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Membrane-targeted strategies for modulating APP and Aβ-mediated toxicity

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by numerous pathological features including the accumulation of neurotoxic amyloid-β (Aβ) peptide. There is currently no effective therapy for AD, but the development of therapeutic strategies that target the cell membrane is gaining increased interest. The amyloid precursor protein (APP) from which Aβ is formed is a membrane-bound protein, and Aβ production and toxicity are both membrane mediated events. This review describes the critical role of cell membranes in AD with particular emphasis on how the composition and structure of the membrane and its specialized regions may influence toxic or benign Aβ/APP pathways in AD. The putative role of copper (Cu) in AD is also discussed, and we highlight how targeting the cell membrane with Cu complexes has therapeutic potential in AD.

Keywords: amyloid • copper • membranes

Introduction

Cell membranes are vital for normal physiology in all living systems. The outer, or plasma membrane of the cell is the first point of contact for the entry of exogenous substances, and the specialized domains of this membrane may regulate the metabolism of those substances that induce changes in cellular functioning. The membranes of the endoplasmic reticulum (ER) and of the endosomes play an essential part in protein synthesis, delivery, targeting and recycling. Relevant to Alzheimer's disease (AD) are the linkages between the roles of these inner membranes and the plasma membrane. The amyloid-β precursor protein (APP) is delivered to the plasma membrane from the ER from where it is retrieved by clathrin-mediated endocytosis and then cleaved by the secretases in late and early endosomes to produce the Aβ peptides. After release into the brain interstitial fluid, redox metal catalysed oligomerization producing toxic entities and amyloid proceeds in the presence of external membrane surfaces [1, 2]. The non-metal binding 25–35 sequence of Aβ is neurotoxic [3] and has been claimed to generate free radicals in the absence of

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metals [4]. However, Dikalov *et al.* [5] showed that this was incorrect and was a consequence of contamination of the commercial spin trap compound used to detect the free radicals. Molecular dynamics studies [6–9] have shown that different sequences of Aβ can sample many different conformations. Any one of these may predominate in a given experimental system depending on peptide concentration, ionic strength, redox potentials and the presence of metal ions and co-factors such as lipid surfaces.

Advances in AD research, particularly the development of biometal based therapies [10–14], indicate that compounds shown to target vital AD-associated pathological pathways *in vivo* have mechanisms of action that may be dependent, wholly or in part, on their initial interaction with cell membranes [15–17]. Thus, a greater understanding of the interaction of metals and metal-complexes with the cell membrane will shed light not only on how these compounds have provided positive outcomes in cell and animal models of AD and in clinical trials, but also on the role of the cell membrane in the biochemical pathways implicated in AD. This review outlines the current information relevant to the cell membrane in AD, and will also review the interactions of the cell membrane with copper (Cu) and Cu-complexes. The importance of this information in relation to AD pathogenesis and the development of new metal-based treatments, in particular Cu-based complexes will be highlighted.

Role of amyloid-β (Aβ) and APP in AD

The AD affected brain is invariably characterized by the presence of amyloid plaques and neurofibrillary tangles [18–21]. Associated with these pathological features are the well-known clinical symptoms of progressive memory loss and cognitive decline. The cumulative neuronal dysfunction in AD ultimately results in death. As the most common neurodegenerative disease amongst the elderly, AD is responsible for significant social and economic costs, making the need to develop efficacious therapeutics urgent.

The major constituent of amyloid plaques in the brain in AD is the amino acid peptide known as amyloid-β (Aβ) [21–25] generated by cleavage of the APP. The peptide in its naturally occurring form ranges in length from 39–43 amino acid residues. Longer Aβ peptides have been found in mouse AD models and in human brain homogenates from carriers of the APP V717F mutation; however, these are not yet well characterized or understood [26]. APP is a transmembrane protein that is normally expressed in the body [27]. Its cleavage to generate Aβ requires activity of the proteolytic intramembranous enzymes β-site APP cleaving enzyme (BACE, also known as β-secretase) and γ-secretase [28–31]. Alternative processing of APP can occur *via* activity of α-secretase, the cleavage site of which is within the Aβ region of APP, thereby precluding Aβ formation [32] (Fig. 1). APP processing by the β and γ secretases to yield Aβ is regarded as the disease associated route because the resultant Aβ is highly amyloidogenic, forms neurotoxic, oligomeric species and aggregates that form the high molecular weight insoluble species present in the amyloid plaques of the AD brain.

APP is expressed in the body under normal physiological conditions, suggesting that it takes part in a normal cell function that is yet to be determined. Because of the metal binding ability of both APP and Aβ [33–35], it is thought that APP may be involved in the regulation of metal levels in the brain [36–38]. Because of the metal binding sites at the APP N-terminal domain and its strong reductase activity, APP has also been proposed as an oxidative stress regulator in the brain [39]. Potential roles for APP in healthy tissues indicate that in addition to the generation of toxic Aβ, aberrant β- and γ-secretase mediated processing of APP may also contribute to AD pathology through loss of normal APP activity. Consistent with this possibility, metal ion dyshomeostasis and oxidative stress are regular features of the AD brain [40–46].

