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Ceramide and Cholesterol: Possible Connections Between Normal Aging of the Brain and Alzheimer's Disease. Just hypotheses or molecular pathways to be identified?

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Abstract Even though it is known that aging is the single most important risk factor for Alzheimer's disease (AD), there is a lack of information on the molecular pathway(s) that connect normal aging of the brain to this form of neuropathology. Because of the rise in average lifespan, the number of individuals that reach the seventh or eighth decade of life and become at high risk for AD is rapidly increasing. Current estimations predict that by 2050 about 45 to 50 million individuals will be affected by AD worldwide. Here, we discuss the need for more age-directed research to understand AD neuropathology. We also elaborate on the possible role of cholesterol and ceramide as molecular connections between aging and AD, and as novel therapeutic targets for the prevention of late-onset AD.

Keywords: Alzheimer's disease; Aging; Ceramide; Cholestrol

1. Introduction

Alzheimer's disease (AD) represents the most common cause of dementia, affecting as many as 15 million individuals worldwide. It is characterized by progressive memory deficits, cognitive impairments, and personality changes accompanied by diffuse structural abnormalities in the brain. The symptoms of the disease include memory loss, confusion, impaired judgment, personality changes, disorientation, and loss of language skills.

Based on the onset of the symptoms, AD is normally divided into 2 groups: early onset (<60 years) and late onset (>60 years). Early-onset AD accounts for approximately 3% of all AD cases and has so far been linked to mutations in the genes for the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) (for review, see [1]). Late-onset AD (LOAD) accounts for about 97% of AD cases and has been associated with both environmental and genetic risk factors. Among the environmental factors, hypercholesterolemia, atherosclerosis, and history of head trauma or stroke seem to be the most important. Among the genetic risk factors, more that 45 putative polymorphisms have been reported so far, but only 1, the ϵ4 allele of the apolipoprotein gene (*APOE*-ϵ4) on chromosome 19 [2], has been consistently found to be associated with AD in several independent studies [3].

The pathologic and histologic hallmarks of AD include extracellular protein deposits termed *senile* (or *amyloid*) *plaques*, neurofibrillary tangles, and amyloid angiopathy accompanied by diffuse loss of neurons and synapses in the neocortex, hippocampus, and other subcortical regions of the brain. The dominant component of the plaque core is the $\beta-$amyloid peptide (Aβ) organized in fibrils of approximately 7 to 10 nm intermixed with nonfibrillar forms of this peptide. The most characteristic form of the amyloid plaque is normally referred to as the "neuritic plaque," in which the dense core of aggregated fibrillar Aβ is surrounded by dystrophic dendrites and axons, activated microglia, and reactive astrocytes. However, in addition to the classical neuritic plaque, diffuse deposits of Aβ (probably a prefibrillary form of the aggregated peptide) are also found without any surrounding dystrophic neurites, astrocytes, or

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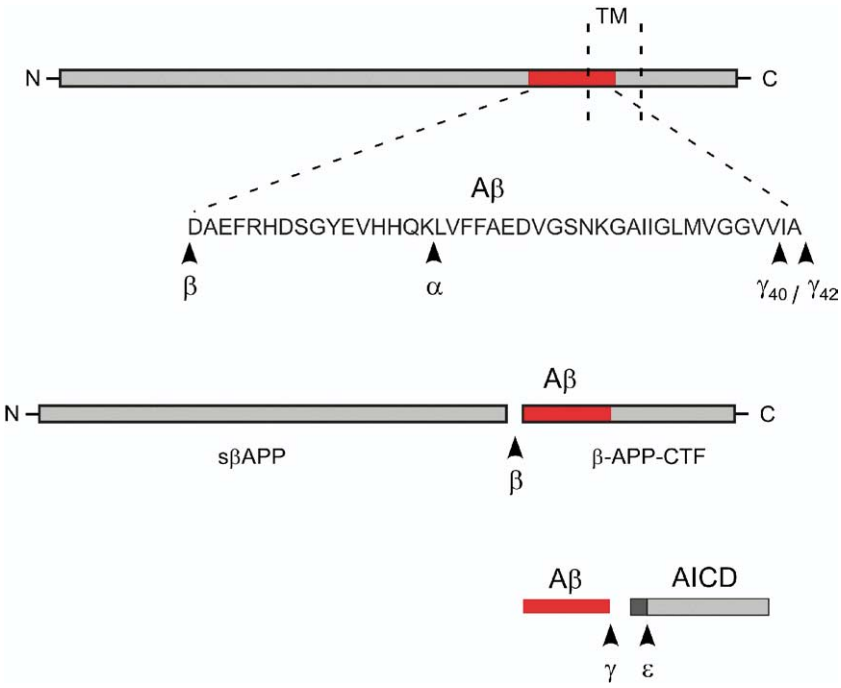

