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The conflicting role of brain cholesterol in Alzheimer's disease: lessons from the brain plasminogen system

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Abstract

Retrospective clinical studies indicate that individuals chronically treated with cholesterol synthesis inhibitors, statins, are at lower risk of developing AD (Alzheimer's disease). Moreover, treatment of guinea pigs with high doses of simvastatin or drastic reduction of cholesterol in cultured cells decrease Aβ (β-amyloid peptide) production. These data sustain the concept that high brain cholesterol is responsible for Aβ accumulation in AD, providing the scientific support for the proposed use of statins to prevent this disease. However, a number of unresolved issues raise doubts that high brain cholesterol is to blame. First, it has not been shown that higher neuronal cholesterol increases Aβ production. Secondly, it has not been demonstrated that neurons in AD have more cholesterol than control neurons. On the contrary, the brains of AD patients show a specific down-regulation of seladin-1, a protein involved in cholesterol synthesis, and low membrane cholesterol was observed in hippocampal membranes of ApoE4 (apolipoprotein E4) AD cases. This effect was also evidenced by altered cholesterol-rich membrane domains (rafts) and raft-mediated functions, such as diminished generation of the Aβ-degrading enzyme plasmin. Thirdly, numerous genetic defects that cause neurodegeneration are due to defective cholesterol metabolism. Fourthly, in female mice, the most brain-permeant statin induces neurodegeneration and high amyloid production. Altogether, this evidence makes it difficult to accept that statins are beneficial through acting as brain cholesterol-synthesis inhibitors. It appears more likely that their advantageous role arises from improved brain oxygenation

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and/or because of their anti-inflammatory properties. At this point, the true role of cholesterol and statins in AD is still to be determined and the final aim of statin-based treatments should be evaluated carefully.

Amyloid accumulation can occur through a loss of function event such as inefficient degradation

Abnormal protein aggregates, whether in the extracellular or intracellular space, can certainly affect cell function. The severity of the effect will depend on the composition of the cumuli, the size and the place of formation. From this basic and obvious standpoint, it is more than justified that researchers who are interested in understanding the cause of AD (Alzheimer's disease) have put so much effort in the analysis of the molecular and biochemical mechanisms responsible for the anomalous Aβ (β-amyloid peptide) and tau protein aggregates that invariably appear in AD patients' brains [1]. In the case of the amyloid cumuli, these can occur by a combination of factors: higher production of the peptide, generation of an abnormal, aggregation-prone form, or decreased rate of degradation. Thus, in some cases, aggregation can be seen as a gain-of-function event, whereas, in others, it reflects a loss of function. In the following sections, we will summarize data from our laboratory showing that loss of function of the brain plasminogen system in AD brains, resulting in a reduction of the levels and activity of the amyloid degrading enzyme plasmin, can account for amyloid accumulation. Yet this is not the only enzyme involved in Aβ clearance, and we recommend to the reader interested in knowing more about this type of mechanism to consult the excellent publications analysing the amyloid-degrading function of other enzymes such as neprilysin, IDE (insulin-degrading enzyme), MMP9 (matrix metalloproteinase 9), endothelin-converting enzyme and elastase (see [2] and references therein).

Among its various roles, the brain plasminogen system is involved in Aβ degradation

The active serine protease plasmin arises following the binding to the cell surface and subsequent cleavage of the inactive pro-enzyme, plasminogen [3]. The cleavage cascade is balanced through activators: tPA (tissue-type plasminogen activator) and uPA (urokinase-type plasminogen activator), and inhibitors: plasminogen-activator inhibitors (i.e. PAI1, PAI2) and plasmin inhibitors (i.e. α-2-antiplasmin, α-2-macroglobulin) [4]. Although the plasminogen–plasmin system is most known in relation to blood coagulation, because of the involvement in fibrin-clot clearance [4], it has been also recognized for years to exist in the brain. In this organ, tPA is the main plasminogen activator, appearing widely expressed in the hippocampus, hypothalamus, cerebellum and amygdala [5]. Interestingly, fibrin has not been found in the brain, indicating that, in this organ, the plasminogen system has different target(s). In this regard, various evidence has demonstrated that the brain tPA–plasminogen cascade is important for proper extracellular matrix degradation during neuronal development [6] and for

