Hypothesis

Cholesterol at the Center of Alzheimer's Disease: A Unifying Hypothesis on the Pathogenic Mechanism

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Cholesterol at the Center of Alzheimer's Disease: A Unifying Hypothesis on the Pathogenic Mechanism

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Abstract

It is hypothesized that in most cases of sporadic late-onset Alzheimer's disease (LOAD), the abnormally-elevated cholesterol level in brain neurons represents a critical causative factor that drives the pathogenic processes of LOAD. Specifically, it is hypothesized that the abnormally-elevated neuronal cholesterol will disrupt mitochondrial structure and metabolic activity, resulting in ATP deficiency as well as reduced formation of neuroactive metabolic intermediates (such as mevalonate and geranylgeraniol) along the cholesterol synthesis pathway in brain neurons. In addition, the abnormally-elevated neuronal cholesterol will cause direct neuronal damage as well as other pathogenic changes in the brain, including increased formation and aggregation of amyloid (Aβ) plaques. It is speculated that Aβ accumulation and plaque formation in a majority of LOAD cases only represent a characteristic secondary pathological change, and are usually not the driving force in the pathogenesis of LOAD. As discussed in this paper, the abnormally-elevated neuronal cholesterol in conjunction with ATP deficiency and lack of neuroactive metabolic intermediates will not only cause learning and memory impairment, but will also reduce the formation of cholinergic vesicles and induce tauopathy. It is expected that these pathogenic changes are more readily seen initially in ischemia-sensitive neurons in the hippocampus and posterior parietal cortex, which is then followed by progressive neurodegenerative and atrophic changes in many other brain regions along with progressive cognitive decline. As explained in this paper, ApoE4 is a major risk factor in LOAD because ApoE4 has a drastically reduced ability than ApoE2 and ApoE3 to efflux excess cholesterol out of neurons. Overall, there is a large body of direct, indirect and circumstantial clinical and experimental evidence which jointly supports the cholesterol-centered hypothesis on the etiology and pathogenesis of LOAD.

Keywords: Alzheimer's disease; pathogenic mechanism; cholesterol; neuronal cholesterol dysregulation; apolipoprotein E; amyloid β; tauopathy

1. Introduction

Alzheimer's disease (AD), first described by German physician Alois Alzheimer in the early part of the 20th century [1], is now recognized as the most common cause of dementia among the elderly [2]. The brain region most vulnerable to neuronal dysfunction and cell loss in AD is the medial temporal lobe, including entorhinal cortex and hippocampus. AD usually begins in the entorhinal cortex and proceeds to the hippocampus, a waystation important in memory formation. As the hippocampal neurons degenerate, short-term memory falters. Often the ability to perform routine tasks begins to deteriorate as well. It then gradually spreads to other regions of the brain, particularly the cerebral cortex, which is the outer area of the brain and involved in functions such as language and reasoning. Disturbing behaviors, such as wandering and agitation, beset many people as the disease progresses. In its final stages, AD wipes out the ability to recognize even close family members or to communicate in any way. All sense of self seems to vanish, and the individual becomes

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completely dependent on others for care. Patients often live for years with this condition, and death usually ensues from a complication of immobility such as pneumonia or pulmonary embolism.

The pathological hallmarks of AD are amyloid plaques, which are the extracellular accumulations of amyloid β (Aβ), and intracellular neurofibrillary tangles (NFTs) composed of the microtubule-associated protein tau [3–5]. The development of amyloid plaques usually occurs earlier, and tangle burden accrues over time in a manner that correlates more closely with the development of cognitive impairment. Although many older individuals develop some plaques and tangles as a consequence of normal ageing, the AD brains have a far higher likelihood of developing plaques and tangles in relevant brain regions [6]. The mechanisms by which Aβ and tau induce neuronal dysfunction and death are still not fully clear at present, but many mechanisms have been suggested, which include direct impairment of synaptic transmission and plasticity, excitotoxicity, oxidative stress, neuroinflammation, and others.

Except for 1–5% cases where genetic differences have been identified, the cause for most AD cases, in particular those sporadic late-onset AD (LOAD) cases, is still unknown, and is believed to be multifactorial. Many interacting risk factors, genetic and environmental, jointly contribute to the onset of the disease. The genetic heritability of AD, based on studying twins and families, range from 49–79% [7,8]. Around 0.1% of the cases are familial forms of autosomal dominant inheritance, which have an onset before age 65 [9]. This form of the disease is known as early-onset familial AD. Studies have revealed that most of the autosomal dominant familial AD cases are attributed to mutations in one of three genes: those encoding amyloid-β precursor protein (APP) and presenilins 1 and 2 [10]. Most mutations in the APP and presenilin genes increase the production of a small protein fragment called Aβ_{42} (the β-amyloid peptide containing 1–42 amino acid residues), which is also a main component of the senile plaques [11]. Notably, the genetic evidence, coupled with the fact that Aβ accumulates in the AD brain, forms the basis for the famous amyloid hypothesis of AD pathogenesis [12–14]. In addition, many other genes have been identified as having alleles that increase AD risk. By far the most important of these is *APOE*, which encodes the apolipoprotein E (ApoE) [9,15]. Individuals inheriting the ε4 allele of *APOE* have a 3-fold or higher risk for developing AD [9]. While these individuals make up less than one-fourth of the population, they account for more than half of all AD cases.

In addition to genetic factors, it has been suggested that the cellular homeostasis of ionic copper, iron and zinc is disrupted in AD. For instance, it was hypothesized that dietary copper excess and zinc deficiency may play a causal role in AD [16]. Presently, it remains unclear whether the observed changes in metals are produced by or causes for alterations in relevant neuronal proteins, such as APP, tau and ApoE [17,18].

In this paper, a unifying hypothesis is proposed, which postulates that in most sporadic LOAD cases, abnormally-elevated cholesterol in brain neurons constitutes a crucial causative factor, driving many key pathogenic processes of LOAD, including learning and memory impairment, cholinergic deficiency, Aβ plaque formation, tauopathy, and ultimately, neuronal death. An in-depth analysis of the proposed hypothesis along with a discussion of the supporting experimental and clinical evidence is provided below.

2. Hypothesis

Cholesterol is involved in many important functions in the central nervous system (CNS), such as learning and memory formation [19–22], synaptogenesis [94], axonal growth and neuronal regeneration [95]. During these processes, brain neurons require an increased supply of cholesterol. It is generally thought that the neighboring astrocytes are the main source of cholesterol for brain neurons [23,24], although neurons also have the ability to synthesize and supply small amounts of cholesterol for their own needs under certain conditions. The cholesterol molecules synthesized in brain astrocytes are carried mostly in lipidated ApoE particles, although other apolipoproteins such as

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ApoA-I, ApoA-II, ApoA-IV, ApoJ, ApoD and ApoH [25,26] are also involved in carrying, to varying degrees, cholesterol in the brain. These lipoproteins are delivered to neurons through receptor-mediated endocytosis. A number of receptors and proteins are involved in mediating the endocytosis of lipidated ApoE particles by neurons (discussed later).

As cholesterol is required for maintaining the normal structure and function of brain neurons, it is understood that if neuronal cholesterol supply becomes severely inadequate, some of its functions will be compromised. In line with this suggestion, an earlier study reported that when cholesterol content in hippocampal neurons is reduced by statin treatment, synaptic density is reduced and synaptic vesicular release is impaired [27]. Similarly, selective loss of astrocytic cholesterol synthesis, which is the chief source of cholesterol in the brain [23,24], alters brain development and synaptic functions, including reduced synaptic vesicle numbers and defective synaptic plasticity [28,29].

Here, it should also be clearly pointed out that when neuronal cholesterol content is abnormally elevated, it can become strongly cytotoxic [30–33]. It is hypothesized that abnormally-elevated cholesterol content in brain neurons constitutes a major causative factor, which drives the pathogenic processes in most cases of LOAD (schematically depicted in Figure 1). Specifically, based on the observations made recently [30] and earlier [31–33], it is hypothesized that elevated free cholesterol (i.e., unmetabolized cholesterol) can disrupt mitochondrial structure and function, resulting in ATP deficiency in neurons as well as other cells. In addition, high levels of neuronal cholesterol will suppress the cholesterol synthesis pathway through feedback inhibition of the HMG-CoA reductase (HMG-CoA-R or HMGR) [34], which then reduces the formation of key neuroactive metabolic intermediates (such as mevalonate and geranylgeraniol) in neuronal cells [35]. These neuroactive metabolic intermediates play a vital role in protein prenylation and synaptogenesis [36]. Jointly, these biochemical changes due to elevated neuronal cholesterol level will gradually lead to learning and memory impairment and tauopathy (explained in latter sections). Additionally, abnormally-elevated neuronal cholesterol is known to drive the formation and aggregation of Aβ_{42} and Aβ_{40} (i.e., amyloid plaque formation) in the brain (reviewed in [37]. As discussed in latter sections, Aβ accumulation and amyloid plaque formation in most LOAD cases only represent characteristic secondary pathological changes, and are not the dominant force that drives the pathogenic process of LOAD.

The above-proposed mechanistic hypothesis on the pathogenesis of LOAD has the following major elements (a detailed discussion of the supporting experimental evidence for each hypothetical element is provided separately in *sections 3–9*):

i. It is hypothesized that in brain neurons, chronically-elevated cholesterol (in particular mitochondrial cholesterol) will cause disruption of mitochondrial structure and metabolic function, resulting in reduced ATP synthesis. This hypothesis is proposed on the basis of recent experimental findings [30].

It is known that adequate supply of cellular ATP is important for maintaining cognitive function and new memory formation in the brain. It is hypothesized that cholesterol-induced decrease in mitochondrial ATP synthesis will create an energy deficiency in neurons, particularly in certain regions of the brain where neurons have a higher demand for oxygen and ATP supply to maintain their normal physiological functions. Inhibition of neuronal mitochondrial metabolic activity by cholesterol is an important early event that subsequently triggers a series of pathogenic changes (discussed in detail later), culminating in learning and memory impairment. Based on this understanding, it is speculated that most LOAD cases begin in brain regions that have a particularly-high demand for oxygen and energy supply (ATP synthesis), and then gradually spread to other brain regions with a relatively lower demand for energy supply, and eventually to most regions of the brain.

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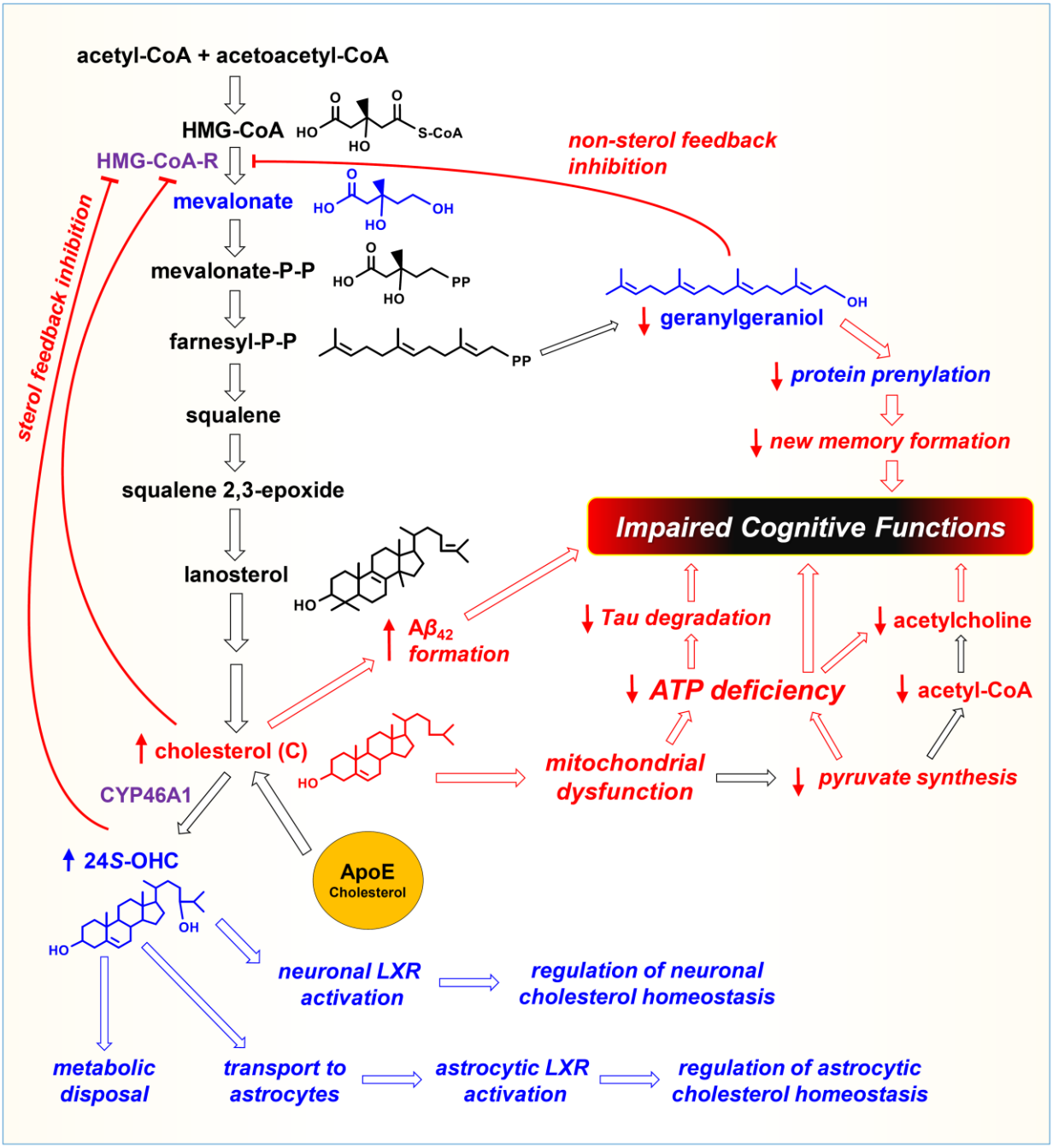

Figure 1. A hypothesis on the etiological role of neuronal cholesterol dysregulation in LOAD. As depicted in the upper left (in black color), cholesterol is synthesized using acetyl-CoA as the initial substrate (all the enzymes involved in cholesterol synthesis and metabolism are separately listed in Figure 2). It is hypothesized that elevated free cholesterol (i.e., unmetabolized cholesterol) can disrupt mitochondrial structure and function, resulting in ATP deficiency in brain neurons as well as other cells. In addition, high levels of neuronal cholesterol will also suppress the cholesterol synthesis pathway through feedback inhibition of HMG-CoA reductase (HMG-CoA-R or HMGR), which then decreases the formation of key neuroactive metabolic intermediates (such as mevalonate and geranylgeraniol) in neurons. These neuroactive metabolic intermediates play a vital role in protein prenylation and synaptogenesis. Collectively, these biochemical changes resulting from elevated neuronal cholesterol will gradually lead to learning and memory impairment (explained in detail later). Additionally, abnormally-elevated neuronal cholesterol will drive the formation and aggregation of Aβ_{42} and Aβ_{40} (i.e., amyloid plaque formation) in the brain. Mitochondrial dysfunction and ATP deficiency will also contribute to reduced degradation of the hyperphosphorylated tau proteins as well as reduced synthesis and release of the cholinergic vesicles. Please refer to the manuscript for a detailed explanation of each of the above hypothetical elements.

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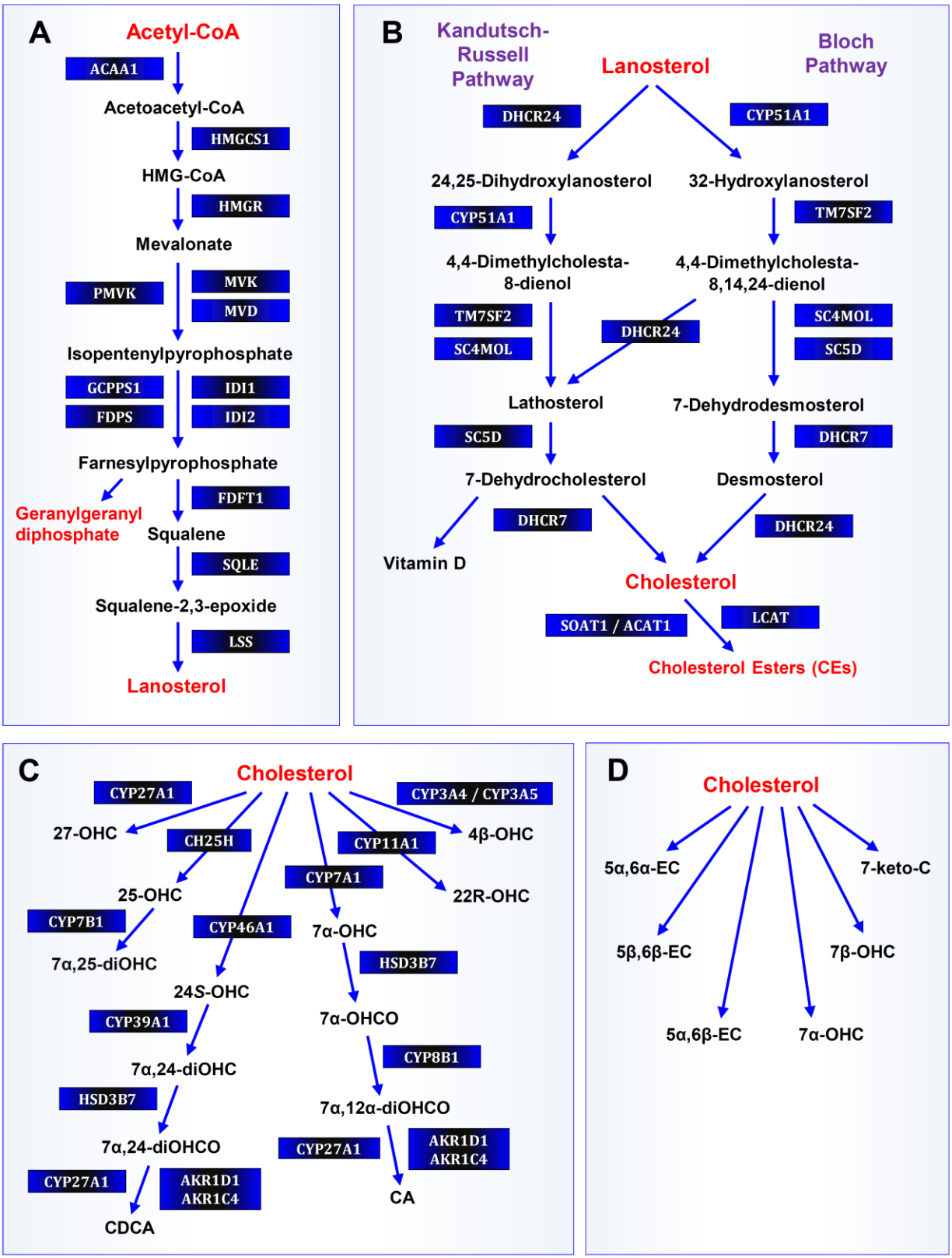

Figure 2. Cholesterol biosynthetic and metabolic pathways. (A). ***De novo*** **cholesterol biosynthesis (pre-squalene mevalonate pathway). Acetyl-CoA**: acetyl-coenzyme A; **ACAA**: acetyl-coenzyme A acetyltransferase; **acetoacetyl-CoA**: acetoacetyl-coenzyme A; **HMGCS1**: 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; **HMG-CoA**: 3-hydroxy-3-methylglutaryl-coenzyme A; **HMGR**: HMG-CoA reductase; **PMVK**: phosphomevalonate kinase; **MVK**: mevalonate kinase; **GGPPS1**: geranylgeranyl diphosphate synthase 1; **IDI1**: isopentenyl-diphosphate delta isomerase 1; **FDPS**: farnesyl-diphosphate synthase; **IDI2**: isopentenyl-diphosphate

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delta isomerase 2; **FDFT1**: farnesyl-diphosphate farnesyltransferase 1; **SQLE**: squalene epoxidase; LSS: lanosterol synthase. **(B). *De novo* cholesterol biosynthesis (post-squalene mevalonate pathway, including the Bloch and Kandutsch–Russell pathways) and cholesterol esterification. DHCR24**: 24-dehydrocholesterol reductase; **CYP51A1**: cytochrome P450 51A1; **24,25 DHLan**: 24,25-dihydrolanosterol; **TM7SF2**: transmembrane 7 superfamily member 2; **SC4MOL**: methylsterol monooxygenase 1; **SC5D**: sterol-C5-desaturase; **DHCR7**: 7-dehydrocholesterol reductase; **SOAT1** (also called **ACAT1**): sterol O-acyltransferase 1 (acyl-CoA:cholesterol acyltransferase 1); **LCAT:** lecithin:cholesterol acyltransferase. **(C). Enzymatic cholesterol catabolism. CYP27A1**: cytochrome P450 27A1; **CYP3A4** or **CYP3A5**: cytochrome P450 3A4 or 3A5, respectively; **4β-OHC**: 4β-hydroxycholesterol; **27-OHC**: 27-hydroxycholesterol; **CH25H**: cholesterol 25-hydroxylase; **CYP11A1**: cytochrome P450 11A1; **22*R*-OHC**: 22*R*-hydroxycholesterol; **25-OHC**: 25-hydroxycholesterol; **CYP7B1**: cytochrome P450 7B1; **7α,24-diOHC**: 7α,24-dihydroxycholesterol; **CYP46A1**: cytochrome P450 46A1; **CYP7A1**: cytochrome P450 7A1; **CYP7B1**: cytochrome P450 7B1; **24S-OHC**: 24*S*-hydroxycholesterol; **CYP39A1**: cytochrome P450 39A1; **7α-OHC**: 7α-hydroxycholesterol; **CYP8B1**: cytochrome P450 8B1; **7α,12α-diOHCO:** 7α,12α-dihydroxycholestenone; **HSD3B7**: 3β-hydroxysteroid dehydrogenase type 7; **7α-OHCO**: 7α-hydroxycholestenone; **CA**: cholic acid; **CDCA**, chenodeoxycholic acid. **(D). Non-enzymatic cholesterol catabolism. 7β-OHC**: 7β-hydroxycholesterol; **5α,6α-EC**: 5α,6α-epoxycholesterol; **5β,6β-EC**: 5β,6β-epoxycholesterol; **5α,6β-EC**: 5α,6β-epoxycholesterol.

ii. The normal learning and memory process requires the supply of certain neuroactive metabolic intermediates, such as mevalonate and geranylgeraniol [35], which are involved in the prenylation of proteins [36]. Offering support for the important role of these neuroactive metabolic intermediates in learning and memory, an earlier study demonstrated that impairments in learning and memory functions in an animal model can be restored by supplying geranylgeraniol, but not cholesterol [35].

Notably, both mevalonate and geranylgeraniol are metabolic intermediates formed in brain neurons as part of the cholesterol synthesis pathway [34,38,39] (see Figure 1). High cholesterol level in neurons will inhibit HMGR and thus suppress the cholesterol synthesis pathway. As a result, it will markedly reduce the levels of these neuroactive metabolic intermediates in brain neurons. Understandably, it is crucial that the upper half of the cholesterol synthesis pathway (depicted in Figure 1) in brain neurons need to remain active for normal learning and memory function as it will provide key neuroactive metabolic intermediates required for the formation of new synaptic connections. Here, it should be noted that activation of the entire cholesterol synthesis pathway will lead to the production of cholesterol, which may be used by neurons to fulfill certain physiological functions when it is so needed; however, when cholesterol is actually not needed by neurons or is already adequately supplied by the neighboring astrocytes, the upper half of the cholesterol synthesis pathway in brain neurons may still need to remain active, which is not for the purpose of synthesizing more cholesterol, but for the purpose of synthesizing neuroactive metabolic intermediates. To achieve this unique function without producing excess cholesterol in neurons, the upper half of the cholesterol synthesis pathway can be, in fact, effectively regulated by geranylgeraniol, which serves as a non-sterol feedback inhibitor of HMGR [34,38] (see Figure 1). In this way, when neurons have already synthesized sufficient amount of mevalonate and geranylgeraniol for protein prenylation, it can effectively shut down HMGR when further synthesis of cholesterol is not needed.

Understandably, when cholesterol level in a neuron is abnormally elevated (for whatever reasons), it will always impose an inhibition of HMGR, a rate-limiting enzyme of the cholesterol synthesis pathway. This inhibition will not only suppress the synthesis of cholesterol, but will also suppress the production of mevalonate and geranylgeraniol. Additionally, elevated neuronal cholesterol will also disrupt mitochondrial structure and metabolic function, and inhibit ATP synthesis (already described above). These pathogenic effects caused by elevated neuronal cholesterol will jointly hamper the normal process of learning and memory formation.

iii. It is known that when the cholesterol level in brain neurons becomes abnormally elevated, it will also lead to increased formation of Aβ plaques (mechanistic explanation is discussed later). In

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addition, it is known that elevated extracellular Aβ_{42} and Aβ_{40} levels can disrupt mitochondrial function, which contributes to reduced mitochondrial ATP synthesis [40,41].

iv. It is well known that the intracellular protein tau is involved in AD pathogenesis by forming intracellular neurofibrillary tangles (discussed in *section 6*). Tau protein degradation in neurons is mediated by the ubiquitin system in an ATP-dependent manner (discussed in *section 6*). Elevated cholesterol level in neurons will lead to reduced ATP synthesis, and it is, therefore, hypothesized that severe cellular ATP deficiency will not only disrupt the process of neurotransmission, but will also slow down proteasome-mediated degradation of tau proteins, which eventually results in accumulation of hyperphosphorylated tau proteins in neurons, resulting in the formation of characteristic intracellular neurofibrillary tangles.

v. It is known that the cholinergic neurotransmitter vesicles contain high levels of ATP, which is a key component of the cholinergic vesicles [42,43]. It is hypothesized that severe deficiency of cholinergic vesicles seen in AD patients is largely due to disruption

of mitochondrial metabolic activity by cholesterol, which reduces ATP synthesis, and the reduced neuronal ATP level is an important factor for the reduced formation of

Cholinergic vesicles. In addition, it is hypothesized that disruption of mitochondrial metabolic activity by cholesterol also reduces the synthesis and cross-mitochondrial transport of acetyl-CoA, a precursor for the synthesis of acetylcholine. It is speculated that these two factors jointly contribute to the reduced synthesis of acetylcholine and the reduced formation and release of cholinergic vesicles in many LOAD cases. Provided below is an explanation of each of the hypothetical elements briefly described above, along with a critical analysis of the available supporting evidence, most of which is scattered in unrelated scientific literature in bits and pieces. In addition, efforts are made trying to apply the proposed mechanistic hypothesis to provide a tentative explanation for some of the interesting, yet poorly-understood, experimental and/or clinical observations related to AD (mostly LOAD).

3. Role of Cholesterol in LOAD

3.1. Cholesterol in Normal Brain Function: A Brief Overview

A very brief review of the relevant knowledge points related to the synthesis, regulation and function of cholesterol in the CNS is provided in this section; it is intended to help non-expert readers more readily understand the proposed hypothesis and the relevant explanations provided in latter sections.

3.1.1. Cholesterol in the Brain

The human brain is enriched with cholesterol compared with other tissues. It is estimated that while the brain only makes up, on average, 2.1% of body mass, it contains 23% of total body cholesterol [44–47]. The cholesterol levels in most animal tissues are around 2 *mg*/*g* tissue, but its level in the brain is 15–20 *mg*/*g* tissue [45].

The majority (70–90%) of cholesterol in the CNS is associated with myelin that surrounds axons. Cholesterol synthesis in the brain is highest in oligodendrocytes during developmental stages involving active myelination, and decreases by approximately 90% in adult brain after myelination is completed [45,48].

It is generally thought that due to the presence of the blood-brain barrier (BBB), cholesterol in the brain does not readily equilibrate with cholesterol bound to lipoproteins in the blood [49,50]. Therefore, cholesterol in the brain is believed to be produced locally [44,45,51], and is synthesized primarily in glial cells, such as astrocytes and oligodendrocytes, although cholesterol can also be synthesized in smaller quantities in neurons [47,52]. Cholesterol transport among different cell types in the CNS is mostly carried out by ApoE-containing lipoproteins [53] (discussed in *section 4.1*).

