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LIPIDS & BRAIN IV: LIPIDS IN ALZHEIMER'S DISEASE

REVIEW

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Topical Issue

Cholesterol and ApoE in Alzheimer's disease

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Abstract – Genetic, neuropathological and biochemical studies suggest strong links between cholesterol, the apolipoprotein E (APOE) and Alzheimer's disease (AD), both in humans and in animal models of the disease. From the literature and our work, we can predict that transient increase of the levels of cholesterol at the membrane of neurons would profoundly affect the processing of the transmembrane Amyloid Precursor Protein (APP) by triggering its clathrin dependent endocytosis and the resulting production of amyloid-β (Aβ) peptides. Here, we will review these data together with structural and molecular dynamic studies that characterized the role of cholesterol on APP conformation and positioning at the membrane. Specifically decreasing brain cholesterol or replacing it with plant sterols crossing the blood brain barrier appear like promising strategies to either delay or counteract the development of sporadic AD.

Keywords: Alzheimer's disease / cholesterol / Apo lipoprotein E / endosome / amyloid

Résumé – **Cholesterol et ApoE dans la maladie d'Alzheimer.** Des études génétiques, neuropathologiques et biochimiques suggèrent des liens étroits entre le cholestérol, l'apolipoprotéine E (APOE) et la maladie d'Alzheimer (MA), chez l'homme et dans des modèles animaux de la maladie. Les études publiées et nos travaux nous permettent de prédire qu'une augmentation transitoire des taux de cholestérol à la membrane des neurones affecterait profondément le clivage de la protéine transmembranaire précurseur de l'amyloïde (APP) en déclenchant son endocytose dépendante de la clathrine et la production résultante de peptides amyloïdes β. Dans cette revue nous colligeons ces données ainsi que des études de dynamique structurale et moléculaire modélisant le rôle du cholestérol sur la conformation APP et son positionnement à la membrane. La diminution spécifique du cholestérol cérébral ou son remplacement par des phytostérols traversant la barrière hémato-encéphalique apparaissent comme des stratégies prometteuses pour retarder ou contrecarrer le développement de la MA sporadique.

Mots clés : maladie d'Alzheimer / cholestérol / Apolipoproteine E / endosome / amyloïde

1 Introduction

Lipids are essential for brain function. Brain is one of the tissues with the highest lipid content, with up to 60% of dry matter. Amongst the various classes of lipids involved, such as fatty acids, phospholipids and cholesterol, membrane of neurons are particularly enriched in fatty acids and complex lipids.

Cholesterol is the major component of cellular membranes. Its insertion between phospholipids forming the membrane bilayer – composed of the internal leaflet facing the cytoplasm and the external leaflet isolating the cell from the extracellular environment – ensures the structure and stability of the membrane. Cholesterol fluidifies the membrane, thus avoiding crystallization of fatty acids. It also decreases membrane permeability to hydrosoluble molecules. In the membranes, cholesterol is enriched in lipid rafts, which are essential nanometric domains involved in anchoring functional molecules such as membrane receptors (Simons and Toomre, 2000; Simons and Gerl, 2010). In neurons, cholesterol is highly enriched in myelin sheaths and allows the propagation of nerve impulses by saltatory mechanisms.

Alzheimer's disease (AD) is characterized by two abundant lesions, namely the extracellular amyloid plaques and the intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein (Duyckaerts et al., 2009). Amyloid plaques are mainly composed of amyloidβ peptides

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(Aβ) produced by processing of the transmembrane Amyloid Precursor Protein APP by two sequential cleavages operated by the βsecretase BACE1 and the γsecretase acting in the membrane bilayer (Selkoe and Hardy, 2016). The amyloidogenic APP processing takes place in the endosomal compartment after internalization of APP where the environmental pH is suitable for enzyme activities (De Strooper and Annaert, 2010).

A large body of evidence from genetic, neuropathological and biochemical studies suggest strong links between cholesterol and AD, both in humans and in animal models of the disease (Cossec *et al.*, 2010a).

