Chapter 2
Cholesterol in Alzheimer's Disease and other Amyloidogenic Disorders

J. Robin Harris and Nathaniel G.N. Milton

Abstract The complex association of cholesterol metabolism and Alzheimer's disease is presented in depth, including the possible benefits to be gained from cholesterol-lowering statin therapy. Then follows a survey of the role of neuronal membrane cholesterol in Aβ pore formation and Aβ fibrillogenesis, together with the link with membrane raft domains and gangliosides. The contribution of structural studies to Aβ fibrillogenesis, using TEM and AFM, is given some emphasis. The role of apolipoprotein E and its isoforms, in particular ApoE4, in cholesterol and Aβ binding is presented, in relation to genetic risk factors for Alzheimer's disease. Increasing evidence suggests that cholesterol oxidation products are of importance in generation of Alzheimer's disease, possibly induced by Aβ-produced hydrogen peroxide. The body of evidence for a link between cholesterol in atherosclerosis and Alzheimer's disease is increasing, along with an associated inflammatory response. The possible role of cholesterol in tau fibrillization, tauopathies and in some other non-Aβ amyloidogenic disorders is surveyed.

Keywords Cholesterol · Alzheimer's disease · Amyloid-β · Aβ · Oligomerization · Fibrillogenesis · Statin · HMG-CoA reductase inhibitor

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid-beta

2.1 Introduction

An understanding of the role of circulatory cholesterol in cardiovascular disease and cerebrovascular disease has long been at the forefront of medical science. Cholesterol is now also thought to impinge strongly upon the field of dementia and neurological disease, in particular its possible role in the development of

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Alzheimer's disease (AD) and other neurological and peripheral amyloidogenic disorders. Because diverse medical, biomedical and basic scientific approaches are being used to pursue studies in relation to AD and the involvement of cholesterol, meaningful correlation of data is not always easy, but in the present state of rapidly expanding knowledge it is nevertheless thought to be appropriate to attempt this review.

Many studies on AD and cholesterol do not relate to the binding of cholesterol to the well-characterized predominantly extracellular amyloid-β (Aβ) protein fragments that occur in vivo, rather they impinge upon metabolic and biochemical considerations. For the sake of completeness it is necessary to include discussion of most of these; furthermore, it is likely that they will have a secondary impact of significance to the structural aspects. The brain synthesizes most of its own cholesterol, but dietary/circulatory cholesterol almost certainly impacts upon cerebrovascular amyloid in the condition termed cerebral amyloid angiopathy (CAA) or vascular dementia. It is not always clear whether one should place emphasis upon free cholesterol, esterified cholesterol or cholesterol oxidation products of both these forms. With the increasing interest in the deleterious effects of free radical and metal ion-induced oxidation within medical systems and the likely benefits to be gained from dietary and therapeutic antioxidants, cholesterol oxidation has emerged as a topic of considerable significance in neurological disease (*see also* Chapter 6).

This subject has been under discussion for some years and has been reviewed extensively, primarily from a metabolic stance (Hartmann, 2005; Koudinov and Koudinova, 2001a; Ledesna and Dotti, 2006; Lukiw et al., 2005; Raffaï and Weisgraber, 2003; Shobab et al., 2005), but less so from a more structural point of view (Yanagisawa, 2005); accordingly, this latter aspect will be given greater emphasis within the present chapter. Studies on the role of cholesterol in Alzheimer's disease range widely from in vivo human and animal experimentation, including dietary, immunological and pharmaceutical approaches and the use of transgenic knock-out and knock-in animals, through to cultures of neuronal and other cells and numerous in vitro biochemical and structural approaches. Overall, a remarkable strength comes from this technical diversity, despite the inevitable instances where data appears to be conflicting. Although the material below is presented as discrete topics, there is considerable overlap of subject matter between the sections.

2.2 Cholesterol Metabolism and Alzheimer's Disease

Implicit in this topic is the underlying concept that cholesterol participates in the control of the membrane-bound amyloid precursor protein (APP) expression, and the expression and activity of the proteases (β- and γ-secretases) involved in APP cleavage, to generate the extracellular soluble amyloid-β peptide fragments that participate in AD. Thus, cholesterolemia is widely considered as a major risk factor in AD (Canevari and Clark, 2007), although the multi-faceted cholesterol interaction in vivo remains far from being fully understood. The possibility that cholesterol

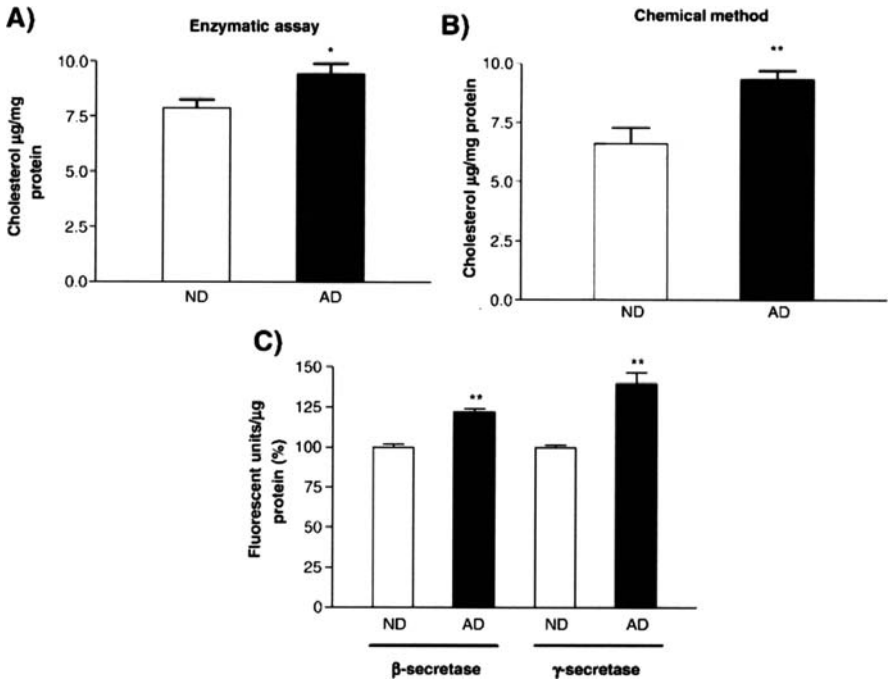

Fig. 2.1 The levels of cholesterol and the activities of β- and γ-secretases in normal and AD brain samples. *Panel* **A** shows that the enzymic determination of cholesterol in AD samples is significantly higher than in normal brain samples. *Panel* **B** shows the chemical determination of total brain cholesterol. AD brains have a significantly higher cholesterol content compared to non-AD brains. *Panel* **C** shows the analysis of brain lysates for β- and γ-secretase activity. The activities of both enzymes are significantly higher in the AD samples, compared to non-AD brain samples. From Xiong et al. (2008), with permission from Elsevier

