT activities assayed on the 23rd day after injection.
mean±SD, *:$p<0.05$, **:$p<0.01$, Student's t-test

Thal-lesioned rats, the decrease in the ChAT activity in the Med Thal was even greater, 60% ($p<0.01$, Student's t-test). However, the activities in the hippocampus and cortex remained almost the same as those of the sham-operated rats.

DISCUSSION

In this experiment, we adopted both passive and active avoidance tests and found a profound deficit in both PPN- and Med Thal-lesioned rats to perform the respective tasks. In PPN-lesioned rats, we tested the spatial memory performance, however, there was no deficit.

IBO produces a marked decrease in the number of nerve cells at the site of injection without damaging axons of passage and nerve terminals of extrinsic origin (McGeer and McGeer, 1983). Furthermore, in order to intercept the cholinergic afferents from the PPN to the Med Thal, we injected the latter with AF64A (Fisher et al., 1982).

In PPN-lesioned rats, the ChAT activity was decreased in the Med Thal, while the activity in the cortex and hippocampus remained the same. The injection of AF64A resulted in a marked decrease in the ChAT activity in the Med Thal, supporting the impairment of cholinergic terminals in this area. From these facts, we conclude that the impairment of the avoidance tasks is due to destruction of the cholinergic PPN-Med Thal projection.

The fact that only impairment of avoidance tasks but no impairment of spatial performance resulted from destruction of the cholinergic PPN-Med Thal projection is noteworthy. In rats, the spatial memory is a function of the hippocampus (O'Keefe and Nadel, 1978). There was no decrease of ChAT activity in the hippocampus in the PPN lesioned rats. In Alzheimer's disease, loss of cholinergic neurons in the basal forebrain is known to be associated with a reduction of ChAT activities in the cortex and hippocampus, but the pathological changes are not limited to the basal forebrain. Also many neurofibrillary tangles are often seen in the PPN in this disease (Mufson et al., 1988). Furthermore, severe cell loss of the PPN has been reported in patients with progressive supranuclear palsy who show dementia as well as motor disturbance (Hirsh et al., 1987; Zweig et al., 1987). Thalamic dementia due to degeneration of the medial thalamus has also been described (Squire and Moore, 1979). Our results, indicating that the cholinergic PPN-Med Thal projection plays an important role in the learning and memory processes, could well explain, at least partly, the pathophysiology of the dementia involved in the above diseases.

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IMPAIRED SPATIAL LEARNING IN RATS WITH A TRIMETHYLTIN-INDUCED HIPPOCAMPAL LESION AND THE EFFECT OF FETAL SEPTAL GRAFTING ON THE IMPAIRMENT

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INTRODUCTION

Trimethyltin (TMT), an organotin compound, is a by-product in the manufacture of dimethyltin chloride, a stabilizing agent for certain plastics. Exposure to TMT causes neuopathological changes in the limbic system of the brain (Brown et al.,1979; Bouldin et al., 1981). An accidental exposure to TMT in humans has been reported to induce a status of mental confusion with generalized epileptic seizures (Fortemps et al., 1987). Among the regions of the central nervous system in rats, the hippocampus subsequently became the focus of research in TMT neuotoxicity. The pyramidal cells of the CA3 and CA4 hippocampal subfields appear to be particularly sensitive to acute toxicity with a single dose of TMT (Dyer et al., 1982). The long-term behavioral sequelae of TMT intoxication have revealed marked hyperactivity and learning impairments (Hagan et al., 1990).

In the present study, TMT-treated rats were further examined in the water maze using place-navigation trials. In addition, fetal septal cells were grafted into the hippocampus of the TMT-treated rats, and the effect of the graft on spatial learning was evaluated.

MATERIALS AND METHODS

Male Sprague-Dawley rats were housed individually in temperature and humidity controlled rooms with free access to food and water on a 12 : 12 light : dark cycle. TMT hydroxide (ICN Biochemicals), dissolved in distilled water, was administered to each rat at the age of 6 weeks as a single peroral dose of 9 mg/kg.

Fetal septal cells were transplanted 1 week after TMT administration. Septal suspension grafts were prepared according to the method of Björklund et al. (1983). The developing septal-diagonal band area was dissected from the ventral forebrain of donor rat fetuses at 15 days of gestation (ED 15). Septal pieces from fetuses were incubated in 0.1 % trypsin solution (in sterile 0.6 % glucose-saline) for 20 min at 37°C, washed 4 times with fresh glucose-saline, and mechanically dissociated in this medium (10μl per piece) to form a milky cell suspension. Three 3-μl aliquots were injected stereotaxically into the hippocampus on each side under pentobarbital anaesthesia at the following coordinates: (1) A = +4.5 mm from the interaural line,

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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L = ±3.5 mm, V = 3.0 mm from dura; (2) A = +3.0 mm, L = ±3.7 mm, V = 3.7 mm; and (3) A = +3.0 mm, L = ±4.8 mm, V = 5.7 mm, with the incisor bar at the interaural line (Gage and Björklund, 1986). Each injection was performed over a 3-min period. The rats in the TMT+sham group received TMT followd a week later by the sham operation.

Spontaneous locomotor activity in an open field was observed for 3 min on an apparatus (80 cm in diameter) with 25 equally divided sectors. The rats were then tested in the Morris water-maze task (Morris, 1984) at 6 weeks after transplantation. The water-maze pool, filled with water maintained at 26°C, was a circular tank (147 cm in diameter) with a nonvisible escape platform. The rats were trained for 10 trials on each day (2 min/trial) for 2 consecutive days.

After completion of behavior tests, the animals were subjected to histological examination. Under pentobarbital anaesthesia, the brain was infused with heparinized saline (100 ml) and then with neutral, buffered formalin (100 ml), excised, and embedded in paraffin. Sections were mounted on glass slides and stained with hematoxylin and eosin.

RESULTS

Open field activity was scored twice at 3 and 6 weeks after the transplantation, but the number of ambulations of TMT-treated rats was not different between animals with and without septal grafts.

Water-maze performance 6 weeks after the transplantation is shown in Fig. 1. Acquisition was measured as the mean total time required to reach the hidden platform in the pool for every 5 trials in each group. TMT-treated rats without grafts (TMT-sham) required a markedly longer time than the controls [$F(1,4) = 63.255$, $p < 0.01$; analyzed by two-factor ANOVA], whereas TMT-treated rats with septal grafts improved significantly as shown

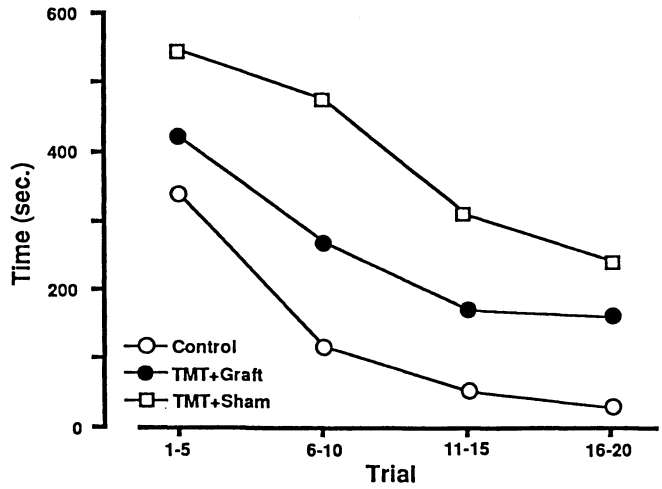

Fig.1 Time required for TMT-treated rats to reach the goal in the Morris water-maze test at 6 weeks after grafting (closed circles, TMT+graft; N=8), as compared with that for TMT-treated rats without grafts (open rectangles, TMT+sham; N=6), and that for naive rats (open circles, control; N=8).

by the shorter time to reach the goal than the TMT-sham animals [$F(1,12) = 9.638$, $p < 0.01$]. In the animals with implants, more than 90 % of the rats reached the goal from the 11th trial onward, while in the animals with only the sham operation, the value remained low (73 %) even over 20 trials [$F(1, 12) = 12.261$, $p < 0.01$]. The treatment effects [$F(1, 14) = 41.132$, $p < 0.01$] and treatment-by-trial interactions [$F(3, 42) = 5.588$, $p < 0.01$] were significant between TMT+sham operated group and the control group (data not shown).

Administration of TMT to rats resulted in marked pathological changes in the hippocampus. The reduction in whole hippocampal volume was obvious in TMT-treated animals. Microscopically, cellular loss was most evident in the CA3 and CA4 regions of the pyramidal cell layer (Fig. 2A). Surviving grafts were found in two or three implantation sites on both sides of the hippocampus in the rats receiving grafts, as shown in Fig. 2B.

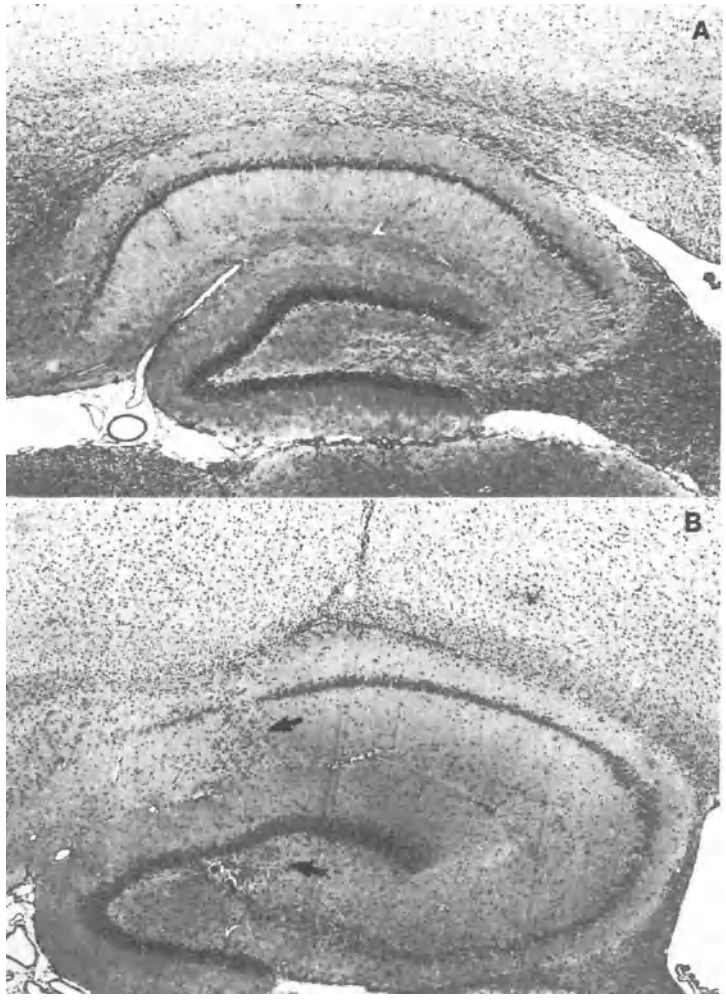

Fig. 2 (A) Brain from a TMT-treated rat. Cellular loss was evident in the CA3 and CA4 regions of the pyramidal cell layer. (B) Brain from a TMT-treated rat with grafts. The animal was sacrificed 6 weeks following the transplantation. Grafted cells are indicated by the arrows(x40).

DISCUSSION

TMT treatment induced a specific loss of hippocampal pyramidal cells in the CA3 and CA4 subfields. Bahavioral tests also revealed marked impairments on the spatial discrimination task in TMT rats, which could be detected effectively by the water maze (Morris, 1984). It is thus suggested that TMT rats with a specific hippocampal lesion, may provide a suitable functional model for the memory loss associated with TMT exposure in humans (Hagan et al., 1988).

The present results also demonstrate the ability of fetal septal grafts, rich in cholinergic neurons, to ameliorate this place-navigation deficit. The mechanism of action of the septal grafts is an intriguing issue for further investigation. A possibility proposed by Tonnaer et al. (1987) is of interest, that cerebral disorders in TMT rats may be caused by a direct action of TMT on particular cholinergic neurons in the forebrain. Fetal septal grafts have been reported to ameliorate spatial learning impairments in aged rats (Gage and Björklund, 1986); and, concerning TMT neurotoxicity, Woodruff et al. (1988) have shown that grafts containing fetal hippocampal tissue reduce activity and improve passive avoidance in TMT-exposed rats.

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SOME EFFECTS OF CNS CHOLINERGIC NEURONS ON MEMORY AND ACH RELEASE

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Summary

The aim of this study is to observe the relationship between the impairment in passive avoidance task induced in rats by the i.p. administration of muscarinic antagonists, scopolamine and methyl-scopolamine, and the changes in acetylcholine (ACh), monoamines output induced by these drugs. Initially we studied the effects of these drugs on the animals' performance of a step-through passive avoidance task. We then measured the change in ACh and monoamines levels after administration of these drugs using an in vivo brain dialysis technique. Scopolamine was effective in impairing the performance of the passive avoidance task, while methyl-scopolamine did not have clear effects on the performance of the task. With regard to ACh output, scopolamine increased ACh dose-dependently and methyl-scopolamine also affected ACh release. Scopolamine did not affect monoamines release except serotonin.

These data suggest that the accumulation of ACh in the synaptic cleft is involved in the memory deficit induced by scopolamine.

Methods

Behavioral Testing

A rat was placed in the lighted compartment of a two-compartment shuttle box. As soon as it entered the dark compartment, an electric shock, A.C.1mA/1sec, was delivered to the feet through the grid. In the retention trial following 24 hr of acquisition, 4 groups of rats received either saline, 0.02, 0.2 or 2 mg/kg scopolamine and 2 mg/kg methyl-scopolamine intraperitoneally, respectively.