2 Genetic risk factors for AD involved in cholesterol homeostasis

At least two genetic risk factors for AD are directly involved in cholesterol homeostasis (Lambert *et al.*, 2013). The ε4 allele of the apolipoprotein gene *APOE* is 2.7 fold more frequent in AD patients (36.7%) as compared to the general population (13.7%) while the frequency of the ε3 allele is lower (59.4%) in AD patients as compared to the general population (77.9%) (Liu *et al.*, 2013). Interestingly the ε2 allele is more frequent (8.4%) in the general population than in AD patients (3.9%). Isoform specific amino acid differences lie at codons 112 and 158 with ApoE4 bearing two Arg, ApoE2 two Cys and ApoE3 a Cys at codon 112 and an Arg at codon 158. *APOE* ε4 non-carriers have AD frequency of 20% with mean age of clinical onset at 84 while *APOE* ε4 heterozygous and homozygous are at 47% or 91%, with mean age of onset of 76 or 68, respectively (Liu *et al.*, 2013). Thus the *APOE* ε4 has a very significant effect both on frequency and age of onset of AD. Recently we found that in a cohort of 318 participants aged 70–85 years with subjective memory complaints but unimpaired cognition and memory, 20% carried at least one ε4 allele. When stratified according to their brain amyloid load assessed by positron emission tomography (PET), 38% were ε4 among participants with Aβ deposition while the percentage dropped to 13% among participants with no Aβ deposition (Dubois *et al.*, 2018). The *APOE* ε4 allele remains the highest genetic risk factor for AD. Besides, genetic meta-analysis identified another risk factor involved in cholesterol homeostasis: the ATP binding cassette ABC transporter 7 (*ABCA7*) gene. Intriguingly, *ABCA7* has one of the strongest association with amyloid deposition, exceeded only by *APOE* ε4 (Apostolova *et al.*, 2018).

3 Neuropathological studies showing increased cholesterol levels in the brain of AD patients

A number of converging studies point towards an increase in cholesterol levels in human autopsy brains of AD patients, compared to age-matched unaffected controls. Evidence from biochemical studies suggested membrane-associated oxidative stress, resulting in perturbed cholesterol metabolism and increased cholesterol levels in the middle frontal gyrus of AD patients (Cutler *et al.*, 2004). Of note, the same authors also demonstrated that the increase in membrane-associated

cholesterol correlated with the severity of cognitive deficits (Cutler *et al.*, 2004). An independent biochemical and histochemical study also showed that cholesterol levels are increased in the occipital cortices of AD patients (Xiong *et al.*, 2008). A liquid chromatography coupled with mass spectrometry approach identified twice as much cholesterol in laser microdissected-Aβ deposits from the isocortex of AD patients compared to the adjacent neuropil (Panchal *et al.*, 2010). Using time-of-flight secondary ion mass spectrometry imaging on cortical human sections revealed that the cholesterol signal was significantly higher in the cortical layers III and IV of AD samples compared to controls (Lazar *et al.*, 2012).

4 Biochemical evidence linking cholesterol, APP and its cleaving enzymes

Cholesterol in the adult brain is produced by astrocytes that can either recapture it from the extracellular compartment or synthesize it using their specific enzyme machinery (Shobab *et al.*, 2005). Synthetized free cholesterol then binds to APOE and is transported across the cell membrane *via* the ABCA1 transporter. Lipoparticles will bind to low density lipoprotein receptor-related protein 1 (LRP1) present at the neuronal membrane and be internalized in the endolysosomal compartment where APOE is degraded and free cholesterol released and relocated into cholesterol enriched membrane domains. APP, BACE1 and the γsecretase protein complex are all localized in lipid rafts, suggesting a role of cholesterol in their processing activities (Bouillot *et al.*, 1996; Cordy *et al.*, 2003; Marquer *et al.*, 2011). Indeed, an increase in cholesterol promoted γsecretase activity and amyloid peptides production (Matsumura *et al.*, 2014). Other studies demonstrated that the palmitoylation of APP regulates its distribution in lipid rafts and its amyloidogenic processing (Bhattacharyya *et al.*, 2013). The palmitoylation of BACE1 also regulates its enrichment in lipid rafts and although it does not affect its steady-state processing activity, it controls the synaptic activity-induced production of Aβ (Vetrivel *et al.*, 2009; Andrew *et al.*, 2017).

