REVIEW ARTICLE

Brain cholesterol and Alzheimer's disease: challenges and opportunities in probe and drug development

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Abstract

Cholesterol homeostasis is impaired in Alzheimer's disease (AD), however, attempts to modulate brain cholesterol biology have not translated into tangible clinical benefits for patients to date. Several recent milestone developments have substantially improved our understanding of how excess neuronal cholesterol contributes to the pathophysiology of AD. Indeed, neuronal cholesterol was linked to the formation of amyloid-β (Aβ) formation and neurofibrillary tangles through molecular pathways that were recently delineated in mechanistic studies. Further, remarkable advances in translational molecular imaging have now made it possible to probe cholesterol metabolism in the living human brain with positron emission tomography, which is an important prerequisite for future clinical trials that target the brain cholesterol machinery in AD patients – with the ultimate aim to develop disease-modifying treatments. This work summarizes current concepts of how the biosynthesis, transport and clearance of brain cholesterol are affected in AD. Further, current strategies to reverse these alterations by pharmacotherapy are critically discussed in the wake of emerging translational research tools that support the assessment of brain cholesterol biology not only in animal models but also in AD patients.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that primarily affects elderly individuals [1]. Due to the rapidly growing prevalence of AD, it has become a leading source of disability, contributing to the mounting healthcare burden in the Western world [2,3]. Histologically, AD is characterized by two major hallmarks, amyloid-β (Aβ) plaques and neurofibrillary tangles, which begin to develop at preclinical disease stages [4]. Along this line, the ability to detect Aβ plaques directly by non-invasive molecular imaging with positron emission tomography (PET) and indirectly with biofluid biomarkers has reshaped the diagnostic landscape and enabled early risk stratification of patients with mild cognitive impairments [5,6]. Although the development of effective Aβ lowering therapy has proven challenging, the recently disclosed findings from the Clarity AD trial with lecanemab – a humanized monoclonal antibody that binds to soluble Aβ protofibrils – revealed a significant attenuation of cognitive and functional decline compared to placebo after 18 months in patients with early AD, together with clearance of Aβ [7]. As such, lecanemab has been granted accelerated approval by the US Food and Drug Administration (FDA) [8]. The development of lecanemab constitutes a critical milestone that is based on decades of strenuous drug discovery efforts, thus channeling the development of donanemab, which was the second Aβ-targeted antibody with significant clinical efficacy [9]. While much remains to be learned about the efficacy and safety of lecanemab and donanemab in larger populations, initial results suggest that the clinical course was only moderately improved compared to placebo [7,9]. According to the Alzheimer's Drug Discovery Foundation, Aβ clearing drugs will likely need to be complemented by combination therapies in the future to achieve improved efficacy [10]. Indeed, given the multifaceted pathophysiology of AD, there is a pressing need for the next generation of drugs that are focused on other targets, and there is a solid body of evidence suggesting that brain cholesterol is heavily implicated in the pathophysiology of AD [11].

Brain cholesterol primarily resides in myelin sheaths of oligodendrocytes and plasma membranes of astrocytes and neurons [12]. Provided that the blood–brain barrier precludes significant exchange between the brain and cholesterol-containing lipoprotein particles in the systemic circulation, the vast majority of brain cholesterol is derived from *de novo* biosynthesis in astrocytes and neurons [13-15]. Under physiological conditions, the brain cholesterol homeostasis is

tightly regulated and represents a balance between cholesterol production, metabolism, transport, and clearance (CNS) [12,13,16]. Some of the key steps involve 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a ubiquitous enzyme responsible for the rate-limiting step in the biosynthesis of cholesterol, apolipoprotein E (ApoE)-mediated cholesterol transport within the CNS, and cytochrome P450 46A1 (CYP46A1) – the CNS-specific enzyme that facilitates cholesterol excess removal from the brain (**Fig. 1**) [17,18]. CYP46A1 is abundantly expressed in neurons and constitutes the primary cholesterol clearance mechanism by catalyzing cholesterol conversion to 24*S*-hydroxycholesterol. This metabolite can readily cross the blood-brain barrier and be eliminated from the CNS [16,19]. In the adult brain, cholesterol biosynthesis is high in astrocytes, therefore brain neurons rely in large part on cholesterol delivery from astrocytes, which occurs via lipid transport on ApoE-containing lipoprotein particles [20,21].

Given the pivotal role of cholesterol in the mammalian brain, it is not surprising that dysfunctional cholesterol homeostasis in the brain can have far-reaching implications for brain physiology and play an important role in the onset and progression of AD. Hence, recent advances in non-invasive technology that quantitatively measure the extent of brain cholesterol metabolism holds promise to broadly impact cholesterol research in AD [22]. This work provides an overview of contemporary concepts that link the brain cholesterol axis to the pathophysiology of AD. Further, challenges and opportunities in the development of brain cholesterol-modulating therapy are critically discussed, thereby highlighting the potential contribution of emerging translational molecular imaging tools in future studies.

Impaired cholesterol homeostasis in AD

A mounting body of evidence points toward detrimental alterations in brain cholesterol homeostasis of the AD brain [23-30]. A summary of selected preclinical and clinical studies discussed in this review is provided (**Tables 1** and **2**). While cholesterol is required for critical physiological brain functions such as synaptic plasticity, learning and memory [14,31-34], recent evidence suggests a multi-layered role of brain cholesterol in the pathophysiology of AD (**Fig. 2**). Specifically, excess neuronal cholesterol can affect lipid rafts (highly specialized and dynamic membrane domains) and thereby promote processing of the amyloid precursor protein

(APP) to amyloid β (Aβ) at the plasma membranes [23-30]. In addition, lipid rafts not only harbor high amounts of sphingolipids, phosphatidylserine and cholesterol, but also represent prominent accumulation sites for glycosylphosphatidyl-inositol (GPI)-anchored proteins, tyrosine kinases and other transmembrane proteins [35-40]. As such, lipid rafts fulfil multiple cellular functions and are involved in numerous signal transduction pathways that affect neuronal signaling and function [41]. There are several lines of evidence pointing towards direct involvement of neuronal cholesterol in AD. First, the landmark discovery by Barrett et al. unveiled that APP is endowed with a flexible transmembrane domain that is capable of binding cholesterol, implicating neuronal cholesterol in amyloidogenic processing (**Fig. 2**) [28,39,41]. This fundamental insight, along with a recent study suggesting a catalytic role of cholesterol for the aggregation of Aβ42 in the presence of lipid membranes [42], provided a solid conceptual basis for the link between neuronal cholesterol and Aβ pathology. In concert with these observations, cholesterol depletion was found to substantially attenuate Aβ generation in hippocampal neurons [23]. Finally, Aβ production in neurons is regulated by cholesterol synthesis and ApoE transport from astrocytes [29]. Taken together, these findings provide support for neuronal cholesterol involvement in Aβ plaque formation.