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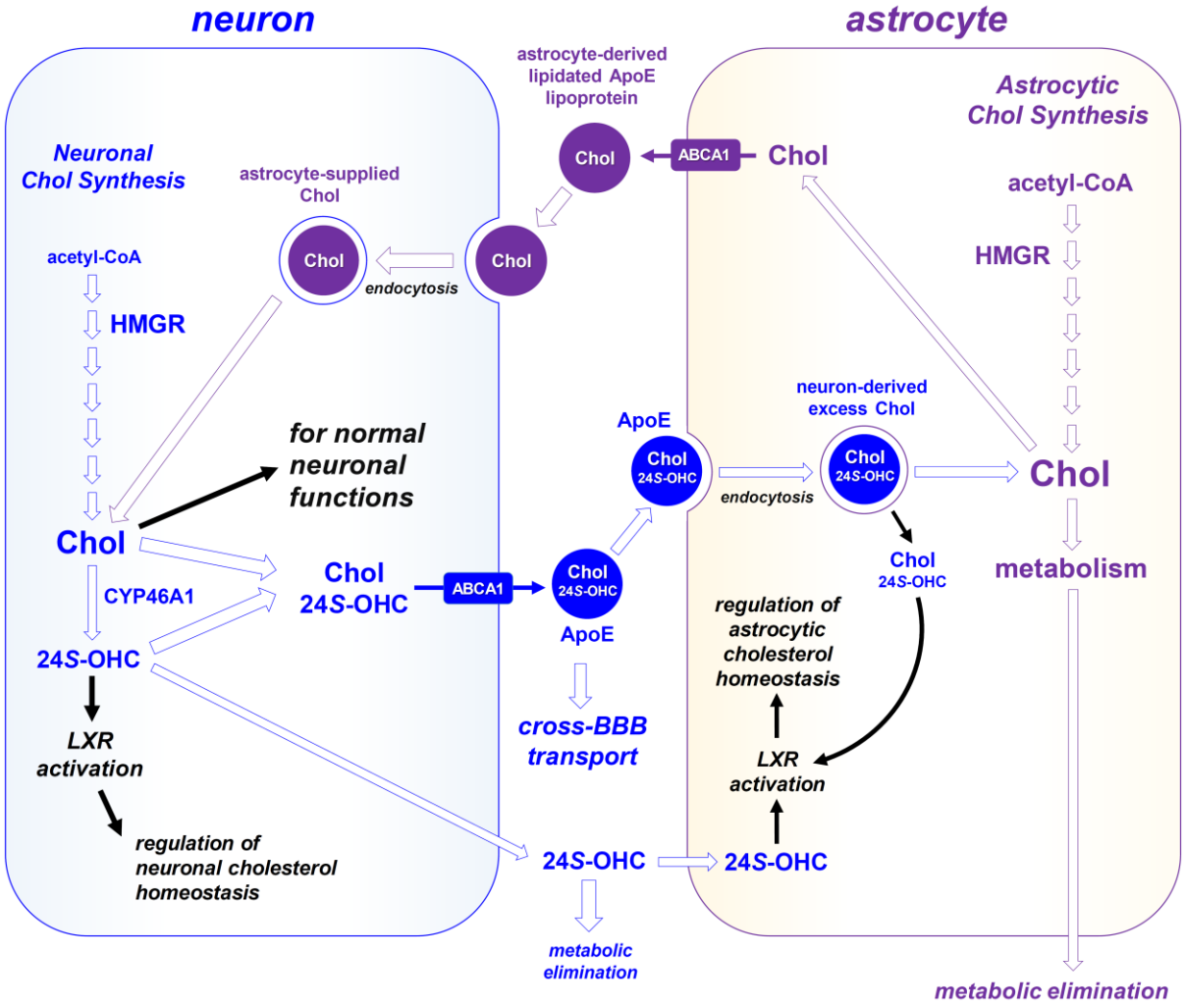

Figure 3. Regulation of cholesterol synthesis and transport in brain neurons and astrocytes. As depicted in the right panel, astrocytes are the main site in the brain for the *de novo* synthesis of cholesterol (abbreviated as **Chol**) using acetyl-CoA as the starting material. In addition, brain neurons also have the ability to perform *de novo* synthesis of a smaller amount of cholesterol using acetyl-CoA as the starting material (depicted in the left panel). Brain ApoE-containing lipoproteins are the main carriers that can transport astrocyte-derived lipids (rich in cholesterol and CEs) to neurons through ApoE receptor-mediated endocytosis. Once internalized, ApoE lipoprotein particles supplies cholesterol (and other lipids) to neurons, which are usually required for fulfilling certain neuronal functions. Importantly, ApoE can also efflux excess neuronal cholesterol to astrocytes for disposal in an ABCB1-dependent manner. As depicted, neurons selectively express CYP46A1; when neuronal cholesterol supply is in excess, CYP46A1 expression will be upregulated, which will convert cholesterol to 24*S*-OHC. 24*S*-OHC is a crucial neuronal regulator, which can activate neuronal LXR to regulate neuronal cholesterol homoestasis. Specifically, under the feedback regulation of 24*S*-OHC, neurons will stop endocytosing astrocyte-produced, cholesterol-rich ApoE particles, and in the meantime, they will enhance the efflux of excess neuronal cholesterol in an ABCA1/ApoE-dependent manner. While these effluxed neuronal cholesterol (in ApoE lipoprotein particles) can be taken up by astrocytes for metabolism and disposal, they can also undergo cross-BBB transport (discussed in *section 8.1*). As an important part of the feedback regulation, the neuronally-produced 24S-OHC can be transported to the neighboring astrocytes (via simple diffusion or ApoE/ABCA1-dependent efflux), which will then activate the LXR in astrocytes to suppress the astrocytic synthesis and release of cholesterol and also will enhance cholesterol metabolism and disposition by astrocytes.

Cholesterol serves as a precursor for the synthesis of neurosteroids, oxysterols and bile acids in the brain [54–56]. Importantly, cholesterol also functions as a membrane reinforcer, regulating cell

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signaling via lipid rafts in neuronal cells. The cholesterol that binds tightly with sphingolipids forms a sterol/sphingolipid-rich domain, commonly referred to as the lipid raft domain, serves as a platform to host various membrane proteins involved in cell signaling and neurotransmission [57,58]. Notably, the amyloid precursor protein (APP) is also richly contained in the lipid raft domain, where enzymatic cleavage of APP by different secretases takes place [59]. In fact, it is known that increasing neuronal cholesterol level can alter APP cleavage, favoring the formation of Aβ_{42} and Aβ_{40} fragments (discussed in detail in *section 5*).

3.1.2. Cholesterol Biosynthesis and Regulation

Cholesterol synthesis in the brain is precisely regulated [34,38]. Cholesterol is synthesized from acetyl-CoA via a complex pathway involving over 30 enzymatic steps (depicted in Figures 1 and 2). Acetyl-CoA, which is transported from mitochondria to cytoplasm, is the building block for cholesterol synthesis. The enzyme acetyl-CoA acyltransferase (ACAA) utilizes acetyl-CoA to produce acetoacetyl-CoA, which is then converted to HMG-CoA by the cytosolic HMG-CoA synthase. The next step, i.e., the reduction of HMG-CoA to mevalonic acid, is catalyzed by HMG-CoA reductase (HKG-CoA-R or simply as HMGR), which is a rate-limiting step in sterol synthesis. Mevalonic acid contains five carbons and is converted through a series of reactions to lanosterol, which is the first sterol precursor with 30 carbons. Through several additional enzymatic reactions that occur mostly at the endoplasmic reticulum (ER) membrane, lanosterol is converted to cholesterol, a sterol with 27 carbons. In addition, mevalonic acid is a precursor for the synthesis of geranylgeraniol, which is needed for covalent modifications of many macromolecules via enzymatic prenylation [36]. Mevalonic acid and geranylgeraniol are considered crucial neuroactive metabolic intermediates [39], and play an important role in learning and memory formation (discussed later).

Important feedback control in cholesterol synthesis involves sterol and nonsterol-mediated rapid degradation of HMGR (Figure 1; reviewed in [60,61]). HMGR is a multi-span membrane protein residing in the ER, and its degradation occurs when sterols accumulate in the ER membranes, which triggers the binding of HMGR to a pair of ER membrane proteins called Insig-1 and Insig-2. Insig binding leads to ubiquitination of HMGR, which undergoes proteasome-mediated degradation in the cytosol.

In addition to sterol's regulation of HMGR degradation, the sterol-dependent transcription factor (SREBP2) is also regulated by sterols (e.g., cholesterol), causing down-regulation of genes involved in sterol synthesis (e.g., HMGR) and transport (e.g., the LDL receptor LDLR) [62]. Geranylgeraniol, which is derived from mevalonate, is a nonsterol that also exerts feedback inhibition of HMGR. In this way, the cholesterol synthesis pathway is precisely regulated when it is activated solely for the purpose of producing neuroactive metabolic intermediates (such as mavalonic acid and geranylgeraniol) but not for the synthesis of more cholesterol.

Additionally, the HMGR activity can be regulated by cellular metabolic state in a sterol-independent manner: the enzyme can be phosphorylated or dephosphorylated in a reversible manner through the actions of protein kinases and phosphoprotein phosphatases, resulting in enzyme inactivation and activation [63].

3.1.3. Sources of Neuronal Cholesterol and Its Intracellular Trafficking

As depicted in Figure 3, cholesterol in brain neurons is synthesized primarily in glial cells (e.g., astrocytes and oligodendrocytes) [44,45,51], although smaller quantities of cholesterol can also be synthesized in neurons [47,52]. Cholesterol transport among different cell types in the CNS is mostly carried out by ApoE-containing lipoproteins [53] (discussed in *section 4.1*). The movement of cholesterol through different subcellular compartments inside a neuron involve multiple metabolic pathways, resulting in different intracellular cholesterol pools that are in slow equilibrium with one another.

i. *Supply by Cholesterol-Rich Lipoproteins.* In the brain, ApoE is a major cholesterol carrier, and

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astrocyte-derived ApoE lipoproteins enter a neuron through receptor-mediated endocytosis [34,38,64] (Figure 3). After endocytosis, lipoproteins first enter a distinct early endocytic compartment which is rich in acid lipase for catalyzing the hydrolysis of cholesterol esters (CEs) [65]. Cholesterol released from lipoprotein-derived CEs then moves to the endosomes that contain a pair of cholesterol-binding proteins designated as Niemann-Pick type C1 (NPC1) and NPC2. NPC2, a soluble protein present in the luminal region of the endosomes and lysosomes [66], binds cholesterol and transfers it to NPC1 [67,68], a protein that contains multiple transmembrane domains, including a sterol-sensing domain [69]. NPC1 then exports cholesterol to the exterior of the late endosomes and lysosomes. Cholesterol exiting from the late endosomes arrives at other membrane compartments, via various transport mechanisms. These recipient membrane compartments include the plasma membrane [70,71], ER [72–74], trans-Golgi network [75–77], mitochondria and peroxisomes.

ii. *De Novo Biosynthesis.* Inside a neuron, the majority of newly-synthesized sterols, including cholesterol, lanosterol and other precursor sterols, move quickly from the ER to the plasma membrane after synthesis. Upon arriving at the plasma membrane, a significant amount of the synthesized sterols, especially lanosterol, is released to the exterior by the sterol efflux process that depends on ABCA1 and apolipoproteins [78,79]. The sterols (including cholesterol, lanosterol and other precursor sterols) remaining at the plasma membrane will recycle between the plasma membrane and various internal compartments, including endosomes and lysosomes [80,81].

iii. *SOAT1-Mediated Esterification.* Cholesterol inside a neuron is also a substrate for sterol *O*-acyltransferase 1 (SOAT1), which is also called acyl-CoA:cholesterol acyltransferase 1 (ACAT1), resulting in the formation of CEs (Figure 2B). CEs are then sequestered in cellular lipid droplets, which are subject to hydrolysis by enzymes collectively designated as CE hydrolases [82]. When SOAT1 is inhibited, part of the cholesterol pool destined for storage as CEs may be shuffled to the plasma membrane where cholesterol may serve as substrate for ABCA1-mediated lipid efflux [79].

iv. *Cholesterol in Mitochondria.* Mitochondrial cholesterol comes from at least three sources: The first source is the plasma membrane. The molecular nature of the plasma membrane–mitochondria cholesterol movement is not clear at present, but this process does not require NPC2 [83]. At the mitochondria, the transfer of cholesterol from the outer membrane to the inner membrane is mainly mediated by steroidogenic acute regulatory (StAR) protein [84]. The second source is from late endosomes and lysosomes. In cells that express low levels of StAR protein, the StAR-related lipid transfer protein domain 3 (STARD3) present in the late endosomes [85,86] works along with the NPC2 protein to transport cholesterol from the late endosomes and lysosomes to the mitochondria [87,88]. This process explains the observation that, in cells with mutant NPC1, cholesterol overloading occurs in mitochondria [89,90]. This information is crucial to understanding the mitochondrial toxicity and cellular ATP depletion as crucial causative pathogenic changes in the NCP disease (discussed in *section 8.4*). The third source of mitochondrial cholesterol comes from a specialized membrane region designated as the mitochondria-associated membranes, which are part of the ER membranes in close physical contact with the mitochondrial membrane [91]. These membranes are enriched with cholesterol and the enzyme SOAT1 for converting cholesterol to CEs [92,93].

3.1.4. Cholesterol and Normal Brain Functions

Cholesterol is involved in many important brain functions, such as synthesis of neurosteroids [54–56], learning and memory formation [19–22], synaptogenesis [94] and axonal growth [95,96]. Being an important component of the plasma membrane,

cholesterol is also critically involved in regulating ion permeability [97] and signal transduction in neurons [98]. An earlier study showed that depletion of cholesterol in cultured rat hippocampal neurons by methyl-β-cyclodextrin, a cholesterol-sequestering agent, leads to reduced excitatory postsynaptic currents (EPSCs) and diminished long-term potentiation (LTP) [21]. Other studies also reported that dysregulation of brain cholesterol homeostasis affects synaptic functions [99–101].

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In the brain, astrocytes are the main source of neuronal cholesterol [23,24] (depicted in Figure 3). Studies have shown that the selective loss of astrocytic cholesterol synthesis can alter brain development and synaptic functions in vivo, with reduced synaptic vesicle numbers and defective synaptic plasticity [28,29]. As cholesterol is enriched in myelin [102] and required for its growth [103,104], oligodendrocytes support neuronal function partly by enwrapping neuronal axons with cholesterol-enriched myelin.

3.2. Neuronal Cholesterol Dyshomeostasis in LOAD

As discussed above, cholesterol has many important functions in the brain. Therefore, some of cholesterol's neuronal functions will be compromised if the supply of astrocyte-derived cholesterol is severely inadequate (more discussion on this subject is provided later). On the other hand, when cholesterol level inside neurons is abnormally elevated, it will also become pathogenic. There is evidence linking elevated blood cholesterol levels to increased risk of LOAD in the elderly [105–109]. Similarly, cholesterol metabolism in LOAD brains is altered compared to normal brains [107,110], and alterations in brain cholesterol metabolism are associated with an increased risk of LOAD [111,112].

Consistent with the human observations, animal studies have more clearly demonstrated that feeding rabbits a cholesterol-rich diet can cause learning and memory impairment, along with increased Aβ production in their brains [113].

The association between impaired cholesterol homeostasis in the brain and neurodegeneration is perhaps best exemplified in the NPC disease, which is caused by mutations in either the NPC1 or NPC2 gene. NPC1 and NPC2 each can bind to cholesterol and act in tandem in late endosomes and lysosomes to mediate the egress of CEs derived from endocytosed lipoproteins [114,115]. Consequently, in NPC1- or NPC2-deficient cells, including neurons [116] and glial cells [117,118], unesterified cholesterol and other lipids become sequestered in late endosomes and lysosomes, and the amount of cholesterol in the plasma membrane and ER (the cellular sites at which cholesterol homeostasis are regulated) is reduced [119]. In the *NPC1*$^{-/-}$ neurons, this defect in cholesterol export from late endosomes and lysosomes to other cellular membrane structures results in a higher-than-normal cholesterol content in neuronal cell bodies (especially inside the mitochondria) and a decreased cholesterol content in the distal axons [116,120].

While the NCP disease is a relatively rare (1/150,000 live births) autosomal recessive inherited disorder in humans, it causes progressive neurodegeneration and premature death (often accompanied by hepatosplenomegaly and lung disease) [121]. A characteristic histological finding in the brain is a massive loss of neurons, particularly Purkinje cells in the cerebellum, consistent with an impairment of motor functions in these individuals [122], although neurons in other regions of the brain are also affected to varying degrees. The precise mechanism underlying the pathogenesis of the NCP disease is still not clear at present, but one thing is certain that dysregulation of the neuronal cholesterol homeostasis (e.g., transport) is a fundamental pathogenic change that can directly result in massive neuronal injury and death. (A more detailed mechanistic explanation on the NCP disease is provided in *section 8.4*).

In addition to AD and NPC disease, other neurodegenerative diseases, such as Huntington's disease and Smith-Lemli-Opitz syndrome, are also associated with cholesterol dyshomeostasis [123,124]. The apparent link between these disease conditions and neuronal cholesterol abnormality underscores the clinical relevance of the proposed cholesterol-centered hypothesis on pathogenesis. A mechanistic explanation on the pathogenic role of cholesterol in LOAD is provided below.

3.3. Mechanistic Explanation

In addition to its involvement in neurosteroid synthesis [54–56], synaptogenesis [94] and axonal growth [95,96], cholesterol also has a unique role in regulating neuronal membrane functions, including membrane fluidity, vesicle formation and fusion, ion channel function, and formation of specialized microdomains involved in neural communication [125,126]. Indeed, cholesterol is required

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presynaptically for formation of neurotransmitter vesicles [127], and postsynaptically for the clustering and stability of neurotransmitter receptors [125]. As changes in the strength of synaptic connections may alter learning and memory formation [19,20], dysregulation of neuronal cholesterol homeostasis had been suggested to affect learning and memory through modulating presynaptic and/or postsynaptic processes of neurotransmission [128]. In addition, neuronal cholesterol abnormalities can alter enzymatic formation of Aβ peptides and amyloid plaques [129–133].

In this paper, a new hypothesis is proposed, which suggests that in most cases of LOAD, abnormally-elevated cholesterol in brain neurons constitutes a major causative factor, which drives the pathogenic processes of LOAD. Mechanistically, it is hypothesized that the elevated neuronal cholesterol (most likely mitochondrial cholesterol) will disrupt mitochondrial structure and metabolic activity, resulting in reduced synthesis of ATP and important neuroactive metabolic intermediates along the cholesterol synthesis pathway which are required for normal neuronal functions of the brain (in particular, learning and memory). These metabolic changes are important initial events that will trigger a series of pathogenic changes culminating in the gradual development of LOAD (depicted in Figure 1). Specifically, elevated neuronal cholesterol will increase the formation of Aβ_{42} and Aβ_{40} (discussed in *section 5*); and neuronal ATP deficiency will enhance tauopathy (discussed in *section 6*) and decrease the formation of cholinergic vesicles (discussed in *section 7*). Additionally, elevated brain cholesterol will suppress macrophage response (due to reduced glial ATP levels), which contributes to the development of neuroinflammation in LOAD.

Offering partial support for this hypothesis, a recent study has shown that exposure of cultured neuronal cells to even very low concentrations of free cholesterol can disrupt mitochondrial structure and metabolic activity (for ATP synthesis) [30]. As shown, a strong disruptive effect of cholesterol on cellular and mitochondrial functions was readily observed when cholesterol was present at 0.1–1 μM, which is really intriguing. These observations are somewhat in line with an earlier study which used a genetic mouse model that selectively overloaded cholesterol in the mitochondria and then examined the effect of mitochondrial cholesterol on Aβ neurotoxicity and AD pathology [134]. Additionally, it was shown earlier that the isolated mitochondria from the cortical neurons of transgenic mice which overexpressed SREBP-2 or lacked NPC1 exhibited mitochondrial cholesterol accumulation, mitochondrial glutathione reduction, and increased susceptibility to Aβ_{42}-induced oxidative stress and release of apoptogenic proteins [134]. Consistent with these observations, the APP/PS1 transgenic AD mice displayed mitochondrial cholesterol loading, glutathione depletion, and functional deficits, and the degree of deficits correlated with the degree of Aβ accumulation [74].

As mentioned earlier, the learning and memory formation needs mevalonate, which serves as a precursor for the subsequent synthesis of geranylgeraniol. Geranylgeraniol plays a key role in the prenylation of proteins in neurons [36]. Both mevalonate and geranylgeraniol are important neuroactive metabolic intermediates that are formed during cholesterol biosynthesis in brain neurons [35] (Figure 1). High levels of cellular cholesterol in neurons will inhibit the cholesterol synthesis pathway, which will then result in reduced biosynthesis of these key neuroactive metabolic intermediates. In support of this hypothesis, an earlier study reported that the learning and memory function could be effectively restored in the AD animal model by giving geranylgeraniol [35].

Based on the above discussion, it is understood that the upper half of the cholesterol synthesis pathway in brain neurons needs to be kept active during the normal process of memory formation as it produces crucial neuroactive metabolic intermediates (mevalonate and geranylgeraniol) required for the formation of new synaptic connections. As such, even if when cholesterol is not needed by the neurons (for instance, when cholesterol is already adequately supplied by the neighboring astrocytes), the upper half of the cholesterol synthesis pathway in neurons may still need to remain active, which is not for the purpose of synthesizing more cholesterol, but for the synthesis of neuroactive metabolic intermediates (Figure 1).

In order to keep the upper half of the cholesterol synthesis pathway active in brain neurons, it is

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important to keep free cholesterol levels relatively low in these cells at all time. When neuronal cholesterol level is abnormally elevated, it will suppress the cholesterol synthesis pathway through feedback inhibition of HMGR. Notably, statins are known to exert their cholesterol-lowering effect through inhibiting HMGR. Based on the above discussion, it is understood that centrally-active statins will not be as beneficial (most likely harmful) for AD because they will always concomitantly reduce the levels of both cholesterol and neuroactive metabolic intermediates in brain neurons. While reductions in neuronal cholesterol likely will be beneficial most of the time, the reduced synthesis of neuroactive metabolic intermediates in neurons will always be harmful for learning and memory formation. Based on the available clinical observations, the net effect of centrally-active statins is mixed, likely depending on the statin doses used and the degree of neuronal HMGR inhibition produced. While most studies reported a lack of improvement in memory function with centrally-active statins, it appears the use of peripherally-acting statins is more likely to have a beneficial effect. More detailed discussion on the complex effects of statins in LOAD is provided in *section 8.3*.

Based on the above explanation, it is also understood that alterations in many factors that affect cholesterol metabolism and transport likely will also alter the risk for LOAD. For instance, CYP46A1, which is also called "cholesterol 24(*S*)-hydroxylase" [51,135,136], catalyzes the conversion of cholesterol to 24(*S*)-hydroxycholesterol (24*S*-OHC) [137,138], which is the most abundant oxysterol found in human brain [139]. This metabolic pathway plays an important role in regulating neuronal cholesterol disposition and homeostasis, and abnormalities are associated with an increased risk for LOAD [137]. Similarly, alterations in factors like ApoE4, ABCA1 and ApoE receptors, which are involved in cholesterol transport (particularly neuronal cholesterol efflux), and SOAT1, which catalyzes CE formation, will also alter the risk for LOAD. Provided below is a discussion of the roles of CYP46A1 and SOAT1 in cholesterol homeostasis and their association with LOAD, while the discussion of other relevant factors (such as ApoE4, ABCA1, lipoprotein receptors, etc.) and their respective pathogenic contribution to LOAD is provided separately in relevant latter sections.

3.3.1. Role of CYP46A1 in LOAD

Neurons are the primary cell type in the brain that expresses CYP46A1 [140]. CYP46A1 oxidizes cholesterol to 24*S*-OHC and helps maintain cholesterol homeostasis in the brain [137]. It was suggested by some researchers that for cholesterol to be transported across the BBB, it needs to be first converted to 24*S*-OHC by CYP46A1 [141]. 24*S*-OHC then diffuses out of cells and crosses the BBB, and is finally cleared in the liver [142]. In addition, it has also been suggested that 24*S*-OHC can be removed from brain neurons in an ABCA1/ApoE-dependent manner (depicted in Figure 3, *left panel*). As discussed below, the newly-proposed hypothesis (i.e., elevated neuronal cholesterol is a key pathogenic factor in LOAD) will help better understand the role of CYP46A1-mediated cholesterol metabolism in both AD animal models and LOAD patients.

i. It has been reported [143] that the adenovirus-mediated selective overexpression of *CYP46A1* in AD animal models can reduce AD severity, such as memory and learning impairment and amyloid accumulation. Similarly, it is predicted that selective knockdown of *CYP46A1* will increase AD severity in experimental AD animal models (if we assume that everything else is unchanged). In fact, the observations made earlier [144] agreed well with the predicted outcomes based on the proposed hypothesis.

The mechanistic explanation for these experimental observations or predictions is quite straightforward. The protective effect of the CYP46A1-mediated cholesterol metabolism in AD animal models likely results from two mechanisms: One is CYP46A1-mediated metabolic disposition of excess neuronal cholesterol. As CYP46A1 is selectively expressed in brain neurons, animals with *CYP46A1* knockdown are expected to have a drastically-lower ability to metabolize neuronal cholesterol and thus will result in higher levels of cholesterol inside brain neurons. Alternatively, if the brain neurons have a heightened ability to metabolize cholesterol, then neuronal cholesterol level will be reduced, which is beneficial for neuronal survival. The other mechanism is related to the

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activation of the nuclear receptor LXR system by 24*S*-OHC, a cholesterol metabolite formed by CYP46A1 [145–147]. Activation of LXR by 24*S*-OHC can regulate a number of genes associated with cholesterol homeostasis [146,148,149], such as reduced expression of HMGR (which reduces cholesterol synthesis) and increased expression of the cholesterol efflux transporters and ApoE (which jointly mediate cholesterol efflux).

ii. While the above explanation of the results from animal studies appears to be quite straightforward and readily understood, there were clinical observations that are more complex than what are seen in animal models. Many earlier human studies reported that the elevated 24*S*-OHC levels in cerebrospinal fluid (CSF) were associated with neurodegenerative diseases (reviewed in [150]). For instance, studies have shown that the CSF 24*S*-OHC levels were higher in LOAD patients, in patients with vascular dementia, and in patients with mild cognitive impairment compared to the control subjects [151–153]. The higher CSF 24*S*-OHC levels in LOAD patients [151,153] were also correlated with elevated CSF levels of ApoE, cholesterol and tau [142,150]. In these clinical studies, there was a clear positive correlation between increased levels of 24*S*-OHC and the risk of LOAD, which gives a false impression that 24*S*-OHC is a causative factor in LOAD. For a better understanding of these human observations, a little more explanation is needed here. In most LOAD patients, it is expected that their average neuronal cholesterol levels are higher than those in non-demented healthy individuals. The higher neuronal cholesterol levels will lead to increased metabolic disposition catalyzed by CYP46A1 (along with activation of other metabolic pathways). As a result, the oxysterol metabolites are higher in their CSF as well as blood circulation, which actually reflects an enhanced effort of the bodies of LOAD patients to dispose the excess neuronal cholesterol. Here, it should be noted that the observed higher levels of 24*S*-OHC found in the CSF and/or blood of LOAD patients compared to non-demented human subjects are largely because a majority of the LOAD patients are expected to have higher levels of neuronal (and blood) cholesterol before the onset of clinical LOAD compared to non-demented control subjects. If two groups of LOAD patients (or two groups of non-demented control subjects) which have the same initial neuronal cholesterol levels are compared, it is quite certain that the expected observations will be opposite, i.e., those who have a higher ability to metabolically convert neuronal cholesterol to 24*S*-OHC (i.e., with higher neuronal CYP46A1 activity) will be associated with a reduced risk for developing LOAD.

iii. In addition to CYP46A1-mediated formation of 24*S*-OHC, there are other enzymes that can also catalyze the formation of other oxysterols (e.g., 27-hydroxycholesterol) and bile acids (Figure 2C). Based on the understanding that elevated neuronal cholesterol is a causative factor in LOAD, it is readily understood that increased metabolic conversion of neuronal cholesterol to other oxysterols and bile acids will also be beneficial for reducing AD risk in animal models, which will be similar to the beneficial effects of enhanced metabolic conversion of cholesterol to 24*S*-OHC seen in animal models (as discussed above).

However, clinical studies again reported an opposite finding, i.e., there appeared to have a "positive correlation" between brain 27-hydroxycholesterol levels and LOAD risk [154]. The levels of 27-hydroxycholesterol in the blood, brain and CSF were found to be markedly elevated in individuals with LOAD [154]. These observations are very similar to the above case with 24*S*-OHC. It is speculated that the increased blood levels of 27-hydroxycholesterol only indirectly reflect an enhanced effort of the LOAD patient's body to metabolically dispose elevated neuronal cholesterol.

Besides, alterations in brain tissue levels of nonenzymatically-produced oxysterols were also observed in LOAD, which include 7α-hydroxycholesterol (which can also be formed enzymatically by CYP7A1) [155], 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, 5α,6β-dihydroxycholestanol, and 5β,6β-epoxycholesterol (Figure 2D). These observations suggest that there is an increased level of free, unmetabolized cholesterol available for both enzymatic and nonenzymatic conversions to various oxysterol derivatives in these patients.

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3.3.2. Role of SOAT1 in LOAD

Besides the conversion of cholesterol to 24*S*-OHC by CYP46A1, a fraction of cholesterol is esterified for storage by two enzymes: SOAT1 and LACT (lecithin:cholesterol acyltransferase) [156,157]. It is of note that when a mouse model of AD was cross-bred with mice lacking *SOAT1*, the amyloid pathology was attenuated in these animals [158]. Similarly, human studies have shown that polymorphism in *SOAT1* gene was associated with reduced brain amyloid load and reduced CSF cholesterol content [159], whereas elevated *SOAT1* expression was associated with more severe amyloid load [160].

How to explain the observed relationship between SOAT1 inhibition and reduced amyloid load? As explained later in *section 5*, enhanced cleavage of APP by β-/γ-secretases resulting in the production of Aβ_{42} and Aβ_{40} (major fragments contained in amyloid plaques) is believed to takes place in the lipid raft regions of the cell membrane when neuronal cholesterol level is elevated. The above experimental observations suggest that the form of cholesterol contained in the lipid rafts of neuronal cells is mostly CEs rather than the free cholesterol. When SOAT1 is absent, the content of CEs in the lipid rafts will not increase proportionally to the elevated cellular level of free cholesterol, and thus will not lead to a proportional increase in APP cleavage through the β-/γ-secretase pathways and a more severe amyloid pathology. A net increase in the cellular free cholesterol pool will naturally lead to increased metabolic disposition by CYP46A1 (due to increased availability of the substrate), thus resulting in increased formation of 24*S*-OHC [158]. Increased formation of 24*S*-OHC will then activate the expression of other genes that further help reduce neuronal cholesterol levels. In addition, an increase in cellular free cholesterol pool may serve as substrate for ABCA1-mediated lipid efflux [79]. However, it should be noted that the learning and memory performance of the SOAT1-deficient transgenic mice (which have a markedly-reduced amyloid pathology) may not be proportionally better as the levels of free cholesterol inside their brain neurons may not be markedly lower. If free cholesterol levels in neurons remain markedly elevated, which will disrupt mitochondrial function and inhibit the synthesis of ATP and neuroactive metabolic intermediates, it is expected that the learning and memory performance of these animals will still be impaired.

Notably, inhibition of SOAT1 enzymatic activity has received attention as a potential therapeutic strategy in LOAD as it can reduce amyloidogenic processing of APP through reducing the formation of CEs plus potentially increasing the conversion of unesterified cholesterol to 24*S*-OHC by CYP46A1 [158]. However, the real therapeutic benefits of SOAT1 inhibitors used alone are expected to be limited since, as discussed above, the free cholesterol levels in brain neurons may not be proportionally reduced as a result of SOAT1 inhibition alone.

3.4. Section Summary

As cholesterol has important physiological functions in the brain, lack of adequate cholesterol supply will impede certain neuronal functions. However, from a pathogenic perspective, it is hypothesized that it is equally or even more important when cholesterol level in neurons is abnormally elevated, because cholesterol can disrupt mitochondrial structure and function and inhibit the synthesis of ATP and neuroactive metabolic intermediates [30]. In addition, elevated neuronal cholesterol is a major cause for increased formation of Aβ_{42} and amyloid plaques (discussed later in *section 5*), and a reduction in neuronal ATP will lead to the development of tauopathy (discussed in *section 6.4*) and reduced formation of cholinergic vesicles (discussed in *section 7.2*). Therefore, a new hypothesis is proposed here which speculates that in most cases of LOAD, chronically-elevated cholesterol inside brain neurons constitutes a major causative factor, which drives the pathogenic process (depicted in Figure 1). There is mounting evidence from human epidemiological studies as well as experimental studies using both animal models and cultured neuronal cells which jointly demonstrate that elevated neuronal cholesterol is associated with mitochondrial inadequacy, neuronal injury, and increased risk of AD. In line with this mechanistic suggestion, an earlier study in a mouse model of AD showed that, in the early stage of AD development, synaptic mitochondria exhibit

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significant functional deficits, and the degree of deficits correlates positively with the degree of Aβ accumulation [74,161].