retention in the Alzheimer's disease brain might be responsible for high β- and γ-secretase activities was advanced by Xiong et al. (2008), in an impressive study on the brains of AD patients, and cultured mouse cells stably transfected with the human APP gene (Fig. 2.1). These workers concluded that cholesterol homeostasis and transport was impaired, leading to increased retention in AD brains, due to altered levels or activities of nuclear receptors, and similar suggestions were made by Burns et al. (2003a). Brain cholesterol accumulation could be expressed by an increase in myelin membrane, neuronal plasma- and cyto-membrane membrane cholesterol content, by intra- or extra-cellular cholesterol inclusions, but the precise location(s) are yet to be defined. Indeed, that Aβ alters intracellular vesicle trafficking and cholesterol homeostasis, resulting in decreased cholesterol esterification and changes in neuronal free cholesterol distribution that are likely to be relevant to neurodegeneration, was advanced by Liu et al. (1998). Cholesterol accumulation in senile plaques of AD patients and transgenic APP (SW) mice was shown by Mori et al. (2001), using filipin fluorometric staining for cholesterol and an enzymatic technique (*see also* Section 1.4). It should, however, be mentioned that this work

has recently been challenged by Lebouvier et al. (2009), who maintained that the purported presence of cholesterol in senile plaque was due to false positive results. Whilst it has to be accepted that cholesterol is naturally abundant in brain tissue, its presence bound within senile plaques could be at a low molecular level, rather than at the gross level of cholesterol crystalline or other lipid-rich deposits know to be present within vascular atherosclerotic plaques.

The presence of an increased amount of cholesterol in Aβ-positive presynaptic nerve terminals from AD brains led Gylys et al. (2007) to suggest that this might underlie neuronal dysfunction (synaptic loss), prior to or independent of subsequent extracellular Aβ deposition. A more general statement on the co-localization of cholesterol and raft lipids with extracellular disease-associated amyloid fibres extracted from tissues was advanced by Gellermann et al. (2005). Also at the neuronal level, earlier studies were performed on the role of cholesterol in synaptic plasticity and neuronal degeneration by Koudinov and Koudinova (2001b).

Independent support for the involvement of cholesterol in AD has come from dietary studies, where animals were subjected to high-fat or high-cholesterol feeding. Zatta et al. (2002) fed rabbits on a high cholesterol diet and found microglial activation and astrocytosis with over-expression of metallothionein-1 and -II, along with intraneuronal Aβ accumulation and occasional extracellular Aβ deposits. Intestinal epithelial cells of mice fed on a high fat diet were found by Galloway et al. (2007) to have an increased APP and Aβ concentration, leading to the interpretation that Aβ could serve as a chylomicron regulatory apolipoprotein, via its hydrophobic domain. A high cholesterol dietary-induced neuroinflammation and APP processing (Thirumangalakudi et al., 2008) was correlated with the loss of working memory in mice. Other supportive data has come from Hooijmans et al. (2009) who showed that cholesterol-containing diets influenced Alzheimer-like pathology, cognition and cerebral vasculature in transgenic mice. With a mouse genetic model for cholesterol loading, Fernández et al. (2009) showed that mitochondrial cholesterol loading enhanced Aβ-induced inflammation and neurotoxicity, modulated via mitochondrial glutathione. The work of Crameri et al. (2006) showed that deficiency in the cholesterol synthesising enzyme seladin-1 increases Aβ generation by increasing the β-secretase (β-site APP-cleaving enzyme: BACE) processing of the APP. Overexpression of seladin-1 had the reverse effect and increased the cholesterol in the membrane detergent resistant domains. Seladin-1 is also neuroprotective against Aβ and shows reduced expression in the AD brain (Greeve et al., 2000).

The muscle disorder termed sporadic inclusion body myositis (IBM) exhibits pathological similarities to AD, with respect to an increased skeletal muscle level of APP and Aβ. Rabbits fed a cholesterol-rich diet were found by Chen et al. (2008) to exhibit increased mRNA and proteins levels of APP and increased secretase activity favouring Aβ production, the pathological features of IBM.

Using the cholesterol-fed rabbit as a model for Alzheimer's disease, Sparks and his colleagues (Sparks and Schreurs, 2003; Sparks, 2004, 2007) have maintained that the presence of trace copper ions is necessary for Aβ to accumulate in the brain. In the absence of copper, rabbits with elevated cholesterol clear Aβ to the blood and liver. Supportive evidence has come from the studies of Opazo et al. (2002) and Puglielli et al. (2005), who claimed that copper-mediated oxidation of

cholesterol might be responsible for AD pathogenesis and plaque formation. In a cholesterol-fed mouse model Lu et al. (2009) have shown that trace amounts of copper induced APP up-regulation, which activated the inflammatory pathway and exacerbated neurotoxicity. Other metal ions, such as those of zinc and iron, have also been implicated in AD (Ghribi et al., 2007; Gehman et al., 2008). Paradoxically, in rat brain tissue Bishop and Robinson (2004) claimed that the complex of Aβ with copper ions was not neurotoxic, whereas Aβ-iron and Aβ-zinc complexes were. The cholesterol-fed rabbit model has also been used by Prasanthi et al. (2008), who determined the extent to which brain hypercholesterolemia-induced Aβ levels were linked to a number of Aβ processing enzymes and receptors.

Even prior to the turn of the 21st century and more recently, cholesterol depletion induced by administration of statins to neuronal cultures and to humans was linked to a reduced risk of developing Alzheimer's disease (Simons et al., 1998, 2001; Fassbender et al., 2001; Wolozin et al., 2000; Wolozin, 2004; Zamrini et al., 2004) and the extended role of Aβ in lipid metabolism has been reviewed in depth by Zinser et al. (2007). Overall, the message that cholesterol-lowering strategies using statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) may provide a useful therapeutic approach to combat AD has emerged strongly, but extremely long-term epidemiological and clinical studies are required to provide the necessary proof. It is generally thought that the more lipophilic statins, such as lovastatin, are likely to carry greater protective potential (Ferrera et al., 2008). Atorvastatin administration to brain-injured rats has been found to be beneficial, in terms of reduced oedema and lipid peroxidation (Turkoglu et al., 2008), possibly mediated by a metabolite of atorvastatin that possesses antioxidant properties and inhibits membrane cholesterol-containing raft/domain formation (Mason et al., 2006).

Apart from the metabolic studies relating to statin reduction of APP production and processing, there is recent evidence that cholesterol depletion reduces Aβ aggregation (oligomer formation and fibrillogenesis) in hippocampal neurons (Schneider et al., 2006) and Aβ fibrillogenesis in macrophages (Gellermann et al., 2006), which provide support for the earlier concept that cholesterol also plays a key role in Aβ fibrillogenesis in vitro (Harris, 2002): *see below*.

2.3 Cholesterol Binding to Aβ and Aβ Fibrillogenesis

The interaction between Aβ and cholesterol has been assessed at the cellular and extracellular level. At the cell membrane level, cholesterol appears to be complexed with ganglosides and sphingolipids within neutral detergent-insoluble *raft domains* where it may influence APP cleavage by membrane-bound β-secretase (β-site APP-cleaving enzyme: BACE) followed by γ-secretase cleavage to release soluble Aβ (the amyloidogenic pathway) (*see* Zinser et al., 2007). In the cytoplasmic and most importantly extracellular compartments, cholesterol may promote the oligomerization and fibrillogenesis of the Aβ peptide. The extracellular deposition of brain senile plaques containing fibrillar Aβ in association with cholesterol, and a number of other proteins and glycoproteins, is of main concern within the present section, but it is necessary to briefly survey the relevant literature on other aspects.