Retention of the passive avoidance task was measured by replacing a rat in the lighted compartment and measuring the time latency to enter the dark compartment before drug administration (preinjection) and 1 min, 40 min and 24 hr after drug administration.

Brain Dialysis Procedure

A guide cannula was implanted in the left striatum for the subsequent insertion of a dialysis probe. Following implantation, the guide cannula was firmly fixed to the skull with two anchor screws and dental cement, and a dummy probe was inserted into the guide cannula. The dummy probe was left in the brain until the dialysis experiment was started.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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We allowed two days to pass after the operation, to avoid the effects of anesthesia, then, the dialysis probe was gently inserted into the guide cannula and was subsequently perfused with Ringer's solution containing 100 μM physostigmine at a flow rate of 2 μl/min (Damsma et al., 1987). Scopolamine and methyl-scopolamine, dissolved in 1 ml of saline, were administered intraperitoneally 3 hr from the beginning of the dialysis experiment.

Biochemical Analysis of Perfusate

The concentration of ACh and monoamines in the perfusate was determined with HPLC-ECD.

Results

Passive Avoidance Task

As snown in Fig. 1, scopolamine was effective in impairing the performance of the passive avoidance task measured either as an increase in the percentage of avoidance failure (left panel), or as a decrease in step-through latency (right panel). Scopolamine administration increased avoidance failure in a dose dependent fashion. As shown in Fig. 3, passive avoidance task was not impaired by methyl-scopolamine.

Measurements of ACh and Monoamines

Although the concentration of ACh in the perfusate obtained every 20 min differed among animals, levels became stable within 3 hr of beginning the dialysis. Data for each individual rat were normalized to the last three preinjection values, the average of which was defined as 100% (Fig. 2). It is obvious from Fig. 2 that ACh release is directly related to the amount of scopolamine injected. As shown in Fig. 4, on the other hand, the ACh levels were slightly affected by 2 mg/kg of methyl-scopolamine. The effects of 2 mg/kg methyl-scopolamine on ACh release were similar to those of 0.02 mg/kg of scopolamine. Scopolamine did not affect monoamines release except serotonine.

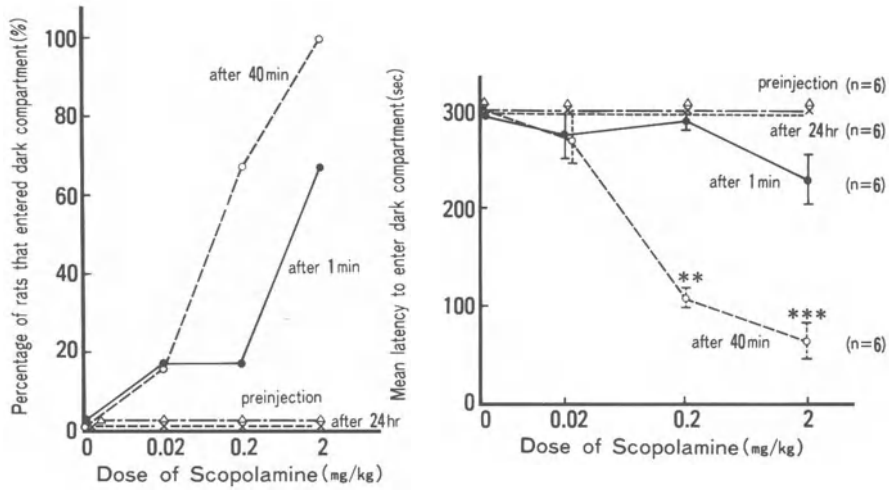

FIG. 1

Effects of scopolamine IP injection on percentage (left) and latency (right) of entering the dark compartment in retention trial.
Results in right panel are shown as mean ± S.E.M. Statistical significance of the differences was judged by Student's t-test: $^{**}p<0.01$; $^{***}p<0.001$
Number of samples examined is given in parentheses.

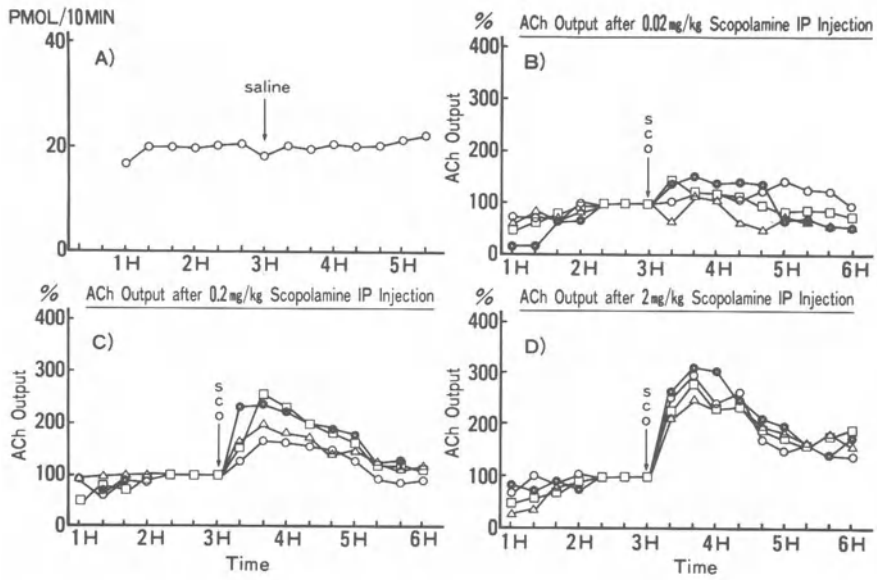

FIG. 2

Effects of scopolamine IP injection on acetylcholine output.
Panel A) shows typical time courses of acetylcholine output in one rat.
Saline did not increase acetylcholine output.

Discussion

Treatment with 2 mg/kg caused a 100% impairment in the passive avoidance task. These results agree with those reported previously (Drachman and Leavitt, 1974; Elrod and Buccafusco, 1988; Peel, 1988). However, the rats administered 2 mg/kg scopolamine became restless in our experiment. Other investigators observed that high doses of scopolamine induced agitated behavior (Ridley et al., 1984; Damsma et al., 1987; Weiner, 1985).

It is therefore difficult to judge the main reason for rats entering the dark compartment following high-dose scopolamine administration, whether it is a true memory disturbance or confusion due to an over-release of ACh. It is known that the memory disturbance in senile

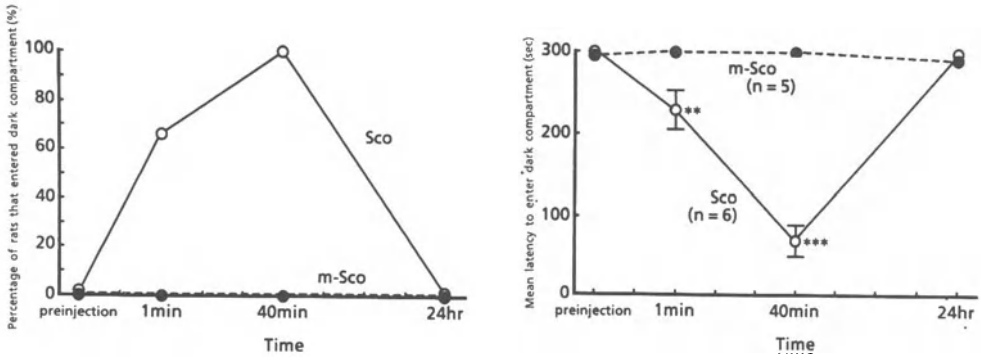

FIG. 3

Comparison of effects of scopolamine and methyl-scopolamine on percentage (left) and latency (right) of entering the dark compartment in retention trial.
Results in right panel are shown as mean±S.E.M. Statistical significance of the differences was judged by Student's t-teat : **p<0.01 ; ***p<0.001
Number of samples examined is given in parentheses.

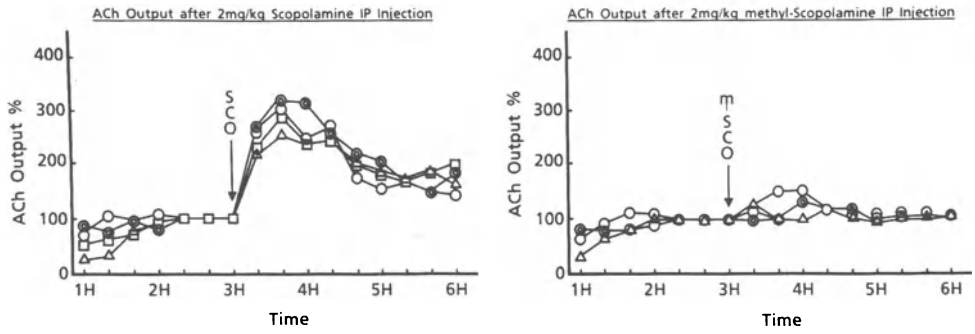

FIG. 4

Comparison of effects of scopolamine and methyl-scopolamine on acetylcholine output

dementia of Alzheimer type (SDA) patients is due to a deficiency of cholinergic neurons. In contrast, scopolamine-induced amnesia may result from an excess of ACh. There is a question as to whether one can use scopolamine amnesia as a model of SDAT without considering the dose.

The results of the present experiments propose a possibility that 1% of methyl-scopolamine can penetrate the blood-brain barrier and is effective in ACh release since the pattern and magnitude of ACh release induced with 2 mg/kg of methyl-scopolamine is similar to those induced with 0.02 mg/kg of scopolamine, although it is generally believed that methyl-scopolamine, a peripherally-acting muscarinic antagonist, does not pass through the blood-brain barrier (Hiraga and Iwasaki, 1984).

It is concluded that the dose of scopolamine appears to play an important role in the amnesia induced by this drug. The development of more selective blockers of either postsynaptic M_1 receptors or of presynaptic M_2 receptors may help us to understand the role of ACh and scopolamine in amnesia and dementia.

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CARBON MONOXIDE (CO)-INDUCED DELAYED AMNESIA AND DELAYED NEURONAL DEATH

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INTRODUCTION

Recent evidence has indicated that ischemia produces several dysfunctions in the central nervous system. For example, in the passive avoidance task, amnesia is induced 24 hours after ischemia in rats (Yasumatsu et al., 1987; Yamazaki et al., 1984). In addition, "delayed neuronal death" is caused even after the recovery from changes in biochemical and electrophysiological parameters induced by ischemic insult (Kirino, 1982; Pulsinelli et al., 1982).

Hypoxia is able to produce brain circulation failure and a deficiency in the supply of oxygen as well as ischemia. It is well known that neuropsychiatric problems may develop insidiously over the days and weeks after recovery from CO intoxication in humans (Choi, 1983; Ginsberg, 1979). Necrosis of the cerebral cortex, hippocampus, substantia nigra and globus pallidus have been discovered by anatomical study (Lapresle and Fardeau, 1967), computed tomography (Sawada et al., 1980; 1983) and magnetic resonance scanning (Horowitz et al., 1987). These results suggest that, like ischemia, CO-exposure produces deterioration of memory and neuronal death. We reported very recently carbon monoxide (CO)-induced deterioration of memory function in mice after a delay, "delayed amnesia" (Kinbara et al., 1989). Here we report a new model of hypoxic brain damage in the mouse. This model has an advantage over the ischemic model in that the method of producing hypoxia is very simple and not time consuming compared with that used to produce ischemia.

MATERIALS AND METHODS

Animals: Male mice of the ddY strain (Nihon SLC, Shizuoka, Japan), 7 weeks of age, were housed in a regulated environment (23 ±1 °C, 50±2% humidity, light on 8 a.m. to 8 p.m.) and given food and tap water *ad lib* .

Step-down type passive avoidance task: The step-down type passive avoidance task was utilized, as previously described by us (Nabeshima et al., 1988). The apparatus consisted of a transparent acrylic rectangular cage (30 ×30×40 cm high) with a grid floor and a wooden platform (4×4 ×4 cm high) in the center of the grid floor. Each mouse was placed on the wooden platform. When the mouse stepped down from the platform and placed all its paws on the grid floor, an intermittent electric footshock (1 Hz, 500 msec, 45 V, D.C.) was delivered for 15 seconds. When an animal was placed on the grid floor, the current resistance

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varied between 100 and 250 KΩ. Therefore, each animal received electric shocks in the range of 0.18-0.45 mA. The step-down latency (SDL) was measured, and the animals in the criteria range (SDL 3-15 seconds) were used for the retention test. This training criterion was also used for animals that were exposed to CO prior to training. After training, each mouse was again placed on the platform, without receiving electric footshock, and the SDL was recorded. An upper cut-off time of 300 seconds was set. When the mice were exposed to CO after training, the interval between the training and the retention test was 1, 3, 5, or 7 days. When the mice were exposed to CO before training, it was 1 day.

Carbon monoxide (CO) exposure: Each mouse was put in a transparent plastic vessel (diameter, 8.5 cm; height, 10 cm) with a pipe feeding into it. Based on a preliminary experiment, CO-exposure at the rate of more than 40 ml/min caused the animal to die and the SDL in the retention test 1 day after training in the groups exposed to CO at the rate of 35 ml/min was significantly shorter than that in the groups exposed at other rates (data not shown). Therefore, the mice were exposed to pure CO gas at a rate of 35 ml/min and then taken out of the vessel immediately after they lost their righting reflex (about 20 seconds). When the mice were exposed to CO after training, CO-exposure was carried out 1 day after training. When the mice were exposed to CO before training, CO-exposure was carried out 1, 3, 5 or 7 days before training. The mice in the control groups received the same treatment except for the CO-exposure.