Based on the mechanistic understanding discussed in this section, it is speculated that neuronal cholesterol is elevated in LOAD patients, which is an important initial change. Subsequently, the elevated cholesterol will suppress neuronal cholesterol synthesis pathway, and will lead to increased metabolic formation of 24*S*-OHC, which will suppress astrocytic cholesterol synthesis, along with increased efflux of cholesterol out of neurons and across the BBB (discussed in *section 8.1*). As a result of these feedback regulations, the net cholesterol level in the brains of LOAD patients often may not be overly too high compared to non-demented control subjects. Here, it is important to point out that while this might be the case, the neuroactive metabolic intermediates in these patients likely are markedly reduced compared to non-demented individuals. The main reason for their decrease is because their synthesis will be suppressed most of the time due to the presence of feedback inhibition by elevated neuronal cholesterol. A recent targeted metabolomic study demonstrated that the concentration of the cholesterol precursor lanosterol, but not free cholesterol, was clearly lower in the brains of LOAD patients than in the brains of control subjects [160]. Similar observations were made with the levels of the enzymes involved in cholesterol synthesis [160], indicating a decrease in major cholesterol-synthesizing enzymes in relevant brain regions of LOAD patients. Notably, the expression of these enzymes was not similarly altered in the *substantia nigra* in Parkinson's patients, suggesting that these changes are relatively specific to the brain regions of LOAD patients [160].

Lastly, it is known that astrocytes are the main site for cholesterol synthesis in the brain [45,51]. An earlier animal study reported that that while selective loss of brain astrocytic cholesterol synthesis in vivo significantly alters brain development, the learning ability of these mice appeared to be largely unaffected (although their memory is reduced) [28]. This observation is intriguing, and suggests that the lack of astrocytic cholesterol supply mostly affects brain development, a process that heavily involves myelination (and also requires a lot of astrocyte-supplied cholesterol), but not learning ability. In fact, the lack of astrocytic supply of cholesterol supposedly will reduce neuronal cholesterol levels, which may actually help maintain a higher level of ATP and neuroactive metabolic intermediates in these neurons, and these changes supposedly are beneficial for the learning process. On the other hand, the long-term memory formation in these animals is still affected, likely resulting from the lack of astrocytic supply of cholesterol which is required for learning-induced *de novo* myelination, an essential process in long-term memory formation [162].

4. Role of ApoE in LOAD Pathogenesis

4.1. ApoE in Normal Brain Function: A Brief Overview

Human ApoE is a 34-kD glycoprotein present in the CNS and periphery, serving as a lipid carrier [163,164]. It consists of 299 amino acids, encoded by the *APOE* gene located on chromosome *19q13.32*. Human ApoE is polymorphic, with three major alleles, *APOE-ε2* (ApoE2: cys112, cys158), *APOE-ε3* (ApoE3: cys112, arg158), and *APOE-ε4* (ApoE4: arg112, arg158) [165,166]. Structurally, ApoE has three main domains: a *N*-terminal (4 helices) receptor-binding domain; a *C*-terminal (3 helices) lipid-binding domain; and an intervening flexible hinge region [167–170].

In the periphery, ApoE is synthesized and secreted mostly by hepatocytes and macrophages, and is associated with various lipoproteins, ranging from small plasma HDL particles (7–14 nm in size) [171] to the larger polyhedral VLDL particles (30–100 nm in size) [88], to the very large chylomicrons (75–1200 nm) [172–174]. Its main function is to transport lipids and regulate plasma lipid levels, and is also involved in immune modulation [175,176].

In the CNS, ApoE is the most abundant apolipoprotein [163], although other apolipoproteins are also present, including the more abundant ApoA-I and the less abundant apolipoproteins such as ApoJ, ApoA-II, ApoA-IV, ApoD and ApoH [25,26]. The majority of ApoE is believed to be produced

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in astrocytes, while microglia, vascular mural cells, choroid plexus and neurons can also produce ApoE [163,177–180].

One of the best-studied functions of brain ApoE proteins is their transport of astrocyte-derived lipids (rich in cholesterol and CEs) to neurons through ApoE receptor-mediated endocytosis [94,163,181–183]. A number of ApoE receptors have been identified, and they belong to the LDL receptor family, such as the LDL receptor, LDL receptor-related protein 1 (LRP1) and ApoE receptor 2 (ApoER2) [184–188]. Once internalized, ApoE supplies cholesterol (and other lipids) to neurons, which are required for certain neuronal processes, such as synaptogenesis [94], elongation of axons [95,189–191] and synaptic plasticity [191,192]. In addition, it was reported that ApoE affects the functions of certain membrane receptors of postsynaptic neurons. For example, the functions of the postsynaptic *N*-methyl-*D*-aspartate (NMDA) receptor in the hippocampus and cortex [193,194], which is important for controlling synaptic plasticity and memory formation (reviewed in [195]), are altered by ApoE4 and cholesterol [196]. Similarly, the functions of some other membrane receptors, channels, and transporters are also affected by ApoE through changes in cholesterol and CE content in neuronal membranes [197].

Offering support for the notion that the astrocyte-derived ApoE in the brains plays an important role in normal cognitive functions, earlier studies have shown that the ApoE knockout mice display learning and memory deficits [198–200]. This phenotype of defective memory function is also evident in mice with selective ApoE knockdown in astrocytes [201].

In addition to supplying neurons with astrocyte-derived cholesterol and CEs, another important function of the brain ApoE is to carry out the efflux of excess neuronal cholesterol. It is known that ApoE2 can promote significantly more cholesterol efflux from both astrocytes and neurons than ApoE3, and ApoE3 can promote more cholesterol efflux than ApoE4 [202,203]. Differences in the lipoprotein-binding preference of the ApoE isoforms are also observed in the periphery: while ApoE3 associates preferentially with the protein-rich HDL particles, ApoE4 associates more effectively to the lipid-rich VLDL particles [169,170,202,204]. These intriguing observations suggest that ApoE3 is more apt to efflux lipids (including cholesterol and CEs) out of neurons, which is akin to HDL's function in the periphery, whereas ApoE4 appears to have a reduced ability to efflux lipids but a good ability to supply lipids to neurons, which is somewhat similar to the function of VLDL in the periphery.

At the molecular level, the three ApoE isoforms differ at positions 112 and 158 in the *N*-terminal domain, which are cysteine–arginine substitutions, altering the charge of the protein and its ability to form cysteine–cysteine dimers [166]. While ApoE4 contains no cysteine residues throughout the protein, ApoE3 contains one cysteine residue (cys112) and ApoE2 contains two cysteine residues (cys112 and cys158). ApoE3 can form ApoE−ApoE and ApoE–ApoA-II dimers through its cys112 residue, and similarly, ApoE2 can more readily form respective dimers as it has two cysteine residues; in comparison, ApoE4 cannot form dimers at all [205–207]. Since the dimeric ApoE2 and ApoE3 can elicit higher lipid efflux than their monomeric forms [202], the inability of ApoE4 to form dimers significantly reduces its ability to efflux excess cholesterol out of neurons.

Consistent with the differential ability of the three ApoE isoforms in neuronal cholesterol efflux, many studies have reported that they also have different levels of lipidation in the CNS [208–210]. While ApoE2 and ApoE3 are better lipidated (with ApoE2 best lipidated), ApoE4 is markedly less lipidated [208,210]. Analysis of the CSF collected from middle-aged and older cognitively-normal individuals showed that ApoE4 is less lipidated than ApoE2 and ApoE3 [208,210]. Selective expression of the human form of ApoE2, ApoE3 or ApoE4 in mice with viral constructs confirmed that ApoE4 forms less lipidated ApoE particles compared to ApoE2 and ApoE3 particles [209].

ApoE-mediated lipid efflux in the CNS takes place in conjunction with the ATP-binding cassette (ABC) proteins, such as ABCA1 and ABCG1, ABCG4 and ABCA7 [211,212]. These proteins are embedded in cell membrane and act to pump lipid molecules into the extracellular space, where they bind apolipoproteins such as ApoE, ApoJ and ApoA-I [212]. Like ApoE, the expression of ABCA1 and

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ABCG1 is increased following activation of the nuclear receptor LXR system, either directly [18] or indirectly [213], to promote ApoE-mediated lipid efflux [211].

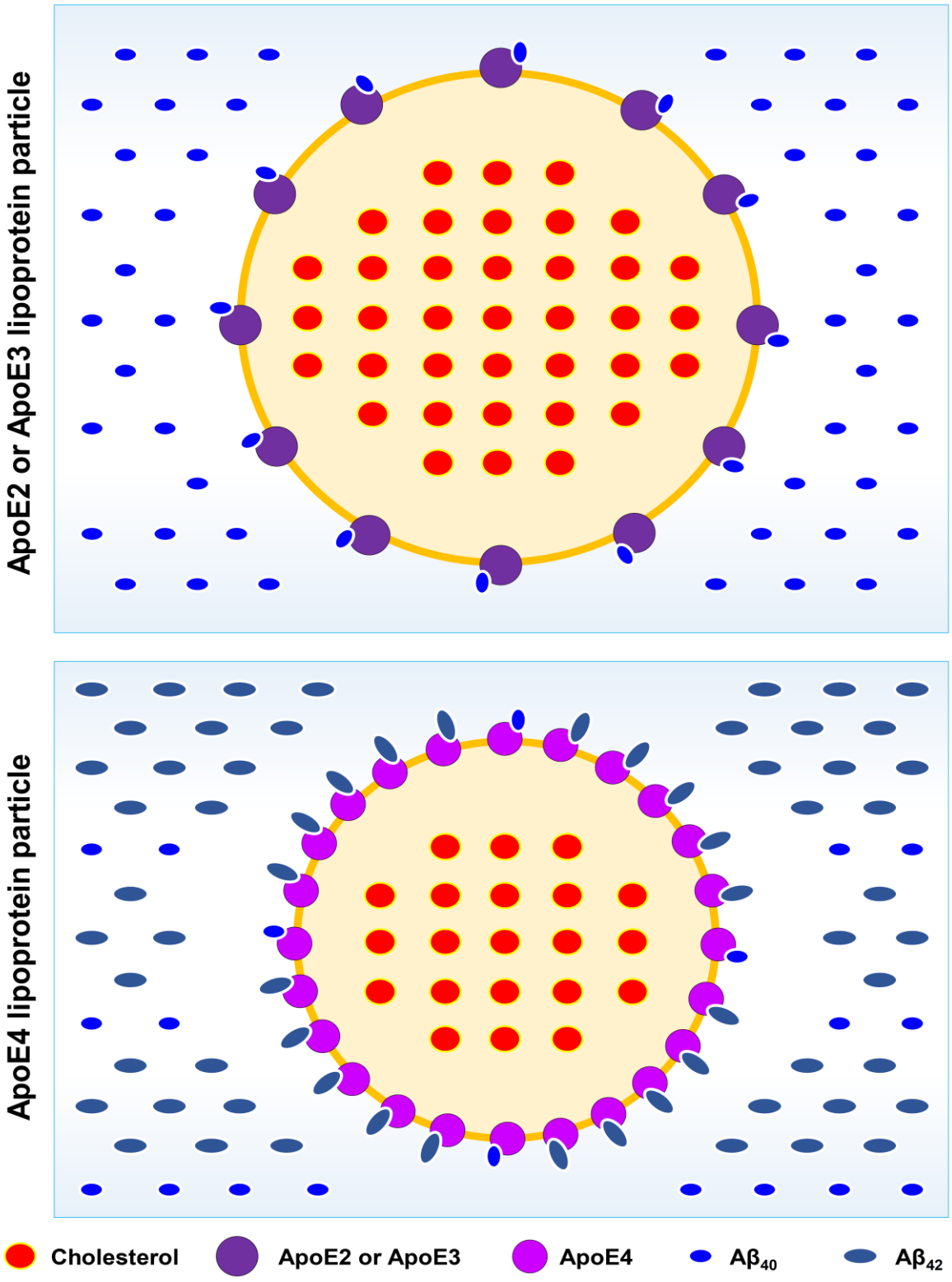

Figure 4. The relative size and density of ApoE2, ApoE3, and ApoE4 molecules in their respective lipoprotein particles. It is known that while the ApoE4 lipoprotein particles have smaller average size than the ApoE2- or ApoE3-containing particles, the ApoE4 particles contain approximately twice the amount of the ApoE4 molecules per particle compared to the E2- or E3 particles. Additionally, ApoE2 and ApoE3 have high binding affinity for

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Aβ40 but very low binding affinity for Aβ42 (**A**). In an opposite fashion, ApoE4 has very low affinity for Aβ40 binding but high affinity for Aβ42 binding (**B**). Because of these unique properties, each ApoE4 lipoprotein particle will bind more Aβ42 fragments. It is hypothesized that the higher density of Aβ42 bound to each ApoE4 lipoprotein particle will accelerate the deposition of the Aβ42-bound ApoE4 particles in the brain (discussed in *section 5*), thereby resulting lower ApoE4 levels in the CSF.

4.2. Pathogenic Role of ApoE4 in LOAD

4.2.1. Observations from Human Studies

ApoE was first identified in 1993 as a genetic risk factor for sporadic LOAD [214,215]. Extensive epidemiological, clinical and pathological studies have since then established the *APOE* gene as perhaps the most important genetic risk factor for sporadic LOAD [216–219]. While ApoE3 is the most common isoform in the general population, ApoE4 occurs in 40–80% percent of all sporadic LOAD patients who possess at least one copy of the *APOE-ε4* allele [16]. People who inherit one copy of the *APOE-ε4* allele is about three times more likely to develop AD, and people who have two copies of the *APOE-ε4* allele (one from the mother and one from the father) are at least eight times more likely to develop AD than those who have two copies of the *APOE-ε3* allele [9]. Conversely, the ApoE2 variant appears to be protective in LOAD. People with one copy each of the *APOE-ε2* and *APOE-ε3* allele have only one-fourth the risk of developing AD as people with two copies of the *APOE-ε3* allele [220–222].

Human studies have shown that the *APOE4* genotype is associated with an earlier onset of LOAD [217]. The PET scan [223] and post-mortem analysis [3,224–226] have shown that AD patients with the *APOE4* genotype have an earlier appearance and more amyloid deposits in their brains.

Functional studies have shown that ApoE4 human individuals are unable to efficiently regulate cerebral glucose metabolism and oxygen utilization compared to ApoE4-negative individuals [227]. Similarly, the glucose uptake (based on FDG-PET scan) was lower in ApoE4 individuals in certain regions of the brain, such as the posterior cingulate, parietal, temporal and prefrontal cortex [228]. Post-mortem analysis of brains from young individuals showed that the *APOE4* genotype is associated with reduced levels of brain glucose and lactate transporters and mitochondrial electron transport proteins [229], clearly suggesting a reduced mitochondrial metabolic activity. Behavioral analysis also showed that the *APOE4* genotype is associated with reduced verbal memory [230] in asymptomatic carriers and reduced visual recall and memory retention even in children [231].

4.2.2. Observations from Animal Studies

Some of the more convincing evidence on the pathogenic role of ApoE4 came from detailed analyses of the transgenic AD mice. These mice selectively expressed the human *APOE* genes from their endogenous *APOE* promoter [232], with the expected glial expression of human ApoE isoforms [233]. Compared to *APOE3* mice, *APOE4* mice were impaired in spatial learning [193,234–239]. They were also impaired in other memory-related functions [193,236,237,240]. More pronounced deficits in behavior and brain functions were observed in older *APOE4* mice [237,241], which are consistent with clinical observations.

The *APOE4* mice also had altered neuronal activity. The electrophysiology of amygdala neurons showed reduced excitatory transmission in the *APOE4* mouse brains [242]. Evoked release of acetylcholine from hippocampal neurons was reduced in older *APOE4* mice [243]. The hippocampal neurotransmission also had fewer shortwave ripples and reduced slow gamma wave activity in aged *APOE4* mice [244].

Structural analysis showed that the brains of *APOE4* mice have simpler structures compared to *APOE*3 mice [242,245–247], including less branching or reduced spine densities. Decreased complexity of neurons in *APOE4* brains was seen in the entorhinal cortex [248,249], consistent with the altered

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functions of that brain region often seen in human LOAD [250]. In older mice, the *APOE* brains appeared to have a lower vascular density, along with white matter damage [251] and smaller hippocampal regions [235].

Here, it is of note that the *APOE*-associated differences in behavior and brain functions in transgenic animals often occurred in the absence of overt amyloid pathology, suggesting that alterations in ApoE-mediated neuronal cholesterol efflux affects cognitive function more predominantly over amyloid pathology.

4.2.3. Mechanistic Explanation

Most previous studies have focused on the role of ApoE in delivering astrocytic cholesterol to neurons. For instance, it was speculated earlier that ApoE isoform-specific effects on learning and memory are partly due to the reduced ability of ApoE4 than ApoE3 in supplying astrocytic cholesterol to neurons and thus a decreased capacity in supporting synaptic functions [252,253]. Recently, an ApoE cascade hypothesis in AD pathogenesis was proposed [254]. Here, a different explanation is entertained. It is hypothesized that the differential ability of the ApoE isoforms in effluxing cholesterol out of neurons plays a more critical role in determining the pathogenesis of sporadic LOAD because a hampered neuronal cholesterol efflux will lead to elevated cellular cholesterol levels, which will disrupt mitochondrial function and result in reduced formation of neuronal ATP and neuroactive metabolic intermediates, along with increased Aβ formation, tauopathy and acetylcholine deficiency. Provided below is a discussion of the available experimental and clinical observations that support the notion that the *APOE4* genotype is a key risk factor for LOAD on the basis of its decreased ability to efflux excess neuronal cholesterol.

i. It is known that ApoE4 has a reduced ability to efflux cholesterol out of neurons in the CNS compared to ApoE3 and ApoE4 [202,255,256]. For instance, ApoE3 has 2.5- to 3.9-fold higher ability to efflux cholesterol than ApoE4. It is known that the dimeric ApoE can induce higher lipid efflux than its monomeric form [202]. As ApoE3 (and also ApoE2) can readily form dimeric forms, ApoE4 cannot form dimers. The inability of ApoE4 to form dimer is a major cause for its reduced ability to remove excess cholesterol from neurons.

Consistent with the reduced ability of ApoE4 to efflux cholesterol, individuals with the *APOE4* genotype also have smaller ApoE-containing lipid particles plus more lipid-depleted particles in the CSF, whereas those with the *APOE3* genotype have larger lipidated ApoE particles [208,257,258]. Similar observations were also made in the transgenic *APOE* mice [176,210,259]. While the *APOE4* mice were associated with smaller ApoE4 lipoprotein particles plus more lipid-depleted particles in their brains [209,236,260,261], the *APOE2* or *APOE3* mice were associated with larger lipidated ApoE particles [209].

ii. Interestingly, studies have shown that humans with the *APOE4* genotype have lower levels of ApoE in their CSF than those with the *APOE3* genotype [208,257,258]. Similarly, the ApoE4 mice also have the lowest levels of ApoE in extracts from relevant brain regions, such as the frontal cortex and hippocampus, whereas the ApoE2 mice have the highest ApoE levels in these brain regions [210].

To explain why people with the *APOE4* allele have low levels of ApoE4 protein in their CSF, earlier studies suggested that ApoE4 proteins can undergo more rapid degradation than ApoE2 and ApoE3 proteins in the brain [210,262]. The potential reasons for the rapid degradation of the ApoE4 particles may include the following: First, despite the smaller size, each ApoE4 particle is known to contain approximately twice the amount of the ApoE4 molecules compared to the ApoE3-containing particles [263,264] (depicted in Figure 4). As such, the relative density of ApoE4 molecules per lipoprotein particle is actually much higher. In addition, it is known that ApoE3 (and also ApoE2) has very high affinity for Aβ_{40} binding but low affinity for Aβ_{42} binding; in contrast, ApoE4 has very low affinity for Aβ_{40} binding but high affinity for Aβ_{42} binding [214,258,265]. As a result, while most of the ApoE3 or ApoE2 particles are bound with Aβ_{40} instead of Aβ_{42}, the majority of the ApoE4 particles are

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bound with Aβ_{42} instead of Aβ_{40} (compare Figure 4A with Figure 4B). Due to the higher ApoE4 density per particle, each ApoE4-containing lipoprotein particle will be bound with a lot more Aβ_{42} peptides. It is speculated that the higher density of Aβ_{42} bound to each ApoE4 lipoprotein particle will accelerate the deposition of the cytotoxic Aβ_{42} peptides in the brain (discussed later in *section 5*). In the meanwhile, these Aβ_{42}-bound ApoE4 lipoprotein particles will stimulate their removal and disposition by different types of cells in the brain, and may even stimulate the cross-BBB transport of these particles, as part of the mechanism that would help remove excess Aβ_{42} peptides (discussed later in *section 8.1*). As a result of these accelerated removal processes, people with the *APOE4* genotype actually will end up with lower levels of ApoE4 in their CSF than those with the *APOE3* (or *APOE2*) genotype.

iii. It has been reported that in general, lower levels of ApoE in the brain are often correlated with an elevated risk of LOAD [266]. Based on the discussion provided above, this phenomenon might be quite readily understood. First, in the case of individuals with the *APOE4* genotype, it is already explained above that these individuals will be associated with lower levels of ApoE4 proteins in their CSF, and they are also associated with an increased risk for LOAD. Second, if two individuals with the same *APOE3* (or *APOE2*) genotype are compared, it is reasonable to suggest that the one with a lower brain ApoE protein level will have a higher risk for LOAD as this individual will have a reduced capacity of ApoE-mediated cholesterol efflux, which will result in elevated neuronal cholesterol (assuming that everything else is the same in these two individuals). Offering partial support for this explanation, an earlier study has shown that administration of bexarotene (an agonist of the RXR nuclear receptor) which can robustly increase ApoE content (likely along with other components of the cholesterol efflux machinery plus cholesterol-metabolizing enzymes) in the hippocampus and cortex of a mouse AD model was associated with improved memory and cognition and reduced Aβ plaques in these animals [267]. These beneficial effects were not observed in ApoE-deficient mice [267].

Therefore, based on the mechanistic explanations provided above, it is understood that individuals with the *APOE4* genotype will have overall clinical manifestations highly similar to those with abnormally-elevated neuronal cholesterol. With this understanding in mind, a brief discussion of a few other risk-modifying factors in LOAD, including ApoE2, ApoJ, cholesterol-efflux transporters (ABCA1, ABCG1, ABCG4 and ABCA7) and the ApoE receptors is provided below. The role of TREM2 in LOAD is discussed separately in *section 8.1.1*.

4.3. Protective Effect of ApoE2 in LOAD

4.3.1. Observations from Human and Animal Studies

The earlier clinical observations that the *APOE-ε2* allele was under-represented in LOAD patients [268,269] had led to the suggestion that the *APOE2* genotype likely is protective in LOAD patients. Compared to *APOE-ε3/ε3* homozygotes, the risk of LOAD in *APOE-ε2* carriers is approximately 50% less [221,270]. Moreover, LOAD patients who are *APOE-ε2* carriers exhibit slower cognitive decline compared with non-carriers [271]. In Down's syndrome patients who have triplicate *APP* gene, *APOE-ε2* protects against the development of AD [272], and also delays the age of AD onset [273–275].

Postmortem LOAD brains from *APOE-ε2* carriers have lower densities of amyloid plaques than those from *APOE-ε3/ε3* individuals [276–278]. PET imaging also showed that the amyloid load in non-demented brains accumulates at a slower pace during aging and has a later onset of amyloid positivity in *APOE-ε2* carriers than in *APOE-ε3/ε3* homozygotes [223]. *APOE-ε2* affects not only the global amyloid load but also the region-specific Aβ deposition [279]. In general, the protective effect of *APOE-ε2* is more pronounced in pathologically-confirmed LOAD than in clinically-diagnosed cases [280].

4.3.2. Mechanistic Explanation

A number of mechanisms have been proposed in the past to explain the protective effect of the

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APOE2 genotype. For instance, it was suggested that hyperlipidation of ApoE2 is a central mechanism for the protective effect of ApoE2, and the main reason for this suggestion is that ApoE2 is capable of transporting more astrocytic cholesterol to neurons. In addition, the lipidation state of ApoE2 affects its binding by Aβ peptides [281–283] and thus may affect Aβ catabolism [284]. Additionally, it has been suggested previously that the protective effect of ApoE2 may be achieved through ApoE2-activated neuroprotective signaling pathways [285–287].

In this article, a new mechanistic explanation is tendered. It is hypothesized that individuals with the *APOE2* genotype will have a reduced level of neuronal cholesterol, which will be protective against the development of LOAD. This mechanistic explanation is rather straightforward, and is supported by a number of experimental and clinical observations, as briefly elaborated below.

First, people with the *APOE2* genotype have a higher overall ability to efflux neuronal cholesterol in an ABC transporter-dependent manner, and this ability is jointly determined by the following two factors: ***i.*** While ApoE4 cannot form dimers, ApoE2 (like ApoE3) can form dimers [205,207], which is associated with a higher ability to carry out neuronal cholesterol efflux, in the rank order of ApoE2 > ApoE3 >> ApoE4 [181,202,288]. In addition, studies have shown that the *APOE2* and *APOE3* mice are associated with larger lipoprotein particles, with ApoE2 particles largest and *APOE4* smallest [289–291]. ***ii.*** Studies have shown that humans with the *APOE2* genotype have higher levels of ApoE in their CSF than those with the *APOE4* genotype [257,258]. Similar observations were also made in the transgenic mice expressing different human ApoE proteins [210].

Second, in addition to the higher ability of ApoE2 to efflux neuronal cholesterol, ApoE2 appears to have a lower ability to deliver cholesterol to neurons compared to ApoE3 and ApoE4, for the following reasons: ***i.*** ApoE2 has far lower binding affinity (approximately 1%) for LDLR (the LDL receptor) than ApoE3 and ApoE4 [264,292,293]. Accordingly, it is speculated that the lipidated ApoE2 particles will have a markedly reduced ability than ApoE3 and ApoE4 to deliver lipids (including cholesterol and CEs) to neurons via LDLR-mediated endocytosis. ***ii.*** LRP1 is another ApoE receptor that mediates the endocytosis of lipidated ApoE particles, and ApoE2 also has a lower binding affinity for LRP1 than ApoE3 and ApoE4 [294]. In fact, the lipidated ApoE4 can bind to LRP1 with a high affinity [295], which further supports the speculation that ApoE4 is highly capable of delivering astrocytic cholesterol to neurons (likely more capable than ApoE3 and ApoE2). ***iii.*** Aβ_{40} has a much higher binding affinity for ApoE2 than for ApoE3 and ApoE4; in comparison, ApoE4 has much higher binding affinity for Aβ_{42} than for Aβ_{40} (depicted in Figure 4) [214,258,265,296]. As a result, ApoE2 lipoprotein particles will be mostly bound by Aβ_{40}, whereas ApoE4 will be mostly bound by Aβ_{42} (Figure 4). As explained in detail later (*section 5.3*), when the APP-binding site on ApoE2 is bound by Aβ_{40}, ApoE2 is effectively blocked by Aβ_{40} and thus no longer be able to perform its cholesterol-delivering function through endocytosis, but the Aβ_{40}-bound ApoE2 lipoprotein can still perform its cholesterol efflux function. As a result, a larger fraction of the ApoE2 particles will be in the cholesterol efflux mode, rather than in the cholesterol-delivery model (i.e., endocytosis mode). This property of ApoE2 also contributes to the reduced ability of the lipidated ApoE2 lipoprotein particles to undergo endocytosis to deliver cholesterol to neurons.

Because of these reasons, it is clear that ApoE2 not only has a higher ability to efflux cholesterol out of neurons, but it may also have a reduced ability to supply astrocytic cholesterol to neurons. A combination of these two properties will beneficially result in reduced brain neuronal cholesterol in individuals with the *APOE2* genotype compared with those with the *APOE3* and *APOE4* genotypes. In agreement with this speculation, it was reported earlier that aged *APOE2* mice had lower cortical cholesterol levels than aged *APOE3* or *APOE4* mice [297].

According to the explanation that ApoE2's neuroprotection results from reduced neuronal cholesterol levels, it is predicted that ApoE2 will be associated with improved mitochondrial metabolic activity (i.e., higher ATP level) and higher levels of neuroactive metabolic intermediates in brain neurons. In addition, the reduced neuronal cholesterol level will also favorably reduce the

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formation of $A\beta_{42}$ (discussed in *section 5*); and the improved neuronal ATP level will help reduce tauopathy (discussed in *section 6*) and improve the formation of cholinergic vesicles (discussed in *section 7*). Additionally, reduced neuronal cholesterol level will also have a reduced macrophage response in the brain [298,299], which may partly explain why the *APOE2* genotype is associated with reduced neuroinflammation — another contributing factor in ApoE2's protective function in LOAD.

Lastly, it should be noted that ApoE2 not only has a protective effect against LOAD [297–301], but it is also associated with longevity [302–306]. It is hypothesized that the improved mitochondrial function and cellular ATP level in neurons (as well as in other somatic cells) resulting from reduced cellular cholesterol levels are important underlying factors contributing to longevity. This notion is certainly in line with the long-held view that healthy mitochondrial function is crucial for general health and longevity [307,308].

4.4. Role of APOJ Genotype in LOAD

Besides *APOE*, genome-wide association studies [309,310] have found a statistically-significant association between a SNP of the *APOJ* gene and the risk of AD [311]. *APOJ* is not as strong a genetic risk factor as *APOE*; the polymorphic site in *APOJ* has an odds ratio of approximately 0.9 for the *APOJ* allele, compared with an odds ratio of approximately 5 for the *APOE-ε4* allele [312].

ApoJ is a component of the CNS lipoproteins [313]. It is functionally similar to ApoE, and involved in neuronal cholesterol efflux [206,314] in an ABCA1-dependent manner [315]. It has been suggested that ApoE and ApoJ can interact with lipid debris in the brain, and are involved in removing degenerating membranes after neuronal injury [316]. It is hypothesized that the polymorphic ApoJ has a reduced ability to efflux neuronal cholesterol and thus increases the risk for LOAD. Given that ApoJ likely plays a lesser role in CNS cholesterol efflux than ApoE, this may determine why the *APOJ* genotype is not as strong a genetic risk factor as the *APOE4* genotype.

