Review

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ACAT1/SOAT1 as a therapeutic target for Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia with no cure at present. Cholesterol metabolism is closely associated with AD at several stages. ACAT1 converts free cholesterol to cholesteryl esters, and plays important roles in cellular cholesterol homeostasis. Recent studies show that in a mouse model, blocking ACAT1 provides multiple beneficial effects on AD. Here we review the current evidence that implicates ACAT1 as a therapeutic target for AD. We also discuss the potential usage of various ACAT inhibitors currently available to treat AD.

Yohei Shibuya[1], Catherine CY Chang[1] & Ta-Yuan Chang*[,1]

[1]Department of Biochemistry, Geisel School of Medicine at Dartmouth, 304 Vail Building, Hanover, NH 03755, USA

*Author for correspondence:
Tel.: +1 603 650 1622
Fax: +1 603 650 1128
ta.yuan.chang@dartmouth.edu

Cholesterol is an essential lipid molecule present in mammalian cell membranes. However, abnormally high levels of cholesterol, especially free (unesterified) cholesterol, are harmful to cells [1]. Thus, within a given cell, cellular cholesterol homeostasis is highly regulated by various control mechanisms as reviewed in [2]. The enzyme ACAT, also known as sterol *O*-acyltransferase, plays important roles in cellular cholesterol homeostasis. ACAT converts free cholesterol to cholesteryl esters to prevent overaccumulation of free cholesterol at cellular membranes. There are two different ACAT genes, *ACAT1* and *ACAT2*, with different tissue expression patterns. In normal human tissues, the expression of *ACAT1* predominates over that of *ACAT2* except in small intestines [3]. In most cell types, cholesteryl esters exist as cytoplasmic lipid droplets. In small intestines, cholesteryl esters form part of the core lipid moieties present in the lipoprotein called chylomicrons for cholesterol transport in the blood.

Alzheimer's disease (AD) is a progressive neurodegenerative disease that causes difficulty in cognitive functions, including memory, speech and perception, etc. AD is the most common cause of dementia in developed countries. Currently available treatments for AD improve symptoms but they do not provide a cure. Cholesterol metabolism is closely associated with AD at different stages, as reviewed in [4,5]. Recent studies have shown that blocking ACAT activity, specifically ACAT1, in mouse models and in cell culture produces several beneficial effects on AD. These studies suggest that ACAT1 can be a novel therapeutic target to treat AD. In this review, we provide a brief overview on the enzyme properties of ACATs, and the experimental evidence supporting the notion that ACAT1 blockage is a promising approach for treating AD. We then describe various mechanisms that may account for the beneficial effects of ACAT1 blockage on AD. We also discuss the potential usage of currently available ACAT inhibitors (Figure 1A) to treat AD.

ACAT as drug targets

ACAT converts free cholesterol to cholesteryl esters by transferring the fatty acyl group of fatty acyl-CoA to the 3β-hydroxy moiety of cholesterol (Figure 1B). ACAT plays important roles in cellular cholesterol homeostasis. The first ACAT gene, *ACAT1*, was cloned by functional complementation of a Chinese hamster ovary (CHO) cell mutant lacking ACAT enzyme activity [6]. ACAT1 is located mainly at the endoplasmic reticulum (ER) [7], and is ubiquitously expressed in all human tissues examined [8]. ACAT1 is an allosteric enzyme; it can utilize a variety of sterols

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Key terms

Cholesteryl esters: Esterified forms of cholesterol where the carboxyl group of a long-chain fatty acid is linked to the hydroxyl group of cholesterol.

Lipid droplets: Lipid-rich intracellular organelles mainly consist of triacylglycerols and cholesteryl esters.

Neurodegenerative disease: A disease that results from structural and/or functional loss of neurons.

Oxysterols: Oxygenated derivatives of cholesterol.

Dyslipidemia: Abnormal elevation of cholesterol and/or triglycerides and/or their protein carriers in the plasma.

Familial AD: A very rare form of Alzheimer's disease, which happens to people who carry a genetic mutation leading to the disease.

(including oxysterols, plant sterols, etc.) as a substrate as well as an activator, with cholesterol being the best substrate and the best activator [9]. The preferred fatty acyl-CoA as substrate for ACAT1 is oleoyl-CoA [10]. The second ACAT gene, *ACAT2*, was identified based on its sequence similarity to *ACAT1* [11–13]. ACAT2 is mainly expressed in the intestines and hepatocytes, and it is also expressed in various other tissues at low level [3]. Its exact subcellular localization remains unknown. Similar to ACAT1, the activity of ACAT2 is controlled allosterically by cholesterol [9,14]. Unlike many enzymes in lipid metabolism, neither ACAT1 nor ACAT2 is transcriptionally regulated by sterols [14].

Traditionally, cholesterol metabolism has long been associated with the disease atherosclerosis. In the early stage of atherosclerosis, under dyslipidemia and chronic inflammation, monocytes adhere to the activated endothelium, and enter the intimal layer of the artery; the monocytes in the intima transform into resident macrophages and begin to acquire a large amount of cholesterol. At the macrophage cell interior, most of the cholesterol is esterifed by ACAT1; the cholesteryl esters accumulate as lipid droplets, causing the macrophages to be foamy in appearance. In human atherosclerotic plaques, ACAT1 is highly expressed in macrophage foam cells [15]. Foam cells may cause the atherosclerotic plaques to be more vulnerable to rupture. Thus, ACAT1 has long been studied as a drug target to treat atherosclerosis. In mouse studies, the *Acat1* knockout (KO) mouse lines, which were independently created by Farese and co-workers, and by Ishibashi and co-workers, were employed to study the roles of ACAT1 in atherosclerosis; the results produced from these two laboratories were equivocal [16,17]. A more recent study showed that in mouse, global deletion of the *Acat1* gene, including cells in the bone marrow, causes an increase in hematopoietic progenitor cell proliferation and lead to leukocytosis [18]. Leukocytosis may alter atherosclerosis progression. To study the roles of ACAT1 in atherosclerosis, tissue-specific *Acat1* KO mice may provide better models. Studies in mouse suggest that ACAT2 is also a potential drug target for treating atherosclerosis [19,20]. In addition, recent evidence suggests that both ACAT1 and ACAT2 may be viable targets to treat various types of cancer, as reviewed in [21].

ACAT1, ACAT2 and acyl-CoA:diacylglycerol acyltransferase 1, which catalyzes the final step of triglyceride biosynthesis, are founding members of the membrane-bound *O*-acyltransferase (MBOAT) enzyme family [22]. There are 11 enzymes in the MBOAT family in humans. MBOATs contain multiple transmembrane domains, and share two similar catalytic sites, with an invariant histidine within a long stretch of hydrophobic domain, and a highly conserved asparagine within a long stretch of hydrophilic domain (Figure 2). MBOATs participate in diverse biological processes [23]. For instance, an MBOAT family enzyme, ghrelin *O*-acyltransferase catalyzes protein acylation of ghrelin [24], which stimulates appetite and increases food intake [25].

Amyloid plaques, neurofibrillary tangles & strategies to produce drugs to diminish amyloid β and pathological tau

The pathological hallmarks of AD in the brain consist of extracellular amyloid plaques, mainly composed of amyloid-β (Aβ) peptides and intracellular neurofibrillary tangles (NFTs), mainly consisting of hyperphosphorylated forms of tau. Aβ and tau can interact with each other synergistically to trigger neurotoxicity [26–29]. Thus, reducing brain levels of Aβ and/or misfolded/aggregated tau and/or hyperphosphorylated tau are attractive strategies to treat AD, as reviewed in [30].