Fig. 1. Schematic view of Aβ generation from APP. APP is a type 1 membrane protein with a large extracellular domain, a single transmembrane domain (TM), and a short cytosolic tail. The Aβ region of APP (in red) includes the first 12 to 14 amino acids of the membrane domain. Aβ generation requires the sequential proteolysis of APP at β and γ sites. β-cleavage occurs at the N-terminus of the Aβ region of APP, generating a large N-terminal fragment (sβAPP) that is rapidly secreted into the extracellular milieu, and a small C-terminal fragment (β-APP-CTF) of 99 amino acids. This event represents the rate-limiting step for the biosynthesis of Aβ, is carried out by BACE1 (a membrane-spanning aspartyl protease), and activates or allows a second cleavage in the membrane domain of APP by the γ-secretase multimeric complex, which generates Aβ. Further cleavage at the ϵ site liberates the signaling active APP intracellular domain (AICD). The α-/γ-pathway is activated by α-cleavage of APP between amino acids 16 and 17 of the Aβ region and generates the secreted N-terminal fragment (sαAPP) and AICD. This pathway precludes the generation of Aβ and liberates a truncated form of APP (sαAPP) that shows neurotrophic activity in culture.

microglia. These plaques appear diffuse—hence the name *diffuse plaques*—and can be found in limbic and association cortices as well as in the cerebellum (where the classical neuritic plaque is almost always absent).

The common pathogenic event that occurs in all forms of AD is the abnormal accumulation of Aβ in the form of amyloid plaques or amyloid angiopathy. In the case of familial AD (FAD), the accumulation is mostly the result of increased production of a specific 42-amino-acid isoform of Aβ ($A\beta_{42}$) that accelerates the aggregation and accumulation of total Aβ into amyloid fibrils. Exceptions are the APP "Swedish" mutation, which elevates total Aβ levels, and trisomy 21 (Down syndrome), where a third copy of the *APP* gene leads to an increased production of total Aβ. In contrast to FAD, the exact mechanisms that lead to the accumulation of Aβ in LOAD are not completely known, but they most likely involve disturbances in the rate of production, clearance, and aggregation of Aβ. Additional information on the mechanisms involved in the abnormal accumulation of Aβ in early- and late-onset AD can be found elsewhere [4–6].

Aβ is a 39- to 43-amino-acid hydrophobic peptide proteolytically produced from a much larger transmembrane precursor, APP (Fig. 1). APP consists of 695 to 770 residues, APP_{695}, APP_{751} and APP_{770} being the most common forms expressed in the brain [7]. They all originate from alternatively spliced mRNAs transcribed from a single gene. APP is a type 1 glycoprotein with its amino terminus on the extracellular surface, a single ~23-residue transmembrane domain and a short cytoplasmic tail (Fig. 1). During its "life-cycle," APP undergoes specific endoproteolytic cleavage: first at the N-terminus of the Aβ region (β-cleavage) and then in the transmembrane domain of APP (γ-cleavage). This proteolytic pathway generates a large NH_2-ectodomain, Aβ, and a signaling active intracellular domain (AICD) (Fig. 1). Additionally, APP can be cleaved between amino acids 16 and 17 of the Aβ region (α-cleavage), producing a small 3-kDa Aβ fragment, which does not aggregate in amyloid plaques. The majority of APP is normally cleaved along the α-, rather than β-, pathway (for review, see [5,7]). The C-terminal fragments of APP, produced by β- and α-cleavage are called β- and α-APP-CTFs, respectively.