Studies have shown that people with LOAD often have more ApoJ in their blood and its levels affect the regional distribution of $A\beta$ [317]. However, ApoJ levels alone cannot be used to predict the onset of LOAD [318]. These observations likely suggest that ApoJ may play a complementary role with ApoE in removing excess neuronal cholesterol. In people with AD conditions, which usually is associated with abnormally-elevated neuronal cholesterol (often associated with the *APOE4* genotype which has a reduced overall ability to efflux cholesterol), it is understood that the body may try to increase ApoJ levels as a functional compensation. Understandably, for a LOAD patient with a faster cognitive decline, it likely means that this patient has much higher neuronal cholesterol levels than usual, and as a result, the body may try to express a lot more ApoJ proteins (along with other brain apolipoprotein isoforms) as a means to help to efflux neuronal cholesterol.

Similarly, it was reported that the brain ApoJ level is elevated in proportion to the *APOE-ε4* allele dose level. The explanation for this phenomenon is related to the fact that the higher *APOE-ε4* allele dose level is associated with lower CSF ApoE4 protein level, and that ApoE4 has a very low ability to efflux neuronal cholesterol, and as a result, a stronger induction of ApoJ in the *APOE-ε4* individuals is required to provide a bigger compensation to efflux neuronal cholesterol.

As both ApoE and ApoJ are involved in neuronal cholesterol efflux, they certainly will also jointly contribute to reducing $A\beta$ deposition in the brain. If an individual with the *APOE4* genotype plus a mutant ApoJ, this person is expected to have a higher chance to have more severe neuronal accumulation of cholesterol, which will be associated with more severe $A\beta$ buildup. The above speculation is supported by earlier studies [319,320]. In addition, earlier studies have suggested that ApoE and ApoJ may also modify $A\beta$ clearance across the BBB (more discussion on this subject is provided in *section 8.1.1*).

4.5. Role of Brain Cholesterol Efflux Transporters in LOAD

The ATP-binding cassette transporters A1 and G1 (i.e., ABCA1 and ABCG1, respectively) are important ApoE-lipidating proteins in the CNS [321,322]. Other members in this transporter family

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(such as ABCG4 and ABCA7) may also be involved in this process. Earlier studies have shown that while ABCA1 preferentially activates the efflux of cholesterol and its binding to unlipidated ApoE, ABCG1 is more selective for partially-lipidated lipoprotein complexes [323,324]. Studies have suggested that ABCA1 and ABCG1 may work in concert to control the efflux of cholesterol and its oxysterol metabolites such as 24*S*-OHC [256,325].

Since these transporters are all involved in neuronal cholesterol efflux, it is understood that a reduced function of any of these transporters likely will affect neuronal cholesterol level, to varying degrees. As ABCA1 is an important and well-studied member of this class, a more detailed discussion of its role in LOAD will be given here as an example. Alterations in the expression of ABCA1 were found to be linked to LOAD (reviewed by [182,326]). Studies of mice with brain-specific deficiency of *ABCA1* manifested mild impairments in neurite morphology, synaptic structures, motor activity and memory formation [327–329]. As ABCA1 is required for the normal acquisition of cholesterol by ApoE, it is expected that the lipidation state of the ApoE particles will be reduced in *ABCA1*-knockout mice [321,322].

When *ABCA1* knockout mice were crossed with the mouse model of AD, it is expected that the AD conditions, including amyloid load and the learning and memory deficits, will be more severe as neuronal cholesterol efflux is more severely hampered [330]. On the other hand, overexpression of ABCA1 in these AD mice is expected to help alleviate amyloid deposition and improve brain function, as observed in an earlier study [330].

It is of note that Tangier disease is caused by mutations in the *ABCA1* gene [331]. In the periphery, these mutations prevent ABCA1 from effectively transporting cholesterol and phospholipids out of cells for pickup by ApoA-I in the bloodstream. Tangier disease is characterized by severe plasma deficiency or absence of HDL, apolipoprotein A-I (ApoA-I, the major HDL apolipoprotein) and accumulation of CEs in many tissues throughout the body. Additionally, the buildup of cholesterol in cells can be toxic, causing cell death or impaired functions. These combined factors lead to the peripheral signs and symptoms of Tangier disease. In the CNS, the absence of ABCA1 is associated with poorly-lipidated ApoE particles, along with elevated neuronal cholesterol, which impairs neuronal functions.

While it has been suggested that 24*S*-OHC may leave neurons and eventually reach peripheral circulation through simple diffusion (due to its slightly higher water solubility than cholesterol), it was also reported that 24*S*-OHC can be secreted by neurons in an ApoE- and ABCA1-dependent manner [256,325]. As the lipidated ApoE particles can be taken up by astrocytes for metabolic disposition, this process may help deliver 24*S*-OHC to astrocytes where it can exert its important regulatory functions. It is known that in astrocytes, 24*S*-OHC can down-regulate the expression of cholesterol synthesis genes while activate the expression of *APOE*, *ABCA1* and *TREM2* [311]. Mechanistically, the nuclear receptor LXR mediates the action of 24*S*-OHC to regulate the expression of these genes [148,332,333]. An earlier study has reported that treatment of APP/PS1-transgenic mice with bexarotene (an agonist of RXR) can stimulate the synthesis of ABCA1, ABCG1 and ApoE, which is associated with enhanced clearance of the brain Aβ and reversal of cognitive deficits [267].

As ABCA1 is involved in the efflux of 24*S*-OHC [256,325], it is expected that neuronal secretion of 24*S*-OHC will be reduced when ABCA1 is deficient, and as a result, astrocytic expression of ApoE (a process regulated by 24*S*-OHC-activated LXR) will be drastically reduced. This may explain why ApoE levels in the brain of ABCA1-knockout mice are 80% lower than in wild-type control mice [321,334].

Deletion of the *ABCA1* gene not only decreases the ApoE level [321], but also increases Aβ deposition [335] in the brain; in comparison, *ABCA1* overexpression in the AD mouse model decreases Aβ deposition [330]. These changes are readily understood on the basis of the expected changes in neuronal cholesterol level under these experimental conditions. Deletion of the *ABCA1* gene will be associated with increased neuronal cholesterol level, whereas *ABCA1* overexpression will reduce

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neuronal cholesterol level. The change in neuronal cholesterol level will then alter the content of CEs in the lipid rafts, which then alters the catalytic activity of β-/γ-secreatases as well as the formation of Aβ_{42} and Aβ_{40} (detailed explanation is provided in *section 5*).

In addition to ABCA1, other ABC transporters (ABCG1, ABCG4 and ABCA7) may also be similarly involved in neuronal cholesterol efflux in the CNS [336,337]. In the light of the functional role of the ABCA1 in neuronal cholesterol efflux and LOAD as discussed above, it is expected that abnormalities in the functions of other CNS-resident lipid transporters may also similarly lead to increased neuronal cholesterol levels, along with other accompanying effects. For instance, studies have shown that mice deficient in ABCG1 exhibit increased neuronal cholesterol level and memory deficits, similar to ABCA1-deficient mice [338,339]. In addition, ABCA7 has also been identified as one of the LOAD susceptibility genes [340], and as expected, studies have shown that deletion of *ABCA7* increases Aβ formation and accumulation in an AD mouse model [341,342].

4.6. Role of ApoE Receptors in LOAD

When ApoE performs its cholesterol delivery functions in the CNS, it needs to bind to its cell surface receptors, such as LDLR [343], VLDL receptor, LRP1, ApoER2 (also known as LRP8), or heparan sulfate proteoglycans (HSPGs) [53,344,345]. The interactions between ApoE and its receptors have clear isoform preference and are affected by the ApoE lipidation status [283,346,347]. For instance, it is known that LDLR is only recognized by lipidated ApoE [283,346,347], indicating that LDLR is chiefly involved in the supply of lipids to brain neurons through endocytosis. This suggestion is consistent with the known functions of LDLR in the periphery, i.e., to deliver lipids to recipient cells. As mentioned in *section 4.3.2.*, earlier studies have shown that ApoE2 has a markedly weaker binding affinity for LDLR compared to ApoE3 and ApoE4 [292,293], indicating that lipidated ApoE2 lipoprotein particles have a lower ability to deliver lipids to neurons than ApoE3 and ApoE4.

In addition to LDLR, LRP1 is also known to mediate neuronal endocytosis of lipidated ApoE particles [348,349]. It is speculated that lipidated ApoE2 particles have a reduced ability to deliver cholesterol to neurons via LRP1-mediated endocytosis. There is some experimental evidence for this suggestion: First, ApoE2 has a lower binding affinity for LRP1 compared to ApoE3 and ApoE4 [294]. Second, Aβ_{40} is known to have a much higher binding affinity (approximately 20-fold higher affinity) for ApoE2 than for ApoE3 and ApoE4 [214,258,265,296], and as such, the APP-binding site on ApoE2 (which is required for endocytosis; discussed later in *section 5*) likely will be effectively blocked by Aβ_{40} and thus will reduce the chances for lipidated ApoE2 particles to undergo endocytosis. This explanation is somewhat in line with the known functional profiles of LPR1 in mediating the endocytosis of various ApoE-containing chylomicron remnants in the periphery (reviewed in [187,350].

At present, much less is known about the receptors involved in neuronal cholesterol efflux. It is speculated that LRP1 may be involved in the efflux of cholesterol, in addition to its ability to mediate endocytosis of ApoE-rich lipoproteins. Similarly, ApoER2 may also be involved in neuronal cholesterol efflux. These are purely speculations, and the experimental evidence for these speculations is mostly lacking at present, except the observations that polymorphisms in LRP1 [288,351,352] and ApoER2 [348] were associated with increased incidence of LOAD.

4.7. Section Summary

ApoE lipoproteins in the brain are responsible for carrying astrocyte-derived cholesterol (and other lipids) to neurons, and they are also responsible for effluxing excess neuronal cholesterol. Past studies have mostly focused on the function of ApoE in delivering astrocytic cholesterol to neurons. Here, a new hypothesis is proposed, which suggests that the differential ability of the ApoE isoforms in effluxing neuronal cholesterol plays a key role in the pathogenesis of sporadic LOAD, as a retarded cholesterol efflux will lead to cellular and mitochondrial cholesterol elevation, which will then disrupt mitochondrial structure and function, and reduce the synthesis of ATP and neuroactive metabolic

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intermediates, along with increased Aβ formation, tauopathy, and cholinergic deficiency.

ApoE4 is known to have a markedly lower ability than ApoE2 and ApoE3 to efflux cholesterol out of neurons, partly due to its inability to form dimers. The reduced ability of ApoE4 to efflux excess neuronal cholesterol will result in markedly elevated cholesterol level in neurons and thus elevated LOAD risk. Based on this mechanistic explanation, it is also understood that individuals with the *APOE4* genotype will have overall clinical manifestations highly similar to those with abnormally-elevated neuronal cholesterol levels.

As ApoE4 has very low affinity for binding Aβ_{40} but higher affinity for binding Aβ_{42}, the ApoE4 particles will be bound with Aβ_{42} instead of Aβ_{40}. Due to the higher density of the ApoE4 molecules per particle, each ApoE4-containing lipoprotein particle will bind with more Aβ_{42} fragments. The higher density of Aβ_{42} bound to each ApoE4 particle will accelerate the deposition of Aβ_{42} peptides in the brain (discussed later in *section 5.3*). By contrast, ApoE2 has a far better ability to promote cholesterol efflux from both astrocytes and neurons than ApoE3 and ApoE4. Besides, ApoE2 has a lower ability to deliver astrocyte-derived cholesterol to neurons than ApoE3 and ApoE4. As a result, it is expected that the cholesterol levels in neurons will be lowest with the *APOE2* genotype, median with the *APOE3* genotype, and highest with the *APOE4* genotype. The low neuronal cholesterol level associated with the *APOE2* genotype is an important mechanistic basis for its protective effect against the development of LOAD.

As the *APOE2* genotype is associated with lower neuronal cholesterol level, it is readily understood that ApoE2 will be associated with improved mitochondrial metabolic activity (i.e., higher ATP levels) and higher levels of neuroactive metabolic intermediates in brain neurons. Additionally, lower neuronal cholesterol content will favorably decrease the formation of Aβ_{42} (discussed in *section 5*); and the improved neuronal ATP levels will help reduce tauopathy (discussed in *section 6*) and improve the formation of cholinergic vesicles (discussed in *section 7*). Lastly, the new mechanism proposed here also offers a good explanation for the observation that ApoE2 not only reduces the risk of LOAD, but it will also promote longevity.

ApoJ is another member of the CNS lipoproteins, and is functionally similar to ApoE in neuronal cholesterol efflux. It is understood that the polymorphic ApoJ with a reduced ability to efflux neuronal cholesterol will be associated with an increased risk for LOAD. Similarly, as the ATP-binding cassette transporters (such as ABCA1 and ABCG1) are involved in neuronal cholesterol efflux, reduced functions of any of these transporters will also affect neuronal cholesterol levels to varying degrees, thus affecting the risk of LOAD.

5. How Do Neuronal Cholesterol and ApoE4 Accelerate Amyloid Plaque Formation?

5.1. Structure and Function of APP

In 1987, APP was cloned and found to be linked to the pathogenesis of familial early-onset AD [353]. APP is a type I transmembrane protein with a large extracellular domain and a small cytoplasmic domain, resembling a transmembrane receptor for an unidentified ligand. The cytoplasmic domain contains an NPXY sequence, which is a consensus binding sequence for a number of adaptor proteins [354]. There are two proteins highly homologous to APP: the amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2) [355].

Presently, the function of APP is still not fully clear. Studies have shown that *Drosophila* lacking their single APP-like protein display impaired synapse formation in neuromuscular junctions [356]. APP knockout mice are viable, but show reduced long-term potentiation in the brain [357], consistent with learning and memory deficits and loss of hippocampal synapses [172,358,359]. APP/APLP2 double knockout mice have impaired neuromuscular junctions [360].

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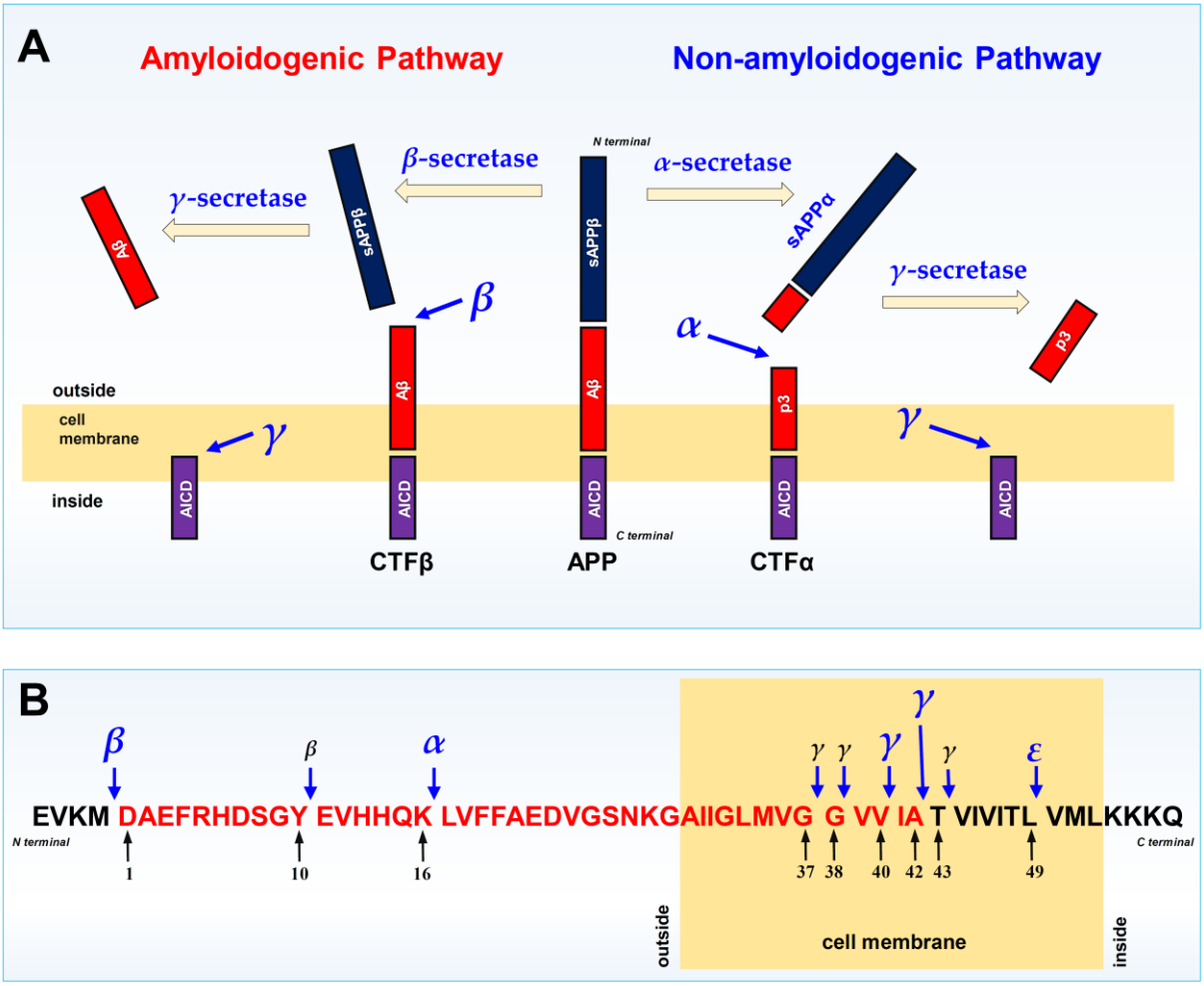

Figure 5. The proteolytic processing of APP. (**A**). The traditional model of APP proteolysis involves APP processing either in the non-amyloidogenic pathway, where sequential cleavage by α-secretase and the γ-secretase liberates sAPPα and p3, or in the amyloidogenic pathway, where sequential cleavage by β-secretase (BACE1) and γ-secretase liberates sAPPβ and Aβ. Both pathways produce AICD, which can be proteolytically degraded or translocated to the nucleus, where it has roles in transcriptional regulation. (**B**). The exact sites where α-, β- and γ-secretases make the cuts. The major sites for cleavage by α-, β- and γ-secretases are labeled with a big blue-colored α, β and γ, whereas the minor sites for cleavage are labeled with a small black-colored α, β and γ. The amino acid residues constituting the Aβ fragment are labeled in red.

It has been extensively characterized that APP is cleaved by a number of proteolytic enzymes which affect the release of the Aβ peptides (depicted in Figure 5A). The α-secretases cleave the extracellular domain of APP's twelve amino acids away from the membrane, and is the quantitatively and functionally most important proteolytic cleavage of APP, which releases the extracellular sAPPα domain, along with the membrane-bound *C*-terminal fragment CTFα. The β-secretase was identified as the β-site APP cleaving enzyme (BACE1) [361], which cleaves the extracellular domain of APP farther way from the membrane. While α-secretase activity occurs primarily at or near the cell surface, β-secretase is more favored in endosomes, consistent with the low pH optimum of BACE1 [361]. The β-cleavage of APP results in the release of the soluble ectodomain sAPPβ, along with the membrane-bound *C*-terminal fragment CTFβ. The γ-secretase, which was identified as a complex of proteins containing the presenilins [362], further cleaves CTFα or CTFβ (following cleavage of APP by α- or β-secretase).

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As depicted in Figure 5A, the sequential cleavage of APP by α-secretase followed by γ-secretase will generate three fragments: sAPPα, a small p3 fragment, and the APP intracellular domain (AICD). The α-secretase pathway also operates in many non-neuronal cell types, generating shorter fragments that are thought to be nonamyloidogenic. sAPPα has been suggested to exhibit neuroprotective and synapse-promoting activities [363]. It was shown that mice that only produce sAPPα did not exhibit the various phenotypes caused by a full APP knockout, including disturbed LTP and memory function. This observation suggests that sAPPα may mediate some of the functions of the APP holoprotein [357].

The β- and γ-secretases cleave APP in the so-called amyloidogenic pathway (Figure 5A,B). β-Secretase releases the ectodomain sAPPβ, and the remaining CTFβ is subsequently cleaved by γ-secretase liberating the Aβ peptide(s) and the AICD. *C*-Terminal heterogeneity is generated by the γ-secretase itself. This protease cleaves APP at different positions, generating a variety of peptides, of which Aβ_{43}, Aβ_{42}, Aβ_{40}, Aβ_{38} and Aβ_{37} variants are detected in cell culture and body fluids (Figure 5B). Aβ_{40} is continuously and abundantly produced in both healthy and AD-afflicted brain tissues, whereas other Aβ peptides are produced at lower levels. Aβ_{42} is the most famous and best studied Aβ peptide, and may serve as a core for the formation of amyloid plaques.

The causative mutations in the APP and presenilin genes which alter APP processing were identified many years ago. Moreover, transgenic APP animal models were successfully developed that can produce pathogenic Aβ deposits [364,365].

Interestingly, ApoE receptors also partly share with APP the pattern of proteolysis [366–368]. ApoE receptors undergo surface cleavage to generate soluble (shed) forms of the receptors [369–371]. Several ApoE receptors have been identified as substrates for β-/γ-secretases, including LRP1 [372,373], and apoER2 [374] and VLDLR [375]. Furthermore, some of the intracellular cytoplasmic adaptor proteins that interact with APP also interact with ApoE receptors [367,368].

5.2. Neuronal Cholesterol and Aβ Production

One of the best-known pathological characteristics of AD is the deposition of extracellular Aβ in neuritic plaques [376]. Cholesterol has been shown to play a crucial role in affecting Aβ production and deposition [132,377]. The first evidence for the role of cholesterol in Aβ production in the brain came from a study demonstrating that dietary cholesterol increased Aβ accumulation in rabbits [113]. Although it is difficult to study the phenomenon in humans, limited human results support the observations made in animal models. For instance, in autopsied AD brains, more severe Aβ deposition was found to be associated with higher levels of blood cholesterol measured earlier during life.

Mechanistically, studies have revealed that the cholesterol content in the membrane (particularly in the lipid raft microdomains) is an important factor that regulates the catalytic activity of β- and γ-secretases [37,129–132]. This finding provides a mechanistic basis for the causal relationship between elevated neuronal cholesterol and increased Aβ production [129,131,133]. Provided below is a discussion on how ApoE4 promotes the formation of Aβ and amyloid plaques.

5.3. Mechanistic Explanation

When neuronal cholesterol level is abnormally elevated, which most likely is due to increased supply of astrocytic cholesterol, a number of regulatory mechanisms will be activated to help its removal. First, elevation in neuronal cholesterol will lead to increased metabolic disposition via CYP enzyme-mediated cholesterol oxidation to 24*S*-OHC and other oxysterols, simply due to the availability of more substrate molecules. Since 24*S*-OHC is an activator of LXR in astrocytes, neurons and other brain cells, its increased levels will activate a number of regulatory pathways in different cell types, which will jointly help reduce neuronal cholesterol. For instance, 24*S*-OHC can increase the expression of its own metabolizing enzyme CPY46A1, the lipid carrier protein ApoE, the lipid transporter ABCB1, and others. In addition to 24*S*-OHC, other oxysterols formed in neuronal cells can also serve as LXR activators, and will have similar regulatory functions.

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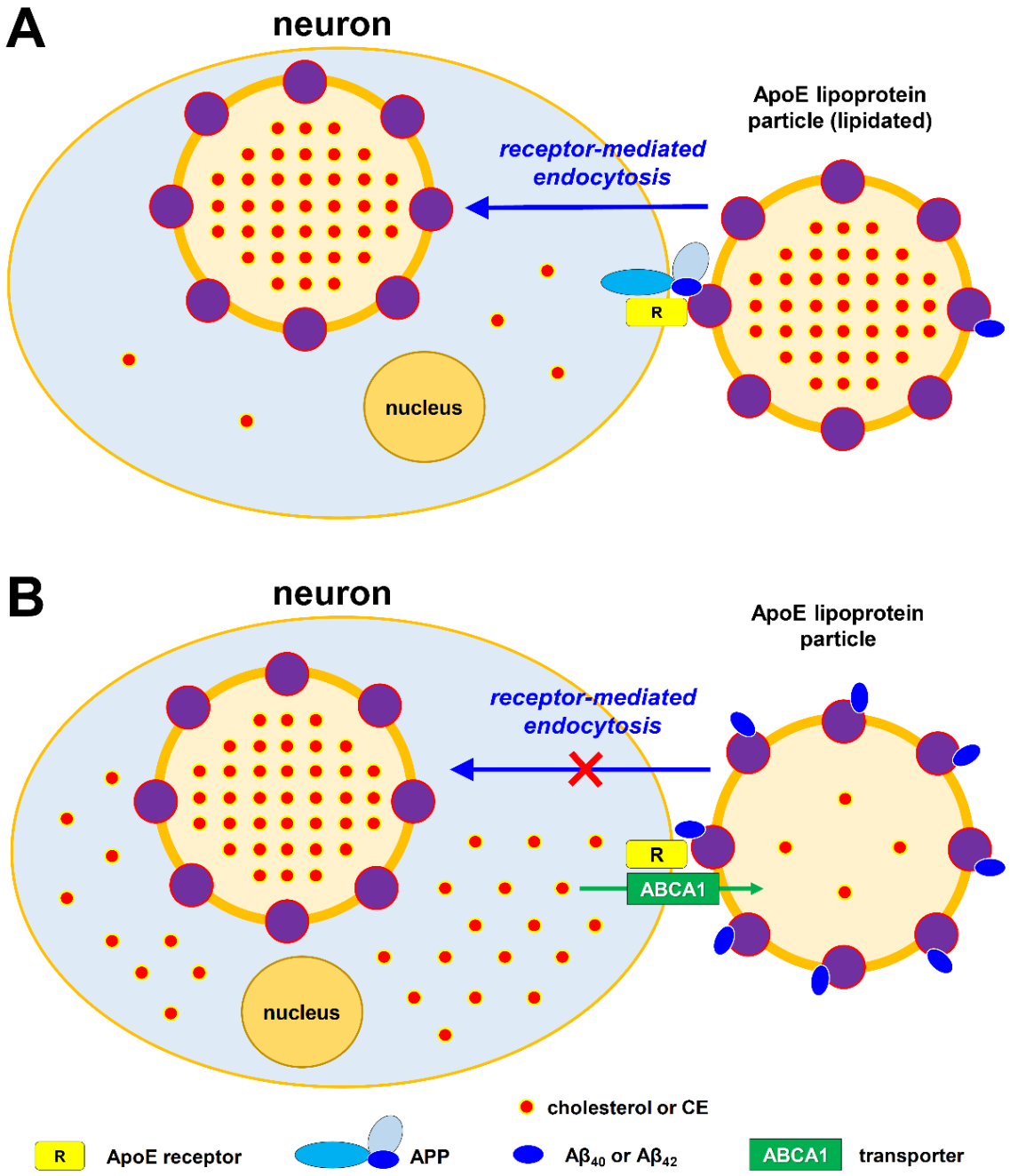

Figure 6. Role of ApoE-containing lipoprotein particles in neuronal transport (i.e., supply and efflux) of cholesterol. As depicted in (**A**), ApoE-mediated delivery of astrocytic cholesterol to neurons is carried out via endocytosis, which involves the binding of lipidated ApoE particles to specific receptors on neuronal surface. It is hypothesized that the intact APP protein is required for the endocytosis of ApoE particles into neurons, and it serves as a "permissive" signal to the neurons. (Here, it is of note that the cleaved CTFα fragment of APP or the APP-related proteins may also share a similar function as the intact APP in carrying out this "permissive" role.) As depicted in (**B**), when the ApoE molecules in the lipoprotein particle are already bound by Aβ fragments (Aβ_{40} or Aβ_{42}), the particle can only bind to its receptor but cannot bind simultaneously to APP (due to competitive blockage). As a result, endocytosis will not be allowed to take place; but under this condition, the ApoE lipoprotein particle can still mediate the efflux of neuronal cholesterol. Therefore, the ApoE particle can be regulated in a precise manner such that it can effectively switch from the function of supplying astrocytic cholesterol to neurons (when neurons are in need of more cholesterol for physiological functions) to the function of removing excess cholesterol from neurons.

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Another important mechanism to effectively reduce neuronal cholesterol is to activate its efflux transport via the ApoE lipoprotein particles in an ABCB1-dependent manner. As discussed in *sections 4.2 & 4.3*, it is known that the unlipidated ApoE2 and ApoE3 particles are highly capable of effluxing cholesterol out of neurons, but ApoE4 is markedly less capable in this respect, which is an important factor determining the pathogenic activity of ApoE4 in LOAD. Here, I will specifically discuss how changes in factors affecting ApoE-mediated neuronal cholesterol efflux contribute to Aβ formation and deposition.

The ApoE-mediated delivery of cholesterol to neurons via endocytosis requires the binding of lipidated ApoE particles to their specific receptors present on neuronal surface (here I will use LRP1 as an example for explanation). ApoE is known to bind to APP [378,379]. It is hypothesized that the intact, uncleaved APP protein is involved in the endocytosis of a lipidated ApoE particle into a neuron by playing a "permissive" role, which means that only when the lipidated ApoE particle simultaneously binds to its specific receptor (LRP1) and APP, then its endocytosis will be permitted to take place. Notably, it is speculated that the cleaved CTFα fragment of APP may also share this permissive role. However, when the lipidated ApoE particle only binds to LRP1 but not simultaneously to APP (or CTFα), then the endocytosis will not be permitted to take place (as depicted in Figure 6A). In so doing, an ApoE particle can be effectively regulated in a precise manner that it can readily switch from its function of supplying cholesterol to a neuron when the neuron is in need of more cholesterol to its function of removing excess cholesterol from a neuron. It is speculated that the APP-related proteins may share a similar function as APP, as the APP knockout mice do not display severe defects unless the APP-related protein APP1 is also concomitantly knocked out.