Histology: Mice were fixed by an intracardiac perfusion of fixative immediately after the retention test 1, 3, 5, or 7 days after CO-exposure. Fixative (150 ml for each mouse) consisted of 3.5 % formaldehyde and 0.9 % NaCl in 0.1 M phosphate buffer (pH 7.2). Immediately after fixation, the brains were removed and kept in the same fixative. They were then cut coronally into small blocks, and these including the hippocampal area were selected. After dehydration through a graded series of alcohol, the blocks were embedded in paraffin. Coronal sections 3 μm in thickness were continually cut from the block on a microtome. The several sections of 1.5 mm in length of the hippocampal CA1 pyramidal cell layer were selected from the serial sections, since the section was approximately at the center of the CA1 pyramidal cell layer and it was 6 mm posterior to the bulbus olfactorius according to the atlas of Sidman et al.(1971). The sections were stained by the Feulgen nuclear reaction with Shiff's reagent. The number of pyramidal cells in the CA1 subfield was automatically counted with a microcomputer imaging device (BRS2, Imaging Research Inc., Canada), capturing the image through a CCD camera (MS-4030, Siera Scientific, Canada) which was attached to a light microscope device (BHT-322, Olympus, Japan).

Statistical analysis: Behavioral data were expressed in terms of median and interquartile range (Q1-Q3). The data were analyzed by 2-way analysis of variance and the Mann-Whitney U-test for multiple and two-sample comparisons, respectively. Biochemical data were expressed in terms of mean and S.E.M. and analyzed by 2-way analysis of variance and Student's t-test for multiple and two-sample comparisons, respectively.

RESULTS

Effect of CO-exposure before and after training on passive avoidance response

As shown in Fig. 1(A), CO-exposure 1 day after training affected SDL in the retention test [$F(1,232)=9.733$, $P < 0.01$]. In the retention test conducted 5 and 7 days after CO-exposure, the SDL in the CO-treated group was significantly shorter than that in the non-CO-treated group, but there was no significant difference 1 and 3 days after CO exposure. Fig. 1(B) indicates that CO-exposure before training affected SDL in the retention test 1 day after training [$F(1,112)=10.012$, $P < 0.01$]. When the mice were exposed to CO 5 and 7 days before training, in the retention test, the SDL in the CO-treated group was significantly shorter than that in the non-CO-treated group, but not when the mice were exposed to CO 1 and 3 days before training.

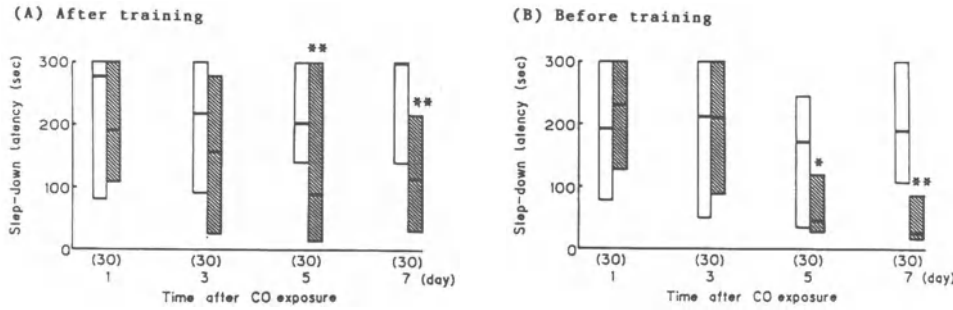

Fig. 1. Delayed amnesia induced by CO-exposure after (A) and before (B) training on the passive avoidance in mice
□: non-CO-treated group, ▨: CO-treated group. The number of animals used is shown in parenthesis. Levels of significance: * $P < 0.05$, ** $P < 0.01$ vs. corresponding non-CO-treated group.

Effect of CO-exposure on the pyramidal cells in the hippocampal CA1 subfield

As shown in Fig. 2, CO-exposure after training affected the number of pyramidal cells in the hippocampal CA1 subfield ($F(1,112)=35.429$, $P < 0.01$) . One day after CO-exposure, the number of pyramidal cells in this subfield was not significantly lower than that in the non-CO-treated group, whereas 3, 5, and 7 days after CO-exposure, it was significantly lower. The death of 17% of the pyramidal cells had occurred by 7 days after CO-exposure. Moreover, the disappearance of the lateral hippocampal CA1 pyramidal cells was noted and atrophy of the medial ones was observed. In addition, the decrement of SDL induced by CO-exposure after training had a significant relationship to the decrease in the number of hippocampal CA1 pyramidal cells (data not shown).

DISCUSSION

In the retention test of the passive avoidance task, CO-exposure after training produced amnesia after a delay of more than 5 days (delayed amnesia). CO-exposure after training also produced a decrease in the number of cells in the hippocampal CA1 subfield after a delay of more than 3 days (delayed neuronal death). Since the delayed amnesia was developed parallel to the delayed neuronal death in the hippocampal CA1 subfield, it is quite probable that the delayed neuronal death may be responsible for the delayed amnesia. That is, the neurodegeneration in the hippocampal CA1 subfield may prevent the retention and/or retrieval of memory; as a result, amnesia is produced. In addition, our results confirmed neuropsychiatric problems and the neuronal death induced by CO-exposure in human (See INTRODUCTION). Furthermore, effects of CO-exposure before training were the same as those of CO-exposure after training. Thus, we suggest that amnesia induced by CO-exposure before training is due to the neurodegeneration in the hippocampal CA1 subfield.

Systemic administration of (+)-MK-801 (0.15 mg/kg, i.p.), a noncompetitive antagonist of N-methyl-D-aspartate (NMDA) receptors, 1 hour prior to CO-exposure ameliorates delayed amnesia (Nabeshima et al., 1989). Gill et al.(1987) have reported that MK-801 protects against the degeneration of hippocampal neurons in the gerbil and rats caused by a brief period of forebrain ischemia. These facts reveal that not only the delayed amnesia but also the delayed neuronal death induced by CO-exposure may be due to an activation of NMDA receptors, since there is a close relationship between delayed amnesia and delayed neuronal death. Moreover, the process of the neuronal death induced by CO-exposure may be similar to that induced by ischemia.

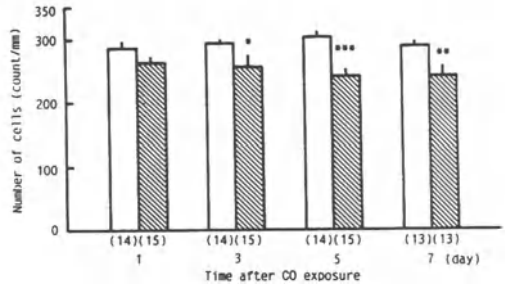

Fig. 2. Delayed neuronal death in the hippocampal CA1 subfield induced by CO-exposure after training
□: non-CO-treated group, ▨: CO-treated group. The number of samples examined is shown in parenthesis. Levels of significance: *P < 0.05, ** P < 0.01, *** P < 0.001 vs. corresponding non-CO-treated group.

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MODIFICATIONS IN THE LEVELS OF ANTIBODIES IN THE CSF OF PARKINSON'S DISEASE PATIENTS FOLLOWING ADRENAL-TO-BRAIN TRANSPLANTATION

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INTRODUCTION

A growing body of evidence suggest that the immune system participates in some neurological disease states such as multiple sclerosis, Alzheimer's and Parkinson's disease (PD) (for ref. see McRae-Degueurce et al., 1987, 1988a)

We have prevously reported that the CSF of PD patients contains immunoglobuline (IgG) species that recognize dopamin (DA)-ergic neuronal populations both in perfusion fixed adult rat brains(McRae-Degueurce et al., 1986, 1988a) and in neuronal cultures from DA cell rich areas of embryonic rat brain (Dahlström et al., 1989). We have also demonstrated that patients having the IgG in the CSF at the time of autologous adrenal medulla transplantation gradually lose the antibody in the months following surgery (McRae-Degueurce et al., 1988b). Thus, CSF samples removed from the patients at various time periods following surgery provide a means to examine effects of these IgG-species on brain cells in culture and determine its possible interaction with these cells.

In an attempt to elucidate these questions, neuronal cultures of DA-ergic cells were employed in this study. The purpose was to investigate the interaction of sequential CSF samples from patients undergoing adrenal medulla to brain transplantation with DAergic cells in culture.

MATERIAL and METHODS

DA-ergic neurons were harvested from the mesencephalic flexure of E15 fetal rats. The dissected CNS tissue was dispersed in a culture medium of nutrient mixture F 12 and MEM (1:1) with 8% inactivated calf serum, 5% glucose, insulin (5μg/ml), L-glutamine (5mM), penicillin (100 IU/ml) and streptomycin (100μg/ml). The medium was changed every 3-4 days. The cells were plated on poly-L-lysine coated cover slips and kept in sterile Petri dishes for 3 weeks. Some cultures were incubated with CSF during the last 72 hours before fixation.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
Edited by T. Nagatsu *et al.*, Plenum Press, New York, 1990

Immunocytochemistry:The cultures were fixed in 4% paraformaldehyde for 1 hour, rinsed several times in PBS, and preincubated with 2% normal serum for 30 min prior to addition of patient CSF or other antibody preparations. To study neurons rabbit-anti-neuronal specific enolase (NSE) and anti-neurofilament 150 (NF 150) antisera, alone or in mixture, were employed. DA-ergic cells were studies with rabbit-anti-DA antiserum. Secondary biotinylated antisera were applied and visualized with streptavidin coupled to FITC or to peroxidase complex (Vector Lab. Burlingame Calif. USA). A NIKON Optophot FX microscope was used both for light- and fluorescence microscopy.

CSF-incubation during culture: Our previous findings (McRae-Degueurce et al., 1988b) have demonstrated that CSF samples removed from 6 of 7 PD patients undergoing adrenal medulla transplantation produced immunocytochemical markings in the rat substantia nigra (SN) region up to one month following transplantation. Then the immunocytochemical markings slowly disappear at around 3 months when the patients showed some clinical improvement. In order to investigate the direct effects of the CSF from these transplanted patients on DA-ergic cells, the cultures were incubated for 72 hours in the presence of sterile CSF samples removed 7 days, 4 months and 1 year, post-operatively, from 3 PD patients who had received adrenal medulla transplantation at Rush Presbyterian Hospital Chicago Illinois. CSF samples from 2 of the patients produced immunocytochemical reaction in the SN up to 3 months after transplantion, whereas the CSF from one patient did not produce markage in the SN at the time of transplantation nor in the following months.

The cultures were observed daily in an inverted phase contrast microscope (NIKON Optiphot), and fixed one week after the beginning of CSF incubations.

RESULTS

There were notable differences on the effect on cultures between, on the one hand, the CSF samples that produced an immunocytochemical reaction in the rat SN and, on the other hand, the one patient samples that did not produce an immunocytochemical reaction to the SN region of the adult rat brain.

CSF samples that produced an immunocytochemical reaction in the SN: When DA cell cultures were incubated with the CSF samples removed 7 days after transplantion the cells retracted their processes and died (Fig. 1a). Dying NSE/NF-positive cells could be detected in the cell culture (Fig. 1b). Previously we have demonstrated that these 7 day CSF samples produced positive labelling of neurons in rat SN (McRae-Degueurce et al., 1988b). In cultures incubated with 4 months CSF samples both dying and alive neurons were observed, but the density of neurons in the cultures were clearly less than in control cultures. The cells incubated with CSF samples taken 1 year after transplantation displayed a survival rate similar to that of control cultures incubated with culture medium alone (Figs. 1 c, d). A large number of NSE/NF-positive cells, immunoreactive for DA, were present in these wells.

Immunonegative CSF samples: The cultures incubated in the presence of CSF samples removed from this "negative" patient 3 days, 7 days and up to 3 months following adrenal medulla transplantation did not destroy the DA ergic cells in culture. A large number of DA-positive NSE/NF-immunoreactive neurons were observed in the culture following incubations with the CSF samples removed at all of the various times following adrenal to brain transplantation.

