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Review

Cholesterol homeostasis in neurons and glial cells

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Abstract

Cholesterol is highly enriched in the brain compared to other tissues. Essentially all cholesterol in the brain is synthesized endogenously since plasma lipoproteins are unable to cross the blood–brain barrier. Cholesterol is transported within the central nervous system in the form of apolipoprotein E-containing lipoprotein particles that are secreted mainly by glial cells. Cholesterol is excreted from the brain in the form of 24-hydroxycholesterol. Apolipoprotein E and cholesterol have been implicated in the formation of amyloid plaques in Alzheimer's disease. In addition, the progressive neurodegenerative disorder Niemann-Pick C disease is characterized by defects in intracellular trafficking of cholesterol.

Keywords: Cholesterol; Alzheimer's disease; Niemann-Pick C disease; Neurons; Glia

Contents

Abbreviations: CNS, central nervous system; ABC, ATP-binding cassette protein; Aβ, β-amyloid; AD, Alzheimer's disease; Apo, apolipoprotein; APP, amyloid precursor protein; HDL, high density lipoprotein; LDL, low density lipoprotein; NPC, Niemann-Pick type C

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1. Cholesterol synthesis and turnover in the brain

Cholesterol is highly enriched in the brain compared to other tissues. In mammals, the brain comprises less than 10% of total body mass yet ~25% of total body cholesterol resides in the brain. The total body pool of cholesterol in a wide range of mammals has been calculated to be ~2200 mg/kg body weight. Thus, whereas the average concentration of cholesterol in fresh tissues of whole animals is ~2.2 mg/g [1] in the brain the concentration of cholesterol is much higher—in the range of 15–20 mg/g [1]. Sterols in the brain are predominantly in the form of unesterified cholesterol with small amounts of desmosterol and cholesteryl esters. The major pool of cholesterol in the central nervous system (CNS[1]) is in myelin although neurons and glial cells also contain cholesterol. It has been estimated that 70–80% of the cholesterol in the brain of adult animals is present in myelin. Consequently, since ~90% of the cells of the brain are glial cells, neurons contribute only a small fraction of the total cholesterol in the brain [1]. In a mammalian cell, 50 to 90% of the cholesterol resides in the plasma membrane [2] where cholesterol is concentrated in detergent-resistant microdomains (lipid rafts) that are also enriched in sphingomyelin and glycosphingolipids.

Dietschy and Turley have calculated that in brains of mice, during the few weeks following birth, the cholesterol pool increases dramatically from 1.5 to 10.6 mg. Similarly in humans, the concentration of cholesterol in the brain increases from ~6 mg/g at birth to 23 mg/g in young adults [1]. The most reliable estimates of the rate of cholesterol synthesis in the CNS have been derived from measurements of the incorporation of either [^{3}H]water or [^{2}H]water into cholesterol. The highest rate of cholesterol synthesis in the CNS occurs during active myelination which, in many mammals, takes place shortly after birth. In the mature animal, when myelination has been completed, the rate of cholesterol synthesis declines to a rate that likely reflects cholesterol synthesis primarily in neurons and glial cells [1,3]. These data indicate that the high rate of cholesterol synthesis that occurs early in life is required for the production of myelin by oligodendrocytes.

The major source of cholesterol in most mammalian cells is de novo synthesis [3–5] although cholesterol can also be acquired from exogenously supplied plasma lipoproteins. Low density lipoproteins (LDLs) that contain cholesteryl esters are internalized by receptor-mediated endocytosis via the LDL receptor and related receptors. In addition, cholesteryl esters can be delivered to cells by selective lipid uptake from exogenously supplied high density lipoproteins (HDLs) via the scavenger receptor, SR-B1 [6]. Cholesterol metabolism in the CNS appears to be distinct from that in other tissues, most likely because the CNS and plasma compartments are separated by the blood–brain barrier. Several studies have demonstrated that essentially all of the cholesterol required for development of the brain and spinal cord is derived from endogenous synthesis within the CNS [7,8].

There is no convincing evidence that cholesterol derived from HDLs and LDLs is transferred from the plasma, across the blood–brain barrier, and into the CNS during either fetal or postnatal development [9]. This conclusion is supported by the following findings. Elimination of the LDL receptor in mice and rabbits does not alter the rate of cholesterol synthesis, or the concentration of cholesterol, in the brain [10,11]. In mice and humans lacking functional ABCG5/G8 intestinal sterol transporters, plant sterols accumulate in the plasma whereas only trace amounts of these sterols are found in the CNS [12,13]. Furthermore, disruption of either the SR-B1 gene or the ABCA1 gene (two genes that are involved in cholesterol homeostasis in plasma) in mice does not alter the rate of cholesterol synthesis or the cholesterol content in the CNS [14]. Taken together, these and other observations imply that plasma lipoproteins do not cross the blood–brain barrier and do not deliver significant amounts of cholesterol to the CNS. Nevertheless, a recent study has demonstrated that a selective uptake of cholesteryl esters from lipoproteins occurs in porcine cerebrovascular endothelial cells, a model of the blood–brain barrier [15,16].

In addition to limiting the entry of plasma lipoproteins into the brain, the blood–brain barrier also restricts the egress of cholesterol from the CNS. In rat brain, cholesterol has a long half-life of 4 to 6 months. However, when the rate of cholesterol synthesis in the CNS exceeds that required for cholesterol homeostasis there is a net export of cholesterol from the CNS to the plasma. The most important mechanism by which cholesterol is excreted from the brain is by conversion to 24(*S*)-hydroxycholesterol via cholesterol 24-hydroxylase (Fig. 1) [17,18]. Murine cholesterol 24-hydroxylase is encoded by the *Cyp46a1* gene, a

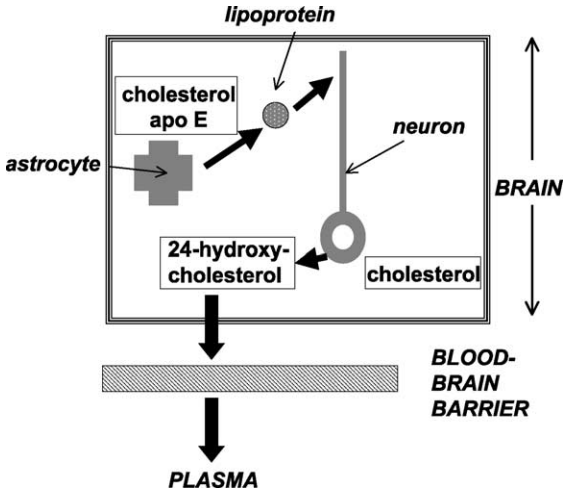

Fig. 1. Model of cholesterol homeostasis in the brain. Neurons synthesize cholesterol in cell bodies. However, additional cholesterol can be supplied to axons from glia-derived lipoproteins. Cholesterol and apo E are synthesized by glial cells (astrocytes) and associate to form lipoprotein particles. These lipoproteins can bind to neuronal surface receptors of the LDL receptor family. Excess cholesterol in the brain is converted to 24-hydroxycholesterol in neurons, crosses the blood–brain barrier, enters the plasma and is delivered to the liver for excretion into bile.

member of the cytochrome P450 family [19]. The expression of this enzyme is restricted to certain types of neurons in the brain, such as pyramidal cells of the cortex and Purkinje cells of the cerebellum [18–20]. There is little, if any, expression of cholesterol 24-hydroxylase in glial cells in the brain [18]. Cholesterol 24-hydroxylase activity is not maximally expressed in mouse brain until myelination is complete. Consistent with the restricted distribution of the enzyme, 24-hydroxycholesterol activity in mouse cerebellum is approximately 100-fold higher than in other tissues, excluding the adrenals [20]. In humans, 24-hydroxycholesterol is the major hydroxylated sterol excreted from the CNS [20]. Although small amounts of 27-hydroxycholesterol can be detected in the CNS, this sterol is thought to be primarily imported from the plasma since the brain contains only trace activities of cholesterol 27-hydroxylase.

Hydroxylation of the side-chain of cholesterol allows the sterol molecule to be transferred across a lipid bilayer orders of magnitude faster than cholesterol per se [21,22]. Nevertheless, the mechanism by which this oxysterol crosses the blood–brain barrier is not completely understood. Bjorkhem et al. have estimated that 6 mg of 24-hydroxycholesterol flow out of the human brain and into the plasma each day [23]. The 24-hydroxycholesterol that enters the circulation is subsequently removed by the liver and is excreted into bile (Fig. 1).

