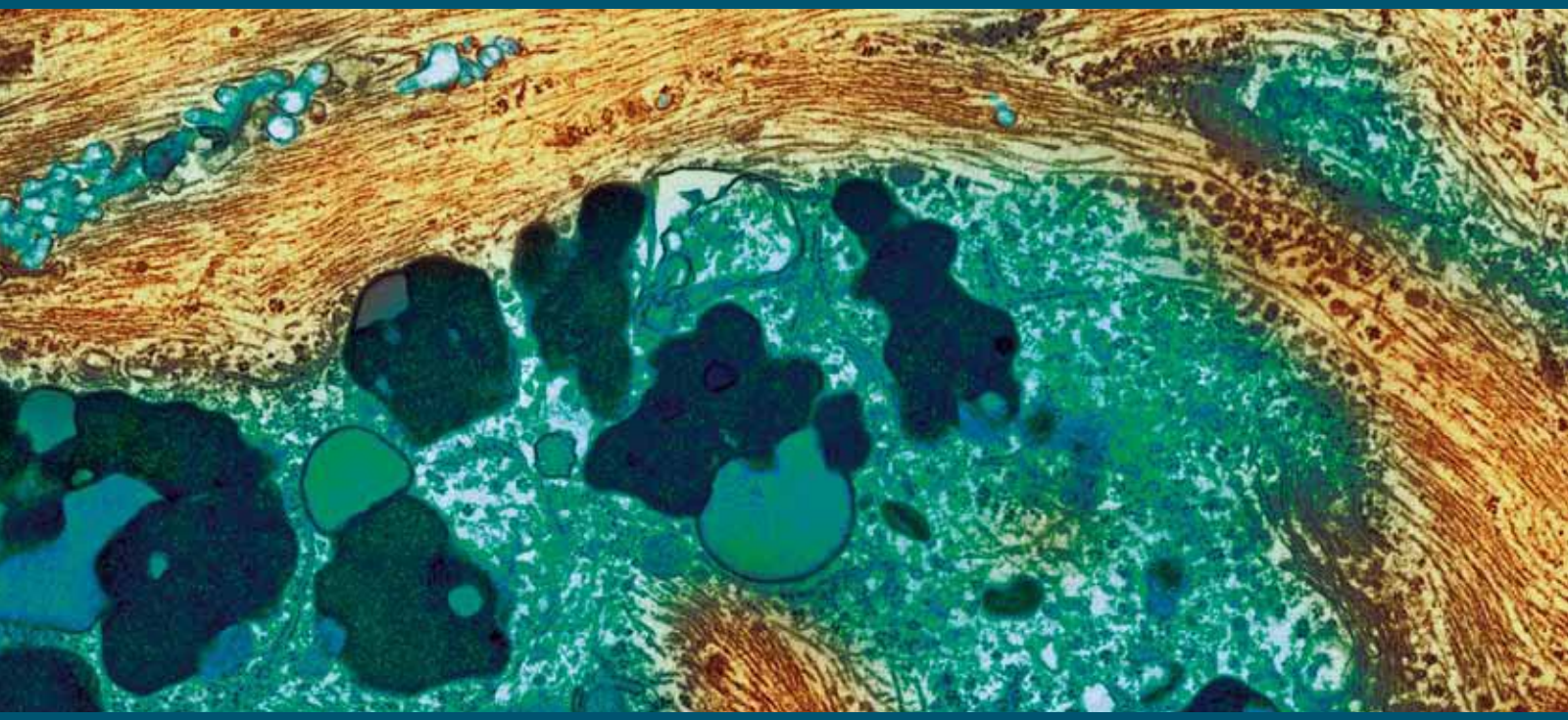
International Journal of Alzheimer's Disease

Aβ Behavior on Neuronal Membranes: Aggregation and Toxicities

Guest Editors: Katsuhiko Yanagisawa, Jacques Fantini, Avijit Chakrabartty, and Anne Eckert



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Editorial Board

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Editorial

Aβ Behavior on Neuronal Membranes: Aggregation and Toxicities

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A growing body of evidence suggests that the aggregation and toxic potentials of amyloidogenic proteins, including amyloid β-protein (Aβ), α-synuclein, and prion protein, emerge through the interaction of these proteins with neuronal and/or glial membranes. The aggregation and deposition of Aβ are the initial events of Alzheimer's disease (AD), and the toxicity of aggregated Aβ is the basis for the neuronal loss in AD brains. Thus, the Aβ behavior on neuronal membranes should be one of the critical issues to be clarified for our further understanding of the pathogenesis of AD and to develop therapeutic strategies. To accelerate studies in this field, we have invited original research articles as well as review articles that will provide novel information for our special issue.

The first three papers of this special issue describe the crucial involvement of lipid rafts, which are specific membrane microdomains on the cell surface that are rich in sphingolipids and cholesterol, in the production, aggregation, and toxicities of Aβ. The subsequent three papers focus on the gangliosides, which are the major constituent of lipid rafts, particularly in terms of their role in the induction of conformational changes of Aβ, leading to their aggregation and emerging toxicities.

The next two articles address how Aβ causes neuronal injury by showing the possibility of formation of amyloid channels in the neuronal membranes, resulting in the disruption of calcium homeostasis that is critical for the function and survival of neurons, and the possibility of generation of radicals. In regard to the Aβ toxicities, much attention has been paid to the argument that the accumulation of Aβ inside neurons may be the critical step. In this context, the next two papers propose a mechanism by which Aβ enters the neurons, which are followed by another two papers showing how the internalized Aβ acts pathologically inside neurons, emphasizing the possibility that the mitochondria may be a target of intraneuronal Aβ.

A further argument for the possible interaction between Aβ and neuronal membranes is presented in the next four papers. In these papers, it is presented how Aβ affects the properties of neuronal membranes or, conversely, how the alteration of membrane properties affects the processing of amyloid precursor protein (APP) leading to Aβ generation. Note that the metabolism of neuronal lipids, particularly sphingolipids and ceramide, can be regulated in association with APP processing.

The final paper of this special issue describes a foresighted aspect of science and technology of nanochemistry with respect to the pathological protein aggregation, which is likely based on the catalysts of membrane lipids, suggesting an opportunity for developing novel nanomedicines and nanodiagnostics for various amyloidoses.

We all look forward to seeing further expansion of studies in this field in the near future.

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Review Article

Lipid Rafts: Linking Alzheimer's Amyloid-β Production, Aggregation, and Toxicity at Neuronal Membranes

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Lipid rafts are membrane microdomains, enriched in cholesterol and sphingolipids, into which specific subsets of proteins and lipids partition, creating cell-signalling platforms that are vital for neuronal functions. Lipid rafts play at least three crucial roles in Alzheimer's Disease (AD), namely, in promoting the generation of the amyloid-β (Aβ) peptide, facilitating its aggregation upon neuronal membranes to form toxic oligomers and hosting specific neuronal receptors through which the AD-related neurotoxicity and memory impairments of the Aβ oligomers are transduced. Recent evidence suggests that Aβ oligomers may exert their deleterious effects through binding to, and causing the aberrant clustering of, lipid raft proteins including the cellular prion protein and glutamate receptors. The formation of these pathogenic lipid raft-based platforms may be critical for the toxic signalling mechanisms that underlie synaptic dysfunction and neuropathology in AD.

1. Introduction

Alzheimer's Disease (AD) is a progressive, neurodegenerative brain disorder which affects over 37 million people worldwide with an estimated global cost of over $600 billion in 2010 [1, 2]. AD is a growing socioeconomic and financial burden due to its strong correlation with ageing; around 1 in 3 people aged over 80 years have AD, which means that a rapid rise in AD cases is anticipated as life expectancy continues to increase. Although several therapeutics are currently available to slow disease progression, there is currently no way to halt or prevent AD [3].

AD is characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles in the brain. The major constituents of senile plaques are the amyloid-β (Aβ) peptides, which are derived from the proteolytic processing of the amyloid precursor protein (APP) within lipid rafts [4]. The Aβ peptide, notably Aβ_{1-42}, is highly aggregation prone and self-assembles to form a heterogeneous mixture of oligomers and protofibrils, ultimately depositing as fibrils in senile plaques. An accumulating body of evidence indicates that soluble Aβ oligomers, which correlate strongly with disease onset and severity, are the major neurotoxic species in AD [5–8]. Although Aβ oligomers are neurotoxic at nanomolar concentrations and cause AD-related memory deficits, the cellular mechanisms of toxicity are poorly characterised. Recently, several neuronal receptors which bind Aβ oligomers have been identified, including the cellular prion protein (PrP^C) [9] and glutamate receptors [10, 11] among others. Interestingly, these receptors reside primarily within, or partition into, cholesterol-rich microdomains within the plasma membrane known as lipid rafts.

The three steps which underlie Aβ oligomer-mediated neuropathology in AD, are (1) Aβ production, (2) Aβ assembly into oligomers and (3) Aβ oligomers interacting with neuronal receptors. These steps therefore represent potential sites of therapeutic intervention in AD. Crucially, all three of these processes occur in lipid raft domains of the plasma membrane which are considered to play a key role in the development of AD [12]. In this paper, we will

outline the pivotal role that lipid rafts play in linking together the generation, self-assembly and toxicity of Aβ oligomers, which underlie the development of the neuropathology in AD. A major focus will be upon the interaction between Aβ oligomers and their putative cellular receptors.

2. Lipid Rafts

2.1. Lipid Rafts as Essential Neuronal Signalling Platforms. The multitude of different lipids and proteins within the plasma membrane were once thought to be distributed homogeneously across the entire lipid bilayer, as proposed by the fluid mosaic model in 1972 [13]. However, the plasma membrane is now known to be more akin to a sea of disordered phospholipids, in which float microdomains with distinct lipid compositions, known as lipid rafts. Lipid rafts are small (10–200 nm), heterogeneous and highly dynamic assemblies that are enriched in specific components, namely cholesterol and sphingolipids (Figure 1) [14, 15]. Biochemically, lipid rafts are defined by their relative insolubility in nonionic detergents at low temperature, conferring upon them the alternative name, detergent-resistant membranes (DRMs). Lipid rafts are also known as liquid-ordered domains because the highly saturated sphingolipid acyl chains enable closer lipid packing, and therefore more restricted lateral movement, than the mainly unsaturated acyl chains of the phospholipids in the surrounding nonraft regions of the membrane.

Functionally, lipid rafts serve to compartmentalise cellular processes by concentrating certain proteins and lipids within the same microenvironment. Lipid rafts are particularly enriched in glycosyl-phosphatidylinositol (GPI)-anchored and acylated proteins due to the preferential intercalation of the saturated acyl chains into the liquid-ordered environment [16]. Other proteins can also associate with lipid rafts either directly or through binding to other cofactors or ligands [17]. The dynamic clustering and pinching off of lipid rafts regulates the spatial and temporal assembly of signalling and trafficking molecules, forming short-lived but vital signalling platforms [17]. Lipid rafts are implicated in various essential cellular functions, including signal transduction, cell adhesion and protein/lipid sorting [18]. Of particular relevance here are cell signalling, sorting and axon guidance, as these processes are essential for neural development and synaptic plasticity [19, 20]. Crucially, neuronal lipid rafts are also required for the maintenance of dendritic spines and healthy synapses, which are vital for neural communication including learning and memory; processes which fail in AD [21]. The observation that lipid rafts are much more abundant in mature hippocampal neurons than in other cell types emphasises their physiological importance within the memory centre of the healthy brain, and may explain why hippocampal neurons are a primary target for Aβ oligomer toxicity and destruction in AD [22].

2.2. Aβ Production Is Lipid Raft Dependent. Lipid rafts are involved in the regulation of APP processing and the generation of the Aβ peptide which is the driving force in AD pathology [23, 24]. For comprehensive reviews detailing the involvement of membrane rafts in AD and Aβ production, see [25–27]. The Aβ peptide is produced by the lipid raft dependent amyloidogenic processing of its precursor protein, APP (Figure 1) [4]. The amyloidogenic cleavage of full-length APP is initiated by the β-site APP cleaving enzyme-1 (BACE1), a transmembrane aspartic metalloprotease. A large, soluble ectodomain (sAPPβ) is released to leave behind a membrane-anchored C-terminal fragment (C99) which retains the intact Aβ sequence. The second amyloidogenic cleavage of APP involves a γ-secretase complex which contains presenilin-1 or presenilin-2 (the catalytic component), presenilin enhancer-2 (PEN2), nicastrin and anterior pharynx defective-1 (APH1). The γ-secretase complex cleaves the remaining C99 stub to release Aβ peptides of between 39–42 residues in length, depending upon the precise cleavage site, along with the APP intracellular domain (AICD).

Although the majority of full-length APP is localised to nonraft regions of the plasma membrane, where non-amyloidogenic cleavage by the α-secretases ADAM 9, 10, and 17 [28] precludes Aβ formation, a subset of both APP and BACE1 partitions into lipid rafts along with γ-secretase components. Both BACE1 and the γ-secretase subunits undergo posttranslational S-palmitoylation which aids their targeting to lipid raft domains [25]. In the case of APP, a direct interaction with cholesterol—the major component of lipid rafts—was recently identified [29]. High cholesterol increases the partitioning of APP, along with BACE1 and γ-secretase components, into lipid rafts [30]. A large body of evidence points towards lipid rafts being the physiological site of amyloidogenic Aβ production by BACE1 and the γ-secretase complex. For example, both the copatching of APP and BACE1 by cross-linking antibodies [31] and the exclusive targeting of BACE1 to lipid rafts by the addition of a GPI-anchor [32] significantly increased APP cleavage at the β-secretase site. Furthermore, enrichments in lipid raft components, namely cholesterol and ganglioside GM1, promote the generation of Aβ [31, 33]. All four of the γ-secretase subunits are also enriched and active within lipid raft fractions derived from human brain [34, 35] and lipid raft-type membranes *in vitro* [36, 37]. In the brain, the majority of Aβ is found within detergent-resistant, glycolipid-enriched rafts, along with γ-secretase components [38].

2.3. Depleting Lipid Raft Components Modulates Aβ Production. The composition of lipid rafts purified from AD brains has been shown to be abnormal, with the rafts being more ordered and more viscous [39], which implies that the modulation of lipid raft composition may present a therapeutic avenue for modulating AD-related neuropathology. This has led to a number of researchers investigating whether depleting lipid raft components could lower Aβ production and therefore prevent AD. Cholesterol, being a major component of lipid rafts and a risk factor for AD, was the obvious choice to target [40]. For a recent review of the involvement of cholesterol in AD, see [41].

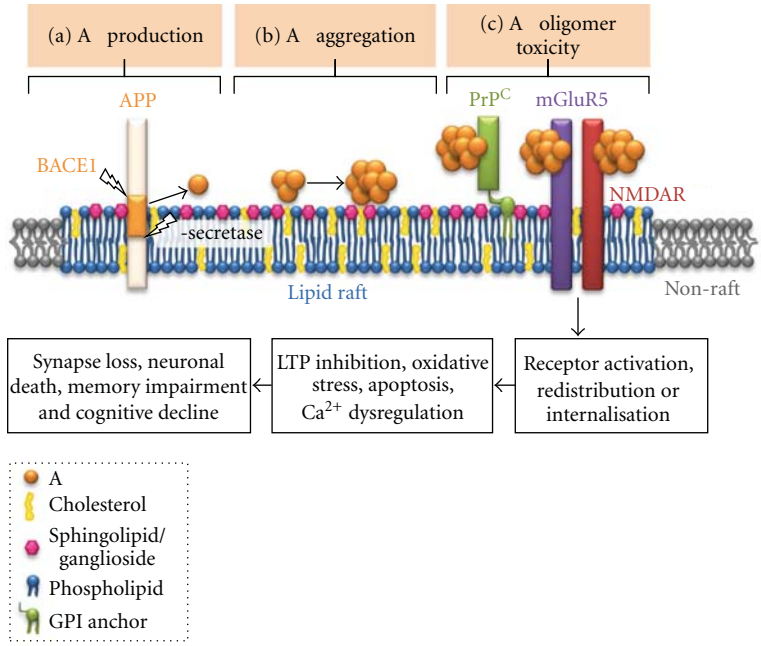


Figure 1: Lipid rafts facilitate the production, aggregation, neuronal binding and toxicity of Aβ oligomers. (a) The Aβ peptide is produced by the lipid raft dependent sequential cleavage of APP, first by BACE1 and then by the γ-secretase complex; (b) Lipid raft components including cholesterol and sialic acid-containing gangliosides promote the aggregation of Aβ to form soluble oligomers; (c) Aβ oligomers bind to specific neuronal receptors within pathogenic lipid rafts, including PrP^C and the NMDA and mGluR5 receptors. The resulting perturbations in neuronal function and survival underlie the memory impairments and cognitive decline which characterise Alzheimer's disease.

Cholesterol depletion has indeed been shown to reduce APP partitioning into lipid rafts which precludes its interaction with BACE1 and γ-secretase components, thus lowering Aβ production [42]. Hypercholesterolaemia is linked to increased Aβ production and deposition in the brain, both in humans [43–45] and in rodents [46–48] and is linked to an increased risk of developing AD. Cholesterol depletion also lowers Aβ production in cultured cells [31] and one study showed that a 70% reduction in cholesterol in living hippocampal neurons was sufficient to completely abolish Aβ production [49].

Taking this into account, cholesterol-lowering drugs known as statins have been evaluated as potential anti-AD drugs, with conflicting results [50]. Some retrospective epidemiological studies have shown that the administration of statins, which lower cholesterol levels, can reduce the incidence of dementia, including AD [51–53]. Cholesterol inhibitors can also lower Aβ levels in cultured neuroblastoma cells [54]. However, other studies have shown no correlation between statin usage and dementia [55] and the effect of statins upon disease progression and cognitive decline in AD patients has been challenged [56]. Intriguingly, it was revealed recently that Aβ production actually reduces cholesterol in cultured cells of neuronal origin by increasing efflux, possibly acting as a chaperone to remove excess cholesterol from the brain to the circulation [57].

Although a reduction in cholesterol may go some way towards reducing Aβ levels in the brain, much longer-term epidemiological studies and clinical trials initiated before significant neuronal loss and cognitive function are apparent are required in order to further elucidate the effects of lowering cholesterol levels upon AD onset and neuropathology. Lipid rafts contain many essential components other than cholesterol, such as sphingolipids, and it is likely that the modulation of just one factor will not completely abolish Aβ production *in vivo*. It is important to remember that cholesterol metabolism in the brain is largely isolated from the rest of the body by the blood-brain barrier. As nearly all of the cholesterol in the brain is synthesised *in situ*, the modulation of cholesterol levels within neurons represents a more difficult pharmaceutical challenge and the blood-brain barrier permeability of the drugs used needs to be considered [29]. Furthermore, even if cholestxerol depletion mediates a reduction in Aβ levels, Aβ oligomers effect neurotoxicity and memory impairments at low nanomolar concentrations [58]. Therefore, residual levels of Aβ production may be sufficient for continued Aβ oligomer-mediated toxicity.

3. Lipid Raft Components Promote Aβ Oligomerisation

3.1. Aβ Oligomers Are the Major Neurotoxic Species in AD. The Aβ peptide is natively unfolded and, under certain conditions, it aggregates to form a heterogeneous mixture of soluble oligomers, protofibrils and fibrils. It was accepted for a long time that the Aβ fibrils that deposit in neuritic plaques, which are observed *post mortem* in diseased brains, were responsible for neurotoxicity in AD [59]. Aβ fibrils have been reported to induce neuronal dysfunction and

cell death, although fibrils are less potent neurotoxins than soluble forms of Aβ [60, 61]. Interestingly, fibrils have been found to become more neurotoxic upon fragmentation [62], raising the possibility that soluble species released from fibril ends may underlie their neurotoxicity. A plethora of studies have now demonstrated that levels of soluble Aβ oligomers in the brain correlate much better than plaques or fibrils with AD onset, progression and severity [5, 6, 8, 63, 64]. Within the last fifteen years, a large number of studies from research groups worldwide have reported the existence of many different oligomeric assemblies from various sources, including AD brain and cerebrospinal fluid (CSF) samples, secreted into the conditioned medium of cultured cells or prepared artificially from recombinant or synthetic Aβ peptides [65]. A heterogeneous range of sizes and peptide conformations have been observed among these natural and artificial Aβ oligomers, including dimers and trimers [66, 67], tetramers, hexamers and the dodecameric Aβ 56 [64], globulomers [68], ring-shaped annular protofibrils [69] and higher molecular weight Aβ-derived diffusible ligands (ADDLs) which can comprise hundreds of monomeric subunits [9, 70] (Figure 2). However, despite the disparity in size and source, Aβ oligomers appear to share important functional properties. Notably, both natural and synthetic Aβ oligomer preparations bind to hippocampal neurons and cells of neuronal lineage, causing a loss of dendritic spines, neurotoxicity, the inhibition of long-term synaptic potentiation (LTP: an electrophysiological correlate of learning and memory) and impairments in working memory at nanomolar concentrations [64, 67, 68, 70–73]. The preferential binding and toxicity of Aβ oligomers towards neurons in the hippocampus may explain why Aβ oligomers correlate with AD severity and disease progression [9, 68, 70]. However, the cellular mechanisms by which these effects are modulated remain poorly understood.

3.2. Aβ Oligomerisation Is Modulated by Lipid Raft Components. Aβ is a physiological peptide which is present in the brain tissue and CSF of healthy subjects throughout life, without necessarily causing neurodegeneration [74–76]. Many studies have shown that monomeric, nonaggregated Aβ does not cause the neurotoxic effects that are mediated by Aβ oligomers. In fact, monomeric Aβ has recently been reported to have neuroprotective roles in the brain [77, 78]. The aggregation of Aβ is necessary for its toxicity [79] and the emerging picture is that soluble Aβ oligomers are the proximate neurotoxins in AD [8, 80]. The aggregation of Aβ is therefore a critical step in the development of AD pathogenesis, and one in which lipid rafts appear to play a fundamental role.

Neuronal sensitivity to Aβ-induced toxicity has been found to be dependent upon Aβ binding to the cell membrane [81] and Aβ has been identified in lipid rafts from cultured cells and from human and rodent brains. Soluble Aβ dimers accumulate rapidly, and have been found at elevated levels, in lipid raft fractions isolated from human and transgenic mouse model AD brains [82]. Importantly, Aβ has been shown to accumulate in presynaptic terminals in AD cortex where it colocalises with the lipid raft markers cholesterol and ganglioside GM1 [83]. Taken together, these data suggest that Aβ accumulation and aggregation within lipid rafts may underlie AD neuropathology.

As cholesterol is a major component of lipid rafts, it was postulated to facilitate Aβ oligomerisation on neuronal membranes. The brain is particularly enriched in cholesterol, harbouring over 23% of the body's total complement but comprising only around 2% of total body mass [84]. However, the role of cholesterol in promoting the assembly of Aβ is controversial and conflicting evidence has been presented in recent years. The main difficulty is being able to distinguish between the key role of cholesterol in building the lipid raft domains necessary for Aβ production and the suggested role of cholesterol in promoting Aβ oligomerisation. As discussed previously, raised cholesterol has been linked to AD; is this solely due to an increase in total lipid raft composition of the plasma membrane which increases amyloidogenic processing of APP to yield more Aβ peptide or due to a direct effect on Aβ oligomerisation?

A growing body of evidence suggests that certain components of lipid raft domains may play a much more sinister role in catalysing the conversion of the aggregation-prone Aβ peptide to its neurotoxic, oligomeric states. Cholesterol is known to modulate the interaction of the Aβ peptide with lipid bilayers [85]. Further, Aβ oligomers isolated from AD patients associate with DRMs in a cholesterol-dependent manner, and cholesterol depletion reduces the aggregation of Aβ [86]. It is currently unknown, however, whether this latter effect is due to a direct interaction between Aβ and cholesterol, or due to the overall depletion in lipid raft domains and/or the subsequent change in composition and properties brought on by a reduction in cholesterol. Conversely, a recent study revealed that increasing the level of cholesterol in human neuroblastoma cells actually reduced the ability of synthetic Aβ oligomers to bind [87], in spite of the colocalisation of the Aβ oligomers with the lipid raft component ganglioside GM1. These data agree with the authors' previous finding that an increased level of membrane cholesterol exerts a protective effect against Aβ oligomer toxicity [88]. In the more recent study [89] it was proposed that a fluctuation in cholesterol levels may alter the physical properties of lipid rafts thereby modulating oligomer binding.

Cholesterol can also facilitate Aβ aggregation through the structural modification of other lipid raft components. A recent study using reconstituted membranes revealed a structural role for cholesterol in modulating the conformation of glycosphingolipids. Depending on the type of glycosphingolipid, cholesterol can either facilitate (such as for ganglioside GM1) or inhibit the interaction of Aβ peptides with lipid rafts through fine-tuning of the glycosphingolipid conformation [90]. This reinforces the notion that Aβ binding to, and aggregation upon, neuronal lipid raft domains cannot be ascribed to a single component, but rather that multiple players are likely to be involved.

In fact, mounting evidence suggests that gangliosides within lipid rafts appear to be the main driving force

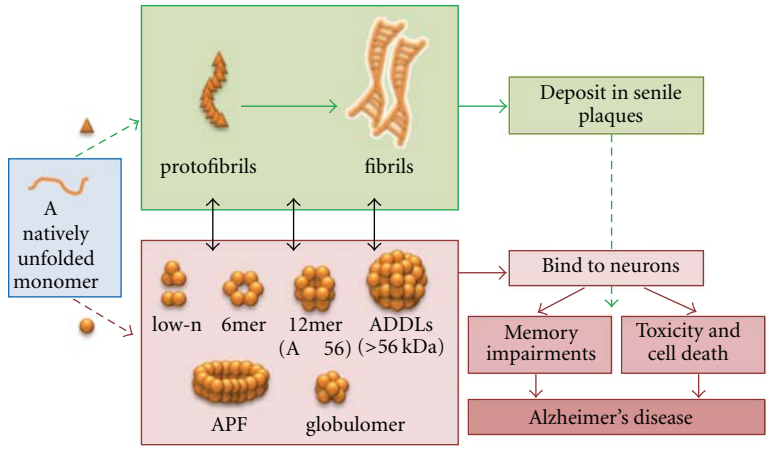


Figure 2: Aβ oligomers are the key neurotoxic assemblies in Alzheimer's Disease. The Aβ peptide is natively unfolded yet conformationally plastic and prone to aggregation. In response to various stimuli, including elevated concentration, Aβ undergoes complex conformational rearrangements to form oligomer-competent or fibril-competent intermediates. A variety of Aβ oligomers can form which include low-n oligomers (dimer and trimers), globulomers, hexameric and dodecameric (Aβ 56) states, higher molecular weight species such as Aβ-derived diffusible ligands (ADDLs) and ring-shaped annular protofibrils (APFs). Some oligomers are stable, off-pathway intermediates whereas others undergo further conformational changes and aggregation to form larger protofibrils and fibrils. Fibrils of Aβ are insoluble and deposit within extracellular senile plaques. Aβ oligomers are soluble and represent the active neurotoxic species in AD. The specific binding of Aβ oligomers to neurons, particularly in the hippocampus, triggers the memory impairments, loss of synaptic functionality and neuronal death which characterise AD.

behind the oligomerisation of Aβ on neuronal membranes. The development of AD within certain brain regions has been found to correlate with increased ganglioside levels [91]. Gangliosides are glycosphingolipids with one or more sialic acid moieties attached to the sugar chain. Gangliosides are found predominantly in the central nervous system, where they are enriched in lipid rafts due to the preferential packing of their saturated acyl chains within the liquid-ordered phase. A study in 1995 revealed that a population of membrane-bound Aβ tightly bound to gangliosides exists in AD brains [92]. More recently, exogenously-applied Aβ was shown to bind to neuronal membranes and to redistribute into lipid rafts where it colocalised with ganglioside GM1 in a time-dependent manner [93]. GM1 facilitated the binding and accumulation of Aβ oligomers at lipid raft domains and appeared to be required for the Aβ oligomer-mediated lipid peroxidation of DRMs [94]. Ganglioside GM1 contains just one sialic acid moiety and plays important physiological roles in neuronal function. Aβ appears to interact with the sialic acid moiety of gangliosides such as GM1 and these bound aggregates can go on to seed further Aβ aggregation [95]. The interaction between sialic acid and Aβ induces a conformational rearrangement of the Aβ peptide chain [96] which may potentiate Aβ oligomerisation. DRMs derived from ganglioside-rich rat brain, but not from liver, were found to promote the oligomerisation of Aβ [97]. Further, this study revealed that the removal of cholesterol or protein from these raft fractions did not prevent Aβ aggregation, providing evidence that neither cholesterol nor protein is essential for this process. However, lipid raft fractions containing very low levels of gangliosides still retained some Aβ oligomerisation ability, and therefore ganglioside-independent aggregation mechanisms cannot be ruled out.

4. Aβ Oligomers Bind to Neuronal Receptors within Lipid Rafts

4.1. Aβ Oligomers Bind to High Affinity Protein Receptors. When the first synthetic Aβ oligomers were prepared from $A\beta_{1-42}$ peptide by the Klein laboratory in 1998, it was observed that their binding to hippocampal neurons and cultured nerve cells was abolished by treating the cells with trypsin [70]. This, coupled with the low oligomer concentration (5 nM) required for neurotoxicity, implied that specific protein receptors were responsible for the binding of Aβ oligomers and for the subsequent transduction and amplification of neurotoxicity. Indeed, a recent study found that Aβ oligomer binding to neurons was saturable with an estimated apparent K_d of 0.4 nM [9]. This finding implied that one or more high-affinity receptors are responsible for Aβ oligomer binding and subsequent neurotoxicity. Immunofluorescence microscopy has revealed that Aβ oligomers bind to dendritic spines of hippocampal neurons where they colocalise with postsynaptic markers [9, 98, 99]. Interestingly, Aβ oligomer binding to neurons has a punctate appearance [100], which is reminiscent of the appearance of lipid raft localised proteins [101]. Several putative neuronal receptors for Aβ have been identified in recent years, namely proteins that are related to mechanisms of memory and neuroprotection in the brain. Noteworthy, all of these receptors either reside primarily within, or can partition into, lipid raft domains at the surface of neurons. Lipid rafts may therefore hold the key to

understanding how the deleterious effects of Aβ oligomers are transduced through binding to specific receptors within these microdomains.

4.2. The Cellular Prion Protein (PrP^{Sc}). In 2009, Laurén and colleagues reported that the cellular prion protein (PrP^{C}) is a specific, high-affinity neuronal receptor for $A\beta_{1-42}$ oligomers [9]. PrP^{C} is a GPI-anchored protein that is expressed at high levels in the brain, particularly at synapses and axons, where it resides in lipid rafts. The misfolded form of the prion protein (PrP^{Sc}) is infamous for being the causative agent in Mad Cow Disease (Bovine Spongiform Encephalopathy, BSE) and its human equivalent, Creutzfeldt-Jakob Disease (CJD). Although the correctly-folded PrP^{C} is critical for prion disease pathogenesis, its physiological function remains enigmatic, with potential neuroprotective roles in oxidative stress defence, metal ion homeostasis and anti-apoptosis [102]. In a search to identify neuronal receptors for Aβ oligomers, Laurén et al. [9] screened a mouse brain expression library of 225,000 cDNA constructs from which only two positive clones, both encoding full-length PrP^{C}, were isolated that were able to bind Aβ oligomers with high affinity and specificity. Interestingly, the PrP^{C} homologues Shadoo and Doppel were found not to bind Aβ oligomers to any significant degree. A further, more focussed screen of 352 clones encoding transmembrane proteins identified amyloid-β precursor-like protein 1 (APLP1) and transmembrane protein 30B (TMEM30B) as weak Aβ receptors, although their specificity for oligomeric Aβ was poor. The α7 nicotinic acetylcholine receptor (nAChRα7) and the receptor for advanced glycation end products (RAGE) were also assayed due to their previously reported affinities for Aβ peptides [103, 104], although neither displayed high-affinity Aβ oligomer binding. Therefore, PrP^{C} was the only identified receptor to display both high affinity and high specificity for Aβ oligomers.

A direct interaction between PrP^{C} and Aβ oligomers was confirmed and the core oligomer binding region of PrP^{C} was narrowed down to amino acids 95–110, a positively charged cluster rich in lysine residues [9]. PrP^{C} was also shown to mediate the inhibition of LTP that is induced when hippocampal slices were incubated with Aβ oligomers at nanomolar concentrations [9]. A follow-up *in vivo* study revealed that the presence of PrP^{C} is required for the Aβ oligomer-mediated memory impairments in an AD model mouse [105]. Taken together, these data indicate a strong association between Aβ oligomers binding to PrP^{C} within lipid rafts of hippocampal neurons and the induction of memory deficits that are characteristic of AD.

Nevertheless, there has been some dispute over the role of PrP^{C} in transducing the deleterious effects of Aβ oligomers *in vivo*, as other studies have reported data which oppose this theory. First, Balducci and colleagues reported that although Aβ oligomers bind tightly to PrP^{C} they cause impairments in long-term memory in mice independently of PrP^{C} [106]. In this study, the effects of synthetic Aβ oligomers upon wild-type mice were observed, whereas Gimbel et al. [72] utilised a mouse model expressing a familial AD mutant APP. Further, the synthetic depsipeptide and the oligomer preparation method utilised by Balducci et al. [106] differed from those used by Gimbel and coworkers [72], raising the possibility that PrP^{C} does not have the same binding affinity for all types of Aβ oligomers. Second, the Aguzzi group crossed an AD mouse model, which suffers from Aβ-dependent memory deficits in the form of LTP impairment, with mice expressing either wild-type PrP^{C}, a secreted form of PrP^{C} (lacking its GPI anchor) or no PrP^{C} [107]. They found that the presence or absence of wild-type PrP^{C} had no effect upon the Aβ-mediated inhibition of LTP. However, expression of the secreted form of PrP^{C} was found to suppress the impairment in LTP, which the authors proposed may be due to the potential chelation and subsequent degradation of Aβ oligomers by soluble PrP^{C} in the extracellular milieu. Third, Kessels and coworkers reported the influence of PrP^{C} upon hippocampal neurons expressing a C-terminally truncated form of APP in a viral expression construct [108]. The same loss of dendritic spines and inhibition of LTP were observed in the presence and absence of PrP^{C}, suggesting that Aβ-mediated synaptic defects do not require PrP^{C}. However, Laurén and colleagues have emphasised the differences in the model system utilised by Kessels and coworkers in their study which may account for the opposing data, namely the viral expression of APP, a higher concentration of Aβ oligomers and a difference in the observed suppression of synaptic plasticity [109].

Further investigation is needed to clarify the role of PrP^{C} in modulating the Aβ oligomer-mediated impairments in memory and LTP. Differences in the oligomer preparations, age and genotype of the mouse models, the nature of the promoter elements driving gene expression and the particular memory tests employed by the different authors may account for the discrepancies in the data.

The binding of Aβ oligomers to PrP^{C} is not the first time that PrP^{C} has been linked to AD. Senile plaques from a subset of AD patients were observed to contain PrP^{C} [110] and abundant Aβ deposits have been observed in some CJD cases [111]. Furthermore, the Met/Val 129 polymorphism in the *PRNP* gene that encodes PrP^{C} is a risk factor for early-onset AD [112]. In 2007, we demonstrated that PrP^{C} negatively modulates Aβ production through inhibition of the APP cleaving enzyme, BACE1 [113]. These data, along with the recent discovery that PrP^{C} binds to Aβ oligomers and transduces their deleterious effects, raises the intriguing possibility of a feedback loop [114]. We propose that, physiologically, PrP^{C} maintains Aβ production at a low level through BACE1 inhibition, but in AD this interaction may be disrupted by Aβ oligomers binding to PrP^{C} and causing its segregation from BACE1. Therefore, Aβ oligomers binding to PrP^{C} may also promote their own production through the ablation of BACE1 inhibition by PrP^{C}. More recently, levels of PrP^{C} have been shown to be reduced in AD brains [115, 116] possibly arguing against PrP^{C} being involved in mediating the neurotoxic effects of Aβ oligomers, at least in the terminal stages of the disease.

It is important to note that Laurén and colleagues reported that the removal of PrP^{C} from hippocampal neurons only reduced Aβ oligomer binding by approximately

50% [9]. This suggests that other receptors not identified in the expression library screen due to nonpreferential binding conditions or a low affinity for the particular type of Aβ oligomers that were used, and/or nonprotein lipid raft components, may play equally crucial roles in Aβ oligomer binding and neurotoxicity. Glutamate receptors, which possibly exist in a complex with PrP^C [117], represent a candidate interacting partner for Aβ oligomers which could explain the deleterious effects upon hippocampal synaptic plasticity.

4.3. Glutamate Receptors. Synaptic failure and impairments in synaptic plasticity are hallmarks of early AD neuropathology [100, 118, 119]. LTP and long-term depression (LTD) are mechanistic dimmer switches which facilitate synaptic plasticity by strengthening or weakening communication across a synapse, respectively, with LTP being essential for hippocampal-dependent learning and memory [120, 121]. Numerous lines of study have confirmed that soluble Aβ oligomers from various sources, including those isolated from AD brains, disrupt hippocampal LTP *in vitro* and *in vivo* and cause impairments in learning and memory [9, 67, 70, 107, 122, 123]. Although not all studies agree, it has also been demonstrated that Aβ oligomers can provoke LTD which opposes LTP [67, 124, 125]. Neuronal receptors which modulate LTP and/or LTD are therefore likely candidates for the specific binding of Aβ oligomers. Glutamate receptors are central to the modulation of LTP and LTD. Additionally, glutamate receptor dysfunction has been implicated in AD which is characterised by memory deficits caused by impaired synaptic plasticity [126]. Glutamate receptors consist of two classes; ionotropic (cation-specific ion channels) and metabotropic (G-protein-coupled). Members of both classes have been implicated as neuronal receptors for Aβ oligomers.

4.3.1. NMDA Ionotropic Glutamate Receptors. N-methyl-D-aspartate receptors (NMDARs) constitute a major class of glutamate receptors in the mammalian brain which localise to the postsynaptic membrane of excitatory synapses [127]. These ion channels play key roles in excitatory synaptic transmission and synaptic plasticity [128]. The membrane channel is usually blocked by Mg^{2+} ions which are displaced when synaptic transmission results in depolarisation and glutamate release and binding. NMDAR channel opening leads to the rapid influx of Ca^{2+} which triggers LTP induction [129]. Longer-term effects which maintain the reinforced synapse include the activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), altered gene expression and kinase activity and the growth of new dendritic spines. Interestingly, NMDAR activation can also stimulate LTD, having the opposing effect of synapse weakening, and this appears to depend upon the nature of the stimulus and the subtype of NMDAR involved [130].

NMDARs localise to lipid raft domains where they interact with flotillins [131, 132] although they can move laterally between raft and nonraft domains in response to cues including phosphorylation [133]. Statins, which deplete cellular cholesterol thus reducing lipid raft formation, have been shown to reduce the localisation of NMDARs to lipid raft domains, which has a neuroprotective effect [134].

Mounting evidence points towards a central role for NMDARs in the modulation of Aβ oligomer toxicity. Soluble Aβ oligomers inhibit NMDAR-dependent LTP [70, 135] and exhibit postsynaptic binding to hippocampal neurons which express NMDAR subunits GluN1 and GluN2B [100]. A reduction in NMDAR subunits GluN1 and GluN2B has previously been observed in the hippocampus of AD brains [136]. Crucially, a recent study has confirmed that Aβ oligomer-mediated early synaptic dysfunction depends upon the activation of GluN2B-containing NMDARs [10]. Aβ oligomers were found to decrease the NMDAR-dependent influx of Ca^{2+} into dendritic spines [137], and to reduce dendritic spine and synapse density [10] in a mechanism which involve the subsequent phosphorylation of tau [138]. NMDAR antagonists, including one which is specific for GluN2B subunits, were able to reverse the Aβ-induced loss of dendritic spine density [100, 137, 139]. These effects are consistent with Aβ oligomers blocking the NMDAR-mediated stimulation of LTP whilst promoting NMDAR-mediated LTD. In addition, Aβ oligomers have been shown to stimulate the excessive generation of reactive oxygen species (ROS) through an NMDAR-dependent mechanism [140], suggesting a link between aberrant ROS regulation and Aβ-induced cognitive impairment.

Furthermore, evidence to confirm a direct interaction between Aβ oligomers and NMDAR subunits has recently been presented. Partial colocalisation was observed between NMDAR GluN2B and Aβ oligomers in hippocampal slices, which increased upon the addition of glutamate, although the maximum colocalisation was less than 50% [141]. Further, Aβ oligomers were recently found to coimmunoprecipitate with NMDAR subunits [117]. However, an indirect model proposed by Venkitaramani and colleagues suggests that the Aβ oligomer-mediated decrease in GluN2B-containing NMDARs results from the former binding to α-7 nicotinic acetylcholine receptors (α7nAChR), which activates striatal-enriched tyrosine phosphatase (STEP), in turn stimulating NMDAR internalisation [142]. More recent data has revealed elevated levels of STEP in a mouse model of AD and in human AD brains, and that the removal of STEP abrogates the Aβ-mediated reduction in NMDARs at the cell surface [143]. Whether or not Aβ oligomers interact with NMDARs directly, growing evidence suggests that NMDARs play an important role in transducing the deleterious effects of Aβ oligomers upon synaptic functionality.

4.3.2. mGluR5 Metabotropic Glutamate Receptor. The mGluR5 metabotropic glutamate receptor plays important regulatory roles in neuronal calcium mobilisation and the modulation of LTP and excitatory postsynaptic potentials in hippocampal neurons [144, 145]. Recently, mGluR5 was identified as a novel Aβ oligomer receptor in a study of the behaviour of fluorescently-labelled Aβ oligomers on hippocampal neurons and their interaction with neuronal receptors [117]. The Aβ oligomers bound to excitatory

synapses where their mobility decreased as they aggregated to form larger clusters over time. Consistent with previous data, Aβ oligomers caused a removal in NMDARs from synapses and were found to coimmunoprecipitate with NMDAR subunits. Interestingly, the Aβ oligomers also formed complexes with mGluR5 receptors, which caused their lateral redistribution into dendritic spines followed by Ca^{2+} dysregulation. Renner and colleagues also observed a time-dependent increase in lipid raft-localised mGluR5s which suggests that Aβ oligomers reduce the mobility of mGluRs, causing their aberrant aggregation within pathological signalling platforms [117]. When mGluR5 was removed from mouse hippocampal neurons, Aβ oligomer binding was reduced by approximately 80% and the loss of NMDARs from the cell surface was prevented.

Metabotropic glutamate receptors have been implicated previously in the pathogenesis of AD and other neurodegenerative disorders [126]. Impaired mGluR signalling in the cortex of AD patients has been shown to correlate with AD-related neuropathological changes [146]. Interestingly, the stimulation of mGluRs can modulate APP processing [147]. A recent study revealed that the Aβ peptide upregulates the expression of mGluR5s in astrocytes, protective nonneuronal cells which are implicated in AD pathogenesis and inflammation [148]. Increased levels of mGluR5s were observed in the brains of Down's syndrome patients [149]; a disease in which elevated levels of Aβ result from the triplication of the APP gene [150].

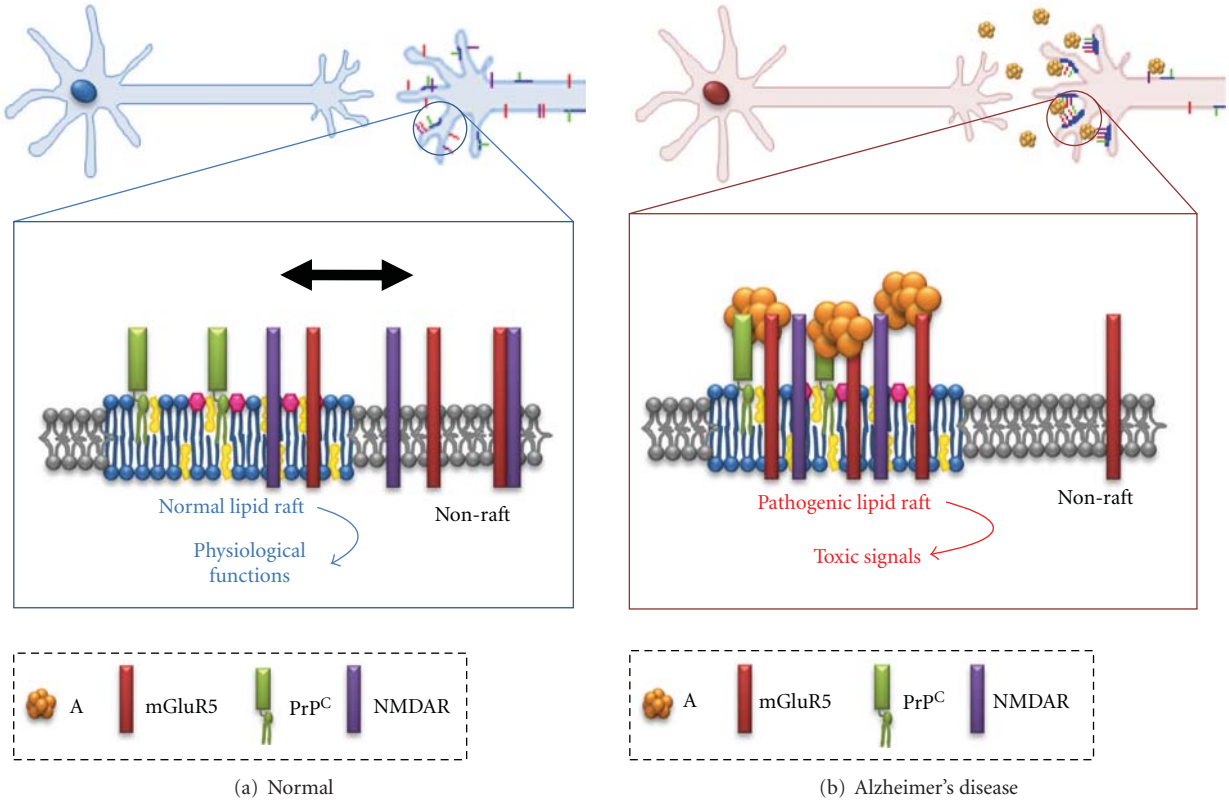
4.3.3. Other Putative Receptors. Various other lipid raft-associated proteins have been reported to effect Aβ-mediated synaptic dysfunction. For instance, the removal of nerve growth factor receptors (NGFRs), including TrkA and p75 neurotrophin receptor, from cells treated with GM1-induced Aβ oligomers caused a significant reduction in oligomer-mediated cytoxicity [151]. NGFR dysfunction and aberrant NGF signalling is associated with AD and increased Aβ production [152, 153]. Although no direct interaction has been shown to our knowledge, it is possible that interplay between Aβ oligomers and NGFRs may form part of a positive feedback loop which serves to reinforce Aβ oligomer production, whilst blocking NGF signalling with deleterious effects upon neuronal survival. Physiologically, NGF binds to TrkA causing the translocation and clustering of receptors within lipid rafts [154]. The binding of Aβ oligomers to TrkA and other NGFRs may therefore cause aberrant lipid raft clustering which prevents or disrupts the formation of the normal signalling platforms.

Recent research proposes that impaired insulin signalling may be involved in AD, even leading to the hypothesis that AD represents a third type of diabetes [155]. Insulin receptors, which are robustly expressed in hippocampal neurons, were found to bind Aβ oligomers and to undergo internalisation from dendritic spines [156]. Perturbations in insulin signalling in the brain caused by Aβ oligomers may impair memory and LTP [157]. Interestingly, insulin receptor subunits are also enriched in lipid raft domains in hippocampal neurons [158].

4.3.4. Multireceptor, Pathogenic Signalling Platforms Are Induced by Aβ Oligomers. The emerging picture is that lipid rafts accommodate multiple receptors for Aβ oligomers, namely PrP^C along with NMDAR, mGluR5 and possibly other, lower affinity receptors. Interestingly, there is evidence to suggest that these three lipid raft-associated receptors interact together. Metabotropic glutamate receptors have been found to cocluster with NMDARs [159]. It has also been reported that PrP^C inhibited NMDAR function in hippocampal neurons and coimmunoprecipitated with NMDAR subunits [160]. The functional and physical links between these Aβ oligomer receptors suggest the existence of a multi-component, Aβ oligomer binding raft complex, comprising of PrP^C, mGluR5 and NMDAR (Figure 3) [117]. Whether the formation of this complex is required for oligomer binding, or whether the interaction of Aβ oligomers with the individual proteins induces its assembly, is a "chicken and egg" situation. One possible hypothesis is that Aβ oligomers promote the clustering of PrP^C and glutamate receptors into pathological mega-scaffolds which induce both toxic loss- and gain-of-function downstream effects. For instance, the aberrant localisation of glutamate receptors may impede neuronal signalling mechanisms including LTP, while the clustering or internalisation of NMDARs may promote their LTD-inducing functionality. The combined effects of oligomer binding upon more than one glutamate receptor is likely to be a large disturbance in Ca^{2+} homeostasis which results in pathological signalling cascades. Interestingly, the PrP^C-mediated response to oxidative stress is thought to induce signalling cascades which can modulate Ca^{2+} flux and synaptic plasticity [161]. Furthermore, Aβ oligomers may cause the internalisation or loss of function of components such as PrP^C thus reducing neuroprotection against oxidative stress at the cell surface. The clustering of Aβ oligomers at lipid raft domains may also cause damage to physiologically important signalling rafts, thus impairing neuronal function. Furthermore, the Aβ oligomer-induced redistribution of neuronal proteins into lipid rafts may influence their nonraft interacting partners, with additional deleterious effects upon neuronal function and integrity.

5. Conclusions

Neuronal lipid rafts are crucial modulators of Aβ production and aggregation, leading to the accumulation of neurotoxic Aβ oligomers in the brain which drive AD pathology. Recent evidence now incriminates lipid rafts as pathological signalling platforms in which Aβ oligomer receptors, such as PrP^C and glutamate receptors, cluster. Aβ oligomer binding appears to induce the aberrant localisation of these proteins with deleterious effects upon their physiological functions including hippocampal LTP, which underlies memory, and defence against oxidative stress. In this way, lipid rafts appear to be directly responsible for the transduction of Aβ oligomer-mediated memory impairments and neurotoxicity which characterise AD. Lipid rafts are not only implicated in AD but may also be the key to a range of neurodegenerative



(a) Normal (b) Alzheimer's disease

Figure 3: Aβ oligomer binding stimulates the clustering of specific neuronal receptors into aberrant pathogenic signalling platforms at the synapse. (a) Synaptic function and neural communication is maintained by the activity of postsynaptic receptors including the neuroprotective PrP^C and the NMDA and mGluR5 glutamate receptors, which modulate synaptic plasticity. In the healthy brain, the dynamic translocation of such receptors between lipid raft and nonraft domains of the plasma membrane modulates their activities; (b) in AD, the binding of Aβ oligomers at postsynaptic membranes causes the redistribution and clustering of receptors including PrP^C, NMDAR and mGluR5 into pathological signalling platforms [117]. The resulting loss of transient lateral movement and subsequently interaction with other components is proposed to cause a loss of normal functionality combined with aberrant signalling by these receptors. The dysregulation of $Ca2^+$ and inhibition of synaptic long-term potentiation likely underlie the memory deficits which characterise AD. Further, the loss of PrP^C depletes neuronal protection against oxidative stress which may partially account for the neuronal death that is observed in AD brains.

proteinopathies, including Parkinson's Disease, Huntington's Disease, amyotrophic lateral sclerosis and prion diseases (reviewed in [12]). Indeed, lipid raft disruption protects neurons against the toxicity of other oligomers besides Aβ [22] and lipid rafts may therefore represent generic platforms for oligomer-mediated neurotoxicity. Understanding the cell biology of the downstream effects of amyloid oligomers binding to neuronal lipid raft proteins may uncover potential therapeutic targets for the prevention of AD and other neurodegenerative diseases.

Abbreviations

α7nAChR:	α-7 nicotinic acetylcholine receptor
Aβ:	Amyloid-beta
AD:	Alzheimer's Disease
ADDL:	Aβ-derived diffusible ligand
AICD:	APP intracellular domain
AMPAR:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APF:	Annular protofibril
APH1:	Anterior pharynx defective-1
APLP1:	Amyloid-β precursor-like protein 1
APP:	Amyloid precursor protein
BACE1:	Beta-site APP cleaving enzyme-1
BSE:	Bovine Spongiform Encephalopathy
CJD:	Creutzfeldt-Jakob Disease
CSF:	Cerebrospinal fluid
DRM:	Detergent-resistant membrane
GPI:	Glycosyl phosphatidylinositol
K_d:	Apparent dissociation constant
LTD:	Long-term synaptic depression
LTP:	Long-term synaptic potentiation
mGluR:	Metabotropic glutamate receptor
NGFR:	Nerve growth factor receptor
NMDAR:	N-methyl-D-aspartate receptor
PEN2:	Presenilin enhancer-2
PrP^C:	Cellular isoform of the prion protein
PrP^{Sc}:	Scrapie isoform of the prion protein

RAGE: Receptor for advanced glycation end products
STEP: Striatal-enriched tyrosine phosphatase
TMEM30B: Transmembrane protein 30B.

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Review Article

Mechanisms of Amyloid-Beta Peptide Uptake by Neurons: The Role of Lipid Rafts and Lipid Raft-Associated Proteins

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A hallmark pathological feature of Alzheimer's disease (AD) is the accumulation of extracellular plaques composed of the amyloid-beta (Aβ) peptide. Thus, classically experiments were designed to examine Aβ toxicities within the central nervous system (CNS) from the extracellular space. However, a significant amount of evidence now suggests that intraneuronal accumulation of Aβ is neurotoxic and may play an important role in the disease progression of AD. One of the means by which neurons accumulate intracellular Aβ is through uptake of extracellular Aβ peptides, and this process may be a potential link between Aβ generation, synaptic dysfunction, and AD pathology. Recent studies have found that neuronal internalization of Aβ involves lipid rafts and various lipid raft-associated receptor proteins. Uptake mechanisms independent of lipid rafts have also been implicated. The aim of this paper is to summarize these findings and discuss their significance in the pathogenesis of AD.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia with a high prevalence rate among the aging population [1]. The clinical symptoms are characterized by loss of selective cognitive functions, particularly memory loss [1]. These traits are accompanied by neuropathological features observed in *postmortem* AD brains, including loss of neurons and synapses in cortical and subcortical regions, as well as extracellular plaques composed of aggregated amyloid-beta (Aβ) peptides and intracellular neurofibrillary tangles that contain hyperphosphorylated Tau protein [2]. Although the precise role of Aβ in AD etiology remains inconclusive, the current consensus is that it is a central player in the development of the disease. This hypothesis is supported by a variety of transgenic mouse lines that contain mutations in the Aβ processing machinery [3]. These mice display some of the neuropathologies and behavioral deficits similar to that observed in AD patients, providing a link between abnormal Aβ production and disease development [3]. Nevertheless, the cellular events that occur between production of the Aβ peptide and degeneration of a neuron remain inconclusive. Aβ is produced from its membrane-embedded precursor, amyloid precursor protein (APP), through sequential cleavage by enzyme complexes β- and γ-secretases [4]. Two distinct cleavage pathways exist. In the nonamyloidogenic pathway, APP is cleaved by α-secretase resulting in an 83-amino-acid C-terminal fragment that is further cleaved by the γ-secretase into a short p3 peptide [5]. In the amyloidogenic pathway, the cleavage occurs at 99 rather than 83 amino acids from the C-terminus and is mediated by the β-secretase [6]. This results in a 99-amino-acid peptide that contains an intact hydrophobic region termed the Aβ region [6]. Subsequent cleavage by γ-secretase releases this peptide region forming the Aβ peptide normally 40-amino-acids in length (Aβ40) [6]. An alternate cleavage by the γ-secretase results in a less abundant form of the peptide 42 residues in length (Aβ42) [7]. Aβ42 is more hydrophobic and has a greater tendency to aggregate into fibrils and plaques compared to Aβ40 [7]. Aβ42 is also the prevalent isoform found in the amyloid plaques of AD patients [8]. Several hypotheses

have been proposed in regards to how Aβ production leads to neuronal degeneration and toxicity: Aβ monomers are known to aggregate into higher-molecular weight oligomers and fibrils [9]. The hydrophobicity of Aβ allows it to target neurons directly; extracellular Aβ aggregates can interact with neuronal membranes resulting in disruptions in bilayer permeability [10]. Aβ oligomers and fibrils also bind to several membrane proteins which may induce change or loss of protein function [11].

2. Sources of Intracellular Aβ

Despite evidence suggesting that Aβ exerts its effects extracellularly, reports have shown that Aβ in the intraneuronal compartment may play an important role. As early as the 1980s, researchers have observed that in AD patients, Aβ deposition inside the cells precedes its accumulation in the extracellular space [12, 13]. The findings in human subjects are corroborated by transgenic mouse models of AD, where intraneuronal Aβ is commonly observed. Various mouse models display Aβ deposits inside neurons well before the appearance of extracellular plaques [14–21]. In the triple transgenic (3xTg) AD mice, the level of intraneuronal Aβ is found to correlate with synaptic dysfunction and memory impairment [21, 22]. In spite of these observations, the concept that intraneuronal Aβ contributes to disease progression has not been ubiquitously accepted. One large-scale human study found contradicting evidence, such that the level of intraneuronal Aβ increases with age even in non-AD individuals [23], suggesting that Aβ inside neurons may not have a pathological role. This might be explained by the fact that some fixation and staining methods detect Aβ in the extracellular space more strongly than inside cells [24–26]. For example, studies have shown that exposure to formic acid, which is a commonly used step in Aβ staining, does not yield the strongest intracellular Aβ signal whereas antigen retrieval by heating enhances the signal [24, 25]. Bayer and Wirths [26] suggested that in transgenic mice studies, brains are normally fixed by cardiac perfusion in a short time frame, leading to more frequent observations of intraneuronal Aβ in mouse models than in human tissues that are exposed to extended periods of *postmortem* fixation.

Although the role of intraneuronal Aβ in disease development is controversial, there is no dispute regarding the presence of Aβ within neurons. The key question that follows is the origin of the accumulated Aβ inside neurons. Thus far it is unclear whether the accumulation originates from direct deposition of Aβ intracellularly or from uptake from the extracellular Aβ pool. In addition to the plasma membrane, APP is also found on membranes of mitochondria, the trans-Golgi network, endoplasmic reticulum (ER), endosomes, autophagosomes, and lysosomes [27]. β- and γ-secretases have been found in these subcellular compartments [27]; hence Aβ production occurs intracellularly as well. Endosomes in particular are hypothesized to be a major site of Aβ production due to their acidic pH and coexpression of both β- and γ-secretases [27]. Studies have shown that internalization of APP from the plasma membrane to endosomes can occur via endocytosis, and that blockade of this process reduces intracellular Aβ levels [28]. Coexpression of β- and γ-secretases is also found in the Golgi [27], suggesting that the secretory pathways are also potential sites of Aβ production. Intracellular trafficking of APP may act as a cellular mechanism that regulates the production of Aβ since some cellular compartments have optimal conditions for APP cleavage while others do not. Nonetheless, there is no question that a significant amount of APP is cleaved at the plasma membrane resulting in extracellular deposition of Aβ. One possible fate of deposited Aβ is re-entry into cells. Glial cells including astrocytes and microglia are the putative phagocytic cells in the CNS. Extracellular Aβ can be internalized by glial cells via phagocytosis as well as pinocytosis and endocytosis [29–31]. A significant portion of deposited Aβ is likely taken up by glia. Neurons, on the other hand, are not generally considered phagocytic cells. Nonetheless, reports have shown that Aβ uptake can occur in neurons contributing to the accumulation of intraneuronal Aβ [6, 26]. Due to their potential significance in AD etiology, the cellular mechanisms that mediate neuronal Aβ uptake have garnered increased attention in recent years. Current data suggest that the majority of Aβ uptake mechanisms in neurons involve membrane microdomains termed lipid rafts.

3. Lipid Metabolism, Lipid Rafts, and AD

Lipid rafts are mobile microdomains in plasma or organelle membranes that are rich in cholesterol and sphingolipids [32]. This specialized lipid composition distinct from the surrounding membrane phospholipids allows lipid rafts to associate with select groups of proteins [32]. Lipid raft-associated proteins have key roles in protein entry and trafficking as well as signal transduction [32]. The idea that lipid rafts may be associated with Aβ metabolism originated from cholesterol studies [32]. In AD patients, serum cholesterol levels correlated with Aβ load in the brain [33]. Individuals with high cholesterol levels during midlife are at a higher risk to develop AD later in life [34], whereas individuals treated with cholesterol lowering drugs have a lower prevalence of AD [35, 36]. Similar trends were observed in animal and cell culture models [32]. Notably, accumulation of intraneuronal Aβ was increased in transgenic AD mice fed with a high cholesterol diet [37, 38]. Despite evidence supporting a role for cholesterol in AD pathogenesis, clinical use of cholesterol-lowering statins to treat AD patients has not yielded consistent results [39]. Furthermore, *postmortem* analyses of AD brains showed that cholesterol levels in the hippocampus and the frontal cortex were not significantly different from that of age-matched controls [40, 41]. Although the precise role of cholesterol in AD is unclear, the discovery of β-secretase, γ-secretase, and APP at lipid rafts suggests that cholesterol is involved in APP processing and Aβ production [42–44]. Indeed, cholesterol depletion in cultured neurons decreases the production of Aβ [45]. Cholesterol depletion is a commonly used method to compromise lipid raft integrity and has been shown to dissociate γ-secretase from lipid rafts [44, 46]. Increased targeting of β-secretase to lipid rafts promoted its cleavage activity of APP [47]. Studies have also found that unlike

APP cleavage, which occurs mostly at lipid rafts, non-APP substrates of the γ-secretase were cleaved at non-lipid raft domains [44]. Lipid rafts may thus serve as a platform for secretases to differentiate APP processing from other secretase-dependent cellular processes.

4. Aβ Binding, Aggregation, and Internalization at Lipid Rafts

In addition to processing of APP, lipid rafts have been proposed as a site for binding of extracellular Aβ and a niche for Aβ aggregation [11, 48]. Exogenously applied oligomeric Aβ has been shown to concentrate at lipid rafts of cultured neurons [49]. Lipid rafts isolated from AD mice are also concentrated with soluble nonfibrillar Aβ [50]. Sphingolipid derivatives such as sphingomyelin and gangliosides can readily bind both soluble and fibrillar Aβ and are thought to be the major Aβ-recruiting component in lipid rafts [51–54]. Cholesterol has also been shown to interact with soluble and fibrillar Aβ [55]. Gangliosides, in particular monosialotetrahexosylganglioside (GM1), is known to form an "Aβ-seed" by complexing with soluble Aβ [51, 52]. GM1-bound Aβ (GM1/Aβ) has a different conformation than that of soluble Aβ and seeds the formation of Aβ aggregates [51, 52]. Binding of Aβ to lipid rafts has been shown to promote its oligomerization and subsequently fibril formation, possibly via the seeding and aggregation-promoting effect of GM1/Aβ [56, 57]. Disruption of lipid rafts in cultured pheochromocytoma cells was able to protect them against toxicity induced by Aβ oligomers [58, 59]. The interaction of Aβ with lipid rafts may induce toxicity via several mechanisms: Aβ aggregation at lipid rafts can induce membrane perturbations by oxidative damage [60, 61]. Alternatively, interaction of Aβ oligomers with GM1 decreases membrane fluidity and in turn stimulates APP processing, resulting in a vicious cycle of Aβ overproduction [62]. It has also been suggested that rather than aggregating on the cell surface, a portion of the Aβ binding to lipid rafts is internalized [63]. One of the earlier studies examining Aβ internalization observed that in human fibroblast cells, Aβ accumulated intracellularly were in a high-molecular weight oligomeric state [64]. A later study using human neuroblastoma cells showed that not all of the applied exogenous Aβ oligomers were internalized; the oligomers bound to the membrane formed aggregates larger than the internalized oligomers [63]. These findings suggest that Aβ uptake may precede its aggregation at the membrane, and that surface aggregation of Aβ takes place as a result of saturation of the uptake pathway. The authors also demonstrated that oligomeric Aβ was internalized more efficiently than fibrillar Aβ [63]. Saavedra et al. [65] in a study using cervical sympathetic neurons found that internalized Aβ oligomers colocalized with cholera toxin subunit B (CTxB), a lipid raft marker that specifically binds to GM1, suggesting that Aβ internalization occurs at lipid rafts, possibly via GM1 binding. The authors further showed that reduction of cellular cholesterol and sphingolipid levels significantly decreased Aβ uptake [65]. A recent study by Singh et al. [66] using mouse neuroblastomas also found colocalization of internalized Aβ with CTxB. The authors pharmacologically inhibited lipid raft-dependent endocytosis which resulted in decreased uptake of soluble Aβ [66]. Thus far it is unclear whether the lipid components or the associated receptor proteins are responsible for initiating Aβ uptake at lipid rafts. Aβ, in addition to lipids, can bind to a wide range of membrane proteins [11], which may act as carriers of Aβ upon endocytosis of the Aβ-receptor protein complex. The following section discusses the various routes of cellular entry for extracellular Aβ.