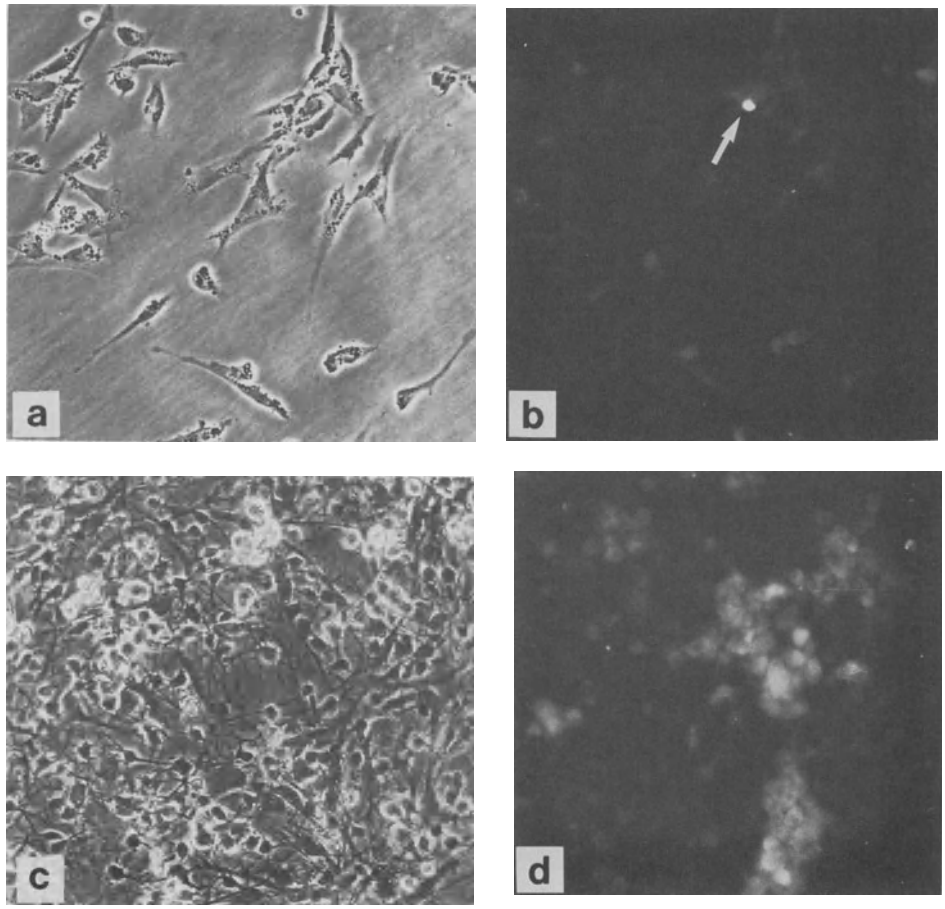

Fig.1: a, b) DA-neuron culture incubated with CSF 7 days after transplantation. Degenerating NSE-positive cell indicated. c, d) After incubation with CSF removed 1 year postoperatively; the culture looks healthy with many NSE-positive neurons (d). Bar is 50 μm.

DISCUSSION

In this study we have observed **1)** that CSF from 2 patients removed 3 and 7 days after adrenal medulla-to-brain transplantation marked DA-ergic cells in the rat SN and also induced cell death in neuronal cultures with DA neurons. **2)** It was also clear that the CSF from the one patient which did not produce marking of the DA-ergic cells in the SN had no deleterious effect on the neuronal cultures. **3)** With time after the adrenal transplantation the immunochemical markage of the SN region gradually disappeared, and in parallel with this also the toxic effect on the cell cultures was lost.

In this experimental set up CSF from the same 3 patients were used for incubating the cultures, and the only difference between the different samples was the time after operation. Thus, the patients are there own controls. The immunocytochemical studies on rat brain sections indicated clear differences in the levels of neuron specific IgG species in the CSF specimens taken at different times postoperatively, and it is reasonable to assume that these changes in antibody levels explain the different effects on the neuronal cultures. This is sup-

ported by results obtained with the immunonegative CSF from the third patient which had no harmful effects on the cultures which looked quite healthy.

It should be noted that the moderate recorded clinical improvement of the patients (Penn et al., 1988) corresponded to the gradual disappearance of the immunocytochemical reaction in the rat SN region (McRae-Degueurce et al., 1988b) and to the decreased destructive ability on the DA-cultures. Other CSF parameters, e.g. levels of catecholamines (CA) and their metabolites, as well as of 5 different CNS peptides did not exhibit any overt trends which could be correlated with the clinical improvement (Carvey et al., 1988). Therefore, it is possible that the adrenal transplants released substances that may influence the immune mechanisms in these transplanted patients, and that the clinical status of the patients is related more to the antibody titre than to the CA levels.

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AUTOTRANSPLANTATION OF PARASYMPATHETIC CHOLINERGIC NEURONS INTO ALZHEIMER MODEL RAT BRAIN

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INTRODUCTION

Senile dementia of the Alzheimer type is a progressive disease in the aged. Based upon the hypothesis relating cholinergic function to Alzheimer's disease,[1] the present study was designed to elucidate the effect of transplantation of cholinergic cells on behavioral abnormalities induced in Alzheimer-model rats. With the goal of clinical applicability, autologous vagal ganglion containing many cholinergic cells were used as a donor tissue.

MATERIALS AND METHODS

1. Immunohistochemistry of the nodosal ganglion

The nodosal ganglion of rats were investigated by choline acetyltransferase (ChAT) immunohistochemistry, the detailed procedure of which has been described elsewhere.[2]

2. Transplantation experiment

Eighteen rats were divided into three groups as follows:

1) Control group (6 rats)
Unoperated rats served as control.

2) NBM lesion group (6 rats)
Lesions were produced in the bilateral nucleus basalis of Meynert (NBM) by ibotenic acid solution injection (2.5μg/0.25μl saline).

3) Transplantation group (6rats)
One week after the lesions were produced in the NBM, these rats underwent autotransplantation surgery of the nodosal ganglion into the right parietal cortex.

Seven weeks after the transplantation surgery, the behavior of the rats was examined by psychological tests, as described below.

3. Psychological examination

Spontaneous activity, a step-down type of passive avoidance response and the Hebb-Williams maze test were recorded for the 18 rats in the 3 groups.

Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Diseases, Volume 1
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4.Morphological study

After the psychological tests, the animals were perfused with a solution containing 2% paraformaldehyde and 0.25% glutaraldehyde, and the brains were processed for acetylcholinesterase histochemistry according to Karnovsky and Roots' method[3] to observe the transplanted tissue and lesions in the NBM.

RESULTS

1.Immunohistochemistry of rat nodosal ganglion

Many ChAT-immunopositive cells were observed in the peripheral part of the rat nodosal ganglion (Fig.1).

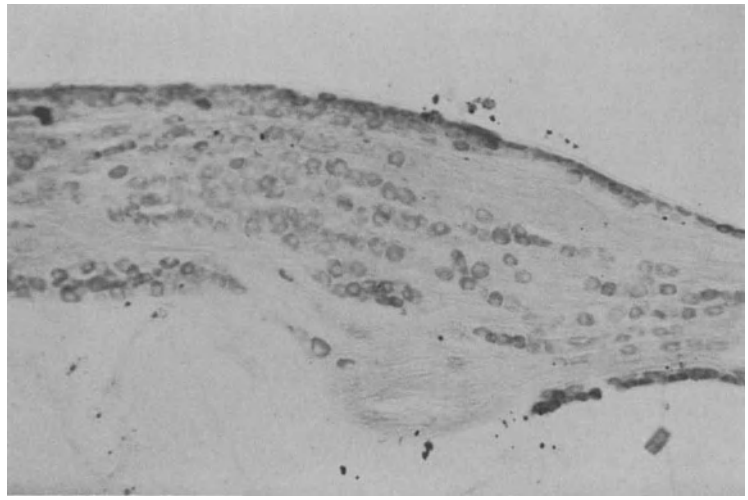
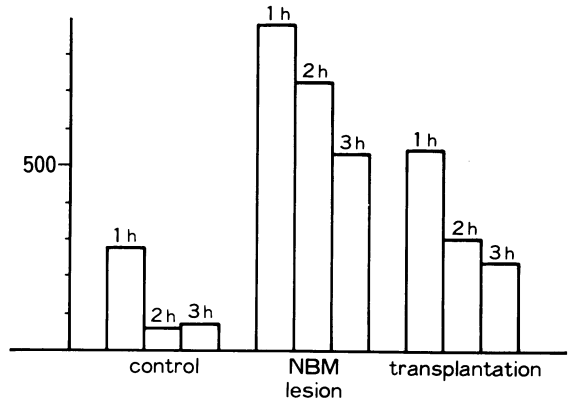


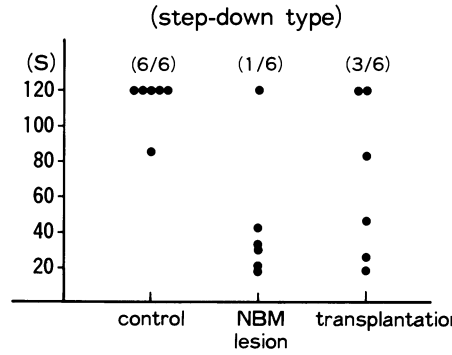
Fig.1. Immunohistochemistry of the rat nodosal ganglion.

2.Psychological examination

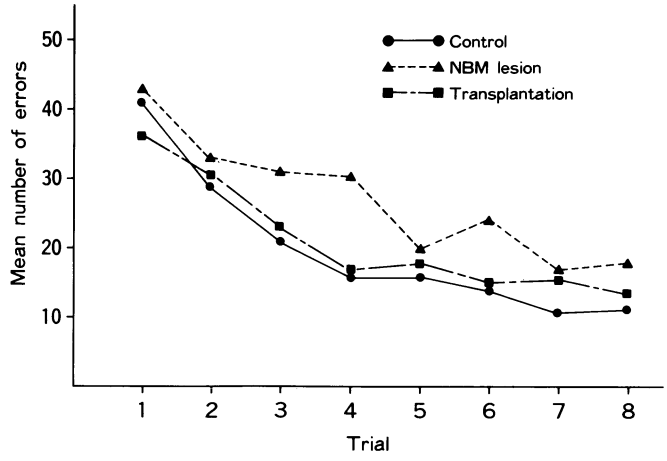
The rats in the NBM lesion group revealed an increase in spontaneous activity (approximately 3 times more than that of the control group). The ganglion-transplanted rats showed a lesser degree of activity than the lesion only group, although it still remained high in comparison with the the control group (Fig.2a). In the passive avoidance test, the latency in leaving the stage was over 120s in 5 out of 6 control rats. The 6 rats with NBM lesions showed a mere 20-30s of latency (disturbance of memory retention). However, 3 of the ganglion-transplanted rats displayed over 80s in latency (improvement in the memory retention) as shown in Fig.2b. In the Hebb-Williams maze test, the rats with the NBM lesion made more errors per trial than the control rats. The transplanted rats manifested slightly lower number of errors than NBM-lesioned rats (Fig.2c).



a) spontaneous activity



b) passive avoidance response



c) Hebb-Williams maze test

Fig.2. Results of psychological examination.

3.Morphological study

In the rats with the NBM lesion, no AChE-positive neurons were observed in the NBM. In the lesioned rats that received the transplant, many AChE-positive cells were observed in the graft (Fig.3). The number of surviving AChE-positive cells in 5 out of the 6 rats were 501, 467, 407, 117 and 89. No AChE-positive cells were observed in the remaining rat.

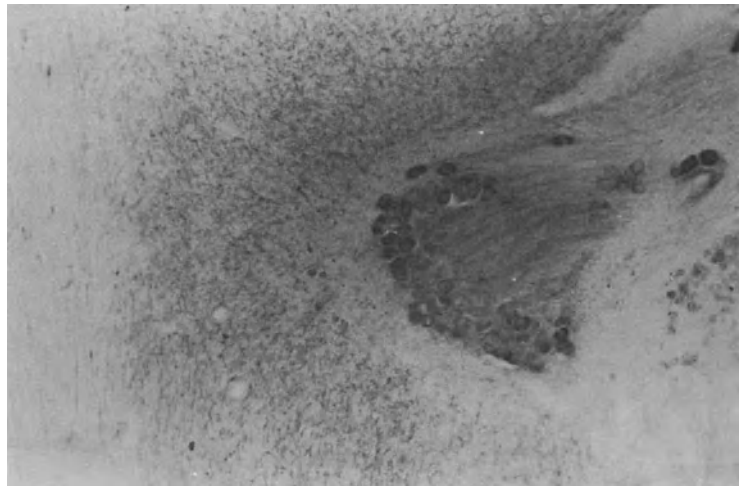


Fig.3. Acetylcholinesterase staining of transplanted tissue.

DISCUSSION

The present experiments have clearly demonstrated that transplanted peripheral cholinergic neurons survived in the brain and probably played a role in improvement of the behavior of the rat following NBM lesions. The mechanism of amelioration of abnormal behavior may be as follows: 1) ACh released from the transplanted tissue led to behavioral improvement. and 2) some trophic factors released from the graft promoted sprouting of residual cholinergic fibers in the NBM-lesioned rats. From the morphological study in this experiment, we suggest that ACh released from the graft may be the main factor for behavioral improvement, because sprouting of residual cholinergic fiber was not observed in the cerebral cortex.

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USE OF MPTP-HEMIPARKINSONIAN (HP) MONKEYS TO EVALUATE EFFICACY OF TISSUE IMPLANTS AS A TREATMENT FOR PARKINSON'S DISEASE

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INTRODUCTION

Although severed peripheral neural axons re-establish functional synapses, in the central nervous system damaged axons fail to regenerate. This had been attributed to the lack of production in damaged brain of "neurotrophic substances" to support axonal growth[1]. During the last 20 years, however, there have been several important discoveries which have made possible serious attempts to promote repair from damage or degeneration in the central nervous system. An increasing number of studies have sought means for retarding degenerative neurological diseases or for replacing degenerated neurons, particularly in Parkinson's disease.

Between 1960 and 1970, the dopaminergic nigrostriatal system was demonstrated and was found to be deficient in patients with Parkinson's disease. L-Dopa, later with potentiation by a peripheral decarboxylase inhibitor, was introduced as a means of effectively treating the motor disorder. Replacement of dopamine, however, affords relief for only a limited period of time; over a number of years the parkinsonian state progressively worsens, control of motor symptoms without prominent side effects becomes increasingly difficult, and in many cases responsivity to the medications is lost. These limitations in the medical treatment of Parkinson's disease and the demonstrations that some neural tissues, particularly when immature, survive implantation into adult mammalian brain, stimulated hopes that dopaminergic tissue implants might be efficacious in the treatment of this progressive motor disorder.