Understanding the factors that affect APP processing to yield Aβ is important for the development of therapies that inhibit excessive Aβ production or alter its oligomerization/aggregation to a more benign pathway. Elucidation of the secretase-mediated pathway of Aβ production for example, has led to a large amount of research into the development of secretase inhibitors for therapeutic intervention. Although the secretases are undoubtedly a worthwhile therapeutic target to inhibit aberrant Aβ production, it is also evident that the processing of APP to yield Aβ may occur *via* other, multifactorial pathways. In particular, the role of the physicochemical properties of the cell membranes as a determinant of APP processing may be the key to understanding Aβ-mediated neurotoxicity in the brain and therefore the development of therapies that target Aβ by acting on the cell membrane or its receptors.

Cell membranes

The structure and composition of the plasma membrane reflects its role as a barrier separating the contents of the cell from the extracellular environment. This membrane also participates in maintaining cell-cell adhesion and adhesion to the extracellular matrix (ECM), and has a regulatory role in endocytosis and signal transduction [47, 48].

The plasma and associated membranes of neurons contain glycerophospholipids, glycosphingolipids, cholesterol and proteins. The lipids are asymmetrically distributed between the two leaflets of lipid bilayers [49–51], which is about 5-nm thick [52]. The glycosphingolipids and the choline-containing lipids, phosphatidylcholine are enriched primarily on the external leaflet of the plasma membrane whereas the amine-containing glycerophospholipids, phosphatidylethanolamine and phosphatidylserine are located preferentially on its cytoplasmic leaflet (Fig. 1). Membrane proteins may be fully or partially membrane penetrant, multimeric and penetrant or associated with the membrane surface.

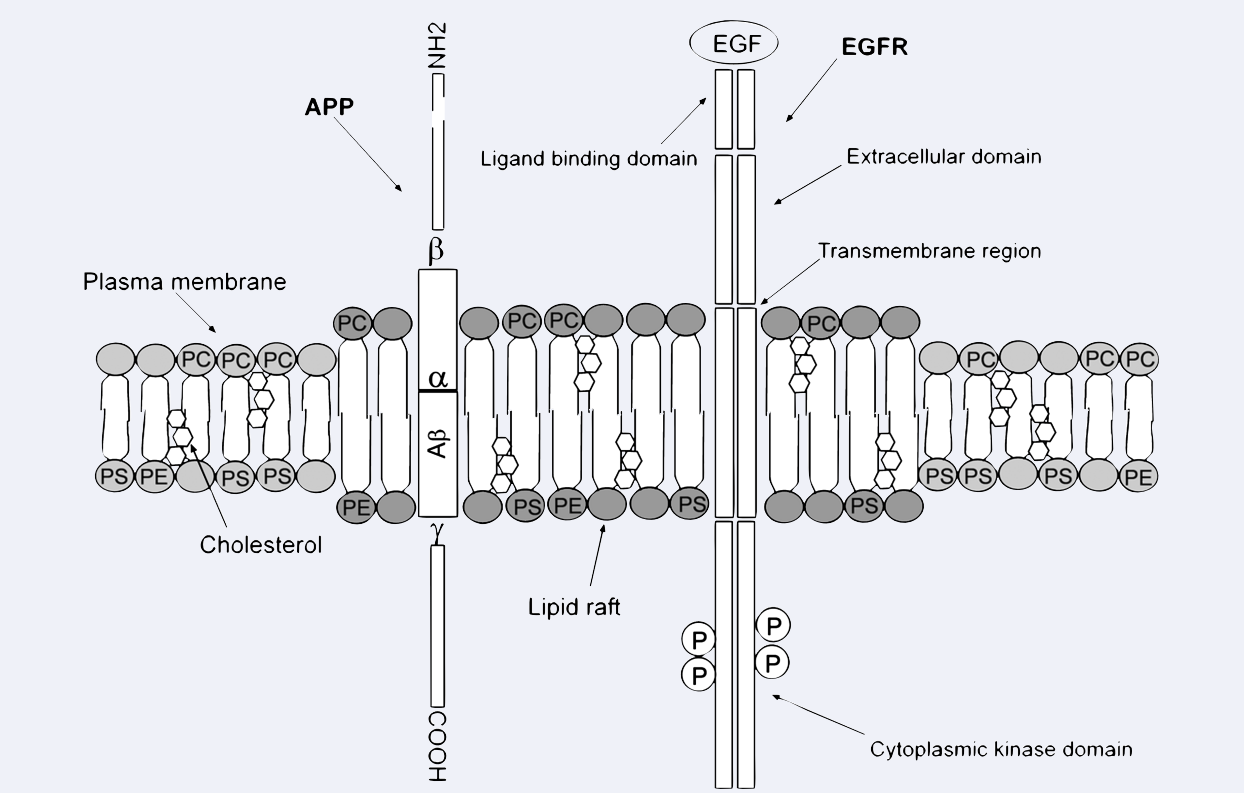

Fig. 1 A representation of the plasma membrane and lipid raft incorporating cholesterol and phospholipid orientation. Phosphatidylcholine is primarily enriched on the external leaflet, whilst phosphatidylserine and phosphatidylethanolamine are located on the cytoplasmic leaflet. Lipid rafts are thicker than the rest of the membrane due to the longer sphingolipid tails, which may facilitate the localization of larger proteins to this site. Proteins and receptors such as APP (with cleavage sites indicated) and EGFR can cluster to lipid rafts, influencing enzymatic processing and cellular signalling. The EGFR phosphorylated dimer is shown above, as indicated by the phosphate groups on the cytoplasmic domain.