The amyloidogenic pathway (or β-pathway) involves the sequential recruitment of 2 enzymes: β-secretase, also called BACE1 (for β-site APP cleaving enzyme) [8–11], and γ-secretase, a multimeric protein complex containing presenilin, nicastrin, Aph-1, and Pen-2 [12–15]. γ-Secretase

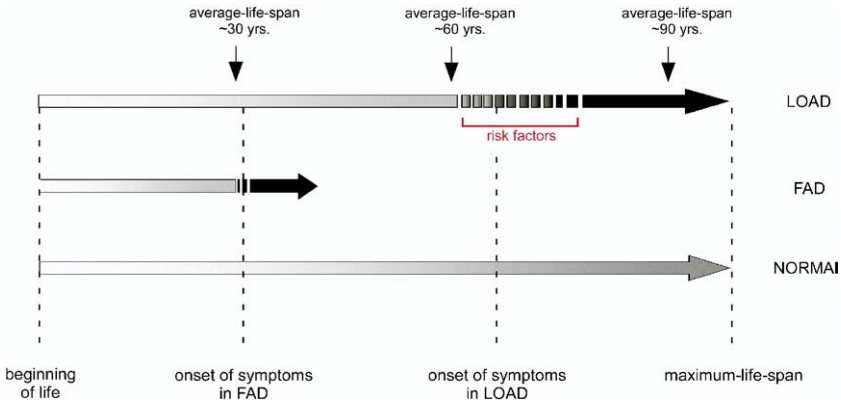

Fig. 2. Schematic view of AD as part of the general programming of aging. The upper arrow depicts normal aging as the product of continuous changes from white (no risk) to different levels of gray (risk), finally converging to a pathologic form of aging—in this case AD—(black part of the arrow). By hypothetically expanding the average lifespan from about 30 to about 90 years, we would expect a dramatic increase in the number of individuals that reaches the black part of the arrow. Indeed, the change in average lifespan observed in many countries is rapidly inducing a marked increase in the incidence of LOAD. In this regard, it is worth remembering that maximum lifespan differs from average lifespan. In fact, in contrast to average lifespan, which can be prolonged by improving environmental conditions, maximum lifespan is increased by actually decreasing the rate of aging. Even though we have experienced a dramatic increase in average lifespan during the last few centuries, our maximum lifespan has not been considerably affected. The transition from normal aging (white–gray part of the arrow) to pathologic aging (black part of the arrow) can be affected —either delayed or accelerated—by both genetic and environmental risk factors (broken part of the arrow). In the case of FAD, the transition from normal to pathologic aging of the brain is suddenly achieved by the gain or loss of function of 1 or more genes (second arrow). The broken vertical lines indicate the average onset of symptoms in both FAD and LOAD, and the theoretical maximum lifespan of humans. The prevalence of AD in individuals that are in the ninth decade of life does not reach 100%, indicating that a successful normal aging—in the absence of any sign of dementia—can be achieved. These individuals do undergo age-associated changes but never experience the transition from normal to pathologic aging (lower arrow).

usually cleaves APP either at position 40 or 42 of the Aβ region generating Aβ_{40} and Aβ_{42}, respectively (Fig. 1). Although Aβ_{40} is more prevalent (75% to 90% of secreted Aβ), Aβ_{42} aggregates far more rapidly into amyloid fibrils and is more toxic. The first enzymatic event (β-cleavage) represents the rate-limiting step for the generation of Aβ and probably induces a change in conformation or a shift of the β-APP-CTF outside the lipid bilayer, which allows the subsequent γ-cleavage.