5. Mechanisms of Aβ Uptake

5.1. Glutamate Receptors. One of the earliest pathological features in AD that coincides with accumulation of intraneuronal Aβ is synaptic aberrations including changes in the shape and the protein composition of synapses as well as an overall decrease in abundance. Application of exogenous soluble Aβ to hippocampal slices leads to Aβ accumulation in CA1 neurons coupled with decreased expression of the synaptic marker synaptophysin [67]. Extracellular Aβ may thus preferentially target synapses. There is a wide range of lipid raft-associated receptors localized at the synaptic membrane. One receptor that is highly expressed at synapses is the N-methyl-D-aspartic acid (NMDA) receptor for the neurotransmitter glutamate. NMDA receptors are associated with lipid rafts [68, 69]. Exposure of the NMDA receptor to Aβ oligomers is known to promote endocytosis of the receptor as well as other signaling events associated with NMDA receptor trafficking [28]. In hippocampal slices treated with soluble Aβ, cotreatment with an NMDA receptor antagonist prevented the neuronal accumulation of Aβ, suggesting that activation of the receptor by Aβ may trigger endocytosis of the Aβ-NMDA receptor complex [70]. However, whether Aβ actually binds to NMDA receptor is unclear. In a study examining the effects of Aβ oligomers on primary hippocampal neurons, Lacor et al. [71] noted that the binding sites of Aβ oligomers overlap with immunoreactivity of NMDA receptor 1 (NR1), a subunit of the NMDA receptor, and postsynaptic density-95 (PSD-95), an anchoring protein associated with the NMDA receptor. This would suggest physical associations between Aβ and the NMDA receptor. However, colocalization and co-precipitation of the two in an intracellular compartment have not been demonstrated. Some hypothesized that the NMDA receptor and its associated signaling cascades interact with Aβ via another Aβ-binding receptor protein. In the study by Bi et al. [70], cotreatment of antagonists against integrins with soluble Aβ nearly doubled the amount of internalized Aβ compared to Aβ treatment alone. As NMDA receptor antagonist yielded opposite trends, the authors hypothesize that membrane integrins and the NMDA receptor modulate Aβ uptake cooperatively [70]. Integrins are associated with lipid rafts as well [72]. Aβ is known to bind integrins directly [73], and in addition to regulating Aβ uptake, integrins also modulate the neurotoxic effects of soluble Aβ [70]. There is currently no data suggesting that integrins physically interact with the NMDA receptor despite evidence showing that integrin signaling modulates NMDA receptor activity

and trafficking [74–76]. Therefore, it is possible that both receptors act as Aβ carriers but at the same time induce other signaling cascades independent of the uptake process.

Another glutamate receptor modulated by integrin signaling is the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor [76–78]. The AMPA and NMDA receptors are often co-expressed by the same glutamatergic synapses. Like NMDA receptors, AMPA receptors are closely associated with lipid rafts and GM1 in particular [68, 79–81]. However, AMPA receptor recycling to and from the synaptic surface is fast compared to that of NMDA receptors, suggesting that AMPA receptors may act as a more efficient carrier of Aβ than NMDA receptors [82]. In a study using primary hippocampal neurons, Zhao et al. [82] showed that AMPA receptor trafficking is regulated by Aβ oligomers. Antagonists against the AMPA receptor inhibited Aβ internalization as in the case with NMDA receptor antagonists [82]. The authors further demonstrated that the glutamate receptor 2/3 (GluR2/3) subunit of AMPA receptors co-immunoprecipitated with Aβ oligomers [82], implicating a role for AMPA receptors as an Aβ carrier. Several other research groups have also reported endocytosis of AMPA receptors induced by Aβ oligomers [83, 84], leading to the speculation that endogenous Aβ at physiological levels may have essential roles in the maintenance of synapses. A recent study demonstrated that in mice overexpressing APP, the density of dendritic spines is increased at a young age before accumulation of Aβ nullifies the effect at an older age [85]. Hence it is possible that APP at synaptic membranes promotes surface expression and stabilization of glutamate receptors, while cleavage of APP and synaptic release of Aβ would promote opposite effects. The uptake of Aβ by glutamate receptors may serve as a regulatory mechanism that prevents Aβ-induced synaptic depression.

5.2. Acetylcholine Receptors. Another neurotransmitter receptor implicated in the uptake of Aβ is the α7 nicotinic cholinergic receptor (α7nChR) for the neurotransmitter acetylcholine. The role of acetylcholine and its receptors have received continuous attention in the AD field due to the high susceptibility of cholinergic neurons to degenerate in AD pathology [86]. Several hypotheses have been formed to explain this regional transmitter-specific vulnerability. It has been proposed that cholinergic signaling modulates APP processing [86]. Conversely, Aβ can affect acetylcholine release from the presynaptic terminal as well as signaling of nicotinic receptors in the postsynaptic compartment [86]. The α7nChR in particular is known to bind soluble Aβ with high affinity [87, 88], leading to the speculation that Aβ may be internalized via complex formation with the α7nChR. Nagele et al. [89] investigated this possibility in a study examining both human AD tissues and human neuroblastoma cells. The authors found that immunoreactivity of α7nChR and Aβ is highly colocalized in neurons of the hippocampus, entorhinal cortex, and cerebellum [89]. They observed similar findings *in vitro*; in neuroblastoma cells transfected with α7nChR, soluble Aβ treatment induced large punctate staining of α7nChR which colocalized with Aβ immunoreactivity [89]. Untransfected cells exhibited slower internalization of Aβ and lower susceptibility to Aβ-induced toxicity, suggesting a role for α7nChR as an Aβ carrier [89]. Although subcellular colocalization of Aβ and α7nChR has not been reported, circumstantial evidence indicates that neurotoxic effects of intracellular Aβ are mediated at least partly by uptake via α7nChR independent of its agonist effects. For instance, binding of α7nChR by Aβ results in primarily neurotoxic effects, yet paradoxically, binding of the same receptor by other competing agonists promotes neuroprotection [90]. Another study showed similar paradoxical results where disruption of lipid rafts attenuated Aβ-mediated α7nChR signaling but not nicotine-mediated signaling [91]. An explanation for this paradox is that the toxic effects of Aβ on cholinergic synapses occur after internalization by cholinergic receptors, and that activation of the receptor upon Aβ binding at common agonist sites triggers other independent pathways. This explanation may also justify contradictory findings in animal studies, where transgenic AD mice deficient in α7nChR expression exhibited exacerbated AD pathology in one report [92] and ameliorated pathology in another [93].

5.3. Apolipoproteins. A group of proteins implicated in the trafficking of Aβ that are not localized to synaptic membranes are apolipoproteins. As implied by the nomenclature, the amphipathic nature of apolipoproteins enables them to bind and transport lipids within a water-soluble milieu. Some apolipoproteins act as soluble chaperones for hydrophobic peptides such as Aβ [94]. In nonfamilial AD, the ε4 allele of the gene encoding apolipoprotein E (apoE) is the most prevalent genetic risk factor [94]. Consequently, the potential link between apoE function and Aβ toxicity has been studied extensively. Transgenic AD mice deficient in apoE display reduced Aβ load, suggesting a role for apoE in promoting Aβ accumulation [95]. Isoform-specific properties of the apoE4 allele were also investigated: binding studies comparing different apoE isoforms showed that apoE2 and apoE3 bind soluble Aβ more efficiently than apoE4 that preferentially binds to an intermediate aggregate form of Aβ [96, 97]. An *in vitro* study on purified synaptosomes showed that soluble Aβ, but not fibrillar Aβ, complexes with apoE, and that formation of the apoE-Aβ complex is crucial in the capacity of apoE3 to promote Aβ internalization [98]. The authors observed the same trend in intact neurons of the dorsal root ganglia [98]. The capacity of apoE to promote uptake of soluble Aβ is isoform-dependent; in the case of apoE4, promotion of uptake requires proteolytic cleavage [99]. In tissues of AD patients, immunoreactivity of apoE strongly correlates with that of intracellular Aβ, suggesting that apoE is internalized with Aβ [100]. Whether Aβ enters neurons as a complex with apoE or as a chaperone-free entity is unclear. The receptor that mediates apoE-dependent internalization of Aβ is also undefined. Similar to Aβ, apoE does not appear to have a dedicated receptor but is known to bind several putative receptor proteins. In transgenic AD mice, apoE is found to accumulate in lipid rafts suggesting that the apoE-Aβ complex may target raft-associated receptor proteins [50]. Moreover, apoE has

been observed to target neurotransmitter receptors such as α7nChR [90], leading to the hypothesis that uptake of Aβ by neurotransmitter receptors results from apoE-receptor binding rather than direct interaction between Aβ and the receptor. The surface receptors most often associated with apoE are the low-density lipoprotein receptor-related proteins (LRPs), a family of multiligand receptors known to have a high endocytosis rate [101]. LRP1 in particular has several known roles in AD. For instance, LRP1 binds to APP at the cell surface and subsequent endocytosis targets APP to the lysosome, modulating Aβ production [101]. The same endocytic mechanism is thought to mediate the uptake of Aβ. Gylys et al. [98] showed that addition of receptor-associated protein (RAP), an antagonist of LRP1, along with the apoE-Aβ complex significantly reduced internalization of soluble Aβ. A recent study in neuroblastoma and neuronal cell lines observed similar trends, such that RNA interference of LRP1 inhibited and overexpression of LRP1 stimulated soluble Aβ uptake [102]. *In vivo* experiments found that AD mice overexpressing LRP had increased intraneuronal accumulation of Aβ [103]. It has been suggested that LRP1 may bind soluble Aβ directly without chaperones. Binding assays, however, showed otherwise [104], and in an apoE-free environment, LRP inhibition did not alter Aβ uptake [65], suggesting that formation of the apoE-Aβ complex is required for internalization by LRP1. Notably, LRP1 associates with lipid rafts transiently such that it traffics between raft and nonraft membrane domains [105]. The motility of LRP1 may serve as a mechanism to regulate its availability to Aβ. The LRP antagonist receptor-associated protein (RAP), in addition to its LRP-modulating activities, has molecular properties similar to chaperone proteins like apoE. Kanekiyo and Bu [106] showed that RAP forms complexes with soluble Aβ much like apoE and similarly promotes Aβ uptake into neuroblastoma cells. They further demonstrated that uptake of soluble Aβ with RAP is independent of LRP1 [106], which is somewhat surprising as RAP is routinely used experimentally to inhibit LRP1 activity. The precise route of entry for RAP-Aβ complexes is thus unclear.

5.4. Other Aβ-Binding Proteins. The proteins implicated in Aβ uptake represent only a small percentage of soluble and membrane proteins that physically interact with Aβ. The list of Aβ-binding proteins is extensive (see [11] for a review). One notable receptor protein implicated in neuronal internalization of Aβ is the receptor for advanced glycation end products (RAGE). RAGE is a member of the immunoglobulin superfamily that interacts with several classes of ligands [107]. Complex formation between RAGE and soluble Aβ has been demonstrated [108]. Neuronal overexpression of RAGE in transgenic AD mice results in exacerbated AD pathology [109], suggesting that RAGE may promote intracellular Aβ accumulation. To demonstrate the role of RAGE in Aβ uptake, Takuma et al. [107] derived primary cortical neurons from RAGE-deficient mice, showing that neurons without RAGE expression internalized markedly less soluble Aβ compared to wild-type neurons. Interestingly, while internalization facilitated by most receptors appeared to localize to lysosomes, the authors showed by electron microscopy that Aβ internalized by RAGE accumulates in the mitochondria [107]. Accumulation of Aβ in the mitochondria is a well-documented occurrence (reviewed in [110]). Mitochondria are closely associated with the ER [111], hence it is possible that Aβ in the mitochondria originates from endosome-Golgi and Golgi-ER vesicular transport. However, since mitochondria are not classically associated with the endosomal pathways, it is proposed that mitochondrial Aβ derives from transport of cytosolic Aβ. Although mitochondria have been shown to import cytosolic Aβ via translocases [112], it is unclear how extracellular Aβ, which enters the cell by endocytosis of RAGE, translocates out of the endo-lysosomal compartments into the cytosol. Localization of internalized Aβ to the mitochondria may not be specific to RAGE endocytosis; therefore, future studies need to determine whether Aβ endocytosed through other receptor proteins also localize to mitochondria.

The serpin-enzyme complex receptor (SEC-R) is another less documented protein implicated in the uptake of Aβ [113]. It was originally identified in leukocytes as a receptor for antiprotease enzymes. Although SEC-R is also expressed in neurons [113], its role in neuronal cells and Aβ-associated processes is unknown. Boland et al. [113] showed that SEC-R binds to soluble Aβ and promotes uptake in pheochromocytoma cells. Whether SEC-R functions similarly in neurons has not been determined. A recent study reported that multiple epidermal growth factor-like domains 10 (MEGF10), a newly identified receptor involved in clearance of cell corpses, also modulates soluble Aβ uptake [66]. Although the authors show expression of MEGF10 in neurons of the hippocampus, it is unclear whether MEGF10 physically interacts with Aβ, and henceforth whether MEGF10 mediates Aβ uptake or merely modulates the process.

5.5. Endocytic Pathways of Aβ. A topic that has generated a significant amount of interest concerns the specific endocytic pathways involved in Aβ uptake. Endocytosis is a well-studied phenomenon in cell biology and to date several distinct pathways involving different protein machinery have been identified (for a review see [114]). The classical endocytic pathway involves invagination of the plasma membrane at the site of the receptor-cargo binding; the invaginated vesicle is then coated with adaptor proteins, which recruit guanosine triphosphatases (GTPases) that provide the necessary energy to facilitate cleavage of the vesicle from the cell membrane and delivery to endosomes or other subcellular compartments [114]. By far the most frequently reported process is dependent on clathrin and dynamins [114]. Clathrin is an adaptor protein that forms the primary component of the vesicle coating complex [114]. Dynamins are responsible for the membrane cleavage to release the invaginated vesicle from the plasma membrane [114]. Endocytosis at lipid rafts, however, is thought to proceed in a clathrin-independent manner (reviewed in [115]). Invaginations formed from lipid rafts, termed caveolae, are rich in cholesterol and sphingolipids and are morphologically distinct from clathrin-coated invaginations [115]. Caveolins are the major protein component of caveolae and are thought to cooperatively regulate lipid raft endocytosis with

Table 1: Summary of receptor proteins involved in the neuronal uptake of Aβ.

	Receptor proteins involved in neuronal Aβ uptake				
	Aβ binding	Aβ species	intracellular colocalization with Aβ	cell type where uptake was observed	endocytic pathway
Membrane receptors					
NMDA receptor	probable	nonaggregated, oligomeric	no data	hippocampal slices	clathrin-dependent
AMPA receptor	yes	Oligomeric	yes	hippocampal neurons	clathrin-dependent
integrins	yes	non-aggregated	no data	hippocampal slices	clathrin-dependent
α7nChR	yes	non-aggregated	no data	neuroblastomas	both clathrin-dependent and independent
LRP1	via apoE	non-aggregated	no data	DRG neurons, neuroblastomas	clathrin-dependent
RAGE	yes	non-aggregated	no data	cortical neurons	no data
MEGF10	no data	non-aggregated	no data	neuroblastomas	raft-dependent/ caveolin-independent
Soluble receptors					
apoE	yes	non-aggregated	yes	DRG neurons, neuroblastomas	clathrin-dependent, via LRP1
RAP	yes	non-aggregated	no data	neuroblastomas	no data

cholesterol and gangliosides [115]. It was originally thought that endocytosis at lipid rafts are caveolin- and dynamin-dependent. However, there is increasing evidence suggesting a caveolin-independent dynamin-dependent pathway as well as a flotillin-dependent dynamin-independent pathway [114]. The notion that lipid raft endocytosis does not involve clathrin has also been challenged as studies have observed clathrin-dependent endocytosis of lipid raft-associated proteins [116, 117].

Among receptor proteins that mediate Aβ uptake, LRP1 has been shown to endocytose Aβ via a clathrin-dependent process; neuroblastoma and neuronal cell lines deficient in clathrin internalized significantly less soluble Aβ as well as LRP1 [102]. Requirement for clathrin has also been shown in endocytosis of the NMDA and AMPA receptors for glutamate [118]. Not all routes of Aβ uptake, however, are clathrin-dependent. In the case of α7nChR, in Chinese hamster ovary cells transfected with α7nChR, endocytosis of the receptor occurs in the absence of clathrin, and in fact, dynamins as well [119]. This contradicts an earlier study in α7nChR-transfected human neuroblastomas that reported inhibition of α7nChR endocytosis along with decreased soluble Aβ internalization by treatment with phenylarsine oxide, an inhibitor of clathrin-coat formation [89]. It is possible that both pathways mediate α7nChR endocytosis and that the specific cell type can determine which pathway is dominant. Clathrin-independent internalization of Aβ has been reported in several different neuronal cell types. In mouse neuroblastoma cells, neither knockdown of clathrin nor inhibition of AP180, a clathrin-associated assembly protein, had an effect on internalization of oligomeric Aβ [120]. The authors reported that inhibition of dynamin and RhoA, a small GTPase, decreased the uptake of Aβ oligomers into the cell [120]. These data suggest that Aβ can be internalized through an IL-2 receptor-β-like endocytic pathway dependent on lipid rafts, dynamins, and RhoA [115]. In cervical sympathetic neurons, Saavedra et al. [65] also reported that uptake of Aβ oligomers is independent of clathrin. A role for dynamins is again implicated, as expression of dominant negative dynamin mutant decreased Aβ internalization [65]. The authors further demonstrate that the internalized Aβ oligomers colocalized with lipid rafts, but not caveolins [65]. This dynamin-/raft-dependent caveolin-independent pathway has also been demonstrated in MEGF10-mediated uptake of soluble Aβ [66] and is likely similar to the IL-2 receptor-like pathway [120]. Collectively these data show that in the absence of clathrin-associated receptors, neurons endocytose Aβ predominantly at lipid rafts via caveolin-independent mechanisms. Clathrin-dependent endocytosis of Aβ can occur, but it is uncertain whether it takes place at lipid rafts. Notably, internalization of Aβ oligomers in cervical sympathetic neurons preferentially occur in axons [65] while in primary hippocampal neurons and neuron-like cell lines described in other reports, the uptake appears to occur in dendritic synapses and cell bodies. These results suggest that endocytic mechanisms are varied not only in different types of neurons, but in different parts of the neuron.

Aside from endocytosis, an unconventional route of Aβ uptake was proposed by Kandimalla and colleagues [121]. By comparing Aβ uptake between primary hippocampal neurons and endothelial cells, the authors reported that while the latter requires endocytosis to internalize soluble Aβ, neurons can take up soluble Aβ by passive diffusion [121]. Neuronal uptake of soluble Aβ was unaffected by low temperature or low glucose, suggesting that neuronal internalization of Aβ

is not mediated by energy-dependent processes including endocytosis [121]. There are biophysical data supporting this conclusion, such that both Aβ40 and Aβ42 intercalate into phospholipid bilayers [122]. It is not unreasonable to propose that endocytosis and passive diffusion of Aβ can coexist in the same neuron. Further investigation is needed to reconcile the seemingly contradicting results in regards to studies showing endocytosis-dependent Aβ uptake.

6. Future Directions and Conclusions

With the recent wealth of novel findings, our understanding of neuronal Aβ uptake has improved since the original discovery of intraneuronal Aβ in AD brains. Cumulatively, the current data implicate several distinct pathways of entry for extracellular Aβ (Table 1). Lipid raft-dependent endocytosis is the predominant Aβ uptake mechanism although lipid raft-independent endocytosis and nonendocytic pathways also exist. Lipid components such as cholesterol and sphingolipids have a role in modulating Aβ endocytosis as well as recruiting Aβ to the lipid raft. Questions remain as to whether lipids can act as carriers of Aβ uptake independent of lipoprotein chaperones and receptor proteins. It may be the case that lipid components and raft-associated receptors synergistically carry Aβ into the cell. Further investigations are needed to address these speculations. There is also little data on the long-term effects of internalized Aβ, which is found not only in endocytic pathways but also in other compartments including the cytosol. *In vitro* studies in general found that internalized Aβ cause lysosomal leakage and neurotoxicity, but whether the same occurs *in vivo* in the presence of physiological buffers is yet to be determined. In fact, most of the mechanisms of Aβ uptake have not been investigated in animal models. The wealth of culture studies has nonetheless demonstrated the potential importance of Aβ uptake mechanisms in the pathogenesis of AD.

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Review Article

Amyloid Oligomer Neurotoxicity, Calcium Dysregulation, and Lipid Rafts

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Amyloid proteins constitute a chemically heterogeneous group of proteins, which share some biophysical and biological characteristics, the principal of which are the high propensity to acquire an incorrect folding and the tendency to aggregate. A number of diseases are associated with misfolding and aggregation of proteins, although only in some of them—most notably Alzheimer's disease (AD) and transmissible spongiform encephalopathies (TSEs)—a pathogenetic link with misfolded proteins is now widely recognized. Lipid rafts (LRs) have been involved in the pathophysiology of diseases associated with protein misfolding at several levels, including aggregation of misfolded proteins, amyloidogenic processing, and neurotoxicity. Among the pathogenic misfolded proteins, the AD-related protein amyloid β (Aβ) is by far the most studied protein, and a large body of evidence has been gathered on the role played by LRs in Aβ pathogenicity. However, significant amount of data has also been collected for several other amyloid proteins, so that their ability to interact with LRs can be considered an additional, shared feature characterizing the amyloid protein family. In this paper, we will review the evidence on the role of LRs in the neurotoxicity of huntingtin, α-synuclein, prion protein, and calcitonin.

1. Introduction

Lipid Rafts (LRs) are highly dynamic, nanoscale domains of the plasma membrane, enriched in cholesterol and sphingolipids (Figure 1). They were originally defined on the basis of their resistance to solubilization in nonionic detergents, which allows their separation and isolation from the rest of the plasma membrane, using sucrose-density gradients [1]. Although their existence has initially been questioned [2, 3], it is now generally agreed that LRs are special membrane domains that act as platforms for the organization and interaction of proteins [4]. They are involved in several cell functions and play crucial roles in signal transduction, phagocytosis, protein sorting, and cell polarity. Besides the role in cell physiology, they are also involved in cell pathology. For example, certain pathogens, such as viruses and bacteria, as well as their toxins, interact with the host cells through LRs [5, 6]. In the pathogenicity of amyloid proteins, LRs have been implicated in amyloidogenesis, in the process of protein aggregation, in the mechanisms of interaction between the cell membrane and amyloid proteins, and in their neurotoxic effect. This paper will first provide an overview on the principal milestones in the history of amyloid proteins. After considering the mechanisms of neurotoxicity of misfolded proteins, it will then focus on the role played by LRs in the interaction between neuronal cells and four amyloid proteins: huntingtin (htt), α-synuclein (α-syn), prion protein (PrP), and calcitonin (CT).

2. The Amyloid Protein History: Breakthrough Discoveries

The history of amyloid proteins has been for a long time, with a few, though remarkable, exceptions, the history of Aβ. Aβ was first isolated in 1984 from brain blood vessels of AD patients and individuals with Down's syndrome

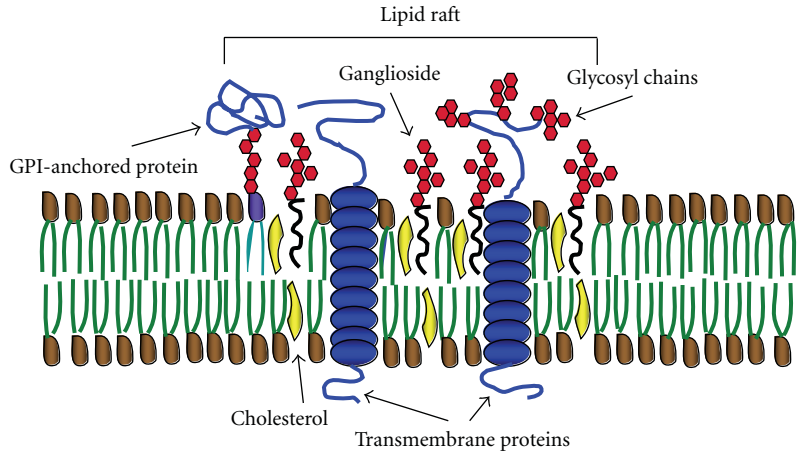


Figure 1: Schematic representation of an LR in the cell membrane, enriched in gangliosides and cholesterol. Glycosylated and non-glycosylated transmembrane proteins and GPI-anchored proteins are also sketched.

[7, 8]. In 1985, Colin Masters identified Aβ as the principal component of amyloid plaques, the hallmark of AD, and in collaboration with Konrad Beyreuther identified the amino acid composition, the molecular mass, and the NH2-terminal sequence of the peptide [9]. In addition, they recognized that the protein was identical to that described for the amyloid deposited in the congophilic angiopathy of AD and Down's syndrome [9]. In the same year, the first evidence was provided that prion proteins (PrP) assemble into filaments within the brain to form amyloid plaques into scrapie-infected hamsters [10]. The discovery of prions dated back to 1982, when the Nobel Prize winner Stanley B. Prusiner described them as novel proteinaceous infectious agents causing scrapie [11]. At the beginning of the 1990s, experiments on primary neuronal cultures showed that aggregated Aβ peptides were neurotoxic *in vitro*, suggesting a link between amyloid formation and neurodegeneration [12]. Since then, primary hippocampal cell cultures have been considered as an ideal cell culture model to study neurotoxic properties of amyloid proteins. In the same years, breakthrough discoveries on the genetics of AD (for a review, see [13]) led to formulate "the Aβ cascade hypothesis." According to this theory, the 1–42 and 1–40 Aβ peptides, deriving from the proteolytic cleavage of the amyloid β precursor protein, operated by the β and γ secretases, are the principal culprits in the development of AD [14, 15]. In the 1990s, the understanding of pathogenic mechanisms of AD dramatically advanced due to the introduction of transgenic animal models, which have provided invaluable insights into several aspects of AD pathophysiology (for a review, see [16]), although mice that precisely model all aspects of AD are not yet available [17]. In the same years, studies on other misfolded proteins started to accumulate. In 1993, the *HTT* gene, associated with Huntington's disease (HD), was identified [18]. It was shown that, in the mutated htt protein, a polyglutamine tract was abnormally expanded, leading to high aggregation propensity. In 1997, Spillantini et al. identified α-syn as the fibrillary component of Lewy bodies (LBs) in Parkinson's disease (PD) and dementia with LBs [19]. In the same years, the first data suggesting a common neurotoxic mechanism of all amyloid proteins were provided [20]. Although aggregation was considered a critical process in the pathogenicity of Aβ from the beginning, it was not until the end of the 1990s that attention focused on the role of amyloid oligomers more than amyloid fibrils [21]. These studies also identified in the synapse a special target of soluble oligomer toxicity [22], providing a biological explanation to the well-known clinical-pathological observation that dementia in AD has a good correlation with the synapse loss, while the amyloid burden is a poor predictor of cognitive decline [23, 24]. Furthermore, they demonstrated that Aβ oligomers can impair long-term potentiation (LTP), an experimental form of synaptic plasticity resulting in long-lasting increase in the strength of synaptic transmission, which is the electrophysiological counterpart of learning and memory [25].

In the same period, attention shifted from the insoluble amyloid fibrils to the soluble oligomeric aggregates also for other amyloid proteins, which were found to be neurotoxic. They included both disease-associated proteins, such as islet amyloid polypeptide (IAPP), α-syn, PrP, and polyglutamine [26], and non-disease-associated proteins, such as HypF-N, a protein that is not associated with any amyloid disease but displays an aggregation-prone behavior [27]. These amyloid oligomers were found to form pores on model membranes with ion channel properties [28], a mechanism originally proposed for Aβ [29], and the induced Ca^{2+} dysregulation was proposed as a common pathogenetic mechanism through which all amyloid proteins lead to neurotoxicity [30].

One of the latest "coups de theatre" in the amyloid history is the observation that PrPC is a high-affinity cell-surface receptor for soluble Aβ oligomers on neurons and is a mediator of Aβ oligomers-induced synaptic dysfunction [31]. This hypothesis, however, has been challenged by several authors [32–34] and has become a highly controversial issue, still far from being settled [35].

3. Amyloid Proteins: A Large Family of Unrelated Proteins with Some Shared Features

Though differing in the amino acid sequences, amyloid proteins share the tendency to adopt an incorrect conformation (protein misfolding) and the propensity to aggregate. Until recently, there was a general agreement on the idea that only a limited number of proteins can undergo aggregation. However, it has been recently shown that the characteristics that enable a protein to become amyloid are present in almost all complex proteins and that the number of amyloid proteins is limited because the region promoting aggregation is generally hidden [36].

The process of aggregation is complex, depending on characteristics intrinsic to the protein and to environmental conditions and proceeds through several organization states, including dimers, trimers, tetramers, low molecular weight prefibrillar oligomers, and linear or annular protofibrils, to reach the final insoluble fibrillar structure, rich in β sheets. The term "amyloid" should more correctly refer to the mature fibrils, which deposit in tissues and are characterized by Congo red and Thioflavin T positivity. In some diseases, such as systemic amyloidosis, these deposits have a pathogenetic role, and the disease is caused by the deposition of mature fibrils. In neurodegenerative conditions associated with protein misfolding, however, it is now generally agreed that the pathogenic forms are not the mature fibrils but the intermediate, soluble oligomeric aggregates [21, 25, 37]. Oligomers of different amyloid proteins have a remarkable structural similarity, evident at Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM), showing an annular morphology with sizes ranging from 8 to 12 nm, a morphology sustaining the amyloid pore hypothesis (see below). In addition, conformation-specific antibodies have been raised, which cross-react with a number of chemically unrelated misfolded proteins, recognizing generic epitopes exposed in similar folding states of the different proteins [38].

Among the shared features, we believe that three characteristics deserve special attention: oligomeric aggregate pathogenicity, synaptotoxicity, and propagation of protein misfolding.

3.1. Pathogenicity of Oligomeric Aggregates. Besides AD, a role for pathogenic oligomeric amyloid species has been suggested for other protein misfolding diseases, most notably for PD, HD, and PrP diseases.

PD is the second most common neurodegenerative disease affecting aging populations, after AD. The characteristic symptoms of PD include rigidity, resting tremor, postural instability, and bradykinesia. The disease characteristically affects the substantia nigra, where dopaminergic neurons accumulate proteinaceous aggregates, referred to as LBs and degenerate. The majority of patients suffering from PD have a sporadic form of the disease, apparently with no genetic cause, while 5–10% of patients have mutations in a series of genes referred to as the PARK genes [39]. Among the proteins encoded by these genes, α-syn has been the object of considerable interest, since it constitutes the principal component of LBs [19]. Intracellular, α-syn-positive inclusions are also present in dementia with LBs and multiple system atrophy, which, together with PD, are collectively referred to as synucleinopathies [19]. α-syn shows a distinctive propensity to aggregate, a phenomenon associated with a conformational change from random coiled to predominantly β-pleated sheet [40, 41]. This characteristic is enhanced when α-syn is mutated or overexpressed, as in some familiar forms of PD and has been correlated to the pathogenesis of the disease [41, 42]. It is assumed that the aggregation process proceeds through progressive stages, from monomers, through partially folded intermediates, up to mature fibrils. As for Aβ, increasing evidence suggests that prefibrillar oligomers and protofibrils, rather than mature fibrils, are the pathogenic species in PD [41, 43]. Two mutations in the α-syn gene, linked to autosomal dominant early-onset PD, have been described to promote the formation of transient protofibrils at a higher rate than wild-type α-syn [42], although both wild-type and mutant α-syn have been shown to form pore-like structures in synthetic vesicles and model membranes [28, 44, 45]. The pore formation, inducing disruption of cellular ion homeostasis, may be responsible for the neurotoxic effect [44]. Although the question is still open [46], data obtained in three established model systems for PD, such as mammalian neurons, the nematode *Caenorhabditis elegans,* and *Drosophila melanogaster*, show a strong correlation between α-syn aggregates with impaired β-structure, neuronal toxicity, and behavioral defects [47], further sustaining a pathogenic role for α-syn oligomers in PD. Evidence on a role of phosphorylation in the oligomerization and neurotoxicity of α-syn has also been provided [48].

HD is a late-onset, autosomal dominant disorder clinically characterized by chorea, cognitive impairment, and psychiatric disorders. The mutation responsible for HD, an expanded CAG repeat sequence in the HD gene, leads to a polyglutamine expansion in the amino-terminal portion of the htt protein. Although the physiopathology of the disease has not been fully clarified, a role for protein misfolding is suggested by the observation that HD occurs when htt expands beyond around 35 glutamine residues, a modification that facilitates protein aggregation and the acquisition of β sheet structure [49]. In lymphoblasts from HD patients and medium spiny striatal neurons of the YAC72 HD mouse model, polyglutamine expansion in htt was accompanied by cytosolic and mitochondrial Ca^{2+} overload, triggering an apoptotic pathway [50, 51]. As for many other misfolded proteins, htt aggregation is a complex process advancing through a variety of different assemblies, eventually leading to the formation of insoluble inclusion bodies. The different aggregative intermediates have probably different biological activities. As described for Aβ and other misfolded proteins, the soluble aggregates, more than the insoluble inclusion bodies, are probably the neurotoxic species [26].

Prion diseases, also known as TSE, are progressive, mostly fatal neurodegenerative diseases. They include Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, kuru and fatal familial insomnia in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep,

and chronic wasting disease in deer and elk. The central pathogenic event in these diseases is the conversion of the PrPC, a normal cellular isoform, into the abnormal PrPSc (where Sc stands for "scrapie"). The conversion determines an increase in the β-sheet content of the protein and is accompanied by changes in biological and biochemical properties of PrPSc, such as increased resistance to proteases and propensity to form amyloid fibrils. The interaction between PrPC and pathogenic PrPSc is supposed to determine a template-induced, progressive deposition of new PrPSc, which accumulates in brain tissue as dense plaque-like amyloid deposits, perivascular deposits, or diffuse, non-fibrillary deposits, reminiscent of synaptotoxic oligomeric β amyloid aggregates. Although the deposition of amyloid plaques is a hallmark of prion diseases, recent studies suggest that, in analogy to Aβ and other amyloid proteins, the soluble oligomeric aggregates of PrPSc are the actual neurotoxic species. For example, prefibrillar oligomers are neurotoxic *in vitro* and *in vivo* [52], and soluble oligomeric species are most efficient in transmitting TSE [53]. It has also been proposed that the fibrillar form of PrP, which is typically observed at autopsy, may actually be neuroprotective [54].

3.2. Synaptotoxicity. Amyloid proteins are, by definition, neurotoxic. Neuronal cell damage induced by various amyloid proteins has remarkable analogies, especially considering one highly specific effect: synaptic dysfunction. Compromised synaptic function is a key event in the pathogenesis of AD. Quantitative evaluation of temporal and frontal cortical biopsies revealed a significant decrease in the density of synapses [55]. At autopsy, synapse loss, as demonstrated by decrease in synaptophysin immunolabeling, showed a clear-cut correlation with the severity of dementia [56]. Later on, it has been shown that synapse loss is an early event in the pathophysiology of the disease [56]. More subtle derangements of synaptic activity, induced by Aβ oligomers, precede synapse loss. The studies by Selkoe and collaborators have shown that natural Aβ oligomers, secreted from cultured cells, when injected in rat brain, potently inhibit LTP, enhance long-term depression (LTD), and impair the memory of learned behaviors in rats [25, 57, 58]. Similar results were obtained from Aβ dimers isolated from the brain of AD patients [59]. Evidence for a role of synaptic dysfunction in PD, HD, and PrP diseases has also been collected, showing modified synaptic activity as a consequence of the interaction with misfolded proteins. Synaptic dysfunction is an early symptom in α-syn-induced pathology [60, 61]. α-syn is localized at synapses, where it is involved in the modulation of synaptic transmission and neuronal plasticity [62], in the regulation of the size of different pools of synaptic vesicles [63], and in the SNARE complex assembly [64]. Recently, it has been demonstrated that α-syn directly regulates the dynamics of actin microfilaments, whose integrity is fundamental in synaptic vesicle mobilization, recycling, and exocytosis. This regulatory activity was profoundly altered in the A30P mutation, associated with familial PD [65]. Using paraffin-embedded tissue blot and protein aggregate filtration assays, it has been shown that the majority of α-syn oligomeric aggregates are located at presynaptic terminals, suggesting an impact on synaptic function [66, 67]. This is also sustained by the observation that, in cultured neurons from brains of transgenic mice overexpressing human α-syn, excessive α-syn induced a decrease in other presynaptic proteins, leading to morphologic and functional changes of synapses [68].

Studies in animal models of HD have clearly shown that synaptic dysfunction precede neuronal loss [69–71]. Decreased pre- and postsynaptic markers and altered glutamate release were found at the corticostriatal synapse before the onset of motor symptoms [72]. Altered LTP and LTD were early electrophysiological signs of aberrant synaptic plasticity [73, 74]. In a *Drosophila* HD model, expanded full-length htt was observed to increase neurotransmitter release efficiency, leading to impairment of synaptic transmission and altered Ca^{2+} homeostasis [75].

In prion diseases, synaptic alterations are among the pathognomonic pathologic features, together with neuronal loss, spongiform change, astrocytosis, and deposition of amyloid aggregates. Immunocytochemical localization of PrPSc has a dot-like appearance around neuronal cell bodies and, along dendrites, reminiscent of synaptic protein localization in synapses [76, 77]. There is evidence that synaptic changes precede neuronal death [78–81], possibly sustained by mitochondrial dysfunction [82]. Mice with prion diseases can be cured at the stage of early synaptic dysfunction and impairments at neurophysiological, behavioural, and morphological levels are reversible [83]. Interestingly, the fact that reversible changes precede extensive accumulation of PrPSc deposits suggest that they may be caused by a transient neurotoxic species [84], in analogy with the effects of soluble oligomeric Aβ in AD.

3.3. Propagation of Protein Misfolding. In PrP diseases, the key molecular event is the conversion of the PrPC into the infectious PrPSc, which serves as template to produce further, aggregation-prone PrPSc. Emerging evidence seems to converge towards the theory that the ability to form auto-perpetuating amyloid aggregation is not exclusive to PrPSc. These findings suggest that several proteins, belonging to the amyloid family, accumulate and propagate through a nucleation-dependent aggregation, starting from what has been defined as an "amyloid seed," whose presence facilitates further oligomerization of the proteins [85].

One of these proteins is Aβ. Injection of brain extracts from human AD brains, but not from control age-matched patients, in transgenic mice overexpressing the AβPP, induced extensive Aβ deposition [86, 87]. The increased Aβ production did not occur when AD brain extracts were depleted of Aβ [87], suggesting that infused Aβ can act as an amyloid seed. The process has also been characterized *in vitro*, showing that homogenates from SHSY5Y cells, which had uptaken Aβ, were capable of seeding amyloid fibril growth [88]. Aggregation of α-syn is also a nucleation-dependent process. *In vitro* biophysical studies have shown that the process is accelerated by the presence of pre-aggregated protein [89]. This mechanism has also been demonstrated in cells, where α-syn aggregates, but not

monomers, can induce the formation of LB-like aggregates [90]. In addition, injection of preformed oligomeric aggregates in cells overexpressing α-syn determines the formation of highly filamentous intracellular, α-syn-positive inclusions [91]. Cross-seeding between different amyloid proteins has also been described. For example, pure α-syn and tau facilitate each other aggregation [92]. Amyloid fibril formation of α-syn is accelerated by preformed amyloid seeds of other amyloid proteins, such as *Escherichia coli* chaperonin GroES, hen lysozyme, and bovine insulin [93]. Susceptibility of different peptides toward cross-seeding is related to the intrinsic aggregation propensity of the peptides [94].

Cell-to-cell propagation of protein misfolding, characteristic of prion diseases, has been described for α-syn in cell cultures and animal models of PD [95]. Furthermore, it has been observed that transplanted neurons in PD patients develop in time LB and PD pathology, suggesting the propagation of α-syn aggregation from host cells to graft cells [96, 97]. This phenomenon may have important clinical implications. For example, efficacy of stem cell therapies in these diseases may be hampered by the risk of propagation of protein misfolding from the host cells to transplanted stem cells [98, 99].

4. Mechanisms of Amyloid Neurotoxicity: The Role of Ca^{2+} Dysregulation

Although there is now wide agreement on the role played by amyloid oligomers in neurotoxicity, the mechanisms through which they induce neuronal cell dysfunction and, eventually, cell death are not fully understood. A number of different possibilities have been explored, including mitochondrial dysfunction, lysosomal failure, and abnormal activation of signalling pathways. These mechanisms may or may not accompany a more general neuronal cell derangement, which is a common effect of the interaction of amyloid proteins with neuronal cells, Ca^{2+} homeostasis dysregulation.

In resting neurons, cytosolic Ca^{2+} concentration is maintained around 100 nanomolar, while the extracellular concentration is about 1 mM and that of intracellular Ca^{2+} stores, the endoplasmic reticulum (ER) and mitochondria, is between 100 and 500 μM. Ca^{2+} entry from the extracellular space occurs through ligand-gated, voltage-gated, and store-operated Ca^{2+} channels, while Ca^{2+} release from intracellular stores, mainly represented by the ER, are regulated by inositol trisphosphate receptors and ryanodine (RyR) receptors. Recently, it has been reported that presenilin I, an integral membrane protein whose mutations cause early-onset inherited AD, also functions as ER Ca^{2+} leak channel [100, 101]. The tight regulation of Ca^{2+} concentration gradient depends on the crucial roles played by Ca^{2+} ions in neuronal cell processes, including neurotransmitter release, generation of action potential, gene expression, synaptic plasticity, and neurite growth. In addition, excessive intracellular Ca^{2+} concentrations may activate a number of pathogenic responses, whose overall effects are modulation of membrane excitability and enzyme/kinase activity, induction of gene expression, formation of reactive oxygen/nitrogen species, mitochondrial dysfunction, and apoptosis/necrosis.

To explain the genesis of Ca^{2+} dysregulation in diseases associated to misfolding and aggregation of amyloid proteins, two main mechanisms have been postulated: the activation of preexisting ion channels and the formation of calcium-permeable amyloid pores.

4.1. Activation of Preexisting Ion Channels. Interaction with several Ca^{2+}-permeable channels has been described for amyloid proteins, potentially leading to an intracellular Ca^{2+} rise. As usual, most evidence derives from experiments on Aβ. The glutamatergic system has been thoroughly studied, on the basis of the role played by glutamate receptors in the excitotoxic neuronal cell damage, whose overstimulation leads to excessive intracellular Ca^{2+} rise [102]. Several *in vitro* studies showed that incubation of neuronal cultures with Aβ oligomers increased Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptors. The moderate-affinity, uncompetitive NMDA receptor antagonist memantine protects against Aβ oligomer toxicity by attenuating intracellular Ca^{2+} increase [103]. Currently, memantine is the only approved treatment for AD, besides acetylcholinesterase inhibitors, although the therapeutic efficacy is limited [104]. Aβ oligomers induce dynamin 1 degradation, which may endanger synaptic integrity. This effect is mediated by NMDA receptor activation [105]. Interactions of Aβ with other Ca^{2+} permeable channels have been documented, such as voltage-gated Ca^{2+} channels [106, 107]. An involvement of nicotinic acetylcholine [108–110], catecholamine [111], and serotonin receptors [112] has also been postulated in Ca^{2+} dysregulation following Aβ treatment.

Intracellular Ca^{2+} stores have also been implicated in Ca^{2+} dysregulation. When presenilin is mutated, its function as ER Ca^{2+} leak is disrupted, contributing to Ca^{2+} dysregulation [100, 101]. Exaggerated intracellular Ca^{2+} levels have also been put in relation to modulation of RyR receptors [113].

A role for calcium-permeable channel has been described for other amyloid proteins. Ca^{2+} influx via N-type voltage-dependent Ca^{2+} channels has been described following α-syn treatment in rat synaptosomes [114]. Overactivation of NMDA receptors, followed by an abnormal neuronal Ca^{2+} signaling, is believed to play a role in HD pathogenesis [50, 115, 116]. Activation of glutamate receptors has been described to be induced by HypF-N [117].

4.2. The Calcium-Permeable Pore Hypothesis. To explain Ca^{2+} dysregulation, a different mechanism has been hypothesized: amyloid oligomers may form nonselective calcium-permeable pores. This ability, originally described for Aβ [29], has also been described for other misfolded proteins and has been proposed as a common property of the amyloid protein family [28, 30]. Several pieces of evidence sustain this hypothesis. Cribbs et al. [118] showed that both D- and L-stereoisomers of truncated form of Aβ were neurotoxic *in vitro*. This observation argues against a role for specific ligand-receptor interaction in the mechanism of toxicity. Morphological studies at TEM and AFM levels have shown that oligomers of many amyloid proteins, such as Aβ and α-syn [44], serum amyloid A, amylin [28], and CT [119], have

a characteristic annular morphology, reminiscent of cation-permeable membrane pores [120]. Furthermore, TEM analysis has also revealed the presence of Aβ pore-like structures in the cell membrane of brains from AD patients but not from age-matched healthy patients [121]. Treatment of SH-SY5Y cells with a wide range of oligomeric, but not fibrillary, amyloid proteins, including Aβ, PrP, IAPP, polyglutamine, and lysozyme, induced increase in intracellular calcium. The increase could not be attributed to activation of endogenous Ca^{2+} channels, because the responses were unaffected by the potent endogenous Ca^{2+} channel blocker cobalt [30]. Electrophysiological recordings using model membranes showed heterogeneous single-channel conductances for several amyloid proteins [28]. Finally, it has been proposed that protein aggregates may mimic bacterial pore-forming toxin, which permeabilize membranes forming oligomeric pores characterized by β-sheet structure [122].

The different hypotheses are not mutually exclusive and can cooperate towards Ca^{2+} dysregulation. Recently, it has been proposed that amyloid oligomers may act at two steps, separated in time, a first, very rapid step, where Ca^{2+} increases due to glutamate receptor stimulation by the oligomers, followed by a second, delayed step, where oligomers permeabilize nonspecifically the cell membrane, possibly via the formation of amyloid pores [117].

5. Lipid Rafts and Amyloid Neurotoxicity

From the original description [123], the concept of LR has remarkably evolved. The introduction of high-resolution imaging techniques (for a review, see [124]) and the progress in lipidomics and proteomics methodologies have revealed that LRs have a highly heterogeneous composition and are characterized by an extremely dynamic structure. LRs can now be defined as nanoscale assemblies of sphingolipid, cholesterol, and proteins, fluctuating in a more fluid phospholipid matrix. By finely tuning lipid-lipid, protein-lipid, and protein-protein interactions, they can coalesce, forming more stable structures and providing functional platforms for crucial membrane activities, such as signaling and trafficking [124].

Considerable amount of data suggest the involvement of LRs in the interactions between amyloid proteins and cell membranes. Some crucial information has been obtained through the use of biophysical techniques on model membranes (for a review, see [125]), which will be briefly illustrated. Most of the work focused on the interactions between LRs and Aβ. However, compelling evidence has also been obtained for htt, α-syn, PrP, and CT, which will be reviewed here.

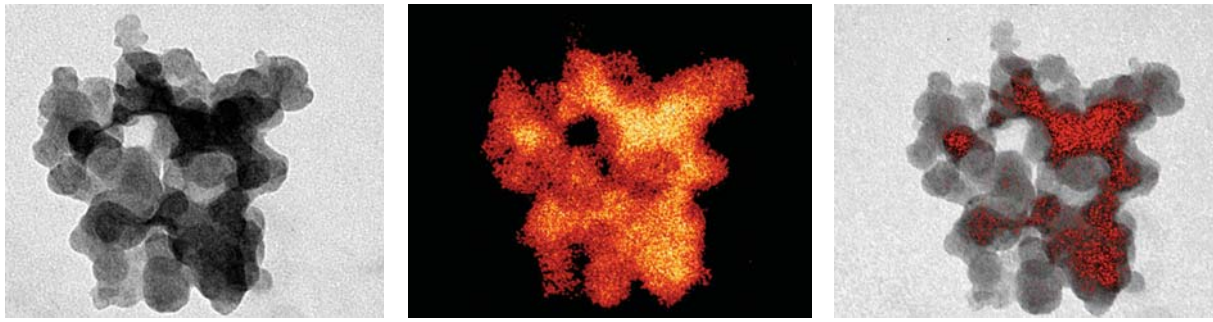
6. Using Model Membranes to Study Amyloid Proteins

6.1. Model Membranes. The use of model membrane systems has remarkably improved our knowledge on the biochemistry of amyloid proteins, providing information about the molecular mechanisms controlling aggregation, the structure of aggregates (oligomeric or fibrillar), and the interactions with cell membranes. Model membranes consist of mono- or bilayers of lipids that can be placed in contact with proteins of biological interest, such as the amyloid proteins. The monolayer model membranes are obtained by depositing at the water-air interface bidimensional molecular films composed of phospholipids, gangliosides, and cholesterol, with and without proteins (Languimur technique) [126, 127]. On these systems, thermodynamic measurements of compression at constant temperature (isotherms) provide useful information on the lipid mosaic phase (solid, liquid, or gaseous) and its modification due to the presence of proteins. Liposomes are vesicular structures, composed of bilayer model membranes. Mono- or bilayer membranes can be deposited onto solid substrates and studied with imaging techniques, such as Energy-Filtered TEM (EFTEM) or AFM, at nanometric resolution [128, 129]. In liposomes, which are suspended in a water solution, the conformation of proteins interacting or not with the lipid bilayer can be also investigated by Circular Dichroism Spectroscopy (CDS) [119, 130].

6.2. Imaging Techniques. EFTEM represents a powerful tool in the study of biological and nonbiological materials. The use of fast electrons (80–120 KeV) and magnetic lenses allows creating images of thin samples with horizontal resolution in the order of 0.4 nm. This is due to the small wavelength associated to electrons of this energy (about 0.005 nm). Using this technique, it is possible to investigate the quaternary structure and aggregation of misfolded proteins and their interaction with model membranes. In this case the image formation is obtained by negative staining with heavy metals such as tungsten and uranium. This technique allows obtaining horizontal resolution in the order of 1 nm. However, using microscopes equipped with energy filters, it is possible to improve the image quality and increase contrast even in unstained samples and perform spectroscopic studies of the transmitted electrons (Figure 2) [128, 131].

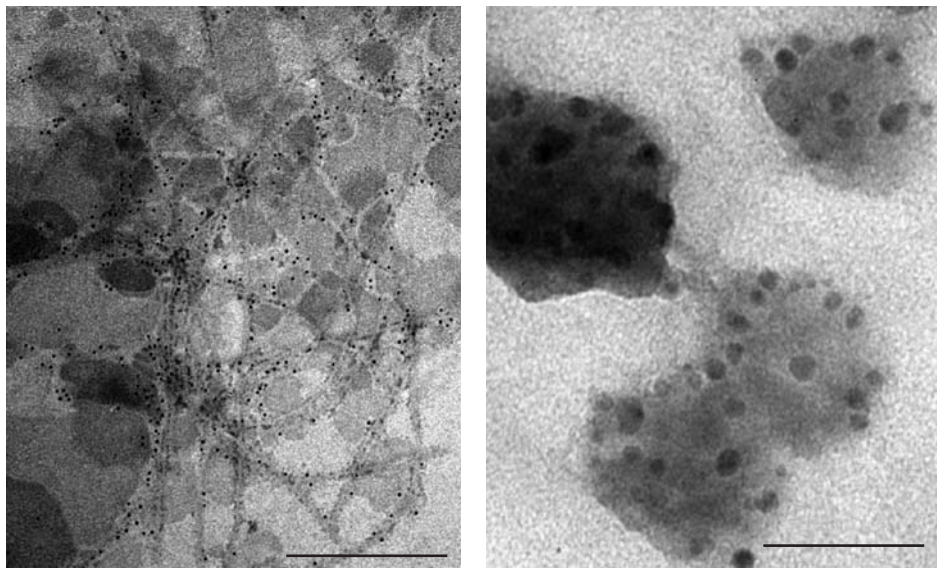
EFTEM can also be combined with immunolabeling techniques to identify proteins by using specific antibodies, conjugated with gold particles (Figure 3). This technique is particularly useful to investigate binding of amyloid proteins to lipid membranes [119].

In AFM, the surface of the sample to be analyzed is scanned by a very sharp tip. The interaction forces occurring between the tip and the atoms of the analyzed surface, in the order of nanonewtons, cause the deflection of the cantilever supporting the tip. Changes in the deflection of the cantilever, due to the morphology of the sample surface, are detected by the reflection of a laser beam. The microscope can operate in static or dynamic mode if the tip is at rest or oscillating vertically, respectively. The structural organization of liposomes or Langmuir films can be imaged by this technique after deposition onto flat substrates of mica, with a resolution up to 1 nm horizontally and 0.1 nm vertically. Morphologic changes induced in model membranes by the incorporation of pore-forming proteins, such as gramicidin A [132], or LR components, such as gangliosides [133], can be analyzed with this technique.



(a) (b) (c)

Figure 2: EFTEM micrograph of a cluster of liposomes (a). The ESI maps show a higher (b) and a lower (c) concentration of Cs entrapped in liposomes. Reproduced with permission from [131].



(a) (b)

Figure 3: Immunogold EFTEM micrographs of sCT in mature fibres ((a) bar = 200 nm) and liposomes, where sCT is inserted in the lipid bilayer ((b) bar = 50 nm). Picture in (b) was reproduced with permission from [119].

6.3. CDS. CDS can be considered a special type of UV absorption spectroscopy and consists in the measure of the difference in the absorbance of left- and right-handed polarized light by optically active molecules, detected in a selected frequency range. This signal depends on the wavelength of the incident light. A dichroic spectrum can be obtained illuminating an optical active sample by light of increasing wavelength. The optical activity of a molecule depends, in the absence of magnetic field, on its chirality: in general, a molecule having an asymmetric charge distribution interacts in a different way with electromagnetic waves characterized by opposite circular polarization.

This type of spectroscopy is generally used to investigate the protein conformation and their change induced by the aggregation process or the interaction with model membranes [119, 132]. In the region of the near UV (250–350 nm), it is possible to obtain information about the tertiary structure of proteins. In this region the aromatic amino acid and the disulfide bonds are excited, giving rise to a dichroic signal depending on the overall three-dimensional structure of the protein. An important application of this technique is to analyze the "folded" state of the proteins; if this is "molten globule" or the protein is incorrectly folded, the near UV spectrum is practically flat. The near UV spectrum is sensitive to small variations of the tertiary structure, due to the interaction of the proteins with other molecules, such as lipids. In the far UV region (190–250 nm), the secondary structure of the proteins can be studied. In this region the chromophore, which is the protein component excited by the incident light, is the peptidic bond. A typical dichroic spectrum for each type of secondary structure exists, and the spectrum of a protein conformed with several secondary structures is formed by the convolution of the base spectra.

7. LRs and htt

Some observations suggest that LRs may be implicated in HD pathogenesis at different levels. From DNA microarray analysis conducted in striatal cells expressing wild-type or mutant htts, genes involved in cholesterol biosynthesis were found to be altered by mutant protein. Since in these cells mutant htt did not form aggregates or cause cell death, this pattern of gene expression may reflect early events in the pathogenetic mechanism [134]. Consistently, dysfunction in the cholesterol biosynthetic pathway was described in mice and cell culture models of HD [135]. In addition, abnormal expression of the genes encoding glycosyltransferases, an enzyme involved in the synthesis of gangliosides, were found in the striatum of the R6/1 transgenic mouse, an animal model of HD, and in postmortem caudate samples from human HD subjects [136]. These observations indicate a disruption in glycolipid metabolic pathways that may alter LR formation. Biochemical analysis of cell membranes from brains and primary neurons of wild-type and presymptomatic HD knockin mice showed that wild-type and mutant htt were recovered in LR-enriched membranes [137]. The association with LRs was stronger for mutant than wild-type htt. In addition, LR from HD mice had a higher content in glycogen synthase kinase 3-beta (GSK). Since GSK activation is involved in neuronal apoptosis, the authors speculate that accumulation of mutant htt and GSK in LRs may have a role in the mechanism of neurodegeneration in HD [137].

8. LRs and α-syn

Although the mechanisms correlating α-syn aggregates to PD pathogenesis remain unclear, there is substantial evidence that binding of α-syn aggregates to lipid membranes is a relevant factor. Oligomeric α-syn binds to model membranes inducing permeabilization of synthetic vesicles, which is considered a potentially cytotoxic event [138]. Dimeric aggregates of wild-type α-syn and its mutants, A53T and A30P, seem to bind to and disrupt lipid membranes more easily than monomeric forms [139], indicating that oligomeric forms are likely to be the pathogenic species. However, even monomeric α-syn can interact with model membranes, undergoing a conformational change from a random coil to an α-helical structure, which may facilitate aggregation [140]. The lipid components seem to have a relevant role in the interaction between α-syn and membranes. α-syn binds to GM1 ganglioside, which are enriched in LRs. This bound is attributed to specific interaction between α-syn and glycidic residues of GM1, such as sialic acid [141]. In addition, α-syn colocalizes with markers of LRs in Hela cell cultures [142]. In the neuronal cells, α-syn is localized in the synaptic terminals, as described above. LR disruption was found to abolish the synaptic localization of α-syn and redistribute it to different cell compartments [142]. Furthermore, association with synaptic LRs is also impaired in the A30P mutation, suggesting that the physiological role of α-syn, lost in the mutated protein, is mediated by LR interaction [142].

9. LRs and PrP

The role of LRs has been the object of considerable interest in prion infectivity. By using model membranes, it has been shown that recombinant forms of the PrPs bind to model LR membranes composed of phospholipids, cholesterol, and sphingomyelin, but not to zwitterionic PC lipids, an artificial model lacking LR components [143, 144]. Inhibitors of the synthesis of cholesterol, a major component of LRs, reduce prion formation *in vitro* [145, 146] and delay the progression of experimental infection [147]. A large body of evidence sustains that LRs are the site where conversion from PrPC to PrPSc takes place [148]. A crucial role has been detected for the PrPC glycosylphosphatidylinositol (GPI) anchor, a complex machinery that has several physiological roles, among which is the targeting of proteins to LRs. Through the GPI anchor, PrPC binds to cell membranes [149]. In absence of the GPI anchor, PrPC redistributes into non-raft regions of the plasma membrane, and the formation of PrPSc is reduced [150]. In addition, synthetic analogues of the GPI anchor [151] and its enzymatic modification [152] reduce the capacity of PrPSc to bind and replicate within neuronal cell lines or primary cortical neurons, suggesting that PrPSc conversion takes place in LR-like microenvironment, following targeting of PrPC to LRs. *In vivo* studies, however, have shown that the role of the GPI anchor is probably more complex than initially assumed. Enzymatic removal of the GPI anchor from PrPSc did not reduce prion infectivity [153], while, in scrapie-infected transgenic mice producing PrPC without a GPI anchor, a high amount of infectious PrPSc was produced, though in the absence of clinical symptoms [154]. However, when mice were engineered to express twofold more anchorless PrP, scrapie infection did induce a fatal disease [155].

10. LRs and CT

Calcitonin (CT), a 32-residue polypeptidic hormone secreted by the C cells of the thyroid gland, belongs to a family of structurally and functionally related regulatory hormones, which also includes amylin, adrenomedullin, and CT gene-related peptide. It plays an important role in Ca^{2+} regulation and bone metabolism. For its activity in reducing bone resorption, it is a therapeutic option in the treatment of osteoporosis. The amyloid nature of CT was unveiled when it was demonstrated that the protein is the principal component of the amyloid fibrils deposited in medullary carcinoma of the thyroid [156]. Later on, its ability to aggregate *in vitro* was studied as a factor limiting its efficacy as pharmaceutical agent [157–160]. The studies of Schubert and coworkers [20, 161] firstly showed that CT, in analogy with other amyloid proteins showing an aggregative behaviour, was toxic to cells in culture. These observations prompted investigators to use CT as a probe to study amyloid formation and neurotoxicity [162–165]. Salmon CT (sCT), which is neurotoxic as CT from other species [20, 159, 166], is characterized by a slower aggregation rate [166], and this

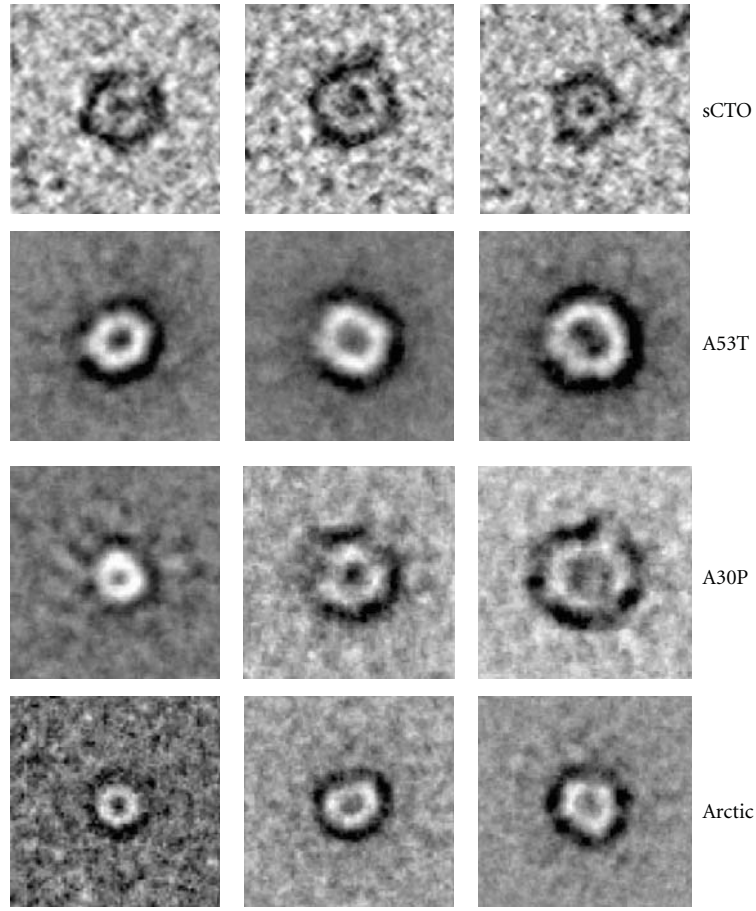
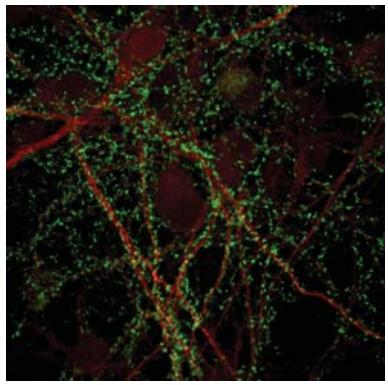


Figure 4: EFTEM of sCTOs. Annular sCTOs show remarkable morphologic similarities with amyloid pores of mutant α-syn (A53T and A30P) and Aβ (Arctic). TEM images of α-syn and Aβ were reproduced with permission from [169].