Aβ is produced from the APP by sequential proteolytic cleavages; there are mainly two forms of Aβ peptide in the brain, Aβ1–40 and Aβ 1–42; Aβ1–42 is much more neurotoxic than Aβ1–40 [31]. Several mutations in the *APP* gene cause an increase in Aβ1–42, or the ratio of Aβ1–42 to Aβ1–40, and lead to familial AD, as reviewed in [32]. APP is a type-I membrane protein, and is highly expressed in neurons. The precise physiological roles of APP are not well understood. On the contrary, APP processing has been extensively characterized, as reviewed in [31,32]. After APP is synthesized at the ER, the nascent APP becomes glycosylated at the Golgi complex, and then is transported to the cell surface via the secretary pathway. At the plasma membrane, APP is internalized via a clathrin-dependent endocytotic pathway and is processed by β-secretase followed by γ-secretase, which is present in intracellular compartments to produce Aβ (Figure 3). Alternatively, APP can

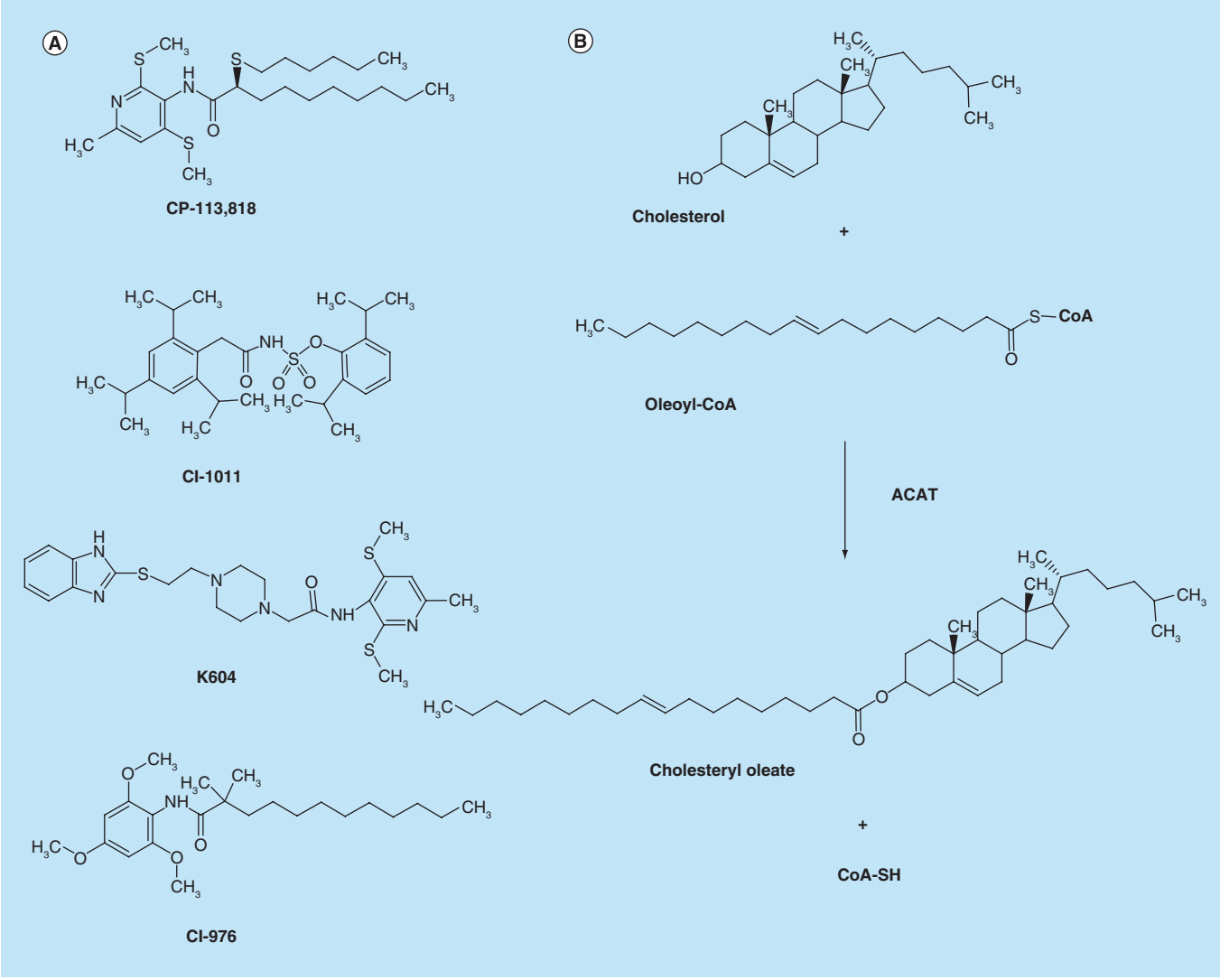

Figure 1. The structures of various acyl-CoA:cholesterol acyltransferase inhibitors and biosynthesis of cholesteryl ester by ACAT. **(A)** The structures of ACAT inhibitors, CP-113,181, CI-1011, K604 and CI-976. **(B)** ACAT transfers the fatty acyl group of long chain fatty acyl-CoA (oleoyl-CoA) to the 3β-hydroxy moiety of cholesterol to produce cholesteryl ester (cholesteryl oleate).

be proteolyzed by α-secretase(s) present at the cell surface, and then internalized and processed by γ-secretase, which generates nonamyloidogenic (i.e., non-neurotoxic) products. Because proteolytic cleavage by α-secretase(s) occurs within the Aβ sequence in APP, this step prevents Aβ generation (Figure 3).

The β-secretase, BACE1, is a membrane-bound aspartyl protease and the rate-limiting enzyme for Aβ generation. Thus, it has been considered as a good therapeutic target for AD (Figure 3). However, *BACE1* KO mice have impaired cognitive functions [33], likely due to abnormal myelination in the CNS [34]. This result raises concerns on usage of a β-secretase inhibitor for AD treatment. The γ-secretase is a multiprotein complex composed of presenilin (PS), nicastrin, anterior pharynx-defective-1 and presenilin enhancer-2 [35]. There are two PS homologs in mammals, PS1 and PS2. Mutations in PS, particularly in PS1, cause familial AD. The γ-secretase has been considered as a drug target for AD (Figure 3). A variety of γ-secretase inhibitors has been developed to treat AD. However, these γ-secretase inhibitors also inhibit the processing of Notch and several other transmembrane proteins, which may cause serious mechanistic side effects [36].

Brain Aβ levels are determined by its production and its clearance. Studies in a mouse model of AD suggest that the brain's ability for Aβ clearance declines with age [37,38]. The clearance of Aβ in late-onset AD

Key term

Late-onset AD: The most common form of Alzheimer's disease that begins after age 65.