events that require synaptic plasticity, such as long-term potentiation [7], memory [8] and motor learning [9]. Although plasminogen is potentially the most likely substrate for tPA, thus making plasmin the putative main modulator of such functions, it is now clear that tPA itself can exert certain roles such as microglial activation [10], mossy fibre outgrowth stimulation [11] and enhancement of NMDA (*N*-methyl-D-aspartate) receptor-mediated signalling [12]. More work is needed to elucidate which of the numerous roles of tPA are plasmin-dependent or -independent. In addition to the physiological functions, the brain plasminogen system has been implicated in neuronal death in conditions such as seizure and stroke [13,14]. In ischaemia and excitotoxicity models, neurotoxicity is mediated by increased tPA synthesis, followed by plasmin generation leading to laminin degradation [14,15]. In contrast, plasmin-independent neurotoxic effects triggered by excess tPA are thought to mediate the neuronal death that occurs after stroke [16].

Recent evidence points to the plasminogen system as a key player in AD pathogenesis. Thus it has been observed that plasmin can efficiently degrade Aβ *in vitro* [17,18] and that the degradation of Aβ in the brain, after injection of the peptide, is significantly less efficient in tPA or plasminogen-knockout mice [19]. That a defect in plasmin activity could lead to reduced clearance of Aβ, and therefore enhanced accumulation in the extracellular milieu in the human disease, came from the finding that brain plasmin levels and activity are significantly reduced in a relevant percentage of AD cases [20]. These three pieces of evidence add the plasminogen system to the complex picture of the molecular defects that can lead to the occurrence of abnormal amyloid aggregates contributing to neurodegeneration. This is supported further by the fact that the Aβ is a strong inducer of tPA expression [18], which, as described above, can lead to cell death in plasmin-dependent or -independent manners [14–16]. We envision that Aβ aggregates and low plasmin would trigger compensatory mechanisms to produce more plasmin relying on increased tPA or uPA production. The involvement of both molecules in cell signalling [12] raises the concern of excessive activation of signalling cascades.

Because of all the above, a reasonable scenario for AD is that an age-dependent down-regulation of the brain plasminogen system could contribute to the appearance of the typical signs of the disease, such as memory loss and learning impairment, because of amyloid accumulation on one hand and increased tPA signalling on the other. Together with our data, the demonstration that a short-term exposure to exogenous tPA and plasminogen can reduce the cytotoxic effects induced by amyloid aggregates [21] indicates further how delicate the balance of these molecules is to play beneficial or noxious roles.

Efficient plasminogen–plasmin conversion is impaired in situations of low neuronal membrane cholesterol

The observation of reduced plasmin levels and activity in certain AD brains raises the question of whether this can be a cause for abnormal amyloid aggregation or simply a consequence of the cell death or other signs that accompany this disease. Against the second premise is the fact that not all AD

brains present low plasmin, despite all of them having similar neurodegeneration and disease signs [20]. Furthermore, the analysis of the level of the precursor plasminogen in the low-plasmin brains revealed that total plasminogen levels are comparable with those of non-AD cases and of the AD cases with normal plasmin [20], pinpointing that disease-associated features, such as inflammation or cell death, cannot be considered in the main responsible for plasmin paucity. Then what could the cause be for such low levels and activity?

The amount and activity of plasmin depends not only on the levels of the pro-enzyme plasminogen, but also on the capacity of the latter to bind to the cell surface [3], an event that requires the proper expression and distribution of different acceptor molecules. Thus defects in the organization of the neurons' membrane and/or on the expression of any of the acceptors may account for low plasmin. In fact, it was shown that exogenous plasminogen binds poorly to the membranes from low-plasmin AD brains [20]. Yet, the levels of expression of the plasminogen membrane-binding acceptors, the ganglioside GM1, and the C-terminal lysine-containing proteins annexin, amphoterin and α-enolase were found to be comparable in all three types of human brain samples: AD with normal and low plasmin levels and non-AD individuals [20]. From these two series of data, it appears that low plasmin in this peculiar AD group is due to a defect at the level of the neuronal plasma membrane.