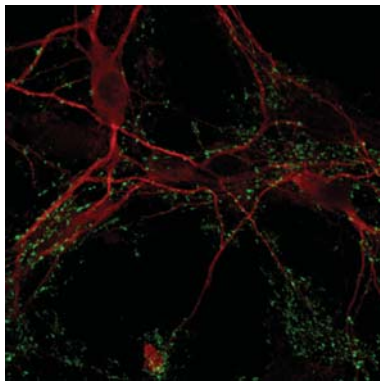
peculiarity is at the basis of its pharmacological use. We have studied the process of sCT oligomerization, focusing on the role of oxidation and time of aggregation [167, 168]. Recently, we showed that sCT oligomers (sCTOs) form Ca^{2+} permeable pores in liposomes [119], highly reminiscent of the ion channels formed by other amyloid proteins, such as Aβ and α-syn [169] (Figure 4). In addition, they damaged neuritic tree and synapses in hippocampal neurons, a behavior highly reminiscent of the effects induced by Aβ [170] (Figure 5).

We used sCT to investigate if a specific neuronal cell susceptibility to amyloid toxicity exists [170]. An issue that has seldom been addressed, in fact, is why misfolded proteins cause diseases so frequently in the CNS, in comparison to other systems or districts. Furthermore, several amyloid proteins, such as CT, are toxic to neuronal cells despite the fact that they are formed outside the CNS. An exception could be represented by amylin, an amyloid protein belonging to CT family. This amyloid protein is considered as a possible pathogenetic species in the development of diabetes, supposedly by damaging pancreatic beta cells, thus exerting a cytotoxic effect outside the brain [171]. If one considers, however, that pancreatic β cells share the same histogenesis of neuronal cells, being neural crest-derived neuroendocrine cells, type I diabetes would not represent an exception. The reasons for this peculiar vulnerability are presently unknown, but several hypotheses may be formulated, which are not mutually exclusive. (1) Neurons may provide a particularly suitable environment for protein misfolding processes, or be more prone to dysfunctions of the machinery deputed to misfolded protein removal. (2) The abundant presence of calcium-permeable ion channels, activated by amyloid proteins, may render Ca^{2+} dysregulation a much more probable event in neurons than in other cell types. (3) Neuronal cells may be more sensitive to the toxic potential of amyloid proteins, a likely event due to the dramatic effects induced by Ca^{2+} dysregulation, as discussed above. (4) Finally, it may be speculated that neuronal cell membrane, due to its intrinsic characteristics, may be more prone to pore formation by oligomers. To address the latter hypothesis, we compared sCTO toxicity in mature, 14-day *in vitro* (DIV) or immature, 6 DIV, hippocampal neurons to that of cultured cells of different histogenesis: MG63 osteoblasts, NIH-3T3 fibroblasts (two immortalized cell lines), and primary astrocytic cultures from rat fetal brain [170].



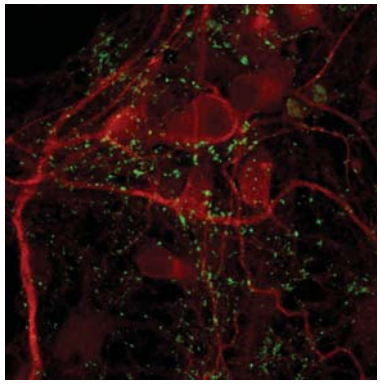
Control

(a)



A

(b)



sCTO

(c)

Figure 5: sCTOs and Aβ oligomers similarly damage the neuritic tree and synapses in mature hippocampal neurons. After sCTO or Aβ treatments, the extension of the dendritic tree, immunolabeled for microtubule-associated protein 2 (red fluorescence), is evidently reduced, while the number of synapses, immunolabeled for synaptophysin (green fluorescence), is decreased.

Among the tested cell types, only mature hippocampal neurons responded to sCTOs with an intense and sustained rise in intracellular Ca^{2+} (Figure 6(a)) and an evident increase in apoptosis. This increase could be due to leakage of intracellular Ca^{2+} stores or sCTO-dependent stimulation of preexisting Ca^{2+} channels, as previously proposed and discussed above. The use of thapsigargin, a specific sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump inhibitor, which depletes intracellular Ca^{2+} stores, showed that sCTO-induced Ca^{2+} rise was mostly due to an extracellular influx (Figure 6(b)). We then considered the activity of NMDA receptor, which, among glutamate receptors, is the one that has most frequently been considered to be involved in the toxicity of amyloid proteins, as already discussed. MK801, a specific NMDA receptor blocker, poorly affected sCTO-induced Ca^{2+} entry. Furthermore, pretreatment with an antibody against the subunit 1 of NMDA receptor (NR1), used to mask possible sites of interactions between sCTOs and the NMDA receptor, again failed to inhibit Ca^{2+} entry (Figure 6(b)). Thus, the different behavior of cell types to sCTO, in terms of Ca^{2+} rise, was conceivably unrelated to an activation of preexisting Ca^{2+} channels and pointed to the formation of calcium-permeable amyloid pores by CT oligomers. We reasoned that the neuronal plasma membrane has other distinctive characteristics, compared to other cell types, such as, for example, a rich content in LRs. This hypothesis was confirmed by our results, where mature neuronal cells showed a much more elevated content in LRs of the other cells types examined (Figure 6(c)). It has also been demonstrated that LRs increase in the plasma membrane during *in vitro* maturation in hippocampal neurons [172]. This could explain why immature neurons were insensitive to sCTO toxicity. Thus, content in LRs higher than the other cell types could render neurons more vulnerable to amyloid toxicity. To further corroborate this hypothesis, we manipulated LRs in mature neuronal cells in the attempt of modifying sCTO-induced intracellular Ca^{2+} entry. Pretreatment of neurons with an antibody against GM1, a ganglioside particularly abundant in LRs, completely suppressed sCTO-driven Ca^{2+} rise, without altering NMDA receptor activity (Figure 6(b)). Furthermore, LR disruption obtained by neuraminidase (NAA), which removes sialic acid from gangliosides, inhibited Ca^{2+} rise and protected against sCTO neurotoxicity, probably modifying the plasma membrane area susceptible to the insertion of the pore-like structures (Figure 6(d)). These results strongly support the conclusion that the intense and protracted Ca^{2+} dysregulation observed after sCTOs treatment is reliably due to the pore formation in a particularly suitable environment, that is, the LR-rich neuronal plasma membrane.

11. Conclusions

LRs are crucial sites in the cell membrane, where pivotal events in the physiology of the cell take place. However, they may also represent areas of fragility of the cell membrane, providing a way into potential cell hosts, such as pathogens and misfolded proteins. The high content in LRs of mature neuronal plasma membrane may render these cells particularly vulnerable to the cytotoxic attack of amyloid proteins and represent one of the reasons for the high vulnerability of CNS to misfolded protein diseases.

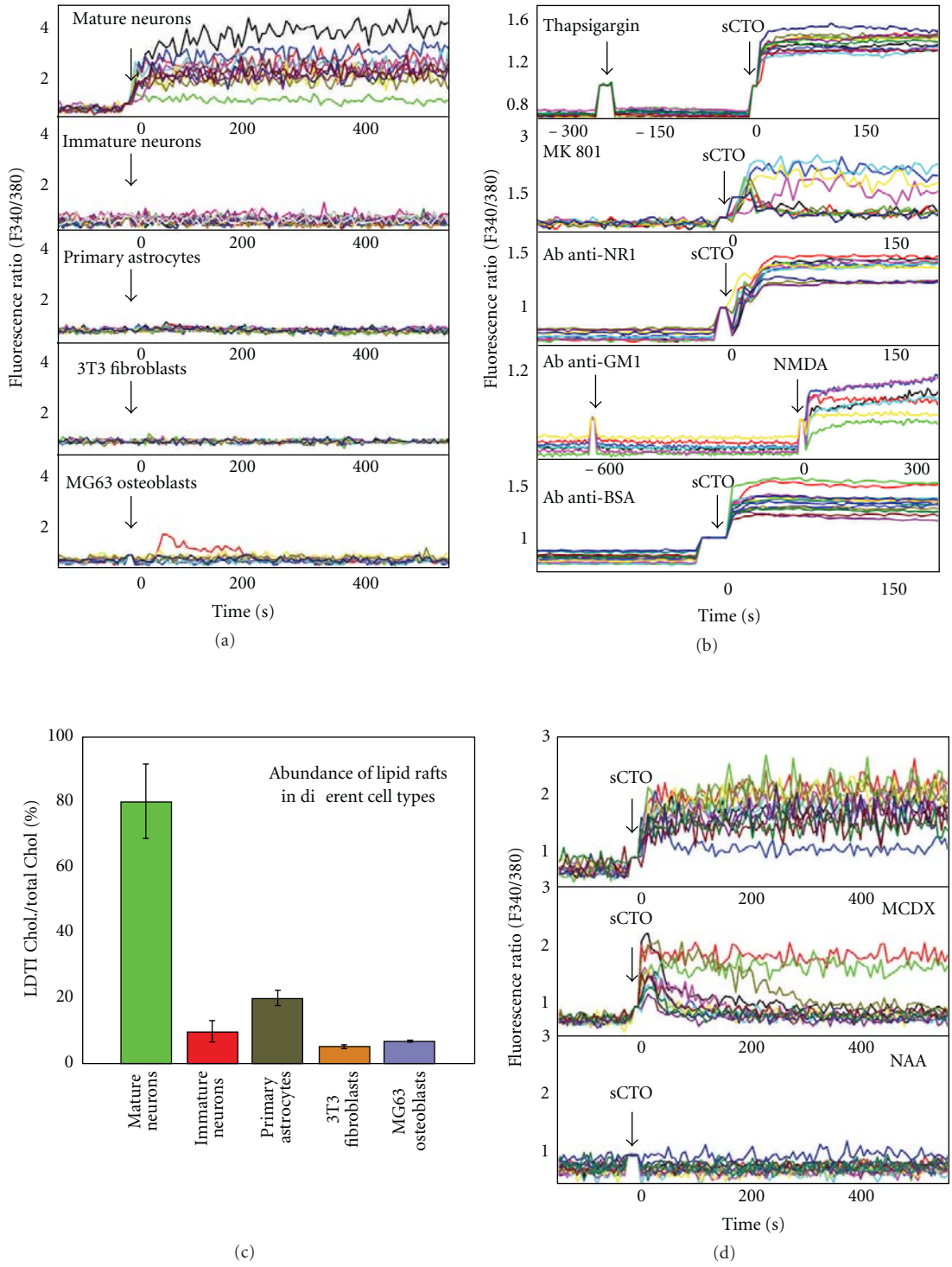


Figure 6: (a) sCTO induces increase in intracellular Ca^{2+} levels in mature hippocampal neurons, but not in immature neurons, primary astrocytes, 3T3 fibroblasts, and MG3 osteoblasts. Ca^{2+} levels were evaluated by optical fluorimetric recordings with Fura-2AM. (b) Depletion of intracellular Ca^{2+} stores with thapsigargin did not affect sCTO-induced Ca^{2+} rise, suggesting that it was mostly due to an extracellular Ca^{2+} influx. MK801, a specific NMDA inhibitor, as well as antibodies against NR1, failed to affect sCTO-driven Ca^{2+} influx, suggesting that the NMDA receptor was not involved. On the contrary, pretreatment with an antibody against the ganglioside GM1, aimed at blocking LRs, completely abolished sCTOs-induced Ca^{2+} increase. Pretreatment with anti-BSA IgGs, an unrelated antibody, did not affect sCTO response. (c) Measure of the weight ratio between cholesterol in LRs (LDTI) and total cholesterol indicates that plasma membrane of mature hippocampal neurons have a much higher content in LRs than the other cell types. (d) Pretreatment of hippocampal neurons with NAA totally suppressed sCTO-induced Ca^{2+} increase. Reproduced with permission and partially modified from [170].

Abbreviations

AD: Alzheimer's disease
Aβ : Amyloid β
AFM: Atomic force microscopy
CT: Calcitonin
CDS: Circular dichroism spectroscopy
DIV: Day *in vitro*
ER: Endoplasmic reticulum
EFTEM: Energy filtered TEM
GSK: Glycogen synthase kinase 3-beta
GPI: Glycosylphosphatidylinositol
htt: Huntingtin
HD: Huntington's disease
LDTI: Low-density, triton-insoluble
LBs: Lewy bodies
LRs: Lipid rafts
LTD: Long-term depression
LTP: Long-term potentiation
NAA: Neuraminidase
NR1: NMDA receptor subunit 1
NMDA: N-methyl-D-aspartate
PD: Parkinson's disease
PrP: Prion protein
RyR: Ryanodine
sCTOs: sCT oligomers
sCT: Salmon CT
TSEs: Transmissible spongiform encephalopathies
TEM: Transmission electron microscopy
α-syn: α-synuclein.

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Review Article

The Pathological Roles of Ganglioside Metabolism in Alzheimer's Disease: Effects of Gangliosides on Neurogenesis

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Conversion of the soluble, nontoxic amyloid β-protein (Aβ) into an aggregated, toxic form rich in β-sheets is a key step in the onset of Alzheimer's disease (AD). It has been suggested that Aβ induces changes in neuronal membrane fluidity as a result of its interactions with membrane components such as cholesterol, phospholipids, and gangliosides. Gangliosides are known to bind Aβ. A complex of GM1 and Aβ, termed "GAβ", has been identified in AD brains. Abnormal ganglioside metabolism also may occur in AD brains. We have reported an increase of Chol-1α antigens, GQ1bα and GT1aα, in the brain of transgenic mouse AD model. GQ1bα and GT1aα exhibit high affinities to Aβs. The presence of Chol-1α gangliosides represents evidence for genesis of cholinergic neurons in AD brains. We evaluated the effects of GM1 and Aβ1–40 on mouse neuroepithelial cells. Treatment of these cells simultaneously with GM1 and Aβ1–40 caused a significant reduction of cell number, suggesting that Aβ1–40 and GM1 cooperatively exert a cytotoxic effect on neuroepithelial cells. An understanding of the mechanism on the interaction of GM1 and Aβs in AD may contribute to the development of new neuroregenerative therapies for this disorder.

1. Introduction

Alzheimer's disease (AD) is an irreversible, slowly progressive neurodegenerative disease that is the most common form of dementia among people age 65 and older and is characterized by cognitive and behavioral problems. The symptoms are initiated by memory loss and gradually lead to behavior and personality changes with impaired cognitive abilities such as decline of decision making and language disability, and eventually disturbances in recognizing family and friends. These losses are related to the worsening lesion of the connections between certain neurons in the brain. Patients often become anxious or aggressive, or wander away from home. Eventually, patients need total care, and the final outcome is always death. Although there is no cure at present, some therapeutic drugs inhibiting acetylcholinesterase have been used to alleviate the disease symptoms and improve the quality of life for patients with AD [1, 2].

Gangliosides are important constituents of cells; they are especially abundant in neuronal membranes and play a variety of biological functions, including cellular recognition and adhesion as well as signaling [3]. The expression of gangliosides is not only cell type specific and developmentally regulated but also closely related to the differentiation state of the cell [3–6]. Numerous studies have indicated that changes of ganglioside expression patterns and levels during cellular differentiation are closely related to their metabolism, particularly their biosynthesis [3, 4, 6]. Notably, gangliosides may have neuroprotective effects to the cell [7]. Gangliosides do not function as a neurotrophic factor themselves, but they potentiate neurotrophic influences present in the nervous system. In this regard, many scientists have reported the beneficial effects of GM1 treatment in animal models of neurodegeneration and diseases. For example, administration of GM1 protects hippocampal progenitor cells from neuronal injury and reduces hippocampal neurogenesis induced by D-galactose treatment [8]. Saito et al. reported that GM1 and LIGA20 can protect mouse brains from apoptotic neurodegeneration induced by ethanol [9]. In clinical applications or animal studies, many studies have

demonstrated the neuroprotective effects of GM1 in diseases such as AD [10], AD model of transgenic mice [11, 12], Parkinson disease [13], stroke [14], and Guillain-Barré syndrome [15].

The pathological hallmarks in the AD brain include senile plaques (SPs) and neurofibrillary tangles. Many scientists believe that the accumulation and aggregation of amyloid β-proteins (Aβs) in SPs in the brain are a central part of the pathogenesis of AD. The conversion of soluble, nontoxic Aβ into aggregated, toxic Aβ rich in β-sheet structures is considered to be the key step in the development of AD. Aβs are able to bind to a variety of biomolecules, including lipids, proteoglycans, and certain proteins [1, 16]. Immunochemical studies revealed that Aβ deposits in AD brain are due to the presence of certain amyloid-associated proteins such as amyloid P component, proteoglycans, and apolipoproteins [17]. The potential significance of the interaction with proteoglycans and Aβs for the pathogenic mechanisms has been reviewed [2].

During aging and neurodegeneration in AD, the physicochemical properties of membranes and lipid metabolism undergo significant alterations [18, 19]. These include multiple changes such as glycosphingolipid (GSL) abnormalities and impairment of neurotrophin signaling, protein trafficking, and protein turnover [20]. These changes can result in imbalances in the proportion of lipids in membranes and/or changed ratios of membrane lipids, which may contribute to the pathogenesis of AD [21–23]. Earlier immunohistochemical studies on the involvement of GSLs using specific monoclonal antibodies revealed that SPs contained GM1 [24], c-series gangliosides [25], and GD1a [26], and these studies implicate that Aβs might be interacting with the above gangliosides. In this paper, we will focus on the role of ganglioside metabolism in the pathogenesis and/or development of AD.

2. Interaction of Amyloid β-Proteins with Gangliosides

A critical question concerning the development of AD is how the soluble, nontoxic Aβ form high in an α-helix-rich structure is converted into an aggregated, toxic form rich in β-sheets in the brain. Terzi et al. first reported that Aβ1–40 undergoes a conformational transition from random coil to aggregated structure rich in β-sheet after addition of lipid vesicles containing negatively charged lipids [27], suggesting that Aβ neurotoxicity may be the result of membrane protein-lipid interactions. Several studies have demonstrated that Aβs bind to gangliosides, especially GM1, resulting in an altered secondary structure of Aβs [28–31]. Aβ binds to membranes containing ganglioside GM1, and upon binding it undergoes a conformational transition from random coil to an ordered structure rich in β-sheet. This interaction appears to be ganglioside-specific because no changes in Aβ1–40 conformation were found in the presence of various phospholipids or sphingomyelin. Aβ binds selectively to gangliosides with a binding affinity ranging from 10^{-6} to 10^{-7} M, depending on the type of the sugar moiety present in the ganglioside molecule. On the other hand, the isolated oligosaccharide moieties of gangliosides are ineffective in inducing alterations in the secondary structure of Aβ1–40 [28, 32], suggesting the involvement of the lipid component in their interaction. The sialic acid (NeuAc) moiety of gangliosides interacts with Aβ to induce conformational changes in Aβ [31]. In this regard, we have reported that Aβ1–40 binds to a number of gangliosides with the following order of binding strength: GQ1bα > GT1aα > GQ1b > GT1b > GD3 > GD1a = GD1b > LM1 > GM1 > GM2 = GM3 > GM4 based on surface plasmon resonance studies [33]. Neutral GSLs, including asialo-GM1, generally have a much lower affinity for Aβ1–40 than do gangliosides. The results suggest that the α2,3NeuAc residue on the oligosaccharide core of gangliosides is required for binding. In addition, the α2,6NeuAc residue linked to GalNAc in the α-series of gangliosides contributes significantly to the binding affinity for Aβ. Although several reports documented the GM1-induced alterations in the β-sheet structure of Aβ, Mandel and Pettegrew, on the other hand, reported that GM1 inhibited Aβ from undergoing α-helix to β-sheet conformational changes [34]. This discrepancy clearly needs further clarification. In addition, asialo-GM1 binds specifically with Aβ in a manner that could prevent β-sheet formation. Nakazawa et al. reported that Aβ1–40 strongly perturbed the lipid bilayer structure of liposomes of dimyristoylphosphatidylcholine and GM1 to form a nonlamellar phase (most likely in the micellar phase) [35]. The α-helical peptide conformation is significantly flexible and is approximately equally partitioned between components penetrated into the bilayer and in liquid phase whereas the β-sheet peptide conformation is rigid and is presumably deposited and stacked at the bilayer surface.

The interaction between gangliosides and Aβ appears to be affected by experimental conditions such as pH, ionic strength [30, 31], and metal ions [36, 37]. For example, McLaurin and Chakrabartty have reported that Aβ1–40/Aβ1–42 disrupts acidic lipid membranes, and this disruption is greater at pH 6.0 than at pH 7.0, at which point gangliosides induce Aβ1–40/Aβ1–42 to adopt a novel α/β conformation [30]. A further study indicated that binding of Aβ1–40 to mixed gangliosides or GM1-containing vesicles induced an α-helical structure at pH 7.0 and β-structure at pH 6.0 [31]. Several lines of evidence have indicated that disruption of the homeostatic balance of redox-active biometals such as Cu and Fe can lead to oxidative stress, which plays a key role in the development of AD. Atwood et al. reported that unlike other biometals tested at maximal biological concentrations, Cu^{2+}-induced aggregation of Aβ1–40 occurred as the solution pH was lowered from 7.4 to 6.8 and that the reaction was completely reversible with either chelation or alkalinization [36]. The aggregation-inducing activity of metals is in the following order, $Cu^{2+} > Fe^{3+}$ > or = $Al^{3+} > Zn^{2+}$ [37].

3. Binding Sites of Amyloid β-Proteins with Gangliosides

The NeuAc residue of the ganglioside head group is important for determining the nature of the conformational change

of Aβ [31, 38] or interaction with Aβ [33]. The isolated pentasaccharide head group of GM1 alone, however, does not bind with Aβ, suggesting the need for a polyanionic membrane-like structure [38]. To provide a structural basis for this pathogenic interaction associated with AD, Williamson et al. have demonstrated using NMR on ^{15}N-labelled Aβ1–40 and Aβ1–42 that the interaction with GM1 micelles is localized to the N-terminal region of the peptide, particularly residues His13 to Leu17, which become more helical when GM1 is bound [38]. The key interaction is with His13, which undergoes a GM1-specific conformational change. Zhang et al. reported that the binding site for GM1 was located within residues 52–81 (N terminus) of amyloid precursor protein (APP), resulting in a conformational change of APP [39]. This phenomenon is specific for GM1, but not for GD1a, GT1b, and ceramide, indicating that specific binding depends on the sugar moiety of GM1.

Utsumi et al. reported the association of Aβ1–40 isotopically labeled with GM1 and lyso-GM1 micelles using 920 MHz ultra-high field NMR analyses [40]. The data revealed that: (a) Aβ1–40, upon binding to the gangliosidic micelles, forms discontinuous α-helices at the segments His(14)-Val(24) and Ile(31)-Val(36), and (b) Aβ1–40 lies on hydrophobic/hydrophilic interface of the ganglioside cluster, exhibiting an up-and-down topological mode in which the two α-helices and the C-terminal dipeptide segment are in contact with the hydrophobic interior whereas the remaining regions are exposed to the aqueous environment. These results suggest that the ganglioside clusters serve as a unique platform for binding coupled with conformational transition of Aβ molecules, rendering their spatial rearrangements restricted to promote specific intermolecular interactions. [40]. Further study of NMR analyses of the Aβ interactions with gangliosides using lyso-GM1 micelles as a model system have revealed that the sugar-lipid interface is primarily perturbed upon binding of Aβ to the micelles, underscoring the importance of the inner part of the ganglioside cluster for accommodating Aβ in comparison with the outer carbohydrate branches that provide microbial toxin- and virus-binding sites [41].

4. Accumulation of Specific Ganglioside-Bound Amyloid β-Protein Complex (GAβ) in AD Brain

Choo-Smith et al. have reported that addition of ganglioside-containing vesicles to the Aβ solution dramatically accelerates the rate of fibril formation compared to vesicles without gangliosides [28]. The mechanism of ganglioside-mediated Aβ-fibrillization likely involves an initial step in which the GSL-bound peptide self-associates on the membrane surface, undergoing a conformational transition to a β-sheet structure. This suggests that gangliosides can mediate Aβ assembly to lead to accumulation in the brain, which may be involved in the development of AD. Yanagisawa et al. first reported the presence of membrane-bound Aβ1–42, but not Aβ1–40, which tightly binds to GM1 in the AD brain [42]. This novel Aβ species, named as ganglioside-bound Aβ (GAβ), may act as an endogenous seed for amyloid [42], and exhibited early pathological changes of AD. It was hypothesized that Aβ adopts an altered conformation following interaction with GM1, leading to the generation of GAβ, and then GAβ acts as an endogenous seed for amyloid in AD brain. GAβ has unique characteristics, including an extremely high aggregation potential and an altered pattern of immunoreactivity, which results in seeding for amyloid fibril formation in brain. Thus, GAβ may serve as a seed for toxic amyloid fibril formation. The formation of GAβ serves as one of the critical factors in the development of AD and may provide new insights into the pathophysiology in AD [43]. The occurrence of GAβ in AD brain was further confirmed biochemically by staining with cholera toxin-B subunit (CTXB) that preferentially binds to GM1, and by immunoprecipitation experiments using several anti-Aβ monoclonal antibodies [44]. Recently, the presence of GAβ was confirmed in sections of cerebral cortices of cynomolgus monkeys of different ages, from 4 to 36 years old; especially, GAβ is significantly increased in the brains at ages below 19 years [45]. In this study, the accumulation of GAβ occurred exclusively in the subcellular organelles that are involved in the endocytic pathway. Since Aβ generation and GM1 accumulation likely occur in early endosomes, it suggests that endosomes are intimately involved in the Aβ-associated pathology of AD [45]. In addition, Aβ aggregation in brain is accelerated through an increase in the level of GM1 in neuronal membranes [46, 47]. The effect occurs in a dose-dependent manner; in the presence of lower concentrations of GM1 (approximately 25 μM), Aβ1–40 forms aggregates much more slowly, indicating that an increase in the concentration of GM1 significantly facilitates the aggregation of Aβ. Further studies have indicated that both GM1 and GT1b promote the aggregation and cytotoxicity of Aβ1–40, and these gangliosides, especially GM1, catalyze the formation of neurotoxic fibrils [48]. Moreover, binding of Aβ to GM1 was dependent on cholesterol-induced clustering of GM1 in the host membranes. An increase in the cholesterol concentration in the neuronal membranes accelerates Aβ aggregation through the formation of an endogenous seed [49, 50], consistent with the notion that cholesterol is also a risk factor for AD development. These results further underscore the importance of control of cellular cholesterol and/or ganglioside contents in the pathogenesis of AD [50–52]. Lin et al. reported the role of GM1 and cholesterol on the Aβ-induced cytotoxicity in the plasma membrane [53]. Depletion of GM1 from the plasma membrane would be expected to block the Aβ-induced cytotoxicity. Decreasing the cholesterol level by around 30% could also attenuate the cytotoxicity of Aβ. These findings validate that cholesterol can stabilize the lateral pressure derived from formation of the GM1-Aβ complex on the membrane surface and that both GM1 and cholesterol are essential for Aβ accumulation. Zha et al. reported that GM1 regulated the expression of Aβ in a dose-dependent manner [54]. Exogenously added GM1 increased Aβ levels in mixed rat cortical neurons containing African green monkey epithelial kidney cells (COS7) and human neuroblastoma cells (SH-SY5Y) that were transfected with APP695 cDNA.

Yanagisawa and coinvestigators developed a novel monoclonal antibody, 4396C, raised against GAβ purified from an AD brain [55]. Using this antibody, the presence of GAβ was confirmed in the AD brain, in which GAβ is endogenously generated. The antibody reacted with GM1-bound forms of two Aβ isoforms, Aβ1–40 and Aβ1–42, but not the unbound forms of Aβ1–40 and GM1. Remarkably, using liposomes containing Aβ1–40 and GM1, this antibody completely blocks amyloid fibril formation in a dose-dependent manner, suggesting that it may act to inhibit the initiation of oligomerization-polymerization of Aβ in the brain and serve in the possible development of a novel therapeutic strategy to the GAβ-dependent amyloidogenesis [55, 56]. The age-dependent high-density GM1 clustering at the presynaptic neuritic terminals is a critical step for Aβ deposition in AD. In amyloid-positive synaptosomes prepared from AD brain, GM1 levels are significantly increased when Aβ deposition begins at presynaptic terminals. The antibody against GAβ, 4396C, suppressed Aβ assembly in the synaptosomes [57]. Moreover, peripheral administration of the Fab fragments of 4396C into transgenic mice expressing a mutant amyloid precursor protein gene, following the conjugation of the protein transduction domain of the Tat protein, markedly suppressed Aβ deposition in the brain [58].

Interestingly, the enhanced GAβ-dependent amyloidogenesis under the endocytic dysfunction was suppressed by pretreatment with a sphingomyelinase synthase inhibitor, suggesting that sphingomyelin is also one of the key molecules for GAβ generation, further implying that the interaction of Aβ with membrane lipids is critical in amyloid fibrillization in the brain [59]. In addition, the expression of apolipoprotein E4 may facilitate Aβ assembly in the brain through an increase in the GM1 content in neuronal membranes, which likely induces GAβ generation [43, 47, 60].

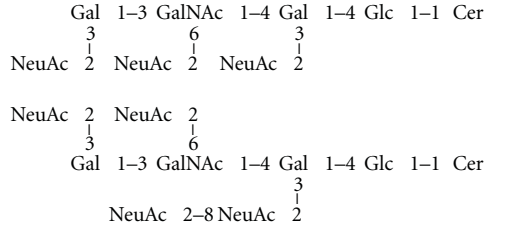
In recent years, evidence has been presented that "lipid rafts" are the preferential sites for the formation of the pathological forms of Aβ [61]. GAβ is generated in the membrane raft-like microdomains, comprised of cholesterol, sphingomyelin, and GM1 [62, 63], in which Aβ undergoes a conformational transition from an α-helix-rich structure to a β-sheet-rich structure or oligomerization with the increase in protein density on the membrane. GM1 induced amyloid fibrillization, especially under β-sheet-forming conditions, leading to the generation and seeding of GAβ. Thus, ganglioside binding with Aβ is the initial and common step in the development of a part of human misfolding-type amyloidoses, including AD [64]. The level of GAβ is increased, and its α-helix structure is converted into a β-sheet structure [65]. Thus, the formation of amyloid fibrils or oligomers is likely mediated by gangliosides in lipid rafts [66], and depletion of gangliosides or cholesterol significantly reduces the amount of amyloid deposits [48, 67].

5. Other Gangliosides May Be Involved In the Generation of GAβ

Other gangliosides have been shown to interact with Aβ, which may lead to Aβ accumulation in the brain. The assembly of wild-type and mutant forms of Arctic-, Dutch-, and Flemish-type of Aβs is accelerated in the presence of not only GM1, but also GM3 and GD3 gangliosides. Dutch and Italian-type Aβs require GM3 ganglioside for their assembly [47]. Arctic-type Aβ, in contrast to the wild-type and other variant forms, shows a markedly rapid and higher level of amyloid fibril formation in the presence of sodium dodecyl sulfate or GM1 ganglioside [68]. These results provide evidence that local gangliosides play a crucial role in the region-specific Aβ deposition in the brain [69, 70]. Gangliosides are located mostly on the cell surface and have been demonstrated to modulate neurotrophic activities. The localization of GD1a in dystrophic neurites suggests that such neurites accumulate GD1a as a membranous component. In addition, the accumulation of GD1a in SPs suggests that it may contribute to SP formation [26]. In a study for the interaction of Aβs with GM1 using rat adrenal medulla pheochromocytoma cells (PC12 cells), Wakabayashi et al. used CTXB for detection of GM1 [71]. However, the ganglioside that interacted with Aβs in PC12 cells may not be definitely GM1 because CTXB also strongly reacted also with fucosyl-GM1 and fucosyl-GD1b [72] and PC12 cells express fucosyl gangliosides including fucosyl-GM1 [73, 74] with little or no GM1 [75]. When PC12 cells were cultured in the presence of Aβ1–40 or Aβ1–42, Aβs accumulated in cells expressing fucosyl gangliosides [72]. Thus, the interaction of Aβ with gangliosides to effect amyloid assembly may not be limited to GM1; indeed, other gangliosides should also be involved in "seeding" [1, 72]. Molander-Melin et al. reported that the detergent-resistant membrane fractions from the frontal cortex of AD brains contained a significantly higher concentration of ganglioside GM1 and GM2 [76]. The increased proportions of GM1 and GM2 in lipid rafts at an early AD stage could accelerate the formation of Aβ plaques, which gradually causes membrane raft disruptions and thereby affects cellular functions that are dependent on the presence of such membrane domains.

6. Ganglioside Metabolism in AD Brains and AD Model Mouse Brains

Aβ changes in membrane fluidity could be induced by chemical interactions of the peptide with membrane components such as cholesterol, phospholipids, and gangliosides [77]. Since gangliosides have a strong affinity to Aβs [33], they could participate in conformational changes of Aβs in membrane fluidity. For this reason, ganglioside metabolism has been considered to be closely associated with the pathogenesis of AD [1, 20]. Several earlier studies showed significant changes of ganglioside patterns in AD brain. The concentration of gangliosides decreased in the majority of brain regions, such as the cerebral cortex, hippocampus, and basal telencephalon, especially in the frontal cortex and white matter [78–80]. Kracun et al. reported that the major brain ganglio-N-tetraosyl-series ganglioside species (GT1b, GD1b, GD1a, and GM1) significantly decreased in the frontal and temporal cortices and basal telencephalon of the brains of patients with AD compared with the respective areas in control brain [81, 82]. Brooksbank and



Scheme 1

McGovern [83] and Crino et al. [84] also reported changes of ganglioside composition in AD brains in which b-series gangliosides, such as GT1b and GD1b, showed a significant decrease, in contrast to a slight increase in GT1a, GD3, GM1, and GM2. These findings suggest that abnormal ganglioside metabolism coincides with the affected cortical areas of neurodegeneration that afflicts AD.

In contrast to these human studies, we found no significant differences in the lipid-bound NeuAc content in the brain slices containing hippocampal/cortical tissue prepared from AD model double transgenic (Tg) mice coexpressing mouse/human chimeric APP with the Swedish mutation and human presenilin-1 with a deletion of exon 9 and age-matched wild-type (WT) mice, even though Aβs were found to be accumulated in the brain (Figure 1) and serum of these Tg AD model mice [85]. In addition, there was no significant difference in the expression levels of major gangliosides (GM1, GD1a, GD1b, and GT1b) in the brains between double Tg and age-matched WT mice. This is consistent with the report by Sawamura et al. [86] who also did not detect notable changes in the major gangliosides in the brain of mutant presenilin-2 Tg mice, despite the remarkable increase in the level of Aβ1–42 and statistically significant lower levels of glycerophospholipids and sphingomyelin. In addition, Bernardo et al. also did not find significant differences in a- or b-series gangliosides between WT and double Tg mice expressing APP with the Swedish mutation and presenilin-1 with a deletion of exon 9 [87]. These studies as well as our recent data indicate no significant changes in the major brain ganglioside metabolism in AD model mice, despite the presence of massive accumulation of Aβ deposits in the brains of these animals. Barrier et al. reported an increase of GM2 and GM3 within the cortices of Tg mice expressing human APP751 with Swedish and London mutations and human presenilin-1 (M1461) [88].

The most consistent and interesting finding of our recent study is the increased expression of cholinergic-specific antigen-1α (Chol-1α) antigens, GT1aα, and GQ1bα (see Scheme 1), especially GQ1bα, in the brain of double Tg mice as compared with those in WT mouse brains (Figure 2). The increase was especially significant in female double Tg mouse brains. No significant differences were found in the expression of GT1aα and GQ1bα between male and female WT mouse brains. These gangliosides are normally minor species in the brain and serve as markers of cholinergic neurons [89, 90]. The expression of Chol-1α antigens in rat brain regions such as the hippocampus is developmentally regulated, and their concentrations increase with aging [91]. Although the functional role of Chol-1α antigens in Tg mice brain has remained obscure, Ando et al. reported that the release of acetylcholine from synaptosomes was inhibited by anti-Chol-1α monoclonal antibody [92]. The memory and learning abilities of rats given anti-Chol-1α antibody were remarkably suppressed. On the contrary, the treatment of Chol-1α antigen induced choline uptake by synaptosomes. As a result of increased choline uptake, acetylcholine synthesis was enhanced by Chol-1α antigens. Chol-1α antigens are specifically expressed in the cholinergic neutrophil and may participate in cognitive functions such as memory and learning. Beneficial effects of Chol-1α antigens were shown to ameliorate decreased functions of synapses from aged brains, suggesting that Chol-1α antigens may play a pivotal role in cholinergic synaptic transmission and participates in cognitive function [93]. Interestingly, Chapman et al. reported the presence of serum antibody in patients with AD that specifically bind to cholinergic neurons [94]. The increasing antibody in the patient's sera may be attributed to the increase of Chol-1α antigens in AD brain. Cholinergic neuronal dysfunction of basal forebrain is observed in patients with AD, and has been linked to decreased neurogenesis in the hippocampus, a region involved in learning and memory [95]. They recently found an increasing number of newborn cells in the dentate gyrus of hippocampus in cholinergic-denervated mice compared to nonlesioned mice, suggesting neurogenesis can occur in Tg mice brain to generate new cells expressing Chol-1α antigens. It would be extremely interesting to enhance neurogenesis in hippocampus of patients and animal models of AD [96–99].

In addition to the above, AD model animals with disrupted ganglioside biosynthesis have been reported to reveal relationships between ganglioside metabolism and aspects of AD. For example, Oikawa et al. crossbred Tg mice expressing human APP having Swedish and London mutations with GM2-synthase knockout mice [100]. The mutant mice expressing GM3 and GD3, but not GM1, GD1a, GD1b, and GT1b, showed a significant increase of Aβ accumulation in vascular tissues and formation of severe dysphonic-form amyloid angiopathy in the brain. In contrast, Bernardo et al. analyzed the AD model of Tg mice expressing APP with the Swedish mutation and presenilin-1 with a deletion of exon 9 that crossbred with mice deficient in GD3-synthase, which catalyzes the synthesis of b-series gangliosides [87]. In the triple mutant mice, b-series gangliosides, including GD3, were completely absent, but GM1 and GD1a were significantly increased. Interestingly, Aβ plaques and associated neuropathology were almost completely eliminated in the triple mutant mice, resulting in cognitive improvement [87]. These observations suggest that b-series gangliosides synthesized by GD3-synthase are one of the major causes of Aβ accumulation and AD. Thus, inhibition of GD3-synthase can be a novel therapeutic target to combat the cognitive deficits, amyloid plaque formation, and neurodegeneration seen in AD.

In this regard, Okada et al. reported that endogenously generated b-series gangliosides may be critical for

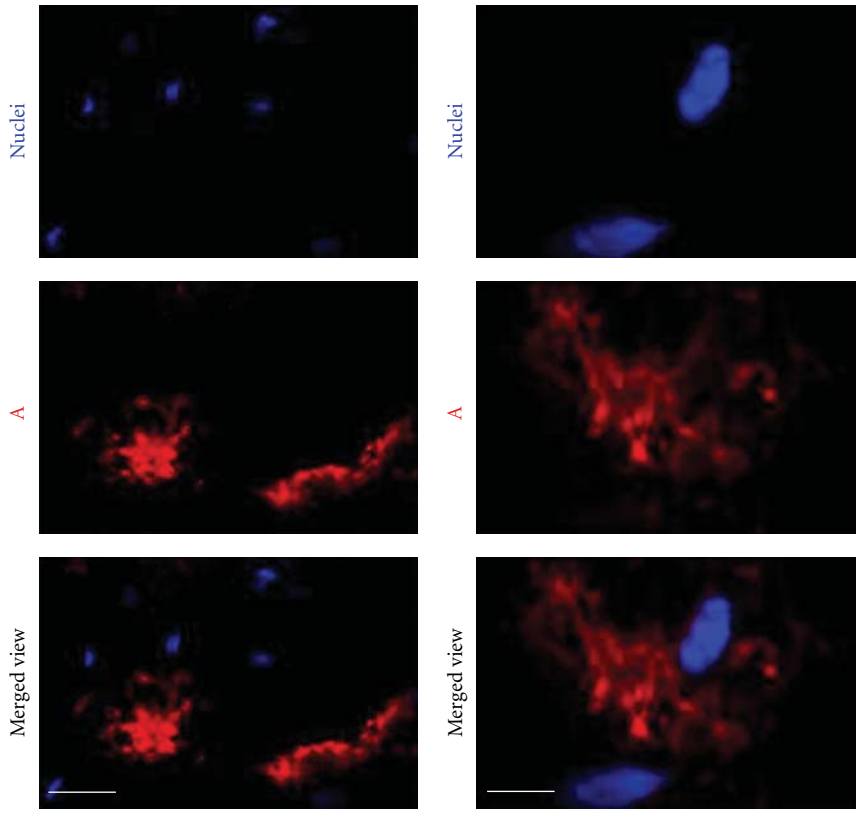


Figure 1: Immunohistochemical localization of Aβs in the cortex of double transgenic mice coexpressing mouse/human chimeric APP with the Swedish mutation and human presenilin-1 with deletion of exon 9. The coronal brain sections are 12 μm in thickness. Nuclei (blue) and Aβ (red) were stained with Hoechst 33258 and antihuman Aβ antibody, respectively. (a) low-magnification view; (b) high-magnification view. Bar = 20 μm in (a); 5 μm in (b). (Reproduced from [85] with permission).

the repair of damaged neural tissues *in vivo* [101]. They established a GD3-synthase gene knockout mouse model in which all b-series gangliosides were deleted. However, animals showed no morphological changes in the brains and apparent abnormal behavior. Moreover, no differences in Fas-mediated apoptotic reaction in lymphocytes compared with the wild type were found. The mutant mice, however, exhibited reduced regeneration of axotomized hypoglossal nerves compared with the wild type, suggesting that b-series gangliosides are more important in the repair of damaged nerves rather than in the differentiation of the nervous system.

7. Neurogenesis and Neural Stem Cells in AD Brain

The neurodegenerative process in AD is initially characterized by synaptic damage accompanied by neuronal loss. Neuronal loss leads to cerebral atrophy, which appears to be hallmarks of cognitive impairment in AD [102]. In addition to the alterations in synaptic plasticity and neuronal integrity in mature neuronal circuitries, the neurodegenerative process in AD has recently been shown to be accompanied by alterations in neurogenesis [103, 104]. The hippocampus is one of the regions in the adult brain where neurogenesis occurs throughout life [5]. Many studies have shown that adult neurogenesis is involved in learning and memory. This has led to the hypothesis that impairment in memory during aging and neurodegenerative diseases such as AD involves abnormal neurogenesis [105]. However, neurogenesis in AD and in animal models is not fully studied yet [106]. In AD brains, there is some controversy whether neurogenesis is increased [107] or decreased [107]. Boekhoom et al. reported an apparent increase of neurogenesis markers in AD brains, which may be related to glial and vasculature-associated changes [107]. A number of mouse models of AD displayed reduced neurogenesis [108–110] or enhanced neurogenesis [97]. Several attributes of adult hippocampal neurogenesis suggest that amyloid deposition may influence neurogenesis [111]. Zhang et al. reported that reductions in dentate gyrus neurogenesis in a murine model of amyloid deposition are linked to the deposition of amyloid [112].

The adult mammalian brain contains neural stem cells (NSCs), undifferentiated neural cells characterized by their high proliferative potential and the capacity for self-renewal with retention of multipotency to differentiate into neurons and glial cells, in the subgranular zones of dentate gyrus

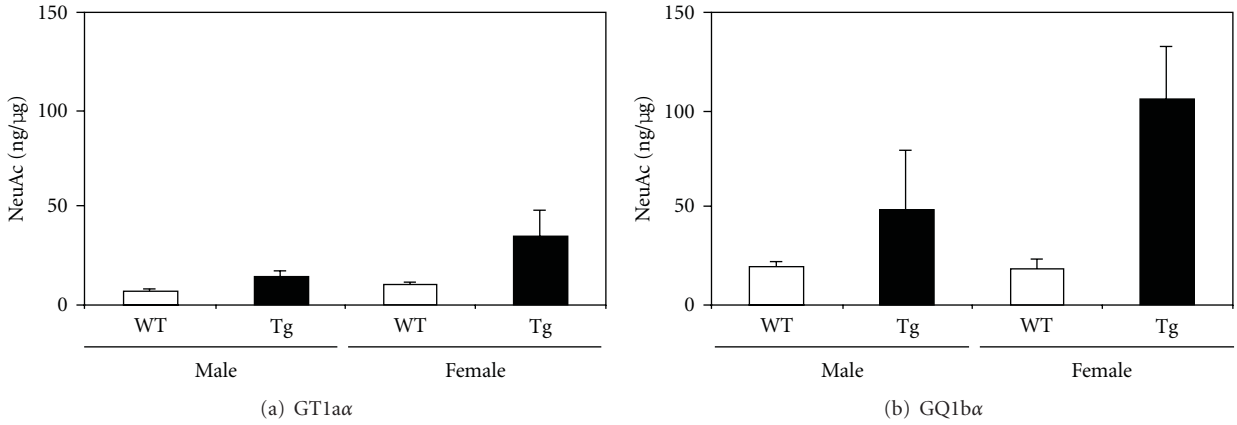


Figure 2: The content of Chol-1α antigens, GT1aα (a) and GQ1bα (b), in AD model mouse brains [85]. GT1aα and GQ1bα extracted from brains of AD model double transgenic mice coexpressing mouse/human chimeric APP with the Swedish mutation and human presenilin-1 with a deletion of exon 9 (Tg) or age-matched wild-type mice (WT) were quantified by densitometric analysis of high-performance thin-layer chromatography immunostaining. n = 3–7. (Reproduced from [85] with permission).

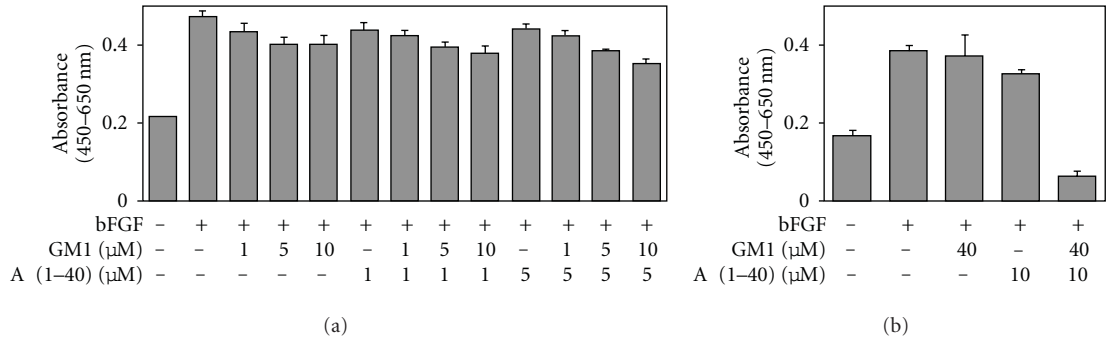


Figure 3: Effects of low (a) and high (b) concentrations of GM1 and Aβ1–40 on NECs. Basic fibroblast growth factor (bFGF; 0 or 5 ng/mL) was added as a mitogen of NECs. The number of NECs cultured in the presence of bFGF for 4 days was estimated by WST-8 assay with (a) GM1 (0, 1, 5 or 10 μM) and Aβ1–40 (0, 1 or 5 μM); with (b) GM1 (0 or 40 μM) and Aβ1–40 (0 or 10 μM). The spectrophotometric absorbance (Abs.) measured at the wavelength of 450 nm (reference, 650 nm) by this assay is highly correlated with the number of living NECs [113]. (Reproduced from [114] with permission).

and the subventricular zone (SVZ) of the lateral ventricles [5]. The possibility that abnormalities in NSCs contribute to the pathogenesis of AD and the cognitive impairments in humans has been suggested [109, 110]. Several papers have described the phenomenon of neurogenesis in hippocampus, and it seems to be enhanced in AD brains. This phenomenon could potentially occur also in the brain of animal models of AD, which points to the possibility of developing strategies for promoting neurogenesis for AD therapy by using NSCs. A number of studies have indicated that Aβs can regulate the proliferation of NSCs and documented the bifunctional roles of Aβs on the cells in a dose-dependent manner. The low concentrations of Aβs have neurogenic effects in some studies [115–117] but cytotoxic effects in other studies [109, 118, 119]. Soluble oligomers of Aβ1–40 and Aβ1–42, but not Aβ40–1, a reversed amino acid sequence, induced neuronal apoptosis [120]. the aggregated form of Aβ1–42 stimulated neurogenesis [117, 121]. In this regard, Aβ1–40 (0.5 μmol/L) significantly reduced proliferation of endothelial progenitor cells by about 65% compared to control whereas Aβ40–1 (0.5 μmol/L), did not affect their proliferation [122]. Gong et al. reported that small, soluble oligomers of Aβ block the reversal long-term potentiation [123]. Controversially, a low micromolar concentration (1 μM) of oligomeric Aβ1–42 increased the proliferation [124] and neurogenesis of adult NSCs [117]. Small peptide, Aβ1–16, had no effect on neuronal proliferation of adult SVZ progenitors [119, 121] Several studies indicated that Aβ25–35 has toxic effects and may induce cell death or apoptosis [109, 110, 118, 125, 126]. In contrast, Li and Zuo reported inhibitory effects of aggregated form of Aβ25–35 (1 mg/mL, 3 μL) on neurogenesis in the SVZ and dentate gyrus after injection into the lateral ventricle of adult mouse [127]. This result indicates that Aβ25–35 could impair neurogenesis in the hippocampus of adult mouse brain.

In neuronal cultures prepared from rat hippocampi (embryonic day 18 to 19), it was reported that 25 μM of Aβ25–35 enhanced the metabolism of lipids such as phospholipids (+52%) and gangliosides (+193%), but not cholesterol [128]. In addition, exposure of rat cultured cortical

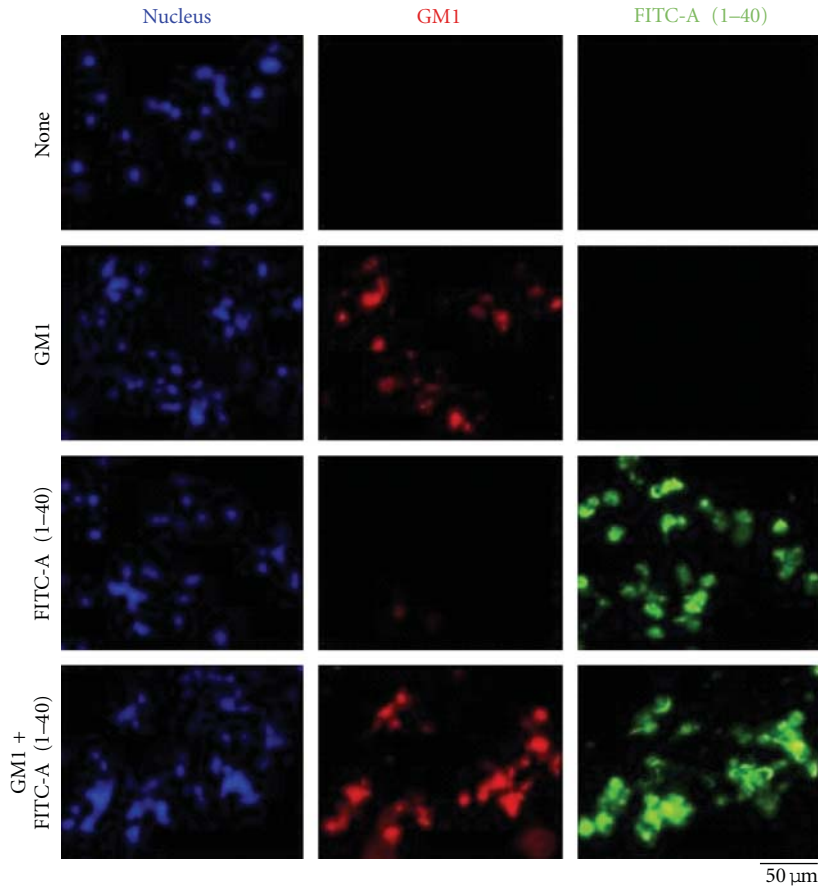


Figure 4: Incorporation of exogenously added GM1 and Aβ1–40 into NECs. NECs were cultured in the presence of GM1 (0 or 40 μM) and/or fluorescein isothiocyanate-conjugated Aβ1–40 (FITC-Aβ1–40; 0 or 10 μM) for 2 days, and then stained with biotin-conjugated CTXB, a probe to detect GM1, and rhodamine-conjugated streptavidin. Nuclei were stained with Hoechst 33258. (Reproduced from [114] with permission).

neurons to Aβ25–35 induced a substantial increase of the intracellular GD3 levels [129]. These reports suggest that Aβ can modulate ganglioside metabolism in NSCs. It has been reported that in NSCs, GSLs, including gangliosides, are involved in cellular proliferation via modulation of the Ras-mitogen-activated protein kinase pathway [114]. These findings prompt us to propose that a combination of Aβ and GM1 induces NSC proliferation. Recently, we evaluated the effects of GM1 and Aβ1–40 on mouse neuroepithelial cells (NECs) that are known to be abundant in NSCs [130]. In NECs cultured in the presence of lower concentrations of GM1 (1, 5 or 10 μM) and/or Aβ1–40 (1 or 5 μM), there was no drastic change of the cell number (Figure 3(a)). However, in NECs cultured in the presence of both 40 μM of GM1 and 10 μM of Aβ1–40, a significant reduction of the cell number was detected (Figure 3(b)). These exogenously added GM1 and Aβ1–40 were efficiently incorporated into NECs (Figure 4). In NECs simultaneously treated with GM1 and Aβ1–40, the Ras-mitogen-activated protein kinase pathway important for proliferation was intact, but caspase-3, an executioner for cell death, was activated. Most NECs treated with GM1 and Aβ1–40 were positive for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, an indicator of cell death accompanied with DNA fragmentation. These results indicate that Aβ1–40 and GM1 cooperatively exert a cytotoxic effect on NECs, likely via incorporation into NEC membranes where the formation of a complex results in activation of cell death signaling.

Several reports have indicated that gangliosides added exogenously in the culture medium have bifunctional effects on neural cell proliferation. Gangliosides added exogenously at the concentration of micromolar levels were found to inhibit neuritogenesis in human neuroblastoma cells, SH-SY5Y [131]. However, under physiological conditions, GM1 enhanced nerve growth factor-induced neurite outgrowth, neurite complexity, and neuronal cell survival following nerve growth factor withdrawal using fetal-chick dorsal root ganglia [132] and induced neurite sprouting in culture neurons [133]. GQ1b induced phosphorylation of cell surface proteins in a human neuroblastoma cell line, GOTO [134]. The effects of gangliosides exogenously added remained obscure and seem to vary from one cell line to another and the culture conditions [7]. Therefore, further studies are needed to clarify the relationship between GM1 and Aβ in the proliferation of NECs. In addition, evaluation of the effects of exogenous GM1 on neurogenesis and pathogenesis of AD in pathological conditions, for instance, using AD model mice [135] will be an interesting and fruitful subject for future

studies. Many studies showed that NSCs improved neuronal survival in cultured postmortem brain tissue from aged and AD patients [136]. Further studies to understand the roles of GM1 and Aβs on NSCs in AD should contribute to the development of new regenerative therapies of this disease.

8. Conclusions

There is increasing consensus that AD is characterized in the brain by aggregated amyloid deposits in SPs. The aggregation of Aβ plays a pivotal role in the pathogenesis of AD that is intimately linked to neuronal toxicity and inhibition of hippocampal long-term potentiation. At present, there is no cure for AD, although some drugs inhibiting acetylcholinesterase have proved to be current treatment to palliate both cognitive and behavioral symptoms within a limited time. Researchers are looking for new treatments to alter the course of the disease and improve the quality of life for patients with AD and related dementia. Aβ is currently clarified to interact with gangliosides with high affinities. In fact, a complex of GM1 (and possibly other gangliosides) with Aβ, termed GAβ, was found to accumulate in the AD brains. An antibody against GAβ was proved to block amyloid fibril formation, suggesting that it can contribute to the development of a novel therapeutic strategy to AD. In this regard, drugs such as nordihydroguaiaretic acid, rifampicin, and tannic acid are found to be potent inhibitors of the binding of GM1 and Aβ, resulting in inhibition of membrane-mediated formation of Aβ fibrils in vitro. These drugs are useful agents for AD therapy [137]. On the other hand, in AD model mice lacking GD3-synthase, Aβ plaques and associated neuropathology are almost completely eliminated, resulting in cognitive improvement. GD3-synthase and its downstream metabolic products, the b-series gangliosides, can be a novel therapeutic target for repressing neurodegeneration and cognitive deficits that afflict AD patients. Another promising therapeutic strategy for AD is cell replacement therapy using NSCs. Although neurogenesis in AD brains is still controversial, transplantation of NSCs into the damaged brain regions may be beneficial for neural regeneration in AD. For therapeutic use of NSCs in AD, however, it should be essential to fully clarify the effects of Aβs and gangliosides on NSC fate regulation. Future therapies for treating AD will include agents that modulate GSL metabolism, either as primary therapeutics or in combination with other drugs.

The nomenclature for gangliosides is based on the system of Svennerholm [138].

Abbreviations

Aβ: Amyloid β-protein
AD: Alzheimer's disease
APP: Amyloid precursor protein
Chol-1α: Cholinergic-specific antigen-1α
CTXB: Cholera toxin B-subunit
Gaβ: Ganglioside-bound Aβ or a complex of GM1 and Aβ
GSL: Glycosphingolipid
NEC: Neuroepithelial cell
NSC: Neural stem cell
SVZ: Subventricular zone
NeuAc: Sialic acid or N-acetyl neuraminic acid
SP: Senile plaque
Tg: Transgenic
WT: Wild type.

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Review Article

Formation of Toxic Amyloid Fibrils by Amyloid β-Protein on Ganglioside Clusters

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It is widely accepted that the conversion of the soluble, nontoxic amyloid β-protein (Aβ) monomer to aggregated toxic Aβ rich in β-sheet structures is central to the development of Alzheimer's disease. However, the mechanism of the abnormal aggregation of Aβ in vivo is not well understood. Accumulating evidence suggests that lipid rafts (microdomains) in membranes mainly composed of sphingolipids (gangliosides and sphingomyelin) and cholesterol play a pivotal role in this process. This paper summarizes the molecular mechanisms by which Aβ aggregates on membranes containing ganglioside clusters, forming amyloid fibrils. Notably, the toxicity and physicochemical properties of the fibrils are different from those of Aβ amyloids formed in solution. Furthermore, differences between Aβ-(1–40) and Aβ-(1–42) in membrane interaction and amyloidogenesis are also emphasized.

1. Introduction

It is widely accepted that the amyloid β-protein (Aβ), which exists in fibrillar forms as a major component of senile plaques, is central to the development of Alzheimer's disease (AD) [1, 2]. The conversion of soluble, nontoxic Aβ monomer to aggregated toxic Aβ rich in β-sheet structures ignites the neurotoxic cascade(s) of Aβ [3]. The mechanism of the abnormal aggregation of Aβ is not well understood. The physiological concentration of Aβ in biological fluids ($<10^{-8}$ M) [4] is much lower than the concentration ($1\,\mu$M) above which Aβ-(1–40) spontaneously forms fibrils [5]. Therefore, there should be mechanisms that facilitate the abnormal aggregation of Aβ under pathological conditions. Although clusterin (Apo J) [6] and Zn^{2+} ions [7] were reported to facilitate the aggregation of Aβ more than a decade ago, their aggregation-promoting mechanisms are yet to be elucidated. In addition to these soluble factors, Jarrett and Lansbury, Jr. suggested that Aβ fibrillizes via a nucleation-dependent polymerization mechanism and lipids could act as heterogeneous seeds for the polymerization [8]. In 1995, Yanagisawa and colleagues discovered a specific form of Aβ bound to monosialoganglioside GM1 (GM1) in brains exhibiting early pathological changes of AD and suggested that the GM1-bound form of Aβ may serve as a seed for the formation of toxic amyloid aggregates/fibrils [9].

We have been investigating the interaction of Aβ with ganglioside-containing membranes for a dozen years and found that not the uniformly distributed but the clustered gangliosides mediate the formation of amyloid fibrils by Aβ, the toxicity and physicochemical properties of which are different from those of Aβ amyloids formed in solution. This review article summarizes Aβ-ganglioside interaction in detail, including latest findings that were not covered in our previous reviews [10, 11]. Especially, differences between Aβ-(1–40) and Aβ-(1–42) in membrane interaction and amyloidogenesis are extensively discussed. Furthermore, a link between Aβ aggregation and lipid metabolism is emphasized. It will shed light on one of the initiation processes of AD.

2. Specific Binding of Aβ to Ganglioside Clusters

Early studies indicated that Aβ-(1–40) associates with GM1 in egg yolk phosphatidylcholine (PC) vesicles only when the GM1 content exceeds 30% [12, 13]. The threshold GM1 content is lowered in a sphingomyelin (SM)/cholesterol mixture [13]. These findings suggest that GM1 molecules only in a specific state can recognize Aβ. To reveal the underlying mechanism, we systematically investigated the interaction of dye-labeled-Aβ-(1–40) [14–16] and -Aβ-(1–42) [17] with membranes of various lipid compositions. The N-termini

Table 1: Parameters for the binding of DAC-Aβs to GM1/cholesterol/SM (1 : 1 : 1) LUVs at 37 C.

Aβ	K (10^6 M^{-1})[a]	x_{max} [b](Aβ/GM1, mol/mol)	Ref.
DAC-Aβ-(1–42)	11.1 ± 2.4	0.0361 ± 0.0021	[17]
DAC-Aβ-(1–40)	8.6 ± 3.6	0.0313 ± 0.0041	[16]
DAC-Aβ-(1–28)	0.0184 ± 0.0007	0.0313[c]	[16]

[a] Binding constant.
[b] Maximal value of x (bound Aβ per exofacial GM1, mol/mol).
[c] Assumed to be the same as that of DAC-Aβ-(1–40).

of Aβs were labeled with the 7-diethylaminocoumarin-3-carbonyl group (DAC-Aβ). DAC-Aβ is useful for fluorometrically monitoring protein-lipid interactions, because a significant blue shift and an enhancement in intensity are induced by a change in polarity upon membrane binding. DAC-Aβs do not bind to major membrane lipids, including electrically neutral PC, SM, cholesterol, negatively charged phosphatidylserine, and phosphatidylglycerol under physiological conditions. On the other hand, the proteins exhibit similar high affinity (binding constant 10^7 M^{-1}) for raft-like membranes composed of GM1, cholesterol, and SM [14, 15]. DAC-Aβ-(1–28) also has a weak affinity for the membrane [16]. Binding parameters are summarized in Table 1. DAC-Aβ-(1–40) also binds to other gangliosides (GD1a, GD1b, GT1b, and asialo GM1) and lactosyl ceramide in raft-like membranes with higher affinity for lipids having larger sugar chains [15, 16]. We have proposed that Aβs specifically bind to ganglioside clusters because a GM1 cluster is formed in GM1/SM/cholesterol membranes but not in GM1/PC membranes. The clustering is facilitated by cholesterol [14].