5 Structural evidence for a role of cholesterol on APP structure and dimerization at the membrane

Although APP is a transmembrane protein that is present in cholesterol enriched domains, its sequence does not contain any cholesterol recognition amino acid consensus sequence (CRAC) or inverted consensus sequence CARC (Fantini *et al.*, 2016). However, a binding site for cholesterol on APP C-terminal fragment (βCTF) originating from the cleavage of APP by BACE1, has been predicted from structural studies using nuclear magnetic resonance (NMR) (Barrett *et al.*, 2012). It was shown that the transmembrane and the juxtamembrane domains of βCTF are important for binding cholesterol, and molecular modelisation predicted that the transmembrane region of APP is a flexibly curved αhelix (Barrett *et al.*, 2012). In addition, binding of cholesterol depends on the charge state of two amino acids (glutamic acid and aspartic acid at positions 22 and 23 of Aβ sequence, respectively) present in the juxtamembrane domain of APP. At

low pH, these amino acids are neutral and bind cholesterol, while at higher pH, they are negatively charged and inserted deeper in the membrane (Panahi *et al.*, 2016).
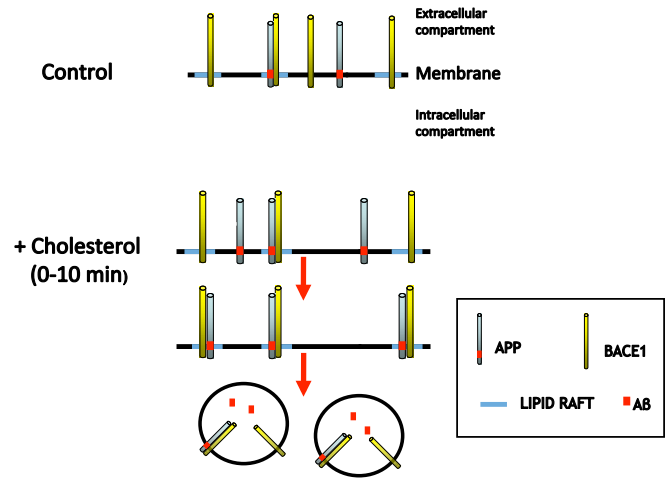
A competition between binding of APP to cholesterol and its dimerization has been suggested (Song *et al.*, 2013), as similar portions of APP are involved in both functions. Indeed, initial studies with Aβ peptides showed that Aβ 29–42 is tilted and forms dimers in the membrane (Pillot *et al.*, 1996). Moreover, dimerization of APP has been found to be due to the presence of GxxxG motifs in the transmembrane region (Kienlen-Campard *et al.*, 2008). More precisely, the trans-membrane motif $G_{33}XXXG_{37}$ and the juxtamembrane motif G25-SNK-G29 (numbering of Aβ sequence) facilitate the dimerization of APP and cholesterol helps to stabilize this interface (Tang *et al.*, 2014). Another study showed that APP dimerization is 5 times higher than with the CTF fragment, highlighting the important role of the APP extramembrane domain (Kaden *et al.*, 2008; Ben Khalifa *et al.*, 2012; Decock *et al.*, 2015; Nierzwicki and Czub, 2015). Modelisation studies showed that the transmembrane helix of APP self-associates in left-handed parallel dimer while the juxtamembrane helix senses dimerization (Nadezhdin *et al.*, 2012). Of note, the dimerization motif GxxxG is not sufficient for cholesterol binding and the juxtamembrane domain is required (Nierzwicki and Czub, 2015). Finally, a recent study showed that the transmembrane motif $V_{39}XXA_{42}T_{43}XXV_{46}XXXV_{50}$ is involved in the dimerization of C99 and binds lipid rafts (Sun *et al.*, 2017).