Mice lacking cholesterol 24-hydroxylase activity were recently generated [18]. These mice appeared outwardly normal, however, the serum level of 24-hydroxycholesterol was 63 to 83% lower, and the amount of this oxysterol in the brain was 98% lower, than in their wild-type counterparts. Disruption of the murine cholesterol 24-hydroxylase gene reduced the rate of cholesterol synthesis in the brain by 40%, whereas the brain content of cholesterol was unaltered [18]. These observations demonstrate that cholesterol 24-hydroxylase is responsible for the catabolism of at least 40% of the cholesterol that is turned over in the brain. Because cholesterol 24-hydroxylase is expressed in only a subset of neurons, these data also indicate that perhaps only 1% of the cells in the brain are responsible for 40% of cholesterol turnover in the brain [18]. Other mechanisms, that have not yet been identified but which perhaps involve processes in glial cells, must also be involved in mediating the turnover of cholesterol in the brain.

2. Roles of apo E- and cholesterol-containing lipoproteins in the nervous system

Many of the proteins involved in transporting cholesterol in lipoproteins in the circulation are also present in the CNS, suggesting that these proteins are involved in cholesterol transport among cells of the brain. For example, several members of the LDL receptor family (such as the LDL receptor, the LDL receptor-related proteins and the apo ER2 receptor), as well as apolipoproteins (apos) E, A1, D and J, and membrane transporters of the ATP-binding cassette (ABC) family (such as ABCA1, ABCG1 and ABCA4) [24,25], are expressed in the CNS. Cerebrospinal fluid, which is separated from the plasma compartment by the blood–brain barrier, contains a population of lipoproteins that are distinct from those in plasma [26–29].

The major CNS apolipoprotein is apo E and apo J is also abundant [30]. Experimental evidence suggests that apo E plays a central role in cholesterol metabolism in the nervous system. Apo E in the brain is synthesized primarily by glial cells (astrocytes and microglia) although a low level of apo E synthesis has also been detected in neurons [31]. Apo E and apo J in the CNS are present in cholesterol-containing lipoprotein particles that are the size and density of plasma HDLs [26–29]. The apo E-containing lipoproteins in the CNS are proposed to bind to neuronal surface receptors of the LDL receptor family, such as the LDL receptor, the LDL receptor-related proteins and the apo ER2 receptor, that can mediate uptake of the apo E-containing lipoproteins. Rothe and Muller have demonstrated that apo E-containing lipoproteins are taken up by Schwann cells and cultured dorsal root ganglion neurons [32]. In addition, distal axons of cultured rat sympathetic neurons take up both the lipid and protein components of human LDLs and HDLs and transport these molecules into the cell bodies [33]. Importantly, some lipoprotein receptors have recently been shown to play other roles in addition to their function in cholesterol uptake. For example, Herz and coworkers have demonstrated that several of these receptors initiate important signaling pathways within neurons upon binding apo E-containing lipoproteins [34]. These signaling pathways are particularly important during development of the nervous system [35,36].

Three common alleles of apo E are found in humans—apo E2, E3 and E4; apo E3 is the most frequently expressed apo E allele. The apo E2 and apo E4 proteins differ from apo E3 by only a single amino acid: apo E3 contains a cysteine residue at position 112 and an arginine at position 158 whereas apo E2 has cysteines at both positions 112 and 158, and apo E4 contains arginines at positions 112 and 158 [37]. Evidence that apo E is a key player in the nervous system comes from several fronts. As discussed in detail below (Section 3.3), inheritance of the apo E4 allele is the strongest known risk factor for the development of late-onset Alzheimer's disease [38–40]. After injury to the CNS or the peripheral nervous system, the synthesis of apo E by glial cells increases dramatically, by up to 150-fold, suggesting that apo E is required for repair processes [41–44]. In addition, the clearance of degenerating nerves from injured brain is impaired in *ApoE*$^{-/-}$ mice [45]. Studies from several research groups have reported that in neuronal cell lines apo E3 stimulates axonal growth whereas apo E4 inhibits growth [46–48]. However, the mechanisms by which apo E isoforms differentially modulate axonal growth are not yet understood.

Cholesterol is synthesized in cell bodies/proximal axons, but not in distal axons, of sympathetic neurons [49]. This observation was made in compartmented cultures of rat sympathetic neurons in which the cell bodies/proximal axons were

located in a fluid environment completely separate from that containing the distal axons [50,51]. Cholesterol that is synthesized in cell bodies is efficiently transported into distal axons [52]. Reduction of cholesterol synthesis in sympathetic neurons by treatment with the drug pravastatin (an inhibitor of the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase), in the absence of exogenously supplied lipids, reduced the rate of axonal extension by ∼50% [52]. The addition of cholesterol to either the axons or cell bodies of pravastatin-treated neurons restored a normal rate of axonal elongation. Similarly, when human plasma LDLs or HDLs were given to cell bodies/proximal axons of pravastatin-treated neurons, axonal elongation proceeded at the normal rate. Axon growth of these neurons also occurred normally when LDLs were given to the distal axons alone. In contrast, the addition of HDLs to the distal axons of pravastatin-treated neurons did not restore a normal rate of axonal growth [52,53]. These findings indicate that in these neurons, the endogenous synthesis of cholesterol supplies sufficient cholesterol for normal axonal extension at a rate (∼1 mm/day) similar to that in the intact animal. However, when the endogenous supply of cholesterol is insufficient, an exogenous source of cholesterol, in the form of lipoproteins, can be delivered to axons or cell bodies and utilized for axonal growth and/or repair. Furthermore, the differential uptake of LDLs and HDLs by axons versus cell bodies suggests that the spectrum of lipoprotein receptors expressed in cell bodies/proximal axons and distal axons is distinct [53].

Glial lipoproteins are thought to be the physiologically relevant source of apo E and cholesterol in the CNS. In recent studies, we have demonstrated that lipoproteins secreted by glial cells stimulate axonal growth of CNS neurons. For these experiments, apo E-containing lipoproteins were isolated from the culture medium of rat cortical glial cells (>90% astrocytes) and provided to distal axons of compartmented cultures rat retinal ganglion cells (CNS neurons) [54]. The presence of the lipoproteins increased the rate of axonal extension by ∼50%. Inhibition of cholesterol synthesis in the glial cells reduced the secretion of both apo E and cholesterol and abrogated the growth stimulatory effect of the glia-derived lipoproteins. These experiments suggested that apo E or cholesterol secreted by astrocytes was responsible for the growth stimulatory effect. Although neither cholesterol nor apo E alone stimulated axon growth, intact glial lipoproteins containing apo E and cholesterol did enhance the growth. Interestingly, axonal extension was stimulated only when the lipoproteins were given to the distal axons, whereas when the glial lipoproteins were provided to cell bodies axon growth was not increased [54]. A receptor of the LDL receptor family was implicated in the growth stimulatory effect since the receptor-associated protein (RAP), prevented the increase in axonal growth induced by the glial lipoproteins [54]. It is not yet clear, however, whether endocytosis of the lipoproteins by the neurons is required for maximal axonal extension or whether axon growth can be enhanced by a signaling pathway that is induced when the apo E-containing lipoproteins bind to the receptor in the absence of internalization of the ligand.

In addition to performing a role in axonal growth, glial lipoproteins also promote synaptogenesis of rat retinal ganglion neurons [55]. The formation of synaptic contacts is important for brain development and for synaptic plasticity in the adult CNS. Upon treatment of cultured retinal ganglion cells with glia-conditioned medium, the cholesterol content of the neurons was increased, as indicated by staining with the cholesterol-binding reagent, filipin. The active component of glia-conditioned medium that was required for the increase in synaptic activity was shown to be cholesterol [55]. Cholesterol is also thought to play a role in generation of action potentials in neurons by altering the permeability characteristics of the plasma membrane surrounding the axon [1].