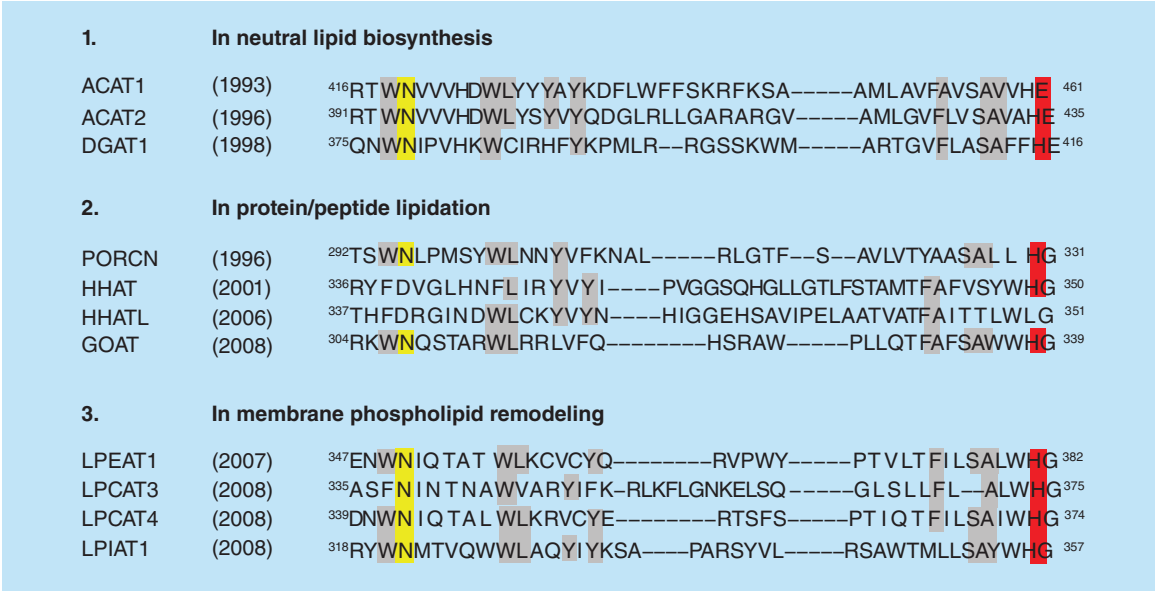

Figure 2. Alignment of the two highly conserved catalytic sites of 11 membrane-bound *O*-acyltransferase enzymes in human. The highly conserved histidine residues and asparagine residues are highlighted in red and in yellow, respectively. The residues highlighted in gray are conserved in more than 50% of the all membrane-bound *O*-acyltransferases. Membrane-bound *O*-acyltransferases can be classified into three groups based on biochemical reactions they catalyze.
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patients is less efficient than control subjects [39]. These lines of evidence implicate that Aβ clearance may be impaired in AD patients. Therefore, enhancing Aβ clearance to reduce brain Aβ levels may benefit AD (Figure 3). There has been intense research interest in developing molecules to enhance the clearance of Aβ. It is estimated that approximately 50% of Aβ is degraded within the brain while the rest is transported to the systemic tissues and degraded there [40]. Aβ immunotherapy is believed to increase the clearance of Aβ. Currently, Aβ immunotherapy using anti-Aβ monoclonal antibodies has been considered as one of the most promising therapeutic approaches for treating AD. In mouse models of AD, treatment with monoclonal antibodies against Aβ dramatically reduces AD pathology and improves cognitive deficits [41–44]. Thus, a variety of anti-Aβ monoclonal antibodies has been developed for the past decade, and some of them have moved to clinical trials [45,46].

NFTs are composed of hyperphophorylated tau. Tau is a soluble microtubule-associated protein, abundantly expressed in the axons of neurons. Tau contains numerous phosphorylation sites; phosphorylations of tau at certain sites modify the ability of tau to stabilize microtubules [47]. Uncontrolled phosphorylations alter the conformation of tau, which eventually leads to accumulation of pathological hyperphosphorylated tau in NFTs [48]. In addition to phosphorylations, tau protein can also be modified by site-specific acetylations [49,50]. In AD, most of the pathological tau is found in neuronal processes known as dystrophic neuritis [51]. In addition to AD, NFTs can be found in various other neurodegenerative diseases collectively called tauopathy, including frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) and Pick's disease. P301L is the most common tau mutation in FTDP-17. Mice expressing human P301L-tau exhibit development of NFTs and memory impairment, similar to those seen in human tauopathies [52,53], which suggests that dysfunctional tau alone without Aβ accumulation can cause neurodegeneration. In mouse models of AD, recent evidence shows that neurotoxicity of Aβ depends on tau, as suppressing tau levels attenuates Aβ-induced deficits [27,28]. These studies suggest that aiming to reduce pathological tau levels is also a potential therapeutic approach to treat AD (Figure 3).

AD, cholesterol metabolism & ACAT

A growing body of evidence suggests that cholesterol metabolism is closely associated with AD at several stages, as reviewed in [4,5]. Epidemiologic studies have identified hypercholesterolemia in mid-life as a risk factor for AD [54,55]. In a mouse model for AD, a high-fat/high-cholesterol diet accelerates AD pathology [56]. These studies implicate that high cholesterol levels in the plasma are correlated with initiation and progression of AD. Studies *in vitro* have indicated that choles-