6 The role of cholesterol on APP endocytosis and processing

Since APP is a transmembrane protein that can interact with cholesterol, and cholesterol levels are increased in AD, we studied the role of cholesterol on APP processing and amyloid pathology. We used methyβcyclodextrin (MBCD) to decrease cholesterol and MBCD-cholesterol complex to deliver cholesterol locally at the plasma membrane of living cells. Indeed, molecular dynamic studies have shown that MBCD can extract cholesterol from the plasma membrane (Lopez *et al.*, 2011). We chose not to modulate the activity of cholesterol synthesis enzymes, such as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), because of strong compensatory effects that take place when knocking down *HMGCR* (data unpublished).

We demonstrated that, in HEK293 cells, membrane cholesterol levels control APP endocytosis and this effect is dependent on clathrin, the small GTPase Rab5 and dynamin2 (Cossec *et al.*, 2010b). Dynamin2 is associated with clathrin-coated pit maturation and fission of the membrane neck of coated pits. Rab5 regulates membrane fusion between early endosomes and their maturation through interaction with phosphatidylinositol 3-phosphate (PtdIns3P) and the early endosomal antigen 1 (EEA1). We found that the effect of cholesterol on APP endocytosis was inhibited by either transfecting dominant negative mutants of Rab5 or dynamin2 that cannot be activated, or by treating cells with siRNA against clathrin (Cossec *et al.*, 2010b).

Using fluorescence activated cell sorting (FACS), we confirmed that APP endocytosis is promoted by cholesterol

Fig. 1. Effects of artificial increase of cholesterol at the cellular plasma membrane.

In the control condition, APP and BACE1 are present inside and outside lipid rafts at the plasma membrane. Raising cholesterol at the plasma membrane induces: i) relocalization of APP and BACE1 in lipid rafts and their rapid internalization assessed by Fluorescence correlation Spectroscopy in enlarged and clustered endosomes (Marquer *et al.*, 2011; Marquer *et al.*, 2014); ii) increase of APP and BACE1 proximity assessed by Total Internal Reflection Fluorescence-Fluorescence Lifetime Imaging-Förster Resonance Energy Transfer (TIRF-FLIM-FRET) microscopy (Marquer *et al.*, 2011); iii) increase of production of A peptides (Cossec *et al.*, 2010a; Cossec *et al.*, 2010b; Marquer *et al.*, 2014).

increase in a dose-dependent manner (Cossec *et al.*, 2010b). To explore the specificity of the effect of cholesterol levels on internalization, we tested it on the cannabinoid 1 (CB1) and the transferrin receptors, and found that an increase of cholesterol at the plasma membrane only slightly promoted their internalization. In contrast, APP endocytosis was much more sensitive to the levels of cholesterol. In addition, we found that cholesterol treatment increased APP internalization in endo-somes that appeared larger than in untreated cells. There was a significant increase of Aβ40 and Aβ42 secretion in HEK293 cells transfected with APP bearing the Swedish mutations and treated with MBCD-cholesterol complex. This effect was dependent on the extent of the cholesterol increase at the plasma membrane and on APP internalization, as demonstrated by clathrin knock-down experiments (Cossec *et al.*, 2010b). A similar effect of cholesterol on endogenous Aβ42 secretion was reproduced in primary rat hippocampal neurons, although, in this case, Aβ40 was unchanged and Aβ38 was decreased (Marquer *et al.*, 2014).