3. Cholesterol and Alzheimer's disease

Alzheimer's disease (AD) [56] is a progressive neurodegenerative disorder that results in cognitive impairment and memory loss late in life. Histological hallmarks of AD are the deposition of extracellular β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles in the brain. AD brains also show a loss of neurons and synapses in the neocortex, hippocampus and sub-cortical regions. As discussed above (Section 2), cholesterol-bearing lipoproteins in the brain are present in the form of HDL-sized particles, many of which contain apo E. These lipoproteins are secreted from glial cells such as astrocytes and microglia. In humans, the three common alleles of apo E are apo E2, E3 and E4, and inheritance of the apo E4 allele is the strongest known genetic risk factor development of late-onset AD [38–40].

3.1. Relationship between the cholesterol content of plasma and brain

Most studies suggest that an increased content of cholesterol in the brain correlates with an increased risk of developing AD. Nevertheless, not all studies support this view and epidemiological studies on the association between plasma cholesterol levels and the development of AD have produced conflicting conclusions [56–60]. The cholesterol content of the brain decreases with advancing age [61] and this decrease appears to be accelerated in individuals with AD [62].

In 2002, Kivipelto et al. reported in a prospective study that an elevated level of plasma cholesterol is an independent risk factor for AD [60]. However, data from the Framingham study indicated that plasma cholesterol levels are not associated with the risk of developing AD [63]. Although considerable uncertainty remains concerning the relationship between plasma cholesterol levels and AD, retrospective epidemiological studies have suggested that the use of cholesterol-lowering drugs, the statins might decrease the incidence of AD [64,65]. Moreover, in studies with a transgenic mouse model of AD, a diet containing a high content of cholesterol

not only increased the level of cholesterol in the brain [66] but also accelerated Aβ deposition [67,68]. In other studies, a high-cholesterol diet resulted in a marked activation of astrocytes and microglia in the hippocampus and cerebral cortex of wild-type and apo E knock-out mice [69]. These findings indicate that the plasma concentration of cholesterol can potentially influence the level of cholesterol in the brain and the deposition of Aβ. Nevertheless, plasma lipoproteins are apparently unable to cross the blood–brain barrier, therefore, the explanation for this correlation is not clear.

As discussed above (Section 1), cholesterol is eliminated from the brain in the form of 24(*S*)-hydroxycholesterol which crosses the blood–brain barrier and enters the plasma far more readily than does cholesterol per se (Fig. 1). Several studies have indicated that the amount of 24-hydroxycholesterol is higher in plasma and cerebrospinal fluid of AD patients than in unaffected individuals [70–72], suggesting that the turnover of cholesterol in the brain is increased during the neurodegenerative changes of AD. In contrast, in other studies the amount of 24-hydroxycholesterol in plasma was found to be lower in advanced AD patients, and also in a murine model of AD, than in controls [73,74]. These apparently contradictory conclusions might be rationalized if one considered that an increased plasma level of 24-hydroxycholesterol reflected ongoing neurodegeneration and/or demyelination, whereas a decrease in the amount of plasma 24-hydroxycholesterol reflected a selective loss of the population of neurons that express cholesterol 24-hydroxylase [75]. Thus, a reduction in cholesterol levels in the brains of AD patients might be the result, rather than the cause, of the enhanced neurodegeneration.

3.2. Cholesterol and β-amyloid production

An increased production of β-amyloid is thought to be centrally involved in the pathophysiology of AD [76,77]. Aβ is a peptide fragment produced upon proteolytic cleavage of the amyloid precursor protein (APP), a transmembrane protein that is cleaved at α, β or γ sites by α-, β- or γ-secretase, respectively (Fig. 2). The normal function of APP is currently unknown. The majority of APP is cleaved by α-secretase and the remainder is cleaved by β-secretase. The C-terminal fragments derived from these cleavages are subsequently cleaved by γ-secretase. The products of α-cleavage do not result in abnormal brain pathology. In contrast, the products generated by the β- and γ-secretases are the 40- and 42-amino acid Aβ fragments that are amyloidogenic and result in formation of senile plaques [78,79]. These plaques in the brain consist of deposits of Aβ surrounded by dystrophic neurites, activated microglia and reactive astrocytes.

Interestingly, cholesterol accumulates in mature plaques in human AD brains as well as in the brains of APP transgenic mice [80]. In rabbits and APP transgenic mice, a high-cholesterol diet induces Aβ deposition in the brain [67]. The observation that a reduced level of cellular cholesterol inhibits the formation of Aβ in hippocampal neurons and enhances

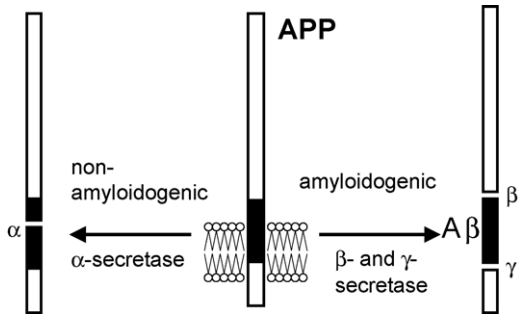

Fig. 2. Processing of amyloid precursor protein (APP). In the major pathway of APP proteolysis, APP is cleaved by α-secretase at the α position within the Aβ region (black box). The products of α-cleavage are non-amyloidogenic (non-pathological). On the other hand, amyloidogenic cleavage of APP is catalyzed first by β-secretase and subsequently γ-secretase cleaves the protein to generate a pathological Aβ fragment.

the non-amyloidogenic cleavage of APP by α-secretase provides further support for the involvement of cholesterol in AD [81,82]. Cholesterol is also thought to stimulate the generation Aβ since the β- and γ-secretase complexes are enriched in cholesterol-rich microdomains (lipid rafts) [83,84]. In contrast, the α-secretase resides outside these domains. Ehehalt et al. [85] reported the existence of two distinct pools of APP. The pool of APP in lipid rafts was cleaved by the β-secretase thereby initiating amyloidogenic APP processing, whereas the pool of APP outside rafts underwent cleavage by α-secretase resulting in non-amyloidogenic processing. Enigmatically, although γ-cleavage of APP occurs in cholesterol-rich lipid rafts, the activity of γ-secretase is cholesterol independent [86].

Brown et al. have reported that cholesterol 24-hydroxylase activity is predominantly expressed in the vicinity of senile plaques and suggested that 24-hydroxycholesterol might increase Aβ generation [87]. On the other hand, 24-hydroxycholesterol was found to inhibit Aβ production in cultured neurons [87]. Thus, the role of 24-hydroxycholesterol in Aβ production and in AD is not clear.

3.3. Apo E and Alzheimer's disease

The three common isoforms of apo E in humans are E2, E3 and E4. Inheritance of the apo E4 allele has been firmly established as a strong risk factor for the development of late-onset AD [38]. Differences among apo E isoforms with respect to their toxicity in neurons [88], and their relative binding affinities to Aβ [89] and tau [90] have been reported, as well as differences in the antioxidant properties [91]. In studies with transgenic mouse models of AD, in which mutant forms of APP were expressed concurrently with the E2, E3 or E4 isoforms of human apo E, the apo E isoform-specific effects on Aβ levels and plaque formation that are observed in AD patients (E2 < E3 < E4) were recapitulated [92,93]. In addition, in aged mice expressing human apo E4, but not apo E3, the amount of in soluble Aβ42 was markedly increased in the hippocampus [94]. Analysis of a cohort of AD patients

showed that apo E4 is associated with a decreased level of soluble Aβ42 in cerebrospinal fluid of healthy control and AD patients in a gene-dosage manner, whereas Aβ deposition in the brain is increased [95]. Moreover, in an APP transgenic mouse model of AD, apo E3 reduced Aβ deposition in a gene-dosage manner [94]. These observations suggest that human apo E can modulate Aβ levels and Aβ deposition in the brain in an isoform-specific manner. The studies also indicate that a potential therapeutic approach for treatment of AD might be to increase the level of apo E in the brain [96]. However, the mechanism by which apo E is involved in AD has not yet been elucidated.

Several murine models of AD are available that are also transgenic for the different apo E isoforms. Each of these models has limitations that complicate our understanding of the link among apo E, cholesterol and AD. For example, Mann et al. [97] recently reported that apo E knock-in mice that expressed human apo E2, E3 or E4 under control of endogenous regulatory elements, had significantly different plasma concentrations of cholesterol and different amounts of apo E in their brains. However, the level of cholesterol in the brains of these mice was the same for each apo E isoform. In addition, in this model of AD, the presence of any of the three human apo E isoforms markedly increased the level of Aβ in the brain [97]. Importantly, however, the animals used in this study were only 4 to 8 weeks old. Thus, further studies using older mice are required to determine whether or not apo E protects against the deposition of Aβ.