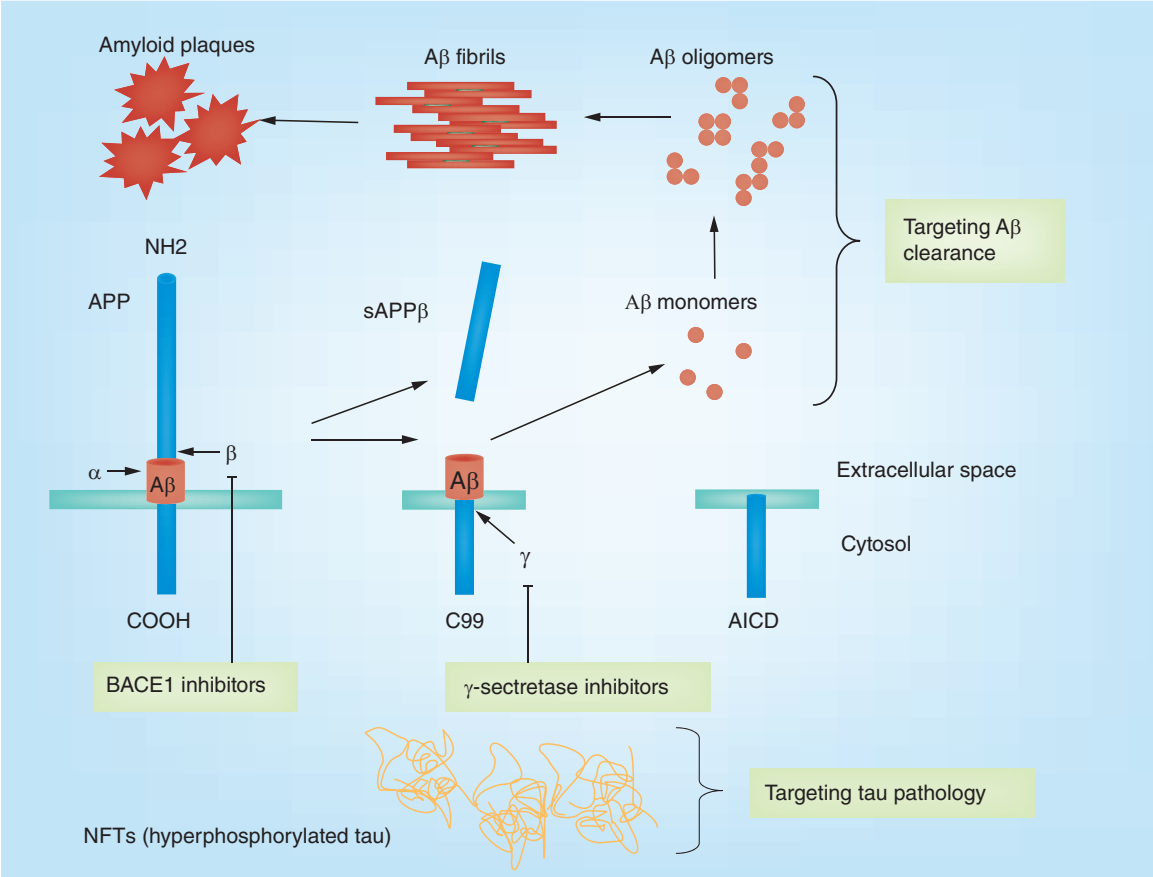

Figure 3. Aβ production and clearance, neurofibrillary tangles and current drug development strategy to treat Alzheimer's disease. The APP can undergo proteolytic processing by secretases. In the amyloidogenic pathway, APP is first cleaved by β-secretase (BACE1), releasing an ectodomain (sAPPβ) and retaining the last 99 amino acids of APP (C99) within the membrane. C99 is subsequently cleaved 38–43 amino acids from the amino terminus to produce Aβ, by the γ-secretase. In contrast, in the nonamyloidogenic pathway, cleavage by α-secretase occurs within the Aβ domain, thereby preventing the generation and release of the Aβ peptide. Current drug development includes targeting BACE1 and γ-secretase to reduce Aβ production. In addition, targeting Aβ clearance aims to increase Aβ turnover in the brain; targeting tau pathology aims to reduce tau toxicity in the brain.
α: α-cleavage site; AICD: APP intracellular domain; β: β-cleavage site; γ: γ-cleavage site.

terol modulates Aβ production; increasing membrane cholesterol levels promotes activity of β-secretase and γ-secretase, which results in increased Aβ production [57–59]; in contrast, reducing membrane cholesterol strongly inhibits Aβ production in neuronal cells [60,61].

ApoE is the major cholesterol/lipid carrier in the CNS and has been most intensively studied as a key molecule that links cholesterol metabolism and AD. In humans, there are three ApoE isoforms (E2, E3 and E4), which differ from each other by only one or two amino acid at positions 112 and 158. The *ApoE4* allele is the strongest genetic risk factor for late-onset AD. Although precisely how ApoE4 causes the increased risk for AD is not fully understood, numerous studies have suggested that ApoE has isoform-dependent effects on Aβ metabolism. For instance, AD model mice expressing human ApoE display an isoform-dependent difference (E4>E3>E2) in Aβ accumulation [62,63]. ApoE also affects Aβ clearance in a manner that likely depends on the isoforms and its level of lipidation [64–67]. The ATP-binding cassette (ABC) transporter ABCA1 plays a key role in the lipidation and the stability of ApoE [68,69]. Thus, increasing ABCA1 levels in the brain to promote ApoE lipidation is a potential therapeutic approach for treating AD [70–73].

It was estimated that in the brain, almost all of cholesterol is unesterified [74]. However, recent analyses have revealed that significant amount of cholesteryl esters exists in human and mouse brains. Importantly, in the brains of AD patients and in several mouse models for AD, the cholesteryl ester content is significantly elevated in the vulnerable regions affected by AD [75,76]. In different regions of the mouse brain, ACAT1 is the predominant enzyme for cellular cho-

lesterol esterification [77]. Thus, ACAT1 may affect initiation and progression of AD. The first experimental evidence that linked ACAT activity and AD was provided at the cell culture level by Puglielli *et al.* [78]. This study showed that in a CHO cell model and in a neuronal cell model, cholesteryl ester levels were correlated with Aβ production; reducing cholesteryl ester content in these cell models by blocking ACAT activity diminished Aβ generation [78]. These results led to the hypothesis that inhibiting ACAT activity benefits AD. This hypothesis has been tested in cell-based and animal-based AD models by using different approaches.

ACAT inhibitors in a mouse model for AD

CP-113,818 (Figure 1A) is a small molecule, isotype-nonspecific ACAT inhibitor that inhibits both ACAT1 and ACAT2 [79]. In a mouse AD model expressing high levels of human APP_{751} containing the London (V717I) and Swedish (K670M/N671L) mutations ($hAPP_{751}$ mice), systemic administration of CP-113,818 for 2 months starting at 4.5 months of age dramatically reduced the Aβ deposition in the brain, and rescued the cognitive deficits [80]. CI-1011 (also known as avasimibe) (Figure 1A) is an ACAT inhibitor structurally distinct from that of CP-113,818. It also inhibits both ACAT1 and ACAT2 [81]. CI-1011 was tested in clinical trials for atherosclerosis but failed to improve disease lesions [82]. When administered to a mouse model for AD, CI-1011 provided interesting result: 2 months treatment of CI-1011 diminished the amyoid plaques and Aβ levels in the brain of young $hAPP_{751}$ mice [83]. Moreover, in old $hAPP_{751}$ mice displaying robust amyloid deposits and sever cognitive impairment, CI-1011 treatment reduced the pre-existing amyloid burden and Aβ levels in the brains [83]. These animal studies suggest that ACAT inhibitors may improve amyloid pathology and ameliorate AD symptoms.

In the brain of $hAPP_{751}$ mice treated with the ACAT inhibitors, there was a decrease in the ratio of mature APP to immature APP; the levels of α- and β-cleavage products of APP were also decreased [80,83]. On the other hand, CP-113,818 and CI-1011 did not affect BACE1 or γ-secretase activity *in vitro* [78,83]. These results suggest that both compounds downregulated the maturation of APP in the early secretory pathway, which led to a decrease in Aβ production [84]. More recently, it has been shown that a small portion of APP receives a post-translational lipid modification called palmitoylation, which plays an important role in APP maturation and subsequent Aβ generation; CP-113,818 and CI-1011 are shown to inhibit palmitoylation of APP [85]. These *in vivo* and *in vitro* data implicate that both CP-113,818 and CI-1011 reduce Aβ generation, possibly by inhibiting APP maturation, and ameliorate amyloid pathology in AD mice.

Genetic ablation of *Acat1* in AD mice

Although the evidence described above suggests that ACAT inhibitors may prevent and/or slow down the progression of AD, several important questions were left unanswered. First of all, it was unclear whether the beneficial actions of CP-113,818 and CI-1011 required these compounds to block both ACAT1 and ACAT2. Secondly, in addition to inhibiting ACAT, these compounds may also inhibit other members of the MBOAT family; that is, the beneficial actions of these inhibitors may be due to their abilities to inhibit other enzymes. Thirdly, it was not clear whether the ACAT inhibitors systemically administrated to the AD mice had actually reached the brain interior. To answer these questions, Bryleva *et al.* [77] took a mouse genetic approach by deleting the *Acat1* gene or the *Acat2* gene in the triple transgenic AD (3XTg-AD) mouse line. The 3XTg-AD mouse model expresses mutant forms of human APP, PS1 and tau, and displays a phenocopy of human AD [86]. The results showed that *Acat1* KO but not *Acat2* KO reduced the Aβ1–42 levels as well as the amyloid plaque load, and improved the cognitive function in 12–month-old 3XTg-AD mice [77]. These data clearly indicate that inactivating *Acat1* alone is sufficient to ameliorate the amyloid pathology in the 3XTg-AD mouse model. Importantly, *Acat1* KO caused a decrease in the protein levels of full-length human APP (both the mature and immature forms); unlike CP-113,818 or CI-1011 described earlier, *Acat1* KO did not alter the ratio of mature APP to immature APP [77]. In addition, *Acat1* KO did not affect the mouse endogenous APP level [77]. This result indicates that the effects of *Acat1* KO are distinct from the effects of CP-113,818 and CI-1011 [80,83]. The different effects between *Acat1 KO* and the ACAT inhibitors on APP may be due to different AD mouse models employed, and/or due to the abilities of CP-113,818 and CI-1011 to inhibit multiple enzymes in the MBOAT family. Other scenarios are also possible.