2.3.1 Cholesterol and Membrane-Associated Aβ Pore Formation and Fibrillogenesis

The membrane disordering effect of Aβ has been largely investigated using cellular, liposomal and lipid monolayer systems, and related to the β-sheet conversion of Aβ and subsequent peptide aggregation/polymerization, in relation to membrane fluidity and neurotoxicity (reviewed by Eckert et al., 2005). That membrane cholesterol can act as a modulator of both membrane-associated Aβ fibrillogenesis and neurotoxicity has been advanced by McLaurin et al. (2002) and Yip et al. (2001). The requirement for cholesterol for the cytotoxic effects of Aβ on vascular smooth muscle cells and for Aβ binding to the muscle cell membrane was shown by Subashinghe et al. (2003), and clearly linked to beneficial drug-induced cholesterol lowering. Although implicit in the above studies, at a biophysical level Wood et al. (2002) have shown more specifically that it is the presence of cholesterol in the outer lipid monolayer of the neuronal cell membrane that is responsible for Aβ accumulation. Synaptic plasma membranes from cerebral cortex and hippocampus were shown by Chochina et al. (2001) to be enriched in cholesterol, compared to cerebellum. This neuronal membrane enrichment with cholesterol was linked to an increase in membrane fluidity, resulting in hydrophobic Aβ accumulation and fibrillogenesis.

The combined influence of metal ions and cholesterol on $A\beta_{1–42}$ interaction with model membranes (Lau et al., 2007; Gehman et al., 2008) has provided further evidence for the conversion of the peptide α-helix to β-sheet structure, the increase in hydrophobicity then enabling the peptide oligomer/pre-pore to penetrate the lipid bilayer as an ion channel. This concept has been advanced to account for the cytotoxicity of amyloidogenic proteins in general (Cheon et al., 2007; Rabzelj et al. 2008), together with the fact that these pore-forming proteins and peptides may share structural and functional homology (Yoshiike et al., 2007). That the dynamic formation of Aβ membrane-penetrating pores can act as calcium-selective ion channels responsible for neurotoxicity is gaining support (Jang et al., 2007b), and the role of cholesterol in this event was implied from studies on planar lipid bilayer membranes (Micelli et al., 2004). Molecular dynamics modelling of putative ion channels formed by neurotoxic Aβ ion channels (Jang et al., 2007a), formed by peptides of different length, has provided support for the overall hypothesis that an intermediate protein unfolding leads to exposure of hydrophobic β-sheet membrane-penetrating peptide hairpins (sometimes termed "U-shaped β-strand-turn-β-strands"; Jang et al., 2007b), in the form of oligomeric β-barrels. It is likely that there is a difference between $A\beta_{1–42}$ and $A\beta_{1–40}$ with respect to oligomer and ion channel formation (Kirkitadze and Kowalska, 2005) that may be responsible for the significantly higher $A\beta_{1–42}$/ $A\beta_{1–40}$ ratio commonly found in familial AD. Using a liposomal model system Qui et al. (2009) have shown that the lateral organization of cholesterol, presumably in raft-like domains, controls the formation of oligomeric Aβ, which in turn could be linked to the toxicity of Aβ in neuronal membranes.

A strong parallel between the various amyloid cytotoxic cation channels and the cholesterol- and other lipid-dependent pore-forming toxins has been drawn by Lashuel and Lansbury (2006) and indeed suggested earlier by Gilbert et al. (1998),

which will be expanded upon in Chapter 21. This concept is supported by the study of Srisailam et al. (2002) who investigated the transformation of the all β-barrel acidic fibroblast growth factor from *Notopthalmus viridescens* and found partially-structured intermediates leading to fibril formation. There are several documented instances where a low concentration of SDS or other alkyl sulfate induces or potentiates amyloid fibril formation, again indicating the importance of a critical level of protein unfolding with exposure of (paired) hydrophobic β-sheets that associate/polymerize in a linear manner as extremely stable (SDS resistant) crossed β-sheets.

2.3.2 Gangliosides and Aβ Fibrillogenesis

The role of monosialoganglioside (GM1) in AD, alone and in association with cholesterol, has been given considerable attention in recent years (reviewed by Yanagisawa, 2005, 2007). Both cellular and biochemical studies have been performed in relation to GM1-containing lipid rafts and Aβ production from APP (Ehehalt et al., 2003; Kalvodova et al., 2005). That the GM1-Aβ complex could act as a *seed* for the production of fibrillar Aβ on the neuronal surface has been proposed (Kakio et al., 2001, 2002), and this concept has been extended to intracellular release of Aβ via a deviant endocytic pathway into endosomes (Kimura and Yanagisawa, 2007; Yuyama et al., 2008).

Support for the involvement of both ganglioside and cholesterol in the formation of cell surface-bound fibrillar $A\beta_{1-42}$ has come from the study of Wakabayashi and Matsuzaki (2007), by showing degenerate neurites on NFG-differentiated PC12 neuron-like cells. Using the same neuronal-like cell line for Aβ cytotoxicity testing, Lin et al. (2008) concluded that both GM1 and cholesterol are essential for the formation of the GM1-Aβ complex on the cell surface and the modulation of the cytotoxicity of monomeric Aβ. In a liposomal model system, containing lipids similar to those in brain cortical membrane, Tashima et al. (2004) assessed Aβ release and fibril formation in the presence and absence of GM1 and cholesterol, and concluded that fibril formation required both these components. Further evidence that an age-dependent GM1 enrichment of neuronal presynaptic terminals forms zones where Aβ binds and promotes fibrillar amyloid assembly has recently been shown by Yamamoto et al. (2008).

2.3.3 Cholesterol and In Vitro Aβ Fibrillogenesis: Structural Studies

Surprisingly, of the numerous biochemical and structural publications dealing with in vitro oligomerization and fibrillogenesis of the amyloid-β peptides (the naturally occurring and chemically synthesised peptides of varying length), rather few have linked the influence of cholesterol to these events. Using fluorescently-labelled lipids, Avdulov et al. (1997) showed that Aβ aggregates, the nature of which was not

fully defined, had a preferential binding for cholesterol rather than for phosphatidylcholine and fatty acids. Although most studies have been performed using the longer Aβ fragments, D'Errico et al. (2008) showed a cholesterol-dependent interaction between $A\beta_{25–35}$ and phospholipid bilayers, apparently due to an increased membrane fluidity. That native, and in particular oxidized, plasma lipoproteins can potentiate Aβ fibrillogenesis was shown by Stanyer et al. (2002), with the suggestion that this might be mediated via reactive aldehyde groups, as fibrillogenesis was inhibited in the presence of an aldehyde scavenger. More recent insights into lipid aldehyde-initiated fibrillogenesis of Aβ has come from Scheinost et al. (2008), who maintained that the ε-amino group of Aβ Lys16 adjacent to the central hydrophobic cluster (amino acids 17–21; Nelson and Alkon, 2007), could be a target for aldehyde adduction.