In addition to apo E, another apolipoprotein, apo A1, is also present in the CNS. Unlike apo E, however, which is synthesized in situ in the brain, apo A1 is thought to be imported into the brain from the plasma although the mechanism of import has not been identified. Fagan et al. [98] reported that a deficiency of apo A1 in a murine model of cerebral amyloidosis (a disease with a phenotype similar to that of AD) markedly reduced the cholesterol content of the brain. However, the absence of apo A1 did not alter the cholesterol level in the CSF, the apo E content of the brain, or Aβ pathology in this AD-like model. Thus, the authors concluded that the level of apo E in the brain, rather than the cholesterol concentration, might be an important factor in regulating Aβ levels.

In addition to the extracellular deposition of Aβ another characteristic hallmark of AD is the presence of intracellular neurofibrillary tangles that result from hyperphosphorylation of the microtubule-associated neuronal protein, tau [79]. In cultured neurons and AD brains, apo E is proteolytically cleaved to generate C-terminally truncated fragments that produce neurofibrillary tangle-like inclusions in cultured neurons and transgenic mice, as well as neurodegeneration in the mice [31,99,100]. The proteolysis of apo E in neurons, and the enhanced phosphorylation of tau were particularly prominent in the neocortex and the hippocampus, regions of the brain that are the most affected in AD. These changes were more extensive in transgenic mice expressing apo E4 than in those expressing apo E3.

In synaptic plasma membranes of young mice, the exo-facial leaflet contains ∼15% of total cholesterol while the remaining 85% of cholesterol is in the cyto-facial leaflet. Although the total content of cholesterol in synaptic plasma membranes does not change with age, the transbilayer distribution of cholesterol is altered upon aging [101]. For example, the cholesterol content of the exo-facial leaflet of synaptic plasma membranes of aged mice is double that of young mice [102]. In addition, in apo E4 knock-in mice the amount of cholesterol in the exo-facial leaflet of synaptic plasma membranes is approximately double that in apo E3 knock-in mice or wild-type mice [103]. These data suggest that a greater abundance of lipid rafts in apo E4 knock-in mice might promote the amyloidogenic processing of APP in those domains [104]. Furthermore, it has been suggested that in brains of aged non-human primates, as well as in AD and Down's syndrome patients, a cholesterol-rich environment, such as that in lipid rafts, accelerates the association of the ganglioside GM1 with these domains thereby providing a "seed" for Aβ aggregation [105,106].

3.4. Apolipoprotein J in Alzheimer's disease

Apo J (also called clusterin) is a heterodimeric, sulfated glycoprotein that is expressed in the CNS. Based on the finding that apo J in the CNS is associated with HDL-sized lipoproteins, this apolipoprotein has been proposed to play a role in lipid transport. In vitro studies have demonstrated that neurons and astrocytes both express apo J mRNA whereas only astrocytes secrete this protein under normal conditions [107]. In human plasma ∼90% of soluble Aβ is associated with lipoprotein particles [108], and in the CSF apo J is associated with Aβ [109,110].

Several studies indicate that apo J might be neuroprotective although the data in support of this idea are not entirely consistent. For example, the levels of apo J protein and mRNA are increased in the brain after injury [111,112]. Apo J has also been reported to prevent the polymerization and aggregation of Aβ as well as the proteolytic degradation of synthetic Aβ in vitro [113]. In addition, apo J has been shown to protect neurons from Aβ toxicity in vitro [114] and to facilitate the clearance of Aβ from the CSF via megalin/grp330, a member of the LDL receptor family [115–117]. In the cortical region of brains of AD patients, the percentage of neurofibrillary tangle-negative neurons that contain apo J is markedly higher than in non-AD controls, and neurofibrillary tangle-positive neurons contain little apo J suggesting that apo J might have a neuroprotective effect [118]. In AD, the amounts of apo J protein and mRNA are increased in the hippocampus and entorhinal cortex but not in the cerebellum [119,120]. In addition, apo J is present in senile plaques [121].

In contrast to the proposed neuroprotective effect of apo J, however, an elevation in the level of apo J in AD did not correlate with the number of Aβ deposits [122]. Nor in another study were differences found in the apo J levels in the CSF of AD patients versus controls [123]. Polymorphisms

in the human apo J gene do not appear to be related to the prevalence of AD [124].

Apo J has also been suggested to promote Aβ formation in the brain and to increase the neurotoxicity of Aβ. DeMattos et al. [125] bred a transgenic mouse model of AD with apo J-deficient mice and found that the absence of apo J decreased the neuritic dystrophy associated with the amyloid deposits. Furthermore, less brain damage was observed in apo J-deficient mice than in wild-type mice following ischemia [126]. One possible explanation for the seemingly contradictory effects of apo J is that apo J in neurons and apo J secreted by glia might play distinct roles in AD.

3.5. Cholesterol lowering as a potential treatment of Alzheimer's disease

Currently, two types of pharmaceuticals are available for treatment of AD dementia—acetylcholinesterase inhibitors and a *N*-methyl-D-aspartate antagonist. Although these drugs delay, and/or temporarily relieve, some symptoms of AD the progression of the disease is not altered [80].

Statins are drugs that inhibit cholesterol synthesis and are widely used for treatment of hypercholesterolemia. These compounds are now also being actively considered for treatment of AD. Two retrospective epidemiological studies have shown that the use of statins for treatment of hypercholesterolemia reduced the risk of AD by 40 to 70% [64,65]. Moreover, when guinea pigs were treated with simvastatin the level of Aβ in the CSF and in brain homogenates was significantly reduced [127]. Simvastatin and lovastatin also reduced the intracellular and extracellular levels of Aβ in primary cultured neurons. Moreover, reduction of cholesterol in peripheral neurons and neuronal cell lines promoted expression of α-secretase and the non-amyloidogenic α-cleavage of APP [82,128]. Recently, Chauhan et al. [129] treated a transgenic mouse model of AD with lovastatin or pravastatin for 1 month and found that the level of Aβ decreased and the α-secretase cleavage of APP in the brain increased in a dose-dependent manner. Moreover, statins reduced the plasma level of 24-hydroxycholesterol in AD patients without lowering the level of apo E [130]. Interestingly, donepeziol hydrochloride, an acetylcholinesterase inhibitor, that is widely used for treatment of AD, reduced the plasma cholesterol concentration [131,132]. On the other hand, two large prospective studies failed to show a beneficial effect of statins for treatment of AD [133,134]. However, since the latter studies focused primarily on cardiovascular disease, the potential beneficial effects of statins for treatment of AD need to be further evaluated [135,136]. Other clinical studies have shown that treatment of individuals with four different statins did not significantly reduce Aβ levels in either the plasma or the CSF [137,138].

An important factor to be considered when evaluating the use of statins for the treatment of AD is the hydrophobicity of the drug. Lovastatin and simvastatin are more hydrophobic than pravastatin and therefore cross the blood–brain barrier more readily than does the more hydrophilic pravastatin [139,140]. However, none of these statins appears to be more beneficial than the others for the treatment of AD patients [64,65] and each statin reduces the level of plasma 24-hydroxycholesterol in AD patients to a similar extent [130]. Statins have also been reported to have anti-inflammatory effects in vitro and in vivo [141–143] which might account for some of their beneficial effects in the treatment of AD. Thus, data concerning the effectiveness of statins for treatment of AD are somewhat contradictory and further studies are required to determine if, and by what mechanisms, statins delay the onset of dementia and/or decrease the risk of developing AD.

3.6. Relationship between other genes of cholesterol metabolism and Alzheimer's disease

A role for lipids, particularly cholesterol, is now strongly implicated in the development of AD. In addition to cholesterol, the level of cholesteryl esters has also been proposed to modulate Aβ production although neurons contain only very small amounts of cholesterol esters compared to most other types of cells. For example, the generation of Aβ was significantly enhanced when the cellular level of cholesteryl esters was increased [144]. In addition, in cells lacking acyl-CoA:cholesterol acyltransferase activity a decrease in the level of cholesteryl esters, and an increase in unesterified cholesterol, occurred and production of Aβ was reduced [144]. Inhibitors of this acyltransferase have also been shown to diminish Aβ formation in neuronal and non-neuronal cells [145]. In support of an association between the amount of cholesteryl esters and Aβ formation, Hutter-Paier et al. recently reported that treatment of a transgenic mouse model of AD with an inhibitor of acyl-CoA:cholesterol acyltransferase not only markedly reduced the level of Aβ in the brain but also decreased the formation of Aβ plaques and improved spatial learning [145]. Moreover, a common polymorphism in the gene encoding acyl-CoA:cholesterol acyltransferase is associated with low cholesterol levels in the CSF, low Aβ levels in the brain, and a reduced risk of AD [146]. Thus, taken together, several studies suggest that inhibitors of acyl-CoA:cholesterol acyltransferase are potential therapeutic agents for treatment of amyloidogenesis in AD patients.