Bryleva *et al.* [77] reported that *Acat1* KO caused an increase in the levels of 24S-hydorxycholesterol (24S-OH). 24S-OH is a key oxysterol in the brain synthesized from cholesterol by the ER resident enzyme Cyp46A1; under normal conditions, Cyp46A1 is almost exclusively expressed in neurons [87]. The *Acat1* KO/3xTg-AD mice had decreased protein levels of HMG-CoA reductase, which is the rate-limiting enzyme in the cholesterol biosynthetic pathway, and decreased rate of cholesterol biosynthesis in the brain;

in primary neurons isolated from the 3XTg-AD mice, *Acat1* KO increased the 24S-OH biosynthesis; treating primary neurons with 24S-OH caused a decrease in the protein levels of full-length human APP and HMG-CoA reductase. Based on these findings, Bryleva *et al.* [77] hypothesized that increased levels of 24S-OH by *Acat1* ablation leads to rapid downregulation of full length human APP protein content, which limits its capacity to produce Aβ in the brain of 3XTg-AD mice (Figure 4). The precise mechanism of 24S-OH-mediated reduction in full-length human APP in neurons is unknown at this point. Other groups have also reported that 24S-OH reduces Aβ production *in vivo* and *in vitro* [88,89]. Thus, increasing 24S-OH content in the brain may be a potential therapeutic strategy for AD treatment.

Anti-*Acat1* siRNA was shown to knockdown (KD) *Acat1* and reduced Aβ generation in a cell culture model [90]. It is important to know whether blocking ACAT1 benefits AD *in vivo*, especially after the disease onset. Thus, Murphy *et al.* from this laboratory tested whether ACAT1 inhibition can still elicit beneficial effects in the 3XTg-AD mice at the postsymptomatic stage [91]. To inhibit ACAT1 specifically, we chose to inject *Acat1* gene KD construct to the brains of 10-month-old 3XTg-AD mice, using a recombinant adeno-associated virus vector to express the anti-*Acat1* siRNA. The 3XTg-AD mouse model displays memory dysfunction by 9 months of age and starts to develop significant Aβ1–42 accumulation approximately at 10 months of age [86]. The results by Murphy *et al.* [91] showed that *Acat1* gene KD reduced the levels of full-length human APP protein and Aβ1–42 in the brains of the 3XTg-AD. These data are consistent with our earlier findings made in the *Acat1* KO/3XTg-AD mice [77], supporting the conclusion that blocking ACAT1 is sufficient to benefit AD. The result of Murphy *et al.* [91] also demonstrated that after the AD onset, partial inactivation of ACAT1 (~40%) in the brain was sufficient to cause a decrease in the levels of Aβ1–42 in the 3XTg-AD mice.

ACAT1 blockage on Aβ1–42 degradation in microglia

Aβ monomers can self-assemble to form small oligomers and then form the larger sized fibrils. Amyloid plaques

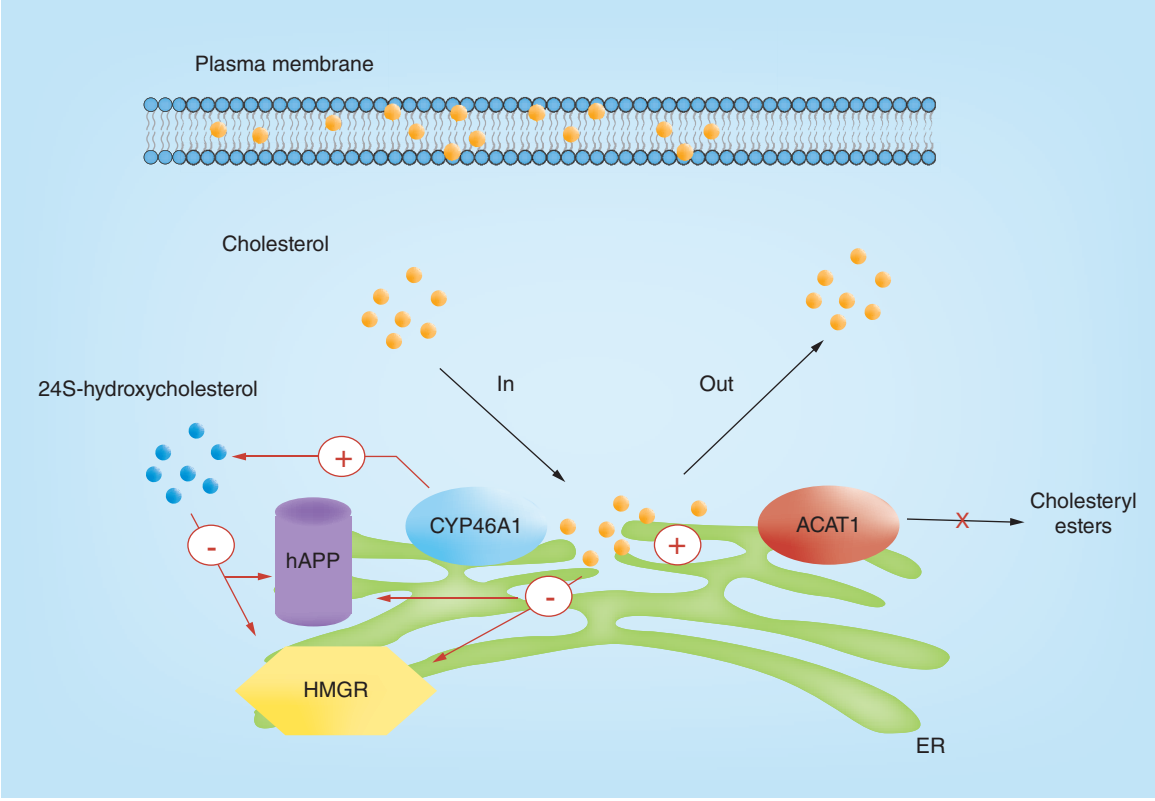

Figure 4. A working model that links cholesterol trafficking with ACAT1, 24S-hydroxycholesterol biosynthesis and downregulations of human APP and HMG-CoA reductase in neurons. This model predicts that ACAT1 blockage provides more cholesterol as substrate for the enzyme CYP46A1 to synthesize more 24S-hydroxycholesterol. The increase in cholesterol and in 24S-hydroxycholesterol at the ER serve as the signal(s) to downregulate the protein contents of hAPP and HMGR.
ACAT1: Acyl-CoA:cholesterol acyltransferase 1; ER: Endoplasmic reticulum; hAPP: Human APP.