2. Alzheimer's Disease and Aging

Late-onset AD is a complex and heterogeneous disease and, together with other common disorders (eg, cardiovascular disease, diabetes, and cancer), is also one of the most common age-related diseases. Aging itself is the single most important risk factor for LOAD. Because of the shift in life expectancy, it is estimated that in 2050 about 25% of the population in the western world will be older than 65 years, one third of whom are likely to experience LOAD. By that time, the total number of patients suffering from AD worldwide is expected to be about 45 to 50 million. Considering that the lifetime cost of care for an individual with Alzheimer's is between US$150,000 and $200,000, it is not difficult to foresee the tremendous economic burden.

Both the prevalence and incidence of LOAD increase progressively without a plateau. The prevalence of LOAD doubles with every decade after the age of 60 and reaches about 50% among individuals older than 85 years [16]. Considering the lifespan of our general population, the prevalence and incidence of LOAD, and the average duration of the disease, it can be predicted that a simple delay of 5 years in the onset of the symptoms would reduce the number of patients affected by AD by about 50%. The tremendous increase in the incidence of LOAD that we are experiencing is almost exclusively explained by the increase in average lifespan of the population and, therefore, in the number of individuals reaching the seventh and eighth decade of life (Fig. 2).

From the biochemical–molecular perspective, we have to envision aging as a product of changes that occur during life. These changes can lead to a "pathologic" form of aging (in our case, the Alzheimer form of dementia) if we live long enough and are exposed to specific risk factors (Fig. 2). The increasing lifespan of the population is obviously working in that direction. The age-associated changes can be affected by either environmental or genetic risk factors, which will ultimately modify (either delay or accelerate) the transition from "normal" to "pathologic" aging. Identification of the multiple risk factors will help delay the onset of AD. However, only the understanding of aging at the molecular level will allow us to influence the molecular interaction between aging and the risk factors, therefore, bypassing the negative effect(s) induced by a specific risk factor (either environmental or genetic). Let us assume, for example, that a polymorphism on a certain ligand (L) is linked to a specific age-associated disease, and that the