Increase of Aβ secretion following cholesterol treatment could be due to either an effect on BACE1 activity or to a facilitation of APP-BACE1 proximity. After showing that induced cholesterol increase at the plasma membrane had no effect on BACE1 catalytic activity, we thought to study the interaction of APP and BACE1 at the plasma membrane of primary neurons in culture (Marquer *et al.*, 2011). Rat hippocampal neurons were transfected with APP-mCherry and BACE1-GFP constructs and imaged using Fluorescence Lifetime Imaging Microscopy (FLIM). When the distance

between the two proteins (BACE1-GFP donor and APP-mCherry acceptor) is below 10 nm, Förster Resonance Energy Transfer (FRET) can occur and the fluorescence lifetime of the donor decreases. By Total Internal Reflection Fluorescence (TIRF) imaging, we could demonstrate that increase of cholesterol favors APP-BACE1 proximity at the plasma membrane. We then wondered whether this happened in specific regions of the plasma membrane enriched in cholesterol (lipid rafts). We used Fluorescence Correlation Spectroscopy (FCS) to measure the diffusion coefficients of APP-YFP molecules (Marquer *et al.*, 2012). We showed that about 7 min after cholesterol treatment, APP-YFP relocalized to lipid rafts where diffusion time is higher as compared to non-raft portions of the plasma membrane (Marquer *et al.*, 2011). This peak was followed by an extinction of fluorescence signal attributed to the internalization of APP-YFP, since this fluorescence decrease was inhibited by dynasore treatment and corresponded to the time of internalization through the clathrin-dependant pathway. Taken together, our microscopy studies strongly suggest that, in normal conditions, APP and BACE1 are distributed inside and outside of lipid rafts. However, less than 10 min after treatment with MBCD-cholesterol, APP and BACE1 are relocalized in lipid rafts that have either higher levels of cholesterol or expand their size with similar levels of cholesterol per surface unit. This event is rapidly followed by internalization in early endosomes that have greater size (Fig. 1).

Altogether, we show that modulating cholesterol levels to the extent of what is observed in human AD autopsy brains (+ 30%) results in increased APP and BACE1 proximity and in their endocytosis in enlarged early endosomes, leading to the overproduction of Aβ peptides. This enlarged endosome phenotype is reminiscent of what has been described in pyramidal cells from postmortem human brain.

7 Increasing neuronal cholesterol levels recapitulates AD phenotypes

We also explored whether, in addition to its effect on Aβ levels, increasing cholesterol could also lead to other cellular phenotypes reminiscent of AD. As previously mentioned, one of the earliest phenotypes of human AD is the enlargement of early endosomes (Cataldo *et al.*, 2000). We found that treatment of primary rat hippocampal neurons with MBCD-cholesterol to reach a 30% increase, induced a change in the endosomal compartment with an increase in the mean volume of early endosomes, as assessed by confocal microscopy (Marquer *et al.*, 2014). Using transmission electron microscopy, we found that not only the surface of early endosomes was increased but they also formed clusters. Another key feature of AD dysfunction is axonal transport deficits (Dai *et al.*, 2002). We thus analyzed axonal transport of APP. Hippocampal neurons were transfected with APP-mCherry and treated with MBCD-cholesterol for 20–35 min and APP-mCherry containing vesicles were followed using live imaging. We found a very significant decrease of APP anterograde transport after cholesterol increase, while retrograde transport remained unchanged (Marquer *et al.*, 2014). Finally and in order to find out whether cholesterol treated neurons had a similar transcripts signature as compared to neurons from postmortem human brain samples, we compared the transcriptome of treated neurons to the ones from brain samples at different Braak stages of AD. Hierarchical clustering analysis highlighted that gene expression profile of cholesterol-treated neurons were similar to early AD stages, suggesting that cholesterol increase could be an early phenotype during the course of the disease, either occurring as a primary or secondary event (Marquer *et al.*, 2014). Among the genes differentially expressed following cholesterol increase, Gene Ontology category enrichment analysis revealed that cholesterol pathway, response to lipid, sterol biosynthetic pathways were the most enriched categories.

Thus, increase in neuronal membrane cholesterol promotes APP processing and endocytosis, enlargement of early endosomes and axonal transport abnormalities, inducing gene expression modifications similar to the ones detected in early stages of AD. Changes in membrane cholesterol linked with age could thus be a starting event triggering sporadic AD.