3. Fibrillization by Aβ on Ganglioside Clusters

Aβ-(1–40) bound to ganglioside clusters assumes different conformations depending on the protein density on the membrane. Circular dichroism measurements revealed that the protein forms an α-helix-rich structure at lower protein-to-ganglioside ratios (0.025) whereas it changes its conformation to a β-sheet-rich structure at higher ratios (0.05) [14, 15]. Aβ-(1–42) also undergoes similar conformational changes [17]. Only the β-sheet form facilitates amyloidogenesis by Aβ-(1–40) [15, 18–20].

Despite very similar initial protein-ganglioside interaction, that is, the binding behavior and the α-helix-to-β-sheet conformational change, a large difference was observed in amyloidogenic activity (amount of amyloids formed under certain conditions) between Aβ-(1–40) and Aβ-(1–42) [17]. Aβs were incubated with GM1/cholesterol/SM liposomes at a Aβ-to-GM1 ratio of 5, and the aggregation of Aβ was monitored as an increase in fluorescence of the amyloid-specific dye thioflavin-T (Th-T) (Figure 1). Aβ-(1–42) formed amyloids without a lag time at 5 μM. In contrast, Aβ-(1–40) at 5 μM did not form amyloids, at least not in 12 h. At a 10-fold higher concentration, Aβ-(1–40) started to aggregate after a lag time of 2 h. The effectiveness of Aβ-(1–42) in fibrillogenesis is at least partly due to the fragility

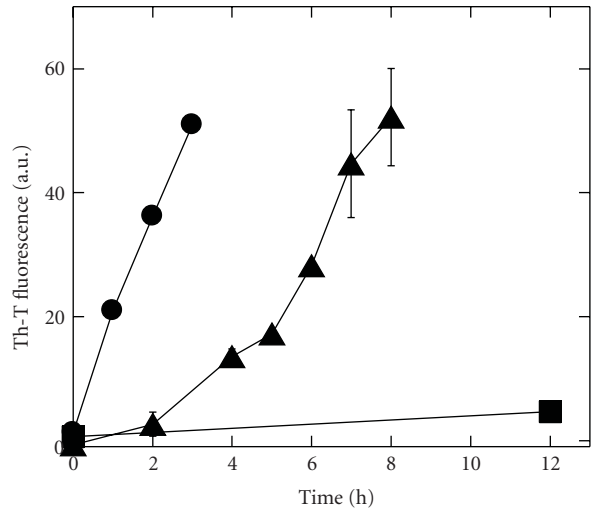

Figure 1: Aβ aggregation in the presence of raft-like liposomes. Aβs (5 μM or 50 μM) were incubated with GM1/cholesterol/SM (1 : 1 : 1) small unilamellar vesicles at a GM1-to-Aβ ratio of 5 at 37 C without agitation, and the aggregation was monitored by the Th-T assay. Symbols: circles, 5 μM Aβ-(1–42); squares, 5 μM Aβ-(1–40); triangles, 50 μM Aβ-(1–40). Data taken from [17].

of fibrils, because the fragmentation greatly facilitates fibril growth [21] (see also Section 5). Other factors, such as the rapid formation of seeds and/or elongation, may also contribute to the difference.

Cell experiments also support the above mechanism of Aβ-ganglioside interaction [22, 23]. Aβ-(1–42) was incubated with neuronal rat pheochromocytoma PC12 cells. Amyloids and gangliosides were detected by the amyloid-specific dye Congo red and the fluorescent-labeled cholera toxin B subunit, respectively. Amyloids were selectively formed on ganglioside-rich domains (Figure 2(a)). Depletion of cholesterol, either by methyl-β-cyclodextrin or the cholesterol synthesis inhibitor compactin, suppressed the accumulation of Aβ. The amyloidogenic activity of Aβ-(1–42) was again more than 10-fold that of Aβ-(1–40) on human SH-SY5Y neuroblastoma cells expressing gangliosides (Figure 2(b)). When cells were incubated with 5 μM Aβ-(1–42), Congo red-positive spots appeared later at 24 h and became prominent with time. In contrast, when cells were incubated with 5 μM Aβ-(1–40), no fibrils were detected even after 72 h. Incubation with a 10-fold higher concentration of the protein, however, resulted in the appearance of Congo red-positive spots at 48 h.

4. Properties of Aβ Fibrils Formed on Ganglioside Clusters

The Aβ fibrils formed on ganglioside clusters (Mem-fibrils) are not identical to those formed in solution (Sol-fibrils) in terms of physicochemical properties and cytotoxicity [20]. Transmission electron micrographs indicate that Mem-fibrils are typical nonbranched fibrils (12.0 ± 0.7 nm, width) whereas Sol-fibrils are thinner fibrils or protofilaments

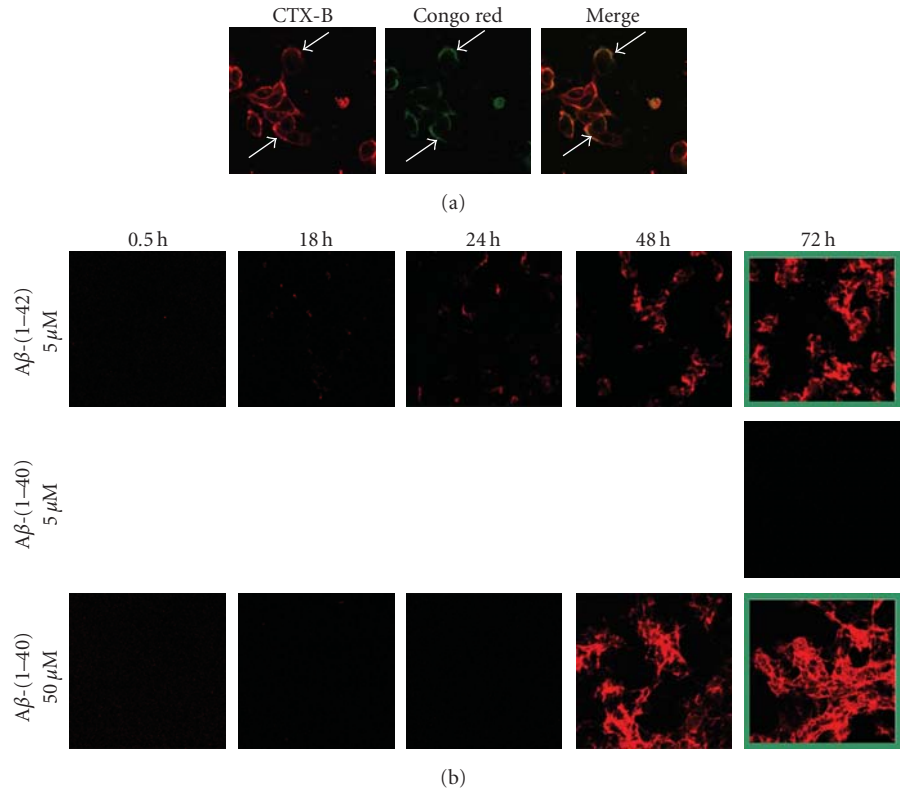

Figure 2: Aβ aggregation on living neuronal cells. (a) Aβ-(1–42) (10 μM) was incubated with PC12 cells for 24 h at 37 C. The distribution of GM1 was detected by using the cholera toxin B subunit conjugated with Alexa Fluor 647 dye (CTX-B, left). Amyloids were visualized by the amyloid-specific dye Congo red (middle). The merging of the two images shows that amyloids were formed in the vicinity of GM1-rich domains of cell membranes (right). Data taken from [10]. (B) Ganglioside-expressing SH-SY5Y cells were incubated with 5 μM Aβ-(1–42) (top), 5 μM Aβ-(1–40) (middle), or 50 μM Aβ-(1–40) (bottom) for 0.5, 18, 24, 48, or 72 h, and the formation of amyloids was detected with Congo red. The conditions under which cell death was observed are framed in green. Data taken from [17].

(6.7 ± 1.3 nm, width), and the protofilaments associate laterally and twist into rope-like fibrils (14.5 ± 0.9 nm, width). The surface hydrophobicity of Mem-fibrils as estimated by the binding of 1-anilinonaphthalene-8-sulfonate (ANS) is larger than that of Sol-fibrils (Figure 3(a)), therefore Mem-fibrils exhibit significantly stronger binding to cell membranes than Sol-fibrils (Figure 3(b)). Consequently, Mem-fibrils are cytotoxic whereas Sol-fibrils are much less toxic (Figure 3(c)). Recently, a correlation between ANS-binding and cytotoxicity was reported for various amyloid species [24].

The structure of Mem-fibrils is suggested to be different from that of Sol-fibrils, in which the cross-β unit is a double-layered structure, with in-resister parallel β-sheets formed by residues 12–24 and 30–40 [25]. The amide I spectrum of the former shows, in addition to a major peak around 1630 cm^{-1} characteristic of a β-sheet, a weak peak at 1695 cm^{-1} whereas that of the latter shows a peak around 1660 cm^{-1} [26].

5. Mechanism of Cytotoxicity by Aβ Fibrils Formed on Ganglioside Clusters

The mechanisms of Aβ-induced cytotoxicity have been controversial. Aβ fibrils were reported to trigger functional disorder in neuronal cells and cell death [27–31] whereas soluble Aβ oligomers have been proposed to play a pivotal role in the onset of AD [6, 28, 32–39]. To obtain an insight into the cytotoxic mechanism of Aβ, we established a multistaining visualization method using unlabeled Aβs and antibodies [17] in contrast to conventional methods using fluorophore-labeled proteins [23, 40]. The accumulation of Aβ, the formation of amyloid fibrils, the formation of oligomers, and cell viability were visualized using the Aβ monoclonal antibody 6E10, the amyloid-staining dye Congo red [22], the antioligomer antibody A11 [34], and calcein acetoxymethyl, respectively. Cell death was detected after the significant accumulation of fibrils (Figure 2(b)) and no A11-positive spot was detected, suggesting that fibril-induced physicochemical stress, such as the induction of a negative curvature [13] or membrane deformation upon fibril growth [41], leads to cytotoxicity. A11-positive oligomers were not formed in the fibrillization with GM1-containing liposomes either [20]. It should be noted, however, that at certain GM1 contents GM1-liposomes generate toxic soluble Aβ-(1–40) oligomers [42]. For both Aβs, similar levels of fibrils were required for cytotoxicity (Figure 2(b)), indicating that the fibrils possess comparable intrinsic toxicity. The fibrillization process and cytotoxicity can be effectively

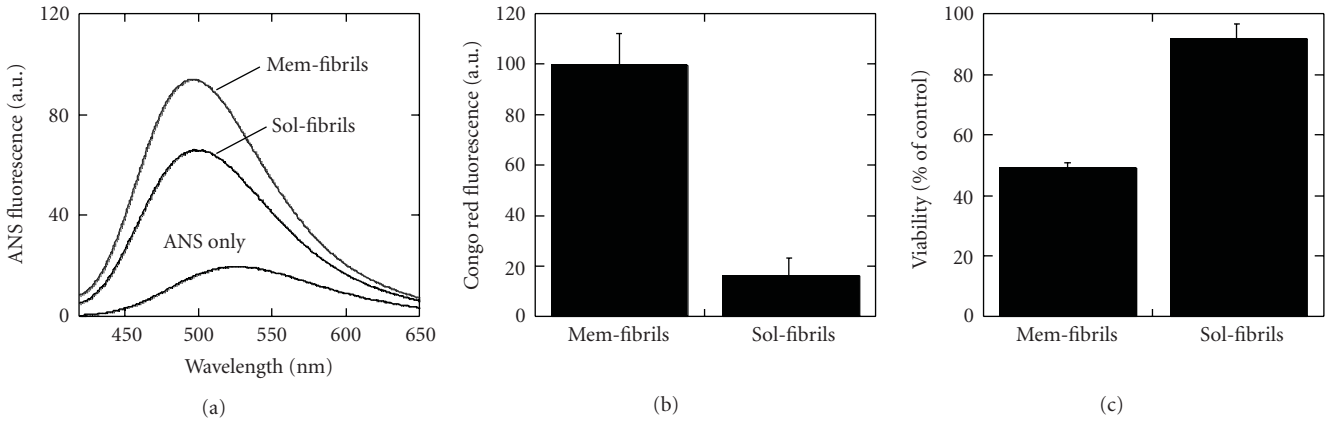

Figure 3: Comparison between Mem-fibrils and Sol-fibrils. Data taken from [20]. (a) Fluorescence spectra of ANS (5.0 μM) in PBS were measured in the absence or presence of Mem-fibrils and Sol-fibrils of Aβ-(1–40) (2.5 μM) with an excitation wavelength of 350 nm. The binding of the dye to a hydrophobic surface results in an enhancement in fluorescence intensity. (b) Aβ-(1–40) fibrils (25 μM) were incubated with NGF-differentiated PC12 cells for 30 min. Binding of Aβ-(1–40) fibrils to cells was evaluated by fluorescence intensity of Congo red per cell (mean ± S.E.; n 100, $P < .001$). (c) Aβ-(1–40) fibrils (25 μM) were incubated with NGF-differentiated PC12 cells for 24 h. Aβ cytotoxicity was estimated with fluorescence intensity of the live cell marker calcein (mean ± S.E.; $n = 6$; $P < .001$ against vehicle treatment).

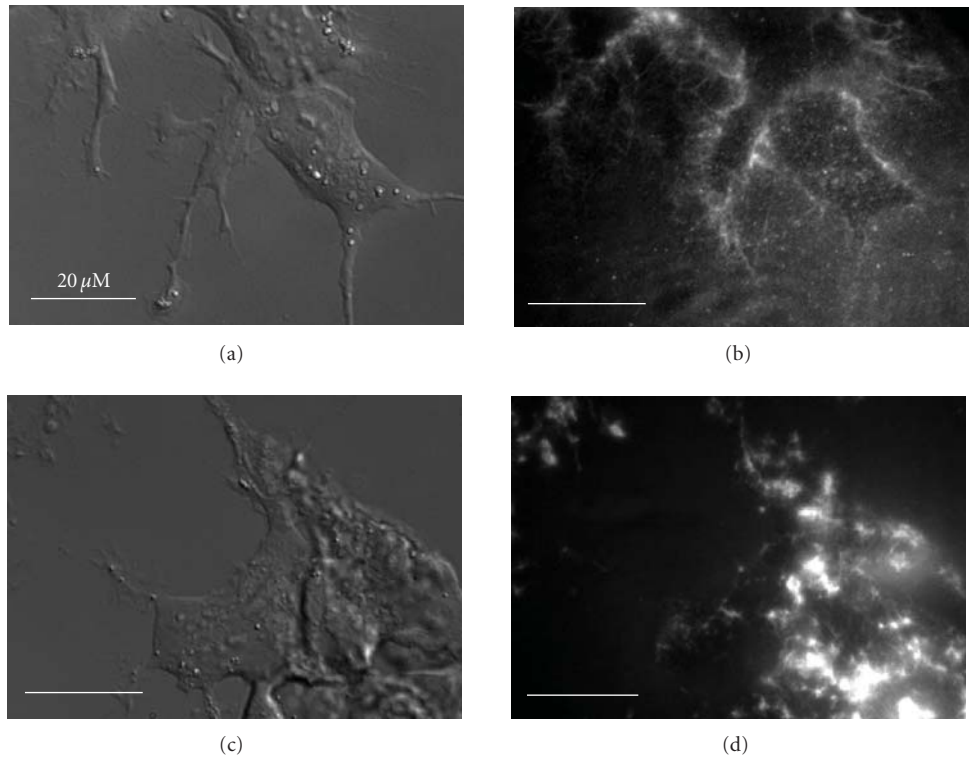

Figure 4: Visualization of amyloid fibrils formed on cell membranes using TIRFM. SH-SY5Y cells were treated with 50 μM Aβ-(1–40) ((a), (b)) or 5 μM Aβ-(1–42) ((c), (d)) for 48 h. Amyloid fibrils were stained with 20 μM Congo red. (a) and (c) are DIC images, while (b) and (d) are TIRF images. Data taken from [17].

blocked by small compounds, such as nordihydroguaiaretic acid and rifampicin [19].

The morphology of amyloid fibrils formed on cell membranes was visualized by total internal reflection fluorescence microscopy (TIRFM) [17]. TIRFM effectively reduces the background fluorescence and therefore is suitable for observing the cell surface. Fibrils were stained with Congo red. Relatively long fibrillar structures were detected around the cell membrane for Aβ-(1–40) whereas relatively short fibrils were coassembled in the case of Aβ-(1–42) (Figure 4). The

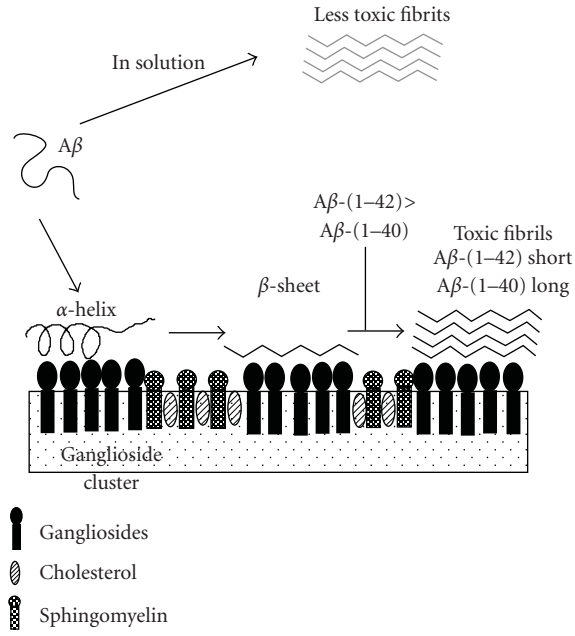

Figure 5: A model for the formation of toxic amyloid fibrils by amyloid β-protein on ganglioside clusters. Aβ is essentially soluble, and takes an unordered structure in solution. Once ganglioside clusters are generated, Aβ binds to the clusters, forming an α-helix-rich structure at lower protein-to-ganglioside ratios whereas the protein changes its conformation to a β-sheet at higher ratios. The β-sheet form facilitates the fibrillization of Aβ, leading to cytotoxicity. The amyloidogenic activity of Aβ-(1–42) is more than 10-fold that of Aβ-(1–40). Amyloid fibrils formed in solution are much less toxic.

latter observation suggests that Aβ-(1–42) fibrils are more readily fragmented. The fragmentation greatly facilitates fibril growth because fibrils grow only at their ends [21].

6. Concluding Remarks

Based on the above observations, we propose a novel model for Aβ-membrane interaction as a mechanism for the abnormal aggregation of the protein (Figure 5). Aβ specifically binds to a ganglioside cluster, the formation of which is facilitated by cholesterol. The cluster can be formed also by late endocytic dysfunction [43]. The Aβ undergoes a conformational transition from an α-helix-rich structure to a β-sheet-rich one with increasing protein density on the membrane. The β-sheet form serves as a seed for the formation of amyloid fibrils, which are more toxic than and have different structures from those formed in solution. Depending on ganglioside contents in the membrane, toxic soluble oligomers may also be generated. The amyloidogenic activity of Aβ-(1–42) is more than 10-fold that of Aβ-(1–40), although the initial interaction with gangliosides is similar between the two proteins.

This model can explain roles of various risk factors in the pathogenesis of AD, especially from a viewpoint of lipid metabolism. Both the aging and the apolipoprotein E4 allele are strong risk factors for developing AD [44]. The amount of cholesterol in the exofacial leaflets of the synaptic plasma membrane increases in aged [45] as well as apolipoprotein E4-knock-in [46] mice. GM1 clustering occurs at presynaptic neuritic terminals in mouse brains in an age-dependent manner [47]. Diet-induced hypercholesterolemia accelerates the amyloid pathology in a transgenic mouse model [48]. A link between cholesterol, Aβ, and AD has been reported [49, 50]. Human AD brains also show abnormality in lipid metabolism in accordance with our model [51, 52]. That is, significant increase in GM1 was reported in Aβ-positive nerve terminals from the AD cortex [51], and lipid rafts from the frontal cortex and the temporal cortex of AD brains were also found to contain a higher concentration of GM1 compared to an age-matched control [52]. It should be noted that, in addition to these modulations of Aβ aggregation by lipids, Aβ also in turn regulates lipid metabolism [53].

The 10-fold higher amyloidogenic activity of Aβ-(1–42) is in accordance with the facts (1) that genetic mutations in the presenilins causing early-onset AD increase the level of Aβ-(1–42) [54] and (2) that the protein is the major species in diffuse plaques, the earliest stage in the deposition of Aβ [55].

In conclusion, in addition to other biochemical cascades, a complex purely physicochemical cascade linked to lipid metabolism (Figure 5) appears to be also involved in the process of Aβ aggregation. Inhibition of one of these steps would be a promising strategy for AD therapy.

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Research Article

Spectroscopic Characterization of Intermolecular Interaction of Amyloid β Promoted on GM1 Micelles

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Clusters of GM1 gangliosides act as platforms for conformational transition of monomeric, unstructured amyloid β (Aβ) to its toxic β-structured aggregates. We have previously shown that Aβ(1–40) accommodated on the hydrophobic/hydrophilic interface of lyso-GM1 or GM1 micelles assumes α-helical structures under ganglioside-excess conditions. For better understanding of the mechanisms underlying the α-to-β conformational transition of Aβ on GM1 clusters, we performed spectroscopic characterization of Aβ(1–40) titrated with GM1. It was revealed that the thioflavin T- (ThT-) reactive β-structure is more populated in Aβ(1–40) under conditions where the Aβ(1–40) density on GM1 micelles is high. Under this circumstance, the C-terminal hydrophobic anchor Val^{39}-Val^{40} shows two distinct conformational states that are reactive with ThT, while such Aβ species were not generated by smaller lyso-GM1 micelles. These findings suggest that GM1 clusters promote specific Aβ-Aβ interactions through their C-termini coupled with formation of the ThT-reactive β-structure depending on sizes and curvatures of the clusters.

1. Introduction

Conformational transitions of unstructured proteins into β-structure-based oligomeric or amyloid states are crucial processes in the onset and development of a variety of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease [1, 2]. Amyloid β(Aβ), a major player in AD, is a 40- or 42-amino acid peptide cleaved from its precursor membrane protein by sequential actions of β- and γ-secretases and has a high propensity for toxic aggregation to form cross-β-fibrils [3, 4]. Accumulated evidence indicates that the GM1 ganglioside, a glycosphingolipid abundant in neuronal cell membranes, interacts with Aβ and promotes its assembly, resulting in pathogenic amyloid formation [5–7]. For example, high-density GM1 clustering, which is exclusively observed in synaptosomes, is suggested to accelerate Aβ deposition [8]. *In vitro* experiments have indicated that the Aβ-GM1 interaction depends on the clustering of GM1, and its carbohydrate moiety alone cannot induce conformational changes of Aβ [15, 30, 31].

Furthermore, it has been suggested that each of the heredity variants of Aβ reported thus far has its own specificities for gangliosides, which have been supposed to be associated with their ectopic deposition [9, 10]. Promotion of amyloid formation in membrane-bound states has also been reported for prion and α-synuclein [11, 12]. For example, prion protein has been reported to be localized in the membrane microdomains and caveolae enriched with ganglioside, which interacts with prion protein and thereby promotes its α-to-β structural conversion [13, 14]. Therefore, detailed conformational characterization of Aβ interacting with the ganglioside clusters not only provides

structural information as cues for drug development in preventing and treating AD but also offers general insights into the mechanisms underlying the disease-associated amyloid formation facilitated in membrane environments.

In previous papers, we have reported nuclear magnetic resonance (NMR) studies of the interactions of Aβ (1–40) with ganglioside clusters using lyso-GM1 micelles (approximate molecular mass 60 kDa) as model systems [15, 16]. Our NMR data showed that Aβ(1–40) is accommodated on the hydrophobic/hydrophilic interface of the ganglioside cluster exhibiting an α-helical conformation under ganglioside-excess conditions. In this state, Aβ(1–40) shows an up-and-down topological mode in which the two α-helices at segments His^{14}-Val^{24} and Ile^{31}-Val^{36} and the C-terminal Val^{39}-Val^{40} dipeptide segment are in contact with the hydrophobic interior of the micelles, whereas the remaining regions are exposed to the aqueous environment. A similar tendency of Aβ(1–40) has been observed using excess amounts of GM1, which forms micelles with an approximate molecular mass of 140 kDa [15, 17]. These findings indicate that ganglioside clusters offer unique platforms at their hydrophobic/hydrophilic interfaces for binding coupled with α-helix formation of Aβ molecules.

To gain further insights into the underlying mechanisms of the amyloid formation of Aβ, it is necessary to characterize the conformational transition from α-helices to β-structures on the ganglioside clusters. On the basis of the circular dichroism (CD) data, Kakio et al. demonstrated that Aβ/GM1 ratios influence the secondary structure of Aβ(1–40) on the raft-like lipid bilayers composed of GM1, cholesterol, and sphingomyelin [18, 19]. Namely, Aβ adopts an α-helical structure at lower Aβ/GM1 ratios (0.025), while it assumes a β-sheet-rich structure at higher ratios (0.05). Although more detailed structural information on Aβ bound to the GM1 cluster is highly desirable, the small unilamellar vesicles used for the CD measurements are still too large to investigate with solution NMR techniques.

In the present study, we attempt to characterize conformational states of Aβ(1–40) in the presence of varying amounts of GM1 aqueous micelles using stable-isotope-assisted NMR spectroscopy in conjunction with synchrotron-radiation vacuum-ultraviolet CD (VUVCD) spectroscopy. We found that GM1 micelles also induce distinct secondary structures of Aβ(1–40) depending on the Aβ/GM1 ratios. On the basis of the spectroscopic data, we will discuss Aβ behaviours on the ganglioside clusters from a structural point of view.

2. Materials and Methods

2.1. Preparation of Aβ(1–40). Recombinant Aβ(1–40) was expressed and purified as a ubiquitin extension. The plasmid vector encoding Aβ(1–40) was constructed and cloned as a fusion protein with hexahistidine-tagged ubiquitin (His_6-Ub) using the pET28a(+) vector (Novagene), subsequently transformed into *Escherichia coli* strain BL21-CodonPlus (Stratagene) [15]. Transformed bacteria were grown at 37 C in LB media containing 15 μg/mL of kanamycin. For the production of isotopically labelled Aβ(1–40) protein, cells were grown in M9 minimal media containing [^{15}N] NH_4Cl (1 g/L) and/or [U-$^{13}C_6$] glucose (2 g/L). Protein expression was induced by adding 0.5 mM isopropyl-β- $_D$ -thiogalactopyranoside (IPTG) when the absorbance reached 0.8 at 600 nm. After 4 hours, cells were harvested and then suspended into buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, subsequently disrupted by sonication. After centrifugation, the pellet was dissolved in buffer A containing 8 M urea. His_6-Ub-Aβ(1–40) was purified by a Ni^{2+}-nitrilotriacetic acid affinity column (GE Healthcare). Recombinant glutathione *S*-transferase- (GST-) tagged yeast ubiquitin hydrolase-1 (YUH-1) was grown until the absorbance reached 0.8 at 600 nm and then induced to express by IPTG. Cell pellets were dissolved in buffer B (50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.5) and disrupted by sonication. GST-YUH-1 was purified by a glutathione affinity column (GE Healthcare). Aβ(1–40) protein was enzymatically cleaved from His_6-Ub by incubation with GST-YUH-1 for 1 h at 37 C at a molar ratio of His_6-Ub-Aβ(1–40): GST-YUH1 = 10 : 1. The cleaved Aβ(1–40) was purified by reverse-phase chromatography using an octadecylsilane column (TSKgel ODS-80T_M, TOSOH) with a linear gradient of acetonitrile. The fraction containing Aβ(1–40) was collected and lyophilized.

Synthetic Aβ(1–40) labelled with ^{15}N selectively at Val^{39} or Val^{40} was purchased from AnyGen Co. Both of recombinant and synthetic Aβ(1–40) proteins were dissolved at an approximate concentration of 2 mM in 0.1% (v/v) ammonia solution then collected and stored in aliquots at – 80 C until use.

2.2. Preparation of Micelles. Powdered lyso-GM1 and GM1 were purchased from Takara Bio Inc. and Sigma-Aldrich, respectively. These gangliosides were dissolved in methanol. Subsequently, the solvent was removed by evaporation. The residual ganglioside was suspended at a concentration of 12 mM in 10 mM potassium phosphate buffer (pH 7.2) and then mixed by vortexing. Micelle sizes were determined by dynamic light scattering using a DynaPro Titan (Wyatt technology).

2.3. Thioflavin T (ThT) Assay. Aβ(1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer (pH 7.2) in the absence or presence of 0.4–9 mM GM1 or lyso-GM1. The samples were kept on ice before measurements. 980 μL of 5 μM ThT (Sigma) solution in 50 mM glycine-NaOH buffer (pH 8.5) was added to an aliquot of 20 μL of each sample. Fluorescence was measured immediately after mixing at the excitation and emission wavelengths of 446 and 490 nm, respectively, [20] using spectrofluorophotometer (Hitachi F-4500) at 37 C.

2.4. VUVCD Measurements. Aβ(1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer (pH 7.2). The CD spectra of Aβ(1–40) in the presence or absence of GM1 were measured from 265 to 175 nm under a high vacuum (10^{-4} Pa) at 37 C using the VUVCD spectrophotometer constructed at beamline

15 (0.7 GeV) of the Hiroshima Synchrotron Radiation Center (HiSOR). Details of the spectrophotometer and optical cell were described previously [21, 22]. The path length of the CaF_2 cell was adjusted with a Teflon spacer to 50 μm or 100 μm for measurements. The VUVCD spectra were recorded with a 1.0-mm slit, a 16-s time constant, a 4-nm min^{-1} scan speed, and nine accumulations. The molar ellipticities of Aβ(1–40) were calculated with the average residue weight of 107.5. The secondary structure contents of Aβ(1–40) were analysed using the modified SELCON3 program [23] and the VUVCD spectra down to 160 nm for 31 reference proteins with known X-ray structures [24, 25]. The secondary structures of these proteins in crystal form were assigned into four classes (α-helices, β-strandes, turns, and unordered structures) using the DSSP program [26] based on the hydrogen bonds between adjacent amide groups. In this analysis, the 3_{10}-helix was classified as an unordered structure. The root-mean-square deviation (δ) and the Pearson correlation coefficient (r) between the X-ray and VUVCD estimates of the secondary structure contents of the reference proteins were 0.058 and 0.85, respectively, confirming the high accuracy of the VUVCD estimation [27].

2.5. NMR Measurements. NMR spectral measurements were made on a Bruker DMX-500 spectrometer equipped with a cryogenic probe as well as a Bruker AVANCE III-400 spectrometer. The probe temperature was set to 37 C. Isotopically labelled Aβ(1–40) was dissolved at a concentration of 0.2 mM in 10 mM potassium phosphate buffer (pH 7.2) containing 10% (v/v) ^2H_2O in the presence or absence of GM1. For ^1H-^{15}N heteronuclear single-quantum correlation (HSQC) measurements, the spectra were recorded using Aβ(1–40) labelled with ^{15}N uniformly or selectively at the amide group of Val^{39} or Val^{40} at a ^1H observation frequency of 500 MHz with 128 (t_1) × 1024 (t_2) complex points and 256 scans per t_1 increment. The spectral width was 1720 Hz for the ^{15}N dimension and 6000 Hz for the ^1H dimension.

One-dimensional carbonyl ^{13}C spectra were recorded using uniformly ^{13}C- and ^{15}N-labelled Aβ(1–40) at a ^1H observation frequency of 400 MHz with a spectral width of 22,000 Hz. In these experiments, 32,768 data points for acquisition and 16,384 scans were acquired. NMR spectra were processed and analysed with the program nmrPipe/Sparky.

3. Results

3.1. ThT Fluorescence Enhancement. We examined whether ThT fluorescence is enhanced by Aβ(1–40) in the presence of varying concentrations of GM1 or lyso-GM1. As shown in Figure 1, GM1 exhibited a bell-shaped dependence on Aβ/GM1 ratios regarding ThT fluorescence enhancement, while lyso-GM1 showed virtually no enhancement. Maximum enhancement was observed at a 1:15 molar ratio of Aβ(1–40) to GM1. The dynamic light scattering data confirmed that the GM1 and lyso-GM1 micelles exhibited an approximate hydrodynamic radius of 6 nm and 4 nm, respectively, irrespective of the Aβ/ganglioside ratios. The

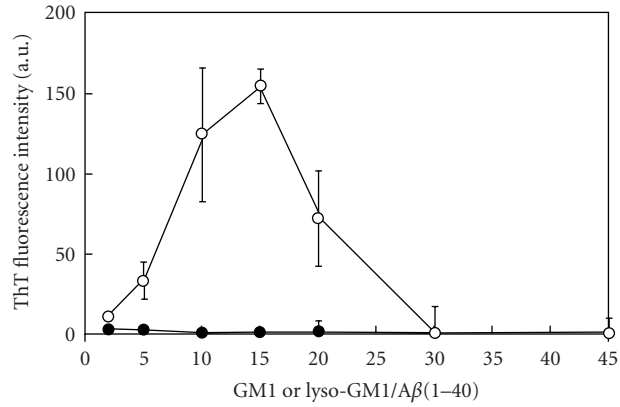

Figure 1: ThT fluorescence enhancement by Aβ(1–40) in the presence of varying concentrations of GM1 (open circle) or lyso-GM1 (closed circle). Each intensity value indicates the average of four values ± S.D.

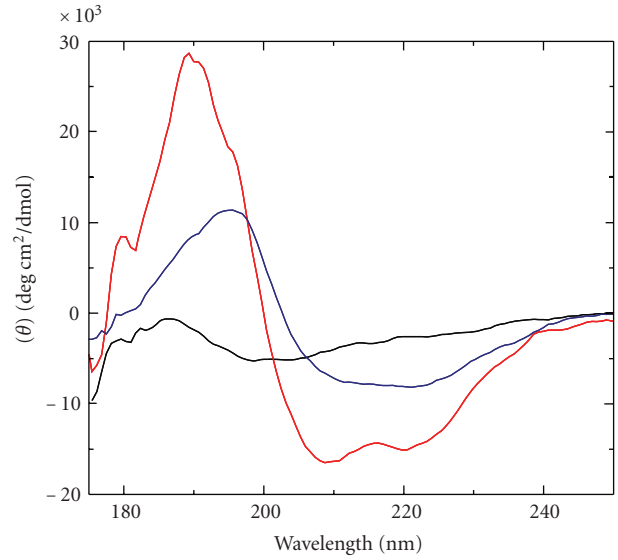

Figure 2: VUVCD spectra of 0.2 mM Aβ(1–40) in the absence or presence of GM1. Aβ/GM1 molar ratios were 1 : 0 (black), 1 : 15 (blue), and 1 : 30 (red).

observed fluorescence intensity remained almost constant up to 12 h. These data indicated that GM1 micelles at appropriate Aβ/GM1 ratios promote some Aβ–Aβ interaction with formation of their β-sheet-like conformation, which, however, does not result in irreversible fibril formation.

3.2. Secondary Structure Transition. We characterized the conformational transition of Aβ depending on Aβ/GM1 ratios by CD measurements. The short-wavelength limit of CD spectroscopy can be successfully extended using synchrotron radiation as a high-flux source of photons, which yields much more accurate data than those obtained with a conventional CD spectrophotometer [28, 29]. The spectral data indicated that Aβ(1–40) undergoes conformational transitions depending on GM1 to Aβ(1–40) ratios (Figure 2).

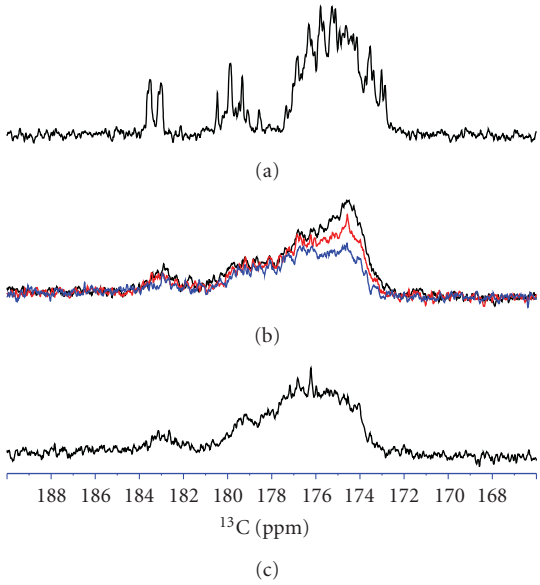

Figure 3: Carbonyl ^{13}C spectra of uniformly ^{13}C-labelled Aβ(1–40). Spectral data were obtained using 0.2 mM Aβ(1–40) titrated with GM1 micelles at Aβ/GM1 molar ratios of (a) 1 : 0, (b) 1 : 15, and (c) 1 : 30. In (b), the spectra measured in the presence of ThT are displayed at Aβ/ThT molar ratios of 1 : 0 (black), 1 : 1 (red), and 1 : 2 (blue).

Table 1: Secondary structure contents (%) of Aβ(1–40) from VUVCD spectra obtained in the presence of varying concentrations of GM1.

Aβ : GM1	α-Helix	β-Strand	Turn	Unordered structure
1 : 0	15.9	17.8	26.3	39.0
1 : 15	23.6	23.6	21.6	29.3
1 : 30	40.0	18.3	14.5	27.9

The secondary structure contents of Aβ(1–40) at Aβ/GM1 molar ratios of 1 : 0, 1 : 15, and 1 : 30 were estimated on the basis of the spectral data (Table 1). The α-helix content of Aβ(1–40) in the presence of GM1 at an Aβ/GM1 molar ratio of 1:30 was calculated to be 40.0%, which is consistent with our previous estimation based on the backbone chemical shift data of lyso-GM1 [15], thus confirming close similarity of the binding modes of Aβ(1–40) between GM1 and lyso-GM1micelles. At an Aβ/GM1 molar ratio of 1:15, where the maximum ThT fluorescence enhancement was observed, the CD data consistently indicated a significantly increased content of β-strands.

The conformation of Aβ(1–40) in the presence of varying amounts of GM1 micelles was further characterized by ^{13}C NMR spectroscopy. The carbonyl ^{13}C NMR spectral data of uniformly ^{13}C-labelled Aβ(1–40) indicated that the peaks shifted upfield, roughly corresponding to β-structures, are more populated at an Aβ/GM1 molar ration of 1 : 15 in comparison with the GM1-excess conditions (Figure 3). Intriguingly, intensities of these peaks were selectively reduced upon the addition of ThT. These NMR data are again consistent with the VUVCD data as well as the results of the ThT assay.

3.3. Local Structure of the C-Terminus of Aβ(1–40). To provide more detailed information on the conformational transition of Aβ(1–40) on GM1 micelles, we observed ^{1}H-^{15}N HSQC spectral changes of Aβ(1–40) upon titration with GM1. Interestingly, at an Aβ/GM1 molar ratio of 1 : 15, Aβ(1–40) exhibited HSQC peaks that were not observed in the spectra of free or fully micelle-bound forms (Supplementary Figure 1). By using site-specifically ^{15}N-labelled Aβ, these extra peaks were assigned to Val^{39} and Val^{40} (Figure 4 and Supplementary Figure 1 available online at doi:10.4061/2011/925073). Namely, the amide groups of these C-terminal residues of the micelle-bound Aβ species show double HSQC peaks under the condition where Aβ/GM1 ratio is relatively high. More interestingly, these double peaks were perturbed upon the addition of ThT, while the corresponding peaks originating from the free and fully micelle-bound forms showed little or no change (Figure 4). On the other hand, many of the ^{1}H-^{15}N HSQC peaks from Aβ(1–40), including Val^{39} and Val^{40}, were not observed at an Aβ/lyso-GM1 molar ratio of 1 : 15 due to intermediate chemical exchange between free and micelle-bound states of Aβ(1–40) (data not shown).

4. Discussion

Accumulating evidence, including our previous reports, indicates that the interaction of Aβ with GM1 involves multiple steps including the initial encounter complex formation and the accommodating process on the hydrophilic/hydrophobic interface of the ganglioside clusters [15–17, 30]. NMR spectral data of Aβ(1–40) titrated with GM1 micelles under Aβ-excess conditions indicated that they form a weak complex presumably through an interaction between the N-terminal segment of Aβ(1–40) and the outer carbohydrate branch of GM1 [15, 30]. Thus, it is conceivable that the outer-branch structures of the carbohydrate moieties of gangliosides influence the association phase of the interaction and thereby determine the ganglioside specificities of Aβ. Nongangliosidic micelles and vesicles are barely or not capable of trapping Aβ(1–40) effectively [15, 18, 31, 32]. On the other hand, the α-helical conformation of Aβ(1–40) accommodated on sugar-lipid interface of the GM1 and lyso-GM1 micelles have been characterized by NMR under ganglioside-excess conditions (Aβ/ganglioside molar ratio of 1 : 30) [15]. Because the structure of the inner part is common among the gangliosides, non-GM1 ganglioside, for example, GM2, can accommodate Aβ and induce its α-helical conformation [16]. Thus, the spectroscopic characterization of the interactions of Aβ with gangliosidic micelles has so far been performed only under the extreme conditions of the Aβ/ganglioside ratios. The present study attempts to bridge the gap in our understanding of Aβ behavior on GM1 micelles by carrying out spectroscopic analyses of Aβ in the presence of varying amounts of GM1 micelles.

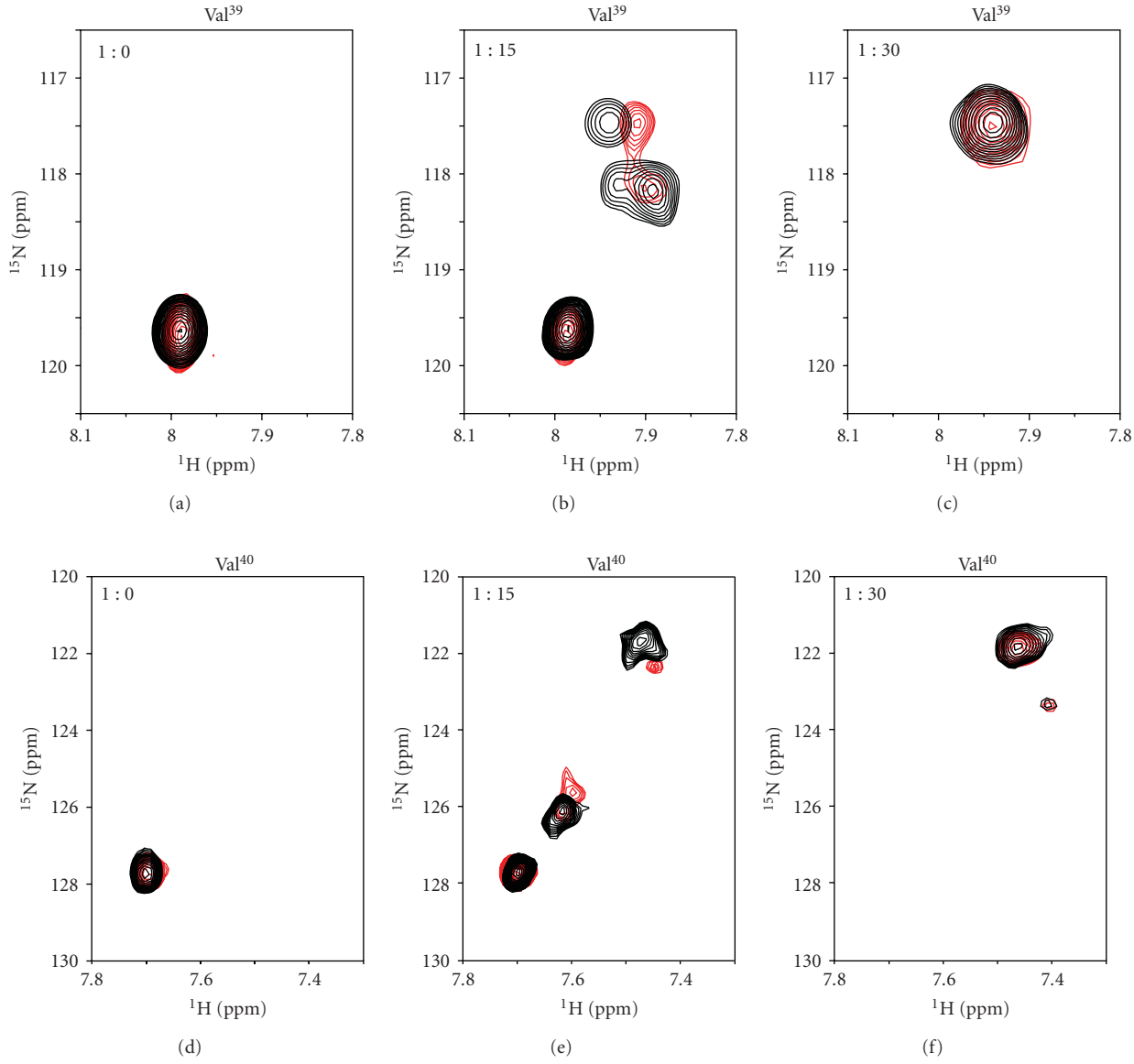

FIGURE 4: ^{1}H-^{15}N HSQC peak originating from Val^{39} (upper) and Val^{40} (lower) of Aβ(1–40) in the presence or absence of GM1 micelles and ThT. Site specifically ^{15}N-labelled Aβ(1–40) proteins (0.2 mM each) were titrated with GM1 at Aβ/GM1 molar ratios of 1 : 0 (a, d), 1 : 15 (b, e), and 1 : 30 (c, f). The spectra measured in the absence (black) and presence (red) of 0.4 mM ThT are overlaid. The peak indicated by asterisk originated from GM1.

The present data all indicated that β-structure is more populated in micelle-bound Aβ(1–40) under the condition where the Aβ/GM1 ratio is higher. It is intriguing that the increased β-structure is reactive with ThT. Although the binding mode of ThT to amyloid fibrils has yet to be fully elucidated, it has been suggested that ThT is more likely to bind perpendicularly to parallel β-strands in a β-sheet [33–35]. In addition, recently reported solid-state NMR data indicate that a ThT-reactive, neurotoxic amyloid intermediate of Aβ(1–40) is composed of parallel β-structures [36]. These data suggest that formation of parallel β-strands is the minimum prerequisite for ThT fluorescence enhancement. With this in mind, the bell-shape dependence of ThT fluorescence enhancement (Figure 1) can be interpreted as follows. At an extremely low concentration of GM1, most of Aβ(1–40) exists as a free form, which is an unstructured monomer and therefore is not reactive with ThT. Fraction of the micelle-bound form of Aβ(1–40) increases with increase of the GM1 amounts. To some extent, the micelles promote intermolecular interaction of Aβ(1–40), giving rise to the ThT-reactive Aβ(1–40) species. Under GM1-excess conditions, however, Aβ(1–40) molecules are presumably relatively isolated from one another and therefore are not capable of forming an intermolecular β-structure. The Aβ/GM1 molar ratio, where the maximum enhancement was observed, was 1 : 15, which corresponds to average number of Aβ/micelle of 11.2 with the assumption of the micellar GM1 aggregation number of 168 ± 4 [37]. Thus, the Aβ density on

GM1 micelles is a crucial factor determining the occurrence of the ThT-reactive Aβ species.

Under the circumstance where the Aβ(1-40) density on GM1 micelles is high, the C-terminal dipeptide of Aβ(1–40) shows, at least, two distinct conformational states that are reactive with ThT. In a previous paper, we demonstrated that the C-terminal Val^{39}-Val^{40} dipeptide is inserted into the hydrophobic interior of the gangliosidic micelles [15]. This C-terminal segment is involved in the parallel β-structure in the amyloid fibril and intermediate [36, 38]. On the basis of these data, we suggest that GM1 clusters promote intermolecular Aβ-Aβ interactions coupled with the conformational transition of their C-terminal hydrophobic anchors into the ThT-reactive parallel β-structure, in which the local chemical environments of the C-terminal segments are different in different β-strands. This may account for the multiple HSQC peaks originating from the C-terminal segments (Figure 4).

It has been reported that Aβ exhibits ThT-reactive β-sheet-rich aggregates in the presence of sodium dodecyl sulfate (SDS) at submicellar concentrations [39, 40]. Under these conditions, all the amide peaks of Aβ(1–40) disappeared from the ^{1}H-^{15}N HSQC spectrum because of the formation of large aggregates, except for those from the C-terminal residues that should still be mobile in this assembly state. On the basis of the NMR data obtained using paramagnetic probes, the C-terminal segment of Aβ(1–40) bound to SDS micelles has shown to be exposed to aqueous environment, exhibiting higher mobility [41]. Taking into account these data in conjunction with our present data, we suggest that different β-like structures of Aβ(1–40) are induced by GM1 aqueous micelles and submicellar concentrations of SDS.

Lyso-GM1 micelles could not induce the formation of the ThT-reactive β-structure of Aβ(1–40) although the micelle-interacting modes of Aβ(1–40) are almost identical between GM1 and lyso-GM1 micelles under ganglioside-excess conditions [15]. By inspection of the dynamic light scattering data on an assumption of their globular shapes, the diameters of GM1 and lyso-GM1 micelles have been estimated as 12 nm and 8 nm, respectively. It is plausible that the sizes and curvatures of the gangliosidic micelles are determining factors for the number of Aβ molecules that can be accommodated on their hydrophilic/hydrophobic interface and the occurrence of Aβ-Aβ interactions coupled with ThT-reactive β-structure formation. Indeed, GM1 clusters with flatter curvature such as GM1-containing unilamellar vesicles induce enhanced Aβ fibrillogenesis [5] in comparison with GM1 micelles. Lipid composition can also be a determining factor for assembly states of GM1 molecules and their interaction with Aβ. Most importantly, there is growing evidence that cholesterol and sphingomyelin contribute to GM1 assembly and thereby influence Aβ deposition promoted by its cluster [8, 18, 42, 43]. Elucidation of the structural basis of these molecular events is an important subject for the forthcoming stage of the research.

In conclusion, in the present study, we firstly identified and characterized the ThT-reactive β-structure of Aβ(1–40) promoted on GM1 micelles. Our findings offer structural insights into the mechanisms underlying the α-to-β conformational transition of Aβ on GM1 clusters, which is associated with the nucleation process in the Aβ aggregation.

Abbreviations

Aβ : Amyloid β
AD: Alzheimer's disease
CD: Circular dichroism
GST: Glutathione *S*-transferase
His_6-Ub: Hexahistidine-tagged ubiquitin
HSQC: Heteronuclear single-quantum correlation
IPTG: Isopropyl-β-$_D$-thiogalactopyranoside
NMR: Nuclear magnetic resonance
SDS: Sodium dodecyl sulfate
ThT: Thioflavin T
VUV: Vacuum-ultraviolet
YUH-1: Yeast ubiquitin hydrolase-1.

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Review Article

Membrane Incorporation, Channel Formation, and Disruption of Calcium Homeostasis by Alzheimer's β-Amyloid Protein

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Oligomerization, conformational changes, and the consequent neurodegeneration of Alzheimer's β-amyloid protein (AβP) play crucial roles in the pathogenesis of Alzheimer's disease (AD). Mounting evidence suggests that oligomeric AβPs cause the disruption of calcium homeostasis, eventually leading to neuronal death. We have demonstrated that oligomeric AβPs directly incorporate into neuronal membranes, form cation-sensitive ion channels ("amyloid channels"), and cause the disruption of calcium homeostasis *via* the amyloid channels. Other disease-related amyloidogenic proteins, such as prion protein in prion diseases or α-synuclein in dementia with Lewy bodies, exhibit similarities in the incorporation into membranes and the formation of calcium-permeable channels. Here, based on our experimental results and those of numerous other studies, we review the current understanding of the direct binding of AβP into membrane surfaces and the formation of calcium-permeable channels. The implication of composition of membrane lipids and the possible development of new drugs by influencing membrane properties and attenuating amyloid channels for the treatment and prevention of AD is also discussed.

1. Introduction

Alzheimer's disease (AD) is a severe type of senile dementia, affecting a large portion of elderly people worldwide. It is characterized by profound memory loss and inability to form new memories. The pathological hallmarks of AD are the presence of numerous extracellular deposits, termed senile plaques, and intraneuronal neurofibrillary tangles (NFTs). The degeneration of synapses and neurons in the hippocampus or cerebral cortex is also observed [1]. The major components of NFTs are phosphorylated tau proteins, and that of senile plaques are β-amyloid proteins (AβPs). Although the precise cause of AD remains elusive, it is widely accepted that oligomerization of AβP and the consequent neurodegeneration might be the cause of neuronal death in AD patients [2, 3].

There is considerable interest regarding the mechanism by which AβPs cause neurodegeneration. AβPs have been reported to cause various adverse effects on neuronal survivals, such as the production of reactive oxygen species, the induction of cytokines, the induction of endoplasmic reticulum (ER) stresses, and the abnormal increase in intracellular calcium levels ($[Ca^{2+}]_i$) [4]. These adverse effects are complex and may be interwoven. Of these effects,

the disruption of calcium homeostasis could be the earliest and primary event, since Ca^{2+} ions are essential for various neuronal functions. The elevation of $[Ca^{2+}]_i$ induces various apoptotic pathways.

There are several mechanisms that account for AβP-induced calcium dyshomeostasis [5–7]. Of these, we focus on the "amyloid channel hypothesis"—direct insertion into membranes of AβP, formation of channels (pores), and disruption of calcium homeostasis *via* unregulated cytotoxic channels may be the molecular basis of its neurotoxicity [8–10]. Other amyloidogenic disease-related proteins, such as the prion protein or α-synuclein, also exhibit similarities in the formation of amyloid channels and in the disruption of calcium homeostasis.

We review here the current understanding of the "amyloid channel hypothesis" based on our recent results and those of other researchers. It is widely recognized that the composition of membrane lipids influences the formation of amyloid channels by affecting the interaction between peptides and membranes. The possible development of new drugs by influencing membrane lipid properties and attenuating amyloid channels for the treatment and prevention of AD is also discussed.

2. Conformational Changes of AβP and Its Neurotoxicity

AβP is a small peptide with 39–43 amino acid residues. It is secreted by the cleavage of the N-terminal of a large precursor protein (amyloid precursor protein; APP) by β-secretase (β-site APP cleaving enzyme; BACE), followed by the intramembrane cleavage of its C-terminal by γ-secretase. This different C-terminal cleavage of APP causes various truncated AβPs, such as AβP(1–40), the first 40 amino acid residues, or AβP(1–42). Genetic studies of early-onset cases of familial AD indicated that APP mutations and AβP metabolism are associated with AD [11]. It was also revealed that mutations in the presenilin genes account for the majority of cases of early-onset familial AD [12]. Presenilins have been revealed to be γ-secretases [13], and their mutations influence the production of AβP and its neurotoxicity [14].

Yankner et al. reported that AβP(1–40) caused the death of cultured rat hippocampal neurons or neurodegeneration in the brains of experimental animals [15]. However, the neurotoxicity of AβP has been a subject of much debate because of its peculiar characteristics. AβP is a hydrophobic peptide with an intrinsic tendency to self-assemble to form oligomers (aggregates). In the aqueous solution, monomeric form of AβP exhibits a random coil structure. Meanwhile, under incubation at 37 C for several days (*aging*), AβPs form aggregates (oligomers) with β-pleated sheet structures. Pike et al. revealed that aged AβP(1–40) was considerably more toxic to cultured neurons as compared to freshly prepared AβP(1–40) [16]. The neurotoxicity of AβP was correlated with their β-sheet contents, as observed by circular dichroism (CD) spectroscopy [17]. Jarrett and Lansbury demonstrated that AβP forms oligomers by a nucleation-dependent process and that AβP(1–42) becomes "seeds" in the aggregates and enhances the oligomerization of AβP(1–40)—suggesting the significance of intracellular N- and C-terminal heterogeneity [18].

Recent detailed analysis using size-exclusion chromatography, gel electrophoresis, and atomic force microscopy (AFM) has demonstrated that there are several stable types of soluble oligomers: naturally occurring soluble oligomers (dimers or trimers), ADDLs (AβP-derived diffusible ligands), AβP globulomers, or protofibrils. Increasing evidence suggests that soluble amyloid oligomers cause synaptic and neuronal degeneration [19–21]. The identification of toxic AβP spices is crucial and has been a subject of scientific debates. Hartley et al. separated aggregated AβP(1–40) into low-molecular-weight (mainly monomer), protofibrillar, and fibril fractions by size-exclusion chromatography, and found that the protofibrillar fraction caused marked changes in the electrical activity of cultured neurons and neurotoxicity [22]. Walsh et al. reported that the naturally secreted (derived from the cerebrospinal fluid of AD patients), SDS-stable low-molecular-weight oligomers (dimers, trimers, or tetramers), but not AβP monomers or larger aggregates, inhibit long-term potentiation (LTP) and cause the loss of dendritic spines and synapses [23]. Lacor and colleagues reported that AβP-derived diffusible ligands (ADDLs) inhibited LTP and exhibited adverse effects on synaptic plasticity, such as abnormal spine morphology, decreased spine density, and decreased synaptic proteins [24]. Recently, Jan et al. found that mixtures of monomeric and heterogenous oligomers AβP(1–42) were more toxic than monomeric, protofibrillar fractions or fibril [25]. They demonstrated that AβP toxicity depends on the ability to grow and undergo fibril formation of prefibrillar aggregates and monomer. The process of fibril formation and its contribution to toxicity is complicated. Mature fibrils are regarded to be less toxic compared to soluble oligomers [26, 27], although there are some cases fibrils direct cause toxicity [28, 29]. It is possible that the toxicity of mature fibrils can result from the leakage of toxic short protofibrils or oligomers [27] or from its size-dependent mechanical properties of accumulations in the normal tissues [30].

As synaptic plasticity is crucial for the process of memory formation, synaptic degeneration (synaptotoxicity) is involved in the early stages of AD. Indeed, the number of synapses is strongly correlated with the level of memory impairment in AD patients, rather than the number of senile plaques or NFTs, [31]. Considering that AβP is secreted in the cerebrospinal fluid (CSF) of young individuals as well as in aged or dementia patients [32], factors that accelerate or inhibit the oligomerization may play essential roles in the pathogenesis of AD. Various factors, such as the concentration of peptides, the oxidations, mutations, and racemization of AβP, pH, composition of solvents, temperature, and trace elements, can influence the oligomerization processes [33]. Among these factors, Al and other trace elements are of particular interest because of the epidemiological link with AD [34].

3. AβP-Induced Neurotoxicity and the Disruption of Calcium Homeostasis

There is considerable interest regarding in the mechanism by which AβPs cause neurodegeneration. Of various adverse effects caused by AβP, calcium dyshomeostasis could be the earliest and primary adverse event, since Ca^{2+} ions are essential for various key enzymes such as kinases, phosphatases, and proteases. Once neuronal calcium homeostasis was disrupted and $[Ca^{2+}]_i$ was changed, various apoptotic pathways such as calpain and caspase activation occurred, leading to neuronal death. The disruption of calcium homeostasis could trigger the membrane disruption, the formation of reactive oxygen species (ROS), and induce other adverse effects which are often observed after exposure to AβP. It is widely known that the increase in $[Ca^{2+}]_i$ induced changes in the number of spines, their morphology, and the number of synapses [35]. Considering that AβP and APP coexist in the synapses [36], calcium imbalances in the synaptic compartment could directly influence neuronal activities and cause synaptic impairment (synaptotoxicity). Ca^{2+} is also implicated in the phosphorylation of the tau protein [37] or in APP sequestration [38]. Fibroblasts derived from AD patients exhibited different Ca^{2+} mobilization compared to those derived from age-matched control subjects [39]. Mounting evidence indicates that calcium dysregulation occurs in AD or in AβP-intoxicated neurons [40, 41].

There are several possible mechanisms by which AβPs interact with neurons and disrupt calcium homeostasis. Demuro et al. reviewed the AβP-induced calcium dyshomeostasis and its toxicity in the context of calcium signaling, and outlined three major mechanisms: the activation of some type of cell surface receptors coupled to Ca^{2+} influx, the disruption of membrane integrity, and the direct incorporation into the membrane to create unregulated cytotoxic channels (pores) [5].

AβPs were reported to bind to NMDA (*N*-methyl D-aspartate-)type or AMPA (α-amino-3-hydroxy-5-methy-lisoxazole4-propionic acid)-type glutamate receptors [42], or nicotinic acetylcholine receptors [43]. All of these receptors were highly Ca^{2+} permeable. Furthermore, AβP influences voltage gated Ca^{2+} channels [44] or inositol triphosphate (IP_3) receptor [45]. It is widely recognized that presenilins are involved in capacitative Ca^{2+} entry, in ER Ca^{2+} signaling, or in mitochondrial Ca^{2+} signaling, and that their mutations affect the calcium-regulated functions [46–49]. Therefore, disturbances of ER Ca^{2+} stress or mitochondrial Ca^{2+} homeostasis may be involved in the pathogenesis of AD.

4. Channel Formation by AβP: Possible Mechanisms of Calcium Dyshomeostasis

In 1993, Arispe et al. first demonstrated that AβP(1–40) directly incorporates into artificial planar lipid bilayer membranes and forms cation selective ion channels [50, 51]. These "amyloid channels" were revealed to be giant multilevel pores and were permeable to Ca^{2+}. Their activity was blocked by Zn^{2+}, which is abundantly present in the brain [52]. Other neurotoxic peptide fragments of AβP, including AβP(25–35) and AβP(1–42), were reported to form calcium-permeable pores on artificial lipid bilayers as well as AβP(1–40) [53, 54]. The characteristics of amyloid channels formed by AβP(1–40) and AβP(1–42) exhibited similarities: multilevel and giant pores (5 nS) and cation (including Ca^{2+}) selectivity. The activity of both channels could be blocked by Zn^{2+}. Fraser et al. reported that the toxic C-terminal fragment of APP(CT_{105}; containing a full length of AβP) induced channel currents on membranes of *Xenopus* oocytes [55].

Durell et al. proposed a 3D structural model of amyloid channels obtained from a computer simulation of the secondary structure of AβP(1–40) in membranes, which showed 5 to 8 mers aggregating to form pore-like structures on the membranes [56]. Strodel et al. proposed a model of AβP(1–42) pores which consist of tetrameric and hexameric β-sheet subunits from the observations in NMR [57]. These models are consistent with morphological observations using high-resolution AFM that demonstrated that AβPs form pore-like structures on mica plates or on membranes [58–60].

A large number of studies have demonstrated that AβP directly binds to membranes, causes membrane perturbation or disruption, and induces the increase in permeability to ions (including Ca^{2+}) or large molecules [61–64]. The findings of Demuro et al. are particularly interest in this context [65]. They investigated effects of AβP and other amyloid peptides in various aggregation states, and revealed that oligomeric peptides caused the rapid increase in $[Ca^{2+}]_i$ or the membrane disruption, whereas monomers and fibrils did not.

Furthermore, the presence of pore-like structures of AβPs was demonstrated in the neuronal cell membrane of the brains of AD patients and of AD-model mice. Using high-resolution transmission electron microscopy, Inoue observed in situ AβP pores in the neuronal cell membrane in AD brains [66]. Kayed et al. reported that the annular protofibrils (APFs) of AβP exhibit ring-shaped and pore-like structures [67]. The age-dependent accumulation of APFs was observed on the membranes of AD model mice (APP transgenic mice; APP23) [68].

To determine whether or not AβPs form channels on neuronal cell membranes as well as on artificial lipid bilayers, we employed membrane patches from immortalized hypothalamic neurons (GT1-7 cells). GT1-7 cells are derived from murine hypothalamic neurons by site-directed tumorigenesis and exhibit various neuronal characteristics, such as the extension of neuritis, and the expression of various neuron-specific proteins or receptors [69]. Within 3–30 min of the addition of AβP(1–40) to the bath solution, the current derived from the amyloid channels appeared across the excised membrane patches [70]. However, AβP(40–1), a peptide bearing the reversed sequence of AβP(1–40), did not form any channels. The characteristics of amyloid channels formed on the GT1-7 cell membranes were considerably similar to those observed on artificial lipid bilayers: cation selective, multilevel, voltage independent, and long-lasting. Its channel activity was inhibited by the addition of Zn^{2+},

and recovered by a zinc chelator–*o*-phenanthroline. Furthermore, Sepulveda et al. revealed that AβP(1–40) formed perforations on membranes excised from hippocampal neurons and induced currents [71]. The effect of AβP was similar to that of gramicidin and amphotericin which are commonly used to perforate neuron membranes.