Native high density lipoprotein (HDL), with and without the three ApoE isoforms, was shown to inhibit Aβ fibrillogenesis (Olsen and Dragø, 2000), but this observation was not linked to cholesterol sequestration by HDL. Although relating more to Aβ production than fibrillogenesis, using a cholesterol-protein binding blot assay Yao and Papadopoulos (2002) showed that cholesterol binds to the hydrophobic amino acid sequence 10–20 of Aβ, thereby blocking the access of the α-secretase and cleavage of APP to $A\beta_{17–40}$, i.e. inhibiting the non-amyloidogenic pathway. Furthermore, the binding of cholesterol to LDL was inhibited by $A\beta_{1–40}$ and the binding of cholesterol to ApoE and LDL was completely abolished by $A\beta_{1–42}$.

The transmission electron microscope (TEM) and to a somewhat lesser extent the atomic force microscope (AFM) can provide detailed molecular information on the structure of Aβ oligomers through to protofibrils and fibrils, together with fibril polymorphism (Harris, 2002, 2008; Milton and Harris, 2009). X-ray fibre diffraction has also provided higher resolution data on the repeating crossed β-sheet structure that underlies fibril formation (Malinchik et al., 1998; Stromer and Serpell, 2005), which is broadly accepted as a structural feature of all known amyloid fibrils. As a slight variant Sinha et al. (2001) proposed a domain swapped interdigitating β-hairpin model for amyloid fibril elongation. Although several of the investigations relating to membrane-bound cholesterol in relation to Aβ fibrillogenesis have utilized AFM and TEM in a serious manner (Yip and McLaurin, 2001; Yip et al., 2001), other studies have only touched upon the possibilities that these microscopies, in particular TEM, can offer for the in vitro study of these interactions, particularly when placed alongside other more indirect biochemical and biophysical techniques (Castaño et al., 1995; Mizuno et al., 1999; Koppaka et al., 2003).

Other than for natural membrane, mixed-lipid liposomal and bilayer model systems, it is difficult to know how one should present cholesterol experimentally for interaction with Aβ in vitro. Cholesterol has a very low solubility in aqueous systems (Harberland and Reynolds, 1973) resulting in the formation of planar cholesterol microcrystals when an ethanolic solution of cholesterol is dispersed in an aqueous phase (Harris, 1988). Esterified cholesterol forms a suspension of globular particles in aqueous solution, similar in size to low density lipoproteins, when prepared in a similar manner (Harris, 2002). A commercially available "soluble" cholesterol (Sigma-Aldrich, termed cholesterol-PEG 600), is a waxy solid in which

the 3β-OH group of cholesterol has been linked to a polyethylene glycol chain, resulting in an average mass of ~600 Da. This product more accurately creates a micellar solution, in all probability with the sterol at least partially buried beneath the surface hydrophilic polyethylene glycol chains. Nevertheless, this synthetic cholesterol derivative has been found to be a useful soluble cholesterol substitute for cellular studies (Ishiwata et al., 1997), as well as for studies on cholesterol binding to Aβ fibrils (Harris, 2008). Examples of cholesterol microcrystals, esterified cholesterol and LDL particles, and cholesterol PEG600 micelles are shown in Fig. 2.2. The clustering of $A\beta_{1-42}$ fibrils on and around cholesterol microcrystals is shown in Fig. 2.3. Detail of the $A\beta_{1-42}$ peptide binding to cholesterol microcrystals, with protofibrils visible on the crystal surface is shown in Fig. 2.4. Other Aβ peptide

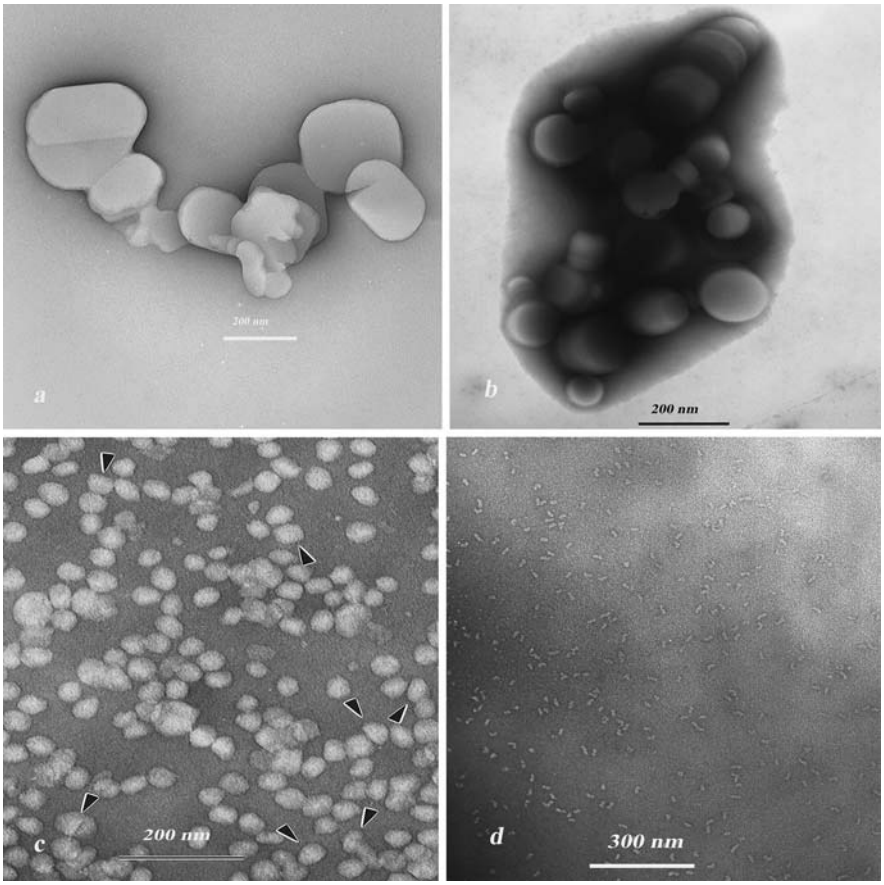

Fig. 2.2 Examples of four different experimental cholesterol substrates, shown in negatively stained TEM images, usable for Aβ fibrillogenesis studies. (**a**) A cluster of cholesterol microcrystals (Harris, 1988); (**b**) cholesterol acetate globular micelles; (**c**) human low density lipoprotein (LDL); (**d**) cholesterol-PEG600 micelles (soluble cholesterol)