Early studies from our laboratory demonstrated that the plasma membrane domains responsible for plasminogen binding and activation are the raft domains [22]. Rafts are small domains of cell membranes, characterized by the enrichment of cholesterol molecules in the exoplasmic leaflet at the hydrophobic–hydrophilic interface of phosphatidylcholine, as well as sphingomyelin that, depending on the cell type and stage of differentiation, have specific membrane and membrane-associated proteins [23]. The presence of cholesterol confers higher rigidity to these areas of the membrane [24]. Proteins that have an especial affinity for this type of lipidic environment perform different functions, either qualitatively or quantitatively, to those when in non-raft domains [25]. Biochemically, such types of proteins can be seen by their flotation to the buoyant density of cholesterol after extraction in cold non-ionic detergents [23]. Such an approach using membranes from cultured hippocampal neurons revealed that the plasminogen-binding molecules α-enolase, amphoterin, annexin and GM1 are enriched in rafts [22]. In agreement with these domains being the precise site of the membrane where plasminogen binding and cleavage occurs, plasmin was found exclusively in the raft fraction of both human and rodent hippocampal membranes [17,20,22]. More importantly, a statin-based reduction of less than 30% of membrane cholesterol in rodent hippocampal neurons that sufficed to cause raft disorganization resulted in a dramatic reduction of plasminogen binding and consequently in low plasmin levels [22]. To us, this last result acquires special relevance when it comes to the use of drugs that may lead to the reduction of cholesterol levels in neurons.

Hippocampal membranes of the AD brains with low plasmin have reduced membrane cholesterol

The reported observation that plasminogen binding is reduced in the hippocampal membranes of the AD patients with low plasmin, added to the demonstration that plasmin is essentially made in the raft membrane domains in a cholesterol-level-dependent manner, led us to the hypothesis that these patients' defect might be a paucity in membrane cholesterol. Indeed, when we measured the cholesterol content of the different samples, we found similar levels of total cholesterol, yet the membrane cholesterol was reduced significantly (30%) in the low-plasmin hippocampi compared to the AD cases with normal plasmin levels or with non-AD samples [20]. Consistently, specific and significant changes in the protein content were evident in the raft fractions of the AD low-plasmin membranes compared with those of the age-matched control individuals or AD patients with normal levels of the protease [20]. The changes in raft organization affected the membrane distribution of plasminogen binding and activating molecules, such as GM1 or uPA receptor. Notably, these alterations were not evident in brain areas that are normally spared from amyloid deposition in AD, such as the cerebellum. Membrane cholesterol measurement in the cerebellum revealed a reduction not higher than 19% in low-plasmin AD samples (M.D. Ledesma and C.G. Dotti, unpublished work), suggesting that cholesterol loss of more than 20% from the membrane would be required to produce defects in rafts and raft-mediated functions. These data also suggest that different brain areas can be more predisposed to cholesterol loss than others and/or that plasmin-independent proteolytic pathways might have a variable relevance in amyloid clearance depending on the brain area.

Low-membrane-cholesterol AD brains: possible relationship with the ApoE4 (apolipoprotein E4) allele

The existence of AD cases with low cholesterol/low plasmin and normal cholesterol/normal plasmin could not be correlated with the amount of amyloid plaques nor tau filament aggregation. They could not be associated either with differences in age of occurrence of the disease, severity of progression, sex, post-mortem brain-sampling delay, hippocampal area under scrutiny or cause of death. The only correlation we observed was the presence of the $\epsilon 4$ allele of the ApoE in AD brains with low plasmin and altered rafts and membrane cholesterol, while AD brains without these alterations all had the $\epsilon 2$ or $\epsilon 3$ alleles [20]. Although a study with a larger population is needed to confirm this correlation, it opens the possibility that the presence of the ApoE4 allele might be relevant in these cases. The fact that ApoE is a protein that participates in the homoeostasis of cholesterol and that the inheritance of the $\epsilon 4$ allele has been confirmed as a risk factor from sporadic AD [26] also supports this notion.