Investigators have also found polymorphisms in other genes that encode proteins involved in cholesterol metabolism in the brain. As discussed above, polymorphisms in the gene encoding apo E, a key lipid transport protein, are associated with the common late-onset familial and sporadic forms of AD [38–40]. Interestingly, the ratio of 24-hydroxycholesterol to cholesterol and the level of Aβ42 in the CSF of AD patients were found to be associated with a polymorphism in the cholesterol 24-hydroxylase gene [147,148]. Moreover, the level of 24-hydroxycholesterol is increased in the plasma and CSF of AD patients [70–72]. Polymorphisms in the cholesterol 24-hydroxylase gene are also associated with an increased deposition of Aβ in the brain, an increased

phosphorylation of tau, and an increased risk of developing AD [149,150].

Another protein that might participate in the removal of cholesterol from cells in the brain is the ATP-binding cassette transporter A1 (ABCA1). This protein is required for the efflux of cholesterol and phospholipids from cells to an acceptor protein such as apo A1 or apo E for HDL formation [151,152]. Wahrle et al. [153] reported that ABCA1 deficiency in mice results in a significant reduction in the level of apo E in the brain and CSF, and a marked decrease in the cholesterol content of CSF. Moreover, primary astrocytes cultured from ABCA1-deficient mice secrete lipoproteins that are smaller in size, and contain a reduced amount of apo E and cholesterol, compared to those from wild-type mice [153]. Clinical studies have indicated that polymorphisms in the ABCA1 gene might be associated with altered levels of cholesterol in the CNS, as well as with an increased risk and decreased age of onset of AD [154,155].

Apo D is another glycoprotein that is associated with lipoproteins and has been implicated in cholesterol metabolism in the brain. Apo D is present in senile plaques in AD brains. The amount of apo D in the brain and CSF is increased in AD patients and in a transgenic mouse model of AD, independently of apo E levels [156–158]. Whereas apo E is localized to the amyloid core of plaques, apo D is found around the amyloid core, suggesting that apo D might function independently of apo E in the deposition of Aβ [159]. An allele of apo D is strongly associated with an increased risk of AD among Finnish and African-American individuals [160,161].

The LDL receptor-related protein is expressed in the CNS and has been suggested to be important for the clearance of Aβ from the brain [162,163]. Moreover, several studies have reported a genetic association between the LDL receptor-related protein and late-onset AD [164–166]. In contrast, in other studies no correlation was observed between polymorphisms in this gene and AD [167,168].

4. Cholesterol and Niemann-Pick type C disease

Niemann-Pick type C (NPC) disease is an autosomal recessive, fatal, neurovisceral lipid storage disorder caused in 95% of cases by mutations in the NPC1 protein. The remaining 5% of cases are caused by mutations in the NPC2 (also called HE1) protein. The clinical manifestations and rate of progression of NPC disease vary among affected individuals. The most severe clinical phenotype is a progressive neurodegeneration that affects primarily the thalamus and the Purkinje cell layer in the cerebellum causing cerebellar ataxia, supranuclear gaze palsy, cataplexy, epilepsy and other neurological impairments. In adult cases, progressive dementia and psychiatric manifestations have also been reported. At the biochemical level, NPC disease is characterized by an accumulation of unesterified cholesterol and other lipids in peripheral tissues, particularly in the liver and spleen (hepatosplenomegaly) [169–172]. At the cellular level, the most prominent characteristic of all cells lacking functional NPC1 or NPC2 is an accumulation of cholesterol, gangliosides and other lipids in endosomal vesicles [173–178]. To date there is no effective treatment for NPC disease beyond symptomatic therapy [179].

Animal models of NPC disease include two murine models (each having a null mutation in the NPC1 gene), a feline model, and a recently described murine NPC2 hypomorph [180–183]. The most widely used, and best-characterized, murine model of NPC disease is the BALB/cNctr*npc*^{N} mouse that completely lacks NPC1 protein [181]. Mice that are homozygous for a mutation in NPC1 are asymptomatic at birth, but by 5 weeks of age they develop ataxia and other neurological symptoms similar to those of human NPC disease. The lifespan of *Npc1*$^{-/-}$ mice is 12 to 15 weeks. The recently generated NPC2 hypomorphic mouse retains up to 4% of the normal level of the NPC2 protein; disease progression in this mouse is indistinguishable from that in the NPC1-null mouse. From cross-breeding studies between NPC1- and NPC2-deficient mice it was concluded that these two proteins act in the same pathway [183].

4.1. Structure and function of the NPC1 and NPC2 proteins

The genes encoding NPC1 and NPC2 have been cloned [181,184]. NPC1 is an integral membrane protein consisting of 1278 amino acids. At steady state, NPC1 resides mainly in the late endosomes/lysosomes but also cycles through the Golgi apparatus [185,186], reviewed in [187]. As depicted in Fig. 3, the NPC1 protein contains 13 transmembrane domains separated by heavily glycosylated lumenal loops. Five of the transmembrane domains exhibit sequence homology to the putative sterol-sensing domains that have been identified in other proteins involved in sterol metabolism, such as 3-hydroxy-3-methylglutaryl-CoA reductase and the sterol-response element binding protein cleavage-activating pro-

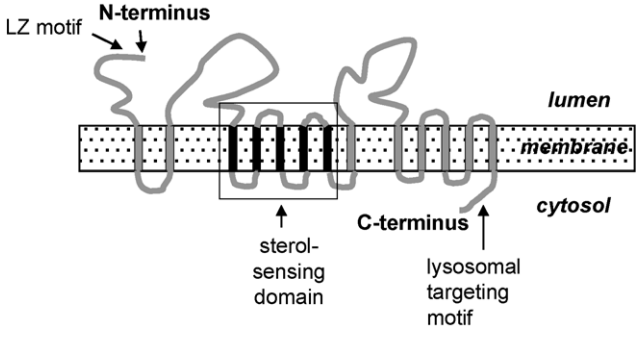

Fig. 3. Topological model of the NPC1 protein. NPC1 is a late endosomal/lysosomal transmembrane protein (membrane shown as stippled rectangle) that is predicted to contain 13 transmembrane domains. Five of the transmembrane regions (black bars) form the sterol-sensing domain. The lumenal N-terminus of the protein contains a leucine-zipper (LZ) motif and the cytosolic C-terminus contains a lysosomal targeting sequence.

tein, SCAP [188]. Other domains of the NPC1 protein include a di-leucine lysosomal targeting motif, and a leucine zipper motif that might indicate an interaction between NPC1 and other, as yet unidentified, proteins [189–194]. In contrast to NPC1, the NPC2 protein is a small, soluble glycoprotein consisting of 151 amino acids that resides in the lumen of late endosomes/lysosomes [195–198]. The crystal structure of NPC2 reveals a hydrophobic binding site [195] that can bind cholesterol with high affinity [196]. The structure–function relationships of both NPC proteins have been the subject of recent reviews [199,200]. It is noteworthy that although the genes encoding NPC1 and NPC2 have been described in detail the exact functions of these proteins remain unknown.