It is known that increased endocytosis of lipidated ApoE particles by a neuron will result in increased supply of cholesterol, which is usually for the purpose of fulfilling certain normal functional needs of this neuron. When neuronal cholesterol level is relatively low, the APP proteins will be cleaved predominantly by α-secretase (which forms A$\beta_{17\text{-}40}$ fragment following further cleavage by γ-secretase). However, when elevated cholesterol level is present in a neuron, its APP will undergo enzymatic cleavages by β- and γ-secretases, resulting in increased production of Aβ_{40} and Aβ_{42} [37,129]. Aβ_{40} and *A*β_{42} fragments are known to bind to ApoE [214,215,258,265,296] (as depicted in Figure 5). Moreover, Aβ_{40} can bind effectively to both lipidated and non-lipidated ApoE3 or ApoE2 particles. As depicted in Figure 6B, it is speculated that the binding of Aβ_{40} to these ApoE3 and ApoE2 particles will prevent them from further binding to APP or APP-related proteins present on neuronal surface, thereby preventing these particles from undergoing endocytosis and thus preventing the further rise in neuronal cholesterol levels. However, the binding of Aβ_{40} to non-lipidated (or less-lipidated) ApoE2 orApoE3 particles will not affect their ability to carry out the cholesterol efflux function, which can help neurons to remove excess intracellular cholesterol. Cholesterol efflux from a neuron is dependent on the ABC transporter ABCA1, although other transporters (such as ABCD1 and ABCA7) may also help with this process, likely to lesser degrees.

At present, it is not known which specific receptors are involved in mediating neuronal cholesterol efflux by the astrocyte-produced ApoE. It is speculated that LRP1 likely is one of the ApoE receptors involved in cholesterol efflux, in addition to its known function in mediating the endocytosis of ApoE-containing lipoprotein particles. As discussed in *section 4.6*, there is clear evidence that LRP1 can mediate ApoE endocytosis; in comparison, its involvement in cholesterol efflux is far less clear (discussed later in *section 8.1*).

LOAD patients with the ApoE4 genotype are known to be associated with more Aβ plaques compared to ApoE2 and ApoE3 carriers. It was suggested earlier that ApoE is involved in the deposition of Aβ through direct protein–protein interactions, and the lipidated ApoE4 binds preferentially to an intermediate, aggregated form of Aβ with a higher affinity than the lipidated ApoE2 or ApoE3 [282]. Here, a different explanation is provided. It is known that Aβ_{40} has a higher binding affinity for the lipidated ApoE2 or ApoE3 particles than for the lipidated ApoE4 particles

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[214,258,265]. In addition, the ApoE2 dimer can form a complex with Aβ_{40} more efficiently than the ApoE3 dimer [296]. By contrast, ApoE4 binds very poorly to Aβ_{40}, but binds more tightly to Aβ_{42} [214,258,265]. It is known that for each individual ApoE-containing lipoprotein particle, the amount of ApoE4 molecules is about twice the amount of ApoE3 or ApoE2 [263,264]. Therefore, in the case of ApoE4 lipoprotein particles, when the same concentration of Aβ_{40} is present in the SCF, it is expected that there are still a lot of ApoE4 proteins not bound (and thus not blocked) by Aβ_{40}. These unblocked ApoE4 lipoprotein particles can still be endocytosed into neuronal cells, which may cause a further rise in neuronal cholesterol level. As such, the neuronal cholesterol level will be higher with ApoE4 than with ApoE3 or ApoE2 (in the order of ApoE4 > ApoE3 > ApoE2). Elevated neuronal cholesterol is known to increase the expression of APP, which will then lead to increased enzymatic cleavage of APP by β- and γ-secretases to form more Aβ_{40} within the cholesterol/CE-enriched lipid raft microdomains of the plasma membrane [37,129,131]. Increased formation and release of Aβ_{40} may partially compensate for its lower affinity for binding to the ApoE4 particles. Such regulatory mechanisms through increased expression of APP and increased formation of Aβ_{40} may or may not be sufficient to effectively bring down the elevated neuronal cholesterol level to a range that is physiologically healthy to neurons. However, if such regulatory measures are still inadequate to bring down neuronal cholesterol level to a healthy range, then it will result in further rises in neuronal cholesterol content (including in neuronal cell membranes). Elevated cholesterol content in lipid raft microdomains will favor the binding of cholesterol to APP, which then increases the formation of Aβ_{42} instead of the usual Aβ_{40} [37,129,131]. In fact, there were both in vitro and in vivo studies showing that neuronal cholesterol accumulation can induce Aβ_{42} formation and accumulation in the brain [37,129,130]. Since Aβ_{42} has a significantly higher binding affinity than Aβ_{40} for the lipidated ApoE4 particles [214,258,265], the increased production of Aβ_{42} is expected to help more effectively block the binding sites on the ApoE4 particles and thus prevent the lipidated ApoE4 particles from endocytosis into neurons. Therefore, it is understood that when ApoE4 is the apolipoprotein, the brain neurons will be forced to produce a lot more Aβ peptides, including both Aβ_{42} and Aβ_{40} (particularly Aβ_{42}), to help prevent ApoE4 particles from endocytosis (Figures 4 and 6B).

Regarding the mechanism by which higher neuronal cholesterol level facilitates enzymatic production of Aβ_{42}, it is known that the β- and γ-secretases that convert APP to Aβ fragments are predominantly localized to the cholesterol-enriched lipid raft microdomains of the plasma membrane [37,129]. It is speculated that increased intracellular levels of cholesterol (likely in the form of CEs) will increase the formation of lipid rafts in the plasma membrane of neuronal cells, which then favorably leads to the activation of enzymatic cleavage of APP catalyzed by β-/γ-secretases, resulting in increased formation of the Aβ_{42} peptide (in addition to Aβ_{40}). Moreover, decrease in cellular cholesterol levels will increase APP cleavage by α-secretase, thereby decreasing the processing of APP into Aβ_{42} and Aβ_{40} peptides that accumulate in amyloid plaques [37,111,132]. Similarly, studies using both in vitro and in vivo models have shown that the use of statins was strongly associated with reduced levels of Aβ_{42} and Aβ_{40} [380,381]. By reducing cholesterol levels in living hippocampal neurons by 70% with lovastatin and methyl-β-cyclodextrin, the formation of Aβ was almost completely inhibited whereas the usual (normal) catalytic process of forming sAPP is unperturbed [382].

Here, it is of interest to note that when neuronal cholesterol level is low, A$\beta_{17–40}$ is the major Aβ peptide formed and the amount of neuronal cholesterol exported by ApoE decreases. Based on this observation, it is tentatively speculated that A$\beta_{17–40}$ may be able to bind preferentially to unlipidated ApoE, and the A$\beta_{17–40}$-bound unlipidated ApoE may not be favored to bind to the ApoE receptors to perform the cholesterol efflux function. This is purely a speculation, and it will be of interest to experimentally examine this possibility in the future.

In addition to LRP1, other ApoE receptors, such as LDLR and VLDLR, can also mediate ApoE endocytosis. As these two ApoE receptors likely are not involved in ApoE-mediated cholesterol efflux, it is uncertain whether the endocytosis mediated by these receptors also requires the binding of APP

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(or similar proteins) to provide a "permissive signal". If, in case, such a permissive signal is not needed, then it is speculated that these ApoE receptors should have effective mechanism(s) for their inactivation when neuronal cholesterol has reached an adequate level; only in this way the undesired further increase in neuronal cholesterol can be prevented in a timely and effective manner. One of the potential mechanisms may be through the secretase (or other proteinase)-mediated cleavage of these membrane-bound ApoE receptors in a manner that their functions can be very sensitively and effectively terminated when adequate amount of cholesterol is already supplied to neurons. There is some indirect evidence in line with speculation—it was shown earlier that the ApoE receptors can be enzymatically cleaved just like APP by secretases [375].

It is known that ApoE4 is selectively bound with a lot of Aβ_{42} peptides, and the ApoE4-bound Aβ_{42} peptides are insoluble and highly cytotoxic. A number of cell types in the CNS were reported to have the ability to internalize ApoE-bound Aβ, including astrocytes [265], microglia [383–385] and neurons [386], albeit with varying capacities. Immunohistological studies of human AD brains using antibodies against various Aβ epitopes have identified the *N*-terminal-truncated fragments of Aβ_{40} and Aβ_{42} inside the lipofuscin-like granules of astrocytes [387,388]. Similarly, in vitro studies using primary astrocytes in culture showed that these cells can internalize and degrade both soluble [389,390] and insoluble Aβ [391]. Interestingly, the ability of astrocytes to engulf and degrade Aβ is compromised in *APOE* knockout astrocytes, or in wild-type astrocytes upon the addition of an antibody against Aβ or ApoE, or an LDLR family antagonist [265]. These results confirm an essential role of ApoE in a receptor-mediated uptake of Aβ-bound ApoE particles by astrocytes. Primary hippocampal neurons were also reported to internalize Aβ in the presence of ApoE [392], but neurons appear to be less efficient in degrading Aβ, resulting in the formation of high molecular weight Aβ aggregates in their endosomal vesicles [393]. Additionally, Aβ can be taken up by smooth muscle cells delineating the arterioles or by endothelial cells that constitute the BBB [394–398]. It was suggested that a fraction of the Aβ fragments is transcytosed and secreted into the bloodstream [399], which may serve as an effective mean of clearance (discussed later in *section 8.1*).

The normal clearance rate for Aβ (likely including both soluble and ApoE-bound Aβ) appears to be very fast, estimated at approximately 8% per hour [394]. Physiological factors, such as the blood and CSF flux rates in the brain, together with a series of clearance receptors, including LRP1 and VLDLR, are all implicated in the removal of Aβ. However, in the case of ApoE4 which are usually bound with a lot more Aβ_{42}, the clearance rate may be reduced when neuronal ATP supply is inadequate, which will gradually lead to Aβ deposition, often associated with heightened neuroinflammation.

5.4. Section Summary

One of the best-known pathological characteristics of AD is the deposition of extracellular Aβ in neuritic plaques. The β- and γ-secretases cleave APP in the amyloidogenic pathway, resulting in the formation of Aβ_{42}, which may serve as a core for the formation of amyloid plaques. Neuronal membrane cholesterol content has been shown to play a crucial role in regulating the catalytic activity of β- and γ-secretases and thus affecting Aβ production and deposition.

When neuronal cholesterol level is abnormally elevated, a number of regulatory mechanisms will be activated to remove the excess cholesterol in neurons. One mechanism is through increasing metabolic disposition via CYP enzyme-mediated cholesterol oxidation; the other mechanism is through the efflux transport of excess cholesterol via the ApoE particles in an ABCB1-dependent manner. The unlipidated ApoE2 and ApoE3 particles have a very good ability to effectively efflux neuronal cholesterol, but ApoE4 has a sharply-reduced ability to efflux cholesterol, which is a key factor contributing to its pathogenic activity in LOAD.

In addition, Aβ_{40} and *A*β_{42} fragments are known to bind to ApoE, including both lipidated and non-lipidated ApoE3 or ApoE2 particles. The binding of Aβ_{40} to these ApoE3 and ApoE2 particles will prevent them from further binding to APP or APP-related proteins present on cell surface, thereby

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blocking these particles from undergoing endocytosis and thus limiting the further rise in neuronal cholesterol level. Notably, the binding of Aβ_{40} to non-lipidated (or less-lipidated) ApoE3 or ApoE2 particles will not prevent them from carrying out the cholesterol efflux function, which will help neurons to remove excess intracellular cholesterol. Notably, Aβ_{40} has a higher binding affinity for lipidated ApoE2 or ApoE3 particles than for lipidated ApoE4 particles. In fact, ApoE4 binds very poorly to Aβ_{40}, but it can bind more tightly to Aβ_{42}. In addition, each individual ApoE4-containing particle contains about twice the amount of ApoE4 molecules compared to that of ApoE3 or ApoE2 particles. Therefore, in the case of ApoE4 lipoprotein particles, when the same level of Aβ_{40} is present in the SCF, there are a lot of ApoE4 proteins still unblocked by Aβ_{40}. These unblocked ApoE4 lipoprotein particles can still be endocytosed into neurons, thus causing further rises in neuronal cholesterol. Elevated neuronal cholesterol will lead to increased expression of APP, which then leads to increased enzymatic cleavage of APP by β- and γ-secretases to form more Aβ_{42}, which has a much higher affinity to bind and thus block ApoE4 than does Aβ_{40}. Therefore, Aβ_{42} is formed as a preferred alternative product when ApoE4 is the apolipoprotein.

While increased formation of large amounts of Aβ is an important initial cause in familial early-onset AD cases, it is understood that Aβ accumulation and plaque formation only represent a secondary pathological change in most cases of LOAD, and is not the dominant force driving the disease pathogenesis and progression. It is clear that cholesterol dysregulation in neuronal cells in ApoE4 carriers is a key factor that drives the Aβ formation and deposition.

6. What Causes Tauopathy in LOAD?

6.1. The Tau Hypothesis

It is generally believed that the microtubules in neurons can serve as tracks, guiding the transport of subcellular organelles and macromolecules from the soma of a neuron to the ends of the axon and back. Additionally, microtubules are involved in other important neuronal functions, such as axonal and dendrite outgrowth, and perhaps neurotransmission. Tau, one of the important microtubule-associated proteins (MAPs), affects microtubule stability and dynamics [400]. A salient feature of many neurodegenerative diseases is the formation of pathological tau aggregates, leading to the formation of NFTs, which mostly contain hyperphosphorylated tau [401].

The tau hypothesis proposes that tau protein abnormalities initiate the AD cascade [402]. In this model, pathologically-phosphorylated tau binds to normal tau and inhibits its functions [403,404]. Eventually, they form NFTs inside nerve cell bodies. When this occurs, the microtubules disintegrate, destroying the structure of the cell's cytoskeleton which would collapse the neuron's transport system. More recently, it was suggested that abnormal tau phosphorylation may subsequently trigger Aβ accumulation in LOAD [405].

While tau is mostly a cytosolic protein, it is also present in the nucleus [406]. Interestingly, the nuclear tau may have a DNA-protective function, which is impaired when tau aggregates [407–409]. In line with this suggestion, tau oligomerization is associated with increased DNA damage [410] and cell death [409]; furthermore, expression of a mutant tau is associated with chromosomal instability [411].

Notably, NFTs have been found in other neurodegenerative conditions including Pick's disease, progressive supranuclear palsy, corticobasal degeneration, and others. The causal relationship between tau dysfunction and neurodegeneration is supported by the identification of over 40 mutations in the tau gene that cause familial frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [412,413]. As discussed in detail below, it is speculated that when tau-based NFTs occur in other neurodegenerative diseases, there may exist severe neuronal energy deficiency as a major underlying cause.

6.2. Hyperphosphorylation and Aggregation of Tau

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Tau in tangles is characterized by a high degree of phosphorylation on many residues (45 serines, 35 threonines and 5 tyrosines) [414]. Some of these sites are typically phosphorylated during embryogenesis or under specific physiological conditions (such as hibernation in certain species), whereas other sites are highly pathological.

In AD, tau phosphorylation is increased and the phosphorylation pattern is also altered, as opposed to normal physiological phosphorylation [415]. Phosphorylation of tau is carried out by multiple enzymes [416,417].

Phosphorylation of tau reduces its binding affinity for microtubules, which is associated with reduced microtubule stability and loss of synaptic functions [418]. In addition, hyperphosphorylated tau proteins are prone to aggregation [416], by a process that is not fully understood yet. Aggregated tau proteins would form oligomers, and eventually form fibrils, which assemble into paired helical filaments (PHFs) and neurofibrillary tangles (NFTs); these are hallmarks of AD [419].

A number of factors have been reported to induce tau hyperphosphorylation, such as dysregulation of kinase–phosphatase activity that can lead to hyperphosphorylation. Several other factors have also been reported to induce tau hyperphosphorylation, such as glycation [420]. However, the mechanism underlying the induction of tau hyperphosphorylation is not clear at present.

6.3. Ubiquitin Degradation of Tau Protein is an ATP-Dependent Process

The 26S proteasome catalyzes the great majority (>80%) of the protein degradation in growing mammalian cells [421], in an ATP-dependent manner. ATP is consumed to make the protein degradation process highly selective through ubiquitination, and also enables substrate unfolding and translocation into an isolated chamber within the proteasome. The rate of hydrolysis of ubiquitinated proteins by the 26S is proportional to its rate of ATP hydrolysis, and the total ATP consumed in degrading ubiquitinated proteins is surprisingly large [422]. Besides, substrate size is also a factor that determines the amount of time and energy needed for proteolysis. The breakdown of large multi-domain proteins will consume much greater time and ATP for the 26S to unwind and degrade each successive domain [249].

Importantly, protein hyperphosphorylation can alter proteasome activity. There is growing evidence that the proteasome's capacity to degrade ubiquitinated proteins is defective in neurodegenerative diseases [423–425]. As the proteasome function decreases, ubiquitinated proteins would accumulate in neurons along with phosphorylated tau.

6.4. Mechanistic Explanation

Neurons are terminally-differentiated, polarized cells, and require a well-developed cytoskeleton and motor proteins to facilitate the trafficking of various vesicles and organelles. Microtubules are also required for neuronal migration, differentiation, axonal extension, and neurotransmission. These important functions of microtubules are heavily dependent on cellular ATP for energy.

As discussed earlier, elevated neuronal cholesterol will lead to reduced neuronal biosynthesis of ATP. It is hypothesized that when there is a severe shortage of cellular ATP supply, the transport of cellular components will not be able to proceed normally, and as a result, some of the transport functions of the microtubules might have to be put on hold to save cellular energy for other, perhaps more important, neuronal processes.

As discussed above, tau protein degradation is mediated by the ubiquitin system present in neurons, which requires large amounts of cellular ATP to degrade unwanted proteins. High cholesterol levels in neurons will lead to reduced synthesis of ATP, and it is hypothesized that when cellular ATP is severely deficient, it will lead to a slowdown in the ubiquitination-associated degradation of tau proteins. When tau proteins cannot be enzymatically degraded in a timely manner, they will accumulate inside neurons. In order to prevent these tau proteins to perform their normal cellular functions (which will further consume cellular ATP), they are extensively phosphorylated in

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certain ways, as hyperphosphorylated tau proteins will have reduced functions. However, hyperphosphorylation of tau proteins also facilitates their aggregation. Therefore, based on the proposed pathogenic mechanism, it is speculated that tau accumulation mostly results from the reduced ability of the ubiquitin-proteosome system to effectively degrade tau due to severe deficiency of neuronal ATP, which results from abnormal elevation of neuronal cholesterol levels.

In support of the above mechanistic explanation, an earlier study has reported that there is a buildup of cholesterol in tangle-bearing neurons, suggesting that increased cellular cholesterol promotes tau phosphorylation [426]. Also, it was shown that synaptic accumulation of hyperphosphorylated tau oligomers in AD is associated with dysfunction of the ubiquitin-proteasome system [427].

Here it is of interest to note that in an Aβ vaccination trial, there were two patients who were almost entirely devoid of Aβ deposition, and yet displayed signs of severe pathology associated with NFTs (Braak stage VI) [428]. As expected, the intellectual capacities of these patients were severely affected, and the clinical progression of mental decline was not significantly different from untreated AD patients [428]. This observation is consistent with the hypothesis that Aβ formation and accumulation often is a secondary event accompanying neuronal cholesterol elevation, which then results in deficiency in neuronal ATP and neuroactive metabolic intermediates. In these two patients, although their Aβ deposition is almost completely removed by Aβ vaccines, if their neuronal cholesterol remains at high levels, then their cognitive functions as well as tauopathy will remain very severe, which are determined by the severity of neuronal ATP deficiency and are largely independent of the severity of Aβ deposition.

In the light of the suggestion that NFT formation is the result of severe neuronal ATP deficiency, it is reasonable to suggest that the appearance and spread of NFTs throughout the brain will closely reflect the degree of ATP deficiency in brain neurons, which can be caused by abnormally-elevated neuronal cholesterol. In other words, the degree of tauopathy is expected to be significantly less severe if neuronal ATP synthesis is not severely inhibited in those AD cases. In agreement with this suggestion, earlier studies have shown that dementia symptoms in AD correlate better with the appearance and spread of NTFs throughout the brain than with the deposition of Aβ in senile plaques [429,430].

Based on the explanation provided above, it is further speculated that factors that can inhibit neuronal ATP synthesis likely will also induce tau hyperphosphorylation and subsequently certain degrees of tauopathy. In partial support of this speculation, earlier studies have shown that oxidative stress and environmental toxins (e.g., arsenite) that are capable of inhibiting mitochondrial metabolic function [431,432] can induce tau hyperphosphorylation [433,434]. Similarly, methanol has also been reported to induce tau hyperphosphorylation and decrease the cognitive ability in animal models [435]. Mechanistically, methanol is known to be metabolically converted to formic acid in the body, which is a strong inhibitor of mitochondrial oxidative phosphorylation [436], thus reducing cellular ATP synthesis. Notably, an earlier study reported that Aβ can increase tau hyperphosphorylation in neuronal cells that take up these secreted peptides [437]. This observation likely is due to the fact that endocytosis of Aβ fragments is usually associated with the endocytosis of Aβ-bound ApoE particles (enriched with cholesterol and other lipids), which will disrupt mitochondrial function and reduce ATP production in neurons.

It is known that the two classic lesions of AD (i.e., Aβ deposits and neuritic tangles) can occur independently in other brain diseases. NFTs composed of tau aggregates that are similar to or indistinguishable from those found in AD have been described in many less common neurodegenerative diseases (e.g., frontotemporal dementia, subacute sclerosing panencephalitis and progressive supranuclear palsy) that essentially lack Aβ protein deposits and neuritic tangles. Conversely, diffuse Aβ deposits can be seen in aged "normal" brains with almost no neuritic tangles. The fact that NFTs composed of aggregated forms of tau proteins occur in certain diseases in the

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absence of Aβ protein deposition is not surprising as severe ATP deficiency is a main driving force for the formation of NFTs in brain neurons. Besides elevated neuronal cholesterol levels, other factors can also cause neuronal ATP deficiency. However, when neuronal cholesterol levels are abnormally elevated, increased Aβ production and deposition may be more readily seen, often jointly with the accumulation of NFTs. During this process, the degree of impairment of memory functions will largely depend on the degree of deficiency of neuronal ATP and neuroactive metabolic intermediates, both of which are the consequences of elevated neuronal cholesterol. In those aged AD patients that lack significant tauopathy, it is expected that their neuronal cholesterol levels are only modestly elevated, which will slowly but chronically increase Aβ formation and deposition, and eventually may end up with rather heavy buildup of amyloid plaques (if their removal is not as efficient). Because neuronal ATP deficiency in these patients may not be as severe, significant tauopathy may not develop as a result; usually these patients are also expected to have slightly better cognitive brain functions.

In line with the above explanations, many earlier studies have noted significant perturbations of neuronal proteolytic degradation machinery in AD. In fact, a striking morphological alteration in neurons of the AD brains is the accumulation of autophagosomes, autolysosomes, and lysosomal dense bodies in dystrophic neurites [438–440]. Similar alterations are also observed in APP and/or PS transgenic AD mouse models [441], even before amyloid deposition is observed. When autophagy is compromised in the brain of an APP-overexpressing AD mouse model following inactivation of the Beclin-1/*BECN1* gene (*BECN1*$^{+/-}$ mice), enhanced Aβ deposition and loss of synapses and neurons are observed, which suggests that decreased autophagy can aggravate or even independently contribute to the overall neurodegenerative process [442]. Interestingly, expression of Beclin-1 using lentiviral vectors attenuated the amyloid load in the injected mouse brain areas.

6.5. Section Summary

As discussed in earlier sections, elevated neuronal cholesterol can lead to increased formation of amyloid plaques. In addition, elevated neuronal cholesterol can reduce the biosynthesis of ATP and its cellular levels, and severe ATP insufficiency then results in tau hyperphosphorylation and aggregation (i.e., the formation of NFTs). Based on this mechanistic explanation, tauopathy will be more readily seen in LOAD as opposed to early-onset familial AD. In early-onset familial AD, neuronal cholesterol elevation and ATP deficiency are not the initial driving causes, therefore it is speculated that hyperphosphorylation of tau likely may not be as severe initially compared to the situation in sporadic LOAD.

According to the proposed hypothesis, modest reduction in neuronal ATP levels is a relatively early event resulting from elevated neuronal cholesterol. As tau accumulation is the result of ATP deficiency, this process will occur relatively early in AD pathogenic process, which is consistent with clinical observations. Notably, a more severe reduction in ATP supply is usually experienced in brain regions that have higher levels of neural activity and thus higher demands for energy supply, such as the medial temporal lobe [443,444]. In fact, this brain region is known to be an early site of tau accumulation (in the absence of evident amyloid accumulation), and its dysfunction may underlie episodic-memory decline commonly seen in aging and dementia [443,444].

While elevated neuronal cholesterol level will increase Aβ_{42} formation, there are also other factors affecting Aβ_{42} formation and deposition. These are the main reasons why the degree of mental functional decline often is not tightly correlated with Aβ pathology. On the other hand, mental functional decline is more directly associated with degree of neuronal ATP deficiency. Since tau accumulation is the result of neuronal ATP deficiency, it is not surprising that mental functional declines are more closely correlated with tau accumulation. Offering partial support for this suggestion, human [^{18}F]flortaucipir PET studies have shown strong associations between regional tau and cognitive decline and neurodegeneration [445].

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7. What Causes Acetylcholine Deficiency in LOAD?

7.1. The Cholinergic Hypothesis

Acetylcholine is a critical neurotransmitter in the brain that is involved in the formation of memories. Moreover, this neurotransmitter is widely used by neurons in the hippocampus and cerebral cortex, regions mostly devastated by AD. In the 1970s, it was found that acetylcholine levels fall somewhat during normal aging, but drop by approximately 90% in people with AD [446,447]. In addition, other neurotransmitters have also been implicated in AD. For example, the levels of serotonin, somatostatin and norepinephrine are also reduced in some AD patients, and deficits in these substances have been suggested to partially contribute to sensory disturbance, aggressive behavior, and neuronal death.

The cholinergic hypothesis is perhaps the oldest hypothesis concerning the potential causes of AD, which postulates that AD is caused by reduced biosynthesis of the neurotransmitter acetylcholine. In fact, some of the currently available drug therapies for AD are based on the cholinergic hypothesis [446,447]. Overall, medications that treat acetylcholine deficiency have not been very effective clinically.

7.2. Mechanistic Explanation

The synthesis of acetylcholine neurotransmitter is a single step reaction catalyzed by the enzyme acetyl-CoA:choline *O*-acetyltransferase (ChAT):

choline + acetyl-CoA ⇌ acetylcholine + CoA

ChAT is found in the nervous system specifically at sites where acetylcholine synthesis takes place. Within cholinergic neurons, ChAT is concentrated in nerve terminals. In subcellular fractionation studies, ChAT was recovered in the synaptosomal fraction, and within synaptosomes it was primarily in the cytoplasmic fraction. In comparison, acetylcholinesterase (AChE), the enzyme responsible for degradation of acetylcholine, is produced by cells that contain cholinoreceptive sites, although it is also produced by cholinergic neurons.

Under usual conditions, synthesis of acetylcholine is mostly determined by the intracellular availability of choline, which is a limiting step determined by the uptake of choline into the nerve ending. Choline is supplied to neurons either from blood circulation or through metabolism of choline-containing compounds. At least half of the choline used in acetylcholine synthesis comes directly from recycling of released acetylcholine, which is hydrolyzed to choline by AChE. Presumably, uptake of this metabolically-derived choline occurs rapidly, before these molecules diffuse away from the synaptic cleft. Another source of choline is the breakdown of phosphatidylcholine. Choline derived from these two sources becomes available in the extracellular space and is then subject to high-affinity uptake into the nerve ending. In the CNS, these metabolic sources of choline are particularly important as choline in the plasma cannot penetrate the BBB. Thus, in the CNS, the high-affinity uptake of choline into cholinergic neurons may be saturated, and as such, acetylcholine synthesis can be limited by the supply of choline, at least during sustained neuroelectrical activity. This suggestion is in line with the observation that acetylcholine stores in the brain are subject to variation, whereas acetylcholine stores in other places such as ganglia and muscles remain relatively constant.

However, under conditions of AD, it is hypothesized that acetylcholine deficiency is mainly due to elevations of neuronal cholesterol levels, which will result in reduced mitochondrial metabolic activity as well as reduced ATP synthesis in neurons and nerve terminals. Since acetyl-CoA is formed as a metabolic intermediate inside the mitochondria, suppression of mitochondrial metabolic activity is expected to result in reduced formation of acetyl-CoA. In addition, since the transport of acetyl-CoA from mitochondria to cytoplasmic compartment is an ATP-dependent process, reduced mitochondrial ATP synthesis resulting from elevated neuronal cholesterol may also reduce the transport of mitochondrial acetyl-CoA to cytoplasmic compartment where acetylcholine biosynthesis takes place.

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In addition to the above two factors, it is important to note that the cholinergic vesicles in nerve terminals not only store acetylcholine, but also ATP and Ca^{2+} [42]. ATP is a required component of the cholinergic vesicles, and the release of ATP has been shown to accompany acetylcholine release from these vesicles during neurotransmission [42]. Under conditions of severe ATP shortage, formation of acetylcholine vesicles is expected to be drastically reduced.

Lastly, the normal process of synaptic neurotransmission itself also demands high ATP supply [43]. When there is an ATP shortage in cholinergic neurons or nerve terminals, the activity of neurotransmission will be significantly reduced, and the level of reduction likely depends on the degree of ATP deficiency in these neurons and their nerve terminals.

8. Explanation of Relevant Clinical and Experimental Observations Based on the Proposed New Hypothesis

8.1. The BBB May Enable Active Transport of CNS Lipidated ApoE Particles into the Peripheral Compartment

It has been generally believed that due to the presence of BBB, cholesterol within the CNS does not readily equilibrate with cholesterol in peripheral circulation [46]. Similarly, it is believed that the peripheral and CNS ApoEs do not cross the BBB and do not exchange their ApoE-containing lipoproteins [448]. Despite these earlier suggestions, it was, however, estimated that a relatively small but significant fraction (up to 0.4%) of the total brain cholesterol pool is "excreted" from the brain every day [45].

To explain the cholesterol turnover in the CNS, it was reported earlier that knockout of *CYP46A1* gene in mice results in over 50% reduction in brain cholesterol excretion and approximately 40% reduction in brain cholesterol synthesis [137]. Based on this observation, it was suggested that CYP46A1 in mice may be directly responsible for approximately 40% turnover of total brain cholesterol [137]. While this explanation is not unreasonable, a different possibility is suggested here. It is speculated that the reduced brain cholesterol turnover in *CYP46A1*-knockdout mice may mostly result from the reduced cross-BBB transport of cholesterol-enriched ApoE and ApoJ lipoprotein particles. It is known that the major components involved in this cross-BBB transport, such as ApoE, ApoJ, ABCA1 transporter and TREM2, are all up-regulated by 24*S*-OHC, which is formed by CYP46A1. As such, when CYP46A1 is deficient in mice, it not only decreases CYP46A1-mediated metabolic disposition of cholesterol, but it also drastically decreases the cross-BBB transport of cholesterol, due to the absence of the key regulatory factor 24*S*-OHC.