We have no scientific data yet to explain how the $\epsilon 4$ allele of ApoE could favour the cholesterol loss in these patients. It is possible that the astrocytes from these individuals are less efficient in providing cholesterol to neurons, as has been described in mice expressing this allele [27]. However, this might not

be the case if it is considered that neuronal cholesterol levels are largely independent from outside cholesterol uptake [28]. In this situation, the presence of the ApoE4 allele might contribute to a more general defect, such as impaired brain oxygenation because of high blood cholesterol that indirectly affects the cholesterol synthesis machinery in the neurons. Still, the question of why not all ApoE4 bearers develop the disease remains. One explanation would be the existence of environmental or associated genetic factors that make some of the bearers more resistant to such indirect effects.

Considerations about membrane cholesterol and rafts, and how statins might help to fight against AD

Besides the possible relevance of the plasminogen system in the prevention of amyloid aggregation, the results detailed above serve to raise the conclusion that a deficit in brain cholesterol can be of extreme danger for proper neuronal function. As an example, cells with reduced cholesterol synthesis might no longer be able to respond efficiently to growth/survival factors, which are known to signal via receptors clustered in cholesterol-rich membrane domains [25].

Different from the above prediction, the opinion exists today that reduction of brain cholesterol can be beneficial to prevent an excess generation of Aβ and thus its accumulation. Indeed, cholesterol-synthesis inhibitors are being proposed for clinical trials for the prevention and cure of AD [29]. Two pillars support this opinion: clinical retrospective studies and basic cell biology/biochemical work in cells and animal models.

Retrospective clinical studies showed that patients with elevated serum cholesterol levels chronically treated with blood-cholesterol-lowering drugs, such as statins, present a decreased incidence of AD [30,31]. Since these drugs have as their basic mechanism of action the capacity to inhibit the early steps of cholesterol synthesis [32], the association between high brain cholesterol predisposing to AD was immediate. Yet, it is predictable that, in addition to a direct effect on the inhibition of cholesterol synthesis, statins might have had a beneficial role through other effects, secondary to the main mechanism of action (lowering blood cholesterol resulting in improved blood circulation and lower blood pressure) or unrelated to it, such as their antioxidant or anti-inflammatory roles [32]. The clinical retrospective studies themselves cannot distinguish between these different possibilities.

Albeit indirectly, cell biological/biochemical data in cells *in vitro* support the view that high brain cholesterol may be at the base of the anomalous amyloid production that occurs in AD. These studies showed that reduction of cholesterol via a combined treatment of cholesterol-synthesis inhibitors, statins, and cholesterol-extracting drugs in cultured cells overexpressing the precursor of Aβ, APP (amyloid precursor protein), or some of the enzymes involved in amyloid generation, inhibited Aβ release [33–36]. Hence, obvious conclusions were that excess amyloid is due to high cholesterol and that the Aβ is produced in cholesterol-rich raft domains. A first disadvantage of this type of approach concerns the fate of the overexpressed protein, whether or not it is targeted and accumulates in the same compartment as the endogenous counter-

part. In fact, the presence of APP in rafts is a controversial issue, with several studies indicating that none or only a minor amount of the protein is in these membrane domains, with variation depending on the experimental approach (i.e. different detergents used in the extraction protocols and conditions of overexpression) [35,37]. Thus, if contrary to the endogenous protein, the overexpressed APP is delivered to rafts, the reduction of cholesterol could result in reduced amyloid generation, but this will reflect an extraordinary situation, not a physiological one. The second drawback is that the cholesterol-reduction treatments produced in those studies resulted in membrane cholesterol loss as high as 70%, raising the concern of disruption not only of raft domains, but also of the membrane as a whole that would affect cell viability. Thus, while useful to raise awareness about the possible role of neuronal cholesterol, we believe that these data do not prove that rafts and high neuronal cholesterol are involved in high amyloid production.

Conceivably, high brain cholesterol could be at the base of excessive amyloid production if increased amyloid production would occur in neurons induced to express high cholesterol. Although many of the enzymes participating in the biosynthesis of cholesterol are known and cloned, this point has not yet been addressed. Support would also come if the brains of AD patients presented higher cholesterol levels than those of non-AD individuals. Contrary to such a prediction, both the mRNA and protein levels of seladin-1, a protein involved in cholesterol synthesis, are down-regulated in the brain areas of AD patients where amyloid plaques form most frequently [38]. In agreement with AD being accompanied by low brain cholesterol, not high, we observed low membrane cholesterol in the hippocampi of a certain population of AD individuals [20]. On the opposite side, it has been claimed that AD brains have higher cholesterol than controls based on the finding that the levels of 24*S*-hydroxycholesterol are high in blood and CSF (cerebrospinal fluid) of AD patients [39]. The major drawback of using this catabolite as a marker for high brain cholesterol content is that it reflects the degree of cell damage and not the level of neuronal synthesis. In fact, its measurement constitutes a standard parameter of analysis to evaluate the degree of neuronal loss in situations such as stroke [40].