The presence of a sterol-sensing domain in NPC1 (Fig. 3), together with the observation that large amounts of unesterified cholesterol accumulate in multivesicular late endosomes/lysosomes in NPC1-deficient cells [173,174], indicate that NPC1 participates in the intracellular trafficking of cholesterol, particularly in the egress of unesterified cholesterol from late endosomes/lysosomes. Current information on the role of NPC1 in the intracellular trafficking of cholesterol has recently been reviewed [201,202]. A model for the mechanism of action of NPC1 has been proposed (Fig. 4) in which exogenously supplied LDLs that contain cholesteryl esters are endocytosed by a member of the LDL receptor family via clathrin-coated pits. Upon reaching late endosomes/lysosomes the cholesteryl esters of LDLs are hydrolyzed to unesterified cholesterol that is subsequently shuttled to the endoplasmic reticulum and the plasma membrane via poorly defined, NPC1-dependent pathways [203–205]. Loss of NPC1 function leads to formation of cholesterol-rich, multilamellar or multivesicular late endosomes/lysosomes,

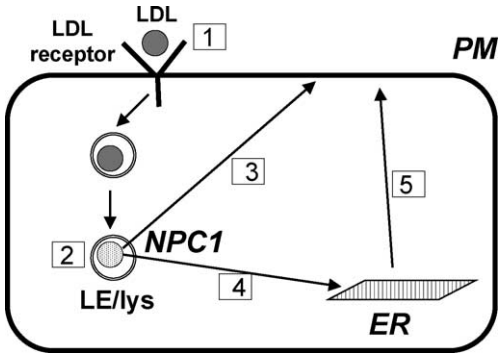

Fig. 4. Involvement of NPC1 in intracellular cholesterol trafficking. (1) Low density lipoproteins (LDL) undergo endocytosis via LDL receptors on the cell surface. In late endosomes/lysosomes (LE/lys) cholesteryl esters within LDL are hydrolyzed to unesterified cholesterol (2) that is subsequently transported throughout the cell, particularly to the plasma membrane (PM) (3) and endoplasmic reticulum (ER) (4) by mechanisms that involve NPC1, a transmembrane protein that is located in late endosomes/lysosomes. In the absence of functional NPC1, unesterified cholesterol is sequestered in late endosomes/lysosomes and the content of cholesterol in the plasma membrane and ER is diminished. The transport of cholesterol from its site of synthesis in the endoplasmic reticulum to the plasma membrane (5) is also impaired in NPC1-deficient cells.

that also contain increased amounts of glycosphingolipids and the unusual phospholipid, lyso-bisphosphatidic acid [174]. Endogenously synthesized cholesterol has also been reported to be sequestered within the endosomal pathway of NPC1-deficient fibroblasts [206].

Although unesterified cholesterol accumulates in lysosomes/late endosomes in cells lacking NPC1, the association between this accumulation and the neurological problems characteristic of NPC disease has not yet been elucidated. Indeed, it is not even clear whether or not disease progression is caused directly by the accumulation of cholesterol. Instead, the major problem caused by the impaired cholesterol trafficking out of lysosomes/late endosomes might be that a deficit of cholesterol is created in other regions of the cell, such as the plasma membrane, mitochondria and the endoplasmic reticulum. Nor is the hypothesis that cholesterol is the primary offending metabolite unanimously accepted since, in addition to cholesterol, gangliosides and other glycosphingolipids also accumulate in late endosomes [87,175–178]. Moreover, in NPC1-deficient brains there is a net accumulation of gangliosides, but not of cholesterol [207]. Thus, it has been proposed that the accumulation of cholesterol occurs secondarily to an accumulation of glycosphingolipids [208]. To test this idea, NPC1-deficient mice were treated with *N*-butyldeoxynojirimycin (NB-DNJ), an inhibitor of complex ganglioside biosynthesis. Disease progression was slightly attenuated in one study with NPC1-deficient mice [176]. This inhibitor has also been used in a human NPC patient [209,210] and is currently being tested in phase 2 clinical trials in the UK. To determine if reduction in the amount of gangliosides in the brain would be beneficial for treatment of NPC disease, *Npc1*$^{-/-}$ mice were crossed with mice deficient in complex ganglioside biosynthesis [208,211]. Although gangliosides did not accumulate in brains of the double knock-out mice, the life-span and neurological symptoms typical of NPC1 deficiency were not changed [211]. This study indicates, therefore, that the accumulation of gangliosides in the brain most likely does not directly cause the neurological abnormalities in NPC disease.

Regardless of the function of the NPC proteins, and the nature of the metabolite that initially accumulates, it is now becoming clear that NPC1-deficient cells exhibit endosomal trafficking defects that go beyond the formation of lipid-laden late endosomes. For example, in NPC1-deficient fibroblasts the mobility of late endosomal tubulovesicular structures is greatly reduced compared to that in wild-type fibroblasts [212]. Moreover, the mannose-6-phosphate receptor becomes trapped in late endosomes suggesting that mistargeting of some proteins might occur [174]. Recent studies have also demonstrated that abnormalities in the late endosomal pathway induced by dysfunctional NPC1 also induce defects in early endosomes. For example, the ganglioside GM1 accumulates in early endosomes of NPC1-deficient Chinese hamster ovary cells [213]. Moreover, the normally rapid recycling of endocytosed material from early endosomes to the plasma membrane is impaired in NPC1-deficient human

skin fibroblasts, possibly because the level of cholesterol is elevated in early endosomes [214]. Interestingly, in NPC1-deficient cells over-expressing Rab7 normal endosomal trafficking is restored and cholesterol no longer accumulates [215].

4.2. Abnormalities in NPC1-deficient brains

Early studies on the distribution of NPC1 in the CNS suggested that this protein is primarily expressed in glial cells, particularly in astrocytic end-feet [216]. However, it is now clear that neurons also express NPC1 [217–220] and that in murine sympathetic neurons NPC1 is present not only in cell bodies but also in distal axons [218]. Messenger RNA hybridization techniques demonstrated that expression of NPC1 is not developmentally regulated and that the level NPC1 mRNA is particularly high in the cerebellum [219]. NPC2 appears to be expressed in cells that express NPC1. Recently, our laboratory found that NPC2 is present in distal axons, as well as cell bodies, of murine sympathetic neurons (B. Karten and J.E. Vance, unpublished data). The NPC2 protein has also been detected in the postsynaptic density although the functional significance of this observation is not yet clear [221].

Investigations of the consequences of NPC disease in the CNS have mainly consisted of histological assessments of brain tissues from human cases and, more frequently, from the murine model of NPC disease. The latter model has been particularly useful in defining the time course of disease progression and for elucidating the primary events that occur during the degenerative process. Ultrastructural and morphological abnormalites are seen in both neurons and glial cells of NPC1-deficient brains [222–225]. Despite the clinical heterogeneity of human NPC disease, similar pathological changes in CNS morphology are consistently observed (reviewed in [226]). Neuronal loss is evident mainly throughout the thalamus and the Purkinje cell layer in the cerebellum [227–229]. Interestingly, not all Purkinje cells are affected to the same extent and a patterned cell loss that correlates with zebrin expression has been described [230]. Little is known so far about which pathways ultimately lead to neuronal death. Moreover, it has not yet been established whether apoptosis or necrosis is the main cause of neuronal death. Several reports have indicated that a “dying back” mechanism occurs in neurons in NPC1-deficient brains and that a slow degeneration of the terminal field of axons and dendrites precedes cell death [216,229]. When *Npc1*$^{-/-}$ mice were crossed with mice expressing a transgene encoding human Bcl-2 in neurons, neuronal death characteristic of NPC disease was not attenuated implying that any apoptosis that occurred was independent of Bcl-2 [231].

Among the most prominent morphological features of NPC brain is the presence of inclusion bodies in neurons and glia [176,207,222–224,227,232]. These inclusion bodies contain cellular material such as lipids, myelin bodies, lysosomal debris and ubiquitinylated proteins. Multilamellar and multivesicular bodies containing unesterified cholesterol, as well as the gangliosides GM2 and GM3, were found within neuronal perikarya [176,232]. The number and size of inclusion bodies increased with advancing age. It is noteworthy, however, that lipid accumulation occurs during the very early stages of NPC disease, prior to the onset of neurological symptoms and neuronal loss. For example, neurons isolated from embryonic and early postnatal *Npc1*$^{-/-}$ mice had already accumulated large quantities of cholesterol as indicated by a characteristic punctate filipin staining [218,220,232]. Although filipin staining is frequently used as an indicator of cholesterol accumulation/redistribution in NPC1-deficient cells, this technique does not provide a quantitative assessment of cholesterol mass. Recently, the compound BC theta has been used as a more sensitive stain for unesterified cholesterol compared to filipin [233]. Using BC theta, Reid et al. detected cholesterol-filled vesicles within neurons in the brains of *Npc1*$^{-/-}$ mice as early as 9 days after birth [234].