The above explanation is reasonable from a theoretical point of view. It is speculated that neuron-expressed CYP46A1 may mostly serve the function of producing a very small quantity of the signaling molecule 24*S*-OHC, for the purpose of regulating neuronal cholesterol homeostasis. The actual metabolic capacity of brain neurons (i.e., the metabolic capacity of CYP46A1) likely is very limited, as these cells are highly specialized for complex and important neuronal functions. If the neuronal CYP46A1-mediated metabolic disposition of cholesterol *per se* were chiefly responsible for brain cholesterol turnover every day, it probably would be more reasonable that some other major cell types in the brain (such as microglia) play a dominant role in this task. This would be akin to the situation that astrocytes, rather than neurons themselves, are largely responsible for the synthesis of lipids required for fulfilling many neuronal functions in the brain. However, if the brain has the ready means to actively transport excess cholesterol across the BBB into the peripheral circulation, then it is readily understood that the selective presence of CYP46A1 in brain neurons (but not in astrocytes) is mostly for the purpose of producing a signaling molecule in very small quantities for regulating neuronal cholesterol homeostasis.

Here, it is of interest to note that earlier studies found that after ApoE particles are secreted by astrocytes, they are initially associated with smaller amounts of lipids forming small discoid particles (8–15 nm in diameter), then they increase in size, becoming spherical as they accumulate more lipids,

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and eventually they are enriched with lipids (12–20 nm with a fraction up to 30 nm) and flow into the CSF [449–451]. These interesting observations have led to the speculation that there might exist a specific mechanism responsible for the active transport of cholesterol-rich ApoE particles in the CSF across the BBB into the peripheral circulation for disposal (note that these ApoE particles are usually also bound with Aβ fragments to prevent them from endocytosis into neurons). Specifically, it is postulated that the BBB can routinely enable the transport of cholesterol-enriched ApoE particles (likely together with ApoJ) into the peripheral circulation in such a fashion that excess neuronal cholesterol can be effectively removed. Mechanistically, it is hypothesized that TREM2 present in microglia is involved in the efflux (transcytosis) of Aβ-bound, ApoE/ApoJ-containing lipid particles across the BBB. In addition, the LRP1 may also mediate the cross-BBB transport of Aβ-bound ApoE particles. Furthermore, under certain conditions, the BBB may even become selectively "leaky", enabling some of the peripheral apolipoproteins to gain access into the CNS to aid in the removal of excess neuronal cholesterol. Some of the supporting observations which are scattered in the literature are briefly discussed below.

8.1.1. Role of TREM2 in Cross-BBB Transport of Cholesterol

Studies have shown that microglial dysfunction is an important pathogenic factor in LOAD [452–455]. Microglia are actively involved in phagocytosis-mediated cholesterol clearance in the brain [456], which is perhaps one of the most important functions of microglia. In fact, it has been suggested that the brain microglia need exogenous cholesterol to maintain their phagocytic capacity and even promote their survival, as the presence of cholesterol will activate the expression of several microglial signature genes associated with the so-called "AD signature phenotype" [457]. Functionally, microglia-mediated myelin remodeling through phagocytosis of lipids (including cholesterol) is part of the process required for memory formation [458].

Large-scale genetic studies have uncovered variants in LOAD risk-associated genes that are highly expressed in microglia [459,460], one of which encodes the triggering receptor expressed on myeloid cells 2 (TREM2), a single-pass transmembrane immune receptor selectively expressed in brain microglia. Individuals carrying rare heterozygous variants of TREM2, such as R47H, have a higher risk for LOAD (average odds ratio of approximately 4.5) [461–463]. Notably, the rare homozygous *TREM2* loss-of-function mutations are known to cause Nasu-Hakola disease, a syndrome characterized by myelin loss, neurodegeneration, and early-onset dementia plus other pathologies such as recurrent bone fractures [464].

Consistent with results from human studies, animal studies have more clearly shown that *TREM2* knockout mice have learning and memory deficits, and transgenic mice that overexpress TREM2 show significant improvements in these functions [465,466]. Additional animal studies have further revealed that the loss-of-function TREM2 variants are associated with increased AD risks [467].

Regarding the protective function of TREM2 in LOAD, it is hypothesized that the observed "microglial clearance" of ApoE and ApoJ [468–471] is a process associated with the cross-BBB transport of Aβ-bound ApoE/ApoJ lipoprotein particles. It is speculated that many of these ApoE/ApoJ particles are cholesterol-enriched, although TREM2 is capable of transporting both lipidated and unlipidated particles. A schematic representation of the proposed hypothesis is depicted in Figure 7. It is hypothesized that TREM2 plays a more important role in guiding the phagocytosed vesicle to re-merge with the cell membrane on the peripheral side. When this function of TREM2 is partially or fully lost (such as due to the loss-of-function mutations), the cross-BBB transport of cholesterol-enriched Aβ-bound ApoE/ApoJ particles will be significantly reduced, which will then lead to elevated brain cholesterol levels, particularly inside microglia, subsequently increasing the risk of LOAD. As discussed below, there are experimental observations offering partial support for the above mechanistic explanation regarding the role of TREM2 in LOAD.

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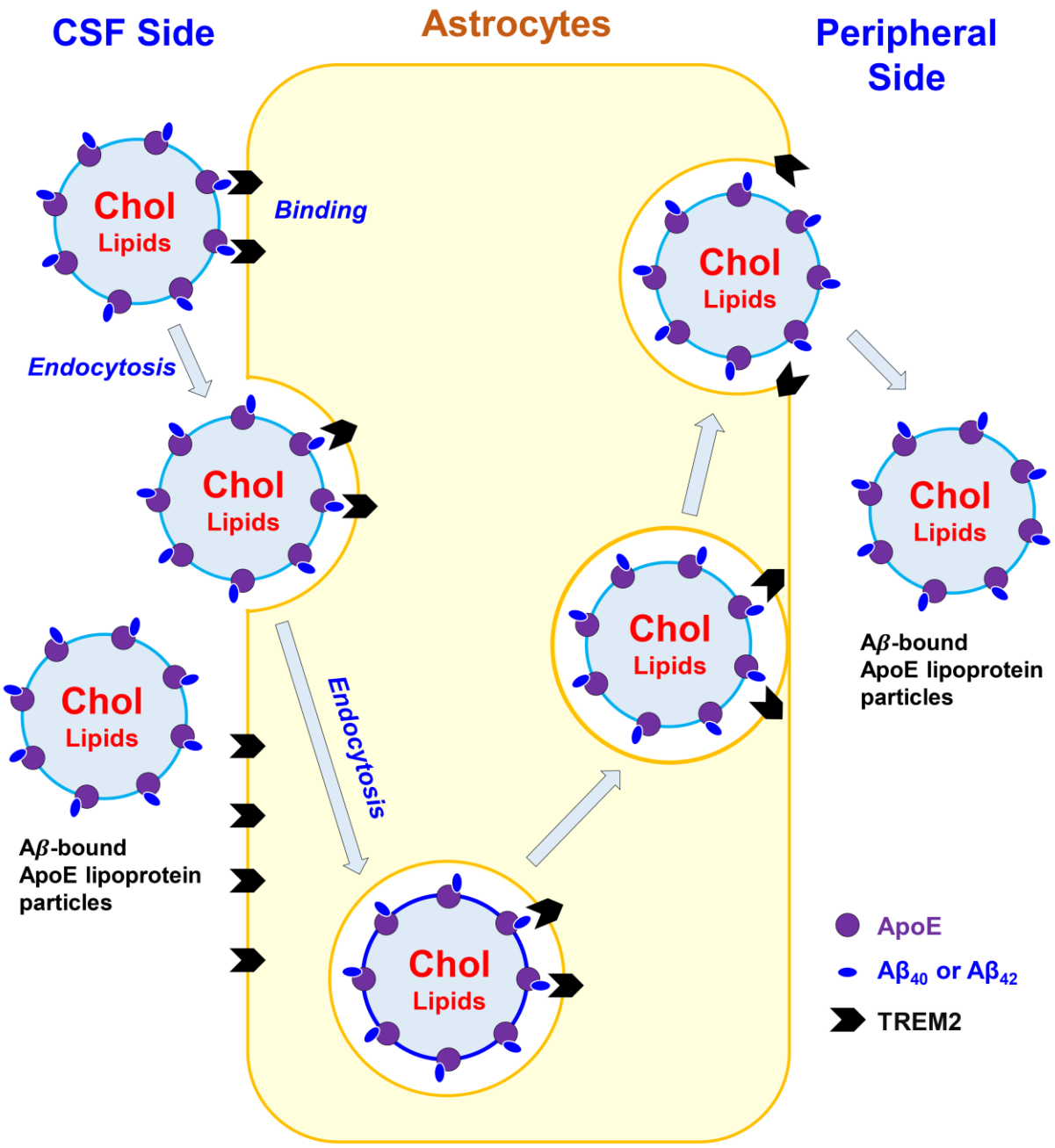

Figure 7. Role of astrocytic TREM2 in mediating the transcytosis of Aβ-bound ApoE lipoprotein particles from the cerebrospinal fluid (CSF) to the peripheral side. As depicted, the extracellular domain (ECD) of the TREM2 protein plays a crucial role in the binding interaction with the Aβ fragments that are tightly bound to the ApoE lipoprotein particles. The is binding interaction between the two will initiate the internalization (endocytosis) of the Aβ-bound ApoE lipoprotein particles. In addition, TREM2 plays an even more crucial role in mediating the fusion of the ApoE lipoprotein particle with peripheral side of the cell membrane, i.e., carrying out the release of the particles to the peripheral side. When TREM2 has loss-of-function mutations, the exiting process will be stopped, and it will result in the accumulation of the Aβ-bound ApoE lipoprotein particles inside astrocytes. As these ApoE lipoprotein particles are loaded with cholesterol, it is expected that the astrocytes will have abnormally-high levels of cholesterol inside, which will inhibit the ATP synthesis, and will also activate the development of the disease-associated microglia (DAM) phenotype, which is an AD signature phenotype. These changes will also facilitate neuroinflammation. (Note that in the diagram, only ApoE is drawn. It is believed that ApoE and ApoJ may be jointly present in the lipoprotein particles, and the presence of both ApoE and ApoJ may facilitate the process of transcytosis from the CSF side to the peripheral side).

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i. The ectodomain (ECD) of TREM2 is a receptor for binding different lipoproteins (e.g., ApoE, ApoJ, ApoA1, and LDL), although ApoE has been the best-studied ligand [468,472,473]. This binding is independent of the ApoE isoforms [468,472–474] and their lipidation states [472–474]. Earlier studies have suggested that ApoJ likely is involved in helping the transport of ApoE-rich lipoprotein particles across the BBB [468,470–472]. Studies have shown that AD-associated TREM2 variants (such as R47H and R62H) indirectly affect the binding of TREM2 with its ligands [463,468,472,473,475,476]. Detailed analysis has revealed the R47H mutation significantly reduces the binding of TREM2 with ApoE [473], including all three isoforms [472] with varying lipidation states [468], with other apolipoproteins [468,473] and lipids [477,478]. It is expected that the reduced ability of TREM2 variants for ligand binding will increase the risk of LOAD [468,472,473,478] as these variants are expected to increase cholesterol levels in the brain, especially in microglial cells.

Notably, studies have revealed that the amyloid mouse models lacking ApoE and the mouse models lacking TREM2 each display similar pathologies in amyloid plaques and microgliosis [479,480]. Their similarities are readily understood as both situations will lead to elevated cholesterol in neurons and microglia. Similarly, it is also understood that individuals simultaneously carrying the R47H variant and the *APOE4* genotype would have a markedly elevated risk for developing LOAD [481].

ii. Studies have shown that under normal homeostatic conditions, TREM2 is primarily located intracellularly [482], in association with the trans-Golgi network [483–485] and in

Exocytic vesicles [483]. These TREM2-containing vesicles continuously shuttle to the membrane, a process which can be rapidly induced by increases in intracellular Ca^{2+} [483]. TREM2 is recycled from the membrane in clatherin-coated vesicles in a beclin-1 [486] and Vps35-dependent manner [486,487]. Vps35 mediates recycling of TREM2 from the membrane via retromer complexes [487]. In the case of loss-of-function variants [475,488–490], TREM2 will have a reduced presence on cell surface but an increased association with lysosomes and is degraded [487].

Consistent with the hypothesis that TREM2 is critically involved in the transcytosis of lipoprotein particles, animal studies have shown that microglia lacking TREM2 have a drastic increase in intracellular vesicles, which resemble autophagic vesicles [487]. This observation was also made in humans, as microglia in AD patients carrying the loss-of-function TREM2 variants have more intracellular vesicles than microglia in AD patients with the common TREM2 variant. These observations support the notion that TREM2 plays a crucial role in guiding the phagocytosed vesicles to re-merge with the cell membrane on the other side. When this function of TREM2 is lost, then it would have a reduced presence on cell surface but an increased association with intracellular vesicles (such as lysosomes) and is quickly degraded [487]. As these vesicles are loaded with cholesterol, it is expected that the microglia with mutant TREM2 will have very high levels of cholesterol inside, which will then inhibit microglial functions and induce neuroinflammation.

iii. It is known that TREM2 serves as a major sensor for extracellular lipids, and mediates cholesterol clearance during demyelination. It detects demyelination by sensing the lipidic components of myelin and promotes myelin debris removal via lipid transport and catabolism [491]. Interestingly, it appears that chronic myelin phagocytosis in *Trem2*$^{-/-}$ brain is not affected, but the lack of TREM2 causes accumulation of CEs and oxidized CEs in the *TREM2*$^{-/-}$ brains. These observations agree with the known functions of TREM2, i.e., it plays a more important role in the transcytosis, i.e., the re-merging of the phagocytosed vesicles with the cell membrane on the peripheral side, whereas its role in mediating the initial phagocytic process is less important.

Studies from recent years have identified the disease-associated microglia (DAM) phenotype, which is a transcriptionally-distinct microglial profile and closely associated with LOAD pathogenesis [452]. In addition to the expression of the microglial marker gene *TREM2*, this subpopulation of microglia is also defined by the expression of genes involved in lipid metabolism and transport, such as *APOE* [452,457]. In fact, increased expression of *TREM2* along with other components is widely

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considered a characteristic shift from the homeostatic phenotype of microglia to the neurodegenerative phenotype [453].

Mechanistically, some earlier studies have suggested that TREM2 may directly control microglial gene expression, resulting in the development of the disease-associated microglia phenotype. While this possibility cannot be ruled out, it is speculated the upregulation of gene expression involved in lipid transport and metabolism most likely is an indirect effect resulting from the functional deficiency of TREM2. It is hypothesized that when neuronal cholesterol is abnormally elevated, the astrocytes will synthesize more ApoE and ApoJ to help efflux cholesterol (and its metabolite 24*S*-OHC). Under conditions when the removal of excess cholesterol is still insufficient (such as due to the presence of ApoE4), more ApoE and ApoJ will be produced. In these situations, there will be a heightened reliance on TREM2-mediated microglial transport of Aβ-bound ApoE/ApoJ particles across the BBB. As such, microglia will be regulated by elevated cellular cholesterol and 24*S*-OHC, further stimulating the expression of TREM2, cholesterol transporters, apolipoproteins (such as ApoE and ApoJ), and metabolizing enzymes (such as SOAT1 and LCAT1), thereby manifesting as the DAM phenotype.

iv. It is known that loss-of-function TREM2 variants can increase the risk of LOAD [479]. These variants under certain conditions (such as chronic demyelination) will lead to accelerated microglial accumulation of intracellular cholesterol in a storage form (i.e., CEs) without altering microglial phagocytic capacity [467]. CEs are known to accumulate in the brains of LOAD patients and AD mouse models [492–495], and the LOAD-linked *TREM2* variants [463] are associated with robust intracellular accumulation of CEs. It is of note that TREM2-deficient and ApoE-deficient microglia show similar abnormalities in cholesterol metabolism, including CE accumulation [479]. Mechanistically, it has been suggested that CE accumulation may mediate cytotoxicity via enzymatic or non-enzymatic formation of oxidized metabolites and lipid peroxides from the mono- and polyunsaturated fatty acid chains the CEs harbor [496,497].

Lastly, it is of note that *TREM2*$^{-/-}$macrophages also exhibit a similar CE storage disorder, resulting in the formation of macrophage foam cells commonly seen in atherosclerotic lesions [498]. Earlier studies of foam cells revealed that cholesterol dyshomeostasis resulting in excess CE accumulation is closely associated with pro-inflammatory responses [496,498,499]. Therefore, it is speculated that abnormal accumulation of cholesterol and CEs in the brain (likely mostly in microglia) is an important factor in neuronal inflammation.

v. Cyclocreatine is a potent bioenergetic agent that helps maintain higher cellular ATP levels during ischemia [500]. Interestingly, dietary administration of cyclocreatine in 5XFAD mice lacking TREM2 prevented the buildup of intracellular vesicles, increased microglia numbers, enhanced clustering around Aβ plaques, and mitigated plaque-associated neurite dystrophy [501]. This observation points to a central role of brain ATP deficiency (presumably caused by elevated neuronal cholesterol resulting from TREM2 deficiency) in microglial dysfunctions and their role in LOAD pathogenesis. This observation also suggests that there are other mechanisms which may compensate for the lack of TREM2 function if adequate microglial ATP supply is maintained.

8.1.2. Role of TREM2 in the Cross-BBB Transport of Aβ

It is known that microglia can directly internalize and degrade Aβ [502]. Studies have shown that TREM2 can serve as a microglial receptor for Aβ, and the Aβ oligomers can bind to TREM2 with higher affinity than the Aβ monomers [503]. In the AD mouse model, elevated microglial *TREM2* gene dosing decreases the Aβ burden, along with improved memory performance [466]. On the other hand, *TREM2*$^{-/-}$ and TREM2-R47H mutant microglia fail to surround and clear amyloid plaques in vivo, resulting in Aβ plaque buildup and accumulation of dystrophic neurites near plaques [463,476,504–509].

As discussed in the preceding sections, the microglial TREM2 likely is involved in transporting the cholesterol-rich ApoE/ApoJ lipoprotein particles across the BBB. It is expected that when these

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ApoE/ApoJ particles are being transported across the BBB, they would also take along with them considerable amount of Aβ fragments that are tightly bound to these particles. In support of this suggestion, earlier animal studies have shown that ApoJ can bind directly to the soluble form of Aβ in a specific and reversible manner, forming complexes that have been shown to cross the BBB [510]. A high-affinity transport system for ApoJ (K_d of 0.2–0.5 nM) was identified at the BBB and the choroid epithelium in vivo, and the ApoJ–Aβ_{40} complex had 2.4- to 10.2-fold higher affinity than ApoJ itself for the same transport system [320].

8.1.3. Role of LRP1/2 in the Cross-BBB Transport of Aβ and/or Aβ-Bound ApoE or ApoJ Particles

In addition to TREM2, earlier studies have indicated that LRP1 is also involved in the vascular transport of Aβ peptides across the BBB [53]. For instance, it was reported that intracerebrally-microinjected ^{125}I-Aβ_{40} was rapidly removed from the brain of young mice ($t_{1/2} \leq 25$ min), largely through vascular transport across the BBB. The efflux transport system for ^{125}I-Aβ_{40} at the BBB was half saturated at 15.3 nM, and the maximal transport capacity was reached between 70 and 100 nM. ^{125}I-Aβ_{40} clearance was substantially inhibited by the receptor-associated protein, and by antibodies against LRP1 and α_2-macroglobulin (α_2M). There was no evidence that Aβ was metabolized in brain interstitial fluid and degraded to smaller peptide fragments and amino acids before its transport across the BBB into the circulation. Similarly, another study suggested that BBB-associated pericytes can clear Aβ aggregates via an LRP1/ApoE isoform-specific mechanism [511].

In addition, an earlier study [512] reported that Aβ_{40} binds to immobilized LRP clusters II and IV with high affinity (K_d = 0.6–1.2 nM) compared to Aβ_{42} and mutant Aβ. Transgenic mice expressing low LRP-clearance mutant Aβ develop robust Aβ cerebral accumulation much earlier than Tg-2576 Aβ-overproducing mice. Aβ_{40} is cleared rapidly across the BBB via LRP1, but Aβ_{42} is removed across the BBB at a slower rate (1.9-fold slower) compared with Aβ_{40}.

The transport pathways for clearance of human Aβ and ApoE/ApoJ in the mouse CNS have been reported [513]. ApoE3 is cleared slowly across the BBB, and after lipidation its transport at the BBB becomes barely detectable. ApoJ is eliminated rapidly across the BBB via LRP2. Aβ_{40} binding to apoE3 reduced its efflux rate at the BBB by 5.7-fold, but Aβ_{42} binding to ApoJ enhanced Aβ_{42} BBB clearance rate by 83%.

8.1.4. The Leaky BBB Phenotype

An earlier study by Zlokovic and colleagues [514] reported that unlike normal mice, the ApoE knockout mice exhibit a leaky BBB phenotype. Interestingly, selective expression of human ApoE2 or human ApoE3, but not human ApoE4, rescued the leaky BBB phenotype of the mouse. It is speculated that the above experimental observations likely are related to the ability of ApoE2 and ApoE3 to remove excess cholesterol from neuronal cells through the ApoE-mediated cholesterol efflux. Under the pathogenic conditions of complete ApoE deficiency, the brains of transgenic mice would not be able to effectively remove excess cholesterol accumulated inside their CNS neurons. As a result, through certain feedback regulatory processes, the brain of these mice would selectively increase the "permeability" of their BBB such that some of the peripheral apolipoproteins may be allowed into the brain to help remove the excess cholesterol from neuronal cells. While the exact mechanism by which peripheral apolipoproteins are allowed into the brain is not clear at present, studies have shown that under certain conditions, the vascular endothelial cells of the BBB can permit the transcytosis of certain peripheral macromolecules (such as apolipoproteins) to cross the BBB [515]. However, when these ApoE-deficient mice are knocked in to express the human ApoE2 or ApoE3, then the functional deficiency in neuronal cholesterol efflux is restored—understandably, the "leaky BBB phenotype" is no longer be needed. However, knock-in of a gene that selectively expresses the human Apo-E4 will not be as helpful since it has a far lower ability to efflux cholesterol out of neuronal cells.

There are some experimental observations offering partial support for the above suggestion that the BBB can become selectively leaky under certain conditions. For instance, it is known that cells in

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the brain do not produce ApoA1, but the cerebrospinal fluid can acquire a significant amount of ApoA1 under certain conditions, most likely from the blood via unknown mechanism(s) [515].

8.2. Why Hypercholesteremia Is Associated with Increased Risk of LOAD?

Cholesterol present in the CNS is mostly synthesized locally in astrocytes, which is delivered to neurons through endocytosis of lipidated ApoE-rich particles. For years, it is widely believed that the two pools of cholesterol and ApoE, i.e., one in the periphery and one in the CNS, are separated, and there is no exchange between these two pools of cholesterol due to the presence of the BBB. However, epidemiological studies have clearly shown that hypercholesteremia is associated with an increased risk for AD [516]. Similarly, in animal studies, it was shown that a cholesterol-rich diet increases Aβ production in the brains of these animals, and an opposite effect was observed in some of the studies when the animals were treated with cholesterol-lowering drugs [113,380,517,518].

The mechanism by which hypercholesterinemia increases the risk of AD is not clearly understood at present. It was speculated earlier that the observed association between hypercholesterinemia and AD likely is because the impermeability of the BBB is compromised in individuals with AD such that cholesterol can be transported into the brain from the plasma [519]. A slightly different explanation is provided here. It is speculated that hypercholesterolemia may largely affect the removal of excess cholesterol from neuronal cells across the BBB into the peripheral circulation. In other words, when the plasma cholesterol level is elevated, it will slow down the net cross-BBB transport of excess cholesterol carried by brain ApoE particles into the peripheral circulation. As a result, reductions in the cross-BBB transport of excess cholesterol will facilitate cholesterol buildup in the CNS (particularly inside neurons) and ultimately result in reduced neuronal synthesis of both ATP and neuroactive metabolic intermediates (mevalonate and geranylgeraniol), contributing to memory and learning deficits. In addition, elevated neuronal cholesterol is expected to facilitate the formation and deposition of Aβ fragments, and the reduced neuronal ATP level will increase tauopathy and decrease the formation of cholinergic vesicles. All these effects will jointly enhance the pathogenesis of AD, in particular LOAD.

8.3. How to Explain the Complex Effects of Statins in AD?

Statins have been used successfully to treat patients with dyslipidemic cardiovascular diseases [520]. They work by reducing cholesterol synthesis through inhibiting HMGR, a rate-limiting enzyme involved in endogenous sterol synthesis [521]. In general, most statins can inhibit HMGR with a very high potency (K_i or EC_{50} <1 nM) [521].

Because high levels of plasma cholesterol are associated with increased incidence of AD (discussed in the preceding section), the use of statins has been suggested as a potential treatment for AD. Studies using both in vitro and in vivo models have shown that statins can reduce the levels of Aβ peptides, including both Aβ_{42} and Aβ_{40} [380,381]. Based on these observations, many human studies had been initiated to investigate the effect of statins on dementia and cognitive functions. While some human studies have noted a decreased risk of dementia or AD in statin users [522–531], there were also studies reporting the lack of a clear relationship between the incidence of AD and statin use [532–534].

The potential benefits of statin use in AD have been controversial for the following reasons: ***i.*** Some researchers felt that the conclusion regarding a beneficial effect of a peripherally-acting statin in AD itself is somewhat "conceptually" controversial, as it has been long held the view that the plasma lipoproteins do not cross the BBB, and that there is no exchange between the central and peripheral pools of cholesterol due to the presence of the BBB. ***ii.*** There are disagreements as to whether it is the peripheral or central lipid-lowering actions of statins that really contribute to the benefits seen in AD. Some researchers strongly felt that if the use of statins is indeed beneficial in AD, then the use of only those statins that can readily cross the BBB will be indicated, rather than the use of peripherally-acting statins for this purpose. ***iii.*** The last and most important controversy centers around the question of

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whether the use of a statin drug is really effective or beneficial in AD, as there are many clinical and animal studies reporting opposite findings. Also, doubts are raised as to whether the potential benefits of statins in AD are really attributable to their cholesterol-lowering effect or might actually be due to some other potential actions of the statin compounds (such as anti-inflammatory and/or antioxidant properties).

As discussed below, the beneficial effects of the statin drugs in AD, and in particular the complexity of their actions in AD, might be more readily understood in the light of the new pathogenic hypothesis proposed in this paper.

First, based on the discussion provided in earlier sections, it is clear that selective inhibition of the peripheral cholesterol synthesis (such as in liver and other peripheral organs) with a statin drug will result in decreased cholesterol levels in circulation, which will be beneficial for the cross-BBB transport of excess neuronal cholesterol to the periphery. Based on this mechanistic understanding, it is clear that the use of a peripherally-acting statin will be beneficial for reducing the risk of AD.

Second, when the HMGR in neurons and astrocytes is inhibited by a centrally-active statin in addition to their inhibition of the peripheral HMGR, the net effect with respect to AD risk will be more complex, as already noted in some of the earlier studies [535], and the underlying reasons for the complexity are explained below.

It is clear that the elevated neuronal cholesterol is "bad" for AD, not only because it will reduce mitochondrial metabolic activity and ATP level in neurons, but it will also inhibit the neuronal HMGR activity, which subsequently reduces the synthesis of important neuroactive metabolic intermediates. Besides, high levels of neuronal cholesterol will also increase Aβ_{40} and Aβ_{42} formation and deposition in the brain. These effects jointly determine the pathogenic role of elevated neuronal cholesterol in AD. Accordingly, reducing neuronal cholesterol levels through reduced cholesterol uptake (influx), increased cholesterol efflux and/or increased cholesterol metabolism will all be beneficial for restoring altered neuronal functions.

However, reducing neuronal cholesterol levels through the use of a centrally-active statin drug will not achieve the same beneficial outcomes as it will constantly suppress cholesterol synthesis even when the neurons need it. Worst of all, the much-needed neuroactive metabolic intermediates that are formed along the cholesterol synthesis pathway will always be suppressed by the presence of a centrally-active statin drug, which is detrimental to learning and memory formation. These combined effects of the centrally-active statins are the underlying reasons for their differential and complex effects on amyloid plaque formation and cognitive functions of the brain. During the use of a centrally-active statin, a strong reduction in neuronal cholesterol level will help decrease amyloid plaque formation, but a decrease in the formation of the neuroactive metabolic intermediates will impair cognitive functions.

Based on the above explanations, it is understood that in real-world clinical settings, the outcomes of a statin drug in AD patients will, in part, depend on the doses of the statin drug used and whether the drug can effectively cross the BBB to exert a central effect. It is speculated that the use of low-dose, peripherally-acting statin drugs is more likely to produce a beneficial clinical outcome for AD. However, some of the peripherally-acting statins may also exert partial central activity when used at higher doses, and under such conditions, the clinical outcomes likely will be mixed. As predicted, many randomized, double-blind placebo-controlled clinical studies reported a lack of significant beneficial effect for most centrally-active statins on the progression of AD symptoms despite significant decreases in plasma cholesterol levels [535–537], while some other studies suggested a potential benefit (such as reduced amyloid load) of the centrally-active statins in AD [107,380,537].

The available results from animal studies have also reflected this complexity. For instance, some studies showed that simvastatin administration ameliorated learning and memory deficits [538–540],

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opposing observations were also reported [541].

Third, it is of note that when statins were used at super-high concentrations (1,000 times higher than the K_i value), they might have off-target effects that are independent of HMGR inhibition [542]. It has been suggested that neuroprotective effect of statins against AD may also be partly attributable to the anti-inflammatory and/or antioxidant properties of the statins [543,544].

Lastly, it is of note that different from statin drugs, the effect of methyl-β-cyclodextrin (which causes acute cholesterol depletion) [545] is expected to be different, since it only helps to reduce cellular cholesterol level without affecting the formation of the neuroactive metabolic intermediates in neurons.

8.4. Pathogenic Mechanism of the NPC Disease: A Tentative Explanation

The NPC disease is a relatively rare autosomal recessive inherited disorder (1/150,000 live births) that causes progressive neurodegeneration and premature death, and is often accompanied by hepatosplenomegaly and lung disease [546]. A characteristic histological feature of the brains of the NPC disease is a massive loss of neurons, particularly Purkinje cells in the cerebellum, consistent with the impairment of motor function in these individuals [122], although neurons in other parts of the brain are also affected to varying degrees.