Beyond amyloidogenic processing, brain cholesterol has been implicated in several other molecular pathways linked to AD pathophysiology. For instance, neuronal cholesterol accumulation boosts the formation of pathogenic neurofibrillary tangles that consist of misfolded phosphorylated tau (p-tau) proteins – independent of Aβ-related pathways [25,43,44]. Indeed, neuronal cholesterol deposits enhanced the accumulation of p-tau through inhibition of its proteasomal degradation (**Fig. 2**) [25]. Given that the reduction of neuronal cholesterol deposits attenuated p-tau levels in isogenic induced pluripotent stem cell (iPSC) lines bearing mutations in the cholesterol-binding domain of APP or APP null alleles, it was concluded that the effect of neuronal cholesterol on p-tau was independent of both APP and Aβ. Additional evidence suggesting a link between neuronal cholesterol and p-tau was derived from studies with transgenic mouse models of tauopathy that lacked an overt Aβ pathology [25,45]. In these animals, cholesterol-lowering therapy attenuated tau pathology, corroborating observations from iPSC-based experiments. In a different attempt to reduce intraneuronal cholesterol, inhibition of acetyl-coenzyme A acetyltransferase (ACAT), the enzyme that catalyzes the conversion of cholesterol to cholesteryl ester, has been suggested as a potential therapeutic strategy in AD and led to the

development of various ACAT inhibitors that are currently in preclinical and clinical development [27,46].

To date, the most extensively investigated link between brain cholesterol machinery and AD is based on the pathogenic role of APOE. Indeed, polymorphic alleles of the *APOE* gene constitute major genetic determinants of AD, and individuals carrying the ε4 allele exhibit a substantially increased risk of developing AD [47]. Despite the strong link between *APOE* polymorphism and AD, it is not entirely clear how the presence of the ε4 allele affects cholesterol transport, metabolism and deposition in the AD brain. Nonetheless, a number of studies have suggested distinct putative mechanisms, some of which directly involve impaired cholesterol metabolism and trafficking in the brain [48]. Studies of rodent and human origin revealed that Aβ levels and amyloid plaque load in the brain depend on the ApoE isoform, and ApoE4 was associated with enhanced amyloid pathology across different species [49-51]. Further, it was shown that different ApoE isoforms exhibit distinct lipidation status, thereby affecting Aβ clearance in an isoform-dependent manner [48]. ApoE4 was found to be less effective in transporting brain cholesterol than other isoforms [52], which may result in impaired cholesterol trafficking in carriers of the ε4 allele and further accelerate cholesterol-dependent amyloidogenic pathways. Collectively, these early observations support a central role of ApoE in Aβ deposition and clearance. More recently, ApoE4 was found to exacerbate tau-mediated neurodegeneration in a mouse model of tauopathy, contributing to a persistent activation of microglial cells and neuroinflammation (**Fig. 2**) [53]. Further, ApoE has recently been shown to regulate cerebrovascular integrity via the cyclophilin A pathway [54], and studies exploring the role of ApoE isoforms concluded that ApoE was associated with an accelerated disruption of the blood-brain barrier and cognitive decline (**Fig. 2**) [55,56]. While these studies did not account for the ApoE lipidation status and cholesterol homeostasis, it remains to be elucidated whether ApoE4 may constitute a viable target for pharmacological therapy to attenuate tau pathology and cerebrovascular impairment in AD. ApoE4 has also recently been linked to impaired neuronal myelination via dysregulation of cholesterol homeostasis in human post-mortem oligodendrocytes [57,58]. While myelin sheaths wrap around neuronal projections called axons, the generation of myelin depends on the expression of myelin basic protein (MBP), which combines with cholesterol to build the foundation of myelin. Remarkably, *APOE4* carriers exhibited a defective cholesterol transport in oligodendrocytes, leading to the accumulation of cholesterol in these cells and ultimately

resulting in a decrease of MBP expression (**Fig. 2**). Pharmacological intervention with cholesterol-lowering agents facilitated cholesterol clearance from oligodendrocytes and resulted in a marked increase in axonal myelination, improving learning and memory in ApoE4 mice [57].

A pivotal role in neural cholesterol homeostasis is attributed to the ATP binding cassette protein A1 (ABCA1). While the *ABCA1* locus has not yielded a prominent hit in large GWAS studies of AD, there are various functional studies linking this *ABCA1* to AD. First, ABCA1 constitutes a cholesterol efflux transporter, which is upregulated in response to excess intracellular cholesterol challenge [59 60]. Of note, the upregulation of ABCA1 offers a spectrum of favorable outcomes, spanning from enhanced APOE lipidation [61] and insulin sensitivity [62,63] to an improved peripheral vascular integrity, blood–brain barrier function [64] and anti-inflammatory signaling [60]. Second, endogenous control mechanisms that respond to excess cellular cholesterol uptake by promoting ABCA1 upregulation are dysfunctional in AD patients [60]. Despite strenuous efforts, the successful translation of therapeutic agents aimed at enhancing ABCA1 activity to clinical applications remains a challenge. Although distinct therapeutic modalities have been developed and validated in animal models, their clinical development is hindered by noteworthy side effects, including lipogenesis and heightened triglyceride production [60,65]. Alternative compound screening approaches, such as by phenotype-based screening, may have the potential for identifying small molecule modulators capable of upregulating ABCA1 without inducing lipogenesis, potentially paving the way for a successful clinical translation [66,67].”