5. Disruption of Calcium Homeostasis Caused by Amyloid Channels

In order to test the validity of the amyloid channel hypothesis, we examined whether AβP alters the $[Ca^{2+}]_i$ levels of GT1-7 cells under the same conditions, using a high-resolution multisite video imaging system with calcium-sensitive fluorescent dye, fura-2 [71–74]. Shown in Figure 1(a) are pseudocolor images of levels indicating the $[Ca^{2+}]_i$ of GT1-7 cells before and after exposure to AβP(1–40). Shortly after exposure to AβP(1–40), a marked increase in $[Ca^{2+}]_i$ occurred among many, but not all GT1-7 cells. Figure 1(b) depicts AβP(1–40)-induced temporal changes of the $[Ca^{2+}]_i$ of 50 randomly chosen GT1-7 cells in the same field of view. Furthermore, we compared responses to AβP and the related peptides (Figure 2(a)). Although a marked increase in $[Ca^{2+}]_i$ was caused by AβP(1–40) (*line (A)*) or by AβP(1–42) (*line (C)*), control peptides such as AβP(40-1) caused no remarkable changes (*line (B)*).

As previously discussed, there are several mechanisms that could account for the elevations in $[Ca^{2+}]_i$ induced by AβP. However, our detailed quantitative analysis of the AβP-induced calcium influx suggests that AβP-induced $[Ca^{2+}]_i$ changes occurred *via* unregulated amyloid channels and not by endogenous receptor-mediated pathways. This is supported by 4 major pieces of evidence.

First, the AβP-induced $[Ca^{2+}]_i$ rise was highly heterogeneous among genetically identical GT1-7 cells. Even in the same field of view, exposure to the same peptide solution produced different change patterns in the $[Ca^{2+}]_i$ levels as shown in Figure 1(b). Although AβP(1–40) induced an increase in the $[Ca^{2+}]_i$ levels either instantly or after some delay, the magnitude and latency differed. Certain other adjacent cells still did not exhibit any responses. It is possible that the membrane binding of AβP is crucial for the cell-to-cell heterogeneity. Simakova and Arispe revealed that the surface phosphatidylserine and the cytosolic ATP levels are important determinants of the binding of AβP to membranes [75]. To analyze AβP-induced calcium influx quantitatively under the cell-to-cell heterogeneous condition, we compared the peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) induced by AβPs and its latency (the lag between the $[Ca^{2+}]_i$ increase and the time of AβP addition) in each cell. This multisite fluorometry system enables the simultaneous long-term observation of temporal changes in $[Ca^{2+}]_i$ of more than 50 neurons. *Second*, the average $\Delta[Ca^{2+}]_i$ was increased in a dose-dependent manner of AβP, while the average latency decreased (Figures 2(b) and 2(c)). It is unlikely that the dose-dependent decrease in the latency occurs through the receptor-mediated pathways. These features are considerably similar to those observed in relation to peptide channels formed on membranes [71, 76]. The concentration of AβP required to form amyloid channels is higher (μM) than the AβP concentration found in the brain. However, it is plausible that it requires a longer period for the lower concentration of AβP to cause changes in $[Ca^{2+}]_i$.

Third, the AβP-induced increase in $[Ca^{2+}]_i$ was not influenced by the addition of the Na^+ channel blocker (tetrodotoxin), the Ca^{2+} channel blocker (nifedipine), the antagonist of NMDA-type glutamate receptor (D-APV), or the antagonist of γ-aminobutyric acid (GABA) receptor (bicuculline) [77].

Fourth, D-AβP(1–40), AβP(1–40) composed of all D-amino acid residues, also caused the elevation of $[Ca^{2+}]_i$ in a manner similar to AβP(1–40) (Figure 2(a) *line (D)*). This is consistent with the findings of Cribbs et al. suggesting that all-D-enantiomers of AβP possess the similar toxicity compared to all-L- AβP [78].

Therefore, it is plausible that AβP-induced $[Ca^{2+}]_i$ changes occurred through amyloid channels by direct incorporation into membranes, but not through some receptor-mediated pathways.

These results strongly support the hypothetical idea termed "amyloid channel hypothesis," namely, that the direct incorporation of AβPs and the subsequent imbalances of calcium and other ions through amyloid channels may be the primary event in AβP neurotoxicity [8–10].

6. Channel Formation and $[Ca^{2+}]_i$ Influx by Other Amyloidogenic Peptides

Pore formation-induced cytotoxicity, such as in the cases of certain toxins or venoms, is commonly observed in our biological system. For example, the α-toxin of *Staphylococcus aureus,* which is secreted as a single-chain, water-soluble 33 kDa molecule, nonspecifically binds to membranes to form pore-like structures composed of hexamers with β-sheet structures, causing Ca^{2+} influx through the pores [79]. Magainin 2, a 26-residue antimicrobial peptide obtained from *Xenopus laevis*, forms transmembrane Ca^{2+}-permeable pores on bacterial cell membranes [80]. Other antimicrobial peptides such as melitin (a bee venom composed of 28 amino acids), or antibiotics such as amphotericin and gramicidin were also reported to form transmembrane pores and to cause cell lysis [81]. In this respect, AβP and other amyloidogenic proteins might share the similar mechanism with these pore-forming peptides. Indeed, Soscia et al. demonstrated that AβP exerts antimicrobial activity against 8 common and clinically relevant microorganisms [82].

Furthermore, electrophysiological and morphological studies have revealed that other disease-related proteins—termed amyloidogenic proteins—exhibit similarities in the formation of amyloid channels as well as AβP.

Prion diseases, including human kuru, Creutzfeldt-Jakob disease, and bovine spongiform encephalopathy (BSE), are associated with the conversion of a normal prion protein (PrP^C) to an abnormal scrapie isoform (PrP^{SC}) [83]. The β-sheet region of PrP^{SC} is suggested to play a crucial role in its transmissible degenerative processes. A peptide fragment

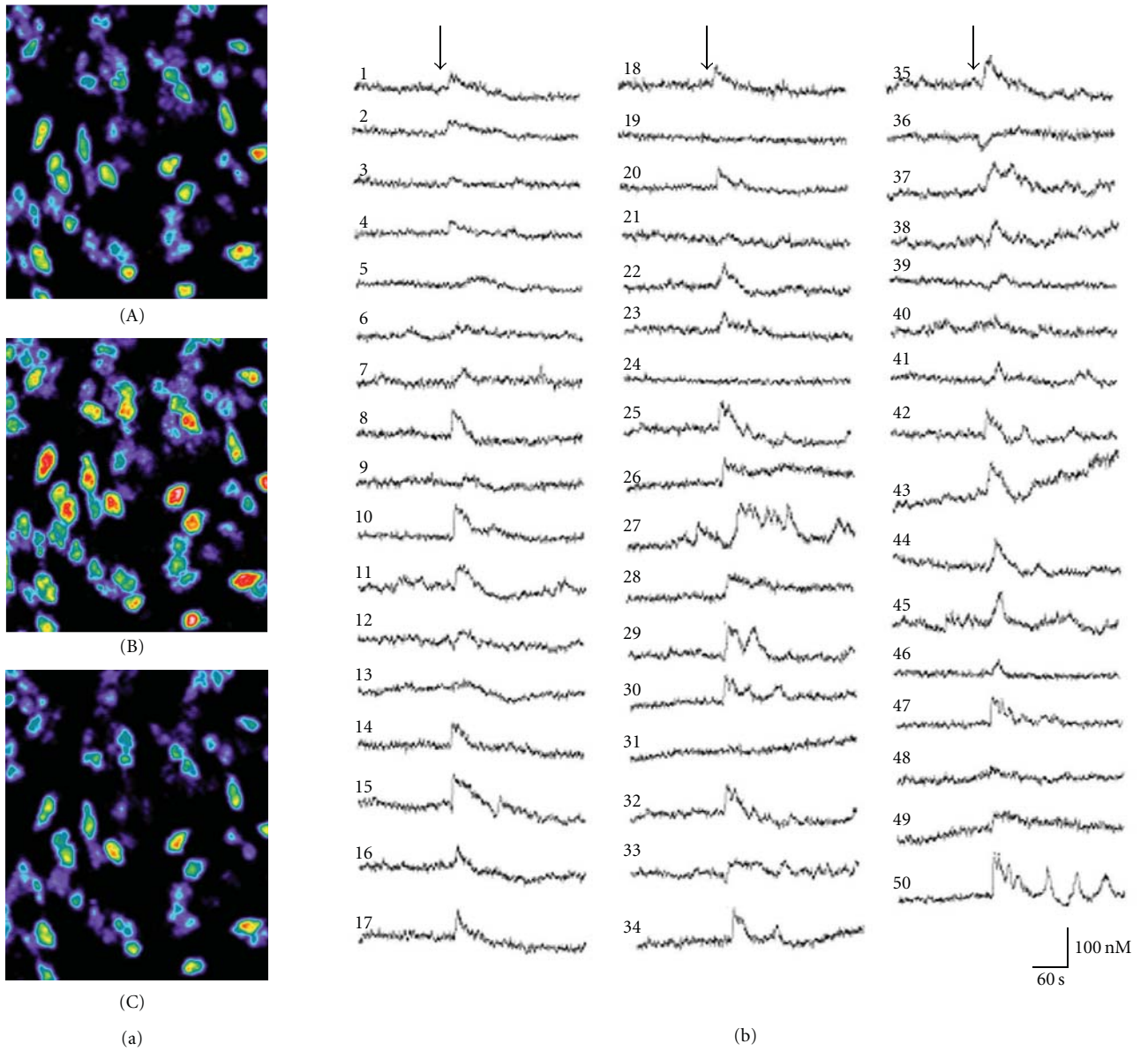

FIGURE 1: Effects of AβP on temporal changes of $[Ca^{2+}]_i$. (a) Pseudocolor images of $[Ca^{2+}]_i$ during exposure to AβP(1–40) in GT1-7 cells. A solution of AβP(1–40) (10 μM) was applied onto fura-2-loaded GT1-7 cells. Temporal changes of fluorescence intensities corresponding to increases in $[Ca^{2+}]_i$ were analyzed. (A) 1 min before exposure to AβP(1–40); (B) 20 sec after exposure; (C) 5 min after exposure. (b) Temporal changes of randomly chosen 50 GT1-7 cells in the same field of view before and after the exposure to AβP(1–40) are depicted. The arrow indicates the time of peptide addition.

of PrP corresponding to residues 106–126 (PrP106–126) coincides with the proposed β-sheet structures and has been reported to cause death in cultured hippocampal neurons [84]. Lin et al. reported that PrP106–126 forms cation permeable pores in artificial lipid bilayers [85]. The activity of PrP channels was also blocked by Zn^{2+}. Kourie and Culverson investigated the detailed characteristics of channels formed by PrP106–126, concluding that it was directly incorporated into lipid bilayers and formed cation selective, copper-sensitive ion channels [86]. They also revealed that quinacrine, a potent therapeutic drug, possibly blocks amyloid channels induced by PrP106–126.

The aggregation and fibrillation of α-synuclein has been implicated in the formation of abnormal inclusions, termed Lewy bodies, and the etiology of dementia with Lewy bodies (DLB) [87]. Nonamyloid component (NAC), a fragment peptide of α-synuclein, accumulates in Alzheimer's senile plaques and causes apoptotic neuronal death [88]. Lashuel et al. demonstrated by electron microscope observation that α-synuclein forms annular pore-like structures [89].

The elongation of a polyglutamine-coding CAG triplet repeat in the responsible genes is based on the pathogenesis of triplet-repeat disease such as Huntington's disease or Machado-Joseph disease [90]. Hirakura et al. reported that

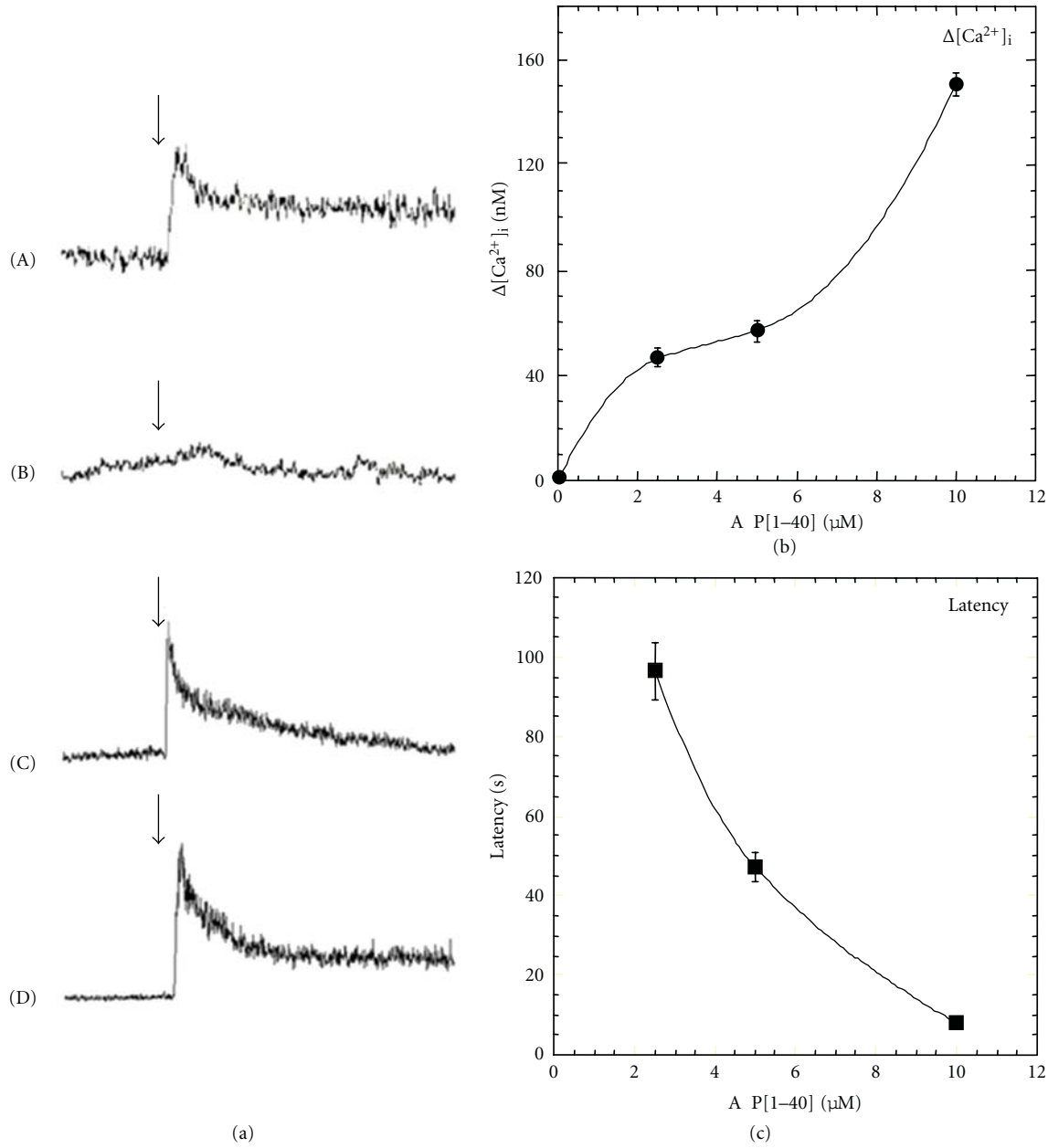

Figure 2: Characteristics of AβP-induced elevations in $[Ca^{2+}]_i$. (a) Typical time course of $[Ca^{2+}]_i$ prior to 2 min and after 3 min of the application of the peptide is depicted. Concentration is 10 μM for all peptides used. (A) AβP(1–40); (B) AβP(40-1); (C) AβP(1–42); (D) D-AβP(1–40). The arrow indicates the time of peptide addition. (b) and (c) Dose-dependence of the increase in $[Ca^{2+}]_i$. Typical responses of $[Ca^{2+}]_i$ in cultured neurons following exposure to various concentrations of AβP(1–40) (2.5 10 μM). The peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) in each cell (b) and the latency after exposure to AβP(1–40) were analyzed in more than 50 neurons in field of view (360 × 420 μm) cultured neurons (mean ± S.E.M., n = 300).

polyglutamine formed ion channels in lipid bilayers [91]. Human amylin (IAAP, islet amyloid peptide) forms amyloid fibrils, accumulates in the islet of patients of type 2 *diabetes mellitus*, and causes cytotoxicity in islet cells or in cultured hippocampal neurons. However, rat amylin did not cause cytotoxicity nor form β-sheet structures, in spite of the 95% similarity in the amino acid sequence [92]. Mirzabeko et al. revealed that human amylin formed ion channels on liposomes, but rat amylin did not [93]. Calcitonin is a 32-amino acid polypeptide hormone, which is produced by the thyroid C-cells. It is involved in calcium homeostasis and is associated with medullary carcinoma of the thyroid [94]. Using transmission electron microscopy (TEM) observation on liposome, Diociaiuti et al. found that calcitonin oligomers exhibit annular pore-like structures [95]. Lal et al. investigated the oligomerization and conformational changes of AβP, synuclein, amylin, and other amyloidogenic proteins using gel electrophoresis and AFM imaging, and demonstrated that these amyloidogenic proteins form annular channel-like structures on bilayer membranes [96].

We have demonstrated that these amyloidogenic peptide also cause the elevations in $[Ca^{2+}]_i$ as well as AβP (Figure 3(a)). A marked increase in $[Ca^{2+}]_i$ was caused by PrP106–126 (*line (A)*), human amylin (*line (C)*), NAC (*line (E)*) and AβP(1–40) (Figure 2(a), *line (A)*) or pore-forming antimicrobial peptide magainin 2 (*line (F)*). However, control peptides such as peptide with random sequence of PrP106–126 (scramble PrP106–126) (*line (B)*) and rat amylin (*line (D)*) caused no remarkable changes. Furthermore, PrP106–126 and human amylin, as well as AβP(1–40), cause disruption of liposome membranes and induce dye release (Figure 3(b)).

These diseases are included in "conformational disease" (protein misfolding disease)—the conformational change of amyloidogenic proteins is suggested to be an important determinant of its toxicity and, consequently, the development of the disease [97]. The disease-related amyloidogenic proteins exhibit similarities in the formation of β-pleated sheet structures, abnormal deposition as amyloid fibrils in the tissues, and introduction of apoptotic degeneration. As shown in Table 1, these amyloidogenic proteins exhibit similarities in the direct incorporation into membranes, formation of calcium-permeable ion channels, and induction of abnormal elevation of $[Ca^{2+}]_i$. It is strongly suggested that disruption of calcium homeostasis *via* unregulated amyloid channels formed by these disease-related proteins may be the molecular basis of neurotoxicity of these diseases.

7. Role of Membrane Lipids in the Formation of Amyloid Channels

It is widely accepted that the direct incorporation of peptides into membranes and consequent channel formation is strongly affected by the membrane lipid composition, particularly the net charges of membrane surfaces and membrane fluidity. Several AβP residues (such as Arg^5, Lys^{16}, and Lys^{28}) have a positive charge at neutral pH, and therefore, AβP has an affinity for negatively charged phospholipids, such as phosphatidylserine (PS) or phosphatidylglycerol (PG), but not for neutral phospholipids, such as phosphatidylcholine (PC) [98]. However, membrane phospholipid distribution is asymmetrical in mammals: neutral lipids (PC, etc.) usually exist on the outer surfaces of plasma membranes, whereas negatively charged phospholipids (PS, etc.) exist in the inner surfaces of the membranes. Thus, the binding of AβP to neuronal membrane surface may seldom occur in normal and young brains.

Further influencing the binding of AβP to membranes are gangliosides—sialic-acid-bearing glycophospholipids. Both APP and AβP are localized in detergent-insoluble, cholesterol-, sphingomyelin-, and ganglioside-rich lipid microdomains, termed rafts [99]. Yanagisawa et al. first demonstrated the existence of membrane-bound AβP tightly bound to GM1 gangliosides in the brains of AD patients [100]. AβP binds to GM1 gangliosides in raft-like membranes *in vitro,* and GM1-bound AβP behave as a "seed" and accelerate the oligomerization of AβP [101]. Numerous studies have indicated the implication of gangliosides in the oligomerization and the binding to the membrane of AβP [102–104].

We have observed the deposition of AβP(1–40) on ganglioside (GM1)/phospholipid (dipalmitoil phosphatidyl choline; DPPC) monolayers by AFM imaging (Figure 4). GM1-DPPC membranes exhibit distinctive, island-like GM1 domains embedded in the DPPC matrix [105, 106]. Aged AβP(1–40) deposited and tightly bound to the membrane surfaces and exhibited the damaged structures of membranes, meanwhile freshly prepared AβP showed few changes.

We have previously demonstrated that AβP causes a marked increase in $[Ca^{2+}]_i$ in a large proportion of long-term (30–35 days *in vitro*; DIV) cultured hippocampal neurons. However, few or no changes were observed in $[Ca^{2+}]_i$ in short-term (8 DIV) cultures (Figure 5(a)) [107]. After several days of exposure to sublethal levels of AβP(1–40) to long-term cultured neurons, AβP binds to some restricted hippocampal neurons and exhibits dotlike depositions on the somata and dendrites. Meanwhile, there is no detectable AβP deposition on the surfaces of neighboring neurons, despite the morphological similarities of these neurons (Figure 5(b)). Malchiodi-Albedi et al. found that lipid rafts increased during the maturation of culture periods of primary cultured rat hippocampal neurons [108]. They demonstrated that Calcitonin, an amyloidogenic peptide, causes $[Ca^{2+}]_i$ changes in mature raft-containing neurons, but not in immature cultured neurons. Williamson et al. found that AβP was not uniformly distributed over the neuronal processes, and was colocalized with GM1 ganglioside [109]. These features are consistent with our results, and it is possible that gangliosides in lipid rafts may regulate the binding of AβP into membranes and its neurotoxicity.

It is widely accepted that cholesterol enhances membrane stiffness, decreases membrane fluidity, and inhibits pore formation by pore-forming peptides [110]. Lin and Kagan found that cholesterol inhibits channel formation by AβP [111]. Cholesterol blocks AβP-induced elevations in $[Ca^{2+}]_i$ [41, 72], aggregation of AβP-containing liposomes [112], and AβP cytotoxicity [112, 113]. Moreover, cholesterol attenuates AβP-induced membrane-disordering effects and calcium increase [114]. Considering that apolipoprotein E, involved in cholesterol transport and metabolism, is present in the senile plaques and NFTs in AD brains and its polymorphism is a risk factor of AD [115], the implication of cholesterol in AD pathogenesis is crucial.

To determine the implications of membrane properties in the formation of amyloid channels, we tested the effects of several lipophilic substances, which modulate membrane properties, on AβP-induced $[Ca^{2+}]_i$ elevations [74, 107]. Phloretin, a plant-derived flavonoid, decreases membrane dipole potential, and inhibits the electrostatic interaction between AβP and membrane lipids, and attenuates AβP-induced neurotoxicity [116]. Meanwhile, 6-ketocholestanol increases the magnitude of the membrane dipole potential and decreases membrane fluidity [117]. Figure 6 shows that the preadministration of phloretin and cholesterol markedly inhibited AβP-induced $[Ca^{2+}]_i$ elevations; meanwhile, 6-ketocholestanol did not cause significant changes, despite the

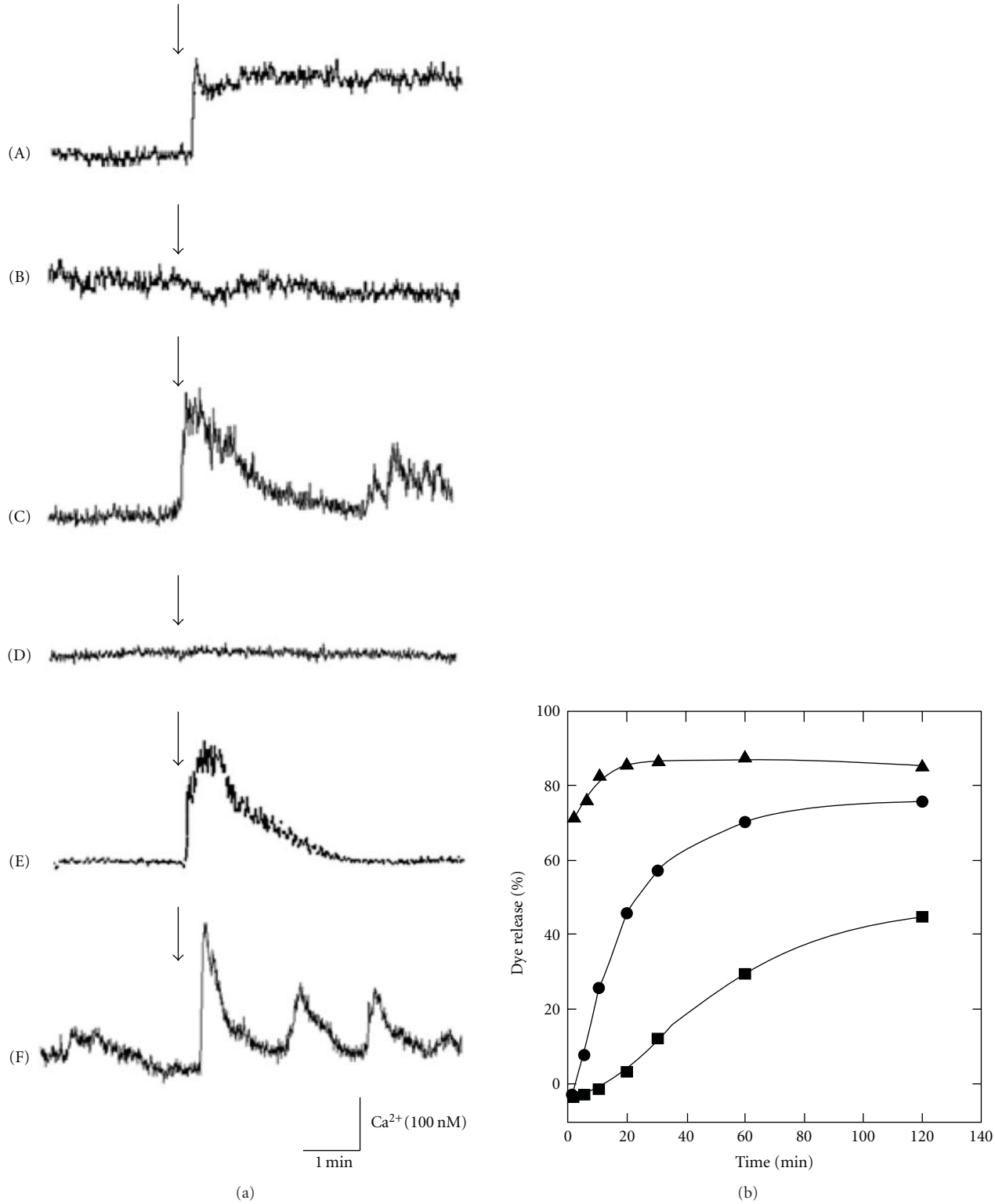

Figure 3: Effects of amyloidogenic proteins on membrane disruption and $[Ca^{2+}]_i$ elevations. (a) Effects of amyloidogenic proteins and their analogues on $[Ca^{2+}]_i$. Typical time course of $[Ca^{2+}]_i$ prior to 2 min and after 3 min of the application of the peptide is depicted. Concentration is 10 μM for all peptides used. (A) PrP106–126; (B) scramble PrP106–126; (C) human amylin; (D) rat amylin; (E) NAC; (F) magainin 2. The arrow indicates the time of peptide addition. (b) Membrane disruption by amyloidogenic peptides. AβP(1–40) (closed circle), PrP106–126 (closed square), and human amylin (open circle) (each 10 μM) were added to negatively charged liposomes containing carboxyfluorescein. The ratio of DPPC (dipalmitoil phosphatidyl choline) : CHOL (cholesterol) : DPPG (dipalmitoil phosphatidyl glycerol) in the liposome was 3 : 4 : 3. The temporal changes of the fluorescence intensity were monitored. The ratio of the released fluorescent dye (carboxy fluoresein; CF) compared to the total amount of CF was described as the percentage of membrane disruption.

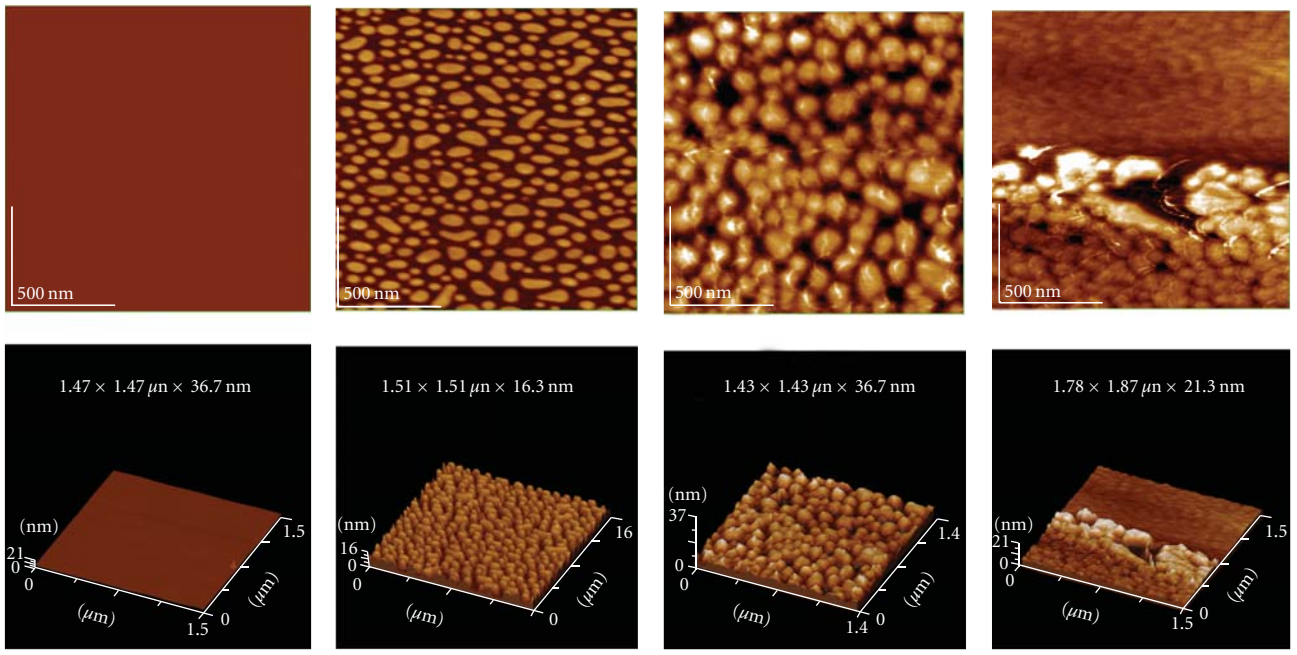

Figure 4: AFM images of AβP(1–40) on monolayer membranes. Lipid monolayer membranes composed by DPPC (a) or ganglioside GM1-DPPC (dipalmitoil phosphatidyl choline) (b) (d) were prepared by bath sonication and reconstitution on mica plates. The ratio of GM1:DPPC was 8 : 2. AFM images were obtained after the exposure to freshly prepared AβP(1–40) (c) or aged AβP(1–40) (d). Scale area: 1.5 × 1.5 μm.

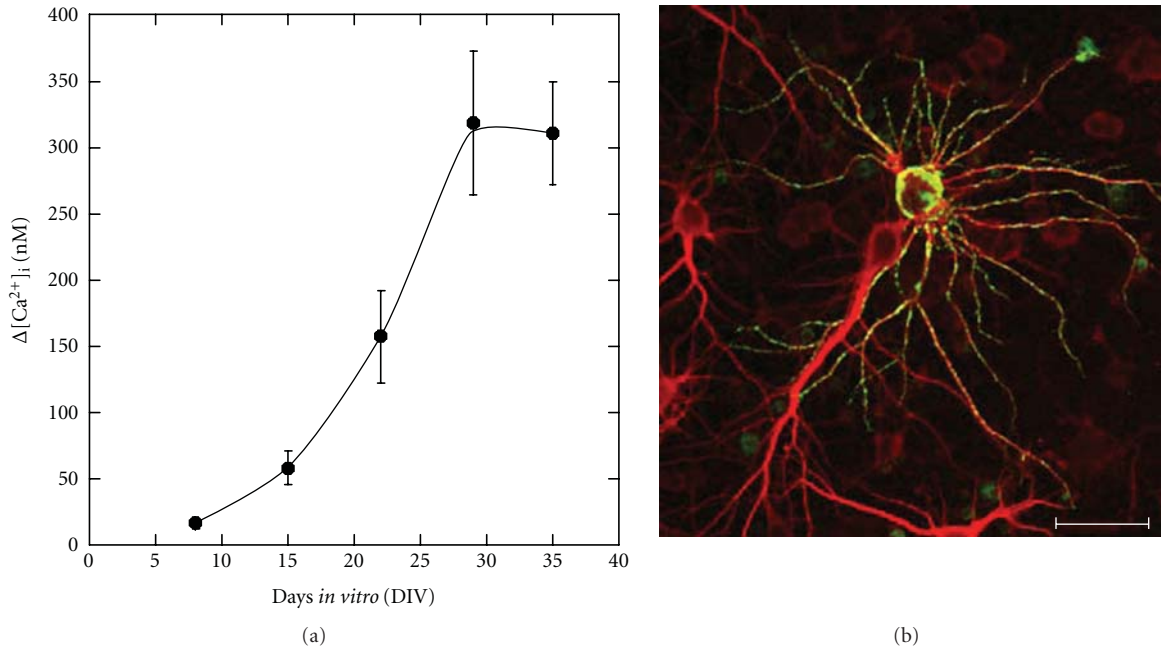

Figure 5: (a) Maturation-dependent increase in AβP-induced $[Ca^{2+}]_i$ changes in primary cultured neurons. (b) Heterogeneous affinity of AβP to mature cultured hippocampal neurons. Long-term cultured rat hippocampal neurons were exposed to 1 μM of AβP(1–40) at 29 DIV and fixed after 4 days. Neurons were double immunostained by anti-MAP2 antibody (Texas Red, red) and anti-AβP antibody (FITC, green), and observed by Laser confocal microscopy. Scale bar represents 50 μm. (modified from [100]).

Table 1: Characteristics of amyloidogenic proteins and the related peptides.

Disease	Amyloidogenic protein or its fragment peptide and the primary sequence	β-sheet formation	Cytotoxicity	Channel formation	$[Ca^{2+}]_i$ rise
Alzheimer's disease	*AβP(1–40)* DAEFRHDSGYEVHHQKLVFFAE DVGSNKGAIIGLMVGGVV	+	+	+	+
	AβP(40-1) VVGGVMLGIIAGKNSGVDEAFFV LKQHHVEYGSDHRFEAD	–	–	–	–
	AβP(25-35) DVGSNKGAII	+	+	+	+
	AβP(1–42) DAEFRHDSGYEVHHQKLVFFAEDV GSNKGAIIGLMVGGVVIA	+	+	+	+
Prion disease	*PrP106–126 (prion protein fragment)* KTNMKHMAGAAAAGAVVGGLG	+	+	+	+
	Scramble PrP106–126 NGAKALMGGHGATKVMVGAAA	–	–	–	–
Parkinson's disease (DLB; diseases with Lewy bodies)	*α-synuclein NAC* (a *fragment of α-synuclein)* EQVTNVGGAVVTGVTAVAQKTVEGAGSIAA-ATGFV	+	+	+	+
Triplet-repeat disease	*Polyglutamine* QQQQQQQQ—	+	+	+	n.d.
Diabetes mellitus	*Human amylin* KCNTATCATQRLANFLVHSSNNFGAILSST-NVGSNTY	+	+	+	+
	Rat amylin KCNTATCATQRLANFLVRSSNNLGPVLPPT-NVGSNTY	–	–	–	–
Medullary carcinoma of the thyroid	*Calcitonin* CGNLSTCMLGTYTQDFNKFHTFPQTAIGVG-AP	+	+	+	+

n.d.: not determined.

structural similarity to cholesterol. Therefore, as expected from other findings, the net charges of membrane surfaces and the membrane fluidity play crucial roles in the elevations of $[Ca^{2+}]_i$ caused by AβP.

Furthermore, numerous studies have demonstrated that gangliosides and cholesterol are implicated in the channel formation of other amyloidogenic proteins. Lipid rafts are considered to be the compartment where the conformational change of PrP occurs [118]. Gangliosides influence the β-sheet formation of PrP106–126 [119] and human amylin [120], or the channel formation of α-synuclein [121]. Cholesterol also inhibits channel formation by human amylin [122].

8. Possible Candidate for the Treatment of AD

The search for protective agents against AβP neurotoxicity is of great importance. Such agents include inhibitors of AβP oligomerization, inhibitors of BACE or γ-secretase, AβP vaccines, and chelators of trace metals; all have been proposed to be effective in the treatment of AD.

Here, we have focused on substances that inhibit the formation of amyloid channels. As discussed, the elevation of $[Ca^{2+}]_i$ by permeation through amyloid channels is considered to be the primary event of AβP neurotoxicity; therefore, such compounds could serve as the seed of new effective drugs with fewer adverse effects.

Zn^{2+} ion, which is abundant in vesicles of presynaptic terminals and is secreted into synaptic clefts with neuronal excitation, inhibits the currents induced by amyloid channels [52, 54, 70]. Zn^{2+} binds to His residues of AβP: Arispe et al. found that histidine-related peptide derivatives such as His-His or polyhistidine are effective in the inhibition of amyloid channels, the attenuation of AβP-induced $[Ca^{2+}]_i$ changes, and the protection of neurons from AβP toxicity [123, 124]. They developed several small amphiphilic pyridinium derivatives which inhibit formation of AβP channels and its neurotoxicity [8, 125].

In line with the search for protective agents, we have screened compounds, which influence membrane properties and inhibit formations of amyloid channels, by observing the AβP-induced Ca^{2+} influx. Among those tested, we

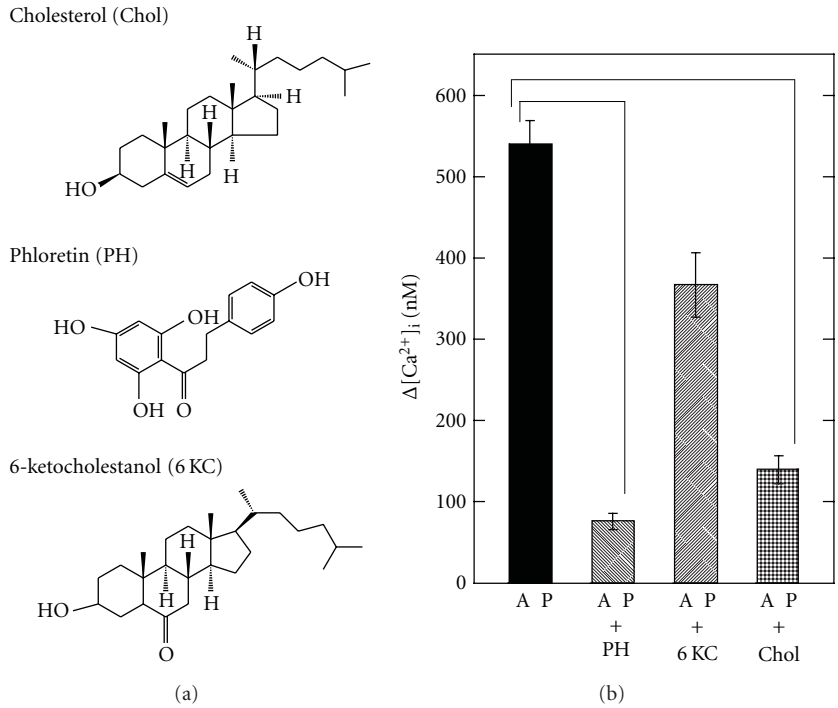

Figure 6: Effect of membrane charges and fluidity on AβP-induced $[Ca^{2+}]_i$ rise. The solutions of phloretin (PH), 6-ketocholestanol (KC), and cholesterol (Chol) were preadministrated on GT1-7 cells; and AβP-induced $[Ca^{2+}]_i$ rise was analyzed. Data are mean ± S.E.M., $n = 250$, $P < .001$. (modified from [67]).

found that several lipophilic substances, such as 17β-estradiol, 17α-estradiol, and neurosteroids (including dehydroepiandrosterone [DHEA], DHEA sulfate [DHEA-S], and pregnenolone) significantly inhibit AβP-induced $[Ca^{2+}]_i$ elevation [74, 107]. 17β-estradiol, a female hormone, is neuroprotective and affects membrane fluidity [126]. Considering that both 17β-estradiol and 17α-estradiol inhibit AβP-induced $[Ca^{2+}]_i$ elevation, the inhibition may not depend on their genomic actions but on their membrane-modifying effects. Neurosteroids are steroid hormones synthesized *de novo* in the central nervous system from cholesterol or from peripheral steroid precursors [127]. Several lines of evidence suggest that neurosteroids modulate various functions of the brain and exhibit neuroprotective activities [128]. Considering that concentrations of plasma DHEA are reduced in AD patients [129], the implication of neurosteroids in the pathogenesis of AD may be important.

9. Amyloid Channel Hypothesis

Based on the results of our studies, together with those of other studies, we propose the following hypothetical scheme: that unregulated calcium influx *via* amyloid channels may underlie the molecular mechanism of AβP neurotoxicity and the pathogenesis of AD (Figure 7).

AβPs are normally secreted from APP, which exists in the synapse, into the cerebrospinal fluid or synaptic clefts. Secreted AβPs are degraded proteolytically by proteases, such as neprilysin [130], within a short period. However, upregulation of the AβP secretion from APP, or an increased ratio of AβP(1–42) to AβP(1–40) may render AβPs liable to be retained in the brain. Mutations of APP or presenilin gene promote this process. The binding of AβP to neuronal membranes is the important determinant for its neurotoxicity. Since AβP seldom binds to normal neuronal membranes with neutral phospholipids such as PC usually existing on the outer surfaces of plasma membranes, it would be less likely to occur in the brains of normal and young subjects. However, when the asymmetrical distribution is disrupted by apoptotic conditions or aging and negatively charged phospholipids such as PS appear on the outer membrane surfaces, AβPs can bind to membrane surfaces (Figure 7(a)). Furthermore, considering that AβPs have affinity to PS in inner membrane surfaces, the intraneuronal accumulation of AβPs may be more toxic [131]. Gangliosides also contribute to the net charge of the outer membrane surface and to the binding to AβPs (Figure 7(b)). Microcircumstances on the membranes, such as lipid rafts, provide suitable locations which facilitate this process from (A) to (B). After incorporation into the membrane, the conformation of AβPs change and the accumulated AβPs aggregate on the membranes. The ratio of cholesterol to phospholipids in the membrane may alter membrane fluidity, thereby affecting these processes. Finally, aggregated AβP oligomers form ion channels leading to the various neurodegenerative processes (Figure 7(C)).

The velocity of channel formation will be regulated by the binding of AβP on membranes and its concentration. Considering that soluble oligomers are more toxic compared to monomer or fibrils [26, 27, 65], it is provable that

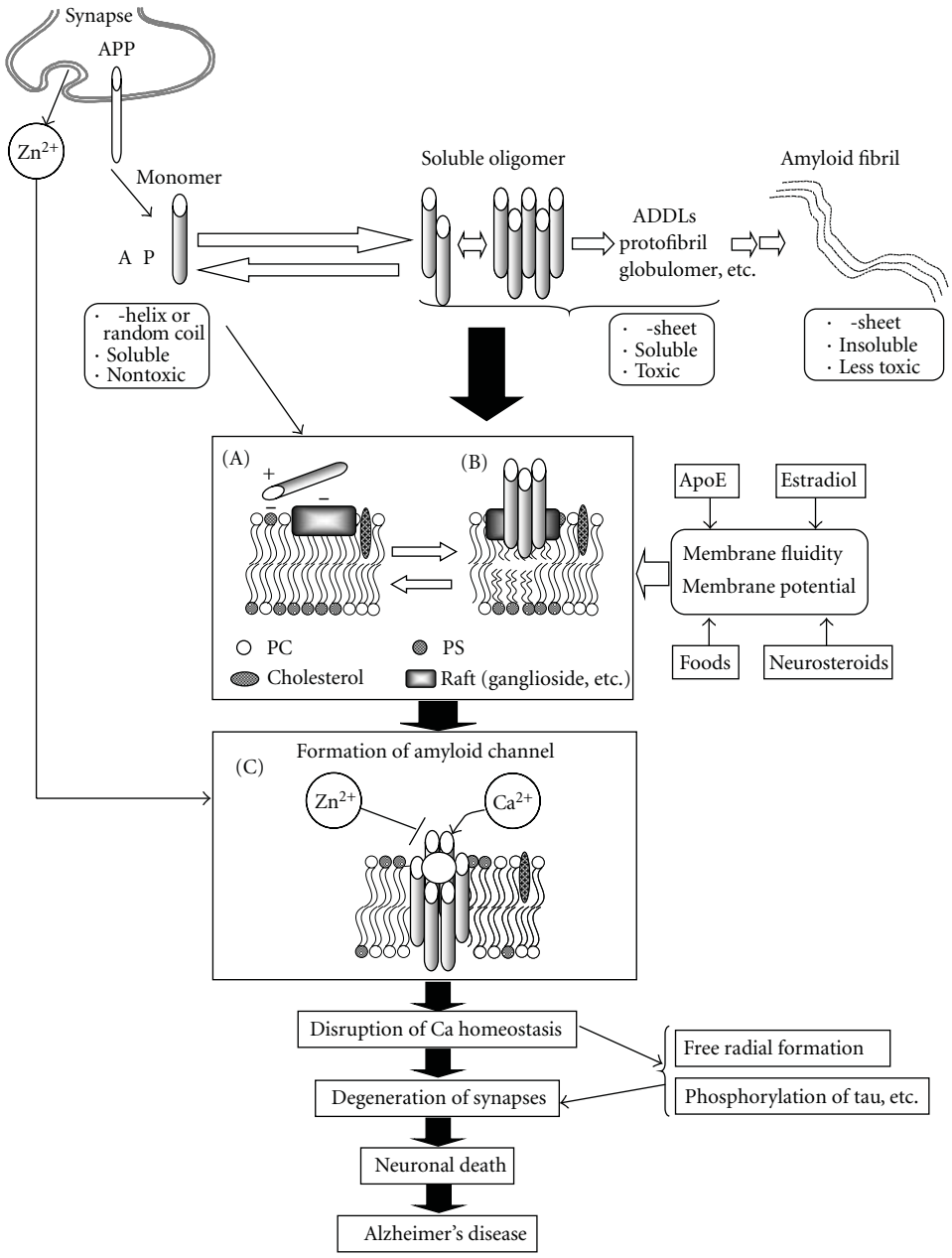

Figure 7: Hypothesis concerning amyloid channels and pathogenesis of Alzheimer's disease. AβPs are secreted from APP in synapses, directly incorporated into membranes. The possible hypothetical scheme of the formation of oligomeric amyloid channels is depicted. Details are shown in the text.

AβP oligomerization *in vitro* accelerates the velocity from (A) to (B), and enhances the formation of tetrameric or hexameric pores on membranes. Indeed, O'Nuallain et al. demonstrated that AβP dimers formed toxic protofibrils more rapidly compared to monomer [132]. However, the proposed structures of AβP channels in membrane mimic conditions are not always similar to the structures formed in the solution such as protofibrils or soluble oligomers. Thus, the conformational changes in membranes may also be significant.

These processes required for channel formation ((A) to (C)) may require a long life span in general and determine the rate of the entire process. Unlike endogenous Ca^{2+} channels, these AβP channels are not regulated by usual blockers. Thus, once formed on membranes, a continuous flow of $[Ca^{2+}]_i$ is initiated.

Disruption of calcium homeostasis triggers several apoptotic pathways and promotes numerous degenerative processes, including free radical formation and tau phosphorylation, thereby accelerating neuronal death. The source of Ca^{2+} may be from extracellular or intracellular Ca^{2+} store (ER or mitochondoria). Considering that presenilins are involved in the capacitive calcium entry, in Ca^{2+} homeostasis in ER or in mitochondoria [46–49] and the implication of ER stress in

AD and other neurodegenerative diseases [133], mutations of presenilins may influence these pathways. Free radicals also induce membrane disruption, by which unregulated calcium influx is further amplified. The disruption of calcium homeostasis influences the production and processing of APP. Thus, a vicious spiral of neurodegeneration is initiated. Meanwhile, zinc ions, which are secreted into synaptic clefts in a neuronal activity-dependent manner, inhibit AβP-induced Ca^{2+} entry, and thus have a protective function in AD.

This hypothesis explains the long delay in AD development; AD occurs only in senile subjects despite the fact that AβPs are also normally secreted in younger or in normal subjects. AD is multifactorial disease. Various environmental factors, such as foods (cholesterol contents) or trace metals, as well as genetic factors will influence these processes and contribute to AD pathogenesis. The amyloid channel hypothesis could explain effects of environmental factors such as cholesterol and other various aspects of AD pathogenesis and may aid in improving a precise understanding of AD and in the development of drugs for AD treatment. Although the findings of channel-like structures *in vivo* [66, 68], it is difficult to determine whether these amyloid channels really exist in the brains of AD patients. Therefore, further *in vivo* studies are necessary.

Abbreviations

AD: Alzheimer's disease,
AβP: Alzheimer's β-amyloid protein
AFM: Atomic force microscopy
AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APP: Amyloid precursor protein
D-APV: 2-amino-5-phosphonovalerate
BACE: β-site APP cleaving enzyme
ER: Endoplasmic reticulum
LTP: Long-term potentiation
NFT: Neurofibrillary tangles
$[Ca^{2+}]_i$: Intracellular calcium levels
NMDA: N-methyl-D-aspartate
ROS: Reactive oxygen species
SDS: Sodium dodecyl sulfate
TTX: Tetrodotoxin.

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Research Article

E22Δ Mutation in Amyloid β-Protein Promotes β-Sheet Transformation, Radical Production, and Synaptotoxicity, But Not Neurotoxicity

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Oligomers of 40- or 42-mer amyloid β-protein (Aβ40, Aβ42) cause cognitive decline and synaptic dysfunction in Alzheimer's disease. We proposed the importance of a turn at Glu22 and Asp23 of Aβ42 to induce its neurotoxicity through the formation of radicals. Recently, a novel deletion mutant at Glu22 (E22Δ) of Aβ42 was reported to accelerate oligomerization and synaptotoxicity. To investigate this mechanism, the effects of the E22Δ mutation in Aβ42 and Aβ40 on the transformation of β-sheets, radical production, and neurotoxicity were examined. Both mutants promoted β-sheet transformation and the formation of radicals, while their neurotoxicity was negative. In contrast, E22P-Aβ42 with a turn at Glu22 and Asp23 exhibited potent neurotoxicity along with the ability to form radicals and potent synaptotoxicity. These data suggest that conformational change in E22Δ-Aβ is similar to that in E22P-Aβ42 but not the same, since E22Δ-Aβ42 exhibited no cytotoxicity, unlike E22P-Aβ42 and wild-type Aβ42.

1. Introduction

Alzheimer's disease (AD) is characterized by amyloid deposition in senile plaques that are mainly composed of 40- and 42-mer amyloid β-proteins (Aβ40 and Aβ42) [1, 2]. These proteins are secreted from amyloid precursor protein (APP) by two proteases, β- and γ-secretases [3]. Aβ42 plays a more critical role in the pathogenesis of AD than Aβ40 because of its stronger aggregative ability and neurotoxicity [3]. Oxidative stress is believed to contribute to neuronal loss in AD [4–6]; one of the proposed mechanisms of Aβ42-induced neurotoxicity is related to the radicalization at both Tyr10 and Met35 accompanied by the generation of hydrogen peroxide [7, 8]. On the other hand, soluble oligomeric assembly of Aβ causes cognitive impairment and synaptic dysfunction in AD [9, 10].

Our previous investigation using solid-state NMR together with systematic proline replacement proposed a toxic conformer with a turn at positions 22 and 23 in Aβ42 aggregates and a nontoxic conformer with a turn at positions 25 and 26; the former showed a potent ability to aggregate, form oligomers, and exhibit neurotoxicity [11]. The turn formation at positions 22 and 23 along with the neighboring β-sheet structure in the toxic conformer of Aβ42 brought Tyr10 and Met35 close together to generate the *S*-oxidized radical cation at Met35, the ultimate toxic radical species, through oxidation by the phenoxy radical at Tyr10 produced by redox reactions [7, 12]. The mutations of Aβ are concentrated at

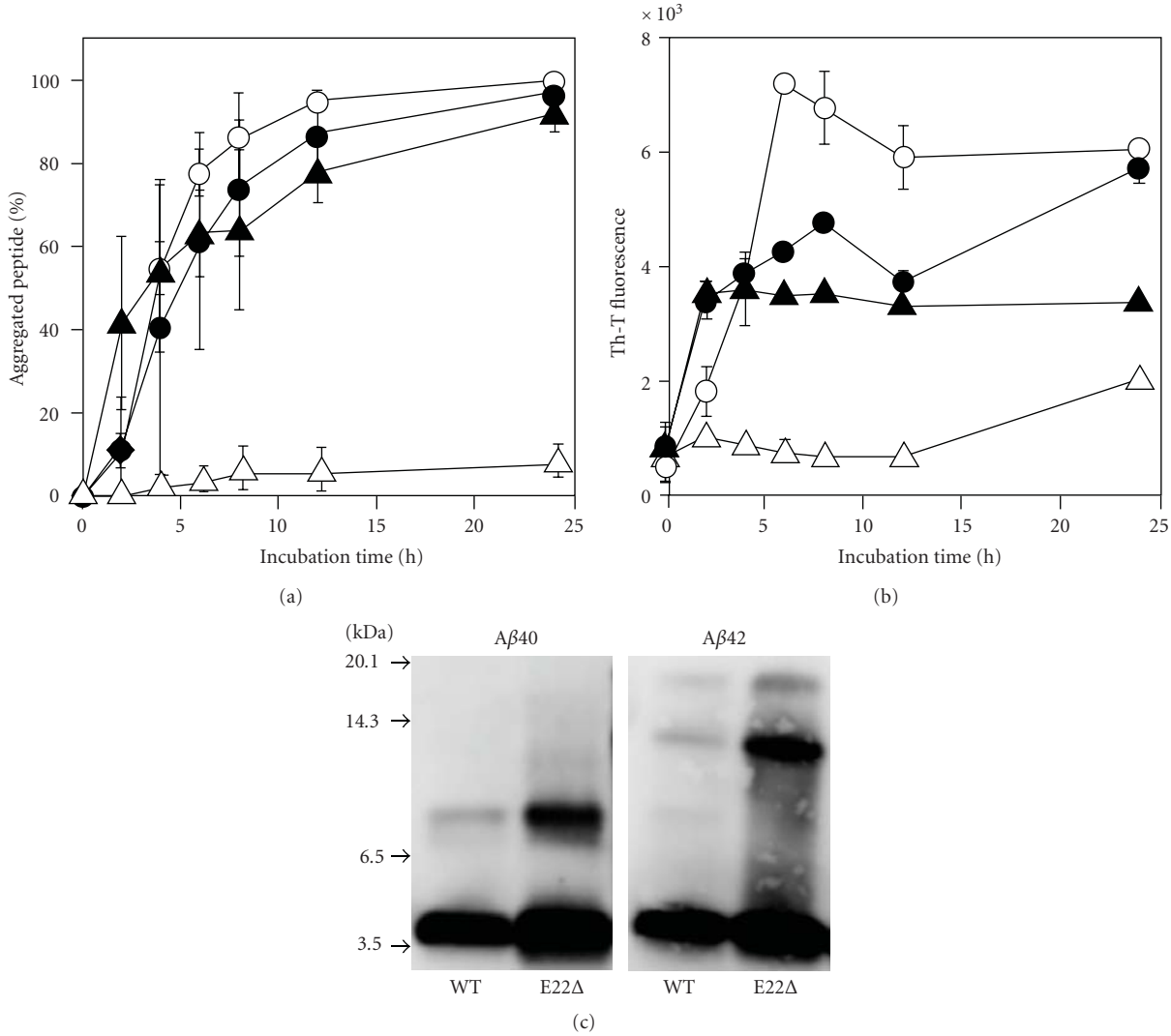

Figure 1: Aggregation profiles of E22Δ-Aβ40 and E22Δ-Aβ42 (25 μM) after incubation at 37 C. (a) Sedimentation assay estimated by HPLC analysis after centrifugation. (b) Th-T fluorescence assay. , Aβ42; , Aβ40; •, E22Δ-Aβ42; ▲, E22Δ-Aβ40. (c) Western blotting without incubation.

positions 21, 22, and 23; A21G (Flemish), E22G (Arctic), E22Q (Dutch), E22K (Italian), and D23N (Iowa) types. These mutations may play a pathological role in cerebral amyloid angiopathy (CAA) or familial AD (FAD) because these mutant proteins induced neuronal death *in vitro* more potently than wild-type Aβ42 [13]. Thus, Glu22 and Asp23 in Aβ are considered to be key residues for neurotoxicity through the formation of radicals.

Recently, Mori and coworkers reported that a novel mutation, in which the Glu-22 residue is defective (E22Δ), induced AD-type dementia without amyloid deposition, and that *in vitro* E22Δ-Aβ42 favorably formed low-molecular weight oligomers to inhibit long-term potentiation (LTP) compared with Aβ42 [14] and to induce synaptic alteration [15]. Therefore, the effects of the deletion at Glu22 on the secondary structure, formation of radicals, and neurotoxicity are interesting from the standpoint of discussing the role of the Glu-22 residue of Aβ42 in the pathogenesis of AD.

This paper describes a comprehensive study of the aggregative ability, secondary structure, radical-generating activity, neurotoxicity in primary rat cortical neuronal cell cultures, and the inhibitory activity of LTP of both E22Δ-Aβ40 and E22Δ-Aβ42. These results were compared with those of E22P-Aβ42 with a turn at positions 22 and 23.

2. Materials and Methods

2.1. Preparation of E22Δ-Aβ. E22Δ-Aβ40 and E22Δ-Aβ42 were synthesized by the method reported previously [16]. Their molecular weights were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS): E22Δ-Aβ40 (*m/z*: calcd: 4201.76; found:

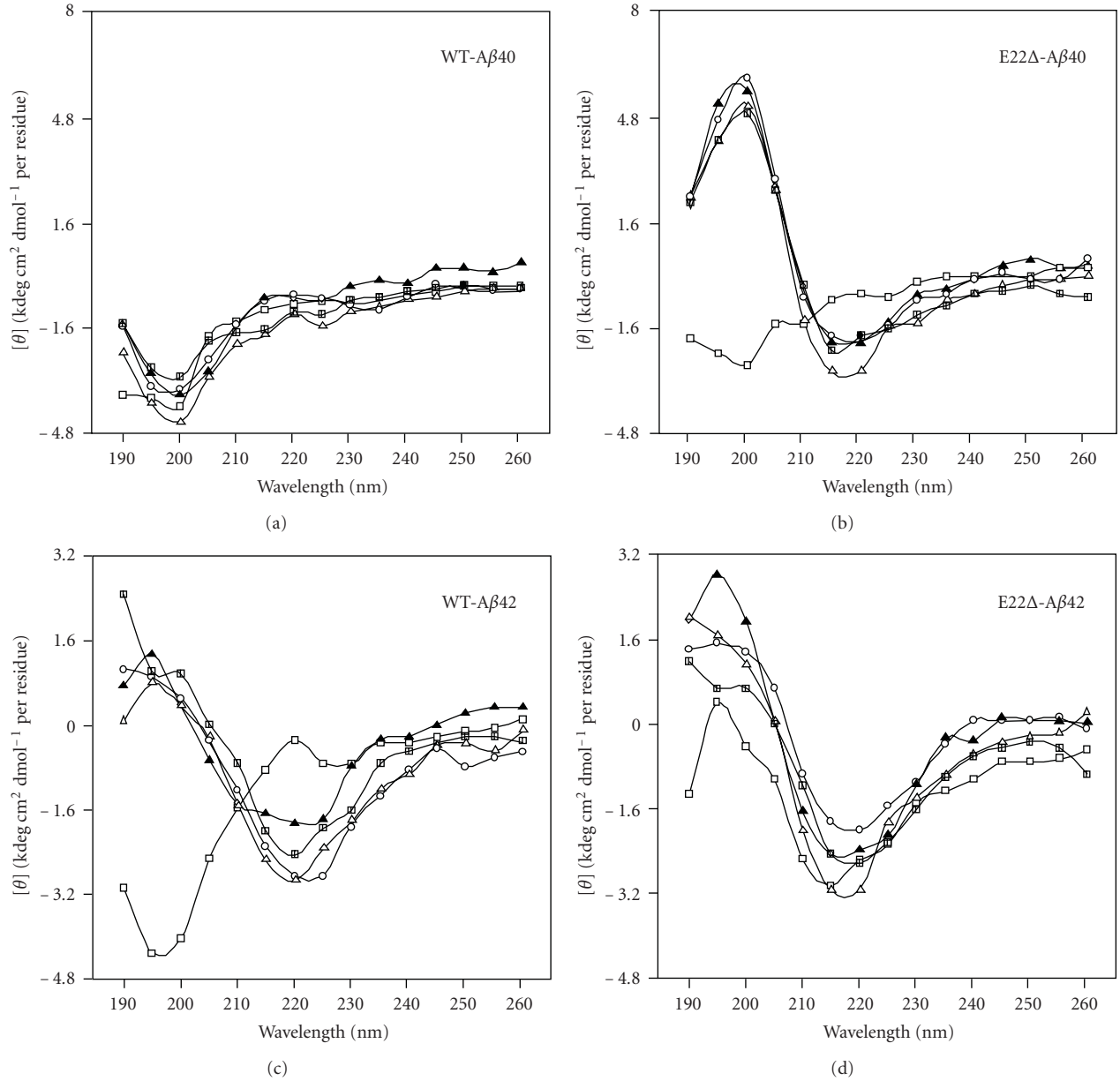

Figure 2: CD spectra of E22Δ-Aβ40 and E22Δ-Aβ42 (25 μM). (a) Aβ40, (b) E22Δ-Aβ40, (c) Aβ42, (d) E22Δ-Aβ42. Each Aβ (25 μM) was incubated in phosphate buffer at 37 C for the following times: □, 0 h; ▲, 4 h; , 8 h; , 24 h; ■, 48 h.

4201.56 $[M + H]^+$), E22Δ-Aβ42 (*m/z*: calcd: 4386.00; found: 4385.98 $[M + H]^+$).

2.2. Sedimentation Assay. The aggregation kinetics of each Aβ (25 μM) was estimated with the sedimentation assay using HPLC. The experimental procedure was described elsewhere [13]. The area of absorption at 220 nm was integrated and expressed as a percentage of the control.

2.3. Thioflavin T (Th-T) Fluorescence Assay. Aggregative ability of each Aβ (25 μM) was evaluated by the Th-T method developed by Naiki and Gejyo [17]. The measurement was performed on a Multidetection Microplate Reader powerscan HT (Dainippon Sumitomo Pharma) at room temperature, as described elsewhere [13]. Fluorescence intensity was measured at 450 nm excitation and 482 nm emission.