are mainly composed of the fibrillar form of Aβ; however, the number and size of amyloid plaques correlate poorly with degree of neurodegeneration or severity of dementia in AD. Instead, recent studies suggest that the oligomeric forms of Aβ are the most toxic molecular species that cause synaptic loss [29,92–93]. Brain levels of Aβ are determined by its production and its clearance. As described above, blocking ACAT1 genetically decreases Aβ levels in the brains of AD mice, at least in part by reducing Aβ generation. However, it is possible that ACAT1 blockage also stimulates Aβ clearance, based on the following observations: *Acat1* KO in the 3XTg-AD mice caused a more striking effect in reducing the Aβ1–42 content (by 80%) than in reducing the full-length human APP content (by 50–60%) [77]. In addition, *Acat1* KD significantly diminished the oligomeric Aβ content in the brains of the 3XTg-AD mice [91]. These observations implicate that, in addition to its effect on reducing Aβ production, ACAT1 blockage may also promote the clearance of oligomeric Aβ. Microglia are the resident tissue macrophage-like cells in the CNS and play important roles in Aβ clearance in the brain. Cultured microglia are able to engulf and degrade Aβ in lysosomes [66,94]. In a mouse AD model, microglia are recruited to newly formed amyloid plaques within 1–2 days [95]. In addition, impaired microglial clearance of Aβ has been shown to be partially responsible for AD pathogenesis [96–98]. These studies emphasize the importance of microglia in Aβ turnover in the brain. Recently Shibuya *et al.* in this laboratory investigated whether ACAT1 blockage enhanced oligomeric Aβ1–42 clearance in microglia. We found that in cultured mouse microglia, *Acat1* KO or an ACAT1 inhibitor K604 (Figure 1A) [81] upregulated lysosome biogenesis and enhanced degradation of oligomeric Aβ1–42 [99]. We also showed that *Acat1* KO in microglia stimulated oligomeric Aβ1–42 clearance in the mouse brain *in vivo* [99]. These results suggest that in addition to reducing Aβ production, blocking ACAT1 may benefit AD by stimulating Aβ clearance in microglia.

ACAT1 blockage on autophagy

Macroautophagy (hereafter referred to as autophagy) is a cellular degradation process that delivers cytoplasmic contents to the lysosome to maintain cellular homeostasis. Autophagy begins with sequestration of the cytoplasm with a double-membrane structure called an autophagosome [100]. Autophagosomes eventually fuse with lysosomes to degrade sequestered cytoplasmic contents, including denatured and/or aggregation-prone proteins/peptides, such as Aβ [101]. Autophagy can be induced by inhibition of the mammalian target of rapamycin (mTOR), which is a serine/threonine protein kinase that regulates cell growth and proliferation [102]. Autophagy is closely associated with lysosome biogenesis [103]. The transcription factor EB (TFEB) coordinates lysosome biogenesis and autophagy. As a master regulator for lysosome biogenesis, it promotes transcription of target genes in the coordinated lysosomal expression and regulation network [104]. TFEB also upregulates the autophagic machinery [105,106]. Inhibition of mTOR signaling activates TFEB pathway to promote both autophagosome formation and lysosome biogenesis [106–108]. Shibuya *et al.* in this laboratory found that blocking ACAT1 in cultured microglia by gene KO or by the ACAT1-specific inhibitor K604 increased autophagy as well as TFEB-mediated lysosome biogenesis [99]. *Acat1* KO microglia freshly isolated from adult mouse brains were also found to have increased mRNA levels of several genes in the coordinated lysosomal expression and regulation network [99]. Interestingly, ACAT1 blockage caused increases in autophagy and in TFEB-mediated lysosome biogenesis in an mTOR-independent manner; in fact, we found that blocking both ACAT1 and mTOR signaling in microglia showed additive effects in autophagy and in lysosome biogenesis [99]. Previous studies have shown that in mouse models of AD, inhibiting mTOR by rapamycin administration increases autophagy in the brain, reduces Aβ1–42 levels possibly by enhancing Aβ1–42 clearance and rescues cognitive deficits [109,110]. Thus, in AD brains, blocking both ACAT1 and mTOR signaling may provide additive effects on reducing Aβ levels.

ACAT1 blockage on autophagy-mediated human tau degradation in neurons

Enhancing autophagy in an mTOR-independent fashion can promote degradation of aggregate-prone proteins in various mammalian cells [111], [112]. Recently, Shibuya *et al.* in this laboratory showed that inhibiting ACAT1 by K604 stimulated autophagy in the mouse neuroblastoma N2a cells without altering mTOR signaling, and reduced human tau protein content ectopically expressed in these cells [113]. *Acat1* KO also caused an increase in autophagy and decreased the P301L-tau content in primary cortical neurons isolated from 3XTg-AD mice [113]. These results suggest that ACAT1 blockage may provide additional benefit to AD by reducing the tau protein.

In the brains of 3XTg-AD mice, hyperphosphorylation of P301L-tau becomes apparent between 12 and 15 months of ages [86]. A previous study showed that in the 3XTg-AD mouse model, upregulating autophagy by rapamycin administration before the disease onset (2 months old), but not after the disease onset (15 months

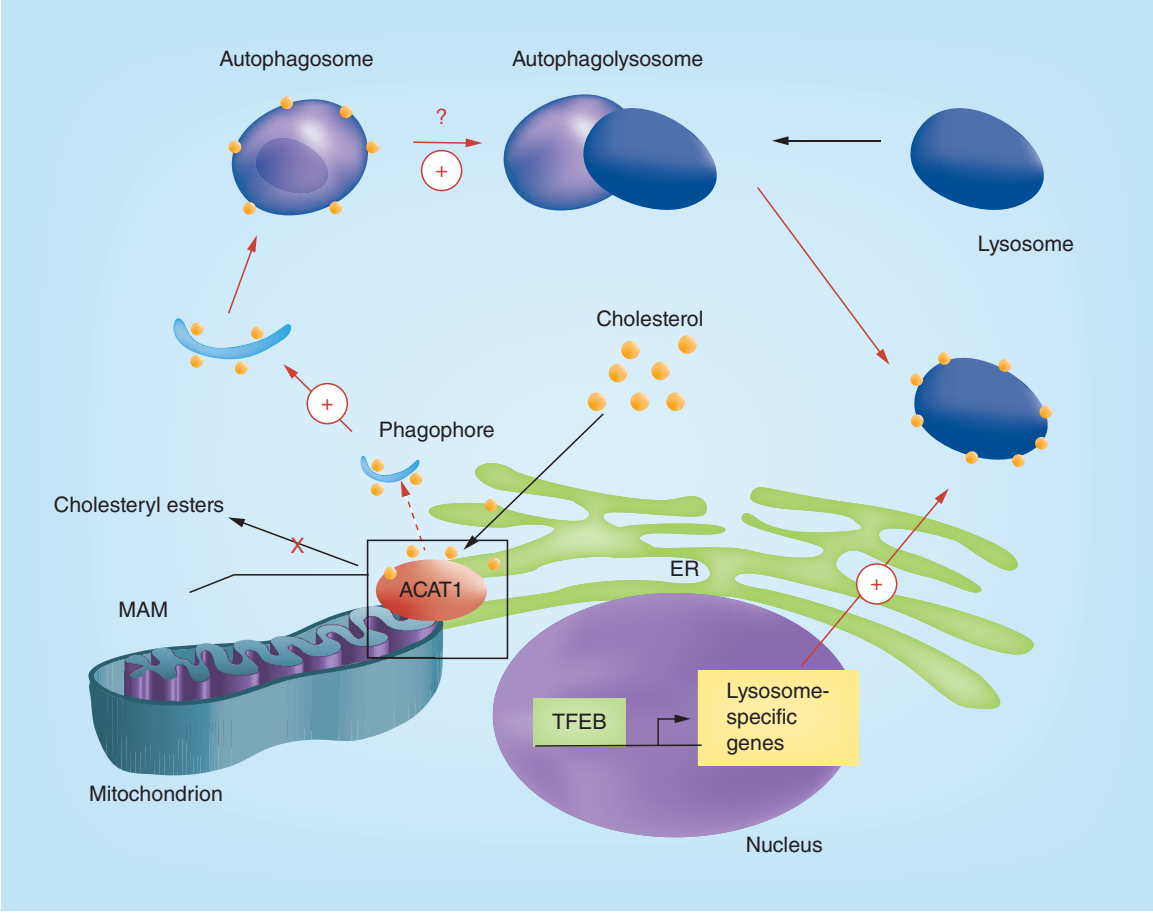

Figure 5. A working model to explain the ACAT1 blockage-dependent increase in autophagy and in lysosome biogenesis in microglia and in neurons. ACAT1 blockage alters the local cholesterol content in MAM, which signals an increase in autophagosome formation. The increase in autophagosome formation leads to TFEB-mediated increase in lysosome biogenesis. ACAT1 blockage may also increase autophagosome-lysosome membrane fusion. MAM: Mitochondria-associated ER membrane.