Cholesterol is an essential component of the plasma membrane, necessary for membrane fluidity, permeability and receptor function [52, 53], and is the main component of neural membranes besides the phospholipids that make up the lipid bilayer [52]. At 15 to 20 mg/g tissue in fresh brain, it accounts for 20% to 25% of the total body cholesterol [54]. The level of cholesterol in the brain is independent of dietary uptake or hepatic synthesis, being maintained almost completely by synthesis *in situ* [55], with mRNAs for cholesterol synthesizing enzymes occurring in adult brain [56, 57]. Removal of the surplus cholesterol can be by conversion to 24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, cholesterol oxides or by esterification *via* acyl-CoA:cholesterol acyltransferase [58]. Thus, these conversions are intimately involved in maintaining brain cholesterol homeostasis [59, 60]. In all cells, cholesterol plays a crucial role in membrane structure, dynamics, function and sorting [61]; however, these functions must be particularly essential to neural function to account for its high level in the central nervous system (CNS).

Sphingolipids consist of a sphingoid base, a straight-chain amino alcohol of 20 to 24 carbon atoms, usually attached to a long acyl chain through an amide bond. Sphingolipids include glycosphingolipids with a sugar(s) as the head group, such as cerebroside and the gangliosides [52], which are abundant in the plasma membrane of nerve cells. Though their specific function there is unclear, they have been implicated in signal transduction in neurons [52], as has been shown for immune cells [62]. They are also an important constituent of membrane rafts, as discussed below.

Other components of the membrane facilitate adhesion to the ECM and other cells, as well as maintain structural integrity. Aside from the head groups of the glycosphingolipids, other similar carbohydrates occur as the prosthetic groups of the membrane's adhesive glycoproteins [52]. Membrane proteins can be found in the form of channels, transporters and receptors, allowing for the cellular uptake of ions and other molecules, or acting as the initiating sites for signal transduction. They may also be found as fibrous proteins such as collagen that are necessary for maintaining the structural integrity of the cell and binding to the ECM. The

adhesive glycoprotein fibronectin can bind collagen, further contributing to the adhesion of the cell to the ECM and it may also be involved in cross-linking of matrix molecules [63].

The microstructure of cell membranes

Although the plasma membrane was initially thought of as a relatively simple, fluid barrier of lipid with embedded proteins as depicted in Singer and Nicholson's 'fluid mosaic model' [64], it is in reality more complex. The inner and outer leaflets are in a dynamic relationship to each other and the maintenance of transbilayer lipid asymmetry is essential for normal membrane function. Disruption of this relationship is associated with cell activation or pathologic conditions. Early models of the cell membranes also did not consider the existence of discrete lipid microdomains in the membranes with distinct biochemical characteristics and functions. These different domains of membranes can have individual roles in cellular signalling, as well as in the maintenance of membrane integrity and other 'housekeeping' duties [65, 66]. It has been proposed that lipid rafts, isolated as a cholesterol and sphingolipid-rich detergent insoluble fraction of the plasma membrane, can serve as platforms for signal transduction, and that this may be their predominant role in the plasma membrane [66, 67] (Fig. 1). Simons and Toomre have commented that studies on rafts in the 1980's were initially controversial owing to the difficulty of proving their existence in living cells [66]. Although improvements in methodology have dispelled most doubts regarding their existence [65, 66], the composition of isolated rafts appears to be influenced by the nature of the detergent used for their separation [68]. Care, therefore, must be taken when comparing results obtained by different methods of raft preparation.

The hydrocarbon tails of the sphingolipids that concentrate in rafts are longer and straighter than those of the phospholipids, making the lipid rafts thicker than the rest of the cell membrane [52]. It has been suggested that this property enables them to better accommodate certain larger membrane proteins [52], possibly explaining why some membrane proteins are enriched in rafts (Fig. 1). Although the properties of the inner domain leaflet of the raft bilayer are still to be adequately characterized [69], Carnie *et al*. [70] theorized that an ordered packing of the outer leaflet of the bilayer would lead to a more ordered packing of the inner leaflet. Although many of the cell surface proteins occur in disordered regions, some proteins preferentially partition into the ordered raft domains. The dynamic nature of lipid rafts means that both proteins and lipids can move in and out of raft domains with different partitioning kinetics, and there is evidence to indicate that lipid rafts influence the processing of membrane-anchored proteins [71]. Pertinent to AD, the proteins that preferentially partition to lipid rafts include the secretases and APP, and changes in raft composition may result in altered APP processing. Findings such as these could provide the basis for novel AD therapeutics that specifically target the membrane.