In rodents, specific dopaminergic denervation may be accomplished by injection of 6-hydroxydopamine (6-OHDA) intracisternally or locally into the region of the substantia nigra. Unilateral administration of this neurotoxin results in asymmetric rotational motor behavior, particularly after administration of drugs which either release endogenous dopamine (e.g., amphetamine) or stimulate directly the supersensitive dopamine receptors (e.g., apomorphine). Reversal of these effects provide an informative and convenient means of examining the efficacy of brain implants. Perlow et al.,[2] showed that when fragments of rat fetal substantia nigra were placed in the lateral ventricle adjacent to the denervated striatum, the implanted tissue survived and partially alleviated the dopamine deficient state; apomorphine-induced rotation

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was reduced. Similarly Bjorklund and Stenevi[3] showed survival and growth of fetal dopaminergic neuroblasts placed in a preformed cavity in the striatum of a 6-OHDA pretreated rat. Later, adrenal chromaffin tissue implants also were found to be effective in reversing rotational behavior in 6-OHDA lesioned rats.

These seminal observations in rodents provided the basis for justifying trials of adrenal medullary implants into the caudate nucleus as a treatment for patients with Parkinson's disease. The first attempts at such treatment[4,5] did not produce prolonged therapeutic benefit, although some transient improvement appeared to have been obtained. After Madrazo et al.,[6] reported spectacularly successful prolonged improvement after chromaffin tissue autotransplants in two young (ages 35 and 39) severely parkinsonian patients, a large number of parkinsonian patients were treated similarly in centers throughout the world. The results obtained have been less remarkable than those reported from Mexico, there have been complications and some deaths have been reported. At present, the benefits of adrenal medullary autotransplants do not appear to warrant the risks and this remains an experimental procedure.

The demonstration[7,8] of survival of human fetal tissues implanted into dopaminergic-denervated rat striatum has encouraged attempts at treating Parkinson's disease using human fetal mesencephalon. Contraversal ethical issues surrounding elective abortion and use of human fetal tissue in treatment of diseases such as Parkinson's disease and diabetes have impeded trials of fetal implants in patients. In the relatively few cases in which human fetal mesencephalic tissue has been implanted in basal ganglia of parkinsonian patients, some efficacy has been suggested.

Studies in experimental animals are clearly necessary to examine the efficacy of neural implants and the mechanisms by which they may contribute to the reversal of parkinsonian motor deficits. The best animal models of human parkinsonism are primates in which the nigrostriatal dopaminergic neurons have been destroyed almost completely by administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydroperidine (MPTP). This substance was first encountered when severe parkinsonian motor deficits rapidly developed in young drug abusers[9,10]. When administered systemically to monkeys, MPTP produces a severe parkinsonian motor deficit which closely resembles human Parkinson's disease[11]. When injected into one internal carotid artery, hemiparkinsonism is produced with characteristic rigidity, tremor, and bradykinesia in the contralateral limbs[12]. Such animals exhibit asymmetric motor activity similar to that found in rodents with unilateral dopamine deficits. It is the purpose of this presentation to summarize the results we have obtained using tissues from various sources as implants in this primate model of Parkinson's disease.

METHODS

MPTP Treatment

Rhesus monkeys were made hemiparkinsonian by intracarotid infusion over a 15 minute interval MPTP (0.4mg/kg) dissolved in 60 ml saline[12]. During the week after this treatment, parkinsonian signs develop in the limbs contralateral to the infusion.

Several other monkeys received MPTP-HC1 intravenously (0.3 mg/kg) daily for five days and an additional two doses (0.2 mg/kg each) one month later. These animals became severely parkinsonian with involvement of all four limbs.

Motor activity was assessed by videotaping recording of spontaneous motor activity and volitional movement in response to food presentation

as well as by electronic monitoring of drug-induced rotations after administration of standard doses of L-dopa/carbidopa (200/20 mg, p.o.), apomorphine (0.2 mg/kg,i.m.) or amphetamine (3 mg/kg,i.m.).

Surgical Procedures for Tissue Implants

After stablization of the motor abnormalities which developed following MPTP administration (generally 1-3 months), to enhance subsequent graft survival, cavities were placed in the heads of the right and left caudate nuclei. Sterile surgery was performed under pentobarbital anesthesia. Under an operating microscope, a small incision was made in the body of the corpus callosum and a portion of the dorsal septum resected, thus exposing both caudate nuclei. Two small cavities were made in the head of each caudate nucleus using pituitary rongeurs; the cavities were filled with trypan blue-stained gelfoam sponges. Using a similar surgical procedure, tissue fragments from various sources were implanted in the cavities 2-5 weeks later. Some animals received adrenal medullary tissue from their own adrenals (autotransplants) or from other monkeys (heterotransplants), others received fetal mesencephalic tissue from 35-42 day old gestational monkey fetuses or fragments of fetal amnion, whereas others served as controls with empty cavities or with implants of other tissues such as fat, adrenal cortex, etc. Motor behaviors of the tissue implanted monkeys and control animals were assessed at various intervals after the tissues were implanted.

Histological Examination

After suitable intervals (5-7 months after placement of tissue implants) the animals were killed and processed for preparation of brain tissues in a manner suitable for immunohistological localization of specific proteins or autoradiographic demonstration of specific binding of radioligands. Immunoreactivity (IR) to tyrosine hydroxylase (TH), particularly in the absence of IR to dopamine-β-hydroxylase (DBH), is indicative of dopaminergic innervation. IR to glial fibrillary acidic protein (GFAP) is highly specific for astrocytic cells. Binding of ^{3}H-mazindol was used to identify presynaptic dopamine uptake sites on dopaminergic terminals and ^{3}H-Sch 23390 and ^{3}H-Spiperone or ^{3}H-Sulpiride to localize D_1 and D_2 dopamine receptors, respectively. Presynaptic binding sites disappear, whereas receptor binding sites are increased in caudate of MPTP-treated hemiparkinsonian monkeys[13]; changes in the receptors appear to be responsive to decreased dopaminergic innervation.

RESULTS AND DISCUSSION

Adrenal Medulla Implants

Bilateral auto- or hetero-implants of adrenal medullary tissue into the caudate nuclei of hemiparkinsonian monkeys produced only a transient diminution in apomorphine-induced turning and partial, transient return of volitional use of the affected limb for retrieval of food[14]. The effects of the implants were no greater than the changes found in control (fat, adrenal cortex implanted or sham operated) animals. After the animals were killed, it was apparent that the implanted tissue did not survive but that the surgical procedure (whether or not adrenal medulla was implanted) had stimulated dopaminergic fiber growth from surviving dopaminergic neurons in other areas into the damaged caudate nucleus, consistent with the observations of Bohn et. al.,[15] in rats. It was suggested that the slight improvement in motor function of operated animals might be attributed to these newly sprouted TH-IR fibres.

Unilateral Fetal Mesencephalic Tissue Implants in a Fully Parkinsonian Monkey

In one monkey which had been made parkinsonian by systemic

administration of MPTP three months earlier, insertion of fetal mesencephalic tissue into a week-old preformed cavity in one caudate nucleus resulted in dramatic bilateral symptomatic improvement in motor function[16]. By three weeks after the implant, tremor and freezing episodes disappeared, grooming, feeding, and general activity increased and the animal appeared normal. Five months after the implant, spontaneous motor activity was attended by increased turning away from the implanted side; this was dramatically enhanced after treatment with amphetamine. Apomorphine administration evoked turning contralateral to the implanted caudate nucleus. These results suggested that the implant had increased dopaminergic innervation on the implanted side.

This animal was killed seven months after the implant and brain examined histologically. In the implant, many TH-IR cell bodies and their processes were evident, but none of the processes could be traced into the host caudate. There were, however, many TH-positive fibres evident in the medial portion of the caudate nucleus of both sides, mainly adjacent to the cavity containing the implant as well as the cavity in the caudate on the opposite side (without implanted tissue). The TH positive fibres appeared to emanate from the ipsilateral ventral striatium, lateral to the N. accumbeus. These observations further support the notion that recovery from motor deficits after tissue implants may be due to reinnervation from host dopaminergic neurons. As in other MPTP-treated monkeys, dopaminergic innervation of the N accumbers, olfactory tubercle, and A 10 areas appeared normal.

Bilateral fetal mesencephalic implants in hemiparkinsonian monkeys

Cavities were made in both caudate nuclei of three hemiparkinsonian monkeys. The cavities were filled with gel foam and 2-5 weeks later, fetal mesencephalic tissue from 35-42 days old gestational monkey fetuses was implanted bilaterally[16]. Two animals received tissue from a single fetus, whereas a third received tissue from two fetuses.

In all three of these hemiparkinsonian monkeys, about one month after placement of the fetal implants, volitional movement in the affected limb returned and there was a marked reduction in apomorphine-induced turning. The animal that received implants from two fetuses, however, had regression of the use of the affected limb after about six weeks; simultaneously apomorphine-induced rotation increased. In the two monkeys which received tissue from a single fetus, recovery was sustained. Histological examination of the region of the implants demonstrated that in the recipient of tissue from a single fetus, the fetal tissue survived; the neurons and associated fibres in the implant expressed TH-IR, but there was no ingrowth of fibres into the caudate. The cavity appeared to have attracted growth of TH-IR fibres from other regions of brain. In the animal which received tissue from two fetuses, the fetal tissue did not survive and there was evidence of inflamation attending immunological rejection. These results suggest that use of tissue from two fetuses may have enhanced the immunological response to the implanted foreign tissue and that reinnervation by dopaminergic fibres was stimulated by the implanted tissue.

Amnion Implants

In studies designed to determine if dopaminergic neurons in fetal tissue implants are essential to effect functional improvement in motor function of MPTP hemiparkinsonian monkeys, consideration was given to other sources of tissue. Since amnion contains neurite outgrowth promoting activity *in vitro*[17], rhesus amnion from 6-14 week pregnancies were implanted in preformed cavities in the caudate nucleus of 4 hemiparkinsonian monkeys[18,19]. Within six (6) weeks, three of the four

amnion-implanted monkeys recovered volitional movement in the affected limb whereas functional improvement did not occur in six other MPTP-hemiparkinsonian monkeys, four of which had preformed cavities and two of which were unoperated. APO-induced turning was diminished by about 2/3 in three of the four amnion-implanted monkeys, was decreased slightly in the four monkeys with cavities but no implant and not reduced in the remaining two control hemiparkinsonian animals. The improvement in amnion-implanted animals persisted for nearly six months when the animals were killed and the caudate examined for TH-IR. Histological examination showed that dopaminergic sprouted fibres, apparently derived from the ventral striatium and N accumbeus, were present in the region of the amnion implant. The fibres appeared to have grown most densely along the adventia of the blood vessels, consistent with *in vitro* demonstration of soluble growth factor which attracted their sprouted processes.

Histological Studies of Implanted Caudate Nuclei

TH-IR was used to identify dopaminergic fibres in the caudate nucleic and surrounding areas. As indicated above, dopaminergic neurons in the host appear to have been stimulated to sprout and extend into the denervated caudate after cavities alone were formed. This response was enhanced markedly when fetal mesencephalic tissue or fragments of amnion were implanted. Amnion tissue becomes vascularized and appears to stimulate, directly or indirectly, axonal sprouting. Parallel fibre-like GFAP-IR suggested that astrocyte growth attended the ingrowth of the dopaminergic sprouts. ^{3}H-mazindol binding demonstrated the presence of dopaminergic fibres growing into the caudate from the region of the accumbens. It is at present unclear whether the astrocytes release growth factors which contribute to ingrowth of the dopaminergic axons. Enhanced binding of ^{3}H-ligands specific for D_1 and D_2 receptors was evident in the caudate of control MPTP-hemiparkinsonian monkeys; this enhanced binding appeared to have been reduced after fetal tissue implants in the caudate cavities, suggesting dopaminergic reinnervation.

CONCLUSIONS

MPTP-parkinsonism in monkeys can be reversed by surgical implants of fetal tissue. Adrenal medullary tissue produces only transient improvement; the tissue does not survive and the level of improvements regresses to that seen with surgical damage alone. Fetal mesencephalic tissue, however, survives in the implant and the functional motor improvement persists. Recovery appears to be due to stimulation in the host of growth of surviving dopaminergic neuronal axons into the caudate, rather than growth of axons from the implant. Soluble growth factors from the implant and/or surrounding damaged tissue appear to be responsible for both glial (astrocyte) proliferation and dopaminergic neuronal sprouting and axonal ingrowth.