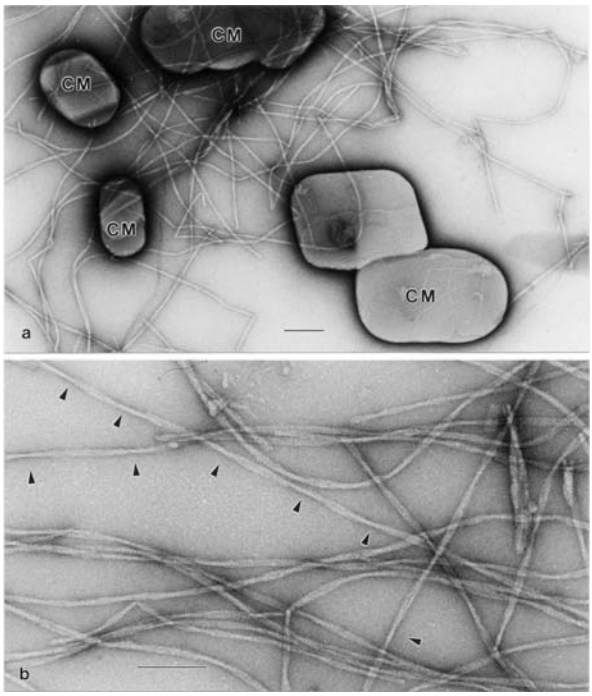

Fig. 2.3 (a) Cholesterol microcrystals (CM) surrounded by a cluster of $A\beta_{1-42}$ fibrils; (**b**) a higher magnification survey showing the double helical nature of the mature $A\beta_{1-42}$ fibrils formed in the presence of cholesterol microcrystals. The scale bars indicate 100 nm. From Harris (2002), with permission from Elsevier

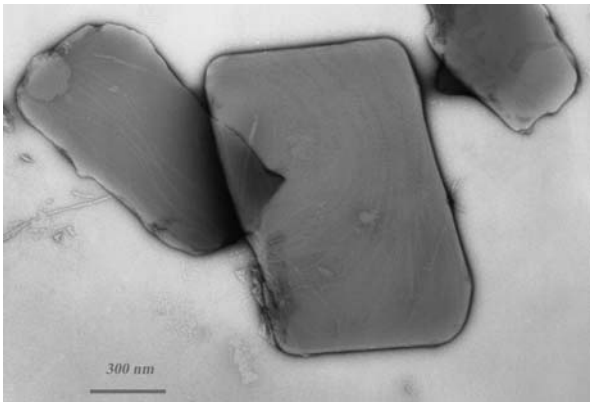

Fig. 2.4 Cholesterol microcrystals showing a thin surface coating of forming $A\beta_{1-42}$ protofibrils and fibrils, strongly indicative of the positive binding of the peptide to cholesterol and the subsequent promotion of fibril formation

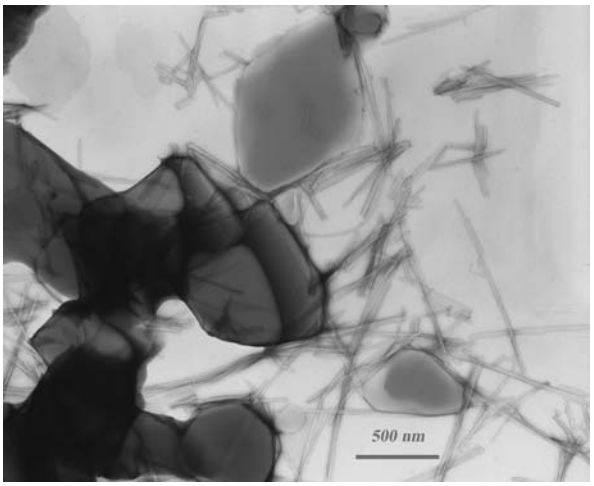

Fig. 2.5 Cholesterol microcrystals surrounded by and binding fibrils formed by the $A\beta_{22–35}$ fragment. These fibrils do not exhibit the characteristic double helical structure shown by mature $A\beta_{1–42}$ fibrils (Harris and Milton, previously unpublished data)

fragments, such as the $A\beta_{25–35}$ fragment, also show an affinity for cholesterol (Fig. 2.5), but it has yet to be demonstrated that Aβ fibril-forming peptide fragments lacking the central hydrophobic domain have lost the capacity to bind to cholesterol. The binding of soluble cholesterol-PEG600 to preformed $A\beta_{1–42}$ fibrils, and fibrils formed in the presence of soluble cholesterol (Harris, 2002, 2008), is shown in Fig. 2.6. However, fibrils formed from the bacterial protease inhibitor Pepstatin A (an eight amino acid bacterial peptide), do not bind soluble cholesterol-PEG600 micelles and similarly, no evidence has been obtained by the authors to indicate that fibrils formed by the peptide amylin (islet amyloid peptide) have any cholesterol-binding potential. When $A\beta_{1–42}$ fibril formation is performed in the presence of both cholesterol and aspirin, fibril formation is prevented, but clusters of short rod-like $A\beta_{1–42}$ aggregates attach to the cholesterol microcrystals (Harris, 2002). This suggests that although aspirin inhibits fibril formation, it may not prevent oligomer formation by the $A\beta_{1–42}$ peptide.

2.4 Apolipoprotein E, Cholesterol and Alzheimer's Disease

There is an extensive literature to link the differing apolipoprotein E (ApoE) isoforms to Aβ binding and late-onset AD (see: Carter, 2005; Crutcher, 2004; Hatters et al., 2006; Sullivan et al., 2008). However, until recently there has been relatively little evidence as to how the known cholesterol-binding of ApoE could modulate this Aβ interaction (Hirsch-Reinshagen and Wellington, 2007; Reiss, 2005). The most significant fact to emerge from the early ApoE studies is that in individuals carrying the ApoE3 allele and most particularly the ApoE4 allele, and thus expressing these

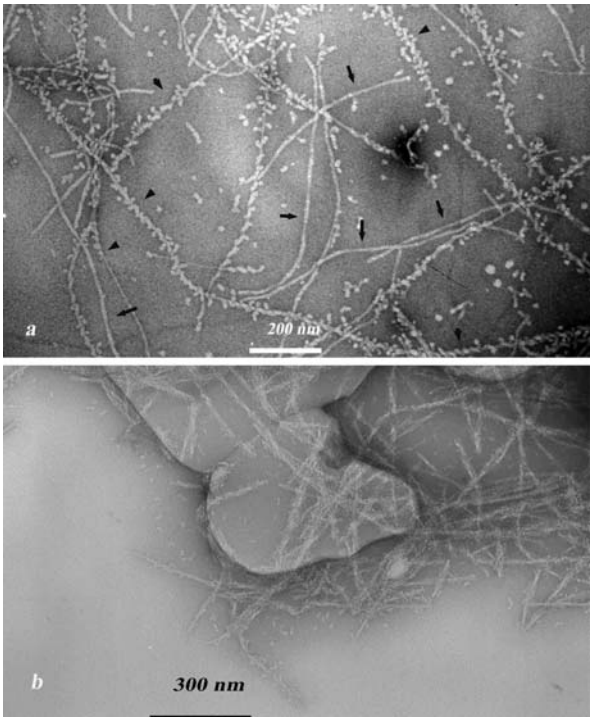

Fig. 2.6 (**a**) Amyloid $\beta_{1\text{-}42}$ fibrils formed in the presence of cholesterol PEG600. The negatively stained image shows that protofilaments have cholesterol-PEG600 micelles clustered obliquely along the length of the fibril (*arrowheads*), whereas the double helical mature filaments appear to have a smooth surface coating of the cholesterol derivative (*arrows*). From Harris (2002), with permission from Elsevier. (**b**) Amyloid $\beta_{1\text{–}42}$ fibrils prepared in the presence of 0.5 mg/ml cholesterol, followed by incubation with cholesterol-PEG600. The mature double helical fibrils, which are clustered around a stack of cholesterol microcrystals, are well-coated with cholesterol-PEG600 micelles, but there is no indication of the periodic binding shown in (**a**). From Harris (2008), with permission from Elsevier

isoforms in higher ratio, are susceptible to an increased AD risk. Those carrying the ApoE2 allele have the lowest AD risk. Although Castaño et al. (1995) showed that in vitro fibrillogenesis of Aβ was promoted by ApoE, this aspect has not be followed further in recent years in relation to the properties of the different ApoE isoforms.