Aβ and cell membranes

APP processing and its relationship with specialized membrane regions

The processing of APP in membranes can be influenced by many factors, each of which can be important determinants of APP-mediated toxicity. Ehehalt and colleagues [71] found that APP localization to lipid rafts resulted in cleavage of APP by γ-secretase, therefore increasing Aβ production. By contrast, APP outside the raft domains was more susceptible to α-secretase mediated cleavage, precluding Aβ generation. These findings suggest that APP exists in two distinct pools, within lipid rafts and outside rafts [71], and this may be decisive for determining the access of proteolytic enzymes to APP within the membrane that can lead to Aβ formation. Similar results were found by Cheng *et al*. [72] in B-cell antigen receptors (BCRs), who showed that the cross-linked BCR would preferentially and rapidly translocate to ganglioside GM1-enriched lipid rafts containing the src kinase Lyn. This led to phosphorylation of Lyn and targeting of the BCR to the antigen processing compartments [72]. Evidence from these studies further highlights the dynamic nature of the interactions between membrane proteins and cholesterol-enriched membrane domains such as rafts in regulating both physiological and pathological processes at this site, and the implications of this for AD are becoming increasingly apparent.

Aβ interactions with the cell membrane

The interaction of Aβ with the membrane once it is cleaved may be an important determinant of its neurotoxicity. The relationship of Aβ with the membrane may be thought of as being played on two stages: (1) As the membrane-penetrant sequence of APP and then as a membrane-penetrant peptide following β- and γ-secretase action in the Golgi apparatus or the plasma membrane or both and (2) as a membrane surface-associated toxic or non-toxic species. These two stages may occur in the same cell or in separate cells. What happens between the stages is important in determining whether Aβ will go down the toxic or non-toxic/aggregation route.

There is increasing evidence that the toxic Aβ species is a soluble oligomer [73–76] of β-structured peptides [77] and that its toxicity is mediated by its capacity to bind to the cell membrane [78]. Aβ binds to the plasma membrane by targeting phosphatidyl serine [79] and, possibly, N-acetyl neuraminic acid [80]. However, the question of whether the formation of the toxic Aβ species, as distinct from its action through the membrane, is also membrane dependent remains unclear.

Cu^{2+} and the formation of the toxic species

Cu^{2+}-mediated toxicity of Aβ has been linked to the metal ion binding site in the Aβ peptide involving the amino acid residues

His6, His13 and His14 [81]. The redox active metal ions Fe^{3+}, and Cu^{2+} coordinated to Aβ [82–85], are capable of Fenton-like redox reactions [86, 87] that can produce reactive oxygen species. The ensuing lipid oxidation [88] could alter normal neuronal membrane composition and be one source of Aβ-Cu^{2+}-mediated cytotoxicity [89, 90]. However, Aβ cross-linking *via* free radical reaction of the Tyr10 residue of Aβ results in dimers [91–93] that may in turn seed higher order toxic oligomeric species. If the imidazole side chain nitrogens of the histidines are methylated, Aβ toxicity is attenuated because of altered metal coordination at the high Cu^{2+}/peptide ratio (1:1) needed for the formation of toxic species [84, 87, 94]. It is also important to note that these studies showed that lack of toxicity correlated with inability of the modified peptides to bind to either cellular or artificial membranes. This finding correlates with the earlier observation that Cu^{2+} coordination facilitated Aβ association with model membranes [85]. Further work is necessary to understand fully the nature of the relationship between Cu^{2+} and Aβ toxicity.

Membrane composition, morphology and Aβ aggregation

Despite increasing knowledge about the interactions of Aβ with the membrane, what drives Aβ aggregation at this site is still poorly characterized. It has been known for a long time that membrane contact favours Aβ fibril formation. To explain this, Aisenbrey *et al.* [2] have argued that membrane association of Aβ induces a surface crowding effect, increasing its effective surface concentration allowing a much higher probability of protein-protein contacts that could accelerate protein transformation from monomers/dimers to aggregates, despite a much lower bulk concentration. Indeed, at physiological concentrations Aβ does not form oligomers/fibrils *in vitro* in the absence of a membrane surface [95, 96]. It seems reasonable to assume that the nature of the membrane surface could determine whether Aβ goes down the toxic oligomer or the insoluble amyloid plaque route. Yip *et al.* [97] found that $Aβ_1$-42 formed fibrils on planar bilayers formed from total brain lipids, and on the other hand Yamamoto *et al.* [98] found that $Aβ_1$-40 formed neurotoxic globules in the presence of GM1-containing phospholipid vesicles or synaptosomes. These results hint that surface curvature and composition may play a part in the toxic/amyloid switch. The plasma membrane surface itself is not a smooth curve but is marked by protrusions and pits with a much sharper radius of curvature than the average of the whole membrane. The membranes of the organelles and the extracellular bodies such as exosomes are also highly curved. Changes in membrane curvature can lead to changes in lipid packing, leaflet asymmetry and surface charge density. The lipid packing constraints imposed by a particular membrane morphology will also be reflected in the composition of the membrane leaflets. So, the influence of membrane curvature and how changes in it could direct Aβ aggregation is worthy of consideration. The influence of membrane curvature on the conformation and activity of certain proteins is already well known. An example is modulation of CTT:phosphocholine cytidyltransferase by membrane curvature elastic stress [99]. Significant curvature in a membrane produces tension between its inner and outer leaflets and the insertion of lipids with a smaller radius of curvature in the outer leaflet can relieve this tension between the membrane leaflets. Gangliosides with their large saccharide head groups (Fig. 2) are one such group of lipids that can modulate this curvature elastic stress.