old), reduces hyperphosphorylated tau levels [114]. Similarly, Shibuya *et al.* [113] showed that *Acat1* KO decreased the P301L-tau protein content in the brains of young 3XTg-AD mice (2–4 months old), but not in those of old mice (17–21 months old), even though *Acat1* KO still promoted autophagy in the brains of the old mice. Thus, ACAT1 blockage could also benefit AD by attenuating tauopathy at early stage. It is currently unknown why enhancing autophagy by *Acat1* KO is not effective in reducing P301L-tau levels in old 3XTg-AD mice. It is possible that in the old 3XTg-AD mouse brain, the hyperphosphorylated/aggregated P301L-tau may become much less susceptible to autophagy-mediated degradation *in vivo*.

Potential mechanisms

What is the mechanism responsible for the ACAT1 blockage-mediated decrease in full-length human APP protein and increase in autophagy? We believe that the answer may be the effect of ACAT1 blockage on local cholesterol content in the ER membranes. ER membranes contain 'regulatory sterol pools' that play important roles in cholesterol homeostasis [115]. Modulating ER cholesterol levels are shown to affect the processing of sterol regulatory element binding proteins [116], which are transcription factors that control cholesterol and fatty acid biosynthesis. ACAT1 resides in the ER, and converts free cholesterol to cholesteryl esters. Blocking ACAT1 is expected to lead to an increase in ER cholesterol pools. In macrophages and in CHO cells, blocking ACAT by using an ACAT inhibitor increases the ER regulatory sterol pool, and leads to downregulation of HMG-CoA reductase and sterol regulatory element binding protein processing [117,118]. In neurons, it is possible that *Acat1* KO or KD increases a local cholesterol pool in the ER, which

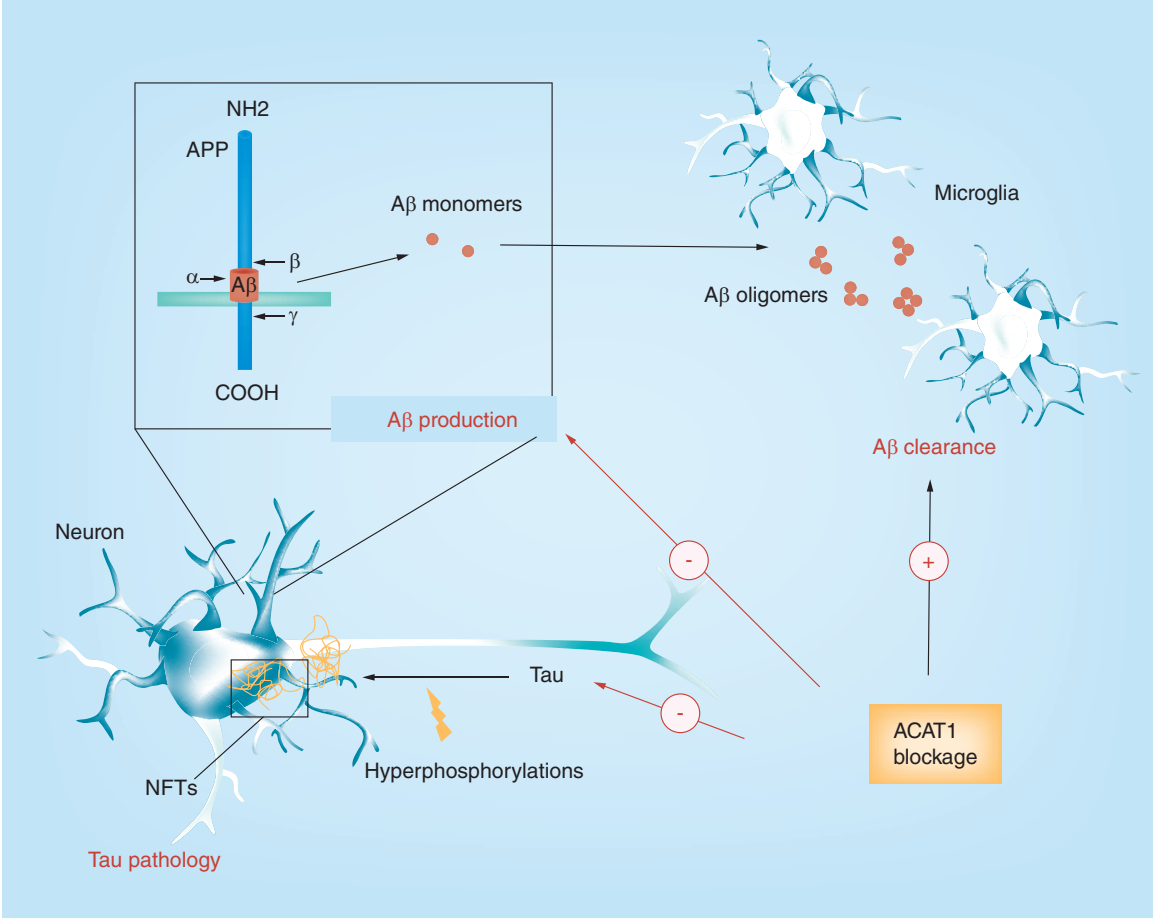

Figure 6. Beneficial effects of ACAT1 blockage on Alzheimer's disease. In neurons, ACAT1 blockage reduces Aβ production and tau protein content; in microglia, it increases Aβ clearance.

may directly downregulate full-length human APP levels (Figure 4). Recent evidence showed that a significant portion of ACAT1 is located at the mitochondria-associated ER membrane (MAM) [119]; MAM is rich in cholesterol and rich in sphingolipid (ceramide) contents [120]. MAM can serve as a platform for autophagosome formation during starvation [121]. Therefore, it is possible that blocking ACAT1 may modify the local cholesterol content in MAM, which signals an increase in autophagosome formation that leads to increase in lysosome volume (Figure 5). In addition, several studies *in vitro* have shown that cholesterol content in membranes facilitates membrane fusion [122,123]. Thus, in addition to promoting autophagosome formation at the MAM, ACAT1 blockage may also increase the cholesterol content in the autophagosome, which may promote fusion between autophagosome and lysosome (Figure 5).

In addition to ACAT1, other resident ER enzymes can also use ER cholesterol as a substrate. In neurons, the ER enzyme Cyp46A1 produces 24S-OH from cholesterol. It is possible that lacking ACAT1 raises the substrate levels for Cyp46A1 and causes an increase in 24S-OH biosynthesis. A similar concept has been reported in macrophages: blocking ACAT enzyme activity by using an ACAT inhibitor increased the biosynthesis of a different oxysterol 27-hydroxycholesterol, presumably because of an increase in the substrate pool for the enzyme 27-hydroxylase Cyp27A1 [124]. In neurons, the increased 24S-OH content in the ER may reduce full-length human APP levels, possibly by upregulating its degradation in the ER (Figure 4).