Brain ApoE is synthesized primarily by glial cells and possibly also by neurons, and is present in the CSF within HLD-like lipoprotein particles. There is little or no evidence for transfer of peripheral ApoE, where it is present in plasma HDL and VLDL particles, across the blood-brain barrier to the CSF. The ApoE molecule has a molecular mass of 34.2 kDa (299 amino acids) and is present in the CSF as a major protein component, at the relatively high concentration of ~5 mg/ml. It is looked upon as a lipid transport protein and in the plasma is considered to have anti-atherogenic properties. The C-terminal domain of ApoE (amino acid residues

216–299) is thought to be responsible for both binding to Aβ and to lipids, whereas the N-terminal domain is responsible for binding of ApoE to the LDL receptor.

As already mentioned, ApoE exists as three main isoforms in man, ApoE2 (Cys^{112}, Cys^{258}), E3 (Cys^{112}, Arg^{158} and E4 (Arg^{112}, Arg^{158}), with the gene for E3 being the most common allele. The blood plasma ApoE4 has a greater lipid-binding capacity, including cholesterol, and tends to locate to VLDL rather than HDL. A conformational change within the ApoE4 molecule exposing amphipathic α-helices results in a change in surface hydrophobicity that is thought to account for increased lipid binding, rather than the direct involvement of the arginine substitution at position 112, since this is out-with the N-terminal lipid-binding domain. The Apo E phenotype can also influence the effectiveness of lipid lowering therapies with more effects observed with fibrates compared to statins (Christidis et al., 2006).

An important finding using Aβ and ApoE immunostaining was the co-localization of Aβ and ApoE in AD-affected brain samples (Aizawa et al., 1997), in addition an antibody against the C-terminal of ApoE showed greater similarity of staining to the anti-ApoE mAb than did an anti-ApoE N-terminal antibody. Furthermore, in an AD transgenic mouse model Burns et al. (2003b) have shown the co-localization of extracellular cholesterol, ApoE and fibrillar Aβ in amyloid plaques. Figure 2.7 shows the co-localization of Aβ and cholesterol, in parallel with thioflavin S staining of fibrillar plaques (courtesy of Marc Burns). Also using transgenic mice, Fryer et al. (2005) investigated cerebral amyloid angiopathy (CAA), which is found in most AD patients, in relation to the ApoE3 and ApoE4 isoforms. They showed that the expression of human ApoE4 in mice led to substantial CAA plaques, but with few parenchymal amyloid plaques. Young ApoE4-expressing mice had an elevated ratio of Aβ 40:42 in the brain extracellular pool, but a lower ratio

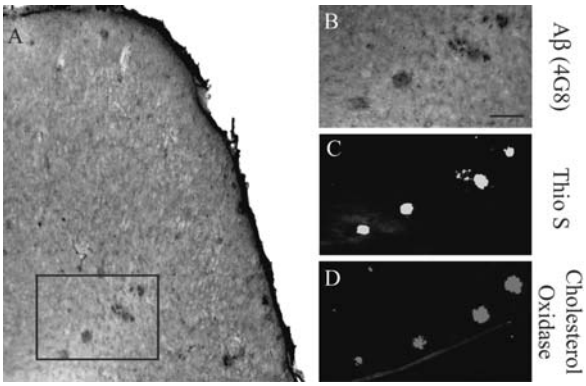

Fig. 2.7 Co-localization of fibrillar amyloid with cholesterol oxidase in the cortex of a 12-month-old PS/APP mouse. (**a**) A low-power overview of amyloid plaques stained with 4G8. (**b–d**) Plaques from within the boxed area double-labelled for Ab (**b**) and thioflavin S (c), and a consecutive section labeled for cholesterol oxidase (**d**). Scale bars 100 mM. From Burns et al. (2003b), with permission from Elsevier

in the CSF, suggestive of altered clearance and transport of Aβ. Although not implicating ApoE isoforms, support for the mediation of ApoE in cholesterol efflux from astrocytes came from the study of Abildayeva et al. (2006), showing that 24(S)-hydroxycholesterol induced ApoE-mediated efflux of cholesterol via a liver X receptor-controlled pathway, of likely relevance for neurological disease.

Cellular studies on ApoE isoforms in primary rat hippocampal neurons and astrocytes (Rapp et al., 2006) have indicated that the ApoE4 isoform is involved to a greater extent in neuronal cholesterol homeostasis than the other isoforms, and that this is more pronounced in neurons compared to astrocytes. However, Gong et al. (2002) showed that astrocytes from ApoE4 knock-in mice had a reduced cholesterol release into HDL-like particles compared to ApoE3 knock-in mice. Extending this study, Gong et al. (2007) have shown that in a neuronal culture system with ApoE bound to the surface of extracellular synthetic lipid particles, the ApoE4 isoform inhibited the release of cholesterol from neurons. However, in an attempt to link the ApoE isoform status to AD, Morishima-Kawashima et al. (2007) using ApoE4 knock-in mice were unable to show an ApoE4-specific effect on the increased association of Aβ with low-density brain membrane domains. In Down Syndrome (DS) the overexpression of APP is thought to contribute to the development of AD symptoms. The cholesterol levels in DS patients are not associated with AD symptoms, however, an Apo E4 allele was associated with susceptibility to hypercholesterolaemia (Prasher et al., 2008).

It has been recently suggested that Aβ binding to ApoE compromises physiological lipid binding and transport by ApoE, which in turn could have implications for amyloid plaque formation and cholesterol accumulation (Tamamizu-Kato et al., 2008). The convergence of risk factors, including the ApoE4 allele, in AD and cardiovascular disease (Martins et al., 2006) has emerged as a significant factor in cholesterol metabolism (*see also* Section 2.6). Other cholesterol-related genes such as those for hydroxy-methylglutaryl-coenzyme A reductase and the cholesterol transporter ABCA1 have also been claimed to modulate the risk of Alzheimer's disease (Rodríguez-Rodríguez et al., 2009).

2.5 Cholesterol Oxidation and Alzheimer's Disease

A link between oxidative stress and AD has been proposed for several years, but the precise mechanisms have yet to be fully defined (*reviewed by* Butterfield et al., 2002; Schöneich, 2002; Pappolla et al., 2002; Nelson, 2007). A broad survey on the role of oxysterols in neurodegenerative diseases has been recently presented by Björkhem et al. (2009). One has to consider the involvement of several reactive oxygen species when producing damage to membrane lipids, to Aβ and other proteins, together with the role of reactive metal ions, in particular copper and iron. Possible protection, particularly from the water- and lipid-soluble antioxidant vitamins, has been given considerable attention (Behl, 2005), yet it remains unclear whether this therapeutic approach really provides significant benefit, in the shorter or longer term. Remarkably, in a cellular study it has been claimed by Yao et al. (2002) that

22*R*-hydroxycholesterol, an intermediate in the production of pregnenolone from cholesterol, protected neuronal cells from Aβ-induced cytotoxicity by complexing with Aβ. Although implicit, it was not shown whether 22*R*-hydroxycholesterol bound to Aβ with a higher affinity than cholesterol.