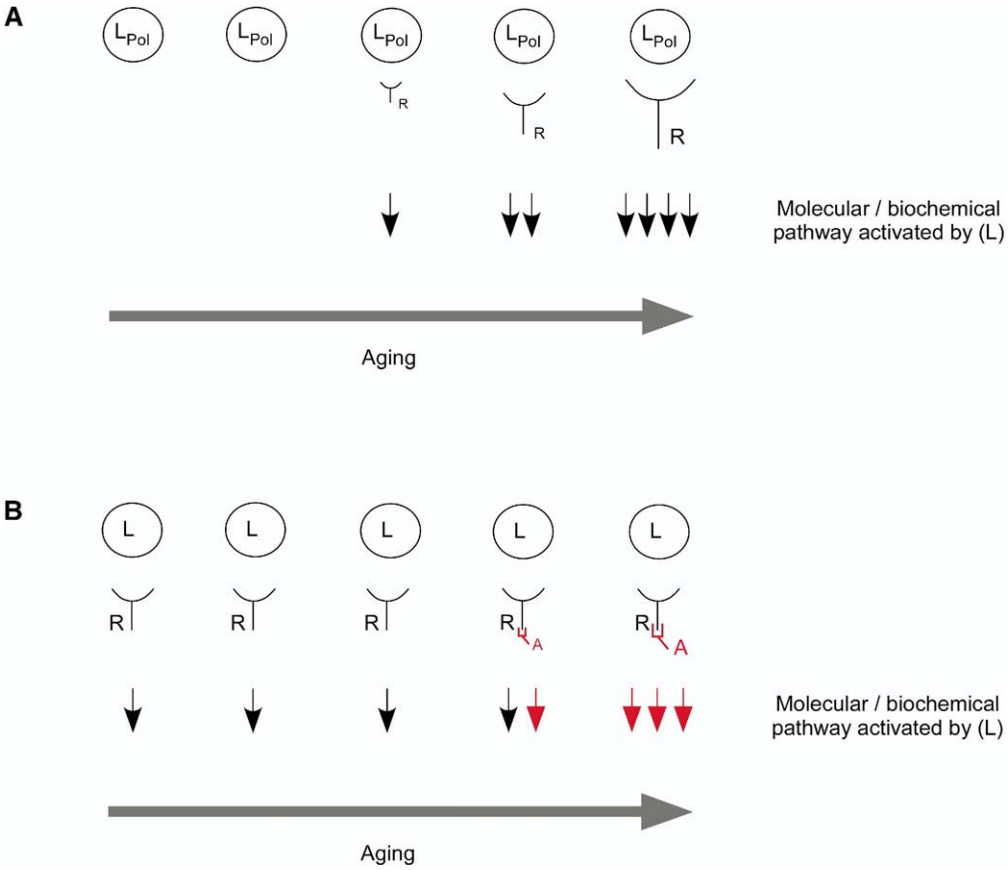

Fig. 3. Possible molecular mechanisms connecting aging to age-associated diseases. (A) A certain ligand (L) can bind to different classes of cell-surface receptors; in these cases, the signal specificity is normally regulated by the different affinities (*Km*) to the receptors. However, such form of regulation can be affected by a polymorphism on the ligand (L_{pol}) that changes the *Km* of the L–R interaction. If the expression of R is activated in an age-dependent fashion, we can expect to observe the biological effect induced by L_{pol} only late in life. The figure depicts R in different sizes to indicate the increased expression produced by aging (horizontal arrow). The increased number of vertical arrows indicates the increased activation of the signaling event down-stream (L–R). (B) In this scenario, neither L nor R expression levels are affected by aging. Aging only activates the expression of an adaptor protein (A) that can influence or change the signaling cascade downstream (R). Obviously, this situation can occur in the presence or absence of polymorphisms on the ligand.
The purpose of this figure is to stress the need to evaluate the molecular events involved in the pathogenesis of age-associated diseases in an age-dependent fashion. Obviously, different variations of the above situations or complete different models (including age-dependent inhibition of protective mechanisms) could be equally possible.

polymorphism itself affects the affinity binding to a receptor (R). If the expression of R is under the general control of aging (ie, expressed late in life), the polymorphism will only be able to act if we reach the appropriate age (Fig. 3A). An increase in average lifespan will obviously favor the incidence of that disease among the population. Under the above conditions, we will be able to identify the exact molecular pathway that is acting downstream (R) only if we envision the disease as part of the general programming of aging (Fig. 3A). Once we achieve that goal, we could design preventive approaches that block or delay either the age-associated expression of R or the specific biochemical events downstream (L–R interaction). The above situation could be even more difficult to analyze if the molecular link between R and L requires an adaptor protein that is expressed in an age-dependent fashion and that, when expressed, changes the signaling cascade downstream (R) (Fig. 3B). If this is the case, we would never identify the biochemical pathway —or the potential pharmacologic targets— unless we envision the disease as a function of age.

The fact that aging is the single most important risk factor for LOAD suggests the existence of specific molecular–biochemical pathways that are activated (or blocked) in an age-dependent fashion and somehow linked to one or more aspects of AD neuropathology. On this regard, it is important to stress the fact that normal aging is also characterized by moderate (or not severe) accumulation of Aβ into senile plaques and by loss of synaptic plasticity. Therefore, we face 2 urgent needs: (1) to use cellular and organismal models of aging to study the different aspects of the molecular pathogenesis of AD, and (2) to analyze the possible role of molecular pathways that control the general programming of aging in the pathogenesis of AD.