Lipidomics studies have revealed that besides sterols, several other lipid classes are dysregulated in AD, including fatty acids, sphingolipids, glycerophospholipids and lipoproteins [68 69]. While alterations in lipid composition often reflect structural changes in the neurodegenerative brain, lipidomics research has provided valuable mechanistic insights into the involvement of various lipids in AD. This includes the identification of inflammatory lipid mediators [70], lipids that play a crucial role in APP processing, biological sensors of oxidative stress and mitochondrial dysfunction [71 72], as well as plasma and CSF markers [73 74 75]. While the discussion of the distinct lipid classes is beyond the scope of this review, evidence to date points towards a broad involvement of dysfunctional lipid metabolism that goes far beyond neural cholesterol. Of note, the contemporary lipidomics landscape in AD has recently been reviewed by various other groups [76-82].

In conclusion, these findings suggest that brain cholesterol deposition and trafficking via ApoE are involved in the mechanisms that independently contribute to amyloid and tau pathology in AD, blood-brain barrier dysfunction, and impaired myelination of axons. While brain cholesterol dysfunction appears to exacerbate the development of AD, the question remains whether removing excess cholesterol from neurons and restoring physiological cholesterol trafficking constitutes a valid approach for a long-sought disease-modifying treatment in AD. First, given that brain cholesterol biology is highly regulated, it is conceivable that pharmacological intervention at one target protein will trigger a cascade of molecular events that may or may not restore a balanced brain cholesterol homeostasis. Second, cholesterol is required for numerous brain functions and the reduction of cholesterol in neurons may harbor a potential risk of causing adverse neurological events. Third, non-invasive assessment of cholesterol homeostasis in the mammalian brain constitutes a major challenge and previous efforts have been hampered by the lack of appropriate tools to accurately quantify cholesterol deposition and trafficking. As such, a better understanding of the brain cholesterol machinery is needed to facilitate monitoring of pharmacological interventions aiming at reducing neuronal cholesterol deposits and restoring appropriate ApoE functions. The ongoing development of improved imaging tools for *in vitro* and *in vivo* quantification of sterols and molecular determinants involved in brain cholesterol biology will substantially facilitate research in the field, potentially paving the way for well-designed studies in experimental models of AD and AD patients.

Genetic evidence implicating cholesterol homeostasis

Genome-wide association studies (GWAS) have been instrumental in characterizing the genetic landscape and identifying gene variants associated with AD. Indeed, two recent large GWAS analyses encompassing a total of 35,274 and 111,326 documented AD cases, respectively, confirmed previously reported risk genes and identified new relevant loci, many of which are directly or indirectly involved in lipid homeostasis [83,84]. Along this line, pathway analyses revealed that some of these genes, including *APOE, TREM2, ABCA7, INPP5D, CLU, SPI1* and *SORL1*, converge on an intriguing interplay between microglial cells and cholesterol-rich cellular structures involved in efferocytosis – the process by which apoptotic cells in the brain are removed via microglial phagocytosis [85]. Of note, the ingestion of apoptotic cells by brain-

resident microglia poses the challenge of internalizing and degrading significant amounts of cholesterol-rich myelin debris. As such, microglia are endowed with an adaptive transcriptional system that allows them to upregulate genes involved in lipoprotein biogenesis and cholesterol efflux [86]. The latter allows microglia to regulate myelin growth and neuronal integrity in the mammalian CNS [87,88], while circumvent the intracellular accumulation of toxic cholesterol levels, which can lead to the formation of cholesterol crystals within lysosomes and contributes to pro-inflammatory microglial priming [89,90]. While the role of *APOE* in AD has been discussed above, the following part will summarize the contemporary body of evidence that substantiates implications of the other AD risk genes associated with lipid homeostasis.

ATP binding cassette subfamily A member 7 (ABCA7) modulates cellular cholesterol content by engaging as a cholesterol efflux transporter [91]. Of note, while ABCA7 has been established as an AD risk gene by several GWAS and functional association studies [92-97], the mechanisms by which ABCA7 confers the risk of AD are not entirely understood. As a member of the ABC transporter superfamily, endowed with an inherent capacity to recognize and transport different lipids across membranes, it is widely expressed in brain-residing microglia [98]. Suppression of endogenous *ABCA7* in several distinct human cell lines resulted in increased β-secretase cleavage and amyloid burden, while augmented ABCA7 protein levels were linked to early- and late-onset AD by post-mortem tissue studies [99-101]. Of note, there is preliminary evidence supporting the concept that ABCA7 promotes phagocytosis in different human cell lines [102,103]. Nonetheless, it should be noted that *Abca7*-null mice exhibit similar serum cholesterol levels to their wild-type counterparts, while cholesterol efflux in macrophages isolated from these mice is not significantly different from that of wild-type macrophages [104]. Further, while *Abca7*-null animals display elevated insoluble Aβ content, they do not show heightened apoE abundancy, indicating that enhanced Aβ levels may be triggered independent of lipid efflux [99]. Taken together, these observations raise the question whether the association of ABCA7 and AD may be rooted in molecular pathways that do not necessarily involve lipid homeostasis.

Triggering receptor expressed on myeloid cells 2 (TREM2) constitutes a microglial surface protein that modulates intracellular protein tyrosine phosphorylation [105]. Dysfunction of TREM2 ultimately results in impaired efferocytosis of myelin debris, thus leading to microglial alterations in cholesterol metabolism [106,107]. Indeed, it was shown that WT microglia acquire a

disease-associated transcriptional state upon demyelination challenge, while TREM2-deficient microglia are plagued by an attenuated priming process, which ultimately results in neuronal damage [107]. Despite their ability to phagocytose myelin debris to some extent, TREM2-deficient microglia are less likely to clear myelin cholesterol, thus leading to intracellular cholesteryl ester accumulation. Notably, this observation holds true not only in TREM2-deficient murine macrophages but also in human iPSC-derived microglia [107], rendering TREM2 a critical modulator of cholesterol homeostasis following neuronal demyelination. While the AD-linked variant, TREM2 R47H, associates with an attenuated microglial proliferation, activation and clustering around Aβ plaques in AD mouse models [108,109], experiments with human iPSCs carrying the R47H mutation has produced inconclusive findings, with some preliminary data suggesting a detrimental phenotype and others indicating that the R47H mutation was not sufficient to cause significant phenotypic defects in human iPSCs [110,111]. Notably, however, the R47H mutation seems to impair the ability of TREM2 to sense damage-associated lipid patterns that occur under neurodegeneration, potentially hampering the microglial response to Aβ plaque formation [105].