Other prominent features of neuronal pathology of NPC disease include the presence of axonal spheroids, meganeurites and ectopic dendrites [176,224–227,235]. Cortical pyramidal neurons in human and feline NPC brains display enlarged axon hillocks and meganeurites that are covered with dendritic/neuritic spines [225]. Numerous axonal spheroids, that appear upon histochemical examination as homogeneous or finely granular focal axonal swellings, are found throughout NPC1-deficient brains [226,227]. Interestingly, similar morphological abnormalities have been reported in individuals suffering from other lysosomal storage diseases [236,237].

All three of the major types of glial cells (astrocytes, oligodendrocytes and microglia) in the brain are affected in NPC disease. Baudry et al. showed that microglial activation is one of the earliest abnormalities seen in brains of the murine model of NPC disease [223]. As early as 2 weeks after birth, BALB/cNctr-*npc1*^{N} mice displayed an increased number of microglia, as well as changes in morphology of microglia in several brain regions [222]. In contrast, activation of astrocytes occurs at later stages of the disease, coincident with an increase in cytokine (IL-1β) expression [223].

A marked hypomyelination has also been observed in the cerebral cortex of NPC1-deficient mice. This abnormality occurs as early as postnatal day 10 and becomes more pronounced with increasing age [238–240]. The hypomyelination has been attributed to a defect in cholesterol trafficking in oligodendrocytes and/or to impaired axon-glia contacts resulting in defects in signaling pathways [238].

Over the past few years the synthesis of neurosteroids in the brain has received increased attention. The rate-limiting step of steroidogenesis is the entry of cholesterol into the mitochondrial matrix for conversion into pregnenolone. Early evidence indicated that steroidogenesis was impaired in NPC1-deficient mice [241], likely because cholesterol could not reach the site of pregnenolone synthesis. Recently, it was found that neurosteroid levels in the brains of NPC1-deficient mice are lower than in wild-type mice and, moreover, that

a bolus injection of allopregnanolone given to 1-week-old NPC1-deficient mice significantly delayed the onset of neuropathological symptoms [242].

4.3. Origin of the accumulating cholesterol in NPC1-deficient brains

Although a lack of functional NPC1 causes an accumulation of cholesterol in all cells that have been analyzed, the cholesterol content of brains of humans and mice lacking functional NPC1 is, surprisingly, not increased. Indeed, the amount of cholesterol in NPC1-deficient brains even decreases with increasing age [207,243,244]. These observations were initially interpreted to indicate that, in contrast to other tissues, cholesterol does not accumulate in cells of the brain as a result of NPC disease. However, this interpretation must be re-evaluated since in glial cells and cell bodies of cultured neurons from *Npc1*$^{-/-}$ mice the cholesterol content is significantly higher than in *Npc1*$^{+/+}$ cells [218]. Moreover, studies by Dietschy and coworkers have demonstrated that the amount of cholesterol in the brains of NPC1-deficient mice immediately after birth, at a time when myelination is incomplete, is higher than in brains of their wild-type counterparts [244]. Thus, a likely explanation for the age-dependent decrease in cholesterol content of NPC1-deficient brains is that the rate of loss of myelin from *Npc1*$^{-/-}$ brains is greater than from *Npc1*$^{+/+}$ brains. Consequently, since myelin is highly enriched in cholesterol, this extensive loss of myelin cholesterol over time masks an increased cholesterol content of neurons and astrocytes.

Several studies have indicated that the cholesterol that accumulates in peripheral tissues of NPC patients and *Npc1*$^{-/-}$ mice is derived from LDLs that are internalized by receptor-mediated endocytosis [243,245] (Fig. 4). The brain, however, is unable to import LDL-derived cholesterol from the circulation because of the impermeability of the blood–brain barrier. In addition, LDL-cholesterol probably does not enter the CNS even during development, prior to closure of the blood–brain barrier [1,9]. These observations imply that most cholesterol accumulating in neurons and glia in NPC1-deficient brains is made endogenously within the CNS. In support of this idea, studies by Cruz et al. showed that in Chinese hamster ovary cells the trafficking of endogenously synthesized cholesterol is impaired by loss of NPC1 function [206]. Moreover, serum-free cultures of NPC1-deficient sympathetic neurons from 1-day-old mice already contained cholesterol-filled vesicles and the accumulated cholesterol did not dissipate even after prolonged culture in serum-free medium [218]. These data show that in neurons endogenously synthesized cholesterol can be, and is, sequestered in late endosomes. As a consequence, the supply of cholesterol to the plasma membrane is reduced [246] and the plasma membrane becomes depleted of cholesterol [173,247].

In some elegant experiments, Dietschy et al. have quantitated the flux of cholesterol in brains of *Npc1*$^{-/-}$ mice and found that NPC1 deficiency decreases the overall rate of cholesterol synthesis and increases the rate of cholesterol excretion. This increased excretion is, however, independent of the 24-hydroxycholesterol pathway [248]. These changes in cholesterol homeostasis might, therefore, contribute to the observed net loss of cholesterol from the brain in NPC disease [244,248].

Although LDLs are absent from the brain, the endogenous synthesis of cholesterol is not the only possible source of cholesterol in the CNS. Under which conditions, and to what extent, cholesterol is exchanged among cells of the CNS is not clear. The brain expresses a wide variety of surface receptors of the LDL-receptor family that can internalize lipoproteins and deliver cholesterol to late endosomes. One mode of cholesterol transport in the CNS involves the delivery of glia-derived lipoproteins to neurons [54]. A similar pathway is thought to operate in the peripheral nervous system in which cholesterol derived from degenerating myelin is re-utilized upon delivery of lipoproteins to neurons for axonal growth and repair. In accordance with this model, an impaired re-utilization of cholesterol from myelin has been reported in the peripheral nervous system of *Npc1*$^{-/-}$ mice [249]. The possibility also exists that plasma lipoproteins can deliver some cholesterol to the CNS via lipoprotein receptors expressed by cells that comprise the blood–brain barrier. However, the introduction of loss-of-function mutations in either the LDL-receptor or of apo E in *Npc1*$^{-/-}$ mice did not reduce the sequestration of cholesterol in the CNS or attenuate the neurological impairment [244].

4.4. Studies in cultured NPC1-deficient neurons and glial cells

Initial studies on the biochemical defect in NPC1-deficient cells were performed with cultures of human fibroblasts or Chinese hamster ovary cell mutants [205,250]. Nevertheless, the most severe consequences of NPC1 deficiency occur in the CNS. Unfortunately, however, only a few studies have utilized primary cultures of NPC1-deficient neurons to investigate the mechanisms by which a loss of NPC1 affects neuronal metabolism and function [218,251], reviewed in [252]. NPC1-deficient embryonic cerebellar and cortical neurons [220,232], as well as cultured sympathetic neurons from *Npc1*$^{-/-}$ mice [218], display a punctate filipin staining pattern, indicative of cholesterol accumulation. This accumulation of cholesterol occurs even when exogenous cholesterol is not present in the culture medium. These observations demonstrate that cholesterol accumulates in intracellular vesicles in NPC1-deficient neurons even during intrauterine development. Interestingly, although the overall cholesterol content of NPC1-deficient sympathetic neurons is not higher than that of wild-type neurons, cholesterol is increased in cell bodies and reduced in distal axons [218]. This redistribution of cholesterol in NPC1-deficient neurons was attributed to an impaired delivery of endogenously synthesized cholesterol from cell bodies, the site of cholesterol synthesis [49,52], to distal axons [251]. In addition, when LDLs

were given to cell bodies of *Npc1*$^{-/-}$ neurons, LDL-derived cholesterol was not effectively used for axonal growth [251].

A similar redistribution of cholesterol occurred when murine sympathetic neurons were incubated with the amphiphilic drug U18666A, a compound that has been widely used to mimic the NPC-like phenotype since it leads to sequestration of cholesterol in late endosomes [205]. In addition, U18666A treatment of hippocampal neurons decreased the amount of cholesterol in axonal plasma membranes, as assessed by the membrane-impermeable probe, BC theta [253]. Although U18666A has been widely used to induce an NPC-like phenotype it is important to note that this drug also inhibits cholesterol biosynthesis and reduces the anterograde transport of cholesterol in sympathetic neurons to a far greater extent than does the loss of NPC1 [251].