3. Ceramide: A Signaling Molecule that Connects Aging to AD?

Findings from our laboratory have recently shown that ceramide can regulate both APP processing and Aβ generation by affecting the molecular stability of BACE1 [17]. Ceramide is a lipid second messenger involved in many biological events spanning from cell growth, apoptosis, lifespan, and vesicular trafficking to neuronal differentiation and functioning. In turn, aberrant metabolism of ceramide has been associated with inflammation, tumorigenesis, diabetes, and neurodegenerative disorders. From a metabolic point of view, ceramide functions as the backbone for sphingolipid metabolism, having roles in regulating the formation of important sphingolipids such as sphingomyelin (SM), acylceramide, glucosylceramide, galactosylceramide, and other complex glycosphingolipids, which are all highly enriched in the brain. From a signaling point of view, ceramide also serves as the precursor to an increasing family of bioactive sphingolipids, including sphingosine, sphingosine 1-phosphate, and ceramide 1-phosphate. However, the most intriguing functions of ceramide are related to its ability to transduce signals that are involved in the overall regulation of terminal differentiation of neurons, cellular senescence, proliferation, and death. Ceramide is normally considered a central regulator of cellular senescence [18,19]. Depending on the cell type and the doses used, exogenously-added ceramide has been shown to either activate or inhibit apoptosis [20,21]. Intracellular levels of ceramide increase during aging in both cultured cells and the entire organ [22–25]. In addition, ceramide levels are found to be increased by more than 3-fold in the brains of AD patients when compared with age-matched controls [25,26]. Under senescence-like conditions, ceramide has been shown to promote outgrowth and survival of cultured neurons [27,28].

An additional and "puzzling" connection between aging and ceramide production comes from the yeast *Saccharomyces cerevisiae*, which shows a marked increase in lifespan when a gene called longevity-assurance gene 1 (*LAG1*) is deleted [29]. *LAG1* resides in the endoplasmic reticulum and shows C26-ceramide synthase activity both in vivo and in vitro [30,31]. The effect of *LAG1* deletion on the lifespan of *S cerevisiae* is rescued by the human homolog *LAG1Hs*, which is highly expressed in the brain, as well as in the testis and skeletal muscle [32]. The possible implications of the yeast phenotype for normal aging of the human brain are very challenging but remain to be analyzed further.

Endogenous active ceramide is mostly generated by either de novo synthesis or hydrolysis of sphingomyelin (SM) at the cell surface, the latter being the most important source of the active pool of ceramide [33,34]. In neurons, ceramide generated by sphingomyelin hydrolysis can be produced by either a neutral- or acid-sphingomyelinase (SMase). The former is localized in the axons, whereas the latter is localized in the body of neurons [27]. Only the neutral SMase (nSMase) generates the signaling active ceramide; the acid SMase (aSMase) is involved in the catabolism of ceramide-containing sphingolipids in the lysosomal compartment [33,34].

Cloning approaches directed against bacterial nSMase have recently identified 2 putative mammalian nSMases capable of hydrolyzing sphingomyelin in vitro, nSMase1 [35] and nSMase2 [36]. Further biochemical characterization of both enzymes has indicated that only nSMase2 fulfills all the characteristics of the long-searched nSMase, including pH and Mg^{2+} dependence, and ability to hydrolyze sphingomyelin both in vitro and in vivo in the same *Km* range observed with purified membrane extracts [37–39]. As predicted, nSMase2 is highly expressed in the brain, localizes in the late secretory pathway (close or at the plasma membrane), and mimics the cellular functions described for ceramide [36]. Interestingly, overexpression of nSMase2 in mammalian cells did not induce apoptosis [39], apparently disputing a possible physiologic role of ceramide in apoptosis. It still remains to be determined whether this is the only nSMase or just a member of a larger family of nSMases sharing substrate specificity and different subcellular localizations.