The NPC disease is caused by mutations in either the *NPC1* or *NPC2* gene. NPC1 and NPC2 each bind to cholesterol and act in tandem in late endosomes and/or lysosomes to mediate the exit of unesterified cholesterol derived from endocytosed lipoproteins [67,114]. Consequently, in NPC1- or NPC2-deficient cells, including neurons [116] and glial cells [117,118], unesterified cholesterol and other lipids accumulate in late endosomes and/or lysosomes. Accordingly, the amount of cholesterol in the plasma membrane and ER (the cellular site at which cholesterol homeostasis is regulated) is reduced [119]. In *NPC1*$^{-/-}$ neurons, this defect in cholesterol export from late endosomes and/or lysosomes likely results in higher-than-normal cholesterol content in neuronal cell bodies and reduced cholesterol content in the distal axons [116,120]. It has been suggested earlier that some of the neurological deficits in the NPC disease might be attributable to a deficiency of cholesterol in axons.

It is speculated that the pathogenesis of NPC disease likely is due to the toxic levels of free cholesterol accumulating in neuronal cells, which will inhibit mitochondrial function and respiration, significantly reduce ATP levels. Therefore, the most pronounced damaging effect is expected to be seen in neurons with a very high density of mitochondria in their cell body. Indeed, recent studies using cultured neuronal cells and cell type-specific *NPC1* knockout mice have demonstrated that the primary cause of neurodegeneration in the NPC disease is due to NPC1 protein deficiency specifically in neurons, rather than in astrocytes or microglia [118,547,548]. It is understood that brain neuronal cells are more sensitive than astrocytes and microglia to the cytotoxicity of abnormal cholesterol accumulation because neurons usually have a higher density of mitochondria and a higher demand for cellular ATP supply to fulfill their neuronal functions. Purkinjie cells in the cellebellum may be especially sensitive to the cytotoxicity of abnormal cholesterol accumulation as these large neuronal cells contain a high density of mitochondria in their cell body [549].

It has been observed that the morphology and composition of synaptic vesicles are altered by NPC1 deficiency [120]. In addition, the *NPC1*$^{-/-}$ neurons have a reduced exocytosis of synaptic vesicles [550]. It is speculated that the following two factors might be part of the reasons contributing to the observed changes in synaptic functions: one is the reduced cholesterol content in presynaptic membranes, as it is known that NPC1 deficiency will reduce the cholesterol content in the plasma membranes of a neuron, which will then affect the recycling and regeneration of the synaptic vesicles. The other factor is the severely-reduced ATP biosynthesis in neuronal cells and particularly in the nerve terminals due to the highly-elevated intraneuronal cholesterol levels resulting from NPC1 deficiency. As discussed earlier, neurotransmission and synaptic functions require large amounts of cellular ATP, and when cellular ATP is deficient, vesicular regeneration and release will be severely

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hampered.

As explained in *section 6.4*, the formation of neurofibrillary tangles and tauopathy are closely associated with severe ATP deficiency in neuronal cells. Also, because of the abnormal accumulation of cholesterol in brain neurons of NCP patients, the neuronal ATP levels likely are severely reduced, and as a result, the formation of neurofibrillary tangles and tauopathy will be markedly increased. Indeed, a number of studies have reported the formation of neurofibrillary tangles and tauopathy in many brain regions of the NPC disease [551,552]. These observations also agree with the suggestion that neurofibrillary tangles and tauopathy are caused by cholesterol-elicited neuronal ATP deficiency.

Based on the proposed mechanistic explanation, it is understood that amyloid pathology will not be as severe in most cases of NPC disease, which will be in contrast to the severe neurofibrillary tangles and tauopathy seen in this patient. It is known that elevated cholesterol content in the plasma membrane will lead to increased enzymatic formation of $A\beta_{40}$ and $A\beta_{42}$ and amyloid deposition in the brain. However, in NPC1/2-deficient neurons, it is expected that while the cholesterol level inside the cells is elevated, its level in the plasma membrane will not be similarly elevated. As a result, the enzymatic formation of $A\beta_{40}$ and $A\beta_{42}$ and the subsequent amyloid deposition in the brain likely will also not be similarly elevated. In fact, this explanation fully agrees with the clinical observations.

Although the amyloid pathology may not be severe in NPC patients, the NPC patients still suffer severe deficits in memory and other cognitive functions [553]. Mechanistically, it is understood that the reduced levels of neuronal ATP and neuroactive metabolic intermediates resulting from high intra-neuronal cholesterol levels in NPC disease are important causes for impaired learning and memory functions. In addition, deficiency in NPC1 results in failed localization of cholesterol in the synaptic membrane, which is involved in synaptic functions, is another contributing mechanism for memory impairments [553].

In addition, as discussed in *section 8.11*, abnormally-elevated cholesterol inside brain microglia will lead to microglial activation (i.e., formation of a neurodegenerative phenotype); in addition, ATP deficiency in brain microglia will also trigger inflammatory responses in the CNS. Certainly, these changes will contribute to neurodegeneration and memory impairments in NPC patients. Indeed, studies have shown that like many other neurodegenerative diseases, neuroinflammation is pronounced in the brains of NPC patients as well as in the brains of mouse models of the disease [554].

At present, no effective treatment is available for the NPC disease. However, recent experiments have suggested a therapeutic approach for the NPC disease. A single subcutaneous injection of the cholesterol-binding compound cyclodextrin into 7-day-old *NPC1*$^{-/-}$ mice significantly slowed the neurodegeneration and extended the lifespan of the mice by approximately 50% [555]. Moreover, the direct intra-thecal delivery of cyclodextrin into the CNS of *NPC1*$^{-/-}$ mice resulted in a concentration of cyclodextrin of 0.1–0.2 mM in the brain [556], and this concentration produced the same beneficial effects on cholesterol homeostasis as observed in neurons and glial cells isolated from *NPC1*$^{-/-}$ mice [557].

Lastly, since the NPC1 and NPC2 proteins are ubiquitously present in all tissues in animals and humans, why only the brain tissue is most severely affected by their deficiency? It is speculated that brain neurons are especially vulnerable to abnormal cholesterol accumulation and its associated damages resulting from NPC1 and NPC2 deficiency; in comparison, the peripheral cells are less vulnerable to the damages caused by cholesterol accumulation as there might be other ways that can more readily help dispose accumulated cholesterol in peripheral cells.

8.5. Why Age Is One of the Most Important Risk Factors in LOAD?

Age remains the most important risk factor for LOAD [558]. Aging is a complex progressive process involving every organ and cell in the body that can span decades. A number of factors that have been suggested to be potential contributors to brain aging and LOAD, which include glucose

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hypometabolism and mitochondria dysfunction, innate immune and inflammatory reactions, β-amyloid processing, dysregulation of cholesterol homeostasis, white matter degeneration and decline in regenerative capacity.

Based on the newly-proposed pathogenic mechanism of LOAD, it is hypothesized that the age-associated hypercholesteremia is an important risk factor in LOAD. In partial support of this hypothesis, many human epidemiological studies have reported that elevated levels of total serum cholesterol measured at midlife is associated with an increased risk of late-life dementia and late-life cognitive decline [105,559–564]. Among the human subjects in a population-based study in Eastern Finland, higher cholesterol levels, measured at a mean age of 50, are associated with an increased risk of dementia, mild cognitive impairment and AD during a 21-year follow-up [565]. In another large observational study, the risk of late-life dementia is increased by 50% among subjects with midlife total serum cholesterol level above 6.2 mM compared to subjects with cholesterol level below 6.2 mM [560]. A recent study performed on twins showed that in twin pairs discordant for dementia, higher cholesterol levels are found in the twins who develop dementia [563]. Most cross-sectional studies showed a correlation between higher HDL levels and lower prevalence of dementia, better cognitive performance, and milder AD pathology [566–570]; in comparison, lower levels of serum HDL and Apo-AI are correlated with a more severe AD condition [566].

Lastly, it is speculated that while the need to remove excess cholesterol from the brain may increase with age as neuronal loss increases with age, the real ability of the brain to remove excess neuronal cholesterol may reduce with aging. The combination of these two factors may also contribute to the accumulation of brain cholesterol in the elderly.

8.6. The Relative Importance of the Amyloid Hypothesis in LOAD Pathogenesis

In 1991, the amyloid hypothesis speculated that the extracellular Aβ deposits are the fundamental cause of AD, and believed that these deposits play a crucial role in all cases of AD [12–14]. Specifically, it was postulated that $A\beta_{42}$ may form aggregates which then initiate a pathogenic cascade ultimately resulting in neuronal loss and dementia. Genetic analysis of the rare familial autosomal dominant AD with early onset has led to the identification of mutations in three genes (i.e., the genes encoding APP, presenilin1 and presenilin2) which are associated with AD and can all increase $A\beta_{42}$ production [4]. Studies have shown that $A\beta_{42}$ is abundantly contained in amyloid plaques of both sporadic and familial AD patients [4,571,572], and it might provide a nidus for amyloid formation [573]. In addition, inheritance of ApoE4 polymorphism was suggested to enhance the stability of Aβ and aid in its accumulation. Based on the similarities in pathology and clinical presentations of familial early-onset AD and LOAD, it became widely accepted that $A\beta_{42}$ accumulation also plays a central role in LOAD.

Regarding the mechanism by which $A\beta_{42}$ causes AD, it was suggested, mostly based on in vitro cell culture studies, that Aβ peptides may enter the cells via multiple mechanisms. One mechanism depends on ApoE and the ApoE receptors, especially the LDLR and LRP1. The Aβ peptides may bind to ApoE lipoproteins first [574,575], and then endocytosed into neurons and other cell types in the brain. Once inside the cells, the oligomeric Aβ peptides may cause numerous functional disturbances, including alterations in mitochondrial morphology and oxidative stress [576–578]; alterations in Golgi morphology by causing fragmentation and malfunctions [579,580]; alterations in mitochondria-associated membranes (ER/mitochondria contact sites) [581,582]; alterations in cellular cholesterol metabolism [583]; and alterations in synaptic organelle transport [584]. In addition, Aβ peptides may also bind to cholesterol directly [585]. Lastly, there were also studies reporting that at high concentrations, Aβ peptides (in particular $A\beta_{42}$) are cytotoxic to neuronal cells in culture and in the brain in vivo [586–589].

Based on the above discussion, it is clear that Aβ or Aβ aggregates are cytotoxic to brain neurons when they are present at high concentrations. However, the real contribution of Aβ or Aβ aggregates to the pathogenesis of AD in vivo appears less convincing [590]. As discussed below, there were some clinical and animal studies suggesting that the abundance of amyloid deposition and plaque

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formation often do not correlate closely with the severity of memory deficits or neuronal toxicity:

i. Human studies have shown that the number of Aβ plaques in the brain does not correlate with the severity of cognitive decrements in patients, and amyloid plaques sometimes are present many years before clinical symptoms are observed. In the process of normal human aging, large amounts of amyloid plaques often are also observed in their brains but with minor neuronal functional alterations, indicating that the relationship between Aβ accumulation and Aβ toxicity is not straightforward [591,592].

ii. In transgenic AD mouse models that overexpress APP and/or presenilin, there lacked a clear relationship between amyloid plaques and cognitive alterations or neurodegenerative changes [593,594].

iii. Animal studies have shown that the transgenic human ApoE4 elicits age-dependent learning and memory impairments in the absence of overt amyloidopathy [234,244,311]. On the other hand, while *APOE-ε2* has a protective effect against Aβ deposition in AD patients, non-demented *APOE-ε2* carriers over 90 years of age (oldest old) have a higher burden of neuritic plaques relative to non-carriers [595,596]. It appeared that *APOE-ε2* carriers are more resilient to Aβ pathology than non-carriers such that the "oldest old" individuals can survive better from Aβ toxicity and thus have their cognitive functions preserved.

iv. An earlier study has sought to determine whether Aβ deposition into plaques is the main mechanism by which ApoE isoforms affect AD. The researchers analyzed the murine ApoE-deficient transgenic mice expressing in their brains the human APP and Aβ together with ApoE3 or ApoE4 [597]. It was found that the cognitive decline in AD correlates better with decreases in synaptophysin-immunoreactive presynaptic terminals, choline acetyltransferase (ChAT) activity, and ChAT-positive fibers than with Aβ plaque load [597].

Based on the literature information reviewed above, it is quite clear that there lacks a clear relationship between the severity of Aβ accumulation in the brain and the severity of cognitive alterations in experimental and clinical situations. To better account for the apparent discrepancy between amyloid plaque load and mental functional decline, a modified amyloid hypothesis was later proposed by some researchers, which speculates that the oligomeric forms of Aβ peptides are probably the more toxic molecular species that cause synaptic loss [598,599]. Over 30 years ago, it was demonstrated in vitro that the nontoxic monomeric Aβ could be converted into a more toxic species after incubation for a few days in buffer [600]. The concept of "Aβ-derived diffusible ligands" (ADDL) [586] or "soluble toxic oligomers" [586,587,589] had received a lot of attention because it provided a potential explanation for the toxicity of the extracellular Aβ peptides and particularly for the lack of a correlation between the deposition of the insoluble Aβ plaques and neuronal loss [601]. Several different oligomeric assemblies of the Aβ peptide have been described, which were generated in vitro [602], or isolated from transfected CHO cells as stable dimers, trimers, and multimers [603], or from transgenic mouse brains as a 56-kDa oligomer [587]. Various oligomeric species have also been isolated from the brains of AD patients; the smallest toxic isolate was reported to be comprised of a dimeric structure [604]. It was reported that the Aβ_{40} dimers, trimers and tetramers are 3-, 8- and 13-fold more cytotoxic, respectively, than Aβ_{40} monomers. Despite all these earlier studies, no consensus exists as to which toxic Aβ assembly is most relevant in vivo, i.e., which oligomeric *Aβ* form contributes to AD development.

While many of the clinical and animal studies discussed above appear to question the amyloid plaque buildup in the brain as a direct driving force for AD development, there is no denial that Aβ_{42} fragments are involved in the pathogenic process. In fact, it is known that in cases of familial early-onset AD, the aberrant formation and accumulation of Aβ_{42} are the single cause driving the pathogenic process. Here the question is: How does the increased formation and accumulation of Aβ alone initiates the pathogenic process of familial early-onset AD? It is known that in these familial AD cases, there is a marked increase in the formation of Aβ_{42} instead of Aβ_{40} due to mutations in related genes.

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Using individuals of the homozygous *APOE3* (*ε*3/*ε*3) genotype as example, it is known that Aβ_{40} has a high binding affinity for ApoE3-containing lipidated lipoprotein particles but Aβ_{42} only has a low binding affinity for these particles; as such, it will lead to more ApoE3-containing particles unbound with *A*β_{42} and thus still available for endocytosis by neuronal cells, leading to the supply of excess astrocyte-derived cholesterol. Consequently, the neuronal cholesterol levels in these individuals with the familial early-onset AD will be abnormally elevated, and this is believed to be an important causative factor that drives the development of AD symptoms, in addition to further enhancing A*β* formation and plaque formation. Since ApoE2 has a similar binding profile for Aβ_{40} and Aβ_{42} as ApoE3, similar outcomes as described above for the homozygous *APOE3* genotype are expected for individuals carrying the homozygous *APOE2* genotype (*ε*2/*ε*2) or the *APOE2*/*E3* mixed genotype (*ε*2/*ε*3).

However, if the familial early-onset AD patient happens to have a homozygous *APOE4* genotype (*ε*4/*ε*4), a different situation is involved. The increased formation of Aβ_{42} in this individual will lead to most ApoE4-ciontaining lipoprotein particles (either lipidated and less lipidated) tightly bound with Aβ_{42}; when the ApoE4 particles are bound with Aβ_{42}, they cannot be efficiently endocytosed (discussed in *section 5.3*). As a result, when the astrocytes need to supply cholesterol to neurons in need, they will have to produce more ApoE-containing particles in order to fulfill this purpose. It is speculated that the amount of excess ApoE4 produced by astrocytes likely will be closely associated with the excess amount of Aβ_{42} being produced by brain neurons. It is known that the presence of ApoE4 can serve as an anchor that binds Aβ_{42} and facilitates the formation of amyloid plaques. In addition, the heavy buildup of cholesterol-containing ApoE4 particles (produced by astrocytes) will overburden the brain microglia and vascular cells to transport these Aβ_{42}-bound cholesterol-containing lipoprotein particles across the BBB. Depending on the excess amount of Aβ_{42} and Aβ_{40} being formed, the efflux capacity of the brain will be overwhelmed to varying degrees. When that happens, a fraction of the A*β*-bound, cholesterol-enriched ApoE4 lipoprotein particles will be stuck in the CSF, which will induce damage because of the excess cholesterol they carry.

Based on the above explanations, it is understood that in the familial early-onset AD individuals carrying the “worst genetic background” (i.e., carrying the homozygous *APOE4* genotype + gene mutations for increased Aβ_{42} formation), their neuronal cholesterol levels actually will be among the lowest compared to individuals with all other genetic background. As a result, these individuals may have exceptional I.Q. levels (particularly in terms of their memory function and related cognitive abilities). The reason is mostly because the astrocyte-produced ApoE4 (and cholesterol) cannot be efficiently delivered to brain neurons, and as a result, neurons in these individuals will have very low levels of cholesterol and thus higher levels of ATP and neuroactive metabolic intermediates. At present, although there lacks rigorous clinical data to offer a strong endorsement of this speculation, many clinical practitioners have the impression that there is a fraction of familial early-onset AD patients that have exceptional intelligence levels before their AD diagnosis. Based on the above explanations, only those individuals who carry the so-called “worst combination” of genetic background (i.e., carrying the homozygous *APOE4* genotype + gene mutations for elevated Aβ_{42} formation) may have the exceptional I.Q. levels, but not the other cases.

In summary, if we put aside the notion that *Aβ* plaque formation is the driving force in AD development, and if we add the component of neuronal cholesterol in AD pathogenesis, then most of the puzzle pieces appear to fit together much better, i.e., the mental functional decline is mostly caused by cholesterol-associated deficiency of ATP and neuroactive metabolic intermediates in brain neurons, whereas A*β* accumulation and plaque formation often may represent a secondary accompanying event, rather than an initial driving force in the pathogenesis of most LOAD cases. As discussed above, even in familial LOAD cases, abnormal cholesterol buildup in brain neurons is still a key pathogenic determinant.

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9. Potential Strategies for Treatment and Prevention of AD

As the number of AD cases keeps rising worldwide, particularly in developed countries, the unmet medical needs for disease-modifying pharmacotherapy continue to grow. Presently, there is no cure for AD; the available treatments offer relatively small symptomatic benefits but remain palliative in nature [605–607]. The widely-used medications for treating the cognitive problems of AD include: acetylcholinesterase inhibitors (e.g., tacrine, rivastigmine, galantamine and donepezil) and NMDA receptor antagonists (e.g., memantine). These agents temporarily relieve some of the AD symptoms for a period of time in a subset of patients, but they do not address the underlying pathological process or substantially slow down clinical progression. The overall benefits from their use are relatively small [605–607], and presently there is no medication that can clearly delay or halt the progression of the disease.

It is of note that much of the past AD research has focused on the amyloid cascade hypothesis. The identification of β- and γ-secretases that generate Aβ_{42} had boosted a race to develop selective chemical inhibitors, antibodies or vaccines in the past two to three decades. However, based on the new understanding that elevated neuronal cholesterol is the key pathogenic factor in LOAD, these treatments, even if developed, may not be as effective as we have hoped for since Aβ_{42} may only represent a secondary change in the pathogenic process of most LOAD.

Provided below is a discussion of some of the potential strategies for treating and/or preventing AD in light of the new pathogenic hypothesis developed in this paper.

9.1. Centrally-Acting Nuclear Receptor Agonists and CYP46A1 Inducers or Activators

9.1.1. LXR Agonists

ApoE, ABCA1 and related macromolecules work together to help remove excess cholesterol from neuronal cells. These macromolecules are regulated by the nuclear receptor system consisting of LXR and RXR in neuronal cells. As such, CNS-acting LXR/RXR agonists are attractive candidates for AD prevention and treatment [608]; these agents can activate the expression of ApoE, ABCA1 and ABCG1 [609], resulting in enhanced neuronal cholesterol efflux. Reduction in neuronal cholesterol will not only improve ATP levels, but also improve the synthesis of neuroactive metabolic intermediates. Moreover, it is expected that reductions in neuronal cholesterol will help reduce the formation of amyloid/neuritic plaques. Indeed, studies have shown that LXR agonists (such as bexarotene) can effectively increase ApoE and ABCA1 levels in the AD mouse brains, which is coupled with improved synaptic plasticity [610] and behavior [611,612] as well as reduced Aβ levels [149,210,267,611,613]. As expected, these beneficial protective effects are only seen in animals that carry both *APOE* and *ABCA1* genes [614]. Similarly, studies have demonstrated that the RXR agonist bexarotene and its derivative OAB-14 each can effectively rescue impaired ApoE4 lipidation and reverse behavioral deficits in *APOE4* mice [236,615]. Based on these observations, it is quite evident that the brain-penetrable LXR/RXR agonists or modulators might be of therapeutic value for LOAD treatment and/or prevention.

Here, it is of note that while 24*S*-OHC is an endogenous ligand (activator) of the brain LXR and can effectively regulate the expression of ApoE, ABCA1 and other proteins involved in cholesterol efflux, it is ineffective in improving AD symptoms, instead it elicits deficits in learning and memory in a rat model [616]. This observation fully agrees with the proposed hypothesis as 24*S*-OHC is an endogenous cholesterol derivative and can strongly inhibit neuronal HMGR, which will then inhibit the formation of neuroactive metabolic intermediates in neurons and thereby inhibit cognitive function and memory formation.

9.1.2. CYP46A1 Inducers or Activators

CYP46A1 catalyzes the metabolic conversion of cholesterol to 24*S*-OHC [136,139]. Earlier studies have shown that overexpression of *CYP46A1* ameliorates amyloid pathology [143] and tauopathy [617] in two different mouse AD models. In addition, overexpression of *CYP46A1* in a mouse model

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for Huntington's disease also decreased neuronal atrophy and improved motor neuron deficits [618]. It is hypothesized that the mechanistic basis for the neuroprotective benefits of *CYP46A1* overexpression is due to increased metabolic formation of 24*S*-OH, which then activates LXR and increases the expression of ApoE, ApoJ, ABCB1 and ABCB7, ultimately enhancing the efflux of neuronal cholesterol.

Based on the above discussion, it is reasonable to suggest that selective induction of neuronal CYP46A1 in humans by centrally-active inducers may provide therapeutic benefits in AD and other related neurodegenerative diseases. While 24*S*-OHC is an endogenous inducer of CYP46A1 in human brains, this oxysterol is not suitable for this particular purpose because it can also inhibit neuronal HMGR. Theoretically, centrally-active synthetic inducers of CYP46A1 which cannot directly inhibit the HMGR would be potentially-useful drug candidates for this particular purpose.

In addition to inducing CYP46A1 expression, studies have shown that the catalytic activity of this neuronal CYP isoform can be activated by some chemicals. Efavirenz, a non-nucleoside reverse transcriptase inhibitor [619], was reported to activate the enzymatic activity of CYP46A1 for cholesterol metabolism [620,621]. An interesting earlier study in iPSC-derived neurons showed that reducing CE levels through CYP46A1 activation by efavirenz can reduce both *p*-tau and Aβ secretion [622]. Efavirenz has also been evaluated in LOAD patients with mild cognitive impairment [623,624].

9.2. Peripherally-Acting Cholesterol-Lowering Drugs

In light of the proposed hypothesis, and also according to many earlier clinical and animal studies (discussed in *section 8.3*), it is suggested that the use of low-dose, peripherally-acting statin drugs which aims to improve peripheral hypercholesterolemia will be of some benefit for reducing the risk of LOAD. In addition, it is expected that their use may also be of benefit for slowing down the progression of LOAD or improving the clinical symptoms. By contrast, the use of centrally-active statin drugs should be avoided because these agents would constantly inhibit the formation of neuroactive metabolic intermediates which are critically needed for learning and memory formation. Moreover, to avoid the potential central effects of statins, it is recommended that relatively-lower doses of the peripherally-acting statins be used.

9.3. Some of the Presently-Approved AD Drugs

9.3.1. Cholinergic Replacement Therapy

The discovery that the acetylcholine neurotransmitter was drastically reduced in the AD brain had led to the earlier hypothesis that replacing acetylcholine would be of some benefit for improving AD symptoms. Many researchers have searched for compounds that can increase the levels of acetylcholine, replace it, or slow its breakdown in affected brain regions.

One obvious target is the cholinesterase, which breaks down acetylcholine after its release (discussed in *section 7*). Many of the AD drugs developed to date are cholinesterase inhibitors, which are designed to suppress cholinesterase such that acetylcholine will not be degraded as quickly, thereby increasing its levels in the brain to compensate for its shortage [625]. There is considerable evidence for their clinical efficacy in mild to moderate AD [626,627], and some evidence for their use in advanced stages. At present, several acetylcholinesterase inhibitors (i.e., tacrine, rivastigmine, galantamine and donepezil) are approved for clinical use for mild to moderate AD, and donepezil is also indicated for advanced AD dementia [628]. The use of these drugs in AD patients with mild cognitive impairment has not shown any efficacy in delaying the onset of the disease [629]. The most common side effects are nausea and vomiting, both of which are linked to cholinergic excess. These side effects arise in 10–20% of users, are mild to moderate in severity, and can be managed by slowly adjusting medication doses [630].

9.3.2. NMDA Receptor Antagonists

Glutamate is an excitatory neurotransmitter of the nervous system, although excessive release in the brain can lead to cell death through a process called excitotoxicity resulting from overstimulation

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of glutamate receptors [631]. Memantine is a noncompetitive NMDA receptor antagonist first used as an anti-influenza agent. It acts on the glutamatergic system by blocking the NMDA receptor and reducing its overstimulation by glutamate [631]. Memantine is moderately efficacious in improving the symptoms of patients with moderate to severe AD [632]. Reported adverse events with memantine are infrequent and relatively mild, including hallucinations, confusion, dizziness, headache and fatigue [633]. The combination of memantine and donepezil was shown to have marginal effectiveness clinically [634].

9.4. Candidate Drugs Targeting the Formation and Aggregation of Aβ and Tau

As mentioned earlier, much of the past AD research has focused on the amyloid cascade hypothesis. As a result, developing prospective mechanism-based treatments such as inhibitors of the β- and γ-secretases and immunotherapeutics (e.g., anti-Aβ monoclonal antibodies and vaccines) has become a major research focus in the past.

9.4.1. β-Secretase as a Drug Target for AD

Studies have shown that knockout of *Bace1* (a gene for β-secretase) in mice drastically reduced Aβ production and reduced amyloid plaque load [635–637], and also improved AD-related symptoms in the AD mouse models [638,639]. Therefore, β-secretase was viewed as a drug target for AD. Orally-effective β-secretase inhibitors have been reported earlier [640]. Notably, the relatively-mild phenotypes in mice deficient for β-secretase compare quite favorably with the severe Notch-related phenotypes caused by broad spectrum γ-secretase inhibition (e.g., gastrointestinal bleedings, autoimmune phenotypes). Additionally, a theoretical advantage of blocking β-secretase instead of γ-secretase is that this would not result in abnormal accumulation of the APP-CTFβ.

9.4.2. γ-Secretase as a Drug Target for AD

In the past, several approaches to increase the therapeutic window, i.e., to find compounds that can efficiently block Aβ production yet without affecting the Notch signaling, have been explored. It was estimated that if the γ-secretase inhibitors could decrease Aβ production by 30–40%, they likely would not detrimentally interfere with the Notch signaling pathway.

A series of non-transition-state γ-secretase inhibitors have been described such as peptide-based inhibitors (DAPT), sulfonamides, and benzodiazepines (compound E). When these inhibitors are used at low concentrations, the ζ-site cleavage is not affected, but the γ-site cleavages are potently inhibited, which is associated with decreased formation of Aβ_{40} and Aβ_{42} [641].

It is of note that an unexpected turn in the development of γ-secretase inhibitors is the finding that these inhibitors represent a viable therapeutic strategy for certain cancers in which the Notch signaling is overly activated, such as T-cell acute lymphoblastic leukemia [642], lung cancer [643] and precancerous adenoma [644].

9.4.3. Aβ Vaccines

It was proposed earlier that an alternative potential approach to secretase inhibition is to use small molecules that can bind to Aβ monomers and prevent their aggregation into potentially neurotoxic oligomers. However, from a theoretical point of view, if an anti-aggregating compound solely blocks amyloid fibril formation, this might actually facilitate the accumulation of the intermediates, such as oligomers, and this effect could potentially aggravate neurotoxicity. Alternatively, an immunologic approach to lowering the levels of Aβ protein monomers, oligomers, and higher aggregates was thus proposed. Studies using the APP transgenic mice have shown that parenteral immunization of mice with synthetic human Aβ_{42} initially led to an antibody response associated with striking clearance of Aβ deposits [645]. Subsequent studies have confirmed and extended this approach by showing that Aβ immunization can indeed lower brain Aβ protein burden in mice, and may also improve their learning deficits [645].

A number of potential mechanisms have been suggested for the beneficial effects of active immunization [645]. First, the anti-Aβ protein antibodies may cross the BBB in small amounts and bind

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to Aβ protein, followed by gradual clearing of the resultant Aβ protein-antibody complexes by local microglia. Second, high titers of anti-Aβ protein antibodies in peripheral circulation may bind and sequester Aβ protein in that compartment, resulting in a gradual redistribution of Aβ protein from brain parenchyma to CSF to plasma. Third, the anti-Aβ protein antibodies might bind to soluble Aβ protein oligomers in the brain and neutralize their synaptotoxic effects.

While no untoward antigen-antibody reactions were reported in active vaccination experiments in the mouse models [645], administration of a $A\beta_{42}$ peptide vaccine (with an adjuvant) to humans with mild to moderate AD resulted in approximately 6% of the patients developing an inflammatory reaction in the CNS that resembled a postvaccinal meningoencephalitis [646,647].

Anti-Aβ monoclonal antibodies such as Aducanumab, Lecanemab and Donanemab were developed as a potential therapy for AD [646,647]. They were reported to reduce Aβ levels in animal and human subjects, and their use was associated with modest improvements in cognitive decline in AD patients. However, there are significant side effects associated with monoclonal antibody therapy. The inflammatory responses produced by monoclonal antibodies on the brain vasculature is associated with the development of edema and hemorrhage within the parenchyma and sulcal spaces.

Based on the understanding that Aβ accumulation and aggregation may only represent a secondary accompanying change in most cases of AD, the expected benefits of β-secretase inhibitors or Aβ vaccines which aim at reducing the overall amyloid plaque load may actually have rather limited clinical benefits in improving the overall cognitive functions in AD patients.