While TREM2 is involved in phagocytosis and processing of cholesterol-rich myelin debris, other AD risk genes that constitute established GWAS hits are indirectly linked to cholesterol metabolism via crosstalk with TREM2. For instance, the inositol polyphosphate-5-phosphatase D (*INPP5D*) gene, which encodes the phosphatidylinositol phosphatase SH-2 containing inositol 5' polyphosphatase 1 (SHIP1), modulates immune stimulatory signaling downstream of TREM2 by catalyzing the hydrolysis of $PI(3,4,5)P_3$ and precluding the recruitment of effector proteins [83,84,112,113]. Another example of an AD risk gene identified by GWAS is the *CLU* locus, which encodes the apolipoprotein clusterin (APOJ) [83,114]. Clusterin binds to TREM2, thereby triggering its internalization into the cell [115]. Of note, binding of Aβ to clusterin-containing lipoproteins facilitates Aβ clearance by microglia [115], highlighting a potential mechanism by which mutations in the *CLU* locus may hamper microglial phagocytosis. Along this line, the presence of clusterin in peripheral macrophages was shown exacerbate efferocytosis [116]. Despite these compelling findings, it should be noted that expression is highest in astrocytes and there is a plethora of open questions on the detailed mechanism by which clusterin modulates AD risk. While there is preliminary evidence supporting the notion that cholesterol and other lipid metabolism may be

involved in the association between *CLU* and AD, further research is warranted to substantiate these claims.

Cholesterol-sensing signal-dependent transcription factors (SDTFs), such as the LXR:RXR nuclear receptors, orchestrate gene expression by activating the transcription factor PU.1, which is encoded by the Spi-1 proto-oncogene (*SPI1*) [85,117]. While the *SPI1* locus has been associated with AD through various GWAS studies, there is mounting evidence mechanistically linking the *SPI1 to cholesterol homeostasis* [84,118-120]. Indeed, liver X receptors constitute oxysterol-activated subunits of LXR:RXR nuclear receptors that regulate cholesterol homeostasis by enhancing the microglial capacity to manage substantial quantities of ingested cholesterol, rendering these nuclear receptors a pivotal player in neurodegenerative disorders such as AD [121]. In microglia, LXR:RXR nuclear receptors target genes are primarily involved in efferocytosis and cholesterol efflux, such as *Apoe* and *Abca1*, thereby suppressing the inflammatory response [122]. Along this line, *Apoe* or *Abca1 knock out prompts a phenotype with* impaired cholesterol efflux, thus hampering myelin debris efferocytosis and remyelination in mouse models of demyelination [122]. Consequently, LXR:RXR nuclear receptors represent promising therapeutic targets for the modulation of APOE/cholesterol metabolism as well as the inflammatory response within microglial populations. Another GWAS hit that is related to

Mutations in the sortilin related receptor 1 (*SORL1)* gene have consistently been linked to AD in large GWAS [83,84,114,123], whereas some coding variants were found in familial and sporadic AD [124]. Notably, the SORL1 protein is thought to act within conventional AD risk pathways by contributing to the preferential trafficking of APP to endosomal recycling pathways, and away from β-secretase cleavage and subsequent β-amyloid formation [125,126]. While SORL1 affects cholesterol trafficking and uptake into neurons by acting as a receptor for APOE, the lack of SORL1 triggers early endosome enlargement, impaired lipid trafficking, and altered APP localization within the endolysosomal neural network [127] [128]. In contrast, *SORL1* knock out microglia do not exhibit an altered APP phenotype, albeit defects in Aβ uptake are observed [85,129]. Although there is accumulating evidence implicating SORL1 in lipid metabolism and APP processing via APOE, its role in microglial efferocytosis has yet to be fully elucidated.

Targeting brain cholesterol clearance

Due to the limited exchange between plasma and CNS cholesterol, a proper balance between cholesterol biosynthesis and metabolic clearance is critical for a healthy mammalian brain. While HMG-CoA reductase catalyzes rate determining step for the synthesis of cholesterol in astrocytes and neurons, cholesterol clearance from the CNS is primarily driven by hydroxylation via CYP46A1 [16-18,130]. Mounting evidence indicates that the coordinated activity of these two enzymes may orchestrate neuronal supply and elimination of cholesterol. For instance, *Cyp46a1*$^{-/-}$ mice show a substantial compensatory suppression of cholesterol biosynthesis in the brain to maintain the same steady-state sterol levels, however, they do not develop AD pathology [131]. Similarly, inhibition of HMG-CoA reductase activity with a statin resulted in a decline of 24*S*-hydroxycholesterol in the cerebrospinal fluid (CSF) of AD patients, thus suggesting a reduced metabolic cholesterol clearance [132]. It should be noted, however, that the reduced amount of 24*S*-hydroxycholesterol in the CSF may, at least in part, reflect a reduced substrate availability, which may occur following statin-induced inhibition of cholesterol biosynthesis. Notably, the balance between cholesterol elimination by metabolism and cholesterol biosynthesis in the brain may be disturbed in AD, potentially accounting for the excess neuronal cholesterol accumulation in the AD brain (**Fig 3**). Historically, brain cholesterol in AD was targeted by HMG-CoA reductase inhibitors, and cholesterol metabolism by CYP46A1 was largely neglected. There is a growing body of evidence implicating CYP46A1 in the pathophysiology of AD. Several clinical studies have revealed that patients with mild cognitive impairments (MCI) and early stages of AD present with augmented levels of 24S-hydroxycholesterol in the CSF [133-137]. Along this line, it was hypothesized that CYP46A1 function is enhanced in MCI and early AD, as an attempt to eliminate excess brain cholesterol [135]. Nonetheless, it should be noted that more advanced stages of AD can be associated with reduced 24*S*-hydroxycholesterol levels, potentially owing to the degeneration of brain areas expressing CYP46A1 [130]. Given the invasive nature of CSF collection, attempts have been made to leverage plasma concentrations of 24*S*-hydroxycholesterol as a surrogate for CYP46A1 function, however, studies assessing the link between circulating plasma levels of 24*S*-hydroxycholesterol and AD have been conflicting [138-142]. An important consideration is that 24*S*-hydroxycholesterol is metabolized in the liver [140,143,144]. The latter has raised significant concerns about the reliability of 24*S*-