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AUTOTRANSPLANTATION OF SYMPATHETIC GANGLION INTO THE BRAIN OF PARKINSONIAN MONKEY

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INTRODUCTION

Recent biochemical and histochemical studies have evidenced that Parkinson's disease results from the loss of dopamine neurons in the substantia nigra. To counteract the dopamine deficiency in the brain, L-dopa is usually administered to patients with Parkinson's disease. However, problems such as "on-off" and "wearing-off" phenomena, or dyskinesia have occurred in patients with long-term administration of L-dopa.[1] As a possible future substitute for L-dopa treatment, we have investigated the effect of transplantation of autologous superior cervical ganglion (SCG) into the brains of monkey in which parkinsonism was induced by 1-methyl-4-phenyl-1, 2, 5, 6 - tetrahydropyridine (MPTP). We used the SCG as a donor tissue, because this ganglion contains not only norepinephrine neurons but also dopaminergic ones.[2]

MATERIALS AND METHODS

Five monkeys (Macaca fuscata), each weighing 3 to 7.6 kg, were used. For induction of parkinsonism, MPTP was repeatedly injected (0.5mg/kg/day, i.v.) into these monkeys. After a total MPTP dose of 3mg/kg had been injected, the monkeys manifested Parkinson's syndrome such as akinesia and muscle rigidity. In three monkeys, the autologous SCG was removed under Fluothane anesthesia, and several pieces of the SCG were transplanted into the bilateral caudate nuclei by stereotactic surgery. One monkey received transplantation of a fragment of temporal muscle as a sham operation, and one monkey having no transplantation after the MPTP treatment served as control. During the experiment, the motor activity of the monkeys was monitored by a telemetric method; the content of homovanillic acid (HVA), a metabolite of dopamine, in the cerebrospinal fluid (CSF) was measured by high performance liquid chromatography (HPLC-ECD) method; and the transplanted tissues were observed by catecholamine (CA) histofluorescence with glyoxylic acid solution.[3]

RESULTS

1. Acute effect of SCG transplantation on MPTP-induced parkinsonism

The three monkeys that had received the SCG autotransplantation

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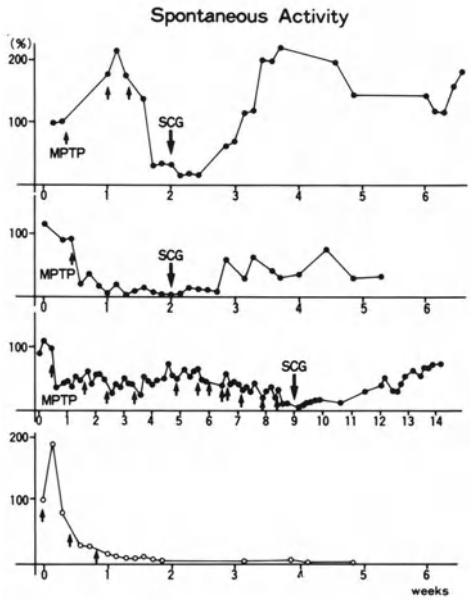

Fig.1. Temporal changes in motor activity in 4 monkeys. Three monkeys recieving SCG transplantation (upper 3 columns) showed increased motor activity, while the one control monkey (lowest column) failed to show any increase in motor activity.

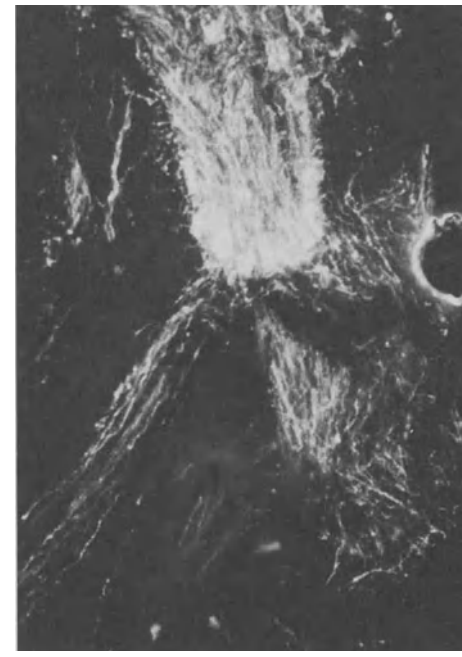

Fig.2. CA histofluorescence shows many CA cells in the graft and CA fiber extension into the host brain.

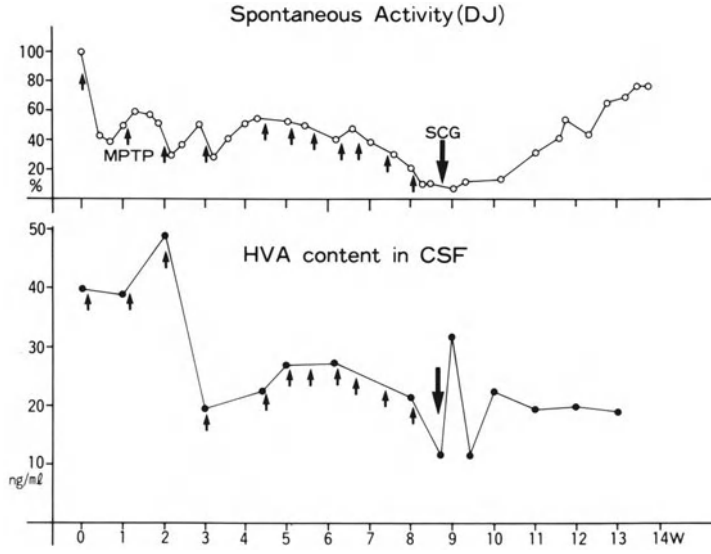

Fig.3 HVA content in the CSF. After transplantation, HVA content in the CSF abruptly increased, which increase paralleled the improvement of motor activity (upper column).

revealed increased motor activity 1 to 3 weeks after transplantation (Fig.1).One of them showed transient hyperkinesia and aggressive moods such as rage when facing the researchers. The sham monkey and one control failed to show improvement in MPTP-induced parkinsonism. CA histofluorescence showed many CA cells in the graft and extension of CA fibers into the host brain (Fig.2). HVA content in CSF decreased after MPTP treatment, which was followed by an increase after the SCG transplantation (Fig.3).

2.Long-term effect of SCG transplantation on MPTP-induced parkinsonism

Two out of 3 monkeys receiving SCG transplantation were carefully observed for more than 2 years after the transplantation. They showed neither recurrence of parkinsonism nor significant side effects such as seizure and psychological abnormalities. HVA content in the CSF continued to be within the normal range in the two monkeys. One monkey was sacrificed 25 months after the transplantation, and its brain was observed by CA histofluorescence. In this monkey, many dopamine-containing cells had been lost in the substantia nigra and no dopamine terminals were observed in the striatum, which suggests no occurrence of sprouting of residual dopamine neurons in this monkey. In the head of the lt-caudate nucleus, the transplanted tissue containing many CA cells and fibers had survived. Many CA fibers in this graft revealed thin varicose appearance similar to the central dopaminergic fibers (Fig.4a). Some small intensely fluorescent cells (probably dopaminergic) were also found in the graft (Fig.4b). However, no CA fibers extended into the host brain.

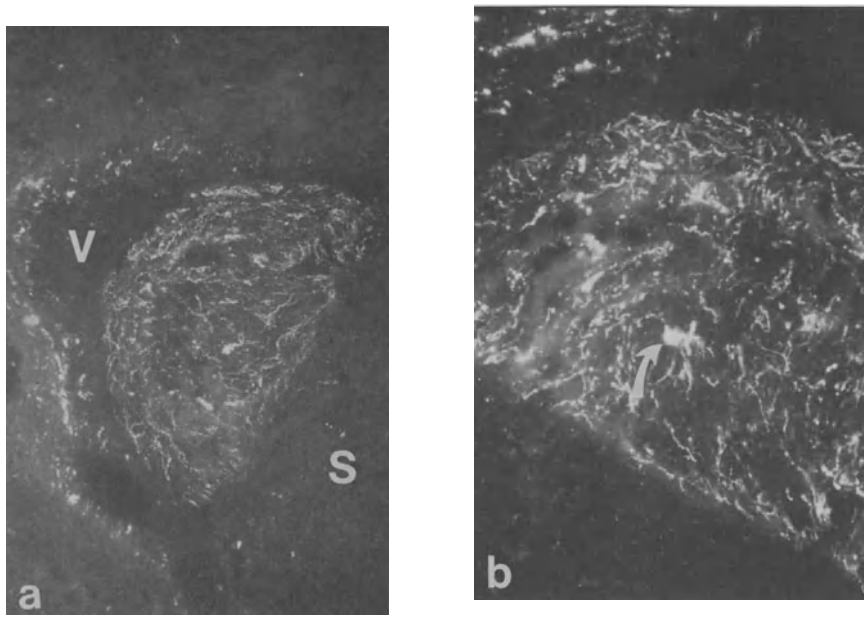

Fig.4 CA histofluorescence showing long-term survival of the grafted tissue.
a) Many CA fibers are observed in the graft. s:striatum, v:ventricle
b) Small intensely fluorescent cells (probably dopaminergic) are also seen in the graft (arrow).

DISCUSSION

The present study clearly demonstrated that autotransplantation of the SCG ameliorated parkinsonism induced by MPTP administration in the monkey. This effect persisted for a long period (at least 2 years) without any unpleasant side effects. The mechanisms underlying this effect may be as follows: Firstly, some trophic factors released from the transplanted SCG promoted sprouting of residual nigro-striatal dopamine neurons.[4] Secondly, dopamine released from the graft acted on target cells in the host striatum, which produced improvement of parkinsonism. Finally, dopaminergic fibers extending from the graft made synaptic connection with the striatal cells, and reconstruction of the neural circuit occurred in the host brain. From the morphological studies in this experiment, we found no sprouting of the nigro-striatal dopamine neurons in the host brain, but we did observe long-term survival of many CA cells and fibers in the graft. HPLC study displayed an abrupt increase in HVA content after the SCG transplantation followed by a continuous increase in the content. These morphological and biochemical data suggest that dopamine released from the transplanted SCG may exert a neurohumoral effect on the striatal cells. In comparison with autotransplantation of the adrenal medullary tissue, the SCG can survive in the host brain and release dopamine for a longer period than the autotransplanted adrenal medulla.[5] In the future, autotransplantation of the SCG should be attempted in patients with Parkinson's disease.

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LONG-TERM SURVIVAL OF GRAFTED CELLS, DOPAMINE SYNTHESIS/RELEASE, RECEPTOR ACTIVITY, AND FUNCTIONAL RECOVERY AFTER GRAFTING OF FETAL NIGRAL CELLS IN MODEL ANIMALS OF HEMI-PARKINSON'S DISEASE

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INTRODUCTION

Grafting of fetal nigral dopaminergic (DAergic) cells, in DA depleted caudate has been reported to produce the recovery of motor disturbances in animal models of hemi-parkinson's disease[1]. In the clinical field, graftings of adrenal medullary tissue into patients with Parkinson's disease have been performed in about two hundred cases[2]. However, the results so far are not always satisfactory. It because the basic problems remain to be solved. In the present study, to make clear how long the grafted cells survive, function, and ameliorate motor performance, we grafted nigral DAergic cells in model rats of hemi-parkinson's disease and investigated the survival of the grafted cells, DA synthesis/release, receptor activity and motor recovery for more than 2 years. Some results have been reported elsewhere[3].

METHODS

6-Hydroxydopamine Lesions, Motor Disturbance and Cell Grafting

Seventy Wistar rats were used for the experiment. 6-hydroxydopamine (6-OHDA, 8μg in 4μl 0.05% ascorbate saline) was injected into the left substantia nigra of 60 rats. Ten served as controls. After the 6-OHDA lesion motor disturbance was assessed by counting methamphetamine (3 mg/kg, i.p.) rotations. Fifty rats that made more than 8 turns/min were regarded as 6-OHDA lesioned rats. They were used as recipient animals for grafting except 5 those kept as 6-OHDA animals. Midbrain areas that include substantia nigra and ventral tegmental area were collected from fetal rats (fetal day 14-16). Cell suspensions (about 10^6 cells/ml) were prepared[4] by pipetting after incubating the tissue in trypsin medium for 30 min at 37℃. 10μl of the suspension was implanted into the DA-depleted head of the caudate nucleus. After the grafting behavior recovery was assessed by the decrease of rotations.

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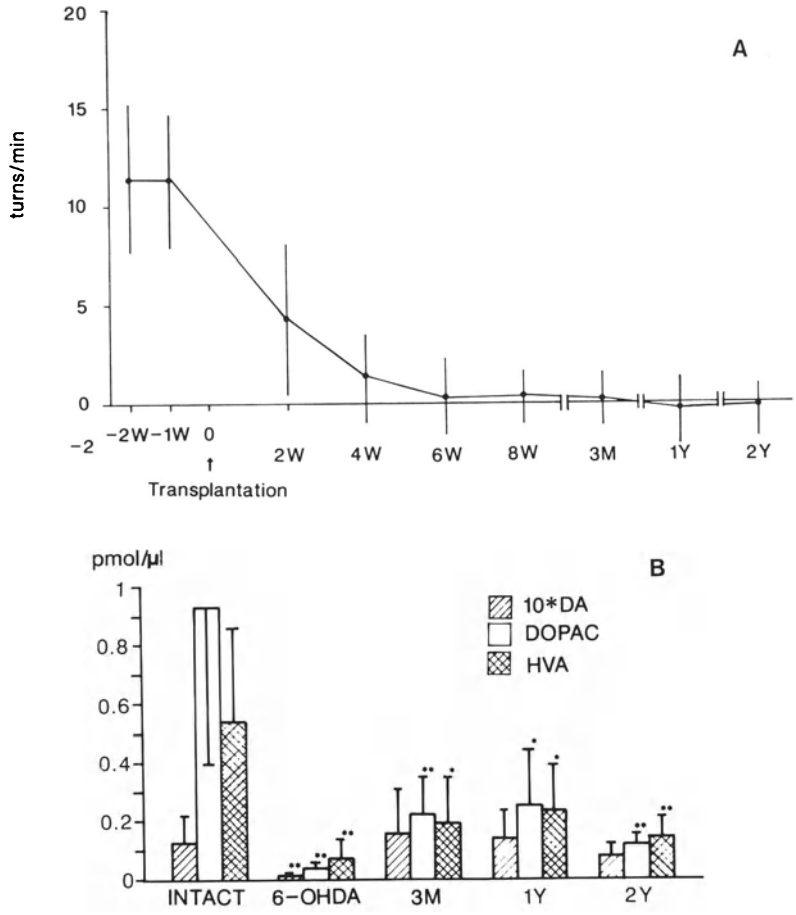

Fig. 1. A, Rotations before and after grafting. W, week ; M, month ; Y, year. B, Concentration of DA, DOPAC and HVA. INTACT, control rat. DA, $\times 10$.