9.4.4. Tau as a Drug Target for AD

Since tau is a major player in AD pathology, research efforts to develop inhibitors of tau aggregation have also been actively considered [648], in the hope that inhibitors of tau aggregation might help reduce NFT formation. Several small molecule inhibitors have shown promising results in laboratory conditions but produced mixed results in clinical trials [648]. Tau aggregation inhibitors can be divided into two classes—covalent and noncovalent. Covalent inhibitors include polyphenols from plants, such as oleocanthal [649]. Non-covalent inhibitors interact with tau through a different mechanism. Methylene blue, a dye, was found to inhibit tau aggregation, with a K_i of 120 nM in a cell-based aggregation system [650]. In a study performed on transgenic AD mice, treatment with methylene blue derivatives resulted in a reduction of tau pathology [651]. A phase II clinical trial reported significant improvements after 24 weeks of treatment [652].

Activity-dependent neuroprotective protein is a peptide that is essential for proper brain function. A small segment of this peptide called NAP (NAPVSIPQ) is thought to have a significant neuroprotective ability and is in phase II clinical trials for schizophrenia [653]. In a mouse model of AD, NAP treatment was shown to reduce the level of hyperphosphorylated tau and to improve behavioral symptoms [654]. NAP also protected microtubules against nocodazole-induced disassembly and stimulated the polymerization of microtubules in cultured cells [655]. Since NAP can reduce the hyperphosphorylated tau and protect microtubules against disassembly, NAP is considered a potential therapeutic agent for AD.

Similar to β-/γ-secretase inhibitors and Aβ vaccines, the expected potential benefits of tau aggregation inhibitors might be limited in AD patients given that tau aggregation is considered a consequence of severe neuronal ATP deficiency. If the situation of neuronal ATP deficiency (resulting from elevated neuronal cholesterol) persists, even a significant reduction in tau aggregation may not improve learning and memory functions of the AD brain.

9.5. Other Potential Candidate Drugs for AD

9.5.1. ApoA1 Mimetic Peptides

Earlier studies have shown that the synthetic ApoA1 mimetic peptides can mimic the effects of ApoE and ApoA1 in stimulating ABCA1-dependent cellular cholesterol efflux (reviewed in [656]). When one of these mimetic peptides, CS-6253, was directly injected into the brains of young ApoE4

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mice, it increased the lipidation of the ApoE4-rich lipoproteins [260]. CS-6253 also significantly reversed ApoE4-associated pathology, including Aβ accumulation and tau hyperphosphorylation in hippocampal neurons, as well as synaptic impairments and cognitive deficits. These results suggest that increasing the lipidation of ApoE4-containing lipoprotein particles by using synthetic ApoA1 mimetic peptides may become a potential strategy to combating LOAD in *APOE4* patients.

9.5.2. SOAT1 Inhibitors

The CE levels in mouse and human brains under normal conditions are very low, making up <1% of the free unesterified cholesterol. However, in the vulnerable brain regions (entorhinal cortex) from AD patients, CE levels are increased by 1.8-fold [493]. In the brains of three different AD mouse models (which express mutant human APP or mutant APP + mutant presenilin 1), the CE levels were elevated 3- to 11-fold compared to the control group [493,657]. In addition, under high-fat diet, the brain CE content in ApoE4 mice was significantly elevated over ApoE3 mice [658]. Together, these results suggest that elevated CE content correlates positively with AD development. In the mouse models for AD, both pharmacological [659–661] and genetic approaches [158,662] showed that inhibition of SOAT1 reduced amyloid plague load and restored cognitive deficits [663].

9.6. NSAIDs

Epidemiological studies have repeatedly shown that the early use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with reduced risk of AD in humans [664–666], but they have been unsuccessful in treating AD in clinical trials [667], or preventing AD in short-term prevention trials of the elderly [668]. Interestingly, the preventive effect of NSAIDs appeared to be more pronounced in those with the *APOE4* genotype [666,669–671], and before the appearance of overt neuropathological changes of LOAD [668]. Similarly, animal studies have also shown that ibuprofen can rescue the effect of *APOE4* genotype on reduced dendritic spine density [248].

The mechanism by which NASIDs protect against LOAD in humans is not quite understood at present. It has also been suggested that some NSAIDs (such as ibuprofen) which have a protective effect against AD may exert its effect through inhibition of the γ-secretase-mediated processing of APP to Aβ_{42} [672]. Here, it is speculated that inhibition of the inflammatory responses resulting from cholesterol-induced neuronal ATP deficiency and cellular damage in microglia likely is a major mechanism underlying NASIDs' neuroprotection in AD.

9.7. Dietary/Nutritional Supplements and Other Natural Neuroprotective Compounds

9.7.1. Boosters of the Synthesis of Neuronal ATP and Neuroactive Metabolic Intermediates

Based on the proposed hypothesis, it is apparent that dietary or nutritional supplements that can safely supply or boost the synthesis of neuronal ATP and/or neuroactive metabolic intermediates (such as mevalonate and geranylgeraniol) will be of great value in helping reduce the risk of LOAD. These food supplements may be potentially used as an adjuvant therapy for LOAD. This is a potentially fruitful area of biomedical research that is presently under-explored.

It is of interest to note that dietary administration of cyclocreatine, a chemical capable of increasing cellular ATP levels [500], was found to effectively mitigate Aβ plaque-associated neurite dystrophy as well as other injuries in TREM2-deficient mice [501]. Similarly, an earlier study reported that impairments in learning and memory functions in an AD animal model can be restored by administration of geranylgeraniol, a neuroactive metabolic intermediate that can readily across the BBB [35].

9.7.2. Acetylcholine Synthesis Enhancers

The earlier finding of drastic acetylcholine deficits in LOAD also raised hope that dietary supplementation of choline and lecithin may help alleviate the conditions in LOAD patients. These two nutrients are used by the body to synthesize acetylcholine. Clinical trials with these two substances have been disappointing so far: while choline supplements showed no effect on cognitive function, lecithin only had a slight effect in a few patients. Researchers are still searching for other

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substances that may promote the synthesis and availability of acetylcholine in the brain of LOAD patients.

Studies have shown that acetyl-*L*-carnitine, a synthetic compound, may activate cholinergic neural transmission and enhance neuronal metabolism in the mitochondria [673] and thus may improve dementia [674] and prevent neuronal degeneration [675]. The exact mechanism by which acetyl-*L*-carnitine exerts its beneficial biological action is currently unclear [673]. Acetyl-*L*-carnitine may enhance the synthesis of acetylcholine [674,676] by facilitating the uptake of acetyl-CoA into the mitochondria during fatty acid oxidation, and stimulate the synthesis of proteins and membrane phospholipids [677,678]. Notably, it was also reported that the plasma *L*-carnitine levels are inversely associated with cognitive impairment in patients with acute ischemic stroke [679].

9.7.3. Estrogens

Gender is an important risk factor in LOAD, as two-thirds of the LOAD patients are women [680]. This proportion is partly attributed to the greater longevity of women over men, making them more susceptible to this and other age-associated diseases [681]. In addition, it has been suggested that the drastic decline in sex hormone levels in women at older age may be an important factor contributing to the increased risk of LOAD in elderly women [682].

In 1993, estrogen made headlines when researchers reported a possible link between estrogen and LOAD. In a study of thousands of women in a southern California retirement community, those who had taken estrogen after menopause had a lower incidence of LOAD than those who had not taken estrogen. However, the neuroprotective effect of estrogens has been controversial. Earlier studies that sought connections between estrogen and mental skills showed mixed results [683,684]. While some studies reported a beneficial effect on cognition in women receiving hormone replacement therapy at different ages after menopause [685–688], other studies reported a lack of beneficial effect on reducing the risk for LOAD [689,690]. Because of the inconsistent epidemiological findings, plus concerns over increased risk for thrombosis, estrogen-based hormone replacement therapy is presently not recommended as a preventive measure for cognitive decline and LOAD [691].

Mechanistically, it was reported that estrogens have neuroprotective effects [692,693] and may prevent mitochondrial dysfunction in nerve cells [693]. Recent studies showed that the 4-hydroxyestrone, an endogenous estrogen metabolite selectively formed in the brain [694], has a far stronger neuroprotective effect than its parent hormones 17β-estradiol and estrone [694,695]. The protective effects of endogenous estrogens and some of their metabolic derivatives (neuroestrogens) may partially explain their beneficial actions in LOAD. In addition, it is hypothesized that the strong cholesterol-modulating effects of estrogens may also contribute to their overall benefits in LOAD [696]. Here it is of note that some of the endogenous estrogen metabolites (e.g., 4-methoxyestrogens) are devoid of meaningful estrogenic activity in vitro and in vivo, but still retain strong cholesterol-lowering activity [696].

9.7.4. Protein Disulfide Isomerase Inhibitors

Recent studies have shown that some of the endogenous estrogen metabolites (e.g., 4-hydroxyestrone) are strong inhibitors of protein disulfide isomerase (PDI) [697], and they exhibit strong neuroprotection against oxidative neuronal death through PDI inhibition [694,695]. Besides, these estrogen metabolites also have strong cholesterol-lowering effects [696], which may jointly contribute to their overall benefits in LOAD.

Recent studies have shown that bazedoxifene and raloxifene, two FDA-approved selective estrogen receptor modulators, are also strong inhibitors of PDI, and they can strongly protect against oxidative neuronal death in vitro and in vivo [698,699]. Additionally, recent studies found that *N*-methyldopamine (an endogenous metabolite of dopamine) and ibopamine (a prodrug which can release *N*-methyldopamine in vivo) each can rescue chemically-induced oxidative ferroptosis [700,701]. These chemicals may hold promise for prevention of oxidative neuronal death.

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9.7.5. Antioxidants

The body has certain lines of defense against oxygen free radicals. Enzymes like superoxide dismutase (SOD) and catalase can disarm the damaging oxygen radicals. Antioxidant vitamins C and E and β-carotene also counter free radicals. The potential protective effect of antioxidants in LOAD has received considerable interest in the past [702,703], but the clinical effectiveness has been controversial.

9.7.6. Vitamin D

Vitamin D is a fat-soluble hormone that is naturally synthesized in the body through subcutaneous synthesis upon exposure to sunlight. This vitamin plays a crucial role in maintaining the health of bones and muscles, and also prevents various diseases such as cancer, diabetes, cardiovascular diseases, and autoimmune diseases. Epidemiological studies have observed a relationship between reduced serum vitamin D levels (especially 25-hydroxyvitamin D) and LOAD risk [704–706]. Vitamin D is an important steroid-derived hormone that acts on calcium metabolism and bone regulation, and also plays a role in the brain in regulating neurotrophic factors, calcium homeostasis, oxidative stress, immune system function and inflammation [707]. In the case of neuroinflammation, it is suggested that vitamin D deficiency can activate the amyloidogenic pathway, resulting in elevation of BACE1 and *APP* cleavage and decrease of Aβ degradation [708,709]; vitamin D supplementation in elderly rats was reported to reduce BACE1 and Aβ formation. It has also been observed that vitamin D can activate macrophages for clearance of Aβ peptides [710,711]. In AD patients, mutations were also observed in vitamin D receptor (VDR) gene, favoring the onset of the disease [712].

In this paper, it is hypothesized that vitamin D may elicit its beneficial effects on LOAD by reducing blood cholesterol levels. Decreased blood cholesterol levels would help reduce neuronal cholesterol in the brain (discussed in *section 8.2*), which is beneficial to the treatment and prevention of AD. Offering partial support for this hypothesis, there were studies in the literature reporting that high blood vitamin D levels are associated with lower LDL levels and higher levels of HDL, but the results from clinical and epidemiological studies are not uniform at present [713]. The mechanism by which vitamin D regulates blood lipid levels is presently also unclear.

To date, there is no large randomized clinical trial to study the effect of vitamin D supplementation on the cognitive functions of AD patients. However, in smaller or cohort studies, the results on the use of high-dose vitamin D and cognitive improvement are divergent [714,715]. It is recommended that vitamin D deficiency should be screened in the elderly population, and vitamin D supplementation would be of value in hypercholesterolemia patients with vitamin D insufficiency and at elevated risk for cardiovascular diseases and AD.

9.8. Physical and Mental Activities

Physical activity is crucial to health regulation since it is significantly linked to obesity, metabolic disease, and atherosclerotic cardiovascular disease [717,718]. The inverse relationship between physical activity and the risk of suffering cognitive decline has been widely documented. Regular physical activity is associated with significant reductions in the risk of developing AD, although there are also discrepancies. Mechanistically, it has been suggested that exercise may cause changes in the brain at the anatomic, cellular, and molecular levels that promote angiogenesis, neurogenesis, synaptogenesis, and stimulation of neurotrophic factors, thereby improving learning, memory, and brain plasticity [719]. It has been reported that exercise increases the gray and white matter of the brain [720], increases cerebral blood flow [721], and reduces Aβ formation and tau phosphorylation [722,723].

Clinical studies have shown that physical activity is associated with reduced risk for LOAD by as much as 45% [724,725]. This protective effect is related to several mechanisms, such as reduction of blood pressure, obesity and proinflammatory activity besides the improvement in lipid profile and

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endothelial function. In addition, adaptations that occur in response to exercise can lead to a better cerebral blood flow and, consequently, better oxygenation of important areas for cognitive function [724]. It has also been suggested that physical activity can prevent LOAD by increasing neurotrophic factors such as BDNF (brain-derived neurotrophic factor), IGF-1 (insulin-like growth factor) and VEGF (vascular endothelial growth factor); stimulating neurogenesis and synaptic plasticity; and lowering free radicals in the hippocampus and increasing superoxide dismutase and eNOS [726]. Studies have shown that physical activities increase hippocampal volume, in addition to increasing plasma BDNF levels in healthy elderly, indicating a possible neuroprotective effect. It was also reported that in the LOAD elderly, physical activity correlates positively with levels of BDNF [727], a growth factor associated with neuronal survival [728]. Recently, it was reported that the liver-derived exercise factor (exerkine) may reverse aging- and AD-related memory loss by targeting the brain vasculature [734].

Notably, earlier studies have shown that physical activity is associated with an elevated release of the neurotransmitters norepinephrine and dopamine in the CNS [729]. Recent studies have revealed that norepinephrine and dopamine have a strong protective effect against chemically-induced oxidative ferroptosis in cultured hippocampal neuronal cells [700,701,730]. Mechanistically, it was shown that PDI plays an important role in mediating chemically-induced ferroptotic cell death through catalyzing NOS activation (i.e., dimerization), which then leads to sequential accumulation of cellular NO, ROS and lipid-ROS, and ultimately, the induction of oxidative ferroptosis [700,701]. Interestingly, norepinephrine and dopamine neurotransmitters are capable of binding to PDI, likely through covalent interactions, and inhibit its catalytic activity, thus abrogating glutathione depletion-associated oxidative neuronal death [700].

Lastly, it is of note that physical activity may exert its beneficial effect on LOAD partly through reducing blood cholesterol levels. A decrease in total blood cholesterol levels would help reduce neuronal cholesterol in the brain, which is beneficial to the prevention and treatment of AD. This may be another reason for the beneficial effects of physical activity in AD. In line with this hypothesis, it is well known that physical activity can help bring favorable changes in plasma lipid profiles, whereas prolonged physical inactivity is associated with a rise in both total cholesterol and LDL-cholesterol [731,732].

9.9. Section Summary

At present, there is still no cure for AD. The available treatments (e.g., cholinesterase inhibitors, NMDA receptor antagonists, and anti-Aβ antibodies) offer relatively small symptomatic benefits but remain palliative in nature. These agents temporarily relieve some of the AD symptoms in a subset of patients, but do not address the underlying pathological process or substantially slow down clinical progression. There is no medication at present that can delay or halt the progression of the disease.

Past AD research has focused heavily on the amyloid cascade hypothesis, and developing inhibitors of the β- and γ-secretases and anti-Aβ immunotherapeutics has become a major research focus in the past. Based on the understanding that Aβ accumulation and aggregation may only represent a secondary change in AD, the expected benefits of β/γ--secretase inhibitors or Aβ vaccines may have rather limited clinical benefits in improving the overall cognitive functions in AD patients. Similarly, the expected potential benefits of tau aggregation inhibitors may also be limited in AD patients given that tau aggregation is a consequence of neuronal ATP deficiency. If neuronal ATP deficiency (resulting from high neuronal cholesterol levels) persists, even a significant reduction in the overall amyloid plaque load and tau aggregation may still not fundamentally improve learning and memory functions of the AD brain.

On the other hands, strategies that aim at reducing neuronal cholesterol level may be highly beneficial in AD prevention and treatment. For instance, agonists for nuclear receptor LXR may activate the expression of ApoE, ABCA1 and ABCG1, resulting in enhanced neuronal cholesterol efflux. CYP46A1 inducers may also have a similar beneficial effect. While 24*S*-OHC is an endogenous LXR activator and inducer of CYP46A1 in human brains, this oxysterol is not suitable for this purpose

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as it also strongly inhibits HMGR in the brain. Theoretically, some of the centrally-active synthetic inducers of CYP46A1 which do not inhibit HMGR likely will serve this particular purpose far better.

It is suggested that the use of low-dose, peripherally-acting statin drugs to improve hypercholesterolemia will be of benefit for reducing the risk for LOAD. Their use may also be of benefit to slow down the progression of LOAD or to improve its clinical symptoms. In this context, it is of note that vitamin D may also exert its beneficial effects on LOAD through reducing blood cholesterol levels.

Based on the proposed hypothesis, it is apparent that dietary or nutritional supplements that can safely supply or booster the synthesis of neuronal ATP and/or neuroactive metabolic intermediates (such as mevalonate and geranylgeraniol) will be of great potential in reducing the risk of LOAD. These food supplements may even be of use as an adjuvant therapy for treating LOAD.

Endogenous estrogens have long been considered a protective factor in AD. Recent studies have shown that some of the endogenous estrogen metabolites (e.g., 4-hydroxyestrone) which have very weak estrogenic activity are strong inhibitors of PDI, and they have strong neuroprotective effect against oxidative neuronal death through PDI inhibition. Besides, these estrogen metabolites also have strong cholesterol-lowering effects, which may jointly contribute to their overall benefits in LOAD. In this context, it is of note that recent studies have shown that bazedoxifene and raloxifene, two FDA-approved selective estrogen receptor modulators, are also strong inhibitors of PDI, and can strongly prevent oxidative neuronal death. These chemicals may hold promise for AD treatment and particularly prevention in women.

Lastly, it is of note that physical activity is associated with a drastically-reduced risk for LOAD. Studies have shown that physical activity is associated with heightened release of the neurotransmitters norepinephrine and dopamine in the CNS, which have a strong protective effect against oxidative neuronal death. In addition, physical activity may exert its beneficial effects on LOAD partly through reducing blood cholesterol levels.

10. Concluding Remarks

Few diagnoses in medicine are more dispiriting for patients and their families than AD. This disease, which causes insidious dissolution of one's most human qualities, namely, reasoning, abstraction, language and memory, now affects over 30 million individuals worldwide. Despite the fact that substantial consensus has been developed that certain biochemical changes in hippocampus and the association cortices occur many years before clinical symptoms, overall the study of AD is presently still fraught with mechanistic ignorance and therapeutic nihilism. Agreements on the temporal sequence of the molecular, biochemical and cellular events leading to dementia and which steps are most amenable to effective intervention have been difficult to achieve.

In this paper, a new hypothesis is developed, which speculates that in most cases of sporadic LOAD, the abnormally-elevated cholesterol level in brain neurons represents a crucial causative factor that drives the pathogenic processes of LOAD. Specifically, the elevated neuronal cholesterol will disrupt mitochondrial structure and metabolic activity, resulting in ATP deficiency as well as reduced formation of neuroactive metabolic intermediates along the cholesterol synthesis pathway in brain neurons. In addition, the abnormally-elevated neuronal cholesterol will cause direct neuronal damage as well as other pathogenic changes in the brain, including increased formation and aggregation of Aβ plaques and tauopathy as well as reduced formation of cholinergic vesicles. It is further hypothesized that Aβ accumulation and plaque formation in most LOAD cases only represent a characteristic secondary pathological change, and are usually not the dominant force that drives the pathogenesis of LOAD.

Genetic factors play a crucial role in the pathogenesis of sporadic LOAD. ApoE is now recognized as the most important genetic risk factor in sporadic LOAD. One of the best-characterized functions of the brain ApoE proteins is the delivery of astrocyte-derived cholesterol to neurons.

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Another crucial function of the brain ApoE proteins is their ability to remove excess neuronal cholesterol. It is hypothesized that the differences in ApoE's ability to efficiently remove excess neuronal cholesterol together with its differential ability to bind Aβ_{40} and Aβ_{42} jointly determines its pathogenic role in many LOAD cases. There are many good reasons for this assertion. First, alterations in ApoE's ability to efflux excess neuronal cholesterol will result in elevation of neuronal cholesterol, which suppresses mitochondrial metabolic activity, accompanied by reduced levels of ATP and neuroactive metabolic intermediates. These effects are detrimental to the normal functioning of neurons as well as their survival. Second, elevations of neuronal membrane cholesterol will lead to activation of the β-/γ-secreatases, resulting in increased formation of Aβ_{42} and Aβ_{40} peptides and subsequently amyloid plaque formation. Additionally, different ApoE isoforms have different ability to bind Aβ_{42} and Aβ_{40} peptides, which also affects amyloid deposition. Lastly, severe deficiency of neuronal ATP will reduce the production of cholinergic vesicles as well as the formation of tauopathy. These pathogenic effects jointly contribute to the development of LOAD. As discussed in considerable detail in this paper, the proposed pathogenic mechanism of AD offers a good explanation for the clinical observations of an elevated AD risk for the *APOE4* genotype, and a reduced risk for the *APOE2* genotype.

It is known that many other genetic risk factors also affect neuronal cholesterol homeostasis and thus contribute, directly or indirectly, to the pathogenesis of LOAD (discussed in ref. [96]). The relationship between these genetic factors and cholesterol dyshomeostasis is really intriguing, which offers additional support for a pivotal pathogenic role of neuronal cholesterol dyshomeostasis in LOAD.

Notably, brain neurons are among a group of cells in the body that have the highest demand for oxygen and energy supply, and adequate supply of cellular ATP in neurons is vital for normal cognitive function and memory formation. Significant reductions in mitochondrial ATP synthesis, which will lead to neuronal energy deficit, are believed to play a crucial role in driving the pathogenic process of all forms of AD. Accordingly, it is speculated that AD usually begins with neuronal injury in brain regions that likely have the highest demand for energy supply (ATP synthesis), and then gradually spreads to other brain regions with a relatively lower demand for energy supply, and eventually to the whole brain. In line with this suggestion, AD usually begins in the entorhinal cortex and then proceeds to the hippocampus, a waystation important in memory formation. As the hippocampal neurons degenerate, short-term memory falters. Often the ability to perform routine tasks begins to deteriorate as well. It then gradually spreads to other regions, particularly the cerebral cortex which is the outer area of the brain involved in functions such as language and reasoning. In the diseased regions, the neurons degenerate, lose their connections or synapses with other neurons, and some of the neurons die as a result.

Since the introduction of the amyloid hypothesis in 1991, it has attracted enormous research interest. It was postulated that Aβ_{42} can readily form aggregates which then initiate a pathogenic cascade ultimately resulting in neuronal loss and dementia. Over the years, there were many clinical and animal studies showing that the abundance of amyloid deposition and plaque formation often does not correlate with the severity of memory deficits or neuronal toxicity. As discussed in this paper, if we put aside the notion that *Aβ* plaque formation is a driving force in AD development, and if we add the neuronal cholesterol as a key component in the pathogenic process, then most of the puzzle pieces appear to fit together much better, i.e., the mental functional decline is mostly caused by cholesterol-induced deficits in ATP and neuroactive metabolic intermediates in brain neurons, whereas Aβ accumulation and plaque formation often only represent a secondary event, rather than the initial driving force in LAOD pathogenesis. As discussed in this paper, even in familial early onset AD cases, abnormal cholesterol buildup in brain neurons is still a key player in the pathogenic process.

It is known that increases in the content of cholesterol (particularly CEs) in neuronal membranes

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increase Aβ formation and deposition, and it is hypothesized that tau accumulation is mostly the result of neuronal ATP deficits. At present, it is difficult to precisely pinpoint which of these two events occurs earlier—it likely depends on the brain regions affected as they have different levels of energy demand. As elevated neuronal cholesterol levels can directly alter β-/γ-secretase activities, it is generally true that increased Aβ formation is usually seen very early on in most LOAD patients. Similarly, tau accumulation may also occur relatively early in selected brain regions where neurons have a particularly high demand for energy supply, and neuronal ATP deficiency resulting from elevated cholesterol will be preferentially experienced and thus will trigger the accumulation of tau. Overall, as mental functions are more closely associated with the degree of neuronal ATP deficiency, this is the main reason why mental functional decline often is better correlated with tauopathy rather than amyloid accumulation in the brain.

Chronic neuroinflammation is a salient feature of AD, which plays a crucial role in AD progression. It is speculated that neuroinflammation is largely caused by two major pathogenic changes: one is neuronal injury and death, which results from elevated cholesterol and reduced ATP synthesis in brain neurons. The other major cause is the accumulation of cholesterol in other brain cells, such as microglial cells, astrocytes and cells that are actively involved in the disposition of cholesterol-loaded, Aβ-bound ApoE particles. An increase in cholesterol levels in these cells is expected to cause mitochondrial dysfunction, energy deficiency, and cell death, which is one of the root causes for neuroinflammation.

At present, there is still no cure for AD. The available treatments offer relatively small symptomatic benefits, but do not address the underlying pathological process or substantially slow down clinical progression. As mentioned earlier, developing inhibitors of the β- and γ-secretases and Aβ-based immunotherapeutics has been a major research focus in the past few decades. However, in light of the new understanding that Aβ accumulation may only represent a secondary change in most LOAD cases, the expected benefit of β- and γ-secretase inhibitors or Aβ vaccines may be very limited for improving the cognitive functions of LOAD. Similarly, the expected benefit of tau inhibitors will also be limited given that tau aggregation is a consequence of severe neuronal ATP deficiency. If the situation of neuronal cholesterol elevation and ATP deficiency persist, even a significant reduction in amyloid plaque load and tau aggregation likely may still not fundamentally improve memory deficits in AD, not to mention the significant side effects associated with these treatments.

On the other hands, strategies aiming at reducing neuronal cholesterol levels may be of great benefits in AD treatment and prevention. For instance, the LXR agonists will activate the expression of ApoE, ABCA1 and ABCG1, resulting in enhanced neuronal cholesterol efflux. CYP46A1 inducers may also have a similar effect. Theoretically, the centrally-active synthetic inducers of CYP46A1 which do not directly inhibit the HMGR would serve this particular purpose much better. Similarly, it is suggested that the use of low-dose, peripherally-acting statins which aim to improve hypercholesterolemia would also be of some benefit for reducing the risk for LOAD. Their use may also be of benefit to slow down the progression of LOAD or improve its clinical symptoms. Based on the proposed hypothesis, it is apparent that dietary or nutritional supplements that can safely booster the synthesis of neuronal ATP and/or neuroactive metabolic intermediates (such as mevalonate and geranylgeraniol) will be of great potential as adjuvant therapies in reducing the risk of developing LOAD. Additionally, recent studies have found that some of the endogenous estrogen metabolites (e.g., 4-hydroxyestrone) which have very weak estrogenic activity are strong inhibitors of PDI and have a strong neuroprotective effect against oxidative neuronal death. Furthermore, these estrogen metabolites also have a strong cholesterol-lowering effect, which may jointly contribute to their overall benefit in LOAD patients. Lastly, it is of note that physical activity is associated with a drastically-reduced risk for LOAD. Physical activity may exert its beneficial effects on LOAD partly through reducing blood cholesterol, in addition to increasing the release of brain neurotransmitters dopamine and norepinephrine, which have a strong protective effect against oxidative neuronal death

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through inhibition of neuronal PDI.

In closing, it is of interest to note that during the initial characterization of AD pathology by Dr. Alzheimer, he also noted the accumulation of "adipose inclusions," likely neutral lipids, in glial cells from postmortem brain samples of patients with dementia (discussed in [733]). The presently-proposed cholesterol-centered hypothesis on AD pathogenesis may finally shed a dawning light on the initial careful observations made by Dr. Alzheimer concerning the potential pathogenic roles of the adipose-like inclusions in various brain cells of dementia patients.

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Abbreviations

The following abbreviations are used in this manuscript:

AD	Alzheimer's disease
Aβ	amyloid β
Aβ_{40}	Aβ fragment containing 1–40 amino acid residues
Aβ_{42}	Aβ fragment containing 1–42 amino acid residues
APP	amyloid-β precursor protein
AICD	APP intracellular domain
ApoE	apolipoprotein E
BACE1	β-site APP cleaving enzyme
ApoJ	apolipoprotein J
CNS	central nervous system
HMGR	HMG-CoA reductase
ATP	adenosine triphosphate
ACAA	acetyl-CoA acyltransferase
SOAT1	sterol *O*-acyltransferase 1 (also called ACAT1)
ACAT1	acyl-CoA:cholesterol acyltransferase
LCAT	lecithin:cholesterol acyltransferase
SREBP2	sterol-dependent transcription factor
ER	endoplasmic reticulum
LDL	low-density lipoprotein
LDLR	LDL receptor
VLDL	very low-density lipoprotein
VLDLR	VLDL receptor
HDL	high-density lipoprotein

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LRP1	LDL receptor-related protein 1
ApoER2	ApoE receptor 2
NPC1 or NPC2	Niemann-Pick type C1 or C2, respectively
NPC disease	Niemann-Pick type C disease
ABCA1	ATP binding cassette transporter A1
StAR protein	steroidogenic acute regulatory protein
STARD3	StAR-related lipid transfer protein domain 3
CYP	cytochrome P450
24*S*-OHC	24*S*-hydroxycholesterol
LXR	liver X receptor
RXR	retinoid X receptor
CSF	cerebrospinal fluid
MAP	microtubule-associated protein
NFT	neurofibrillary tangle
ChAT	acetyl-CoA:choline *O*-acetyltransferase
AChE	acetylcholinesterase
BBB	blood-brain-barrier
TREM2	the triggering receptor expressed on myeloid cells 2
DAM phenotype	disease-associated microglia phenotype
NSAIDs	nonsteroidal anti-inflammatory drugs
BDNF	brain-derived neurotrophic factor
IGF-1	insulin-like growth factor
VEGF	vascular endothelial growth factor
NOS	nitric oxide synthase
PDI	protein disulfide isomerase

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