In embryonic striatal neurons from NPC1-deficient mice the signaling of brain-derived neurotrophic factor via the TrkB receptor was impaired and resulted in reduced neurite outgrowth and branching [254]. The TrkB receptor is located in cholesterol-rich membrane domains and the signal propagated by this growth factor is believed to be conveyed to the cell body via retrograde transport of phosphorylated TrkB in signaling endosomes [255,256]. The observed impairment in signal transduction in NPC1-deficient neurons might, therefore, have been caused by disruption of TrkB-containing plasma membrane domains as a result of alterations in cholesterol homeostasis. This finding also suggests that a defect in retrograde endosomal trafficking of brain-derived neurotrophic factor might play a role in the aberrant signaling via TrkB in NPC1-deficient striatal neurons. In contrast to striatal neurons, no defects were observed in survival, axonal growth or in the response to nerve growth factor in NPC1-deficient sympathetic neurons [218]. Signaling of nerve growth factor via the MAP kinase pathway also appeared to be normal in NPC1-deficient sympathetic neurons (B. Karten and J. Vance, unpublished observations). Interestingly, MacInnis and Campenot have recently demonstrated that internalization and retrograde transport of nerve growth factor are not required for the survival signal induced by this neurotrophin in rat sympathetic neurons [257].

The role of astroglial cholesterol metabolism in NPC disease has recently received increased attention. As mentioned above (Section 2), lipoproteins and/or cholesterol secreted by astrocytes enhance axonal growth [103], as well as synapse number and the efficacy of pre-synaptic neurotransmitter release in retinal ganglion cells [55,258,259]. Since unesterified cholesterol is sequestered intracellularly in NPC1-deficient cells we investigated the possibility that this accumulation of cholesterol in astrocytes would impair lipoprotein formation by these cells. To test this hypothesis, glial cells (>95% astrocytes) were cultured from the cerebellum [260] and cortex [261] of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. The secretion of neither sterols (cholesterol and desmosterol) nor apo E was compromised by the lack of NPC1. In addition, the lipoprotein particles generated by *Npc1*$^{-/-}$ glia were essentially indistinguishable in size and composition from those secreted by *Npc1*$^{+/+}$ glia [261,260]. Moreover, NPC1-deficient cerebellar astroglia produced lipoproteins that functioned normally in an assay designed to test their ability to support axonal growth of CNS neurons [260]. Interestingly, astroglia also secrete the NPC2 protein but the amount of NPC2 secreted was neither increased nor decreased by NPC1 deficiency [261].

Although apo E secretion is unaffected by NPC1-deficiency, the secretion of apo D, a member of the lipocalin family that can bind sterols and steroid hormones, by *Npc1*$^{-/-}$ astroglia was significantly diminished despite an increased amount of apo D within the cells [262]. In other studies, the mRNA and protein levels of apo D have been reported to be enhanced in several neurological conditions and following traumatic brain injury [156,263]. However, the physiological significance of these findings remains unclear since the function of apo D in the brain is unknown. Further investigation of factors secreted by astrocytes is warranted since such studies might shed light on the reason why NPC1 deficiency leads to such profound consequences.

The lack of significant abnormalities in lipoproteins produced by *Npc1*$^{-/-}$ glia suggests that the neurodegeneration characteristic of NPC disease can more likely be ascribed to defects in neurons rather than to defects in cholesterol homeostasis in glial cells or in lipoproteins produced by the glia.

4.5. Similarities between NPC disease and Alzheimer's disease

Over the last few years, interest in NPC disease has increased, in part at least, because of similarities between the histopathological characteristics of NPC disease and AD [264]. Evidence that the intracellular trafficking of cholesterol might modulate APP processing has been provided from experiments performed in mice lacking NPC1. The activities of α- and β-secretase are not altered by NPC1 deficiency whereas γ-secretase activity is significantly enhanced, and Aβ40 and Aβ42 levels are increased [265]. Moreover, when neuronal cells were treated with the cholesterol transport inhibitor U18666A, γ-secretase activity and the production of Aβ40 and Aβ42 were increased [266].

As in AD, neurofibrillary tangles have been detected in brains of juvenile and adult cases of NPC disease. These tangles are immunocytochemically and morphologically indistinguishable from the tangles in AD brains and show the same pattern of tau hyperphosphorylation [224,232,267,268]. It is noteworthy that NPC1-deficiency is the only known neurological disorder that leads to neurofibrillary tangle formation as early as the first or second decade of life. The murine model of NPC disease does not develop neurofibrillary tangles in the brain [229] but tau protein is hyperphosphorylated at the same epitopes that are phosphorylated during the early stages of AD [220,269]. Increased APP immunoreactivity is seen in brains of *Npc1*$^{-/-}$ mice as early as 10 days after birth [238]. In addition, tangle-bearing neurons in AD contain more unesterified cholesterol than do tangle-free neurons suggesting that a sequestration of unesterified cholesterol,

such as occurs in NPC disease, might influence tangle formation [270]. Although the mechanism by which cholesterol modulates neurofibrillary tangle formation has not been established, Bu et al. proposed that the generation of tangles depends on the concentration of tau [271]. These researchers also observed that NPC1-deficient cerebellar neurons do not contain significant numbers of neurofibrillary tangles even though early markers of tangle formation, such as tau hyperphosphorylation, are evident [271]. Furthermore, the tau content of Purkinje neurons is markedly lower than that in other brain regions which might, in turn, reduce the aggregation of tau and inhibit neurofibrillary tangle formation [271]. A possible explanation for the aberrant phosphorylation of tau in NPC disease is that the activity of cyclin-dependent kinase 5 (cdk5) is increased in brains of adult *Npc1*$^{-/-}$ mice [271]. Moreover, the phosphorylation of several other cytoskeletal proteins is increased [271]. Consistent with these findings, it was recently reported that intracerebroventricular treatment of *Npc1*$^{-/-}$ mice with the cdk5 inhibitor roscovitine ameliorated the motor defects and slightly attenuated Purkinje cell loss [272].

Tau is not the only protein that is hyperphosphorylated and forms aggregates in neurodegenerative disorders. The accumulation and phosphorylation of α-synuclein, and its aggregation to form Lewy bodies, are hallmarks of Parkinson's disease as well as AD and other neurodegenerative disorders. It has been proposed that synuclein and tau act synergistically to promote aggregation [273]. Interestingly, phosphorylated α-synuclein is frequently found in brains of NPC patients, and in some cases Lewy bodies have also been detected [274]. The isoform of apo E is another factor that might influence neurofibrillary tangle generation in NPC disease. Neurofibrillary tangles and the deposition of phosphorylated synuclein and Aβ are increased in brains of NPC patients that carry one or two apo E4 alleles [274,275].

Another common feature of AD and NPC disease is the presence of endosomal abnormalities. Thus, an increased leakage of proteases, such as cathepsin D, a candidate APP secretase, from endosomes might occur thereby initiating the apoptotic cascade and promoting neurodegeneration [276]. In addition, in NPC1 deficiency, the number of cathepsin D-positive vesicles, that were identified as early endosomes by their Rab5 reactivity, was increased in the dendritic arbour of Purkinje cells but not in hippocampal neurons [277].

The loss of NPC function also appears to modulate APP metabolism. Jin et al. detected an abnormal accumulation of Aβ42 in the hippocampus and cerebral cortex of an adult with NPC disease [277]. In addition, treatment of APP-expressing neurons with U18666A (to induce cholesterol sequestration in late endosomes) altered the pattern of APP cleavage and increased the deposition of Aβ-positive material in endosomal compartments that were distinct from the late endosomes that sequestered cholesterol [277]. These observations suggest that in NPC disease, changes of cholesterol levels within the endosomal trafficking system might contribute to abnormal APP processing and Aβ42 deposition.

5. Concluding statement

Many questions remain concerning the mechanisms of cholesterol homeostasis in the brain. Research has already demonstrated that lipoprotein metabolism in the brain is important for a normal functioning of neurons and glial cells. Since lipoprotein metabolism in the brain is distinct from that in the plasma, because of segregation of the CNS from the plasma compartment, novel mechanisms for the regulation of cholesterol homeostasis in the brain are likely to be discovered. The importance of understanding cholesterol metabolism in the brain is underscored by the accumulating and convincing evidence that an association exists between cholesterol and development of neurodegenerative disorders such as AD and NPC disease.

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