2.4. Western Blotting. Gel electrophoresis using 10–20% Tricine gel (Invitrogen, Carlsbad, CA) and Western blots analysis were carried out according to the manufacturer's protocol. The experimental procedure was described elsewhere [12]. Briefly, each Aβ was dissolved in 0.1% NH_4OH at 250 μM. After a 10-fold dilution by 50 mM sodium phosphate containing 100 mM NaCl at pH 7.4, the resultant peptide solution (25 μM) was incubated for 0, 2, or 4 hr at

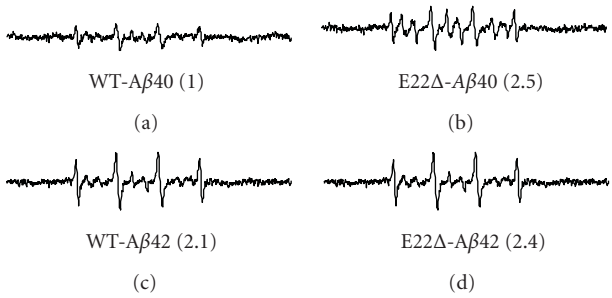

Figure 3: ESR spectra of E22Δ-Aβ40 and E22Δ-Aβ42 (100 μM) after 48-hr incubation at 37 C. (a) Aβ40, (b) E22Δ-Aβ40, (c) Aβ42, (d) E22Δ-Aβ42. The spectra of Aβ are shown after subtraction of the background spectrum in the presence of PBN without Aβ. Numbers in parentheses represent relative integral intensities of ESR signals, where the intensity of Aβ40 was taken as 1.0.

37 C. The anti-N-terminus of Aβ antibody, 82E1, (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) was used at 1 μg/mL as the primary antibody.

2.5. CD Spectrometry. Each Aβ was dissolved in 0.1% NH_4OH at 250 μM and diluted 10 times with 50 mM phosphate buffer (pH 7.12). The procedure was described elsewhere [16].

2.6. ESR Spectrometry. A reliable method for estimating the ability of Aβ (100 μM) to produce radicals using ESR was developed by Butterfield's group [18]. ESR spectrometry was performed on an EMX ESR spectrometer (Bruker BioSpin K.K., Karlsruhe, Germany) at room temperature, as described elsewhere [19].

2.7. Estimation of Cell Survival. To evaluate the neurotoxicity of Aβ using an MTT assay, we used undifferentiated PC12 cells, which have the potential to differentiate into neural cells, are sensitive to Aβ, and are generally used for detecting neurotoxicity as a neurotoxicity model [20]. The experimental procedure was described elsewhere [15].

2.8. Preparation of Primary Culture and Estimation of Cell Survival. Near-pure neuronal cultures were obtained from the cerebral cortices of fetal rats (17–19 days of gestation) as described [21, 22]. Cultures were maintained in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum or 10% heat-inactivated horse serum at 37 C in a humidified 5% CO_2 atmosphere. To prevent the proliferation of nonneural cells, 10 μM cytosine β-arabinofuranoside hydrochloride was added after 5 days of plating. In all experiments mature cells used after 11–13 days *in vitro*. Animals were treated in accordance with the guidelines of the Kyoto University animal experimentation committee and the guidelines of the Japanese Pharmacological Society.

Each Aβ was dissolved in 0.02% NH_4OH at 200 μM and diluted on ice immediately before treatment. After 48 hr treatment, neurotoxicity was evaluated by lactate dehydrogenase (LDH) release assay and MTT assay.

2.9. Long-Term Potentiation. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 region of rat hippocampal slices (Wistar rats, male, 6 weeks old) by electrically stimulating the Schaffer collateral [23]. Hippocampal slices were soaked in E22Δ-Aβ40, E22Δ-Aβ42, and E22P-Aβ42 solution [20 μg/200 mL phosphate-buffered saline (PBS)] before high-frequency stimulation (5 trains consisted of four 100-Hz pulses with an intertrain interval of 200 ms). fEPSPs were measured in the presence and absence of each Aβ.

3. Results and Discussion

3.1. Aggregative Ability of E22Δ Mutants. E22Δ-Aβ40 and E22Δ-Aβ42 were examined for their aggregative ability by a sedimentation assay: HPLC analysis after centrifugation of each Aβ solution. Both E22Δ-Aβ40 and E22Δ-Aβ42 aggregated at a velocity similar to Aβ42, while Aβ40 hardly aggregated even after 24-hr incubation (Figure 1(a)). This suggests that the ability to form aggregates of both E22Δ-Aβ40 and E22Δ-Aβ42 would be comparable to that of Aβ42 though soluble Aβ assemblies (oligomers) could not be distinguished from high-molecular weight fibrils in this assay condition (centrifugation: 20,000 g × 10 min). In the Th-T assay, which can estimate the β-sheet structure in Aβ aggregates [17], E22Δ-Aβ40 showed higher fluorescence than Aβ40. In contrast, the maximum fluorescence of E22Δ-Aβ42 did not exceed that of Aβ42, although the velocity of E22Δ-Aβ42 showing fluorescence was slightly higher than that of Aβ42 (Figure 1(b)). These data suggest that the E22Δ mutation accelerates the aggregation of Aβ.

Western blotting was carried out to estimate accurately the oligomerization state of Aβ. E22Δ-Aβ42 formed trimers exclusively, but E22Δ-Aβ40 produced dimers immediately after incubation (Figure 1(c)), as did the cases in the paper by Tomiyama et al. [14]; however, our Th-T assay results do not coincide with their results [14]; under Tomiyama's conditions, both mutants showed almost no fluorescence, even after 7 days. This discrepancy of the Th-T test may be due to the different conditions to make aggregates, presumably resulting in the generation of oligomers containing a β-sheet structure, as Ishii and coworkers suggested [24, 25].

3.2. Secondary Structure of E22Δ Mutants. To investigate the secondary structure of E22Δ-Aβ40 and E22Δ-Aβ42, their CD spectra were measured. In the control experiment using Aβ42 (Figure 2(c)), the positive peak at 200 nm and the negative peak at 220 nm gradually increased during the 48-hr incubation, suggesting that transformation of the random organization into a β-sheet structure occurred, while Aβ40 remained mainly random (Figure 2(a)). In contrast, E22Δ-Aβ42 formed a β-sheet-rich structure immediately after dissolution (Figure 2(d)). The velocity of the transformation of E22Δ-Aβ40 was also higher than that of Aβ42 (Figure 2(b)).

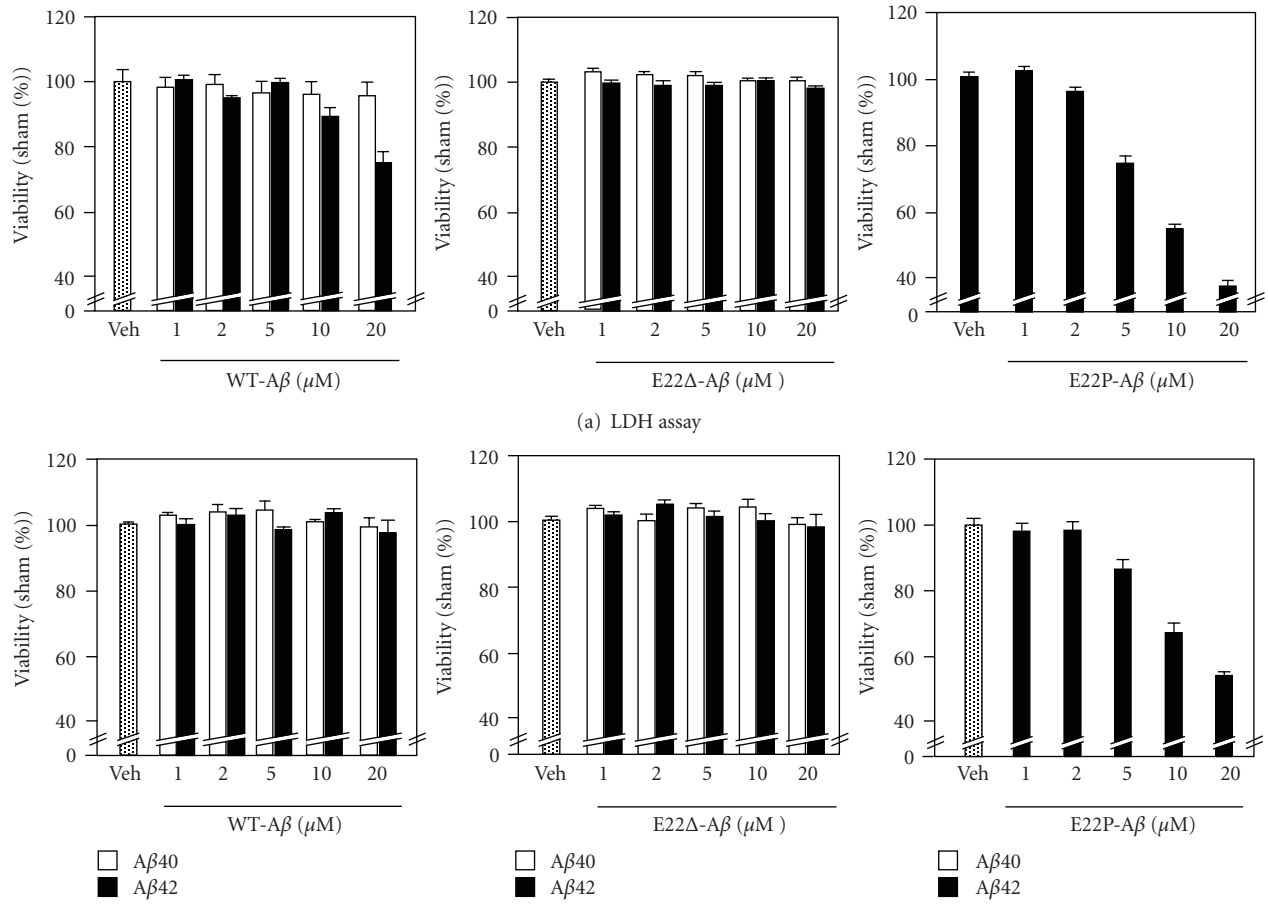

Figure 4: Neurotoxicity of Aβ40, Aβ42, E22Δ-Aβ40, E22Δ-Aβ42, and E22P-Aβ42 with the indicated concentration (1, 2, 5, 10, and 20 μM) using primary rat cortical neuronal cell cultures after 48-hr incubation at 37 C. Data are expressed as the mean ± s.e.m. $P < .05$ *versus* vehicle, $P < .01$ *versus* vehicle. Veh: vehicle.

These results suggest that the E22Δ mutation induces β-sheet transformation to form Aβ oligomers under our condition.

3.3. Radical Production by E22Δ Mutants. Our previous studies suggested that the radical productivity of Aβ42 mutants at position 22 such as E22P-, E22K-, E22Q-, and E22G-Aβ42 correlated with their aggregative ability and neurotoxicity [7]. To investigate the effect of E22Δ mutation in Aβs on the radical-generating activity, ESR was measured using phenyl-*N*-*tert*-butylnitrone (PBN) as a spin-trapping reagent (Figure 3). ESR signals of E22Δ-Aβ40 were twice more potent than those of Aβ40, and E22Δ-Aβ42 also showed slightly stronger signals than Aβ42. The radical productivity of the E22Δ-Aβs correlated basically with their ability to form oligomers and a β-sheet structure (Figures 1(c), 2).

3.4. Neurotoxicity of E22Δ-Aβs in Primary Rat Cortical Neuronal Cell Cultures. Having demonstrated that E22Δ mutation enhanced the β-sheet structure and radical productivity, we assessed the effect of this mutation on the neurotoxicity in primary rat cortical neuronal cell cultures by LDH and MTT assay (Figure 4). Treatment of the neurons with 1–20 μM of wild-type Aβ42 for 2 days induced neurotoxicity in a dose-dependent manner in the LDH test (Figure 4(a), left), in which the released LDH of the damaged cells (mainly neurons) was measured in the medium. E22P-Aβ42 with a turn at positions 22, and 23 induced stronger damage to the neurons than wild-type Aβ42; cell viability was less 40% at 20 μM (Figure 4(a), right). On the other hand, the difference in cell viability between the vehicle and wild-type Aβ42 did not reach a significant level in the MTT assay even at 20 μM (Figure 4(b), left). The cell viability of E22P-Aβ42 in MTT was also about 50% at 20 μM. In the MTT assay, total cells containing neurons, astrocytes, and microglia damaged by Aβs were counted. Since the neurons are more sensitive to damage than astrocytes or microglia in the primary cell cultures [26], the "neurotoxicity" estimated by the LDH test is often stronger than that evaluated by the MTT test.

It is worth noting that E22Δ-Aβ40 and E22Δ-Aβ42 as well as Aβ40 at 20 μM failed to show neurotoxicity against the primary cultures both in the LDH and MTT tests (Figure 4). These results are consistent with those reported by Takuma

et al.; the neurotoxicity of E22Δ-Aβ42 was very weak against mouse neuroblastoma Neuro-2a and human neuroblastoma IMR-32 [15]. In our MTT test using rat neuroblastoma PC12 cells, the IC_{50} of E22Δ-Aβ42 and wild-type Aβ42 was 4.6 ± 1.1 μM and 0.65 ± 0.11 μM, respectively, showing that E22Δ-Aβ42 was significantly less toxic than wild-type Aβ42. The neurotoxicity of E22Δ-Aβ40 (IC_{50} = 10 ± 1.0 μM) was weak as expected, but slightly stronger than that of Aβ40 (IC_{50} = 20 ± 1.0 μM).

3.5. Synaptotoxicity of E22Δ Mutants. Selkoe and coworkers suggested that Aβ dimers are the smallest synaptotoxic species inhibiting the LTP in the pathogenesis of AD and that plaque cores are largely inactive but sequester and release dimers [27]. Tomiyama et al. reported the more potent inhibition of LTP by E22Δ-Aβ42 than by wild-type Aβ42 [14]. We tested the inhibition of LTP by E22Δ-Aβ40 using rat hippocampal slices. Figure 5 shows that E22Δ-Aβ40 is not such a potent inhibitor of LTP as E22Δ-Aβ42, whose inhibitory potency was stronger that of than wild-type Aβ42, as Tomiyama et al. reported [14]. This coincides with the previous datas that the 42-mer Aβ showed more potent neurotoxicity than 40-mer Aβ [13]. Notably, E22P-Aβ42, which can more readily form a toxic conformer with a turn at positions 22 and 23 than wild-type Aβ42 [11], inhibited the LTP more strongly than E22Δ-Aβ42 at an almost undetectable level after 60 min (Figure 5(b)). This suggests that the conformation at positions 22 and 23 of E22P-Aβ42 might be similar to that of E22Δ-Aβ42 at positions 21 and 23.

3.6. Relevance of E22Δ Mutation to Turn-Induced Neurotoxicity. The present results suggest that E22Δ mutation in Aβ accelerates the transformation of a random form into a β-sheet structure (Figure 2) and radical productivity (Figure 3) but does not increase neurotoxicity in primary rat cortical neuronal cell cultures (Figure 4). E22Δ-Aβ42 synthesized in our laboratory showed the significant formation of oligomers (Figure 1) and synaptotoxicity (Figure 5), as reported by Mori and coworkers [14]. In addition, E22P-Aβ42 inhibited LTP more severely than E22Δ-Aβ42 (Figure 5). We previously reported that E22P-Aβ42, with a turn at positions 22 and 23 as a Pro-X corner (X: variable amino acid residue) [28], could form significant oligomers [11] with a β-sheet-rich structure [16] and radicals to result in potent neurotoxicity with the formation of radicals [7]; therefore, E22Δ-induced synaptotoxicity might be in part related to turn-induced radical formation. This implies that conformational change in E22Δ-Aβ is similar to that in E22P-Aβ42, but is not the same since E22Δ-Aβ42 exhibited no neurotoxicity, unlike E22P-Aβ42 and wild-type Aβ42.

It should be noted that the effects of E22Δ mutation on the physicochemical properties of Aβ40 are significantly higher than those of Aβ42. This tendency is similar to cases of other CAA or FAD mutant Aβs. We previously reported a comprehensive study on the aggregation, neurotoxicity, and secondary structure of Aβ mutants at positions 21–23 (A21G, E22G, E22Q, E22K, and D23N) [13]. Since Aβ40

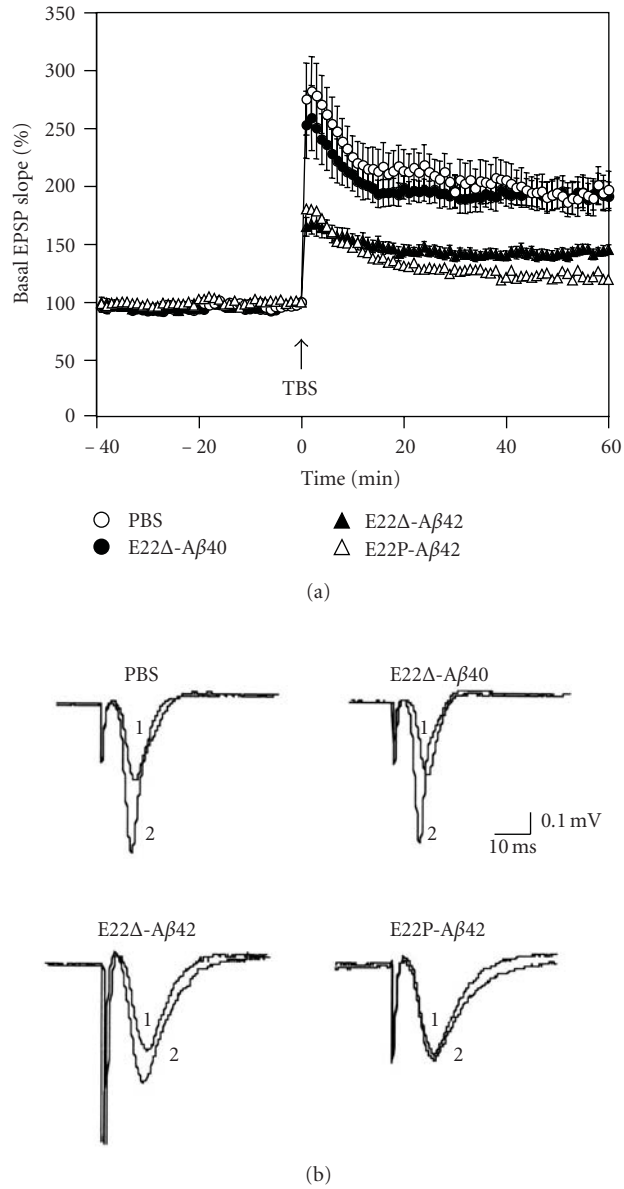

Figure 5: *In vivo* synaptotoxicity of E22Δ-Aβ40, E22Δ-Aβ42, and E22P-Aβ42 estimated by LTP expression. (a) Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 region of rat hippocampal slices (Wistar rats, male, 6 weeks old) by delivering theta burst stimulation (TBS) to the Schaffer collateral/commissural pathway. LTP was induced by high-frequency stimulation (5 trains consisted of four 100-Hz pulses with an intertrain interval of 200 ms) in the presence and absence of each Aβ (20 μg/200 mL PBS), to be injected into the lateral ventricle 20 min before stimulation. Each point on the graph represents the mean ± s.e.m. of basal fEPSP slope (0 min); n = 12 for PBS, n = 9 for E22Δ-Aβ40, n = 10 for E22Δ-Aβ42, and n = 10 for E22P-Aβ40. $P < .0001$ versus PBS; $P = .4258$ between PBS and E22Δ-Aβ40. $P < .0001$ versus PBS, when fEPSP slopes were compared with 1 to 60 min after TBS. The means between the four groups were compared using analysis of variance followed by Fisher's protected least significant difference (PLSD) test. , PBS; •, E22Δ-Aβ40; ▲, E22Δ-Aβ42; , E22P-Aβ42. (b) Typical field excitatory postsynaptic potentials at (1) 0 and (2) 60 minutes after TBS.

is secreted in neurons about nine times more abundantly than Aβ42 [3], in some cases Aβ40 may play a critical role in the pathogenesis of CAA or FAD. In addition, the E22Δ mutant of Aβ40 [14] as well as the CAA- or FAD-related Aβ40 mutants at positions 21, 22, and 23 have been reported to be more resistant than wild-type Aβ40 against degradation by insulin-degrading enzyme [29]; however, it remains controversial whether E22Δ is a familial type of AD or AD-type dementia.

Mori, Tomiyama, and coworkers implied the intracellular accumulation of Aβ oligomers using cultured cells [30] and their own developed mouse model [31] with E22Δ mutation. This mutation also caused apoptosis induced by stress in the endoplasmic reticulum [30]. Quite recently, we proposed the involvement of a turn at positions 22 and 23 of Aβ in intracellular amyloidosis [32]. Thus, the increase of radical productivity by E22Δ mutation is in good agreement with the turn-induced oxidative stress of Aβ42 [7], presumably *via* the interplay between Tyr10 and Met35 [12]. The deletion mutation of the residue at position 22 might promote Tyr10 in close proximity to the sulfur atom of Met35, inducing the effective production of radicals.

4. Conclusion

In summary, E22Δ-Aβ42 effectively induced the transformation of a random form to a β-sheet structure and the formation of radicals accompanied with oligomerization. However, the molecular mechanism of the pathology of AD of E22Δ-Aβ42 might be different from that of wild-type Aβ42 since E22Δ-Aβ42 showed more potent synaptotoxicity but weaker neurotoxicity than wild-type Aβ42.

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Research Article

Cell Surface Binding and Internalization of Aβ Modulated by Degree of Aggregation

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The amyloid peptides, Aβ40 and Aβ42, are generated through endoproteolytic cleavage of the amyloid precursor protein. Here we have developed a model to investigate the interaction of living cells with various forms of aggregated Aβ40/42. After incubation at endosomal pH 6, we observed a variety of Aβ conformations after 3 (Aβ^{3}), 24 (Aβ^{24}), and 90 hours (Aβ^{90}). Both A$\beta 42^{24}$ and A$\beta 40^{24}$ were observed to rapidly bind and internalize into differentiated PC12 cells, leading to accumulation in the lysosome. In contrast, A$\beta 40/42^{90}$ were both found to only weakly associate with cells, but were observed as the most aggregated using dynamic light scattering and thioflavin-T. Internalization of A$\beta 40/42^{24}$ was inhibited with treatment of monodansylcadaverine, an endocytosis inhibitor. These studies indicate that the ability of Aβ40/42 to bind and internalize into living cells increases with degree of aggregation until it reaches a maximum beyond which its ability to interact with cells diminishes drastically.

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disorder resulting from the deposition of Alzheimer β-Amyloid peptide (Aβ) as senile plaques, the appearance of neurofibrillary tangles, and selective neuronal loss. The most abundant forms of Aβ are 40 and 42 amino acid residues long and referred to as Aβ40 and Aβ42, respectively [1].

The endocytic pathway has been implicated in the extracellular secretion of Aβ40 and Aβ42 [2, 3]. These peptides are derived from the endoproteolytic cleavage of the Amyloid precursor protein (APP) with β-secretase, followed by γ-secretase. β-secretase cleavage occurs in the acidic late endosomes [4, 5]. After γ-secretase cleavage, Aβ40 or Aβ42 is free in the endosomal lumen [6]. The endosomal contents can be either secreted from the cell [7–9] or transferred to the lysosome [10].

The endosome has been found to be quite acidic (pH 6) with the recycled endosome slightly less acidic (pH 6.5) [11, 12]. Exposure of Aβ to endosomal pH conditions has been found to induce various conformational and oligomeric states [13–15]. Many oligomeric forms of Aβ have been proposed and characterized as intermediates in the pathway to forming the Amyloid fibre. Some of these structures include trimers, pentamers, high molecular weight Aβ-derived diffusible ligands (ADDLs), protofibrils, and fibrils [16–21].

Here we present a method for generating a mixed population of Aβ conformations using model endocytic conditions. Using this method, we demonstrate that when Aβ is exposed to endosomal conditions for an extended period of time, the ability of the peptide to bind and internalize into living rat adrenal pheochromocytoma (PC12) cells increases with time until it reaches a maximum beyond which its ability to interact with PC12 cells diminishes drastically.

2. Materials and Methods

2.1. Peptide Synthesis and Purification. Aβ40 and Aβ42 were synthesized and purified as described previously [22]. Before cleavage from the resin, the fluorophore, Nα-(9-Fluorenylmethoxycarbonyl)-Nε-tetramethylrhodamine-(5-carbonyl)-L-lysine (Molecular Probes, Eugene, OR) (abbreviated TMR), was coupled to the N-terminus via a glycine linker. The crude peptides were purified by HPLC using a Superdex Tricorn 10/300 GL Peptide column (Amersham Biosciences, Piscataway, NJ) with 30 mM NH_4OH running buffer. To maintain stock peptide solutions free from fibril seeds, solutions were stored at pH 10 and 4 C immediately after chromatographic separation of monomeric peptides. Aβ preparations were never lyophilized, as this process may allow for seeds to form. These solution conditions have been previously shown to maintain the monomeric state [16, 23, 24]. Peptide purity and identity was confirmed using both MALDI mass spectrometry and amino acid analysis. Concentrations of stock peptide solutions were determined using amino acid analysis and confirmed by either tyrosine absorbance (275 nm, $\varepsilon = 1390\,\text{cm}^{-1}\,\text{M}^{-1}$) or TMR absorbance for labelled peptides (550 nm, $\varepsilon = 92000\,\text{cm}^{-1}\,\text{M}^{-1}$). At least three separate synthesized lots of Aβ were used in this study and each displayed identical cell association rates when compared for quality assurance. Oligomeric samples were prepared by diluting stock Aβ samples to 30 μM with 30 mM NH_4OH and reducing to pH 6 with 0.2 M HCl and incubating for zero (Aβ^0), 3 (Aβ^3), 24 (Aβ^{24}), or 90 hours (Aβ^{90}) in the dark at 20 C.

2.2. Dynamic Light Scattering (DLS). Hydrodynamic radius (Rh) measurements were made at 20 C with a DynaPro DLS instrument (Protein Solutions Inc., Piscataway, NJ). Peptide samples (30 μM) were reduced to pH 6 using 0.2 mM HCl, centrifuged at 12000 × g for 3 minutes and then rapidly added to a 1 cm path length cuvette and left in the instrument. DLS data was collected at various time points over 90 hours. Particle translational diffusion coefficients were calculated from decay curves of autocorrelation of light scattering data and converted to hydrodynamic radius (Rh) with the Stokes-Einstein equation. Histograms of intensity versus Rh were calculated using Dynamics data analysis software (Protein Solutions Inc., Piscataway, NJ).

2.3. Filter Assay. Aβ samples (30 μM) were either not filtered or spin-filtered for 30 minutes at 14,000 × g using 10, 30, 100 kDa (Amicon ultra cellulose KMWO), or 0.1 μm (Amicon PVDF) spin filters. Absorbance at 550 nm was collected on a Molecular Devices SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA) and graphs were created normalizing the absorbance signal from each filtered sample to the corresponding unfiltered sample.

2.4. Thioflavin-T Assay. Fluorescence measurements were obtained using 200 μL of 30 μM Aβ samples, within a 96-well plate, after addition of 5-fold molar excess of thioflavin-T and incubation at room temperature for 30 minutes. Emission at 485 nm was collected using 440 nm excitation on a Molecular Devices SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA).

2.5. Cell Culture. PC12 cells were maintained in DMEM/F12 containing 10% fetal bovine serum (HyClone, Logan, UT) with 100 units/mL penicillin and 100 μg/mL of streptomycin. To induce differentiation of PC12 cells and for cell imaging, they were plated at 2.2×10^4 cells/cm^2 in Lab-tech chambered cover glass chambers and suspended in phenol red free DMEM/F12 containing N2 supplement and 10 ng/mL NGF. Cells were differentiated for 72 hours before media was replaced and peptide treatments (final concentration 1.5 μM) were performed. Cells were maintained at 37 C in a humidified incubator with 5% carbon dioxide.

2.6. Analytical Ultracentrifugation. TMR-labelled Aβ samples (6 μM) were prepared in phenol red free DMEM/F12 media containing N2 supplement and 10 ng/mL NGF. Either fresh media or cultured supernatants, obtained from cell culture after 72 hours, were used. To avoid interference from cell culture components, molecular weights of TMR-labelled Aβ were obtained by selective monitoring of TMR absorbance at 550 nm. Sedimentation experiments were performed at 20 C on a Beckman XLI analytical ultracentrifuge using an AN50-Ti rotor. Molecular weights were calculated using Beckman XLI data analysis software in which absorbance versus radial position data were fitted to the sedimentation equilibrium equation using nonlinear least-squares fitting.

2.7. Confocal Microscopy. Three-dimensional stacks of fluorescence micrographs were taken at 20 C with a confocal laser-scanning system consisting of an LSM 510 Zeiss META NLO confocal microscope with a C-APO 40X water immersion objective (numerical aperture 1.2) and HeNe laser with a 543 nm laser line. The displayed images were captured using Zeiss LSM Image version 4 and prepared using ImageJ version 1.37v and represent a single cross-section through the cells.

2.8. Inhibition of Endocytosis. Differentiated PC12 cells were treated with 50 μM monodansylcadaverine (MDC) for 20 minutes at 37 C with 5% carbon dioxide. After inhibitor treatment, Aβ^{24} (final concentration 1.5 μM) was added to the media containing MDC and imaged after 4 hours at 37 C. Aβ^{24} (final concentration 1.5 μM) was also added to differentiated PC12 cells media for 4 hours at either 37 C or 4 C prior to imaging.

2.9. Intracellular Localization. Differentiated PC12 cells were first treated with Aβ^{24} (final concentration 1.5 μM) for 6 hours at 37 C followed by treatment with 40 nM Mito-Tracker Deep Red and 50 nM LysoTracker Green DND-26 for 20 minutes. After treatment, the media was exchanged with fresh phenol red free DMEM/F12 media containing N2 supplement and 10 ng/mL NGF with 1.5 μM DAPI for nuclear staining. Cells were imaged using Argon laser with 488 nm laser line for LysoTracker, HeNe laser with 633 nm

laser line for MitoTracker, HeNe Laser with 543 nm laser for TMR-labelled Aβ^{24}, and a tunable Chameleon laser at 730 nm for two-photon excitation of DAPI. 2D histograms and correlation coefficients were determined using Image J version 1.42q with colocalisation threshold plugin [25].

2.10. Toxicity. Differentiated PC12 cell media was replaced with media containing 0.6 to 20 μM Aβ^{0}, Aβ^{24}, Aβ^{90}, or 10 μM Melitin as a positive control for 48 hours. Cell survival was quantified using the Sulforhodamine B assay [26], and absorbance was measured at 560 nm using Molecular Devices SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA). LC50 values were determined as the concentration of Aβ required to kill fifty percent of the cells from an absorbance versus Aβ concentration plot.

3. Results

3.1. Aβ Aggregation Is Mediated through Cell Interaction. To study the interaction between Aβ and live cells, we synthesized and fluorescently labelled Aβ40/42. The synthesized Aβ was maintained in solution from purification to storage and was never lyophilized, as these solution conditions are known to significantly reduce the formation of Aβ aggregation seeds [16, 23, 24]. We covalently attached tetramethylrhodamine (TMR) to the N-terminus of Aβ via a flexible glycine linker to generate TMR-Aβ. The N-terminus of Aβ is highly accessible even in the fibril state [21, 27, 28] and attaching a fluorescent label to this site has been shown to neither alter its amyloidogenic properties [16, 29, 30], nor its solubility behaviour [31, 32].

We have previously shown that treating cultured cells with 1.5 μM monomeric TMR-Aβ42 leads to the formation of visible aggregates on the surface of PC12 cells within one hour of treatment [29]. We initiated our current study by investigating whether Aβ aggregation could occur in cell culture media alone. Using analytical ultracentrifugation, we measured the molecular weights of Aβ40 and Aβ42 present in phenol red free cell culture medium, both freshly prepared and conditioned media taken from differentiated PC12 cells 3 days postdifferentiation. Following the addition of 6 μM Aβ to each medium and subsequent 24-hour incubation at room temperature, both media preparations were centrifuged to equilibrium at room temperature in an analytical ultracentrifuge in order to determine the molecular weight of Aβ conformations. The molecular weights of Aβ40 and Aβ42 from both media preparations were measured to be approximately 4103 Da and 4425 Da, respectively. These values both correspond to the expected monomeric molecular weights of Aβ, falling within the 95% confidence intervals of 3650–4570 Da for Aβ40 and 3650–4770 Da for Aβ42. Thus, the aggregation of Aβ seen by confocal microscopy apparently occurs only after interaction with the differentiated PC12 cells and not with cultured supernatants. It should be noted that the concentration previously used to treat cells (1.5 μM) and the concentration used for ultracentrifugation (6 μM) are considerably lower than the reported 20 to 50 μM range required for *in vitro* aggregation [33–35].

3.2. Aβ Oligomerizes at Endosomal pH. We have previously shown that Aβ40 and Aβ42 aggregate significantly at pH 6 [29]. Since Aβ40 and Aβ42 are generated through endoproteolytic cleavage [7–9], and the pH of the endosome and recycled cellular vesicles is equivalent to pH 6 [12], we characterized the Aβ conformations formed under endosome conditions. Aβ (30 μM) was reduced to pH 6, and hydrodynamic radius calculations were collected after zero (Aβ^{0}), 3 (Aβ^{3}), 24 (Aβ^{24}), and 90 (Aβ^{90}) hours using dynamic light scattering (Figure 1). The average hydrodynamic radius of Aβ40 was found to increase from 2.0 nm at time zero (Aβ40^{0}) to 216 nm after 90 hours at pH 6 (Aβ40^{90}). A more striking increase was found with Aβ42, beginning with 1.7 nm at time zero (Aβ42^{0}) to 451 nm after 90 hours at pH 6 (Aβ42^{90}). For each of these samples, the development of increasingly higher ordered aggregates was observed over time and the samples that were treated for 90 hours contained particles over 1000 nm in radii.

To further investigate the relative levels of peptide aggregation at endocytic pH, we filtered the Aβ40 and Aβ42 samples through various molecular weight cutoff (MWCO) spin filters (Figure 2). Approximately 67% of Aβ40^{3}, 60% of Aβ40^{24}, and 55% of Aβ40^{90} were recovered through the 10 kDa MWCO spin filter and approximately 80% were recovered through the 100 kDa MWCO filter, except for Aβ40^{90} with only 60% recovered. In contrast, only 55% of Aβ42^{3}, Aβ42^{24}, and Aβ42^{90} were recovered through the 10 kDa filter and approximately 60% were recovered through the 100 nm filter. These results indicate that the majority of peptide conformations present under these conditions were able to pass through a 10 kDa molecular weight filter, but that just over 40% could not be recovered through the 100 nm filter for the Aβ40^{90}, Aβ42^{3}, Aβ42^{24}, and Aβ42^{90} samples.

We also used thioflavin-T to assess the time-dependence of the extent of Amyloid fibril formation at endocytic pH. Thioflavin-T is a dye known to shift its fluorescence from 430 nm to 490 nm upon binding specifically to the cross-β-structure of Amyloids but not to monomeric or small oligomeric complexes [36, 37]. We observed enhanced thioflavin-T fluorescence at all time points (Figure 3); however, Thioflavin-T bound most strongly to Aβ40^{90}, Aβ42^{24}, and Aβ42^{90}. The high thioflavin-T binding to Aβ^{90} samples suggests that these late stage Aβ conformations are the most aggregated.

3.3. Endocytic Aβ Undergoes Rapid Cellular Interaction. Using confocal microscopy, cell surface association and internalization of peptides can be monitored. We have previously shown that monomeric Aβ42 associates with cells more rapidly than Aβ40, with significant staining observable after six hours of treatment [29]. To determine whether the aggregation state of Aβ affects cell association, we exposed each of the Aβ40 and Aβ42 samples to differentiated PC12 cells and monitored the kinetics of association by confocal microscopy. Upon treating cells with Aβ40^{24}, the cell surface association was observed within one hour of treatment (Figure 4). Moreover, Aβ40^{24} was observed to significantly internalize into these cells after only 6 hours, whereas significant internalization of Aβ40^{0} was only visualized around

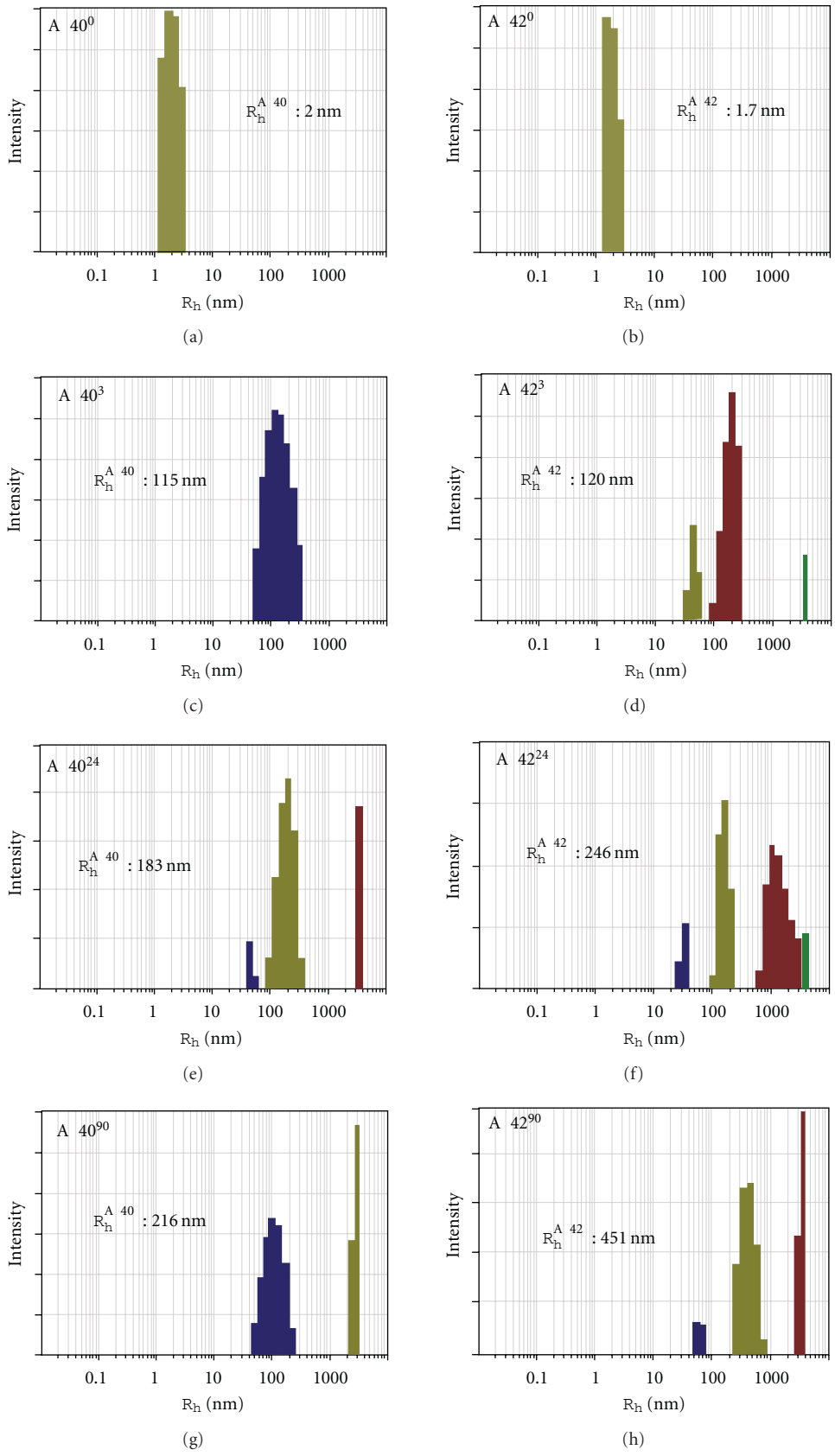


Figure 1: Dynamic light scattering of endocytic Aβ. A time-dependent increase in the average hydrodynamic radius (R_h) was observed with incubation of Aβ under endocytic conditions. At each time point, a variety of aggregates are present.

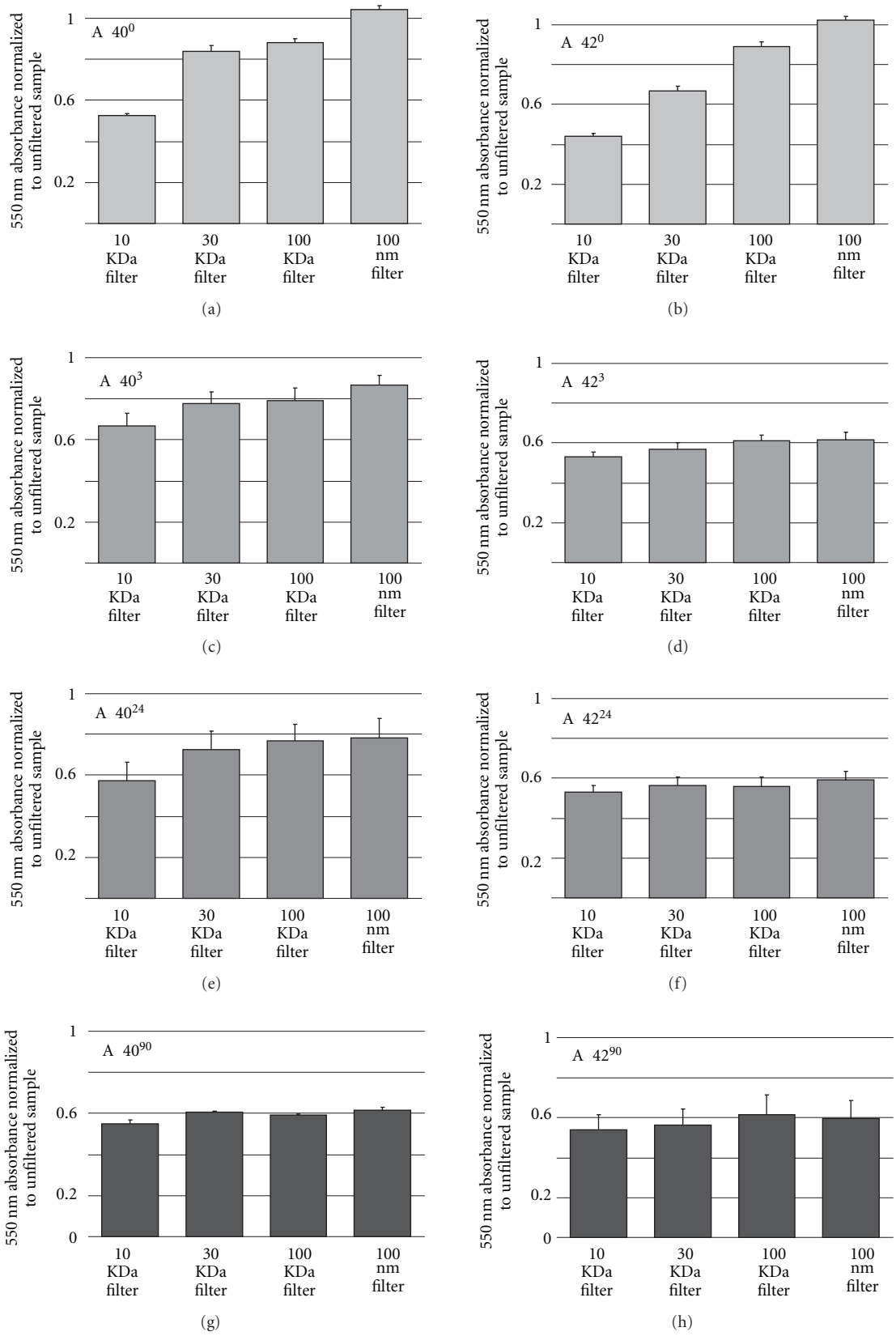


Figure 2: Separation of Aβ by ultrafiltration. Graphs show the TMR absorbance of the filtrates for each Aβ sample divided by the absorbance of the unfiltered sample at 550 nm. Error bars represent the range from two independent experiments.

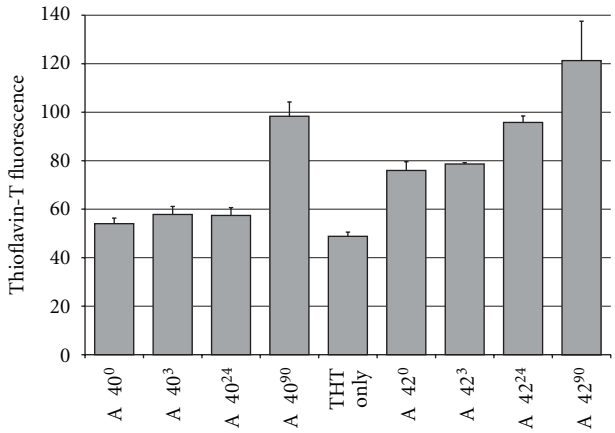


Figure 3: Thioflavin-T fluorescence of Aβ exposed to endocytic conditions. Significant thioflavin-T fluorescence for the Aβ40^{90} and Aβ42^{90} samples indicates the highest level of aggregation present. Error bars represent the range of two independent experiments. Statistical significance is indicated ( ) $P < .05$.

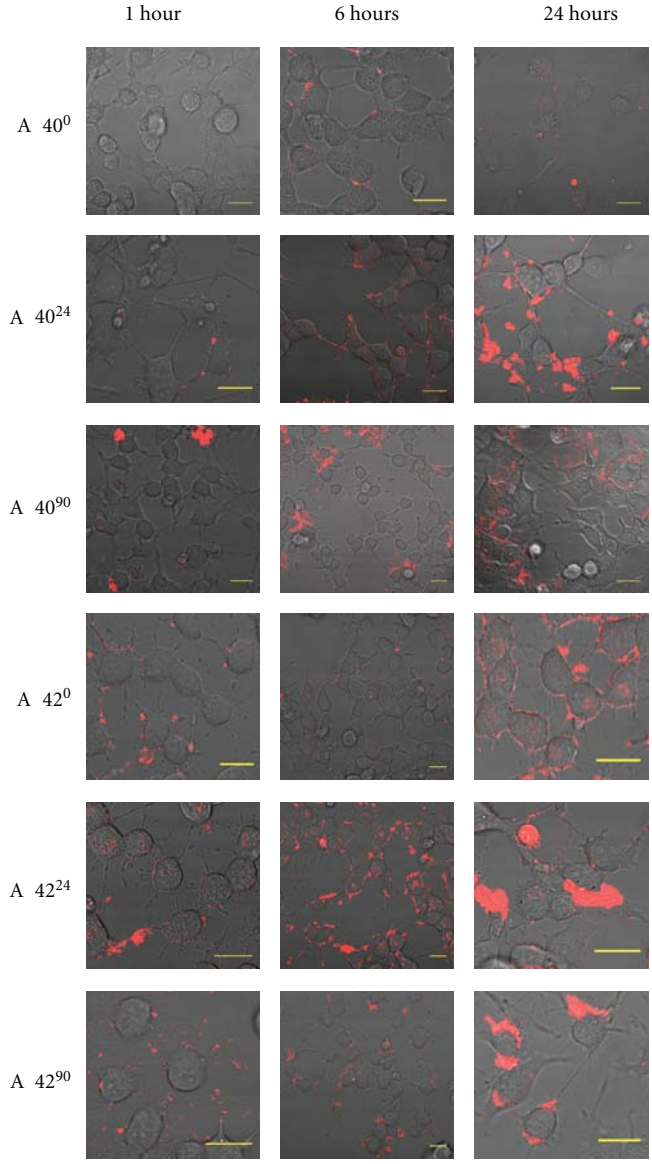


Figure 4: Confocal Microscopy images showing Aβ time course of cell entry. Confocal microscopy images of differentiated PC12 cells treated with various Aβ samples exposed to endosomal conditions after 1, 6, and 24 hours. All scale bars are 20 μm in length. Aβ40^{24} associates rapidly to differentiated PC12 compared to Aβ40^{0}, whereas Aβ40^{90} displays weak cell association over its treatment course. Aβ42^{24} displays rapid internalization compared to Aβ42^{0}. Aβ42^{90} displays reduced cell interaction compared to Aβ42^{0} and Aβ42^{24}.

24 hours (Figure 4). Similarly, Aβ42^{24} internalized into differentiated PC12 cells after only one hour of treatment, whereas Aβ42^{0} treatment only became observable at 24 hours (Figure 4). Interestingly, when differentiated PC12 cells were treated with late-stage Aβ40^{90} or Aβ42^{90}, very few cells underwent internalization or even exhibited cell surface interaction with these aggregated peptide forms (Figure 4). To illustrate the contrast between these treatments, we collected the three-dimensional image slices through a cell from the base to the apex for the 6-hour treatment with Aβ42^{0}, Aβ42^{24}, and Aβ42^{90} (Figure 5). Aβ42^{0} was only observed around the periphery of the cell, whereas some Aβ42^{24} was located inside the cell. By contrast, Aβ42^{90} treatment seemed to localize to extracellular regions and did not produce the punctate pattern as observed with the Aβ42^{0} sample.

To quantify the frequency of cells that exhibited peptide internalization, we randomly selected five fields of view from at least three separate 6-hour treatment experiments and plotted the percentage of cells having internalized Aβ (Figure 6). Approximately 25% of cells internalized Aβ40/42^{3}, whereas more than 90% of cells internalized Aβ40/42^{24}. In contrast, very few cells were found to internalize Aβ40/42^{90}. Since late Aβ40/42^{90} was found to have a large number of aggregates with hydrodynamic radius over 1000 nm (Figure 1) and were found to significantly bind thioflavin-T (Figure 3), then these aggregates may favour self-association over cell association.

3.4. Internalization of Aβ Is Mediated through Cellular Import Mechanisms. To determine if the internalization of endocytic Aβ is mediated through cellular processes, such as receptor-mediated endocytosis or through direct peptide-mediated processes like membrane pore formation, we monitored the effects of temperature and monodansylcadaverine (MDC) on internalization. MDC is a known inhibitor of receptor-mediated clathrin-dependent endocytosis [38, 39]. At physiological temperature (37 C), after a 4-hour treatment of cells with Aβ40^{24} and Aβ42^{24}, internalization was observed (Figure 7). At 4 C membrane vesicle formation is inhibited, preventing endocytosis of extracellular and cell surface components [40]. When Aβ40^{24} or Aβ42^{24} association with differentiated PC12 cells was monitored at 4 C, none of the cells were found to have internalized these peptides

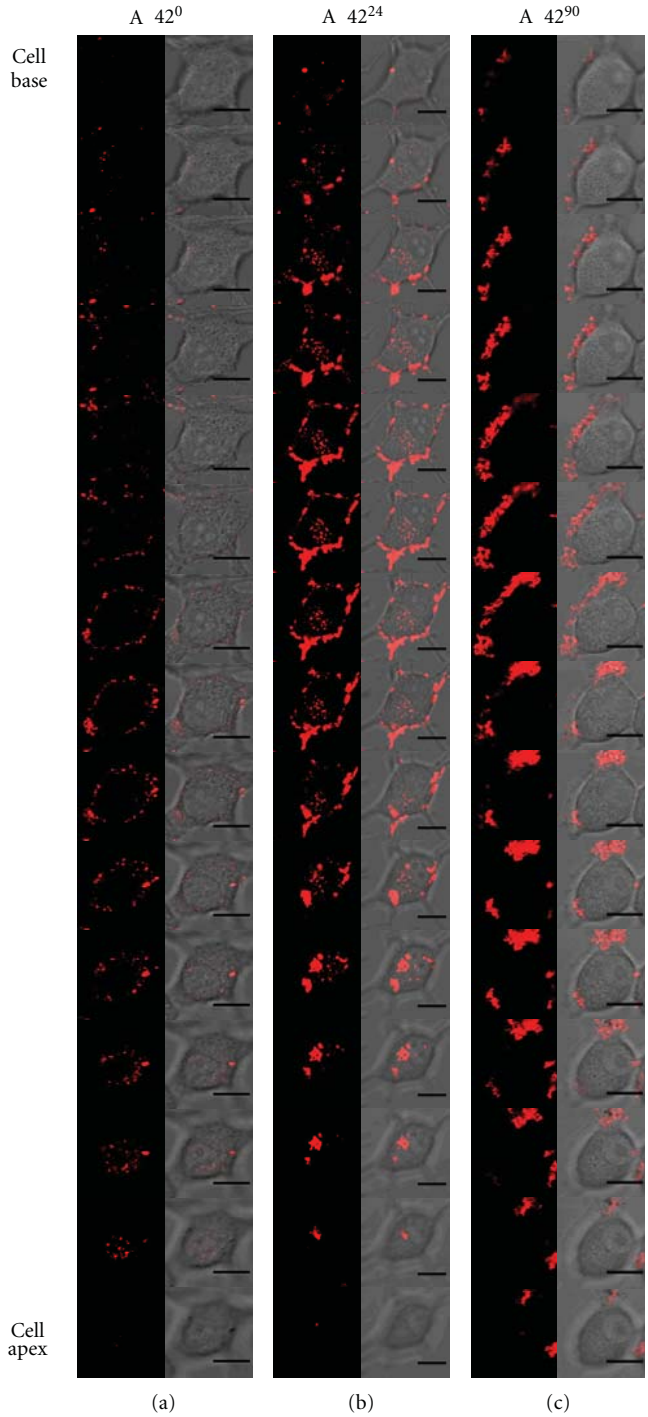


FIGURE 5: Image planes though a single cell for each Aβ42 treatment. Confocal microscopy images from differentiated PC12 cell base to cell apex for A$\beta42^{0}$, A$\beta42^{24}$, and A$\beta42^{90}$, with the TMR signal only (a) and TMR cell merged image (c). All scale bars are 10 μm in length.

(Figure 7). Similarly, when differentiated PC12 cells were treated with MDC, neither A$\beta40^{24}$ nor A$\beta42^{24}$ were observed within the cells. These observations indicated that Aβ^{24} was internalized through a cell-directed import mechanism, rather than an independent penetration route through the cell membrane.

3.5. Internalized Aβ Is Targeted to the Lysosome. The location of deposited intracellular A$\beta40/42^{24}$ was examined using intracellular organelle markers in differentiated PC12 cells. Cells treated with A$\beta40^{24}$ and A$\beta42^{24}$ for 6 hours were visualized using LysoTraker Green DND-26 for the lysosome, MitoTracker Deep Red for the mitochondria, and DAPI for

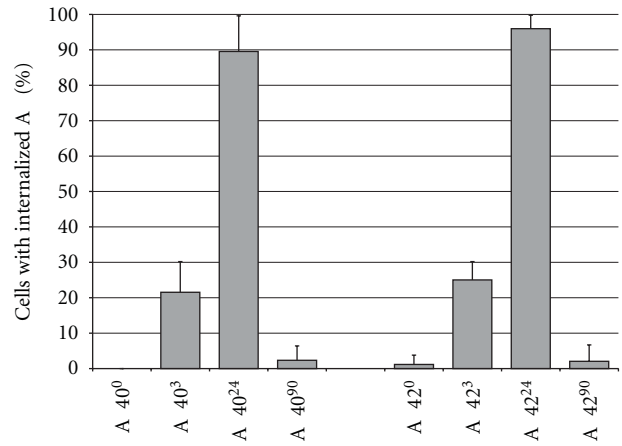


Figure 6: Percentage of PC12 cells with internalized Aβ. Aβ40^{24} and Aβ42^{24} were more significantly internalized into differentiated PC12 cells than the other forms of Aβ. Aβ40^{90} and Aβ42^{90} display a striking drop in the amount of internalization. Error bars represent the standard deviation from at least three individual experiments with $n > 200$ cells per condition. Statistical significance is indicated ( ) $P < .005$.

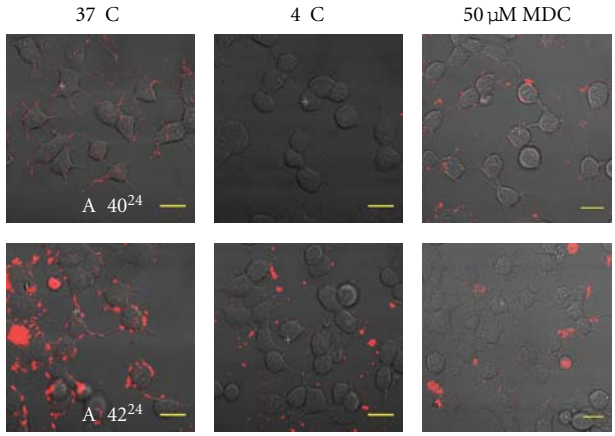


Figure 7: Inhibition of Aβ internalization. Confocal microscopy images of differentiated PC12 cells treated with Aβ40/42^{24} for 4 hours. All scale bars are 20 μm in length. Internalization was only observed at 37 C. Membrane integrity was maintained throughout all treatments, indicating that internalization is mediated through a cellular process such as clathrin-dependent endocytosis.

nucleus staining (Figure 8). The staining pattern of each cellular organelle marker in the same image plane as the Aβ40^{24} and Aβ42^{24} treatment was determined (Figure 8) and quantified (Figure 9). Only the signal from the lysosome marker costained with both internalized Aβ40^{24} and Aβ42^{24}. The red signal from the peptides costained well with green signal from the lysosomes, resulting in the yellow signal in the merged image. When quantified, 71% of the lysosome signal intensity colocalised with the Aβ40 channel and 80% with the Aβ42 channel (Figure 9). Whereas only 9% of the mitrochondria signal intensity colocalised with Aβ40 channel and less than 1% with the Aβ42 channel (Figure 9). These findings suggest that the internalized vesicles containing Aβ40/42^{24} are directed to the lysosome. The intensity of both Aβ40^{24} and Aβ42^{24} fluorescence signals was found to increase in the lysosomes over time, which may reflect the accumulation of Aβ40/42^{24} in the lysosome.

To determine the relative toxicity of these Aβ conformations, a cytotoxicity assay was performed where differentiated PC12 cells were treated with various peptide concentrations for 48 hours (Figure 10). We calculated the lethal concentration at which 50% of cells were killed (LC50), for each of the Aβ conformations. We found that Aβ40^{24} was moderately more toxic than Aβ40^{0}, with a lower LC50 value (Figure 10). Surprisingly, Aβ40^{90} was also found to have a lower LC50 value compared to Aβ40^{0}. Aβ42^{24} was also found to be moderately more toxic than Aβ42^{0}, whereas Aβ42^{90} had a much higher LC50 value compared to Aβ42^{0} (Figure 10).

4. Discussion

We have developed a method to produce a collection of Aβ conformations that differ in their extent of aggregation and investigated the interaction between these states of Aβ40 and Aβ42 and differentiated PC12 cells. Others have described the methods to isolate individual soluble oligomeric forms of Aβ, using various chemical reagents and protocols [41–44]. In addition, purified oligomeric Aβ molecules from cell culture [45, 46] or transgenic mice [47] have also been monitored. These purified sources of oligomeric Aβ offer great potential in understanding the progression of Aβ aggregation; however, they cannot be directly visualized over the time course of their effects on cells during maturation from earlier to later conformations. Since it is well established that Aβ monomers can oligomerize under the physiological pH of the endosome [13–15], and Aβ is formed through endoproteolytic cleavage, we have utilized this condition to determine which conformations are present and how these mixed conformations interact with differentiated PC12 cells. It is known that the extent of oligomerization/fibrillation is very dependent on experimental conditions. Necula et al. [48] have indicated that Aβ can be induced to form fibrils via dilution from 100 mM NaOH to neutral pH in the presence of 10 mM HEPES/100 mM NaCl buffer, while dilution into phosphate buffered saline results in oligomer formation. In our experiments, the peptide was diluted from 30 mM ammonium hydroxide (pH 10) to pH 6 with final condition of 1 mM ammonium chloride as the only additional chemical. We do note that the normal human physiological concentration of ammonium chloride in blood and cerebrospinal fluid is approximately 20 to 50 μM [49, 50], and that hyperammonemia has been linking to Alzheimer type II astrocytosis [51, 52].

When extracellular monomeric Aβ associates to the surface of cells, we speculate there are three possible outcomes: (1) it can act as a stable template to allow further Aβ aggregation; (2) the peptide can penetrate through the cell membrane depositing in the cytoplasm; or (3) the peptide may be internalized into the cell within endocytic vesicles, which would result in a reduction in the surrounding pH. In the third case, the endocytic vesicles containing Aβ can

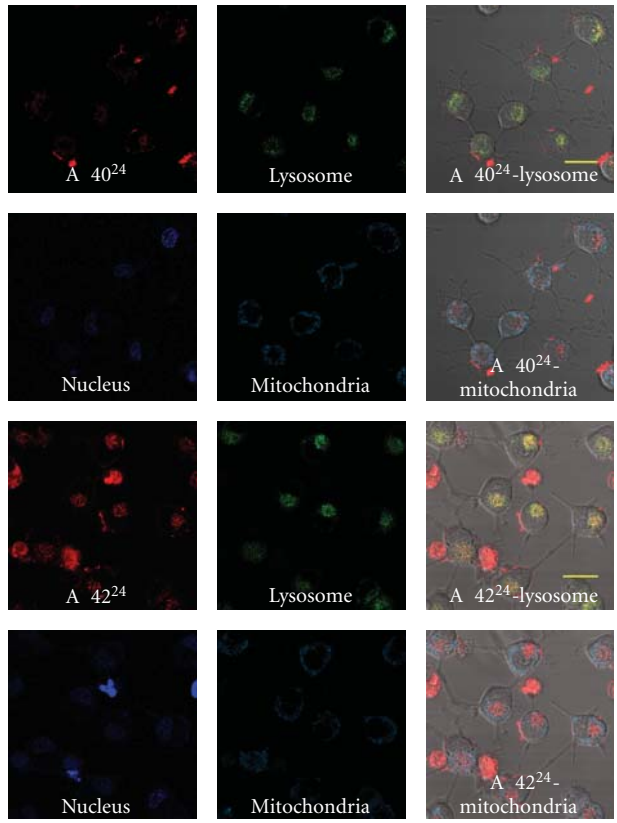
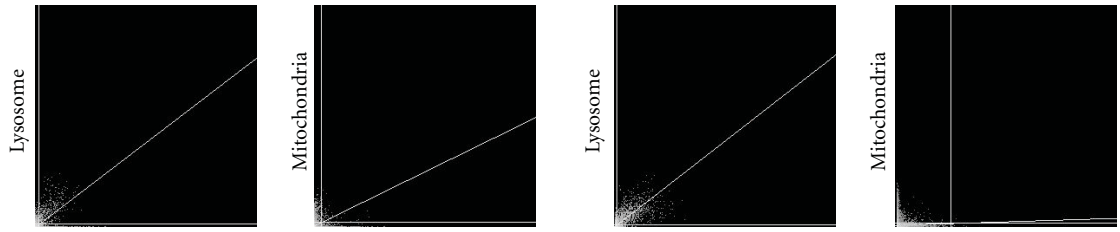


Figure 8: Intracellular localization of endocytic Aβ. Confocal microscopy images through a single plane of cells treated with either Aβ40^{24} or Aβ42^{24} and intracellular markers. All scale bars are 20 μm in length. Colocalization between the red Aβ40/42^{24} signal and the green lysosome signal is shown in yellow in the merged image. Colocalization was not observed with either mitochondrial or nuclear stains.