hydroxycholesterol as a plasma biomarker of brain cholesterol metabolism [140]. Nevertheless, serum and CSF 24*S*-hydroxycholesterol quantifications suggested that CYP46A1 activation by enzyme overexpression or positive allosteric modulation could be beneficial in AD [145]. Indeed, CYP46A1 was shown to be endowed with an allosteric site that can be targeted by a small dose of the anti-HIV drug, efavirenz [146,147]. A neuroprotective role of CYP46A1 has been corroborated in various mouse models of AD [30,148-151]. Similarly, efavirenz treatment also reduced deposition of cholesterol in tissue cultures of induced pluripotent stem cell (iPSC)-derived AD neurons and attenuated Aβ and tau pathology [25]. A clinical trial assessing efavirenz safety and CYP46A1 engagement in patients with early AD has been recently completed (NCT03706885), and identified efavirenz doses that enhance CYP46A1 activity and brain cholesterol metabolism [152]. This proof-of-concept investigation created a conceptual paradigm for larger clinical studies to refine efavirenz dosing for optimal CYP46A1 activation and therapeutic effects.

More recently, a novel mechanism has been suggested, linking CYP46A1 with AD via a molecular pathway that involves the ATPase family AAA-domain containing protein 3A (ATAD3A) [153]. This work supported a key role of CYP46A1 in the pathophysiology of AD, which seems to be consistent across different mouse models of AD, as well as in human cell cultures and post-mortem brain samples from diseased AD patients. Thus, monitoring for changes in cholesterol elimination by CYP46A1 seems critical for the elucidation of underlying causes of impaired brain cholesterol homeostasis in AD. Given recent advances in the development of CYP46A1-targeted translational molecular imaging probes, it has now become possible to visualize CYP46A1 in the living human brain using positron emission tomography [22,154]. PET is an imaging modality that allows the quantification of biological processes non-invasively, which is particularly useful for CNS applications in humans. CYP46A1-targeted PET creates new possibilities to study the impact of therapeutic intervention on neuronal cholesterol metabolism and clearance from the CNS in AD patients, potentially serving as a predictive biomarker and allowing the identification of patient subpopulations that may benefit most from therapeutic intervention. In a proof-of-concept study, it was shown that the novel PET tracer, ^{18}F-Cholestify, was sensitive to differences in brain cholesterol metabolism between the 3xTg mouse model of AD mice and respective control animals [22]. Employing PET to elucidate how neuronal cholesterol metabolism is affected by cholesterol-lowering therapy may shed light on

the statin controversy as a therapy in AD, which is discussed in the next chapter. Further, insights gained from a CYP46A1-targeted PET in AD patients may improve our mechanistic understanding of AD-related aberrations of brain cholesterol homeostasis, potentially paving the way for the design of novel therapeutic strategies in AD.

Statin controversy as a therapy in AD

Statins lower cholesterol levels by inhibiting HMG-CoA reductase, the rate-limiting step in the biosynthesis of cholesterol [155]. HMG-CoA reductase has been successfully validated as a therapeutic target in cardiovascular medicine, and statins have become a fundamental tool of cardiovascular disease prevention [156-159]. Yet, it is debated whether statins affect the risk of dementia. Although the concept of reducing neuronal cholesterol deposits by lowering *de novo* cholesterol biosynthesis in the brain seems plausible, the impact of statins on neuronal cholesterol accumulation in humans remains poorly understood. Despite early evidence from cohort and case-control studies indicated that statin therapy was associated with a reduced risk of dementia [160-165], randomized controlled trials failed to establish a convincing link between statin treatment and cognitive improvement to date [166-168]. Hence, statin treatment is not recommended for the prevention or treatment of dementia in contemporary clinical guidelines [169]. Moreover, the FDA issued a black box warning in 2012 outlining that statins may be associated with transient cognitive impairment in a small number of individuals, which typically disappeared following discontinuation of the respective statin therapy [162]. The underlying cause is currently not understood. Nonetheless, one trial showed a significant cognitive improvement in a subpopulation of patients with mild-to-moderate AD who carried the *APOE* ε4 allele [48]. In addition, an ongoing large-scale randomized controlled trial involving 81 medical centers in the United Stated of America will test the efficacy of atorvastatin in preventing dementia, persistent disability and death in community-dwelling adults ≥75 years of age (NCT04262206). The conflicting findings from clinical trials assessing the use of statins, as well as the observation that *APOE* ε4 allele carriers may benefit from statin therapy, emphasize the need for an improved understanding of the mechanisms by which statins affect brain cholesterol homeostasis in distinct AD subpopulations. Such data is currently lacking, constituting a critical knowledge gap in the field. A fundamental breakthrough could be achieved by the validation of novel predictive

biomarkers to identify AD subpopulations that benefit most from cholesterol lowering therapy. Further, assessing the impacts of cholesterol lowering therapy on neuronal cholesterol biosynthesis, transport by APOE and CYP46A1-mediated metabolic clearance from the CNS may deliver key insights into how conventional statin therapy modulates brain cholesterol in humans. While pleiotropic statin effects have been primarily described in cardiovascular studies [170-175], these effects in the brain are poorly understood. In particular, pleiotropic statin effects have not yet been elucidated within the context of AD. Future studies aimed at elucidating how pleiotropic statin effects manifest in the mammalian brain would be particularly useful. Of importance are concerns about the ability of the FDA approved cholesterol lowering agents to cross the blood-brain barrier and inhibit HMG-CoA. Accordingly, the availability of a suitable HMG-CoA reductase-targeted PET probe could provide crucial information about the extent of brain penetration for statins in humans by means of target occupancy studies [176]. Such mechanistic insights are of paramount translational relevance to validate the notion that the brain cholesterol levels could be altered, thus paving the way for the development of novel cholesterol lowering agents that are tailored for CNS-targeted therapy.