Although the lack of clearly indicative data showing that high brain cholesterol occurs in AD brains or that high neuronal cholesterol increases amyloid production weakens the need for using a cholesterol-synthesis inhibitor to treat this disease, it can be argued that what statins do in hypercholesterolaemic people is reduce the circulating cholesterol that is later exposed to the neurons. While this cannot be ruled out completely, it appears unlikely because neuronal cholesterol content is mostly, if not exclusively, dependent on internal synthesis, not from extracellular uptake [28]. This implies that neurons have a very strict control of their own cholesterol levels that, in principle, would make these cells able to cope with an extrinsic increase of the lipid with transcriptional shut-down. Although it can certainly be that hypercholesterolaemia perturbs such a feedback loop, this would be reflected in the presence of high circulating cholesterol in AD brains, an event that has not been documented.

All the above data lead necessarily to question whether it is worth trying to reduce cholesterol synthesis with statins in neurons to prevent AD and how dangerous it could be if neuronal cholesterol synthesis is impaired.

Animals treated with different types of statins should be useful to address these questions. Unfortunately, the data so far are contradictory. Thus guinea pigs treated with high doses of simvastatin have less Aβ in the CSF and in brain homogenates [34]. Since these are not hypercholesterolaemic animals, there is no reason to think that the anti-amyloidogenic effect involved inhibition of neuronal cholesterol synthesis. In fact, brain cholesterol levels are unchanged compared with control animals, suggesting that the statins utilized do not cross the blood–brain barrier efficiently. Quite the contrary: when Park et al. [41] used a blood–brain-barrier-permeant statin, an enhancement of amyloid production and senile plaque deposition was observed in female, but not male, mouse brains. From these results, it comes that statins can have noxious effects. This is in agreement with our data on raft disturbance due to moderate cholesterol loss in AD patient brains [20].

A number of genetic disorders also support the view that low, and not high, brain cholesterol is harmful to brain function. Thus mice bearing a defect in cholesterol homoeostasis due to mutations in the NPC (Niemann–Pick type C) 1 gene, that in humans causes NPC disease, present neurodegeneration. In NPC cells, cholesterol accumulates in late endosomes and lysosomes, while its synthesis is reduced as well as its anterograde transport to the axonal membrane [42]. Interestingly, an enhanced generation of Aβ, like in AD, has been reported in this disease [43]. Another example is the genetic disorder RSH/Smith–Lemli–Opitz syndrome, where the accumulation of 7-DHC (7-dehydrocholesterol) and paucity of cholesterol are thought to be responsible for neurodegeneration and early death [44]. Importantly, the gene responsible for this disease is homologous with seladin-1, which, as mentioned above, is abnormally down-regulated in the neurons of patients with AD in the areas of the brain that show the highest levels of amyloid deposition [38]. The obvious conclusion from these last series of observations is that the maintenance of appropriate cholesterol levels is essential for proper neuronal function.

Conclusion

The lack of a clear demonstration that amyloid plaques in AD form because of anomalously high brain cholesterol, added to the numerous data, biochemical and genetic, showing that a reduction of neuronal membrane cholesterol can be deleterious, make us conclude that only statins that do not cross the blood–brain barrier should be used. These drugs can still be extremely useful to fight the disease, either because of the improvement of brain oxygenation that a reduction in circulating cholesterol would produce, or because of their anti-inflammatory roles. Because of the last possibility, it would be interesting to establish precisely if chronic treatments with non-steroidal anti-inflammatory compounds, which were shown to also be beneficial against the occurrence of the disease in retrospective clinical studies [45], could replace

statins, as these would not have the risk of producing undesired cholesterol synthesis inhibition, as has been the case for similar drugs in the recent past.

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