This postulate based on evidence collected in neuronal cultures is supported by *in vivo* studies. For example, inhibiting CYP46A1, the enzyme that hydroxylates cholesterol at position 24, allowing its passage through the blood brain barrier and the decrease of cholesterol levels in the brain, worsens AD phenotypes. Specifically, CYP46A1 inhibition leads to brain cholesterol accumulation, endoplasmic reticulum stress, increase of βCTF and Aβ production, tau hyperphosphorylation and hippocampal atrophy linked to neuronal death, supporting that high neuronal cholesterol could induce or accelerate the course of sporadic AD (Djelti *et al.*, 2015). In addition, crossing APP/PS1 mice with mice overexpressing sterol regulatory element-binding protein-2 (SREBP2) induced mitochondrial cholesterol accumulation, oxidative damage and Aβ accumulation as well as, very interestingly, tau phosphorylation and neurofibrillary tangle formation that had never been detected before in any mouse model reproducing the amyloid pathology (Barbero-Camps *et al.*, 2013).

8 Towards decreasing neuronal membrane cholesterol to delay/counteract sporadic AD

Specifically decreasing neuronal cholesterol thus appears like a promising strategy to either delay or counteract the development of sporadic AD. Several studies in mouse models convincingly support this hypothesis. Injection of adenoviral constructs expressing CYP46A1 in mouse models of AD reproducing either the amyloid or the tau pathologies improves cognitive function and reduces amyloid pathology as well as tau hyperphosphorylation (Hudry *et al.*, 2010; Burlot *et al.*, 2015). CYP46A1 knock-out mice have severe memory deficits (Kotti *et al.*, 2006) while older transgenic mice overexpressing CYP46A1 show improved cognitive performance (Maioli *et al.*, 2013). In addition, treatment of AD mice overexpressing APP with the Swedish mutations with hydroxypropyl-β-cyclodextrin reduced the number of amyloid plaques, increased the expression of genes involved in cholesterol transport and Aβ clearance, and improved spatial learning and memory deficits (Yao and Papadopoulos, 2002), thus showing clear neuroprotective effects.

Finally replacing membrane cholesterol with stigmasterol, a plant sterol crossing the blood brain barrier, decreased APP processing and the production of Aβ peptides. Mice under stigmasterol-rich diet had decreased amyloidogenic APP processing (Burg *et al.*, 2013). On the contrary, high cholesterol diet in a transgenic mouse model expressing Swedish and London mutations disturbs the integrity of the BBB, increases Aβ in the brains and decreases Aβ in the plasma of these mice (Loffler *et al.*, 2016).

In total, results from these studies, using various strategies to decrease neuronal cholesterol, converge towards the conclusion that lowering cholesterol levels in the brain is indeed beneficial and alleviates an array of AD phenotypes.

9 Conclusions

Since the identification of the cholesterol transporter APOE ε4 haplotype as the highest genetic risk factor for AD (Mayeux *et al.*, 1993), much has been done to understand the role of cholesterol metabolism in the pathogenesis of AD (Allinquant *et al.*, 2014). We deliberately omitted all the work performed with statins, since most of the clinical and molecular studies have not been conclusive so far (Yasar and Whitmer, 2018). A significant body of work points to a deleterious role of neuronal cholesterol in the development of sporadic AD. Amongst these, molecular studies and new microscopies allowing the analysis of sub compartments of the plasma membrane provided definitive evidence of this role. Measuring *in situ* cholesterol levels in *postmortem* human brain samples remains a challenge. Without any selective antibodies, we are left with very few markers that remain extremely difficult to utilize. Some therapies have been tested preclinically in mouse models of AD but much remains to be done in clinical research to validate possible targets and food supplements in order to delay or counteract sporadic AD. Finally, a more precise molecular analysis of the APP cholesterol binding site and the important yet misunderstood interplay with APOE genotype might bring new insights into the field and pave the way to unprecedented therapeutic avenues.

Disclosure

The authors disclose any conflict of interest.

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