Using lipid monolayers, oxidative damage to membrane lipids was claimed by Koppaka et al. (2003) to be linked synergically via $A\beta_{1–42}$ to the promotion of fibril formation by $A\beta_{1–40}$. That Aβ induces oxidation of membrane lipids emerged from a number of studies, exemplified clearly by Cutler et al. (2004), who claimed that in hippocampal neurones oxidative stress led to the accumulation of ceramides and cholesterol, preventable by inclusion of α-tocopherol. In several studies, the exact chemical nature of the oxysterols and cholesterol oxidation products are not defined, however Vaya and Schipper (2007) provided a detailed analysis of the range of oxysterol intermediates that can act as ligands for the liver X-activated receptor (LXR) nuclear receptors, regulators of genes involved in cholesterol homeostasis (*see* below). The principle cholesterol oxidation metabolites, derived from hydrogen peroxide and oxygen free radical interaction, are water soluble 24S-hydroxycholesterol and 7β-hydroxycholesterol; indeed, it has bee shown that hydrogen peroxide is produced catalytically by interaction of Aβ with cholesterol (Ferrera et al., 2008). Although 24S-hydroxycholesterol can cross the blood-brain-barrier (Björkhem et al., 2009) and is the primary cholesterol elimination product of the brain (with an increased level in the brains of AD patients) it has also been suggested that 24S-hydroxycholesterol has protective properties, by complexing with Aβ (Krištofiková et al., 2008). Differential expression and polymorphism of the gene encoding cholesterol 24S-hydroxylase, cytochrome P450 46 (*CYP450 46)*, has been associated with AD, with a predisposition in certain genotypes (Kölsch et al., 2002; Papassotiropoulos et al., 2003; Borroni et al., 2004; Brown et al., 2004); this association has been challenged by others (Desai et al., 2002; Tedde et al., 2006). However, from a proteome analysis of cortical neurones Wang et al. (2008) concluded that 24S-hydroxycholesterol is a down-regulator of cholesterol synthesis and thereby important for brain cholesterol hemostasis. The crystal structure of the principal brain cholesterol hydroxylase, CYP450 46A1, has been determined by Mast et al. (2008) at 2.6 Å and at a slightly improved resolution, with and without bound substrate (White et al., 2008). This structural and biochemical data may ultimately lead to the development of therapeutically useful stimulatory and inhibitory agents. The alternative hydroxycholesterol, 27-hydroxycholesterol, synthesized by sterol 27-hydroxylase (CYP27A1), is known to facilitate the flow of cholesterol from the circulation across the blood brain barrier into the brain, and is likely to have a significant impact upon brain APP processing and Aβ production (Scott Kim et al., 2009). Further emphasis on the importance of 27-hydroxycholestrol has come from the studies of Prasanthi et al. (2009) and Ghibi et al. (2009).

At the genetic level, there has also been interest shown in a link between cholesterol and the enzyme heme oxygenase-1, which stimulates oxysterol production, but also activates the liver X receptor-β (Infante et al., 2008).

Other cholesterol oxidation products include 7-ketocholesterol and cholesterol epoxides (Ong et al., 2003), but their likely pathological roles have yet to be fully

defined. Another reactive cholesterol oxidation metabolite that may be involved in inflammatory atherogenesis (Stewart et al., 2007) and Aβ aggregation (Zhang et al., 2004), is cholesterol *seco*-aldehyde (3β-hydroxy-5-oxo-5, *seco*cholestan-6-ol), produced by ozonolysis of cholesterol. Sathishkumar et al. (2007) found that although cholesterol *seco*-aldehyde-induced neurotoxicity could be prevented by *N*-acetyl-l-cysteine, this compound did not prevent Aβ aggregation. In their detailed in vitro study, Scheinost et al. (2008) found that fibrillogenesis of both $A\beta_{1–40}$ and $A\beta_{1–42}$ was accelerated by cholesterol *seco*-aldehyde, involving a site-specific adduction of the aldehyde to the ϵ-amino group of Lys16 pf Aβ. Cholesterol inhibited this cholesterol *seco*-aldehyde-induced fibrillogenesis of Aβ, perhaps unexpectedly.

That Aβ, and APP, possess an inherent copper-dependent enzyme-like activity in the presence of cholesterol and other substrates (Opazo et al., 2002; Yoshimoto et al., 2005), resulting in the catalytic production of hydrogen peroxide, with oxidation of the cholesterol at the C3β –OH group to produce 4-cholesten-3-one (Puglielli et al., 2005) or alternatively 7β-hydroxycholesterol (Nelson and Alkon, 2005), appears to be a highly significant observation. The correlation of this catalytic activity with the pathogenic mechanism leading to accumulation of cholesterol and cholesterol oxidation products remains to be clarified, but the fact that oxidative mechanisms are emerging strongly as mediators for both atherosclerotic and amyloid plaque formation indicates the likely importance of future research in this area (*see below* and Chapter 5).

2.6 Atherosclerosis and Alzheimer's Disease

That deviant cholesterol metabolism and deposition might be a link between peripheral vascular disease, cardiovascular disease and cerebrovascular disease, i.e. cerebral amyloid angiopathy, Alzheimer's disease and dementia, has been suggested for several years (Hofman et al., 1997; Li et al., 2003), and the topic has been widely reviewed (Sparks et al., 2000; Casserly and Topol, 2004; Kalman and Janka, 2005; Martins et al., 2006; Cechetto et al., 2008). That this link might also involve both peripheral and cerebral proinflammatory events is also apparent (Finch, 2005). In the periphery there is long-standing evidence that blood vessel macrophages accumulate free and esterified cholesterol (Klinknera et al., 1995), but despite the fact that brain astrocyte proliferation is associated with AD there is limited evidence for cholesterol accumulation by these cells or microglia. With circulatory macrophages, Aβ bound to modified LDL has been found to enhance cholesterol accumulation, foam cell formation and Aβ deposition in blood vessel walls (Schulz et al., 2007), but it is not clear whether this is due to the peptide monomer or to an oligomerized/fibrillar form of Aβ. On the other hand, microglial inflammatory activation has been found under conditions of cholesterol embolization (Rapp et al., 2008) and in dietary-induced hypercholesterolemia (Streit and Sparks, 1997; Xue et al., 2007). Modulation of this inflammatory response by liver X receptors has indicated that LXRs have the capacity to maintain phagocytosis in fibrillar Aβ-stimulated microglia (Zelcer et al., 2007). An overall improvement of cerebrovascular function, including reduced

inflammation and soluble Aβ levels, following the administration of simvastatin to aged APP mice was shown by Tong et al. (2009). However, no reduction in the number of Aβ-containing plaques and memory improvement was detected.