The generation of ceramide in neurons is mostly regulated by the p75 neurotrophin receptor ($p75^{NTR}$), which controls the activation of endogenous nSMase [21]. Oxidative and metabolic stresses, together with interleukin 1 and 1-α,25-dihydroxyvitamin D3, have also been proposed as additional ways to activate nSMase, but final proof in vivo is lacking. Additional identified nSMase activators, including the p55 tumor necrosis factor receptor (TR55) and the B cell CD40 glycoprotein receptor, do not play any role in the regulation of ceramide metabolism in the brain. Binding of neurotrophins to $p75^{NTR}$ activates nSMase and increases the levels of intracellular ceramide. The increase in ceramide levels is followed by axonal growth and inhibition of cell death [28,40]. Withdrawal of the nerve growth factor (NGF) from neuronal cultures activates apoptosis; this effect is inhibited by exogenously added ceramide, which rescues neurons from cell death [40,41]. In apparent contrast to the above studies, a chronic increase in intracellular ceramide can inhibit axonal elongation, receptor-mediated internalization of NGF, and activate cell death [20,27]. In addition, it also reduces receptor-mediated internalization of lipoprotein-associated cholesterol [27], which is involved in the regulation of synaptogenesis [42]. Such effects may be part of a delicate set of events that occur during senescence, with an age-dependent reduction of neuronal plasticity as a final result. Obviously, it still remains to be addressed whether ceramide regulates one or more molecular events linked to AD pathogenesis in an age-dependent fashion. If this is the case, we will also need to identify both the upstream and downstream events to advance toward the understanding and prevention of LOAD.

4. Cholesterol Homeostasis/Distribution in Neurons: A Possible Connection Among Aging, AD, and Synaptogenesis?

The last few years have witnessed the emerging of many results implicating cholesterol with the pathogenesis of AD (reviewed in [5]). The initial observation that high levels of cholesterol in the brain and in cell lines could increase Aβ generation has been followed rapidly by more detailed analyses of several molecular aspects of cholesterol metabolism, which have identified at least 5 different possible connections with AD neuropathology: (1) clustering of APP and BACE1 into lipid rafts, which facilitates β-cleavage of APP [43]; (2) intracellular cholesterol distribution, which regulates the amyloidogenic processing of APP [44,45]; (3) ozonolysis of cholesterol, which generates peroxi-derivatives of cholesterol that accelerate the aggregation of Aβ monomers [46]; (4) Aβ-mediated oxidation of membrane cholesterol, which liberates H_2O_2 and aggravates oxidative stress [47,48]; and (5) biosynthesis of nonsterol isoprenoids that can affect APP processing without involving the "classical" cholesterol metabolic pathway [49–51]. This is further complicated by the complex interplay between the different members of the "cholesterol efflux" machinery, including apolipoproteins, ATP-binding cassette A1 transporter (ABCA1), and the LXR family of nuclear receptors that could dramatically affect cholesterol homeostasis in the brain [52].

However, many other aspects of cholesterol homeostasis and distribution in neurons still remain to be analyzed. Unfortunately, neurons (and the brain) have been almost elusive in our effort to study in detail the molecular pathways that regulate cholesterol distribution and metabolism and their possible role in regulating neuronal functioning and age-associated modifications. The role of cholesterol metabolism in the brain has so far been studied using 2 main approaches: in vivo radiolabeling and transgenic/knock out models. Unfortunately, neither of them has provided exhaustive answers to our questions. Radiolabeling experiments are limited by the fact that they are performed in conditions of nonequilibrium (the blood– brain barrier impedes functional equilibrium between the cerebrospinal fluid and the plasma), whereas transgenic or knock-out models are complicated by the fact that the brain possesses many "redundant" systems to regulate cholesterol homeostasis and distribution, which compensate or overlap with the function of other molecules. In addition, the half-life of cholesterol metabolism in the brain is in the range of several months, which is enormous when compared with that in the liver (~1 day) [5,53]. Therefore, biochemical dissection of the many different molecular pathways is required.