Ch1	Ch2	Pearson's correlation coe cient whole image	m	b	Ch1 threshold	Ch2 threshold	Pearson's correlation coe cient above threshold	Pearson's correlation coe cient below threshold	Mander's coe cient Ch1	Mander's coe cient Ch2	Mander's coe cient Ch1 threshold	Mander's coe cient Ch2 threshold	Ch1 colocalized %	Ch2 colocalized %	Ch1 intensity colocalized %	Ch2 intensity colocalized %
A 40	Lysosome	0.356	0.764	− 0 3	4	3	0.5323	0.007	0.5355	0.8996	0.4869	0.7578	43.72%	51.55%	43.77%	71.34%
A 40	Mitochondria	− 0 205	0.49	1.1	8	5	− 0 1885	0.009	0.2702	0.6454	0.1539	0.1302	11.33%	9.7%	7.82%	9.24%
A 42	Lysosome	0.782	0.781	− 1 1	4	2	0.4755	0.054	0.6235	1	0.5736	0.8464	63.02%	57.01%	51.34%	80.61%
A 42	Mitochondria	0.01	0.032	1.9	64	4	NA	0.009	0.4781	1	0.1681	0.0008	9.09%	0.07%	0.17%	0.04%

Figure 9: Quantitative colocalisation analysis of intracellular Aβ. Quantitative analysis indicates significant colocalisation of Aβ40^{24} and Aβ42^{24} with the lysosome marker within live cells.

theoretically be recycled back to the cell surface or directed to the lysosome where a further reduction in pH would occur. We have previously visualized oligomeric Aβ on the surface of neuronal cell lines [29], and here sought to determine first whether this aggregation occurred prior to cell surface deposition in the cell culture mediaor are aggregation and cell surface binding concomitant and linked processes. Using ultracentrifugation analysis of Aβ preparations either in freshly prepared cell culture media or in conditioned media removed from cultured cells after 3 days, we did not observe any conformation larger than the monomer. These results indicate that components in our cell media or secreted factors from cultured cells are not responsible for the observed Aβ aggregation present on the surface of neuronal cells. After adding monomeric Aβ to cells, we noted a maturation time for the visual appearance of Aβ on

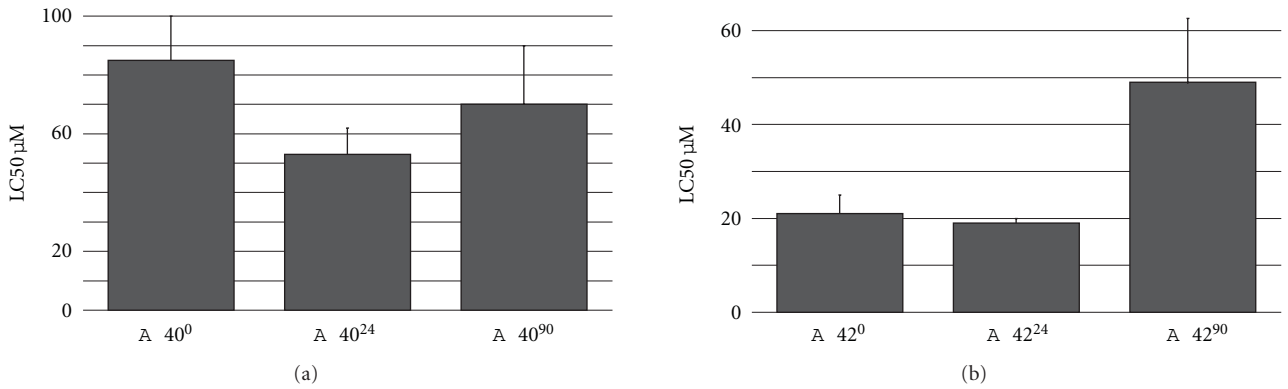


Figure 10: Relative toxicity of endocytic Aβ. Lethal concentration of endocytic Aβ required to kill 50% of differentiated PC12 cells at 48 hours is shown. Error bars represent the standard deviation from the mean using three replicates. Statistical significance is indicated ( ) $P < .05$.

the cell surface and in the cell interior (Figure 4; [29]). We postulate that the peptides on the cell surface might undergo a series of pH reductions through endocytic recycling, before becoming visible punctae on and inside the cell. Since pH 6 is approximately endocytic pH, we characterized the kinetic effects of this condition on peptide conformations. Exposing Aβ40 for up to 24 hours at pH 6, we observed an increase in the average hydrodynamic radius from 2.0 nm (A$\beta40^0$) to 183 nm (A$\beta40^{24}$) (Figure 1). In addition, approximately 60% of these peptides were recovered through a 10 kDa MWCO filter and 80% were recovered through a 100 kDa MWCO filter (Figure 2), and only minor thioflavin-T binding was found (Figure 3). Following exposure of up to 24 hours at pH 6, Aβ42 had an increase in the average hydrodynamic radius from 1.7 nm (A$\beta42^0$) to 246 nm (A$\beta42^{24}$) (Figure 1), approximately 55% were recovered through 10 kDa MWCO filter and only 60% were recovered through 100 nm filter (Figure 2) and more significant thioflavin-T binding was measured (Figure 3). Both Aβ40 and Aβ42 treated for 90 hours at pH 6 were observed to have large average hydrodynamic radius particles, with 40% of these samples being withheld by a 100 nm filter and both displayed significant thioflavin-T staining. Also, these early-and late-stage Aβ samples were observed to have an ensemble of Aβ conformations (Figure 1).

The interactions of these early-and late-stage Aβ conformations were monitored with differentiated PC12 cells. Whereas late stage A$\beta40^{90}$ and A$\beta42^{90}$ exhibited very few interactions with cells (Figures 4, 5) and internalized at a very low frequency (Figure 6), early stage A$\beta40^{24}$ and A$\beta42^{24}$ interacted with cells rapidly (Figure 4) and internalized into the majority of the cells present (Figure 6). Interestingly, A$\beta40^{24}$ showed a more rapid cellular association and internalization compared to A$\beta40^0$ (Figure 4). Similarly, A$\beta42^{24}$ was found to internalize more rapidly than A$\beta42^0$, indicating that a specific conformation may play a role in cell binding and internalization. Furthermore, our model of endocytic Aβ toxicity (Figure 10) is in agreement with previous findings utilizing chemically-produced oligomers of Aβ which showed that Aβ fibrils were less toxic than soluble oligomers [41, 53]. Our results with Aβ^{90} are also in agreement with studies that have indicated that regions with large Amyloid plaques do not correlate directly with regions of significant neuronal loss [54, 55].

Many studies have reported that certain oligopeptides are freely imported into cells. For example, the peptide sequences corresponding to Tat (48–60), penetratin, and oligoarginine are known to internalize into live cells. Cellular import of Tat (48–60) and penetratin was shown to be temperature dependent, indicating the possible role of endocytosis for internalization [56, 57]. In contrast, oligoarginine containing C-terminal tryptophan was shown to follow a nonendocytic pathway, independent of energy requirements and temperature [57]. Another type of internalization process has been studied in the bacterium *Clostridium septicum*, whose alpha toxin contains functional domains responsible for oligomerization and cellular pore formation. Using specific domains that bind cell surface receptors, alpha toxin monomers oligomerize to form pores in human cells and thus impose direct entry [58]. By exposing Aβ to endosomal conditions, we were able to observe internalization of A$\beta40/42^{24}$, which was inhibited at 4 C or with the endocytosis inhibitor MDC (Figure 7). Throughout the course of our confocal experiments, we did not observe any disruption of the cell membrane, which would have been expected if membrane channels or pores were being formed by Aβ. Thus, the structural conformation of Aβ that was observed to internalize into cells seems to follow a cell-mediated import mechanism.

Our results with the various Aβ conformations correlate directly with previous reports that indicate the deposition of Aβ in the lysosome [15, 59, 60] and with the previous study that internalized Aβ can persist undegraded for days [61]. From our findings taken together with previous studies, we present the following model for the interaction of Aβ with neuronal cells (Figure 11). As monomeric Aβ binds the cell surface, this event could lead to a conformational change allowing for the further catalytic aggregation of Aβ. Recently, it has been shown that oligomeric Aβ binds to GM1 ganglioside and alters physical properties of the plasma

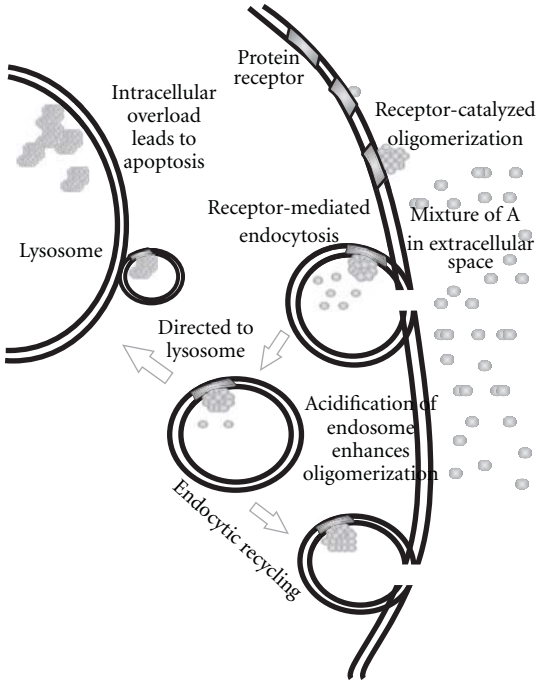


Figure 11: A plausible mechanism of Aβ neurotoxicity.

membrane which stimulates the amyloidogenic processing pathway of APP [62, 63]. Aβ that is bound to the cell surface may become internalized and recycled, allowing for acidification and further aggregation, which in turn may stimulate additional Aβ generation [62]. Once the surface Aβ reach some specific structural form, they may be internalized and directed to the lysosome for potential degradation. However, the reduced pH of the lysosome may instead cause further aggregation and persistence of Aβ, resulting in lysosomal overload and cell death. The recently reported high turnover rate of monomeric Aβ [64, 65] and the formation of large aggregate pools of Aβ may indicate that the human body uses these mechanisms to sequester and remove the toxic Aβ oligomers from neuronal cells. With age, the turnover rate for monomeric Aβ may slow down, which could then result in the initiation of our proposed mechanism and may contribute to the late onset of Alzheimer's disease. It is also possible that endocytosis of Aβ and lysosomal targeting are mechanisms by which the cell clears Aβ aggregates from the cell surface. Also, certain large aggregates of Aβ may just be too large for the cell to internalize, and they may represent the precursor for extracellular Amyloid formation.

5. Conclusion

Our study shows that using a simple method to generate various Aβ conformations, the rates of cellular interaction and targeting can be followed with live cell cultures. Using this model, we found that early endocytic conformations, rather than highly aggregated late forms, serve as the major contributors to rapid cell internalization. The mechanism of internalization likely involves a cell surface receptor-mediated process instead of peptide-mediated direct entry, resulting in accumulation in the lysosome. This method allows for conformation-specific therapeutics and conditions to be screened with live cells, circumventing the need to purify specific Aβ conformations.

Abbreviations

AD:	Alzheimer's disease
Aβ:	Amyloid β
ADDLs:	Aβ-derived diffusible ligands
APP:	Amyloid precursor protein
DAPI:	4 ,6-diamidino-2-phenylindole
DMEM/F12:	Dulbecco's modified eagle's medium: nutrient mixture F-12 1 : 1 mixture
D-PBS:	Dulbecco's phosphate-buffered saline
MDC:	Monodansylcadaverine
MWCO:	Molecular weight cut-off
NGF:	Nerve growth factor
PC12:	Rat adrenal pheochromocytoma
TMR:	Tetramethylrhodamine.

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Review Article

Aβ Internalization by Neurons and Glia

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In the brain, the amyloid β peptide (Aβ) exists extracellularly and inside neurons. The intracellular accumulation of Aβ in Alzheimer's disease brain has been questioned for a long time. However, there is now sufficient strong evidence indicating that accumulation of Aβ inside neurons plays an important role in the pathogenesis of Alzheimer's disease. Intraneuronal Aβ originates from intracellular cleavage of APP and from Aβ internalization from the extracellular milieu. We discuss here the different molecular mechanisms that are responsible for Aβ internalization in neurons and the links between Aβ internalization and neuronal dysfunction and death. A brief description of Aβ uptake by glia is also presented.

1. Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia in the elderly. The increase of the average age of the population is causing a significant rise in the number of people afflicted with this devastating disease, and it is predicted that the incidence of AD will approximately triplicate by 2040 [1] if more effective therapeutic strategies are not made available. In order to develop better therapeutic approaches, the molecular pathways leading to the pathological alterations of the disease must be fully understood.

Major neuropathological and neurochemical hallmarks of AD traditionally included the extracellular accumulation of amyloid-β peptide (Aβ) in brain senile plaques, the intracellular formation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated Tau protein, the loss of synapses at specific brain sites, and the degeneration of cholinergic neurons from the basal forebrain [2]. The original amyloid cascade hypothesis had proposed that the key event in AD development is the extracellular accumulation of insoluble, fibrillar Aβ [3–5]. This "extracellular insoluble Aβ toxicity" hypothesis was later modified to acknowledge the role of soluble Aβ oligomers as pathogenic agents. Only more recently the importance of intraneuronal Aβ accumulation in the pathogenesis of AD has been recognized, despite the fact that the original reports showing Aβ accumulation inside neurons are dated more than 20 years ago. The "intraneuronal Aβ hypothesis" does not argue against a role for extracellular Aβ but complements the traditional amyloid cascade hypothesis [6–8].

The intraneuronal pool of Aβ originates from APP cleavage within neurons and from Aβ internalization from the extracellular milieu. Here we focus on the mechanisms that mediate Aβ internalization in neurons and glia, and we discuss the consequences of Aβ uptake by brain cells.

2. Intraneuronal Aβ

Evidence from several immunohistochemical studies suggested the accumulation of intraneuronal Aβ in AD. Yet, the acceptance of this concept was hampered by the fact that in many studies, antibodies that could not distinguish between APP and Aβ inside the neurons were used. This problem and other experimental issues have been addressed in detail elsewhere [9–11]. Despite these initial technical complications, several studies using antibodies specific for $A\beta_{40}$ and $A\beta_{42}$ have confirmed the presence of intraneuronal Aβ and suggested a pathophysiological role for this Aβ pool [12–14]. In the past few years several excellent reviews have discussed the evidence available on accumulation of intracellular Aβ in brains of AD patients and animal models of AD and its impacts on pathogenesis of AD, synaptic impairment, and neuronal loss [6, 9, 11, 15–17]. Here we

just mention the most salient aspects of intracellular Aβ accumulation without reviewing the evidence exhaustively.

Intraneuronal accumulation of Aβ is one of the earliest pathological events in humans and in animal models of AD. Intraneuronal Aβ_{42} immunoreactivity precedes both NFT and Aβ plaque deposition [12, 13], and in the triple transgenic mouse model, Long-Term Potentiation (LTP) abnormalities and cognitive dysfunctions correlate with the appearance of intraneuronal Aβ, prior to the occurrence of plaques or tangles [18, 19]. Moreover, when Aβ is removed by immunotherapy, the intracellular pool of Aβ reappears before tau pathology [20]. Importantly, Aβ accumulation within neurons precedes neurodegeneration in nearly all the animal models in which intracellular Aβ and neuronal loss have been reported, and all models in which intracellular accumulation of Aβ was examined and was present showed synaptic dysfunction [21]. Studies in cultured cells also showed accumulation of intracellular Aβ [22–24].

The observation that cortical neurons that accumulate Aβ_{42} in brains of AD and Down syndrome patients are apoptotic [25, 26] and that microinjections of Aβ_{42} or cDNA-expressing cytosolic Aβ_{42} rapidly induce cell death of primary human neurons [27] indicated the importance of intracellular Aβ in neuronal death. In support of this notion, generation of transgenic mice harboring constructs that target Aβ either extracellularly or intracellularly has demonstrated that only intracellular Aβ-producing transgenic mice developed neurodegeneration [28]. Furthermore, a recent quadruple-mutant mouse has shown neuronal loss in association with intracellular accumulation of Aβ [29]. There is also mounting evidence that intracellular Aβ accumulation is associated with neuritic and synaptic pathology [24, 30, 31] and with alterations of synaptic proteins [32]. Besides, the internalization of Aβ antibodies reduced intraneuronal Aβ and protected synapses [33] as well as reversed cognitive impairment [19].

With respect to the specific form of Aβ that accumulates intracellularly, the use of C-terminal-specific antibodies against Aβ_{40} and Aβ_{42} in immunocytochemical studies of human brains with AD pathology, indicated that it is Aβ_{42} the peptide present within neurons [12, 13, 34–38]. Furthermore, using laser capture microdissection of pyramidal neurons in AD brains, Aoki and collaborators showed increased Aβ_{42} levels and elevated Aβ_{42}/Aβ_{40} ratio in neurons from sporadic as well as from familial cases of AD, whereas Aβ_{40} levels remained unchanged [39].

An interesting development of the "intracellular Aβ" cascade is the possibility that Aβ plaques would originate from death and destruction of neurons that contained elevated amounts of Aβ [13, 40, 41]. Indeed, the release of Aβ from intracellular stores by dying cells seems responsible for the reduction or loss of intraneuronal Aβ_{42} immunoreactivity in areas of plaque formation [12]. Recently, a model was presented in which internalized Aβ starts fibrillization in the multivesicular bodies (MVBs) upon spontaneous nucleation or in the presence of fibril seeds, thus penetrating the vesicular membrane causing cell death and releasing amyloid structures into the extracellular space [42].

The contribution of intracellular Aβ to formation of NFTs has also been proposed. The intracellular pool of Aβ associates with tangles [43], and intracellular Aβ may disrupt the cytoskeleton and initiate the formation of aggregated intracellular Tau protein [12]. Contrary to the concept that intracellular Aβ is linked to NFTs, one report found that intracellular Aβ is not a predictor of extracellular Aβ deposition or neurofibrillary degeneration, although in this study mostly an N-truncated form of Aβ was examined [14].

3. Origin of Intraneuronal Aβ

Based on the evidence presented above, it is now well accepted that two pools of Aβ exist in the brain: intracellular and extracellular. Both Aβ pools are important, and a dynamic relationship between them exists [9, 44].

The intraneuronal pool of Aβ has a double origin: slow production from APP inside the neurons and uptake from the extracellular space. These two mechanisms are quite distinct and are regulated differently. Hence, understanding which pathway, if any, is more relevant to AD pathogenesis may help in the identification of potential targets to treat the disease. There is extensive evidence that indicates the production of Aβ_{42} from APP "in situ" inside the neurons [23, 45–53]. We are not going to discuss this mechanism of intracellular Aβ accumulation, which has been reviewed recently [9, 15].

Several studies favor a mechanism that involves uptake of Aβ from the extracellular pool [13, 37, 54, 55]. This mechanism of internalization occurs selectively in neurons at risk in AD as demonstrated using organotypic hippocampal slice cultures in which Aβ_{42} gradually accumulates and is retained intact by field CA1, but not by other subdivisions [40, 56]. Moreover, Aβ from the periphery enters the brain if the blood brain barrier is compromised and accumulates in neurons but not in glia [57]. Recent work also favored a mechanism of Aβ uptake from the extracellular pool based on the fact that intracellular Aβ was always accompanied by increased extracellular Aβ, while in subjects without increased extracellular Aβ there was no detection of intracellular Aβ [10].

Aβ uptake from the extracellular space and Aβ generation from APP inside neurons have been linked in what can be considered an autocatalytic vicious cycle or loop. According to this concept, intracellular accumulation of Aβ_{42} causes pronounced upregulation of newly generated Aβ_{42} within neurons. Glabe's group has shown that internalization of exogenous Aβ_{42} by HEK-293 cells overexpressing APP resulted in accumulation of amyloidogenic fragments of APP [58]. The effect was specific since the amount of nonamyloidogenic α-secretase carboxy-terminal fragments was only slightly affected. The accumulation of the amyloidogenic fragments did not result from an increase in APP synthesis, but instead it was due to specific enhancement of peptides stability, possibly by interaction of the fragments with stable Aβ aggregates causing evasion of the normal degradation pathway. Glabe's group also demonstrated that the amyloidogenic fragments can be further cleaved to

produce Aβ, further supporting the hypothesis that amyloid accumulation is a process mechanistically related to prion replication [41, 59]. Exogenous Aβ_{42} might initiate the cycle in the multivesicular bodies or lysosomes, where Aβ_{42} accumulates [40, 58]. The induction of amyloidogenic APP fragments by Aβ_{42} was also documented in the field CA1 of hippocampal slices [40], and the accumulation of intracellular Aβ upon Aβ_{42} uptake was demonstrated in dendrites of primary neurons [60]. Importantly, the Aβ-induced synaptic alterations demonstrated in this last study required amyloidogenic processing of APP. Indeed, the decrease in synaptic proteins caused by extracellular Aβ [32, 61] is reversed when Aβ is provided together with a γ-secretase inhibitor or given to APP knockout neurons [60]. A link between extracellular Aβ-induced neuronal death and APP cleavage has been suggested [60] based on the evidence that extracellular Aβ causes death of wild type neurons but not APP-knock out neurons [62] and that point mutations in the NPXY motif in the C-terminus of APP block Aβ toxicity [63].

4. Aβ Uptake by Neurons

The molecular events involved in neuronal Aβ internalization in AD are unclear. Aβ is internalized by dissociated neurons, neuron-like cells, and other cells in culture [64–71] (Song, Baker, Todd, and Kar, resubmitted for publication) and in cultured hippocampal slices [40, 56, 72]. In neurons, as in other cells, several forms of endocytosis exist (reviewed in [73–75]). Clathrin-mediated endocytosis has been considered the major mechanism of Aβ internalization until recently but many other endocytic processes independent of clathrin may mediate Aβ uptake.

4.1. Uptake of Aβ through ApoE Receptors. The first discovered mechanism of clathrin-mediated Aβ endocytosis involved receptors that bind to apolipoprotein E (apoE) and belong to the Low-Density Lipoprotein Receptor (LDLR) family. ApoE is a polymorphic protein that transports extracellular cholesterol. We [76] and others [77] have reviewed the role of apoE in AD, including the increased risk of developing AD in individuals who express the apoE4 isoform. ApoE receptors themselves play important roles in processes related to AD such as neuronal signaling, APP trafficking, and Aβ production (reviewed in [78]).

Studies in human brain indicated that intracellular Aβ accumulation in damaged cells correlates with apoE uptake [54], and neurons with marked intracellular Aβ_{42} immunoreactivity also stain positively for apoE [12]. Furthermore, the presence of one or two apoE4 alleles strongly correlates with an increased accumulation of intraneuronal Aβ [79]. The finding of apoE inside neurons has been taken as evidence of receptor-mediated uptake [80, 81]. In support of this concept, intraneuronal Aβ is significantly decreased in brains of PDAPP mice lacking apoE [82].

From the several receptors that belong to the LDLR family and bind apoE, the evidence available points at the low-density lipoprotein receptor-related protein 1 (LRP1) as the most important in Aβ uptake. LRP1 is required for Aβ endocytosis in several cell types including cortical neurons from Tg2576 mice [67], glioblastoma [68] and neuroblastoma cells [83], fibroblasts [72], human cerebrovascular cells [69], synaptosomes and dorsal root ganglion cells [84], and brain endothelial cell lines [85]. Moreover, overexpression of the LRP minireceptor mLRP2 enhanced Aβ uptake in PC12 cells [82], and increased extracellular deposition of Aβ (which was considered as indication of reduced internalization, although this is questionable) was detected in mice that have reduced levels of LRP1 due to deficiency of the chaperone receptor-associated protein (RAP) [83].

Binding of apoE to Aβ increases or decreases Aβ endocytosis depending on the cell type and other environmental conditions [84–90]. ApoE4, in particular, seems to cause a switch to a mechanism independent of LRP1, mediated by other receptors, which in the blood-brain barrier seems to be VLDLR [85, 87]. Whether the formation of a complex Aβ-apoE is required for the regulation of Aβ uptake is still unclear. Some studies showed evidence that LRP1 binds and mediates Aβ endocytosis directly (reviewed in [78, 91]), thus apoE would not be required. However, Yamada and colleagues found that Aβ does not interact directly with LRP1 and suggested that a coreceptor might be needed for Aβ internalization [85]. A fragment of apoE increased Aβ uptake without binding Aβ directly or without inducing up-regulation of LRP1 [92]. As apoE, α2-macroglobulin (α2M) has been linked to AD and is a ligand of LRP1. α2M promotes Aβ uptake by cortical neurons [67] and fibroblasts [72] in culture.

4.2. Uptake of Aβ in the Absence of ApoE. We have speculated that Aβ would exist in the brain in equilibrium between a complex with apoE (or other chaperones) and free Aβ (Figure 1). That equilibrium would be affected by the affinity of apoE for Aβ, which is isoform specific. In addition, during AD, especially when soluble Aβ accumulates in the brain parenchyma, the pool of free Aβ would increase. We demonstrated that neurons are able to internalize free Aβ in the absence of apoE [66]. ApoE-free Aβ is endocytosed by a mechanism that does not involve receptors of the LDLR family, since it is insensitive to RAP. Interestingly a similar RAP-independent Aβ uptake mechanism has been previously observed in synaptosomes, although it was interpreted as nonspecific internalization by constitutive membrane endocytosis [84]. In our case however, it occurs selectively in neuronal axons and, albeit it is independent of clathrin it requires dynamin suggesting that it is a regulated mechanism of endocytosis. A common form of clathrin-independent endocytosis that requires dynamin also involves caveolae, but in our studies we found that Aβ endocytosis does not require caveolin [66]. We reached this conclusion not because neurons do not express caveolin, in fact the neurons used in our studies (except those isolated from caveolin null mice) do express caveolin, as demonstrated for many other neurons [93], but neurons seem to lack caveolae. N2A cells internalize Aβ by another clathrin-independent, dynamin-mediated endocytosis that requires RhoA [65] suggesting that Aβ might also use the pathway of the IL2Rβ receptor [74].

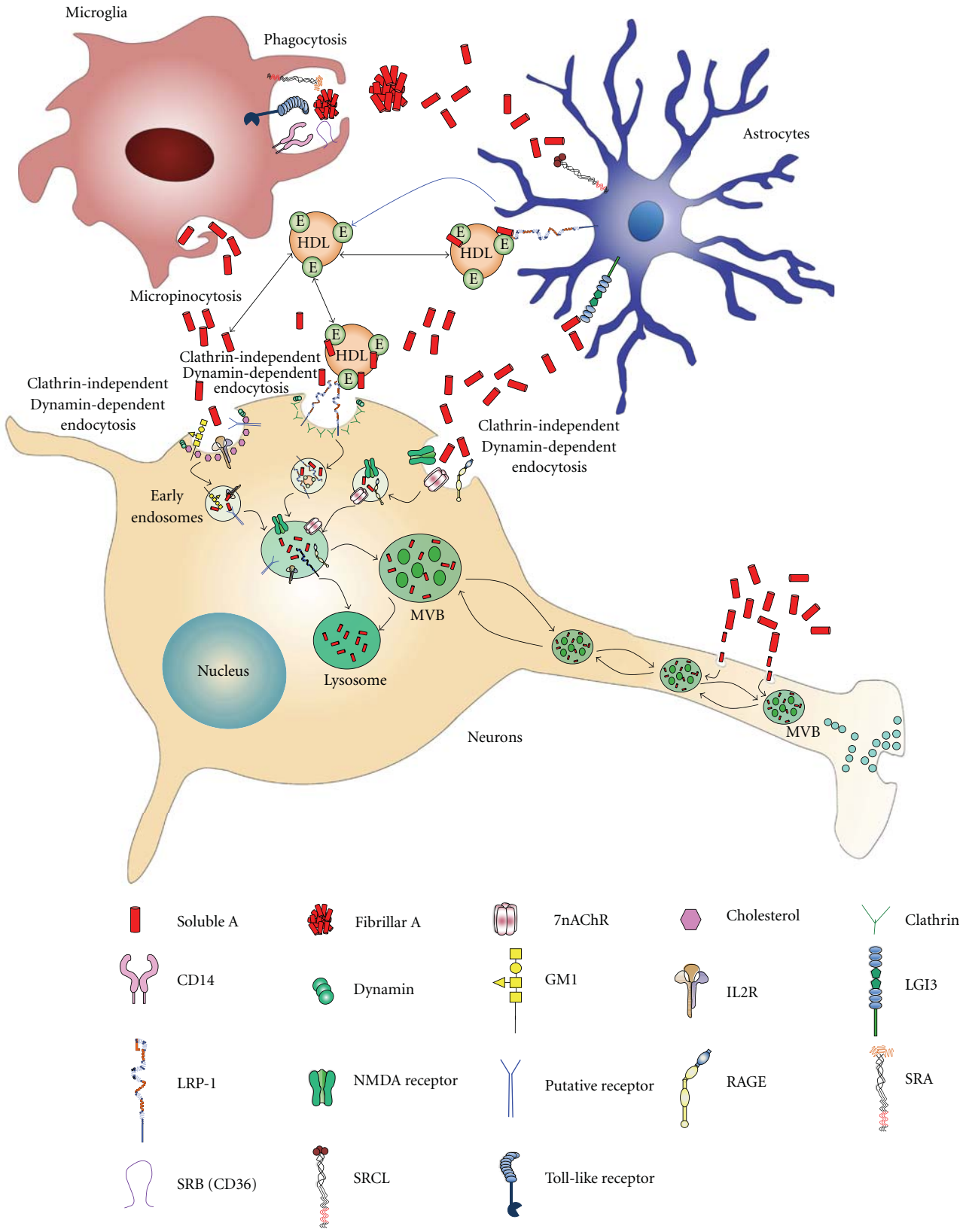

Figure 1: Mechanisms of Aβ internalization in neurons and glia.

4.3. Lipids and Aβ Endocytosis. Our work implied that at least one mechanism by which neurons internalize apoE-free Aβ involves noncaveolae, GM1-containing rafts [66]. Lipid raft endocytosis occurs in cells with and without caveolae [94]. Aβ uptake by this mechanism is impaired by the simultaneous inhibition of cholesterol and sphingolipid synthesis, and, under these conditions, there is also decreased uptake of cholera toxin subunit B (CTxB). CTxB binds specifically to the ganglioside GM1 and is a known marker for clathrin-independent endocytosis in many cells [73]. Raft-mediated endocytosis is regulated by plasma membrane cholesterol and sphingolipid. Cholesterol regulates several processes that take place in AD including APP cleavage, Aβ production and/or aggregation, and intracellular APP trafficking [95, 96]. Likewise, sphingolipids and gangliosides participate in key events that involve Aβ [96, 97]. Previous work demonstrated that the level of cholesterol at the cell surface regulates Aβ binding and Aβ toxicity [98–100]. None of these studies investigated the role of cholesterol in Aβ internalization. The inhibition of Aβ uptake under low cholesterol and sphingolipid levels could be explained by the disorganization of lipid rafts with the consequent misslocalization of a putative Aβ receptor. Alternatively, Aβ could be internalized in a complex with GM1. Our studies support this last possibility for two reasons; internalized Aβ partially colocalizes with CTxB, and treatment with fumonisin B1 causes decrease of GM1 synthesis [101] and blocks Aβ endocytosis [66]. Our results argue for a concerted role of sphingolipids/gangliosides which is in agreement with extensive evidence and with the model proposed by Dr. Yanagisawa's group [97].

4.4. Nicotinic Acetylcholine Receptors. Other receptors implicated in Aβ internalization are the nicotinic acetylcholine receptors (nAChRs), which have been linked to AD in several other ways (reviewed in [102, 103]). The most vulnerable neurons in AD appear to be those that abundantly express nAChRs, particularly neurons of the hippocampus and cholinergic projection neurons from the basal forebrain that express the α7nAChR. α7nAChR colocalizes with amyloid plaques and more importantly, α7nAChR regulates calcium homeostasis and acetylcholine release, two key events in cognition and memory. In addition, α7nAChR seems to mediate at least some of the toxic effects of Aβ and Aβ-induced tau phosphorylation.

nAChRs seem to be internalized by endocytosis independent of clathrin and dynamin, in a process that requires the polymerization of actin through activation of Rac-1 [104]. Several studies have suggested the involvement of α7nAChR in the internalization of $A\beta_{42}$. Work in brains from patients with AD and in neuroblastoma cells expressing α7nAChR suggested that $A\beta_{42}$ accumulates selectively in neurons that express this receptor as the result of internalization of the Aβ in a complex with α7nAChR [55]. It is unclear if the role of α7nAChR on Aβ uptake depends on the direct binding of Aβ to the nAChR, although Aβ interacts with α7nAChR with high affinity [105, 106]. S 24795, a novel selective α7nAChR partial agonist decreases the interaction between Aβ and α7nAChR in vitro and reduces the intraneuronal Aβ load in organotypic frontal cortical slices [107]. However, in our studies using cultured primary rat neurons, $A\beta_{42}$ was unable to compete with α-BTx nicotinic receptor binding sites in neuronal membranes, and α-BTx did not affect $A\beta_{42}$ internalization, despite the expression of α7nAChR, especially in the axons of these neurons [66]. Our results are in agreement with evidence obtained using three different systems namely membrane preparations from rat hippocampus, brain slices and neuroblastoma cells expressing α7nAChR [108]. The difference in the results may be explained by the use of different Aβ preparations and the presence or absence of lipoproteins (and therefore Aβ chaperones) in the different studies. Recently, it was shown that the loss of α7nAChR in Tg2576 mice (A7KO-APP mice) enhances Aβ oligomer accumulation in the extracellular space and increases early cognitive decline and septohippocampal pathology in young animals [109], but improves cognitive deficits and synaptic pathology in aged A7KO-APP mice [110]. It would be interesting to assess the intraneuronal levels of Aβ in the brain of those animals at different ages.

4.5. Integrins and NMDA Receptors. Two receptors present in many synapses are integrins and N-methyl-D-aspartate (NMDA) receptors. Both receptors regulate clathrin-mediated endocytosis. Several links between Aβ and NMDA receptors have been reported. Aβ-induced neurodegeneration [111, 112], disruption of axonal transport [113], and impairment of synaptic transmission [61] are mediated, at least in part, by NMDA receptors. In agreement, neurons are protected against neuronal degeneration and Aβ toxicity by transient inactivation of NMDA receptors [114, 115]. Memantine is a noncompetitive NMDA receptor antagonist used for the treatment of moderate to severe AD patients. Memantine protects against neuronal degeneration and Aβ toxicity [111, 116]. Importantly, new evidence from Kar's laboratory indicated that the protective role of memantine in cultured cortical neurons are independent of endocytosis since memantine was unable to inhibit Aβ uptake (Song, Baker, Todd, and Kar, resubmitted for publication). In other systems, however, the uptake and the effects of $A\beta_{42}$ on hippocampal neurons were blocked by the NMDA receptor antagonist APV [56]. Moreover, it has been reported that Aβ mediates and promotes NMDA receptor endocytosis possibly via the α7nAChR [61, 117].

The uptake of Aβ by neurons in hippocampal slices is also regulated by integrins. Bi and colleagues found that integrin antagonists enhance Aβ uptake [56]. They propose the following mechanisms of action for integrin antagonists: (i) the increase in peptide availability for uptake, due to disruption of the interaction of Aβ with integrins, which might represent the first step in Aβ extracellular proteolysis, (ii) the facilitation of endocytosis, by reducing the binding of integrins to the extracellular matrix and submembrane cytoskeleton which would slow invagination and endocytosis and (iii) a change in lysosomal proteolysis of Aβ since adhesion receptors can change the rate at which primary lysosomes are formed. Moreover, they suggested that the selectivity in Aβ uptake could be explained by the different

types of integrin subunits expressed in each area of the brain or even in specific neurons.

4.6. Receptor for Advanced Glycation End Products (RAGEs). The receptor for advanced glycation end products (RAGEs) is considered a primary transporter of Aβ across the blood-brain barrier into the brain from the systemic circulation [118], but some evidence exists that RAGE binds monomeric, oligomeric, and even fibrillar Aβ at the surface of neurons [119–121]. Recently, it was reported that RAGE cointernalizes with Aβ and colocalizes with Aβ at the hippocampus of mouse model of AD and that blockade of RAGE decreases Aβ uptake and Aβ toxicity [122].

5. Consequences of Intraneuronal Accumulation of Aβ

The cellular uptake and degradation of Aβ have been originally considered as mechanisms that reduce the concentration of Aβ in interstitial fluids. However, Aβ_{42} is degraded poorly, and its accumulation inside neurons has dramatic consequences. Intraneuronal Aβ accumulates within the endosomal/lysosomal system, in vesicles sometimes identified as lysosomes [13, 40, 56, 64, 71, 82, 123] and some others as late endosomes/multivesicular bodies (MVBs) [30, 124–126]. In sympathetic neurons we found that Aβ_{42} causes sequestration of cholesterol (Figure 2(a)), which colocalizes with LAMP-1 and is the site of Aβ accumulation (Figure 2(b)).

Aβ_{42} internalized from the extracellular milieu is quite resistant to degradation possibly due to formation of protease resistant aggregates. Shorter Aβ peptides are degraded and do not accumulate after endocytosis [58, 59, 123, 127]. In one study Aβ_{42} was shown to be cleared rapidly after delivery to lysosomes, although it previously concentrated and aggregated within the cells, possibly serving as a seed for further Aβ aggregation [71].

Aβ accumulation in lysosomes may cause loss of lysosomal membrane impermeability and leakage of lysosomal content (proteases and cathepsins) causing apoptosis and necrosis [13, 55, 123, 128–130] (Song, Baker, Todd, and Kar, resubmitted for publication). The release of lysosomal contents into the cytoplasmic compartments has been considered one of the earliest events in intracellular Aβ-mediated neurotoxicity in vitro [123], and inhibition of lysosomal enzymes protects against Aβ toxicity in cultured cells [131]. ApoE4 potentiates Aβ-induced lysosomal leakage and apoptosis in N2A cells by a mechanism that requires endocytosis by LRP1 [132]. Immunogold studies suggested that the disruption of MVBs could release enough Aβ_{42} to induce neurotoxicity [30].

An increase in cathepsin D levels secondary to Aβ internalization has been reported in hippocampal slices [56, 133] and cultured cortical neurons (Song, Baker, Todd, and Kar, resubmitted for publication). Elevation of cathepsin D levels is a characteristic of AD brains [134–136]; endosome dysfunction occurs early in AD, before amyloid deposition (reviewed in [128]) and is enhanced in persons expressing apoE4 [137]. Abnormal endosomes are also detected in Down syndrome and Niemann-Pick type C, in which Aβ peptide accumulates intracellularly [138].

Endosomal dysfunction, however, might not necessarily involve lysosomal leakage in all cases but could involve defects in intracellular trafficking. MVBs are considered late endosomes, which form by fusion of early endosomes with signaling endosomes and serve as vehicle for the transport of receptors and signaling molecules [139]. MVBs are important vesicles in retrograde transport, and accumulation of Aβ within MVBs would impair their degradative and trafficking functions. MVBs contain inner vesicles with lower pH in the lumen. Aβ interacts with, and partitions into negatively charged membranes [140] and there is evidence that Aβ_{42} is localized to the outer membrane of the MVBs in brains of patients with AD [30], and is inserted in the membrane of lysosomes in cultured cells that internalized Aβ [130]. The MVBs represent a good location for Aβ aggregation because MVBs are rich in membranes and have low pH [30]. In addition, Aβ accumulation in MVBs membranes will likely disrupt intracellular trafficking as mentioned above.

In neurons, axonal retrograde transport is essential for neuronal life since it secures the delivery of growth factors and/or their survival signals to the soma. This requires the normal function of the endosomal system in axons [141, 142] and will likely be affected by Aβ accumulation in axonal MVBs. We demonstrated that axons are entry points of Aβ and apoE [66, 143] suggesting that accumulation of Aβ in axonal MVBs could impair retrograde transport. Our new evidence suggests that cholesterol accumulation in MVBs could worsen intracellular trafficking in neurons. The impairment of retrograde transport has been proposed to play an important role in degeneration of basal forebrain cholinergic neurons in AD [144, 145]. Recent work has shown impairment of BDNF-mediated TrkB retrograde transport in Tg2576 axons and in cultured neurons treated with Aβ [146].

Protein sorting into MVBs is a highly regulated event. One of the mechanisms of MVB sorting is the ubiquitin proteasome system (UPS) [147]. Aβ inhibits the proteasome [148–150]. Important in the context of this review, part of Aβ internalized by neurons appears in the cytosol, where it could get in contact with the proteasome [149]. LaFerla's group demonstrated an age-dependent proteasome inhibition in the triple transgenic mice model of AD [150]. This inhibition was responsible for tau phosphorylation and was reversed by Aβ immunotherapy. Inhibition of the UPS was responsible for impairment of the MVB sorting pathway in cultured Tg2576 neurons challenged with Aβ [124]. Inhibition of fast axonal transport by Aβ by mechanisms that do not involve MVBs directly has also been reported [151].

6. Neuronal Death Secondary to Aβ Uptake

The role of Aβ in neuronal death and dysfunction has been investigated extensively. The attention has focused mainly on how extracellular Aβ causes neuronal death. On the other hand, whether the intracellular accumulation of Aβ

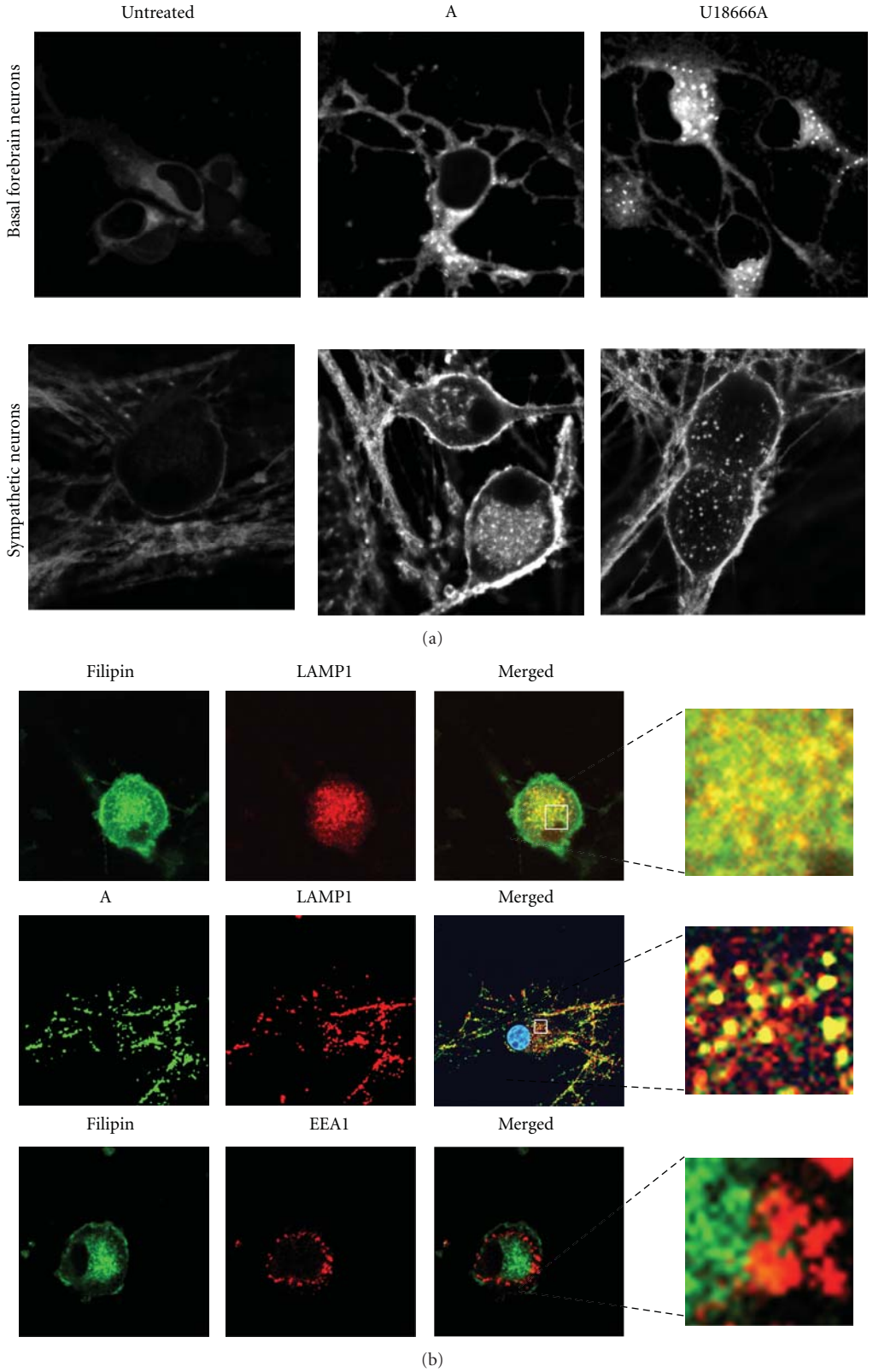

FIGURE 2: Aβ causes cholesterol sequestration in primary neurons. (a) Rat primary neurons (forebrain and sympathetic) cultured in serum-free medium, were treated for 24 h with 20 μM oligomeric Aβ_{42} (prepared according to [66]) or with 1.5 μM U18666A, a drug extensively used to induce cholesterol sequestration. Cholesterol was examined by confocal microscopy, using filipin. (b) Neurons were treated as in (a) but fluorescent oligomeric Aβ_{42} was used. Intracellular localization of cholesterol and Aβ was examined by double indirect immunofluorescence confocal microscopy using LAMP1 as a marker of late endosome/MVBs and EEA1 as a marker of early endosomes.

is a cause of neuronal death has been a matter of debate. Some groups consider that intracellular accumulation of Aβ is not responsible for neuronal loss. For instance, the appearance of Aβ immunoreactivity in neurons in infants and during late childhood, adulthood, and normal aging, suggests that this is part of the normal neuronal metabolism [14]. Moreover, Aβ did not produce clear signs of cell death upon infusion in hippocampal slices [40] although in combination with transforming growth factor-β (TGF-β) it induced neuronal degeneration in field CA1 [152]. On the other hand, evidence that supports the importance of intracellular Aβ in cell death includes the observations that different mice models of AD show dramatic intraneuronal Aβ accumulation and neuronal cell death that correlates with intraneuronal Aβ accumulation and precedes Aβ deposition [7, 26, 29, 55, 126, 153]. Moreover, the abnormalities and cognitive dysfunctions in several models of AD correlate with the appearance of intraneuronal Aβ, before the appearance of plaques or tangles [18, 19]; markers of apoptosis are present in the subset of neurons that accumulate Aβ in Down syndrome brains [25], and microinjections of Aβ_{42} or a cDNA-expressing cytosolic Aβ_{42} rapidly induces cell death of primary human neurons [27]. In addition, treatment of cultured neurons or neuron-like cells with Aβ_{42} causes Aβ internalization and death [55, 65, 66, 116, 123, 154, 155] (Song, Baker, Todd, and Kar, resubmitted for publication).

The evidence above opens the question whether Aβ internalization is required for toxicity. Inhibition of Aβ endocytosis in N2A cells [65], primary cortical neurons (Song, Baker, Todd, and Kar, resubmitted for publication) and sympathetic neurons (Saavedra and Posse de Chaves, unpublished observations) resulted in significantly less intracellular Aβ accumulation and reduced Aβ toxicity. Besides, the selective toxicity of Aβ oligomers versus Aβ fibrils has been explained by the preferential oligomeric Aβ uptake by receptor-mediated endocytosis [156]. As indicated above, the endocytic mechanisms used by Aβ in different cells or under different conditions seem to be different, but in all cases the fate of internalized Aβ is similar, being delivered to MVBs or lysosomes.

7. Aβ Internalization by Astrocytes and Microglia

The accumulation of activated astrocytes and microglia close to Aβ deposits suggests that these cells play a role in AD pathology [157–159]. Astrocytes are the most abundant type of cells in the CNS. Upon exposure to Aβ, they become activated and play a neuroprotective role by extending their hypertrophic processes to physically separate the neurons from Aβ fibrils [160]. In addition, activated astrocytes can internalize and degrade Aβ [161], possibly in an attempt to reduce Aβ availability to neurons. Nevertheless, exposure of astrocytes to Aβ could have detrimental consequences. Aβ upregulates inflammatory cytokines and increases the release of nitric oxide in cultured astrocytes [162]. Moreover, Aβ induces not only astrocytic cell death [163], but also neuronal cell death indirectly [164].

Microglia are mononuclear phagocytes of the innate immune system in the CNS. MicrogIia can act as a dual sword in AD pathology. Aβ deposition activates microglia, which release proinflammatory cytokines and other cytotoxic compounds that cause neurodegeneration [165, 166]. Some studies, however, suggested a neuroprotective role of microglia via their ability to internalize and degrade Aβ [167–170].

The evidence of Aβ accumulation in brain glia in AD is contentious. Aβ accumulation in areas with high Aβ deposition has been shown in astrocytes and microglia [171] or astrocytes but not microglia or neurons [172, 173]. Blood-derived Aβ_{42} is able to cross a compromised blood-brain barrier, is internalized, and accumulates in cortical pyramidal neurons but not in glia [57]. But continuous intracerebral infusion of Aβ in rat brain resulted in Aβ accumulation in astrocytes but not microglia [174]. The lack of intracellular Aβ in microglia cannot be interpreted as microglia being unable to take up Aβ, since it could also reflect that they are highly efficient in degrading it [174]. A theory that opposes this concept establishes that, instead of accumulating Aβ intracellularly, microglia release fibril Aβ contributing to the growth of amyloid plaques [160, 175]. Aβ internalization by microglia in vitro has been shown in several studies [176, 177]. 3D reconstruction of ultrathin sectioning of microglia cells in the vicinity of dense-core amyloid plaque showed that amyloid plaques were exclusively extracellular deposits suggesting that microglia do not internalize fibril Aβ [178]. On the contrary, Bolmont et al. found that plaque-associated microglia internalize a fluorescent dye binding amyloid injected systemically. The intracellular dye particles were positive for Aβ and were not continuous with the amyloid plaque, suggesting true Aβ internalization by microglia [179].

As discussed for neurons, the intracellular pool of Aβ in microglia and astrocytes could be derived from increased endogenous production or increased internalization of exogenous Aβ. Some studies showed that Aβ production in these cells is very low due to reduced APP expression in microglia and reduced beta-secretase activity in astrocytes compared to neurons [180–182]. Nevertheless some stimuli induce expression of APP, beta-secretase, γ-secretase and production of Aβ in astrocytes and microglia [183–185].

7.1. Aβ Internalization by Astrocytes. The involvement of LDLR/LRP1 in Aβ internalization by astrocytes is controversial. The ability of astrocytes to degrade Aβ deposits demonstrated in brains of transgenic PDAPP mice depends on apoE secretion and is blocked by RAP suggesting a mechanism mediated by a member of the LDLR family [186]. Unfortunately, Aβ internalization by astrocytes was not examined in this study [186], and in view that Aβ degradation by astrocytes could be mediated by extracellular matrix metalloproteinases [187], Aβ internalization in this paradigm is not granted. One study showed that Aβ-induced activation of cultured astrocytes is mediated by LRP [188] suggesting that LRP participates in Aβ uptake, although

Aβ internalization was not directly examined under these conditions either. Conversely, another study demonstrated that Aβ internalization by astrocytes is not affected by RAP treatment [69] arguing against the involvement of LDLR/LRP1.

The accumulation of fibrillar Aβ in cytoplasmic vesicles of human astrocytes is associated with increased cellular level of apoJ/clusterin [189]. Since apoJ/clusterin binds to fibrillar Aβ [190] and is involved in LRP1- and scavenger-receptor-mediated endocytosis/phagocytosis [191], it was hypothesized that human astrocytes can take up fibril Aβ via apoJ/clusterin-mediated endocytosis [189]. Recently, it has been shown that astrocytes can take up oligomeric Aβ better than fibrillar Aβ [192]. ApoE and apoJ/clusterin reduced oligomeric Aβ positive astrocytes without affecting fibril Aβ uptake [192]. This indicates that Aβ uptake by astrocytes depends on Aβ aggregation status and that oligomeric Aβ internalization by astrocytes could be mediated by the LDLR family.

Scavenger receptors (SRs) are cell surface receptors expressed by diverse cell types that bind to a variety of unrelated ligands [193]. Based on the ability of fucoidan and polyinosinic acid, known ligands for SR, to reduce Aβ binding to and internalization by astrocytes SRs have been recognized as possible mediators of Aβ internalization by astrocytes [164, 194, 195].

Formyl peptide receptor-like 1 (FPRL1) is a G protein-coupled receptor regulating the immune responses [196]. FPRL1 mediates Aβ internalization in astrocytes. Immunostaining of Aβ-treated astrocytes shows colocalization of internalized Aβ and FPRL1. In addition, cotreatment with a FPRL1 agonist (fMLF) or antagonist (WRW4) reduces Aβ internalization. This indicates that Aβ binds to FPRL1 stimulating the complex internalization [197].

Another type of receptors that has shown to be involved in Aβ internalization by astrocytes is leucine-rich glioma inactivated protein 3 (LGI3), a type I transmembrane protein containing leucine rich repeat (LRR) [198, 199]. Aβ induces the expression of the Lib gene in astrocytes, which encodes for LRR-containing type I transmembrane proteins [200]. These LRR containing proteins are thought to mediate protein-protein or protein-matrix interactions [201]. LGI3 colocalizes with Aβ at the plasma membrane and intracellularly in astrocytes suggesting that LGI3 could be playing a role in Aβ internalization [198]. This was supported by the ability of LGI3 downregulation to reduce Aβ internalization by astrocytes [199]. LGI3 is involved in clathrin-mediated endocytosis in astrocytes and neuronal cell lines [199]. It interacts with flotillin regulating APP intracellular trafficking in neuronal cells [202].

Phagocytosis is another mechanism that could mediate Aβ internalization by astrocytes. Astrocytes that accumulate Aβ in AD brains also have high levels of neuron-specific choline acetyltransferase (ChAT) and α7nAChR [163], which suggest that astrocytes are able to internalize Aβ-loaded neurons via phagocytosis. However, the evidence that cytochalasin B, an inhibitor of phagocytosis, does not block Aβ internalization in astrocytes is in conflict with this notion [203].

7.2. Aβ Internalization by Microglia. With respect to the mechanisms that mediate Aβ uptake in microglia, the evidence suggest that different mechanisms exist for soluble and aggregated Aβ (reviewed in [204]). Soluble Aβ internalization by microglia does not depend on the presence of apoE [205] and is not blocked by RAP treatment [168, 170] excluding the involvement of LDLR/LRP-1. Internalized soluble Aβ does not colocalize with internalized transferrin further excluding clathrin-mediated endocytosis [168]. Moreover, soluble Aβ internalization by microglia is non-saturable excluding receptor-mediated internalization [168, 170]. Soluble Aβ internalization by microglia has been classified as fluid phase macropinocytosis, a process dependent on cytoskeletal structures. Aβ-containing macropinocytic vesicles fuse with late endosomes and later with lysosomes, where they are degraded [168]. Blocking microglial surface receptors that mediate fibril Aβ internalization do not affect internalization of soluble Aβ [168] confirming that the two mechanisms are different.

Fibril/aggregated Aβ internalization by microglia seems to proceed by receptor-mediated endocytosis and receptor-mediated phagocytosis [177, 206]. The surface receptors involved are Pattern Recognition Receptors (PRRs). These are the receptors used by the innate immune system to recognize pathogen associated molecular pattern, including SR-type A, CD14, CD47, SR-type B (CD36), α6β1 integrin, and toll-like receptors (TLRs) [177, 206–211]. Microglia take up fibril Aβ into phagosomes, which then enter the endosomal-lysosomal system for degradation [177, 206, 207]. Fibril Aβ internalization by microglia is blocked by the scavenger receptor agonists Ac-LDL or fucoidan, but not by RAP indicating the involvement of scavenger receptors but not LDLR/LPR-1 [177]. Microglia that do not express CD14 have lower ability to take up Aβ [207]. The microglial Aβ cell surface receptor complex, composed of α6β1 integrin, CD47 (integrin-associated protein), and the B-class scavenger receptor CD36 [210], mediates microglial uptake of fibril Aβ via a receptor mediated nonclassical phagocytosis [206]. Activation of toll-Like Receptors (TLRs) increases microglial ability to internalize Aβ [207–209, 212]. TLRs activation increases the expression of G protein-coupled mouse formyl peptide receptor 2 (mFPR2), mouse homologue of FPRL1, in microglia. Increased Aβ uptake by microglia upon TLRs activation was blocked by pertussis toxin PTX, Gαi-protein coupled receptor deactivator, W peptide, mFPR2 agonist, anti-CD14, as well as scavenger receptors ligand. This indicates that mFPR2, CD14 and scavenger receptors work together to increase Aβ internalization by microglia upon TLR activation [208, 209]. In addition, formyl peptide receptor-like 1 (FPRL1) was also found to mediate Aβ internalization in microglia [197].

In addition, microglia can internalize fibril Aβ by phagocytosis stimulated by Aβ-antibody complex interaction with Fc-receptor [177, 213] and/or fibril Aβ interaction with the complement system C1q (antibody dependent) or C3b (antibody independent) [204, 214–216].

8. Conclusions

The intracellular accumulation of Aβ has been confirmed, and evidence of Aβ internalization from outside the cells exist. Neurons seem to use different mechanisms than glia to take up Aβ. The existence of phagocytic processes in glia suggests that these cells participate mostly in the clearance of Aβ. More research is required to understand if neurons take up Aβ under physiological conditions and whether this is part of Aβ normal metabolism. Regulated endocytosis is the main process by which neurons internalize Aβ. The participation of a number of receptors suggests that more than one mechanism exists. The challenge ahead is to understand the significance of this diversity in the development and progression of AD.

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Review Article

Amyloid-Beta Interaction with Mitochondria

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Mitochondrial dysfunction is a hallmark of amyloid-beta(Aβ)-induced neuronal toxicity in Alzheimer's disease (AD). The recent emphasis on the intracellular biology of Aβ and its precursor protein (AβPP) has led researchers to consider the possibility that mitochondria-associated and/or intramitochondrial Aβ may directly cause neurotoxicity. In this paper, we will outline current knowledge of the intracellular localization of both Aβ and AβPP addressing the question of how Aβ can access mitochondria. Moreover, we summarize evidence from AD postmortem brain as well as cellular and animal AD models showing that Aβ triggers mitochondrial dysfunction through a number of pathways such as impairment of oxidative phosphorylation, elevation of reactive oxygen species (ROS) production, alteration of mitochondrial dynamics, and interaction with mitochondrial proteins. In particular, we focus on Aβ interaction with different mitochondrial targets including the outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and the matrix. Thus, this paper establishes a modified model of the Alzheimer cascade mitochondrial hypothesis.

1. Introduction

Although the hallmark lesions of the disease were described by Alois Alzheimer already in 1906—extracellular amyloid plaques mainly composed of amyloid-beta (Aβ) and intracellular neurofibrillary tangles (NFTs) built up of hyperphosphorylated tau—the molecular mechanisms underlying the disease are still unknown. However, more recently, energy deficiency and mitochondrial dysfunction have been recognized as a prominent, early event in Alzheimer's disease (AD) [1–11]. Successful development of Alzheimer cell culture models as well as single, double, and recently triple transgenic mouse models that mimic diverse aspects of the disease facilitated the investigation of pathogenic mechanisms in AD and particularly assisted in an understanding of the interaction of amyloid-beta (Aβ) with mitochondria. Mitochondria were found to be the target both for amyloid precursor protein (APP) that accumulates in the mitochondrial import channels and for Aβ that interacts with several proteins inside mitochondria and leads to mitochondrial dysfunction [12].

2. Intracellular Localization of AβPP

Amyloid precursor protein (AβPP) is a type 1 integral 110–130 kDa glycoprotein containing a 40 or 42 amino acid sequence, respectively, called Aβ_{40} and Aβ_{42}. AβPP is ubiquitously expressed in human tissues and localizes at the plasma membrane as well as in several organelles, such as endoplasmic reticulum (ER), Golgi apparatus, and mitochondria (Figure 1) [13, 14]. AβPP can undergo two pathways of cleavage by secretases: a nonamyloidogenic pathway and an amyloidogenic pathway. In the nonamyloidogenic pathway, a first cut of AβPP is catalyzed by α-secretase, an enzyme that belongs to the ADAM family of disintegrin and metalloprotease and is particularly present in post-Golgi compartment or at the plasma membrane [15]. α-secretase cleaves AβPP within the Aβ sequence [16], forming the small membrane-anchored C83 fragment and sAβPPα. The C83 fragment is subsequently cleaved by γ-secretase, a multimeric complex of presenilin proteins PS1 and PS2, nicastrin, anterior pharynx defective1, and presenilin enhancer 2 [17], to form P3 fragment and AβPP

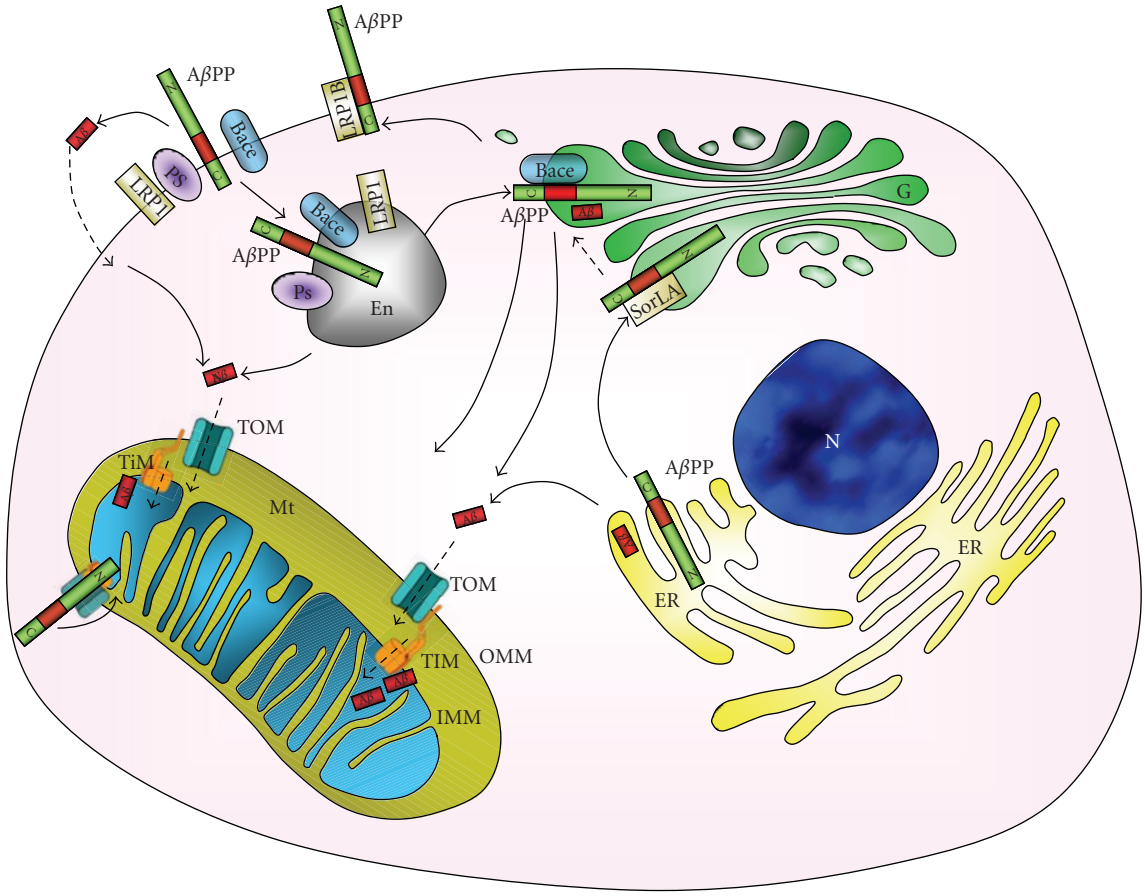

Figure 1: Intracellular localization of AβPP and Aβ. AβPP is synthesised in the endoplasmic reticulum (ER) and is trafficked through Golgi network (G), to the cell surface or to mitochondria (Mt). In the plasma membrane the apolipoprotein (Apo) receptor LRP1 forms a complex with AβPP, inducing the internalization of the amyloid precursor protein, together with other plasma membrane enzymes, such as the β-secretase BACE and the γ-secretase presenilin (PS): Aβ is produced through amyloidogenic cleavage of AβPP. The plasma membrane complex AβPP with another Apo receptor, LRP1B, decreases the cellular uptake of AβPP. Sources of mitochondrial Aβ are the endosome (En) but also G and ER. G production of Aβ is decreased by binding of AβPP to another Apo receptor SorLA that blocks AβPP in the early G, counteracting AβPP cleavage pathways. Aβ enters into the mitochondrial matrix through TOM and translocase of the inner membrane (TIM) or is derived from mitochondria-associated AβPP metabolism. N = nucleus, OMM = outer mitochondrial membrane, IMM = inner mitochondrial membrane.

intracellular domain (AICD) [18, 19]. In the amyloidogenic pathway, AβPP generates Aβ through the activity of β-secretase first and then γ-secretase, especially present in Golgi or in late endosomes following the reuptake from the cell surface. β-secretase whose activity was attributed to BACE [20] cleaves AβPP at the N-terminal of Aβ sequence forming a 99 amino acid fragment C99 and sAβPPβ. C99 is subsequently cleaved by γ-secretase producing Aβ fragment and AICD [18]. Lipoprotein receptor LRP1 can interact with AβPP (Figure 1), influencing AβPP cleavage pathway: the interaction accelerates endocytosis of the complex AβPP-LRP1 and the adaptor Fe65 via clathrin-coated pits and the delivery to the late endosomal compartments for cleavage by β-secretase, to generate Aβ [21, 22]; this process can be counteracted by the slower rate of endocytosis LRP1B that sequestrates AβPP at the plasma membrane, increasing α-secretase activity [23, 24]. SorLA, a further lipoprotein receptor, is also involved in AβPP processing by binding AβPP to the Golgi compartments, impairing the transition to the plasma membrane and blocking β-secretase activity (Figure 1) [25, 26].