Concluding remarks

Several lines of evidence indicate that brain cholesterol homeostasis is impaired in AD. Although there seems to be a consensus that excess neuronal cholesterol contributes to the pathology of AD, molecular mechanisms that prompt the accumulation of neuronal cholesterol are largely unexplored. Clinical studies assessing the efficacy of HMG-CoA reductase inhibitors in AD patients have yielded conflicting results and there is a plethora of unanswered questions regarding the effects of statins on brain cholesterol homeostasis, particularly in humans. Recent breakthrough discoveries provided novel mechanistic insights into how APOE and CYP46A1 could contribute to AD pathology. Achieving therapeutic benefits in AD patients by targeting brain cholesterol requires an in-depth understanding of the molecular mechanisms that contribute to enhanced neuronal cholesterol accumulation. Leveraging the rapidly growing body of literature on APOE and CYP46A1, along with insights from extensive GWAS and advanced lipidomics, has the potential to pave the way for innovative combination therapies that could alleviate the suffering of millions of AD patients.

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Competing interests

S.H.L., and A.H. are listed as inventors on the provisional patent application "Novel PET ligands for imaging cholesterol homeostasis" (application number 63/397,463).

Supplementary material

Supplementary material is available at *Brain* online.

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Figure legends

Figure 1 Simplified model of brain cholesterol biology. In the adult mammalian brain, cholesterol is mainly derived from de *novo synthesis* in astrocytes. HMG-CoA reductase constitutes the enzyme responsible for the rate-limiting step of cholesterol biosynthesis. Cholesterol is delivered from astrocytes to neurons via ApoE-mediated transport. Excess neuronal cholesterol is primarily eliminated via CYP46A1 – a key enzyme that mediates the reaction of cholesterol to 24S-hydroxycholesterol, which readily crosses the blood-brain barrier and can be eliminated from the central nervous system (CNS). Impaired neuronal cholesterol homeostasis can lead to an enhanced formation of neuronal cholesterol deposits, as observed in isogenic induced pluripotent stem cells (iPSCs) derived from Alzheimer's disease (AD) patients. Neuronal cholesterol has been linked to the development of amyloid β and tau pathology in AD.

Figure 2 Putative mechanisms by which brain cholesterol can contribute to pathophysiology of AD. A. *Extraneuronal mechanisms that involve the high-risk ApoE4 variant.* 1) ApoE4 has been linked to impaired axonal myelination. Excess cholesterol in oligodendrocytes of ApoE4 carriers and reduces myelin basic protein (MBP) ultimately hampering the ability of oligodendrocytes to carry out axonal myelination. 2) ApoE4 inhibits the cyclophilin A (CypA) pathway in pericytes, which involves activation of nuclear factor kappa B (NF-κB) and matrix metalloprotease 9 (MMP9) and is required for a healthy function of tight junctions in the endothelium. 3) The presence of ApoE4 associates with enhanced microglial activation and release of proinflammatory cytokines. **B.** *Intraneuronal mechanisms that implicate neuronal cholesterol in AD.* 4) Cholesterol trafficking from neurons to other cells in the CNS is hampered in ApoE4 carriers due to the reduced capability of this particular isoform to transport brain cholesterol. 5) Neuronal cholesterol can be esterified by the enzyme, acetyl-coenzyme A acetyltransferase (ACAT), and is stored in form of lipid droplets. 6) Notably, the

amyloid precursor protein (APP) is endowed with a flexible transmembrane cavity that binds cholesterol, 7) triggering amyloidogenic processing and generating Aβ monomers. 8) Aβ monomer nucleation and formation of Aβ fibrils is accelerated in the presence of membrane-associated cholesterol. Cholesterol accumulates in specialized membrane substructures known as lipid rafts. 9) Aβ plaque formation requires cholesterol, whereas considerable amounts of cholesterol can be found in Aβ plaques. 10) Aβ pathology boosts the formation of neurofibrillary tangles. 11) The formation of neurofibrillary tangles is further accentuated by the attenuation of proteasomal p-tau (hyperphosphorylated tau) degradation through neuronal cholesterol deposits.

Figure 3 Balance between in situ cholesterol biosynthesis and clearance from the brain. A potential hypothesis to conceptualize the enhanced neuronal cholesterol accumulation in Alzheimer's disease constitutes a disturbed balance between *de novo* biosynthesis and metabolic clearance of neuronal cholesterol. If this concept is validated in future studies, pharmacological therapy that aims at restoring the balance between production and clearance of neuronal cholesterol holds promise to provide therapeutic benefit in AD patients.

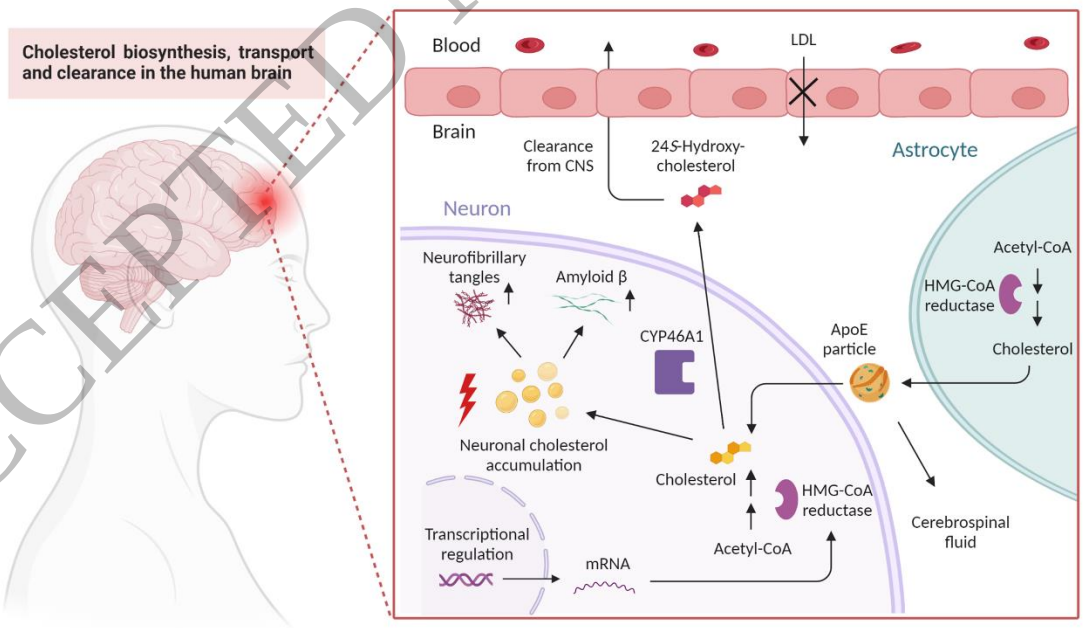

Figure 1
339x190 mm (x DPI)