Because neuronal membranes are rich in gangliosides there has been a long history of studies where Aβ has been associated with these glycosphingolipids under varying conditions. None of these considered lipid packing affects, rather they concentrated on specific sugar-Aβ interactions. These experiments also provided evidence that the membrane surface could have a seeding effect on Aβ association [100–103]. One of the earliest findings was of an SDS soluble Aβ1–42 fraction associated with GM1 ganglioside in the amyloid plaques of brains from Down's syndrome patients and normal aged individuals [104]. These authors suggested that this species might act as a seed for amyloid formation and that intracellular abnormalities in membrane recycling may already exist at the stage of amyloidogenesis. At this stage, amyloid itself was considered to be the toxic entity. These findings were followed by the discovery of the ganglioside bound Aβ species in AD brain [104]. The GM1 seeding hypothesis was pursued by Kakio *et al.* [105] who showed that increases in the content of both ganglioside and cholesterol enhanced the uptake of Aβ1–40, and later found that the generation of GM1Aβ seemed to be associated with sphingolipid, cholesterol-rich rafts [106, 107]. These findings are consistent with those of Ehehalt *et al.*, who showed that APP processing to Aβ is enhanced by raft localization [71].

The conventional α-helical view of the membrane penetrating conformation of Aβ is based on NMR structure determination in SDS [108, 109], and is supported by complex ^{31}P magic angle spinning nuclear magnetic resonance (NMR) and circular dichroism (CD) studies on the peptides incorporated in phospholipid bilayer systems [110]. There is abundant evidence that membrane surface associated plaque peptide is in the β sheet conformation [111–114] and there is increasing detail of the various supramolecular assemblages that the peptide might adopt [115, 116]. Matsuzaki and Hirikiri [117] studied the interactions of Aβ1–40 with ganglioside containing membranes by using CD and Fourier transform infrared-polarized attenuated total reflection (FTIR-PATR) spectroscopy. They found that at physiological ionic strength Aβ1–40 bound to ganglioside-containing membranes and induced a two-state, unordered β-sheet transition above a threshold intramembrane ganglioside concentration, depending on the host lipid bilayers. FTIR-PATR spectroscopy experiments also showed that Aβ1–40 forms an antiparallel β-sheet, the plane of which lies parallel to the membrane surface, inducing dehydration of lipid interfacial groups and perturbation of acyl chain orientation. These authors suggested that Aβ1–40 imposes negative curvature strain on ganglioside-containing lipid bilayers, disturbing the structure and function of the membrane. Yanagisawa *et al.* [104] extended these studies to cultured rat phaeochromocytoma cells

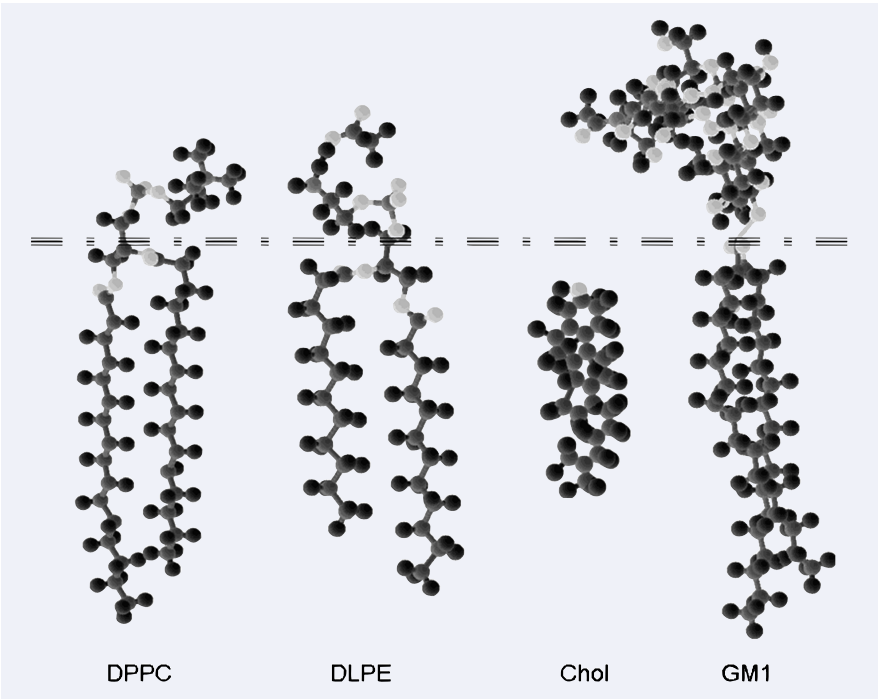

Fig. 2 Membrane lipids vary widely in the size of their headgroups. Lipids such as gangliosides are incapable alone of forming bilayers, forming micelles instead. Incorporated into membranes' outer leaflets they are associated with phospholipids with smaller headgroups, such as dipalmitoyl phosphatidyl choline and dilinoleoyl phosphatidyl ethanolamine which may have saturated or unsaturated acyl chains. Depending on the membrane conformation, packing strain is relieved by the insertion of cholesterol molecules into the outer leaflet.

and showed that fluorescently labelled Aβ1–40 co-localized with GM1-rich domains, and was cytotoxic depending on concentration and length of exposure. The cytoxicity was partly rescued by cholesterol depletion.