When analyzing the relationship between cholesterol and Aβ, we also need to consider that Aβ is able to oxidize cholesterol [47,48], therefore, affecting both intracellular cholesterol metabolism (oxysterols are powerful modulators of cholesterol metabolism) and neuronal functioning (H_2O_2, one of the bio-products of sterol oxidation, is a powerful inducer of apoptosis). The identification of the role that the acylCoA: cholesterol acyltransferase (ACAT) plays in APP metabolism and Aβ generation [44] seems to further strengthen the importance of the identification of molecular determinants that control intracellular cholesterol homeostasis and distribution in the brain. In fact, ACAT is an allosteric enzyme that is mainly regulated by the availability of its 2 substrates, free cholesterol and fatty acids. Therefore, the molecules that regulate the intracellular pool of free cholesterol and fatty acids will ultimately regulate ACAT activity.

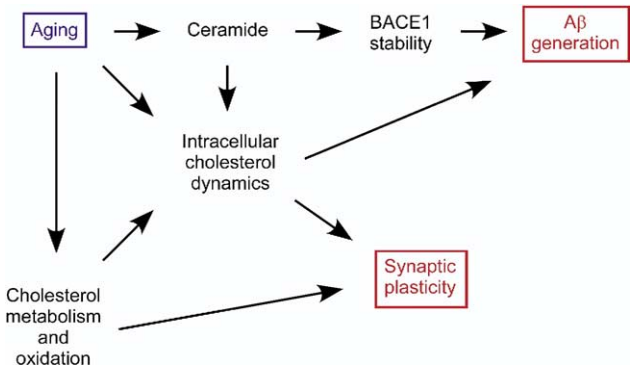

Fig. 4. Schematic overview of possible age-dependent interactions between ceramide and cholesterol, which could influence one or more molecular events linked to Alzheimer's disease.

The above considerations are further complicated by the fact that we have very little understanding of how aging influences neuronal cholesterol homeostasis and distribution. In this regard, it is important to consider that transgenic or knock-out models of "cholesterol dyshomeostasis" develop brain alterations only late in life, suggesting either a time- or an age-dependent effect. In the first case, the initial noxa would require time to develop a phenotype that is only delayed by the high plasticity of the brain; in the second case, instead, the noxa would need the activation (or inhibition) of biochemical pathways to strike. The therapeutic repercussions of the above situations are obviously very different.

5. Conclusions

Studies from several groups, including ours, are starting to delineate a complex network of biochemical pathways that can affect both Aβ biogenesis and the ability of the brain to sustain or generate synapses. Some of these pathways often interact with each other and are influenced or modulated by aging itself (Fig. 4). For example, aging induces a progressive increase of ceramide content in the brain [25], which, in turn, can stabilize BACE1 and activate Aβ production and secretion [17]. At the same time, cer-

amide can regulate receptor-mediated uptake of lipoprotein-associated cholesterol [27] and can affect maturation of the sterol regulatory element–binding protein (SREBP) [54], therefore, influencing cholesterol metabolism and distribution in the brain. Both events control the generation of Aβ and the ability of neurons to generate and sustain their synapses [5,42]. In addition, aging is also accompanied by progressive oxidation of circulating lipoprotein, which can lead both to intracellular accumulation of enzymatically resistant cholesterol esters and to changes in cholesterol dynamics [55]. Some of these events may be able to potentiate themselves, becoming difficult to dissect. However, the great challenge that lies ahead is the identification of all the molecules and biochemical events that are involved in the above pathways during normal aging of the brain. Only then we will able to understand how they influence the progression of Alzheimer's disease and design specific treatments for the prevention of this devastating form of dementia.

Acknowledgments

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