Blocking ACAT1 activity also raises the overall free cholesterol levels in cells, which promotes excretion of excess cholesterol to various acceptors present in the cell exterior, such as high-density lipoprotein apoAI, and other proteins that bind to cholesterol [2]. A previous study showed that treating macrophages with isotype-nonspecific ACAT inhibitors causes ER stress and induces apoptosis, when the macrophages are cultured in the growth medium without any cholesterol acceptor [125]. Because ER stress can activate ER-associated

degradation [126], it is possible that blocking ACAT1 may induce ER stress, which in turn leads to an increase in ER-associated degradation of APP in neurons. ER stress is also known to induce autophagy [127]. To test these possibilities, we examined the possible link between ER stress and ACAT1 blockage in our cell systems. The results showed that neither the ACAT1 specific-inhibitor K604 nor *Acat1* gene KO-induced ER stress in mouse microglia and in mouse neuronal cells [99,113]. Thus, ER stress is unlikely to mediate the effects of ACAT1 blockage describe in neurons and in microglia. Earlier, Huttunen *et al.* [84] also showed that the isotype-nonspecific ACAT inhibitor CP-113,818 did not induce ER stress in CHO cells or in rat neuroblastoma cells.

Pitfalls of currently available ACAT inhibitors

Studies described in this review suggest the use of small molecule ACAT1-specific inhibitor to treat AD. However, there are concerns to use currently available ACAT inhibitors for clinical use. Recent evidence has shown that many ACAT inhibitors also inhibit other MBOAT family enzymes. This is because most of these inhibitors were discovered before the first *ACAT* gene (*ACAT1*) was identified in 1993 [6]. In 2000, the MBOAT family was discovered, with ACAT1 as the founding member [22]. Thus, the ACAT inhibitors developed prior to year 2000 may also inhibit other MBOAT members, which may cause undesirable side effects. For instance, the 'ACAT inhibitor' CI-976 (Figure 1A) was shown to inhibit multiple membrane trafficking steps, at least in part by inhibiting the enzyme activity of lysophospholipid acyltransferase 3 [128], which is an MBOAT member [23]. The 'ACAT inhibitor' CI-1011 orally fed to animals or to men decreased the plasma concentrations of total triglyceride [129], presumably because CI-1011 also inhibits the enzyme activity of diacylglycerol acyltransferase 1, a different MBOAT member [23]. Currently K604 is the only small molecule ACAT1-specific inhibitor available. In an *in vitro* study, K604 at 0.5 μM was shown to inhibit ACAT1 enzyme activity by 70% without significantly affecting the ACAT2 enzyme activity [81]. In cultured neurons and cultured microglial cells, we showed that when used at 0.5 μM, the effects of K604 faithfully mimic the effects of *Acat1* KO [99,113]. However, these results do not eliminate the possibility that at higher concentration, K604 may also inhibit other member(s) of the MBOAT family.

A second concern for using the currently available ACAT inhibitors to treat AD is that many ACAT inhibitors are very hydrophobic compounds and possess 'membrane-active' property; the 'membrane-active' compounds are known to be sequestered within the lipid bilayer; they can reach high local concentration to affect the biophysical properties of membranes [130]. The short-term and long-term effects of these membrane active compounds in the CNS are unclear. To treat AD, it would be desirable to develop new small molecule ACAT1-specific inhibitors that are permeable to the blood–brain barrier without causing various off-target side effect(s) described above. In addition, we believe that the development of nonviral delivery of anti-*Acat1* siRNA [91] for treating AD should also be seriously considered.

Conclusion & future perspective

Over the past decade, a large amount of effort has been devoted to find agents for treating AD. At present, only a few of these agents have shown efficacy in clinical trials. As summarized in this review, in a mouse model for AD, blocking ACAT1 elicits multiple beneficial effects (Figure 6): first, it diminishes Aβ production by reducing full-length human APP protein. Second, ACAT1 blockage promotes autophagy-mediated lysosome biogenesis to enhance clearance of Aβ1–42 in microglia. Third, blocking ACAT1 upregulates autophagy in neurons and promote degradation of tau before it becomes hyperphosphorylated. Thus, we believe that ACAT1 can be a prominent therapeutic target for AD treatment. In the future, it will be important to test whether ACAT1 blockage also produces beneficial effects in the presence of ApoE4, because approximately 40% of all late-onset AD patients carry at least one copy of the *ApoE4* allele [131]. A common event that occurs in several major neurodegenerative diseases in humans, including AD, Parkinson's disease, frontotemporal dementia, etc. is the presence of certain specific misfolded/aggregated proteins in a given region of the CNS; these misfolded/aggregated proteins are subjected to autophagy-mediated degradation [101]. Thus, in the future, it would be interesting to test if blocking ACAT1 can also benefit other neurodegenerative diseases.

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Executive summary

- ACAT converts cholesterol to cholesteryl esters.
- There are ACAT1 and ACAT2 in human with different tissue expression patterns.
- Blocking ACAT1 can be a promising therapeutic approach for Alzheimer's disease (AD).

ACAT as drug targets

- ACAT1 and ACAT2 are members of the membrane-bound *O*-acyltransferase enzyme family.
- ACAT1 and ACAT2 have been studied as drug targets to treat atherosclerosis.

Amyloid plaques, neurofibrillary tangles & strategies to produce drugs to diminish Aβ & pathological tau

- Reducing Aβ levels and/or tau levels in the brain are currently the main therapeutic strategies for AD.

AD, cholesterol metabolism & ACAT

- Cholesterol cholesterol metabolism is closely associated with AD.
- The E4 of allele of apolipoprotein E, which is the major cholesterol/lipid carrier in the CNS, is the strongest genetic risk factor for late-onset AD.

ACAT inhibitors in a mouse model for AD

- Treating a mouse AD model with isotype-nonspecific ACAT inhibitors, CP-113,818 and CI-1011 diminishes Aβ deposition in the brain.

Genetic ablation of ACAT1 in AD mice

- *Acat1* gene knockout (KO) and *Acat1* knockdown in a mouse model for AD mouse model reduce Aβ levels in the brains.

ACAT1 blockage on Aβ1–42 degradation in microglia

- Blocking ACAT1 by gene KO or by using an ACAT1-specific inhibitor K604 in microglia promotes lysosome biogenesis, and enhances oligomeric Aβ1–42 degradation in lysosomes.

ACAT1 blockage on autophagy

- Blocking ACAT1 by gene KO or by using K604 stimulates autophagy and lysosome biogenesis in microglia.

ACAT1 blockage on autophagy-mediated human tau degradation in neurons

- Inhibiting ACAT1 by K604 or by gene KO stimulates autophagy and promotes degradation of human tau in mouse neuronal cells.

Potential mechanisms

- ACAT1 blockage alters local cholesterol content in the ER membranes, which may cause various beneficial effects on AD.

Pitfalls of currently available ACAT inhibitors

- Many ACAT inhibitors currently available also block other membrane-bound *O*-acyltransferase family enzymes in addition to blocking ACAT1, which may cause undesirable side effects.

Future perspective

- To develop new ACAT1 inhibitors without side effects.
- To test whether blocking ACAT1 elicits beneficial effects to AD in the presence of ApoE4.

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