Microdialysis

To detect the content of DA and its metabolites in the extracellular space in the caudate, microdialysis was performed and the content of DA, DOPAC and HVA in the dialysate was measured by HPLC.

Immunocytochemistry and In Situ Hybridization

To detect implanted DAergic cells and the extent of 6-OHDA lesion, tyrosine hydroxylase (TH) immunocytochemical staining was performed at 3 months, 1 year and 2 years after grafting. The expression of TH mRNA in grafted cells was investigated by in situ hybridization. Tissue sections (15μm in thickness) were hybridized with 1×10^5 dpm of TH oligonucleotide probe (1223-1252 TCA-AAG-GCT-CGG-ACC-TCA-GGC-TCC-TCT-GAC-[dA^{35}S]n, DUPONT). Following hybridization, sections were dipped in nuclear emulsion and processed for autoradiography.

Receptor Activity

To investigate the variations of receptor activity following 6-OHDA

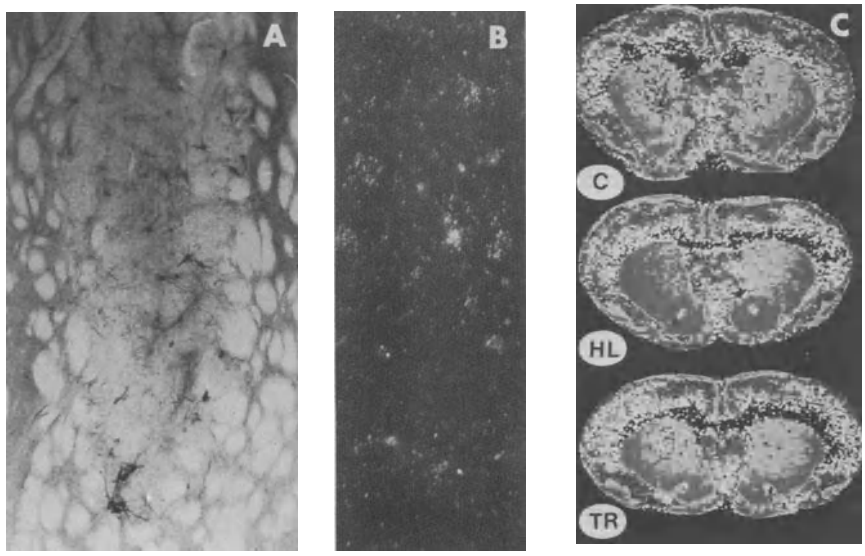

Fig. 2. A, TH positive cells around the grafting track (1 year after grafting). B, TH mRNA in situ hybridization. C, D_2 receptor binding. C, control; HL, hemi-lesioned; TR, grafted rat.

lesion and grafting, D_2 receptor binding was studied using ^{3}H-spiperone under autoradiography.

RESULTS

Fig. 1 A summarizes the time course of motor recovery up to 2 years. Before grafting, rats made more than 10 turns per min. After the grafting, however, rotation reduced and finally disappeared in 40 among 45 rats.

In control rats the concentration of DA, DOPAC and HVA were 9.5 ± 5.5, 768 ± 410 and 455 ± 254 f mol/μl, respectively. In 6-OHDA rats, DA was undetectable, DOPAC decreased to less than 5%, and HVA less than 15% of those of controls. In grafted rats, DA recovered to almost control level. DOPAC and HVA also recovered up to about one third and a half of those of controls. There were no significant differences in the concentration of DA, DOPAC and HVA among 3 periods (3 months, 1 year and 2 years) (Fig. 1 B).

In 40 rats that showed motor recovery, more than 400 TH positive cells per animal (maximum, 2755 cells per animal) survived around the grafted area in the caudate. They extended neurites with abundant ramifications (Fig. 2 A). Fig. 2 B shows dark-field picture taken from a section processed for TH mRNA in situ hybridization-autoradiography in an animal 2 years after grafting. Fine radioemitted grains aggregate on several somata in the grafted area.

Fig. 2 C shows autoradiograms after binding of specific D_2 receptor antagonist (^{3}H-spiperone). After 6-OHDA lesion in unilateral nigrostriatal DAergic pathway (hemi-lesion), D_2 receptor binding increased (Fig. 2 C, middle). Following the grafting, the increase disappeared and the level of binding restored to almost control level (Fig. 2 C, lower).

DISCUSSION

Following the grafting of fetal DAergic cells into the caudate nucleus of hemi-parkinson model animals, motor imbalance (methamphetamine rotation) decreased significantly in the 4th week, then finally disappeared for more than 2 years. In functionally recovered animals, DA recovered up to almost control levels, and DOPAC and HVA to one third of the controls. No significant differences were found in the concentration of these 3 substances at different periods (3 months, 1 year, 2 years) though they decreased a little at 2 years after the grafting. TH immunocytochemistry revealed that, in behavior recovered animals, many TH positive cells survived around the grafting track, and again there were no differences in the number of TH positive cells at different period. In situ hybridization-autoradiography revealed that, around the grafted sites, even at 2 years after grafting, many cells express TH mRNA. The distribution of these neurons expressing TH mRNA corresponds closely with the pattern of TH immunocytochemistry. Thus it was found that once grafted DAergic cells survived they remained stably and produced TH that promotes DA synthesis, and consequently released DA stably for more than 2 years. However, all these evidences are presynaptic events when evaluated from the standpoint of caudate output. On the other hand, the functional recovery depends on the thorough integration of input and output systems, i.e. both pre- and postsynaptic events. In the present study we evaluated the postsynaptic activity by the intensity of binding of ^{3}H-spiperone to D_2 receptor. The increase of D_2 receptor binding in caudates following 6-OHDA hemi-lesion disappeared and the intensity of binding returned to normal level after the grafting of DAergic cells. Bringing all these data together, it can be concluded that both presynaptic (survival of TH positive cells, TH mRNA expression, DA release) and postsynaptic (receptor activity) events were restored by the grafting of DAergic cells.

In conclusion, grafted DAergic cells in DA depleted rat caudate survive, synthesize/release DA, normalize receptor activity, and restore motor imbalance for more than 2 years. Since there were no untoward side effects, neuronal cell grafting in the brain is one of promising approaches to improve disturbed function.

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INTRA-ACCUMBENS DOPAMINERGIC GRAFTS RESTORE METHAMPHETAMINE-INDUCED LOCOMOTOR HYPERACTIVITY RESPONSE AND THE RELEASE AND METABOLISM OF DOPAMINE IN RATS HAVING 6-OHDA LESIONS IN THE VENTRAL TEGMENTAL AREA

Yasushi Ishida,[1] Hiroyuki Hashiguchi,[1] Teruchika Ikeda,[1] Takeshi Hashitani,[2] and Hitoo Nishino[2]

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INTRODUCTION

An impairment of dopaminergic (DAergic) transmission in the mesocorticolimbic system results in deficiencies such as hypoexploration, loss of amphetamine-induced hyperactivity, supersensitive locomotor responses to apomorphine, and other sensorimotor deficiencies. DAergic cell transplantation into the nucleus accumbens (NAC) is reported[1,2] to alleviate these deficiencies. In the present study, methamphetamine-induced locomotor activity in an open-field was investigated in order to determine the effects of 6-hydroxydopamine (6-OHDA) lesions in the ventral tegmental area (VTA) and intra-accumbens DAergic grafts. Using an *in vivo* microdialysis-HPLC (high-performance liquid chromatography) detection system, we aimed to clarify the extent to which a deficiency in locomotor activity and its subsequent recovery are related to dopamine (DA) metabolism in the NAC and the degree to which DA is released. Our present study shows that DAergic grafting restores metabolism and release of DA in the NAC.

MATERIALS AND METHODS

Twenty-five male Wistar rats (body weight, 100-120 g) were provided at the beginning of the experiment. Each animal was administered desipramine hydrochloride (20 mg/kg, i.p., Sigma) 30 min before surgery, and then anesthetized with sodium pentobarbital (40 mg/kg, i.p.). 6-OHDA (Sigma) was dissolved in sterile isotonic saline, supplemented with 0.1% ascorbic acid, at a concentration of 2 mg/ml. To produce DAergic neuron lesions, we injected 20 animals with 2 µg 6-OHDA (2µg/1µl/site) over a 2-min period into each of 4 sites in the bilateral VTA (A=1.7 and 2.4 mm rostal to the interaural line, L=±0.3 mm, V=7.3 mm below dura). Control animals (5 rats) received bilateral injections of the same amount of saline. Five weeks later, 8 animals that displayed a decrease in methamphetamine-induced locomotor activity (locomotor activity less than 80% of the control) were grouped as 6-OHDA-lesioned animals, and 4 of them were chosen for transplantation.[2] Ventral mesencephalic areas were dissected out of rat embryos at 16 days gestation and ground into cell suspensions.[3,4] Four µl of the suspension was injected bilaterally into the NAC (A=1.2 mm rostal to the bregma, L=±1.0 mm, V=8.3 mm under the surface of the skull). Controls did not receive grafts.

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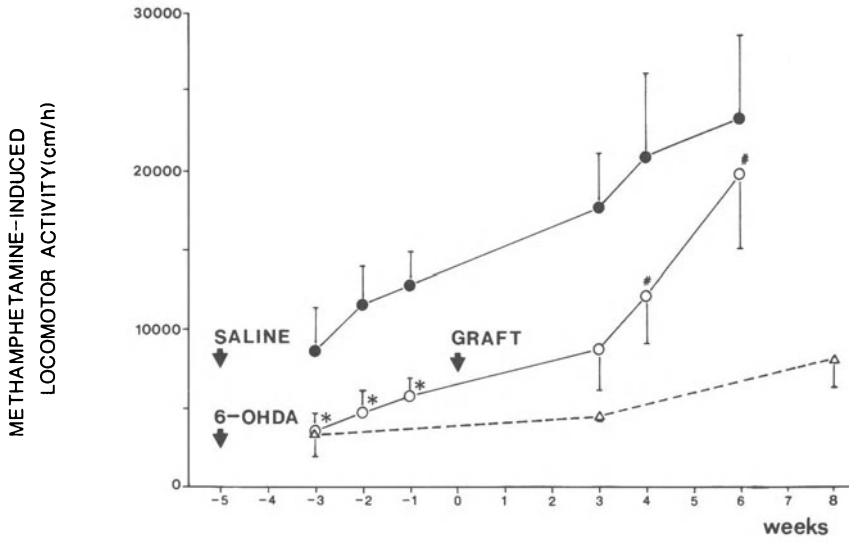

Fig. 1. Effects of 6-OHDA lesions and DAergic cell grafting on methamphetamine-induced locomotor activity measured for 1 h in an open-field. Abscissas, weeks before and after grafting. -●-, control (n=5); -○-, lesion plus grafted rats (n=4); -△-, lesioned rats (n=4) from another experiment of ours, for reference. Results are expressed as mean±SEM. *P<0.05, compared with control group (Mann-Whitney U-test). #P<0.05, compared with the values at 1 week before grafting (Wilcoxon's test).

Locomotor activity was measured as the total distance/h covered during the daytime (09.00 to 17.00 h) in an open-field (50 x 50 cm square with walls 40 cm high) equipped with photocells (Muromachi; Japan). After a 30-min habituation to the apparatus, methamphetamine (1.5 mg/kg, i.p.) was injected and locomotor activity was measured for 1 h. Behavioral tests were conducted three times before grafting (at 2, 3, and 4 weeks after the VTA lesion), and then three times, at 3, 4, and 6 weeks, after grafting.

The microdialysis method used was as described by Nakahara et al.[5] Between 8 and 9 weeks after transplantation, while under sodium pentobarbital anesthesia, the animals were stereotaxically implanted with 22-G guide cannulae and dummy cannulae into the grafted area in the left NAC. Two to three days after implantation, the dummy cannula was replaced with the dialysis cannula consisting of a 24-G introducer needle the tip of which was covered with cellulose hollow fiber tubing (10 μm thick, 0.25 mm outside diameter, 2.0 mm long, cutoff mol. wt. 5000). Ringer's solution was perfused through the dialysis probe at a flow rate of 2.0 μl/min using a microinfusion pump (BRC; Japan). Following a 2.5-h stabilization period, two dialysate samples were collected at 20-min intervals to establish the baseline level. Subsequently, methamphetamine (3.0 mg/kg, i.p.) was injected and successive 20-min samples were collected for 80 min. Dialysis was also performed in control and VTA lesioned animals. DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the collected dialysates were assayed immediately with an HPLC apparatus fitted with an electrochemical detector (EICOM; Japan).