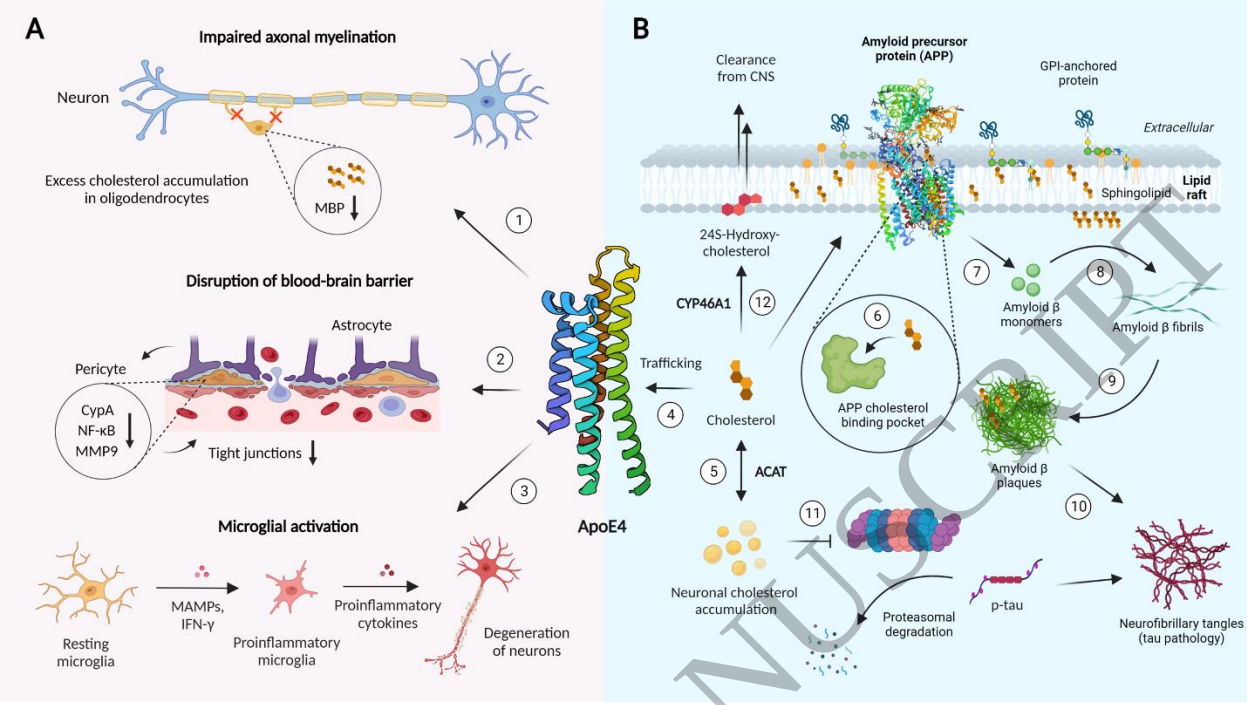

Figure 2
339x190 mm (x DPI)

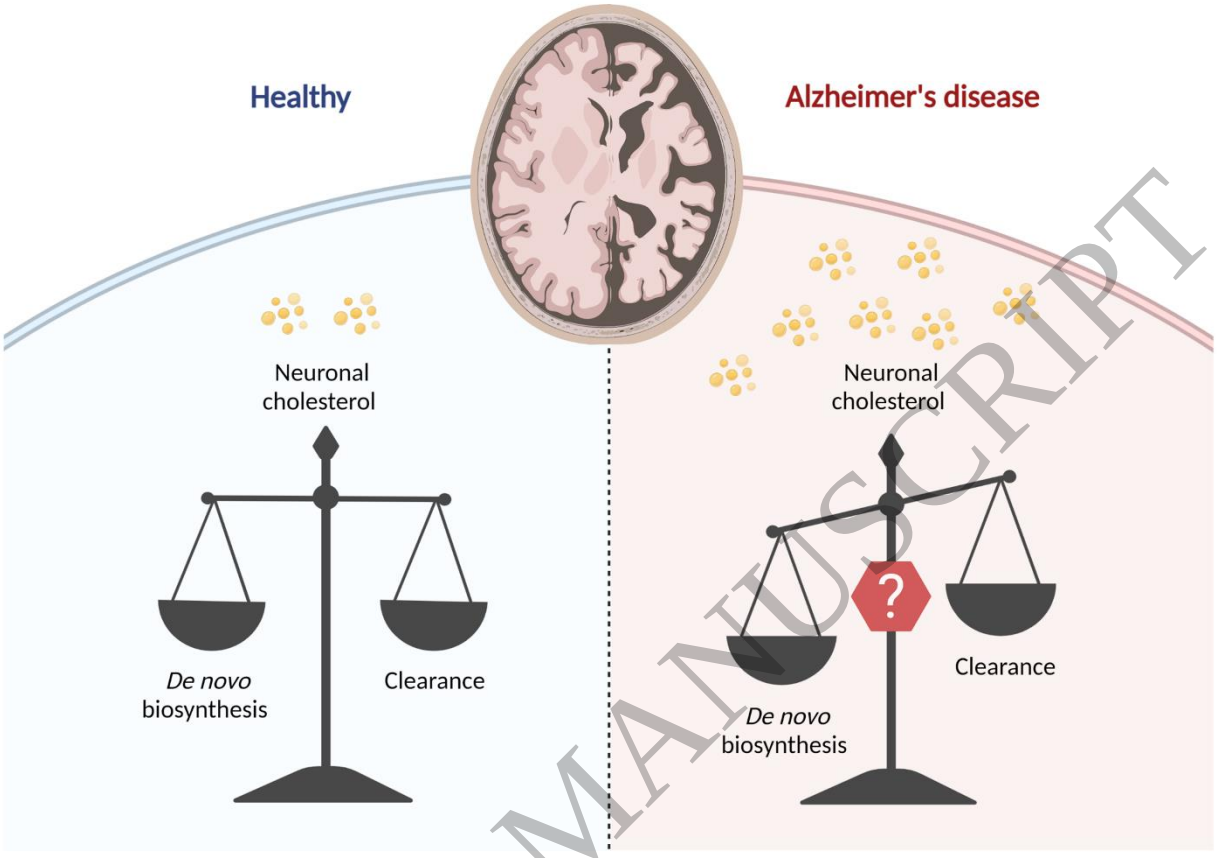

Figure 3
270x192 mm (x DPI)

Table 1 Selected preclinical evidence implicating brain cholesterol homeostasis in Alzheimer's disease.