Membrane composition and conformation and the relationship with Cu

The internal membranes and the exosomes (40–90 nm diameter), the latter with their cup-shaped morphology, have much sharper radii of curvature than the outer membranes of cells. They also contain a variety of sphingolipids and cholesterol [118–120]. Since Aβ is released with exosomes [121] , it is in the extracellular space but membrane associated in a more highly strained *milieu* than the cell surface. The Cu^{1+} inside the cell is redox inactive, and metal-catalysed oxidation will not affect newly formed Aβ and the dimers necessary to form aggregates/toxic oligomers will not form intracellularly. On the other hand in the external medium, Cu^{2+} is available from other substrates, such as the gangliosides that Fainerman-Melnikova *et al.* [122] have suggested to contribute to the biologically available pool of Cu^{2+} with which the peptide is associated in the membrane rafts and exosomes. The relative ion binding coefficients and the molar ratios of the Aβ and the gangliosides and other Cu^{2+} providers would, therefore, determine the rate of the redox reaction that would lead to the formation of Tyr-Tyr linked Aβ dimers. Another factor influencing dimer formation could be the changed steric relationship between the Cu^{2+} binding N-acetyl neuraminic acid residues of glycosphingolipids' head groups and the peptide N-terminus depending on the lipid packing, which could also affect the rate of dimer formation [123]. The looser lipid packing related to the smaller radius of curvature of the exosomes' membranes would affect the degree of penetration of the peptide's hydrophobic side chains contained in residues 28 to the C-terminus (Fig. 3). The smaller the radius of curvature the greater the penetration of these chains, decreasing the probability of their self-associating to form larger assemblages, while leaving free the hydrophobic cluster consisting of Val18, Phe19, Ala21 and Gly25 [123] where Ala21 has been particularly implicated in Aβ association [124], while inhibitors targeting residues 15–25 prevent association. Association occurring in this region could lead to the formation of peptide micellar structures associated with the membrane assembling into toxic oligomers. These could then be delivered to the membranes of target cells by endocytosis. Although this hypothesis is yet to be tested experimentally, the best evidence for the effect of membrane curvature on protein folding pathways comes from experiments on the different conformations adopted by actin during its lipid-induced polymerization on monolayers *versus* bilayers [125]. In pure physical terms, this influence of curvature could be explained by differences in the dynamics of the two systems, where the amplitude of surface tension thermal fluctuations are orders of magnitude smaller on the actin filament scale for the monolayer than for the bilayer. This difference, which reflects changes in lipid packing, would also exist for the cell and exosome surfaces and would have a differential effect on the direction of Aβ oligomerization.

The complex studies described above demonstrate that the interactions of the cell membrane and Aβ peptide may potentiate