RESULTS

In the open-field study, lesioned animals showed lower locomotor activity (41-45% of that of controls, P<0.05). However, after grafting, activity

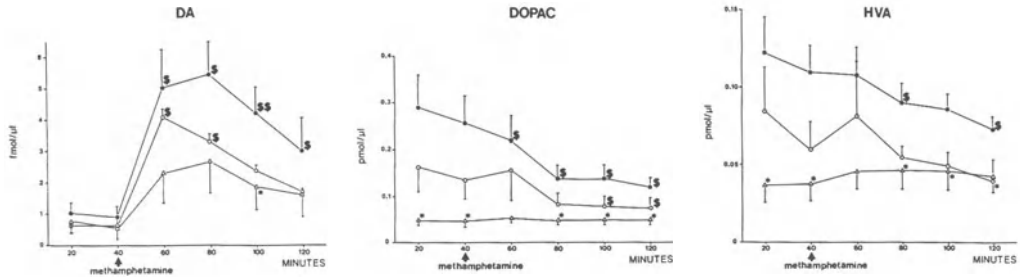

Fig. 2. Time courses of extracellular concentrations of DA, DOPAC, and HVA in the NAC measured by in vivo microdialysis method. Methamphetamine (3 mg/kg, i.p.) was administered 40 min from the start of dialysate collection. -●-, control (n=5); -△-, lesioned (n=4); -○-, grafted rats (n=4). Results are expressed as mean±SEM. Data were analyzed by ANOVA. *$P<0.05$, compared with control group (the least significant difference test for multiple comparison). \$$P<0.05$, \$\$$P<0.01$, compared with the baseline values (during 20-40 min from the start of dialysate collection for each group) (paired t-test).

was gradually restored, and no significant differences were found between grafted animals and controls at 3, 4, and 6 weeks after grafting (Fig. 1).

Figures 2 shows the time courses of extracellular concentrations of DA, DOPAC, and HVA measured by the microdialysis method before and after methamphetamine. In lesioned animals, the mean content of DA was about a half of the control level, but this was not significant because the individual variation was rather large. However, DOPAC and HVA levels were substantially lowered (DOPAC, 18%; HVA, 34% of control value, $P<0.05$). In grafted animals, the DA, DOPAC and HVA levels were about half of the control levels. In control animals, methamphetamine (3 mg/kg, i.p.) induced an increase in DA efflux (maximal response, 605% over the basal level) and decrease in DOPAC (47%) and HVA efflux (66%). These effects continued for over 80 min. In lesioned animals, methamphetamine did not increase the DA efflux significantly; in addition, both DOPAC and HVA failed to decrease significantly. In grafted animals, methamphetamine induced a significant increase in DA efflux. However, the increase was transient and became non-significant at 60 min after methamphetamine administration. A significant decrease in efflux of DOPAC (57%) was observed in grafted animals after methamphetamine administration. The time course of decrease in efflux of DOPAC and HVA following methamphetamine administration was similar to that observed in control animals, though the decrease was much more pronounced for DOPAC than for HVA.

DISCUSSION

Four and 6 weeks after DAergic cell grafting in the NAC, locomotor activity (which was decreased to about 50% of the control value) increased significantly, and no difference could be found between control and grafted animals. These data agree with those reported previously.[1,2,6] After grafting, basal concentration of DOPAC and HVA increased. The release of DA increased while those of DOPAC and HVA decreased after methamphetamine treatment. As a result, the time courses of the methamphetamine response became similar to those seen in the controls. Methamphetamine causes DA release and an inhibition of DA re-uptake. Therefore, the decrease in DOPAC and HVA after methamphetamine administration might due to the inhibition of DA re-uptake. Another possibility is a reduction in DA synthesis after methamphet-

amine administration. This would probably occur as the result of a feedback mechanism. In the grafted animals, the maximal response of DA efflux to methamphetamine occurred earlier than in the controls, and was short-lived. This might be due to the relatively small number of surviving DAergic somata or terminals in the grafted NAC, and DA could easily be depleted by a strong sustained stimulus such as methamphetamine. Another possibility is that the feedback mechanism on grafted DAergic cells is a little weak, or the mode of action of newly formed synapses or circuits between grafted DAergic cells and host neuronal elements[4] are somewhat different from those of natural ones. The recovery of basal levels of DOPAC and HVA in grafted animals probably reflects an ongoing DA synthesis and turnover rather than a release.[7]

In conclusion, grafted mesencephalic DAergic neurons in the NAC can compensate for deficiencies in mesolimbic DAergic system with behavioral and biochemical manifestations, as has been described for the nigrostriatal DAergic system. However, the methamphetamine-induced DA increase and DOPAC/HVA decrease in the grafted animals suggest that the recovery is not complete.

ACKNOWLEDGMENTS

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INVESTIGATION OF MHC ANTIGENS ON NEURAL GRAFT IN MOUSE PARKINSON'S MODELS

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INTRODUCTION

Parkinson's disease is one of the most common disorders that affect the nigrostriatal system in human. Loss of neurons in the substantia nigra and severe depletion of dopamine (DA) in the corpus striatum are the hallmarks of this condition. The most effective treatment of Parkinson's disease, particularly in its early phase, is the administration of both the L-isomer of 3,4-dihydroxyphenylalanine (L-DOPA) and dopamine-receptor agonists. However, it is clear that L-DOPA and the receptor agonists provide only limited ameliorable treatment and that most patients inexorably become progressively debilitated regardless of the type of therapy. On the other hand, an alternative approach in the therapeutic management of Parkinson's disease is surgical implantation of either DA neurons or adrenal chromaffin cells that have the ability to release DA.[1]

The present studies demonstrate that (1) both grafted syngeneic and allogeneic embryonic DA neurons survive in the host brain and express H-2 and Ia antigens, that (2) the dopaminergic fibers grow predominantly into the ipsilateral striatum, and that (3) these embryonic cells do not express the H-2 and Ia antigens before the neural transplantation.

MATERIALS AND METHODS

Lesion Surgery and Behavioral Testing

6-OHDA (7.5ug) was stereotaxically injected into the right nigrostriatal DA pathway of female C57BL/6 mice (8-12 weeks, $H\text{-}2^b$). Two to three weeks after the lesion, the mice were given 5 mg/kg of methamphetamine intraperitoneally, and their ipsiversive rotational behavior was counted for one minute every 5 minutes in 25-cm-diameter bowls. Mice that exhibited more than five ipsilateral full body turns per

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minute after the administration of methamphetamine were selected for neural transplantation.

Donor Tissues and Inplantation Procedures

DA-rich cells of syngeneic C57BL/6 mouse (H-2^b) embryos or allogeneic C3H/HeN mouse (H-2^k) embryos were used as donor cells. Both embryos were taken at embryonic day 15 (ED15). Several pieces of ventral mesencephalon of these embryos were dissected under a microscope, and the donor cells were prepared as cell suspensions by a published method.[2] Briefly, several pieces of the ventral mesencephalon were cut into six to eight smaller solid pieces and were kept gently in 0.1% trypsin-containing 0.01 M phosphate buffered saline (PBS) for 20 minutes at 37°C. After these pieces were washed with 0.6% glucose-containing saline, they were gently dissociated in 50-100 ul of the same solution with a Pasteur pipette.

Recipients were anesthetized with sodium pentobarbital and were placed in a David Kopf stereotaxic apparatus. Five to 10 ul (containing about 10^6 cells) of the cell suspension was slowly injected into the head of the caudate-putamen through a 10-ul Hamilton syringe stereotaxically. After the operation, the overlying skin was sutured and the animals were allowed to recover.

Immunohistochemistry

Fifty to 75 days after the neural transplantation, mice were anesthetized with sodium pentobarbital and perfused transcardially with 60 ml saline and an equal volume of 4% (wt/vol) paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were dissected from the cranium and postfixed in the same fixative overnight. The brains were sliced 4-10-um thick with a cryostat, and were mounted on several slides. These slides were incubated with monoclonal antibody to tyrosine hydroxylase (TH)[3] for 16-24 hours at 4°C. After washing, they were incubated with FITC-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin-G (GAMIgG) for 16-24 hours at 4°C. Finally, the slides were washed three times with 0.01 M cold PBS and coverslipped. For detection of class I (H-$2K^k$) antigens on fetal donor cells in the recipient's brain, monoclonal anti-K^k antibodies (11.4.1), which were IgG_{2a}, were used. For detection of class II (Ia^k) antigens in the brain, monoclonal anti-Ia^k antibodies (10.2.16), which were IgG_{2b}, were used. These primary antibodies were incubated with several slice of the host brain for 16-24 hours at 4°C. Then, as secondary antibodies, horseradish peroxidase (HRP)-conjugated GAM IgG_{2a} and IgG_{2b} were reacted with the primary antibody overnight at 4°C.

Flow Cytometry of H-2 and Ia Antigens on Mouse Embryonic Brain Cells

Dissociated cells (10^6) of the ventral mesencephalon of C3H/HeN mouse embryos were incubated with 50 ul of diluted (x 100) mouse monoclonal antibody to H-$2K^k$ antigens or Ia^k for 30 minutes at 4°C. After washing, they were incubated with 50

ul of diluted (x 50) FITC-conjugated GAM-IgG_{2a} or -IgG_{2b} for 30 minutes at 4°C. Then they were resuspended in 1 ml 0.01 M cold PBS containing 20 ug/ml propidium iodide and were analyzed on a fluorescence-activated cell sorter (FACS-IV, Becton Dickinson, CA, USA).

DA-rich cells of the ventral mesencephalon of C3H/HeN mouse embryos were cultured in vitro for 14 days and then were co-cultured with 2000 units/ml of mouse interferon (IFN)-gamma for 2 days. These cells were analyzed for $H\text{-}2^k$ and Ia^k antigens by flow cytometry in the same way.

RESULTS

Motor Asymmetry

All mice were examined for the amphetamine-induced rotational behavior until 90-110 days after 6-OHDA lesioning. Nongrafted mice, which had been lesioned by 6-OHDA previously, showed no recovery of amphetamine-induced ipsiversive rotational behavior. On the other hand, the syngeneic dopaminergic cell-grafted lesioned C57BL/6 mice exhibited complete behavioral compensation in the amphetamine-induced rotation test 65 days after neural transplantation. The behavioral compensation in the lesioned mice that were transplanted with the DA-rich cells of allogeneic C3H/HeN mouse embryos appeared about 60 days after transplantation, in the absence of any immunosuppressive agents.[4]

Morphological Finding

Four to 5 months after the lesions, the mice were sacrificed by transcardial perfusion with 4% paraformaldehyde in 0.1 M PBS. Microscopic examination of the section demonstrated the TH-immunoreactive cell clusters in the parenchyma around the needle tracts in either syngeneic or allogeneic embryonal cells-grafted mice showing functional recovery. Fine TH-immunoreactive fibers were detected within the host ipsilateral striatum.[5]

H-2 and Ia antigens were at first detected in the host's brain one week after the neural transplantation. And these antigens were shown in the recipient's brain until at least two months after transplantation (Table 1).

Detection of H-2 and Ia Antigens with Flow Cytometry

The FACS-IV analysis revealed that neither $H\text{-}2K^k$ nor Ia^k antigens were detected on the DA-rich cells of the ventral mesencephalon of C3H/HeN mouse embryos (ED15).[4] However, these antigens were induced on the DA-rich cells cultured in vitro for two weeks followed by a 2-day-incubation with mouse IFN-gamma (Table 1).

DISCUSSION

These experiments demonstrated that C57BL/6 mice with DA deficiency exhibited the same ipsiversive rotational behavior

as rat Parkinson models in the amphetamine-induced turning tests. They showed complete behavioral compensation 60 days after transplantation of the dissociated cells of either syngeneic C57BL/6 or allogeneic C3H/HeN mouse embryos in the absence of any immunosuppressive agents.[4,5]

Mouse Parkinson models exhibited complete behavioral compensation by 10 weeks after neural transplantation. The behavioral compensation occurred a little later than that in rat models utilizing rodent donors, in which functional effects can be observed two to four weeks after grafting.[2,6] However, this compensation is much faster than that seen with similar human-to-rat grafts, in which functional effects can be first observed at 12.5 weeks.[7] The behavioral recovery in the mice that were transplanted with syngeneic embryonal cells, appeared to be equivalent to that in the mice grafted with allogeneic embryonal cells without any immunosuppressive treatment.

H-2 and Ia antigens could not be detected on the dissociated cells of the ventral mesencephalon of the mouse embryos (ED 15).[4] But, when these cells were cultured in vitro for 14 days and then were incubated with mouse IFN-gamma for 2 days, both H-2 and Ia antigens could be detected on the cells. And also $H\text{-}2K^k$ and Ia^k antigens were found in the brain of behaviorally compensated C57BL/6 mice ($H\text{-}2^b$) that had been transplanted two months earlier with allogeneic DA-rich cells from C3H/HeN mouse ($H\text{-}2^k$) embryos. These findings indicate that the immune surveillance system could not detect the allogeneic H-2 antigens, as the brain is a so-called the immunologically privileged site.

Table 1. H-2 and Ia Antigens on DA-rich Cells from C3H/HeN Mouse Embryos (ED 15).

normal development		Fetus (15ED[a])	Newborn (2-day-old)			Adult (10-wk-old)
in vivo brain cells	H-2	−	−(→+)[b]			−
	Ia	−	−(→+)[b]			−
after cell culture		2 days (17ED[a])[c]	7 days (1-day-old)[c]	14 days (8-day-old)[c]	21 days (15day-old)[c]	
in vitro brain cells	H-2	−(→−)[b]	−(→−)[b]	±(→++)[b]	−(→++)[b]	
	Ia	−(→−)[b]	−(→−)[b]	±(→++)[b]	−(→+)[b]	
transplanted transplantation			1 wk (1-day-old)[c]	2 wks (8-day-old)[c]	5 wks (4-wk-old)[c]	8 wks (7-wk-old)[c]
transplanted brain cells	H-2		++	+	+	+
	Ia		++	+	+	+

a, embryonal day(ED); b, co-cultured with mouse interferon-gamma; c, age of brain cells.

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INDEX

AD = Alzheimer's Disease
PD = Parkinson's Disease