Type of study	Key findings	References
Evidence from animal studies		
3xTg-AD mouse model	Cholesterol clearance via CYP46A1 is enhanced in experimental AD	Haider *et al.*[22]
	ACAT inhibition enhances autophagy and reduces P301L-tau protein content	Shibuya *et al.*[43]
hAPP mouse model of AD	ACAT inhibitor, CP-113,818, reduces amyloid pathology in experimental AD	Hutter-Paier *et al.*[27]
APP23 mouse model of AD	Adeno-associated virus vector encoding short hairpin RNA directed against mouse *Cyp46a1* mRNA triggers Aβ pathology and neuronal death	Djelti *et al.*[30]
5XFAD mouse model of AD	Pharmacological activation of CYP46A1 by efavirenz reduces amyloid burden and attenuates microglial activation	Mast *et al.*[148]
5XFAD mouse model of AD with heterozygous knock-out for *Atad3a*	ATAD3A oligomerization restores neuronal CYP46A1 levels and brain cholesterol turnover, attenuating APP processing and reducing AD pathology	Zhao *et al.*[153]
Double-mutant P301S/K257T mouse model of tauopathy	Simvastatin decreased NFTs and improved T-maze performance	Boimel *et al.*[45]
P301S tau transgenic mice with distinct ApoE isoforms	ApoE4 exacerbates tau-mediated neurodegeneration, independent of Aβ	Shi *et al.*[53]
Evidence from animal cell cultures		
Hippocampal neurons from fetal rats	Cholesterol depletion disrupts synaptic transmission and plasticity	Frank *et al.*[32]

	Depletion of cholesterol with lovastatin attenuates Aβ formation	Simons *et al.*[23]
Neuronal cultures from embryonic mice	Cholesteryl ester levels, modulated by acyl-CoA:cholesterol acyltransferase (ACAT), are linked to Aβ production	Puglielli *et al.*[24]

Table 2 Selected clinical evidence implicating brain cholesterol homeostasis in Alzheimer's disease

Type of study	Key findings	References
Evidence from clinical trials		
Imaging and biomarker study with cognitively normal individuals and early-stage AD patients	MRI-based assessment of blood-brain barrier (BBB) permeability revealed a link between *APOE4* and dysfunction of the BBB, predicting cognitive decline	Montagne *et al.*[55]
Amyloid PET study with cognitively normal *APOE4* carriers and non-carriers	*APOE4* gene dose was associated with higher fibrillar Aβ in frontal/posterior cingulate-precuneus and temporal, parietal and basal ganglia	Reiman *et al.*[49]
Plasma biomarker study in patients with AD	Statin treatment reduces the plasma levels of 24S-hydroxycholesterol without affecting the levels of ApoE	Vega *et al.*[132]
Prospective cohort study assessing the impact of statin use in cognitively normal individuals on the risk to develop subsequent AD	Statin therapy associates with a lower risk of AD in early age, but not in late age. The link between statin use and AD is consistent across *APOE* isoforms	Li *et al.*[161]
Prospective study to assess whether lipophilicity of statins affects the association with AD	The use of statins was linked to a lower risk of developing AD – independent of statin lipophilicity	Haag *et al.*[163]
Randomized controlled trial of atorvastatin in mild to moderate AD	Atorvastatin was not associated with significant clinical benefit over 72 weeks	Feldmann *et al.*[167]
Randomized controlled trial of with simvastatin in individuals with high risk for vascular disease (MRC/BHF Heart Protection Study)	Five-year treatment with simvastatin did not affect cognitive function	Heart Protection Study Group[177]
Population-based cohort study to assess the effect of statins on a range of health outcomes including AD and non-AD dementia	Statin therapy exhibits a protective effect against AD and non-AD dementia	Smeeth *et al.*[162]
Case control study to assess the impact of untreated hyperlipidaemia on the association between statins and AD	Statins substantially attenuated the risk of developing dementia, independent of the presence or absence of untreated hyperlipidaemia	Jick *et al.*[178]
Evidence from GWAS		
GWAS analysis included 111,326 clinically diagnosed/'proxy' AD cases and 677,663 controls	75 AD risk loci were identified, of which 42 were new at the time of analysis. A new genetic risk score for the development or progression of AD/dementia was developed. Several hits are involved in lipid homeostasis.	Bellenguez *et al.*[83]
Genome-wide AD meta-analysis with 898 AD cases, 52,791 AD proxy cases and 355,900 controls	Identified 37 risk loci, including novel associations. Several hits are involved in lipid homeostasis.	Schwartzentruber *et al.*[179]
Meta-analysis on data from 13 cohorts, totaling 1,126,563 individuals	Identified 38 LOAD-associated loci, including seven previously unidentified loci. Several hits are involved in lipid homeostasis.	Wightman *et al.*[113]
Meta-analysis of 94,437 clinically diagnosed late-onset AD cases	Confirmed 20 previous risk loci and identified five new genome-wide loci. Several hits are involved in lipid homeostasis.	Kunkle *et al.*[84]
Evidence from post-mortem studies and human iPSCs		
Post-mortem human brain & iPSC-derived neurons	ApoE4-mediated cholesterol dysregulation in oligodendrocytes results in impaired myelination	Blanchard *et al.*[57]
Human iPSC-derived microglia	Microglia promote brain organoid maturation via cholesterol trafficking	Park *et al.*[88]
Human iPSC-derived neurons	Cholesteryl esters enhance Aβ and tau pathologies via independent pathways	van der Kant *et al.*[25]