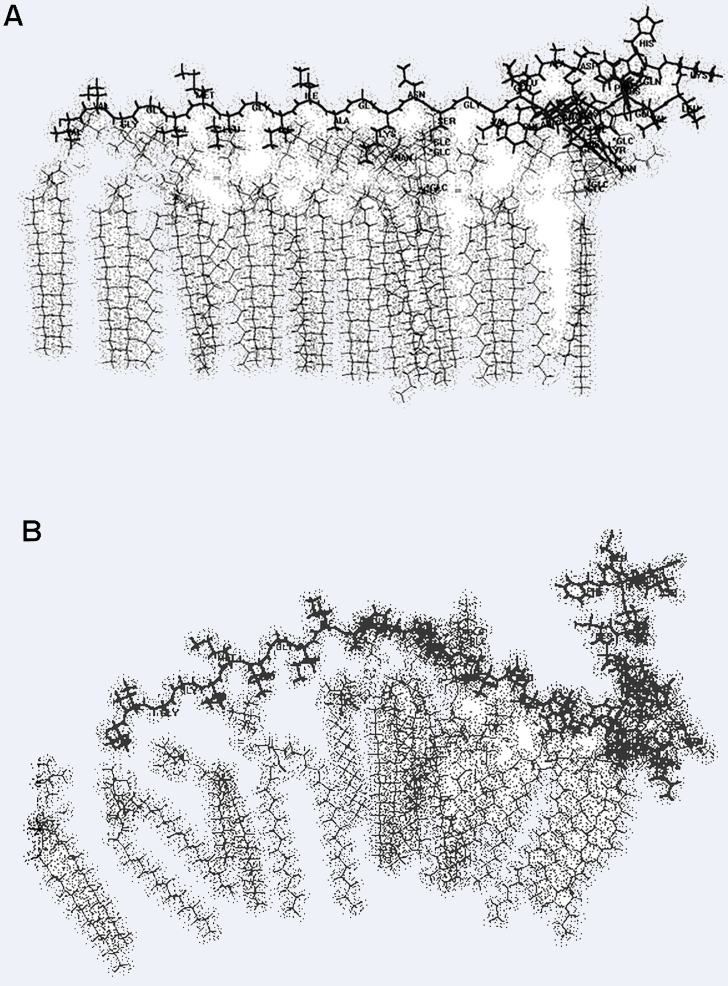

Fig. 3 (**A**) Aβ42 with its β structured C-terminal sequence lying parallel to the outer leaflet of a planar bilayer. Lys 27 could be electrostatically associated with a ganglioside N-acetyl neuraminic acid. There is a high thermodynamic barrier to the insertion of the C-terminal side chains into the bilayer. Thus, they are rendered available to self-associate and form plaques. The unstructured N-terminus can [141] be associated with ganglioside headgroups, primarily through interactions with its aromatic groups. Intriguingly, Phe 18, 19 in the region that has been implicated in Aβ aggregation could also associate with a ganglioside, adding to the 'anchor points' in the N-terminus. The dotted line represents the approximate middle of the broad boundary between the polar and not polar regions of the bilayer. Note, that the labelled lipids represent gangliosides. (**B**). Aβ42 associated with a curved membrane surface. The looser packing lowers the thermodynamic barrier to the insertion of the hydrophobic C-terminal residues. This insertion renders their side chains less available for self-association, while association of the C-terminus with the gangliosides may be substantially unchanged. The peptides with their extended β-strand conformation were based on the magic angle spinning solid-state NMR data of Gehman *et al.* [141]. Since these data only give an indication of the conformation, the structure was optimized using the AMBER force field (distance dependent dielectric ε), the molecular mechanics algorithm used was Polak-Ribiere conjugate gradient and the structure finally annealed with molecular dynamics options of heat time 0.1 ps, run time 0.5 ps, step size 0.0005 ps, starting temp 0 K and simulation temperature 300 K with a temperature step of 30 K. In Fig. 3B, residues 29–32 were inserted at a tilt into a lipid environment as described by Ravault *et al.* [142] and the geometry of the whole structure reoptimized. Both peptides are illustrated against a cartoon of a planar bilayer (**A**) and a curved bilayer (**B**) formed from the structures given in Fig. 2. The C-terminus of the Aβ peptide is on the left-hand side of each schematic.

its accumulation at specific sites and enhance its neurotoxicity. Overall, this review of the dynamic relationship of Aβ with the inner and outer membranes of the cell emphasizes the need for new therapeutics strategies for AD to target the membrane. The relative successes of some AD therapies that have targeted the membrane are described below.

AD therapeutics targeting the membrane

Recent evidence indicates that the development of AD therapeutics that specifically target the membrane are efficient in abrogating the pathological features of AD. This corresponds with the studies discussed throughout this review which suggest that enhanced toxicity of Aβ occurs when interacting with particular constituents of the membrane. Recently, Rajendran *et al.* [121] described the significantly enhanced inhibition of γ-secretase by an inhibitor that is anchored to the membrane. Their study compared the efficacy of a γ-secretase inhibitor that was anchored to a sterol moiety in the endosomal membrane (where γ-secretase is active) with a free γ-secretase inhibitor. The study showed that the sterol-linked inhibitor was significantly more efficient in preventing the formation of Aβ1–40 and Aβ1–42, and increased the soluble pool of APPα, thereby precluding Aβ formation in cultured cells and *in vivo* [121]. Using scanning fluorescence correlation spectroscopy and avalanche photodiode imaging, they also showed that the sterol-linked inhibitor facilitated partitioning of the inhibitor to lipid rafts, which can function as APP processing sites [121].

The importance of membrane-targeting for potential AD therapeutics is also evident from the large body of literature that points to high blood cholesterol as a risk factor for AD. Several studies have shown that elevated CNS cholesterol in middle-age is linked with AD susceptibility [126, 127], and other clinical studies have shown significant decreases in the likelihood of developing AD in cohorts taking cholesterol-lowering drugs (statins) compared with groups taking other lipid-lowering drugs [128, 129].

Statins inhibit cholesterol synthesis in the CNS by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), an enzyme involved in the *de novo* synthesis of cholesterol [54]. The clinical data from these studies is aligned with research that shows that APP processing is facilitated by localization to cholesterol and sphingolipid rich membrane rafts. Several groups have showed that inhibition of *de novo* cholesterol synthesis can lower Aβ accumulation and secretion both *in vitro* and *in vivo* [130–133]. Some of these studies also used methyl-β cyclodextrin (MβCD) combined with statin treatment to further deplete cholesterol [130–133]. Methyl-β cyclodextrin is a water soluble cyclic heptasaccharide [134], with a high specificity binding pocket for cholesterol [135]. It has been used in numerous corresponding studies to examine the effects of cholesterol depletion on membrane-associated signalling and raft localization in cell models. These data correlate with those of Ehehalt *et al.* [71] described earlier, which showed that MβCD treatment of N2a mouse neuroblastoma cells depleted cholesterol from lipid rafts and led to a reduction in proteolytic cleavage of APP. Significantly, decreasing cholesterol by 85% totally abolished Aβ secretion in N2a cells [71].