Phagocytosis by activated microglia has also been implicated in demyelination disease (Smith, 2001), but this aspect has not received any emphasis in relation to the established presence of microglia within amyloid plaques. However, Stadler et al. (1999) concluded from their study on amyloid plaques in the brains of APP23 transgenic mice that microglial activation and phagocytosis might be associated with neuronal loss. In an attempt to relate the influence of Aβ on the cholesterol content of the Golgi complex in astrocytes, Igbavboa et al. (2003) concluded that extracellular $A\beta_{42,}$ in oligomeric rather than fibrillar state, disrupted cellular cholesterol homeostasis. Extending this study, Igbavboa et al. (2009) have shown that Aβ stimulates the trafficking of both cholesterol and caveolin-1 from the plasma membrane of primary astrocytes to the Golgi complex. The likely importance of the precise distribution of cholesterol, rather than just total brain cholesterol, was shown by Burns et al. (2006), who found that reduction of cholesterol level by statins also caused translocation of cholesterol from brain membrane cytofacial lipid monolayer to the exofacial monolayer. Cholesterol lowering by statins and the link between atherosclerosis and AD has been given due emphasis by Panza et al. (2005) and Orr (2008), and at the genetic level (Papassotiropoulos et al., 2005; Carter, 2007; Reiman et al., 2008) the likely association of multiple gene polymorphisms associated with cholesterol and lipoprotein metabolism in peripheral and cerebral vascular disease, and AD susceptibility, has been assessed.

2.7 Cholesterol and Tau Fibrillization in AD, the Tauopathies and Non-Aβ Amyloidogenic Disorders

The intracellular neuronal accumulation of paired helical filaments of the hyperphosphorylated tau protein represents a well-studied parallel aspect of AD and Niemann-Pick type C disease (NPC), in addition to Aβ studies, which in both diseases appears to be significantly influenced by cholesterol or cholesterol metabolism (*see also* Chapter 11). Despite the increasing interest in the similarities of AD and NPC, and the value of this comparison (Distl et al., 2003; Ohm et al., 2003; Burns and Duff, 2002; Michikawa, 2004; Adalbert et al., 2007), it is clear that the genetic lesion in NPC is clearly defined as a fatal autosomal recessive neurovisceral cholesterol storage disorder. In NPC there is intracellular tau fibrillization and secondary intracellular Aβ accumulation, which presents a significantly different feature to AD, where neuronal cholesterol accumulation although significant is less pronounced and where fibrillar Aβ formation is predominantly extracellular.

Dietary-induced cholesterol-dependent hyperphosphorylation of tau is common to both AD and NPC, and is ApoE isoform dependent (Saito et al., 2002; Rahman et al., 2005; Ghribi et al., 2006; Michikawa, 2006). Contrary to this Fan et al. (2001) had earlier claimed that inhibition of cholesterol synthesis in cultured neurons resulted in hyperphosphorylation of tau. The more widely accepted current point

of view is that cells containing neurofibrillary tangles contain more free cholesterol than tangle-free NPC neurons (Distl et al., 2001). Contrary to AD, which is a neuronal disorder, in NCP the cholesterol storage defect is expressed by neurones, astrocytes and glial cells.

Early neuronal cholesterol accumulation is present within Purkinje neuronal dendritic trees (Reid et al., 2004), but subsequent age-dependent cellular changes in NPC are more clearly linked to the endosomal/lysosomal pathway, with respect to both cholesterol accumulation, and APP processing and Aβ aggregation (Yamazaki et al., 2001; Nixon, 2004). In an elegant cellular and subcellular study Liao et al. (2007) presented convincing evidence in $Npc^{-/-}$ mouse brain that autophagic dysfunction is linked to cholesterol accumulation, with the presence of neuronal vacuole-like structures and multivesicle bodies (*see also* Chapter 11). Also, in a *Drosophila* model for NPC1, Phillips et al. (2008) provided evidence for progressive filipin-staining of cholesterol aggregates in brain and retinal cells during ageing.

Over-expression of the protein α-synuclein is associated with Parkinson's disease, Lewy body formation, and other neurodegenerative α-synucleinopathies.

That normal membrane localization of cholesterol-containing lipid rafts is modified in the Parkinson's-associated A30P mutation, due to raft disruption and redistribution away from synapses, has been claimed by Fortin et al. (2004) to underlie the pathogenesis of Parkinson's disease. Further indication of the involvement of cholesterol has been shown in a cellular model of Parkinson's disease, where statins reduced α-synuclein aggregation and cholesterol supplementation increased α-synuclein aggregation (Bar-On et al., 2008). Similar to AD, it has also been claimed that cholesterol oxidation products are closely involved in α-synuclein fibrillization, of relevance to the development of Parkinson's and Lewy body disease (Bosco et al., 2006). Contrary to the above, Karube et al. (2008) have shown that the N-terminal region of α-synuclein is essential of fatty acid-induced oligomerization of this protein.

The involvement of cholesterol in the generation of other amyloid diseases is limited. Hou et al. (2008) presented evidence that cholesterol and anionic phospholipids are important for transthyretin fibrillogeneis and the resulting cytotoxicity of this protein, which is responsible for familial amyloidotic polyneuropathy. Calcitonin, a 32-aminoacid peptide involved in bone calcium metabolism, undergoes a structural transformation similar to other amyloidogenic proteins (Avidan-Shpalter and Gazit, 2006). The pore-like oligomers formed by calcitonin have an affinity for cholesterol containing rafts in membranes, "termed hydrophobicity-based toxicity" (Diociaiuti et al., 2006), which act as calcium channels. Similarly, in type II diabetes, amylin, the islet amyloid protein which leads to pancreatic fibrillar deposits, undergoes a cytotoxic membrane interaction (Jayasinghe and Langen, 2007), but the evidence presented indicated that phosphatidyl serine was the required lipid for the partly unfolded amylin hydrophobic β-sheet interaction within the membrane bilayer, rather than cholesterol. However, Cho et al. (2008) have produced evidence to suggest that cholesterol regulates amylin non-fibrillar aggregation and deposition within planar cholesterol-containing raft lipid membranes. These workers have recently extended their studies by using model membranes (Cho et al.,

2009), by showing amylin clustering and aggregation on cholesterol-containing membranes, whereas on cholesterol-depleted membranes amylin formed smaller oligomeric structures.

Acidic phospholipids were shown to be necessary for pore formation by human stefin B in model membranes (Rabzelj et al., 2008). Oxidized cholesterol aldehyde products, present in atherosclerotic lesions also have the ability to promote apolipoprotein C-II amyloid fibril formation (Stewart et al., 2007).

2.8 Conclusions

Whole animal and human metabolic studies, together with genetic, cellular and biochemical studies have provided a wealth of information supporting a link between cholesterol and Alzheimer's disease. Increasing evidence suggests that cholesterol interaction is involved in the oligomerization, membrane pore formation and fibrillogenesis of the Alzheimer's Aβ peptide and other amyloid peptides. Undoubtedly, further structural studies will have much to contribute, and are likely to correlate well with the other diverse approaches being used extensively for the study of Alzheimer's disease and other amyloidopathies.

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