AD therapeutics targeting membrane receptors

Additional studies performed by Lambert *et al.* [53] and Chen and Resh [134] have similarly shown that lipid raft disruption by MβCD resulted in ligand-independent activation of the epidermal growth factor receptor (EGFR) present at the membrane. Lambert *et al.* found that MβCD treatment of HaCaT cells caused a clustering of EGFR out of lipid rafts to non-raft regions of the membrane, and these clusters contained the phosphorylated, or activated form of the receptor (p-EGFR). Lambert and colleagues argued that the large clusters of EGFR on the surface of the membrane may permit ligand-independent activation as a result of their high concentration in a restricted region, or possibly even through the physical absence of inhibitory factors remaining in raft domains [53]. Chen and Resh also argued that treatment of cells with MβCD may lead to EGFR expression changes, leading to an increased concentration of EGFR on vital regions of the cell surface [134]. In the context of AD, EGFR activation has been shown to lead to degradation of Aβ1–40 *via* the downstream extracellular-signal regulated kinase (ERK) [13, 136]. This was achieved using a clioquinol-Cu (CuCQ) metal complex to deliver Cu into CHO-APP cells, which was then acting *via* EGFR and ERK and causing activation of the proteolytic matrix metalloproteases (MMPs) to degrade Aβ [136]. A crucial link can therefore be made between specific regions of the membrane and the pathological processes occurring in AD that are highly dependent upon membrane interactions, and this link further highlights the need for membrane targeting to be an important therapeutic strategy in the disease. Additionally, the findings of studies such as these provide the rationale for the idea that delivery of metals such as Cu into the cell may be the key to the application of metal-complexes in AD therapy, and that their therapeutic potential for AD may reside in their capacity to initiate neuroprotective effects though modulation of membrane receptors.

Potential for metal complex delivered metal ions to alter membrane-mediated activation pathways

We have already mentioned Cu^{2+} binding by the neuraminic acid residues of the gangliosides as a potential source of metal ions for Aβ in the immediate extracellular *milieu.* Lau *et al.* have shown that Cu^{2+} and Aβ mutually modulate their interaction with model phospholipid membranes and that this relationship is changed by the metal attenuating drug clioquinol [16, 137]. Furthermore, metals like Cu and Zn may influence membrane interactions and activity by activating membrane receptors such as EGFR. To do this, metal delivery into the cell appears to be vital. Findings from studies by White *et al.* [13] that showed the neuroprotective potential of metal delivery into the cell by CuCQ have lead to the development of a new generation of metal-complexes in which metal delivery into the cell does not depend on the ligand itself, but instead depends on the ligand's ability to increase intracellular metal bioavailability. These bis(thiosemicarbazones) (BTSCs) are a family of low molecular weight compounds capable of crossing the blood brain barrier. They are structurally stable and consist of a diimine backbone and a varying number of alkyl and methyl groups [138] upon which the metal delivery capacity of the complex is highly dependent. Though initially developed as cancer imaging agents, their potential as a therapeutic for neurodegenerative diseases such as AD became evident because of these key structural and biochemical properties. The key point regarding the BTSCs is that their varied chemical properties allows for the determination of what characteristics are favourable for the delivery of metal into the cell and membrane targeting, and in turn which of these may be useful in AD therapy.

The effects of these BTSCs in AD models were investigated in a recent study by Donnelly *et al.* using CHO-APP cells [138]. In particular, the metal-BTSC (M-BTSC) CuGTSM was found to be taken up into cells, with Cu^{2+} undergoing reduction to Cu^{1+}, followed by dissociation of the metal ion from the ligand and an increase in cellular Cu bioavailability [138]. The ability of CuGTSM to increase intracellular Cu bioavailability correlated with its effects on Aβ levels, which were reduced in cell culture medium in a dose-dependent manner [138].

Importantly, the mechanisms involved in Aβ reduction following delivery of metals intracellularly by the M-BTSCs was found to be the same mechanism hypothesized by White *et al.* in the CuCQ studies. The delivery of Cu into CHO-APP cells resulted in the activation of the PI3K-Akt and JNK pathways, which similar to White *et al.*, was found to promote the up-regulation and activation of MMPs and subsequent Aβ degradation [13, 138]. Price *et al.* extended these studies to characterize the upstream part of this pathway, and found that activation of MMPs was mediated by the membrane tyrosine kinase receptor EGFR [139]. The intricacies of this process are poorly characterized, but are thought to involve complex interactions of the metal with the membrane. There is very little information about how metal-complexes such as the M-BTSCs may affect the metabolism and dynamics of membrane cholesterol and phospholipids, as well as specialized membrane regions such as lipid rafts.

Another recent novel approach to metal-based therapeutics is that of Barnham *et al.* [140] who have shown that 1,10-phenanthroline complexes of Pt^{2+} coordinate to the histidine imidazole side chains of Aβ altering its biophysical properties in a way that potently inhibits its neurotoxicity and synaptotoxicity. This advance was based on the earlier findings that modification of the Cu^{2+} binding site of Aβ made the peptide unable to bind to the cell membrane and therefore non-toxic [84] and opens the door to other lines of attack on the metal-related membrane binding properties of Aβ.

New investigations in this area and extensions of these published data are critical for a more thorough understanding of how metal-complexes are taken up into cells, their interactions with the membrane, and in turn how they are able to potentiate their neuroprotective effects.

Conclusions

The literature reviewed here indicates that the success of novel AD therapies will depend upon an understanding of the cell membrane, in particular the role of the membrane in mediating the processing of the APP to the Aβ peptide. This conclusion is rational considering that the Aβ peptide, which has been at the centre of AD research since its discovery, is borne out of the membrane and ultimately exerts its toxicity at the membrane. More research is necessary in order to fully understand the complexity of Aβ interactions with membrane constituents such as the lipid rafts, potentiation of Aβ toxicity *via* metal-mediated oxidative stress at the membrane, and also the membrane curvature hypothesis. Novel data indicating that metal delivery into the cell can activate membrane-mediated neuroprotective pathways further demonstrates the centrality of the membrane to the pathogenesis of AD, and this information should be exploited in the development of efficacious AD therapies.

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