With regard to localization of AβPP in mitochondria, recent evidence is provided. Mainly the group of Devi and coworkers [27] demonstrated that AβPP harbours a chimeric targeting signal consisting of an N-terminal hydrophobic ER followed by a mitochondrial targeting signal. The positively charged residues at 40, 44, and 51 of AβPP are critical components of the mitochondrial targeting signal. In addition, they showed that AβPP formed stable approximately 480 kDa complexes with the translocase of the outer mitochondrial membrane (OMM) 40 (TOM40) import channel and a supercomplex of approximately 620 kDa with both mitochondrial TOM40 and the translocase of the inner mitochondrial membrane 23 (TIM23) import channel

TI M23 in an N (in mitochondria)-C (out cytoplasm) orientation, probably due to a 70 amino acid long acidic domain [13]. Also in brain tissues of AD affected subjects AβPP localized with mitochondria fraction, associated to TOM40 and TIM23 [27], in a translocation-arrested manner, that may prevent import of *de novo* synthesised nuclear-encoded mitochondrial protein, such as subunits of the electron transport chain (ETC) [27]. Nevertheless, until very recently [12], it was believed that mitochondrial Aβ has to arrive from other sources, since AβPP mitochondrial orientation presents Aβ outside the mitochondria [27].

3. Intracellular Localization of Aβ

In agreement with the intracellular localization of AβPP, cell biological studies have demonstrated that Aβ is generated in the ER, Golgi, and endosomal/lysosomal system (Figure 1). Reports indicate that Aβ*x*-42(truncated Aβ peptides with "*x*" generally ranging from 1 and 11) is preferentially generated within the ER, whereas Aβ1–40/42 peptides are predominantly made in the Golgi/trans-Golgi network (TGN) and packaged into post-TGN secretory vesicles [28, 29]. It is thought that the N-terminal truncation extends to a maximum length around amino acid 11 which renders Aβ even more insoluble, the latter pool of Aβ42 not being secreted [29].

Moreover, mitochondrial accumulation of Aβ has been shown in AD patient and AβPP transgenic mouse brain [4, 28, 30]. In transgenic AβPP mice expressing AβPP V717/F and the AβPP Swedish mutation, mitochondrial Aβ accumulation increased at around 4 months of age, well before the formation of plaques [30]. In total, these findings are further in line with the recently proposed hypothesis of an intracellular Aβ toxicity cascade which suggests that the toxic Aβ species intervening in molecular and biochemical abnormalities may be intracellular soluble aggregates instead of extracellular, insoluble plaques [4, 31].

How can Aβ access mitochondria? On the one hand, Aβ accumulation in mitochondria might derive from the ER/Golgi (Figure 1), since inhibition of protein secretion can modulate mitochondrial uptake [30, 32] or might derive from mitochondria-associated AβPP [12] (Figure 1). It has been hypothesized that oligomeric Aβ, with its sharp morphology in contrast to monomeric Aβ, has the ability to permeabilize cellular membranes and lipid bilayers thereby entering organelles such as mitochondria [33, 34]. Of note, already early reports about the action of aggregated Aβ on membranes implicated increased membrane permeability elicited by fibrils [35, 36]. These mechanisms might explain why aggregated Aβ preparations elicit effects on mitochondrial function, but not disaggregated Aβ. Recent findings, however, indicate a specific uptake mechanism for Aβ by mitochondria rather than simply being adsorbed to the external surface of mitochondria [37, 38]. In this model, Aβ is taken up by mitochondria via the TOM complex. On the other hand, a new mechanistic view of mitochondria-related AβPP metabolism was suggested very recently indicating that AICD, P3 peptide, and potentially Aβ are produced locally at the mitochondria. Hereby, mitochondrial AβPP is cleaved by Omi in the intermembrane space and a concerted action of cytosolic α/β and mitochondrial γ-secretases [12].

4. Mitochondria

Mitochondria are dynamic ATP-generating organelles which contribute to many cellular functions including intracellular calcium regulation, alteration of reduction-oxidation potential of cells, free radical scavenging, and activation of caspase-mediated cell death. ATP generation is accomplished through oxidative phosphorylation [7, 39–42]. ATP is subsequently used for a large repertoire of functions like intracellular calcium homeostasis, neurotransmitter production, and synaptic plasticity. Mitochondrial number is indeed very high in neurons, and mitochondria are especially enriched in synapses. Due to the limited glycolytic capacity of neurons, these cells are highly dependent on mitochondria function for energy production [43]. However, as the Pandora's Box, mitochondria are full of potentially harmful proteins and biochemical reaction centres. They may liberate reactive oxygen species (ROS) and free radicals. Thus, mitochondria are the major producers of ROS and at the same time major targets of ROS toxicity.

Mitochondria are composed of a double lipid membrane which structures four compartments, distinct by composition and function. The porous outer membrane (OMM) encompasses the whole organelle. It contains many proteins like import complexes and voltage-dependent anion channels (VDAC) responsible for the free passage of low molecular weight substances (up to 5000 Da) between the cytoplasm and the intermembrane space (IMS) (Figures 1 and 2) which represents a reservoir of protons establishing a proton electrochemical gradient across IMM that is needed for the production of ATP via ATPase (complex V). IMS contains proapoptotic proteins like cytochrome c, Smac/Diablo, EndoG, and Htra2/Omi. In contrast to the permeable OMM, the inner mitochondrial membrane (IMM), rich in cardiolipin, provides a highly efficient barrier to the flow of small molecules and ions, including protons. This membrane is invaginated into numerous cristae increasing cell surface area. It houses the respiratory enzymes of the electron transport chain (ETC), the cofactor coenzyme Q, and many mitochondrial carriers. In the matrix, different metabolic pathways take place including the tricarboxylic (TCA or Krebs) cycle (Figure 2).

Mitochondria generate energy by two closely coordinated metabolic processes: TCA and the oxidative phosphorylation (OXPHOS) (Figure 2). OXPHOS is made up of the ETC assembled in four enzymes (complex I to IV) as well as the F1F0-ATP synthase (complex V). Complex I, III, and IV are located in IMM as integral proteins whereas complex II is attached to the inner surface of IMM. These five enzymes are connected functionally by mobile electron acceptors and donors: ubiquinone and cytochrome c. Electrons from NADH and $FADH_2$ are fed into complex I and II, respectively. Ubiquinone Q carries electrons from both complexes to complex II, and cytochrome c does it from complex III to IV reducing molecular oxygen to

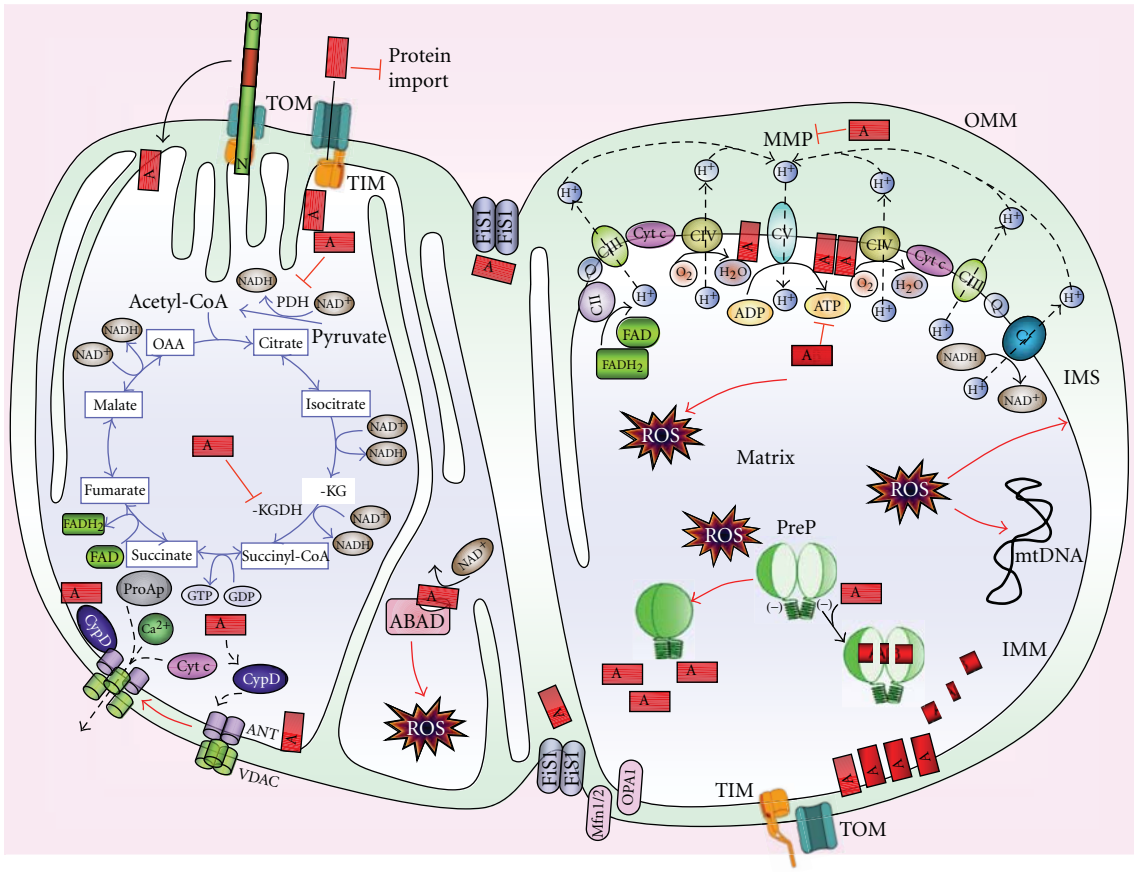

Figure 2: Mitochondrial targets of Aβ. Aβ associated with mitochondria may be deposited at several locations. Although not present exclusively on the outer mitochondrial membrane, Aβ that might be present at that site might influence the interaction of multiple cytosolic proteins (including those of the bcl2 family) with mitochondria, as well as affect the receptor binding of cargo targeted for import into the organelle via the TOM import machinery impeding mitochondrial entry to neosynthesised nuclear-encoded proteins such as subunits of the electron transport chain (ETC) complex IV (CIV). In the intramembrane space, Aβ might affect the functions of both the inner and outer mitochondrial membrane by multiple mechanisms including modulating their permeability. In the mitochondrial matrix, Aβ might interact with important components of metabolic or antioxidant mechanisms. The interaction of Aβ with the inner mitochondrial membrane would bring it into contact with respiratory chain complexes with the potential for myriad effects on cellular metabolism. Thus, Aβ affects the activity of several enzymes, such as pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH), decreasing NADH reduction, and the ETC enzyme CIV, reducing the amount of hydrogen that is translocated from the matrix to the intermembrane space, thus impairing the mitochondrial membrane potential (MMP). The dysfunction of the ETC leads to a decreased CV activity and so to a lower ATP synthesis, in addition to increasing reactive oxygen species (ROS) production. ROS negatively influences presequence P (PreP) activity, blocking Aβ degradation, exacerbating mitochondrial Aβ presence. Moreover, ROS induce peroxidation of several mitochondrial macromolecules, such as mitochondrial DNA (mtDNA) and mitochondrial lipids, additionally impairing mitochondrial function. Aβ binds NAD^+ pocket in ABAD, blocking its activity and inducing further ROS production. Aβ also influences mitochondrial dynamic, by improving Fis1 presence and activity, thus increasing mitochondrial fragmentation (fission protein: Fis1; fusion proteins: Mfn1/2 and OPA1). Furthermore, Aβ binding to cyclophilin D (CypD) enhances the protein translocation to the inner membrane, favouring the opening of the mitochondrial permeability transition pore, formed by ANT and VDAC. Calcium storage in mitochondria is impaired, altering neuronal function; calcium is exported to the cytosol, as well as other apoptotic factors (ProAp) such as cytochrome c, apoptosis-inducing factor, Smac/DIABLO, endonuclease G, and procaspases, activating cellular apoptosis. IMM: inner mitochondrial membrane, IMS: intermembrane space, OMM: outer mitochondrial membrane.

water (Figure 2). As electrons are transferred along ETC, a fixed number of protons are pumped from the matrix into IMS establishing a electrochemical gradient characterized with a specific electrical potential (mitochondrial membrane potential = MMP: negative inside – 150 to – 180 mV). The redox energy drives the synthesis of ATP from ADP as protons are transported back from IMS into the matrix via complex V.

5. Mitochondrial Targets of Aβ

Growing evidence indicates that Aβ toxicity can be associated with the outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and the matrix.

5.1. OMM-IMM-IMS

5.1.1. TOM-TIM Import Machinery. A working model postulates that Aβ and/or AβPP interact with mitochondria by inhibiting protein import [13, 44]. Import deficits are initially insufficient to impair mitochondrial integrity but over time cause mitochondrial dysfunction and further import deficits. Aβ may inhibit protein import by direct interactions with the import machinery or by indirect mechanisms. Given that multiple mitochondrial activities, including protein import itself, are dependent upon the import of nuclear-encoded proteins, it seems likely that even a modest decline in import could have potentially catastrophic consequences in the long-term. Furthermore, a decline in protein import seems to precede increased ROS and decreased mitochondrial membrane potential suggesting a gradual failure of mitochondria. Indeed, recent data from our group demonstrated mitochondrial dysfunction in a novel triple transgenic mouse model (pR5/AβPP/PS2)—$^{\text{triple}}$AD mice—that combines both pathologic features of the disease in brain [1]. Using comparative, quantitative proteomics (iTRAQ) and mass spectroscopy, we found a massive deregulation of 24 proteins, of which one-third were mitochondrial proteins mainly related to complexes I and IV of the oxidative phosphorylation system (OXPHOS). Notably, deregulation of complex I was taudependent, while deregulation of complex IV was Aβ dependent, both at the protein and activity levels. Together, these findings emphasize that Aβ and tau synergistically impair complex I function with aging. Notably, changes in the expression of complex IV subunits seem to be mainly related to Aβ. Indeed, a down regulation of several subunits of complex IV is essentially seen between pR5 and $^{\text{triple}}$AD mice, but not between AβPP/PS2 and $^{\text{triple}}$AD mice. Interestingly, all of these deregulated proteins are nuclearencoded in agreement with the assumption of Sirk and coworkers [44].

5.1.2. Fusion and Fission Proteins. Mitochondria are dynamic organelles, undergoing continual fission, mediated by the protein Fis1 and dynamin-like protein1 (DLP1), and fusion, mediated by OPA1, Mfn1, and Mfn2 [45] (Figure 2). Unbalanced fusion leads to mitochondrial elongation, and unbalanced fission leads to excessive mitochondrial fragmentation, both of which impair the mitochondrial function [46, 47]. Mitochondrial fusion and fission have a different impact in mitochondria physiology: fission allows mitochondrial renewal, redistribution, and proliferation into synapses maintaining a pool of healthy mitochondria, while fusion facilitates mitochondrial movement and distribution across axons and synapses, suggesting a protective mechanism helping the maintenance of sufficient bioenergetic levels adjusted to situations with high-energy demands [48–50].

Several findings indicate that mitochondrial Aβ might play a role in impaired mitochondrial dynamics [6]. It could be demonstrated that neuroblastoma cells overexpressing human AβPP bearing the Swedish double mutation (AβPPsw) showed a higher percentage of highly fragmented and slower mitochondria compared to control cells. Moreover, MMP and ATP produced by mitochondria were reduced in AβPPsw cells compared to control cells, suggesting vice versa a direct effect of mitochondrial dynamic on the function of the organelle. In particular, proteins involved in mitochondrial dynamics were present at different levels in AβPPsw cells compared to control cells. Thus, in AβPPsw cells, the balance between mitochondrial fusion and fission was shifted to enhanced fission which was accompanied by increased protein levels of fission proteins such as Fis-1 and reduced levels of fusion proteins like OPA1 and DLP-1. In agreement with these in vitro findings, an abnormal distribution of mitochondria was also found in pyramidal neurons of AD-affected individuals [51–53].

Altered mitochondrial dynamics might be due to enhanced nitrosative stress generated by Aβ, such as S-nitrosylation of DLP1. This modification can disturb the balance between fission and fusion of mitochondria in favour of mitochondrial fission followed by mitochondrial depletion from axons and dendrites and subsequently synaptic loss [54, 55].

5.2. IMM

5.2.1. ETC: OXPHOS, ATP, and ROS. Early energy dysfunction characterized by a decreased mitochondrial membrane potential, ATP level, and complex IV activity has been reported for 3- and 6-month-old AβPP transgenic mice (AβPP; Swedish (KM670/671NL) and London (V717I) mutation) [3]. These mice showed also increased levels of 4-hydroxynonenal (HNE), a marker of lipid oxidation, and reduced activity of Cu/Zn superoxide dismutase (SOD) [56]. Interestingly, mitochondrial defects such as the decrease of complex IV activity in 3-month-old AβPP transgenic mice were already observed in the absence of plaques, but in the presence of increased Aβ levels in brain [3, 57]. Furthermore, an age-dependent impairment of oxygen consumption such as a decrease of state 3 and uncoupled respiration were observed in AβPP transgenic mice compared to age-matched controls [3, 30, 58]. In addition, AβPP/PS1 transgenic mice, which in contrast to AβPP transgenic mice exhibit Aβ plaques already at an age of 3 months, presented stronger reductions in mitochondrial membrane potential and ATP levels compared to age-matched AβPP transgenic mice. Consequently, Aβ-dependent mitochondrial dysfunction starts already at a very young age and accelerates substantially with increasing age as does Aβ plaque load [59].

The development of sophisticated proteomic methods allowed the examination of synaptosomal fractions from AβPP transgenic mice (Tg2576) and revealed a massive neuronal decay and synapse loss as the final consequence from all pathological changes occurring in AD [18]. Additional studies revealed significant differences in mitochondrial hsp70 and protein subunit composition of respiratory

chain complexes I and III in this transgenic mouse model [58].

In a new ^{triple}AD (pR5/AβPP/PS2) mouse model, we showed first of all that Aβ and tau act synergistically in amplifying mitochondrial respiratory deficits, mainly of complex I and IV activities. Thereby, hyperphosphorylated tau may drive a vicious cycle within the Aβ cascade. Remarkably, deregulation of complex I was related to tau, whereas deregulation of complex IV was Aβ dependent, both at the protein and activity levels. The synergistic effects of Aß and tau led already at the age of 8 months to a depolarized mitochondrial membrane potential in the ^{triple}AD mice. Additionally, we found that age-related oxidative stress at 12 months of age may exaggerate the dysfunctional energy homeostasis and synthesis of ATP and, in turn, take part in the vicious cycle that finally leads to cell death [60]. Our data complement those obtained in another triple transgenic mouse model 3xTg-AD (P301Ltau/AβPP/PS1) [61]. Yao and colleagues described age-related bioenergetic deficits in female 3xTg-AD mice aged from 3 to 12 months [62]. They found a decreased activity of regulatory enzymes of the OXPHOS (pyruvate dehydrogenase (PDH) and cytochrome *c* oxidase (COX)) and increased oxidative stress and lipid peroxidation. Most of the effects on mitochondria were seen at the age of 9 months, whereas mitochondrial respiration was significantly decreased with 12 months of age. Importantly, mitochondrial bioenergetic deficits precede the development of AD pathology in the 3xTg-AD mice.

5.2.2. Cyclophilin D. Mitochondrial Aβ may interact with Cyclophilin D (CypD), an integral part of the mitochondrial permeability transition pore (mPTP) which potentiates free radical production, causes synaptic failure, and promotes the opening of the mPTP leading to apoptosis [63].

CypD, a peptidylprolyl isomerase F, normally resides in mitochondrial matrix and is involved in apoptosis and necrosis. It has been demonstrated that CypD is able to form complexes with Aβ within mitochondria of cortical neurons from APP transgenic mice, increasing the translocation of CypD from the matrix to the inner membrane (Figure 2) [64]. The translocation of CypD represents a first step in the opening of mPTP and involves the binding of CypD with adenine nucleotide translocase [65–69]. mPTP opening can lead to matrix swelling, dissipation of the inner membrane potential, and generation of ROS, with subsequent rupture of the outer membrane and a nonspecific release of intermembrane space proteins into the cytosol, such as cytochrome c, apoptosis-inducing factor, Smac/DIABLO, endonuclease G, and procaspases, that will activate several signal transduction pathways such as apoptosis [70–75]. Furthermore, in AβPP transgenic mice the abrogation of CypD was able to attenuate Aβ-mediated abnormal mitochondrial dysfunction, such as calcium-induced mitochondrial swelling, lowered mitochondrial calcium uptake capacity, and impaired mitochondrial respiratory function [64]. Moreover, a correlation was found between levels of CypD in mitochondria and resistance to the opening of mPTP induced by calcium [76, 77], further supporting an active role of CypD in neuronal loss caused by Aβ.

5.3. Matrix

5.3.1. The Tricarboxylic Acid Cycle (TCA or Krebs Cycle). Impairment in the activity and levels of several mitochondria enzymes involved in the Krebs cycle was reported since the early 80's (Figure 2). Perry and colleagues observed a reduction in pyruvate dehydrogenase (PDH), ATP-citrate lyase, and acetoacetyl-CoA thiolase in postmortem brain tissues of AD-affected subjects and correlated the decrease of these enzymes to the decreased production of acetylcoenzyme A and cholinergic defects that are observed in AD [78]. A frontal cortex deficiency in PDH activity was also observed in another cohort of brain tissues from AD patients [79]. Moreover, a reduced activity of thiamine-pyrophosphate-dependent enzymes, such as α-ketoglutarate dehydrogenase (KGDH), was observed in brain tissues [80, 81] as well as in peripheral cells from AD subjects [81, 82]. The brain's reduction in α-ketoglutarate dehydrogenase presence and activity was region specific and observed mainly in temporal cortex, parietal cortex, and hippocampus [83]. Very interestingly, the loss of α-ketoglutarate-dehydrogenase-positive neurons could be correlated to the total loss of neurons, suggesting a possible reason for the selective vulnerability in AD brain [84]. In the central nervous system, the high metabolic demand can lead to a higher level of oxidative stress via the production of free radicals. KGDH is sensitive to a wide range of oxidants. Under pathological conditions, Aβ further increases oxidative stress leading directly or indirectly to a decline in the activity of KGDH [85, 86].

5.3.2. ABAD. The involvement of mitochondria in the pathogenic pathway of Aβ was confirmed by specific binding of Aβ and AβPP to mitochondrial proteins which causes energy impairment and cell physiology defects. Firstly, Aβ specifically binds to the mitochondrial Aβ-binding alcohol dehydrogenase (ABAD) [4], a mitochondrial matrix protein which is upregulated in the temporal lobe of AD patients as well as in AβPP transgenic mice [87–89]. The Aβ-ABAD interaction caused elevated reactive oxygen species (ROS) production and cell death as well as spatial learning and memory deficits in 5-month-old AβPP/ABAD double-transgenic mice. The investigation of the crystal structure of ABAD-Aβ demonstrated that the formation of the complex prevents the binding of NAD^+ to ABAD, thereby changing mitochondrial membrane permeability [90] and reducing the activities of respiratory enzymes [4] which then may lead to mitochondrial failure. ABAD, a member of the short-chain dehydrogenase/reductase family, shows enzymatic activity toward a broad array of substrates including n-isopropanol and beta-estradiol. Thus, ABAD is important for mitochondrial function via facilitation of ketone body utilization by promoting the generation of acetyl-CoA to feed into the TCA cycle, an effect that is particularly important in situations of stress.

5.3.3. mtDNA. Some of the alterations that are found in mitochondrial function in AD have been attributed to mutations of mtDNA [91]. Although most mitochondrial

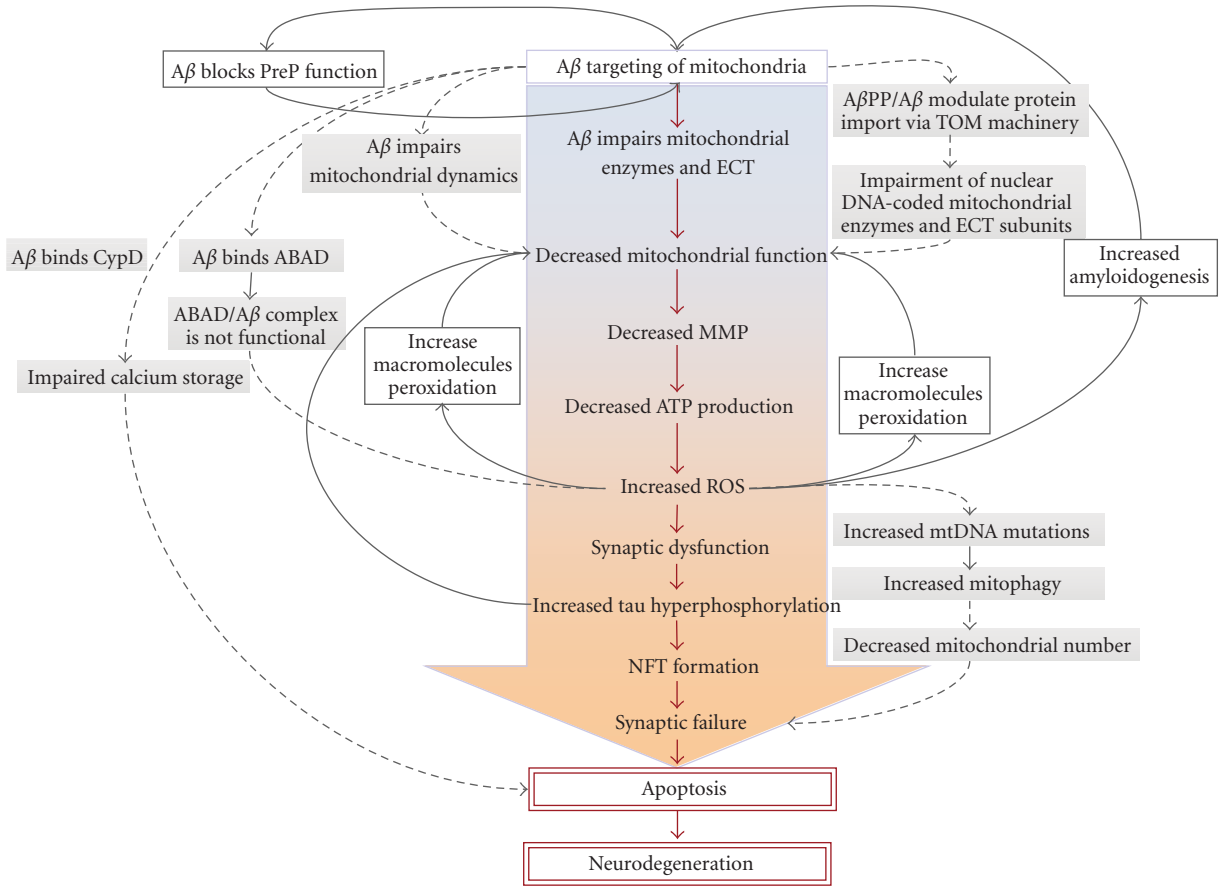

Figure 3: A hypothetical sequence of the pathogenic steps of the Alzheimer mitochondrial cascade hypothesis. The main cytotoxic pathway of Aβ (red arrows) involves Aβ-induced mitochondrial function, increased ROS production, activation of neurofibrillary tangles (NFT) formation, synaptic failure, and neurodegeneration. Several other pathways feed this cascade via feeding back (black arrows) or forward (dashed arrows) revealing several vicious cycles within a larger vicious cycle. All of them, once set in motion, amplify their own processes, thus accelerating the development of AD.

proteins are encoded by the nuclear genome, mitochondria contain many copies of their own DNA that encodes for 13 polypeptide complexes of the respiratory chain. The increased number of mtDNA mutations can be explained by the proximity of mtDNA to oxidative stress generated by the respiratory chain itself, the lack of mtDNA to any protective histone covering, and a deficient repair mechanism compared to nuclear DNA. Therefore, mitochondria themselves are extremely sensitive to oxidative stress. For instance, mtDNA np4336 mutation in the tRNAGln gene was reported in sporadic AD patients [91–95]. In addition, it was shown that AD brains exhibited a striking increase in a 1,112-np mtDNA control region, an element known to be involved in mtDNA transcription and/or replication [96]; mutations in this region may be responsible for a decreased number of mitochondria. In the light of these data, a mitochondria cascade hypothesis was developed to explain the prevalence of AD by hypothesising that the pathology will easily develop when a mitochondria starting line, in conjunction with genetic and environmental factors, exhibits increased mtDNA mutations (Figure 3).

5.3.4. PreP. Several enzymes were identified to degrade Aβ. Neural endopeptidase (NEP) was shown responsible for the extracellular degradation of Aβ [97], while the two members of the pitrilysin oligopeptidase family, human insulin degrading enzyme (IDE) and presequence protease (PreP), are responsible for the intracellular degradation of Aβ. PreP is a metalloprotease containing an inverted zinc-binding motif and was first identified in *Arabidopsis thaliana* as the enzyme responsible for mitochondrial degradation of targeting peptides up to 65 amino acids [98–100]. Intramitochondrial localization studies demonstrated PreP to be localized within the mitochondrial matrix [101, 102]. In case, Aβ reaches the matrix, it can be degraded by PreP (Figure 2). Two highly conserved cysteines (Cys527 and Cys90) are able to form a disulphide bridge in oxidizing condition,

locking PreP in an inactive form. Since oxidizing conditions are present with high amount of Aβ in mitochondria, this may prevent the clearance of Aβ (Figure 2), exacerbating mitochondrial dysfunctions by accelerating its interactions with CypD and/or ABAD [37].

6. Conclusion

Rigorous scientific research has identified multiple mechanisms of Aβ interaction with mitochondria at different mitochondrial compartments: the outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and the matrix. With regard to the involvement of Aβ-induced mitochondrial dysfunction in AD pathogenesis, a vicious cycle as well as several vicious circles within the cycle, each accelerating the other, can be drawn emphasizing the Alzheimer mitochondrial cascade hypothesis (Figure 3). AβPP and/or Aβ may block mitochondrial translocation of nuclear-encoded proteins [27], such as components of the ETC [103–106], impairing mitochondrial function. Intramitochondrial Aβ is able to perturb mitochondrial function in several ways by directly influencing ETC complex activities [1], impairing mitochondrial dynamics [6] or disturbing calcium storage [107, 108], thus increasing apoptotic pathways [64], as well as via interaction with CypD. Moreover, Aβ interacts with mitochondrial matrix components, such as enzymes of the Krebs cycle [109] and ABAD [87, 110], as well as PreP [37]. An improper mitochondrial complex function leads to a decreased mitochondrial membrane potential of the organelle [111], impairing ATP formation [3, 59, 112–118]. Increased ROS levels act at multiple levels to impair mitochondrial function: they induce mtDNA mutations [91] that consequently negatively influence mitochondrial function [119], enhance Aβ production by guiding AβPP cleavage pathway toward the amyloidogenesis [120], increase lipid peroxidation [121, 122], activate mitophagy [123], leading to a reduced mitochondrial number [123], and augment tau hyperphosphorylation and NFT formation impairing organelle trafficking and neuronal function finally leading to apoptosis.

Finally, the critical role of mitochondria in the early pathogenesis of AD may make them attractive as a preferential target for treatment strategies. Transgenic mice modelling some pathological aspects are hence very valuable in monitoring therapeutic interventions at the mitochondrial level. In agreement, recent data suggest that natural plants such as a standardized *Ginkgo biloba* extract or the green tea component epigallocatechin-3-gallate may be promising treatment strategies. Of note, in addition to their antioxidative properties, these compounds stabilize mitochondrial functions such as the mitochondrial membrane potential, ATP levels, and mitochondrial respiratory complexes [124–127]. Moreover, in APP transgenic mouse models, an antiamyloidogenic effect of these compounds was reported by inhibiting amyloid fibril formation either by a direct interaction with Aβ [128, 129] or indirectly by reducing ROS levels [127]. However, the precise actions and, in particular, the mitochondrial targets of these drugs at the molecular level are unclear and need further clarification. In view of the increasing interest in mitochondrial protection as a treatment strategy in dementia, besides strategies with regard to the treatment and/or removal of both Aβ and tau pathology, the findings of a substantial protection of mitochondria against Aβ-induced dysfunction deserve further attention.

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Research Article

ApoE4-Driven Accumulation of Intraneuronal Oligomerized Aβ42 following Activation of the Amyloid Cascade *In Vivo* Is Mediated by a Gain of Function

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Activating the amyloid cascade by inhibiting the Aβ-degrading enzyme neprilysin in targeted replacement mice, which express either apoE4 or apoE3, results in the specific accumulation of oligomerized Aβ42 in hippocampal CA1 neurons of the apoE4 mice. We presently investigated the extent to which the apoE4-driven accumulation of Aβ42 and the resulting mitochondrial pathology are due to either gain or loss of function. This revealed that inhibition of neprilysin for one week triggers the accumulation of Aβ42 in hippocampal CA1 neurons of the apoE4 mice but not of either the corresponding apoE3 mice or apoE-deficient mice. At 10 days, Aβ42 also accumulated in the CA1 neurons of the apoE-deficient mice but not in those of the apoE3 mice. Mitochondrial pathology, which in the apoE4 mice is an early pathological consequence following inhibition of neprilyisn, also occurs in the apoE-deficient but not in the apoE3 mice and the magnitude of this effect correlates with the levels of accumulated Aβ42 and oligomerized Aβ42 in these mice. These findings suggest that the rate-limiting step in the pathological effects of apoE4 on CA1 neurons is the accumulation of intracellular oligomerized Aβ42 which is mediated via a gain of function property of apoE4.

1. Introduction

Apolipoprotein E (apoE) is a major brain lipoprotein and is expressed in humans as three common isoforms that differ from each other by one or two amino acids; these isoforms are termed apoE2 (Cys112, Cys158), apoE3 (Cys 112, Arg158), and apoE4 (Arg112, Arg158) [1, 2]. Genetic and epidemiological studies revealed that the allele ε4 of apoE is a strong genetic risk factor for early and late onset of Alzheimer's disease (AD) [3, 4]. More than 50% of AD patients carry the apoE ε4 allele which increases the risk for the disease by 2-3-fold in individuals who express one such allele and by more than 10-fold in subjects with two ε4 alleles [5, 6]. Histopathologically, apoE4 is associated in AD with increased amyloid deposition [7], and corresponding animal model and *in vitro* studies revealed synergistic pathological interactions between Aβ and apoE4 [4, 8–12] that are associated with cognitive deficits [13, 14]. This led to the suggestion that apoE4 potentiates the neurotoxic effects of Aβ and the amyloid cascade and drives them above a pathological threshold. The molecular mechanisms underlying the pathological cross-talk between Aβ and apoE and the extent to which they also mediate other pathological hallmarks of apoE4 in AD, such as impaired neuronal plasticity and repair [15–17] and increased brain inflammation [18], are currently not known. Another key unresolved issue is whether the pathological effects of apoE4 are due to the gain of a pathological property by apoE4 or to the loss of a protective function by this molecule, that the other apoE isoforms have.

We have recently shown that activation of the amyloid cascade by inhibiting the Aβ-degrading enzyme neprilysin

in targeted replacement mice that express either apoE4 or apoE3 results in the isoform-specific accumulation of Aβ42, oligomerized Aβ42, and apoE in CA1 neurons of the apoE4 mice, which in turn trigger mitochondrial pathology, neurodegeneration, and activation of cell death process [13, 19]. We presently investigate the extent to which the apoE4-driven intracellular accumulation and oligomerization of Aβ42 and the resulting mitochondrial impairments are due to either gain or loss of function of apoE4 relative to the AD benign isoform, apoE3. This was performed by inhibiting the Aβ-degrading enzyme neprilysin in apoE-deficient mice and in corresponding apoE4- and apoE3-targeted replacement mice. This was then followed by investigation of the resulting effects of apoE deficiency on the accumulation and oligomerization of Aβ42 and the associated neuropathology in CA1 neurons and of the extent to which they are similar to those observed in either the apoE4 or the apoE3 mice.

2. Materials and Methods

2.1. Transgenic Mice and Implantation of Alzet Miniosmotic Pumps. ApoE-targeted replacement mice (apoE mice), created by gene targeting [20], were purchased from Taconic. The mice were back-crossed to C57BL/6J mice for eight generations and were homozygous for either the apoE3 (3/3) or the apoE4 (4/4) allele. ApoE-deficient mice were purchased from Jackson Laboratories and were on the same background. The experiments were performed utilizing 4-month-old male apoE3, apoE4, and apoE-deficient mice whose genotypes were confirmed by PCR analysis [13, 21]. All experiments were approved by the Tel Aviv University Animal Care Committee, and every effort was made to minimize animal usage and reduce animal stress. Alzet miniosmotic pumps (model 2001, which deliver their contents at 0.25 μl/h for up to 14 days) were loaded with the neprilysin inhibitor thiorphan (0.5 mM; Sigma) in artificial cerebrospinal fluid containing 1 mM ascorbic acid or with a similar solution without thiorphan "sham." The Alzet pumps were implanted with a brain infusion canola inserted into the lateral ventricle as previously described [13].

2.2. Immunofluorescence and Confocal Microscopy. Thiorphan and sham-treated mice were anesthetized with ketamine xylazine at the indicated times following implantation of the Alzet pumps. The brains of these mice were then processed for immunoflourescence after which free-floating frozen coronal sections (30 μm) were treated with 70% formic acid for 7 min and then immunostained as previously described [13]. Aβ42 and oligomerized Aβ42 were detected utilizing rabbit anti-Aβ42 (dilution 1 : 500 from Chemicon) and biotinylated I-11 (dilution 1 : 2000) provided by Dr. Rakez Kayed, whereas the mitochondrial marker COX-1 was detected utilizing goat anti-COX-1 (1 : 400, from Santa Cruz Biotechnology). The bound primary antibodies were then visualized by incubating the sections with the appropriate fluorescently labeled second antibody, or with fluorescently labeled streptavidin as previously described [13]. The sections (between bregma – 2.0 and – 3.0) were visualized using a confocal scanning laser microscope (Zeiss, LSM 510). Images (1024 × 1024 pixels) were obtained by averaging eight scans per slice. Control experiments revealed no staining in stained sections lacking the first antibody. The intensities of immunofluorescence staining, expressed as the percentage of the area stained, were calculated utilizing the Image-Pro Plus system (version 5.1, Media Cybernetics) as previously described [13]. Two sections were analyzed per brain, and each staining was performed at least twice. All the images for each immunostaining were obtained under identical conditions, and their quantitative analyses were performed with no further handling. Moderate adjustments for contrast and brightness were performed on the images when the figures were prepared but were the same for the images of the different mouse groups. For the Aβ42 and I-11 and the COX-1 and Aβ42 double labeling colocalization experiments, each image was first analyzed separately to determine the percentage of the area stained and then to determine the percentage of the area of the two images that colocalize. Measurements of the contribution of stochastic processes to the co-localization data, which were performed by shifting the channels of one of the images laterally by 1 μm^2, revealed that the contribution of stochastic processes in all the experiments was negligible.

2.3. Immunoblot Analysis. Hippocampi were homogenized (20% w/v) in PBS, pH 7.4, with protease inhibitor cocktail (Roche, # 1 836 153) and centrifuged at 10,000 rpm for 5 min, after which the supernatant (PBS extract) was collected. The resulting pellet was extracted by resuspension in an equal volume of 10 mM Tris pH 7.6 containing 150 mM NaCl, 2 mM EDTA, protease inhibitor cocktail (Roche), and 1% NP-40, after which it was centrifuged for 5 min at 10,000 rpm and the resulting supernatant (NP-40 extract) was collected. SDS gel electrophoresis utilizing 12% SDS and immunoblotting with mAb 266 (Elan pharmaceuticals) were then performed as previously described [19]. Intensities of the immunoblot bands were quantified by using EZQuant-Gel software (EZQuant, Tel-Aviv, Israel).

2.4. Statistical Analysis. It was performed using SPSS version 14. The effects of treatment in the apoE-deficient mice (e.g., sham and thiorphan-treated mice) and in the thiorphan-treated apoE3-, apoE4-, and apoE-deficient mice at the 7-day time point were each analyzed by one-way ANOVA. In the 10-day experiment, in which both sham- and thiorphan-treated mice in all three mouse groups were investigated, the results were analyzed by two-way ANOVA. When appropriate, these results were further subjected to post hoc analysis by Student's t-test utilizing Bonferroni correction for multiple comparisons.

3. Results

Immunofluorescence measurements of the levels of Aβ42 in hippocampal CA1 neurons of apoE-deficient mice and their comparison to those of the corresponding apoE3 and apoE4 targeted replacement mice are depicted in Figure 1(a). As shown, the Aβ42 levels of the apoE deficient mice were not affected by the thiorphan treatment at day 7; they were

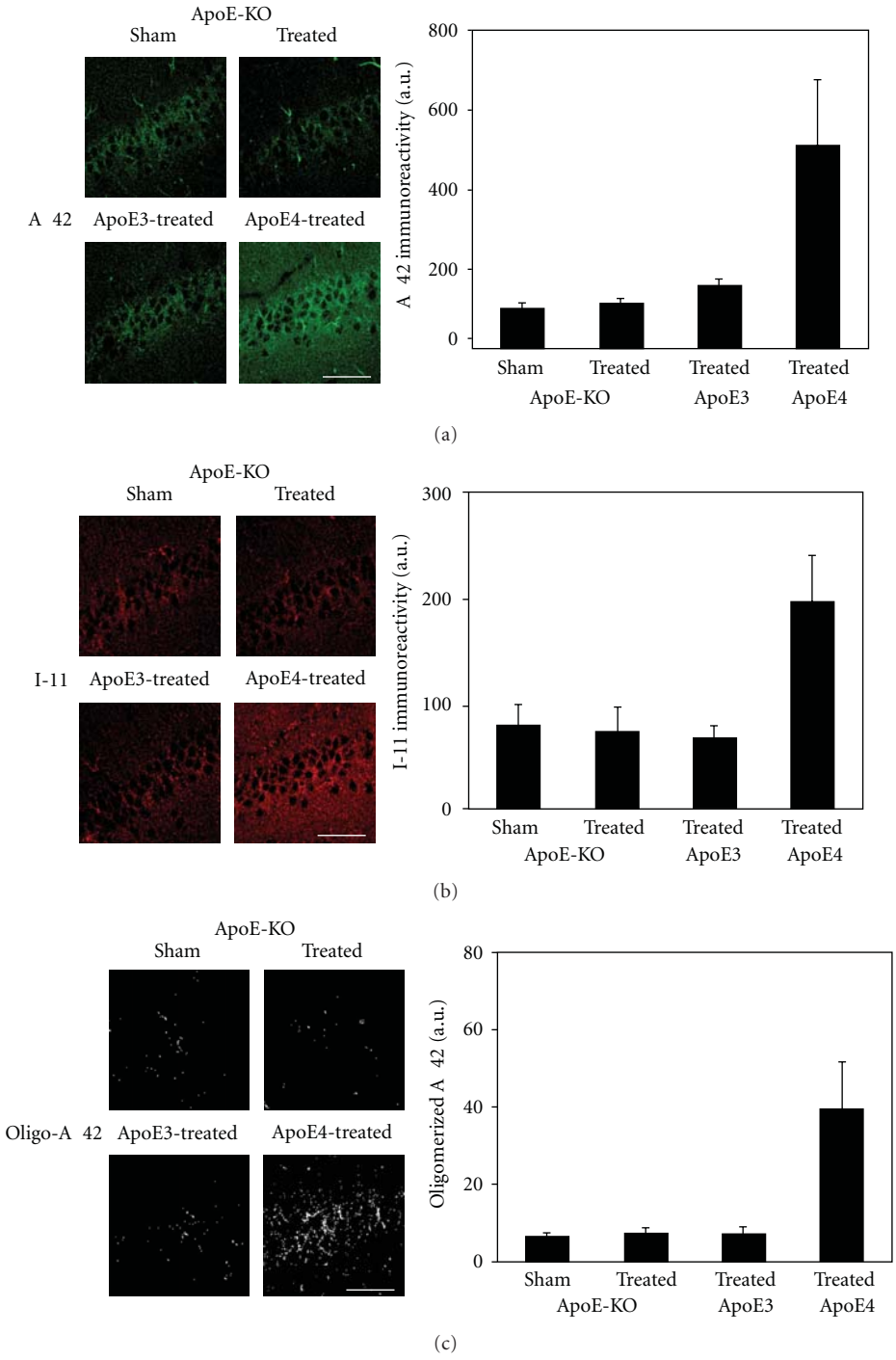

Figure 1: The effects of apoE3, apoE4, and apoE deficiency on the levels of Aβ42 and oligomerized Aβ42 in CA1 hippocampal neurons following inhibition of neprilysin. ApoE3, apoE4, and apoE-deficient male mice were injected i.c.v. with the neprilysin inhibitor thiorphan or sham-treated for 7 days, after which their brains were excised and subjected to anti-Aβ42 and anti-I-11 immunofluorescence, as described in "Materials and Methods." (a) Representative coronal sections of sham- and thiorphan-treated apoE-deficient mice (upper row) and thiorphan-treated apoE3 and apoE4 mice (lower row) immunostained with anti-Aβ42 are shown on the left (bar = 50 μm). Quantification of the density of Aβ42 staining (mean ± SEM; n = 5–6 mice/group in the sham- and thiorphan-treated groups) in the CA1 neurons of the indicated mice is shown on the right. $P < .05$ for the effects of treatment on the three mouse groups by one-way ANOVA. (b) Representative confocal images of I-11 of the CA1 area of the indicated mouse groups treated for 7 days with thiorphan (left) and quantification (right) of the density of I-11 staining (mean ± SEM; n = 5–6 mice/group in the sham- and thiorphan-treated groups) (mean ± SEM; n = 4–5). $P < .03$ for the effect of treatment on the three mouse groups by one-way ANOVA. (c) Representative masked oligo-Aβ42 images of the CA1 area of the indicated mouse groups treated for 7 days with thiorphan (left) and quantification (right) of the density of oligo-Aβ42 staining (mean ± SEM; n = 5–6 mice/group in the sham- and thiorphan-treated groups). $P < .05$ for the effect of treatment on the three mouse groups by one-way ANOVA.

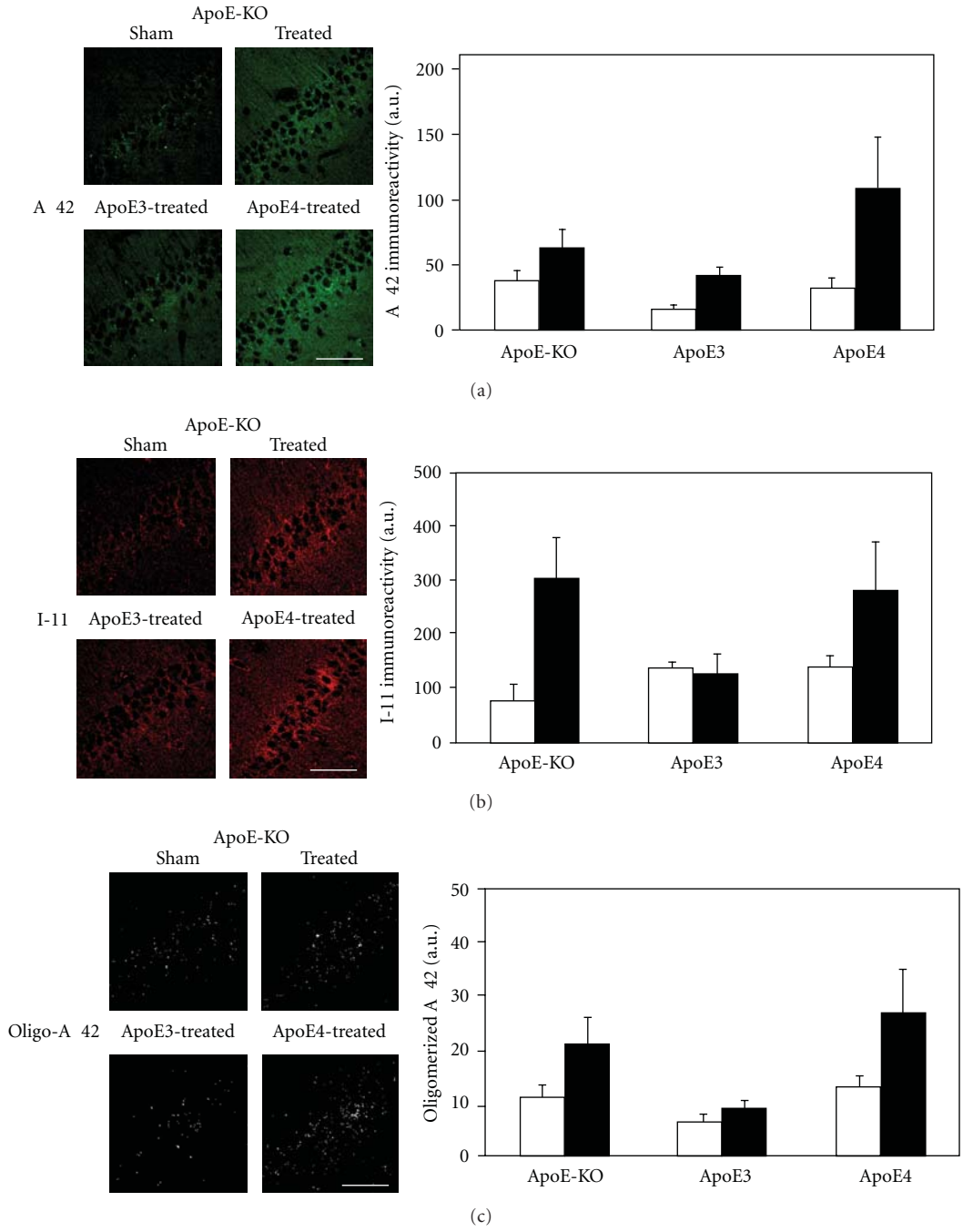

Figure 2: The effects of apoE3, apoE4, and apoE deficiency on the levels of Aβ42 and oligomerized Aβ42 in CA1 hippocampal neurons following inhibition of neprilysin. ApoE3-, apoE4- and apoE-deficient male mice were injected i.c.v. with the neprilysin inhibitor thiorphan; or sham-treated for 10 days, after which their brains were excised and subjected to anti-Aβ42 and anti-I-11 immunfluorescence, as described in "Materials and Methods." (a) Representative coronal sections of sham- and thiorphan-treated apoE-deficient mice (upper row) and thiorphan-treated apoE3 and apoE4 mice (lower row) immunostained with anti-Aβ42 are shown on the left (bar = 50 μm). Quantification of the density of Aβ42 staining (mean ± SEM; n = 4–5 mice/group) in the CA1 neurons of the indicated mice is shown on the right (empty and filled bars correspond, resp., to sham- and thiorphan-treated mice). $P < .02$ for the effects of treatment on the three mouse groups by two-way ANOVA. (b) Representative confocal images of I-11 of the CA1 area of the indicated mouse groups treated for 10 days with thiorphan (left) and quantification (right) of the density of I-11 staining (mean ± SEM; n = 4–5 mice/group). Empty and filled bars correspond, respectively, to sham- and thiorphan-treated mice and $P < .02$ for the effect of treatment by two-way ANOVA. (c) Representative masked oligo-Aβ42 images of the CA1 area of the indicated mouse groups treated for 10 days with thiorphan (left) and quantification (right) of the density of oligo-Aβ42 staining (mean ± SEM; n = 4–5 mice/group). Empty and filled bars correspond, respectively, to sham- and thiorphan-treated mice and $P < .02$ for the effect of treatment by two-way ANOVA.

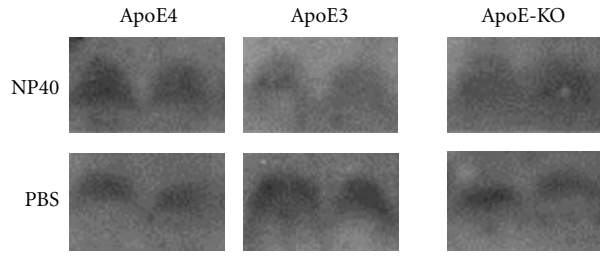

Figure 3: Aβ42 immunoblot of the hippocampal CA1 field of apoE4, apoE3, and apoE-deficient mice. The mice were treated for 10 days with thiorphan after which they were killed and their hippocampus was extracted and immunoblotted with the anti-Aβ42 Ab 266 as described in "Materials and Methods." Membrane bound NP40 extractable Aβ42 (apparent molecular weight 4.5 kD) is shown in the upper panels whereas the corresponding PBS extractable soluble Aβ42 is shown in the lower panels.

similar to those of the thiorphan-treated apoE3 mice and were significantly lower than those of the thiorphan-treated apoE4 mice ($P < .05$; Figure 1(a)). Further measurements of the effects of apoE on the accumulation of oligomerized Aβ42 were performed utilizing Ab I-11, which is directed specifically at the backbone of amyloid oligomers [22, 23]. This revealed that at day 7, and in accordance with the Aβ42 results, the thiorphan treatment induced I-11 immunoreactivity to accumulate in the CA1 neurons of the apoE4 but not in those of either the apoE3 mice or the apoE-deficient mice ($P < .03$; Figure 1(b)). The I-11 and Aβ42 immunoreactivities of the individual apoE4 mice were highly correlated ($R^2 = 0.9$). I-11 recognizes amyloid-like structures derived from Aβ as well as non-Aβ peptides [23]. Accordingly, the levels of I-11 in the different mouse groups that correspond to oligomerized Aβ42 were determined by double labeling confocal experiments utilizing I-11 and anti-Aβ42. This revealed significant co-localization of these stains and of their merged image, which we will term here "oligo-Aβ42," in the CA1 neurons of the apoE4 mice (Figure 1(c)). In contrast, and in agreement with the single labeling experiments, the levels of oligo-Aβ42 in CA1 neurons of the apoE-deficient and apoE3 mice were similar and low.

Additional measurements of the levels of Aβ42, I-11, and oligo-Aβ42 in the different mouse groups were also performed on day 10 after the thiorphan treatment began. This revealed that, unlike at the earlier time point, the levels of Aβ42, I-11, and oligo-Aβ42 of the apoE-deficient mice were now elevated and comparable to those observed with the apoE4 mice (Figure 2; $P < .02$ for the effect of treatment by 2-way ANOVA). Importantly, the corresponding levels of Aβ42, I-11, and oligo-Aβ42 in the apoE3 mice were not increased by the thiorphan treatment even at 10 days (Figure 2).

Previous immunoblot experiments utilizing hippocampal homogenates revealed that the thiorphan-treated apoE3 and apoE4 mice have similar Aβ42 levels but that in the apoE4 most of the Aβ42 is membrane-bound whereas in the apoE3 mice it is soluble [19]. Accordingly, we next investigated the extent to which apoE deficiency affects the levels and solubility of the accumulated Aβ42. As shown in Figure 3 the levels of the membrane-bound NP40 extractable Aβ42 and of the soluble PBS extractable Aβ42 pools of the apoE-deficient mice were intermediate to those of the apoE3 and apoE4 mice. Furthermore, the total levels of soluble and insoluble pools were similar in the apoE4, apoE deficient, and apoE3 mice (resp., 100 ± 30%, 91 ± 17% and 91 ± 18%). Comparison of these results to the immunoflourescence findings (compare Figures 2 and 3 both of which were obtained at day 10) revealed that the relative levels of the NP40 extractable Aβ42 pools and of the accumulation of intracellular Aβ42 in the different mice groups have the same rank order (apoE4 > apoE deficient > apoE3) suggesting that the accumulated intracellular Aβ42 is membrane bound.

We have recently shown by electron microscopy that the specific accumulation of Aβ42 in CA1 neurons of apoE4 mice following inhibition of neprilysin is associated with marked mitochondrial deformation and with the colocalization of Aβ in the affected mitochondria [19]. Complementary immunofluorescence confocal microscopy experiments revealed that the mitochondrial pathology is associated with increased levels of mitochondrial COX-1 immunoreactivity and with the colocalization of Aβ42 with COX-1 [19]. Utilizing COX-1 as a marker of mitochondrial pathology, we investigated the extent to which inhibition of neprilysin in the mice affects their mitochondria. This revealed that mitochondrial pathology at 7 days, like the accumulation of Aβ42 and oligo-Aβ42, occurs only in the apoE4 mice (not shown). In contrast, at day 10 it occurred in both the apoE4 and the apoE-deficient mice but not in the apoE3 mice (Figure 4(a)). Furthermore, the magnitude of the mitochondrial effect and the levels of Aβ42 in the apoE-deficient were both similarly lower in the apoE-deficient than the apoE4 mice (compare Figures 2 and 4). Co-localization confocal microscopy revealed that the Aβ42 which accumulates in CA1 neurons of the apoE-deficient mice, like that of the corresponding apoE4 mice [19], colocalizes with mitochondria (Figure 4(b)). The findings that the levels of mitochondrial pathology and of Aβ42 and oligomerized Aβ42 in the CA1 neurons of the different mice groups correlate suggest that the main and rate-limiting effect of apoE4 on the mitochondria is due to stimulation of the accumulation of Aβ42 and oligomerized Aβ42.

4. Discussion

The present study revealed that apoE4 triggers the accumulation of Aβ42 in hippocampal CA1 neurons during the early phase (i.e., 7 days), following activation of the amyloid cascade *in vivo* and that this effect is specific to apoE4 and does not occur in either apoE3 or apoE-deficient mice. This effect reflects differences in the extent of accumulation of Aβ42, since the total hippocampal Aβ42 contents, determined by immunoblots, were similarly elevated following inhibition of neprilysin in the ApoE3 and apoE4 mice [19] and in the apoE-deficient ones (not shown). The present findings are in accordance with previous *in vitro* cell culture studies [24–26] and suggest that the rate-limiting step in the apoE4-driven accumulation of Aβ42 is due to a gain of function.

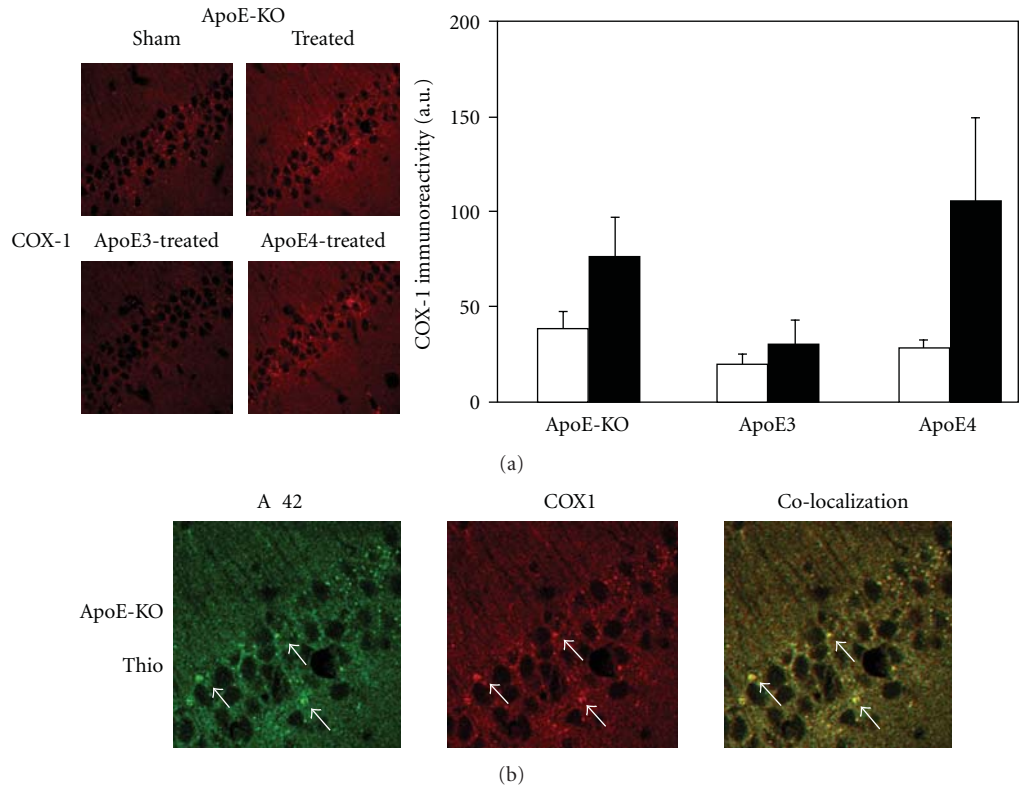

Figure 4: The effects of apoE3, apoE4, and apoE deficiency on the mitochondria of hippocampal CA1 neurons following inhibition of neprilysin. ApoE3, apoE4, and apoE-deficient male mice were injected i.c.v. with the neprilysin inhibitor thiorphan or sham-treated for 10 days, after which their brains were excised and subjected to COX-1 immunofluoresence as described in "Materials and Methods." (a) Representative coronal sections of sham- and thiorphan-treated apoE-deficient mice (upper row) and thiorphan-treated apoE3 and apoE4 mice (lower row) immunostained with anti-COX-1 are shown on the left (bar = 50 μm). Quantification of the density of staining (mean ± SEM; n = 4–5 mice/group in the sham- and thiorphan-treated groups) in the CA1 neurons of the indicated mice is shown on the right (empty and filled bars correspond, resp., to sham- and thiorphan-treated mice). $P < .03$ for the effects of treatment on the three mouse groups by Two-way ANOVA. (b) Representative confocal images of the co-localization of Aβ42 and COX-1 in the CA1 area of apoE-deficient mice treated with thiorphan for 10 days.

The finding that the I-11 immunoreactivity levels of the CA1 neurons in the apoE-deficient mice, like their Aβ42 levels, did not rise during the initial 7 days following the thiorphan treatment (Figure 1(b)) is in accordance with the observation that these molecules colocalize in the thiorphan-treated apoE4 mice (Figure 1(c)). Moreover, it suggests that thiorphan treatment in the apoE-deficient mice does not stimulate the production of non-Aβ amyloid molecules in the CA1 neurons.

At longer time intervals, following activation of the amyloid cascade by inhibition of neprilysin (i.e., 10 versus 7 days), this treatment also induces the accumulation of oligomerized Aβ42 in the CA1 neurons of the apoE-deficient mice but not those of the apoE3 mice (Figure 2). This may be a reflection of time-dependent differences in the apoE dependencies of the kinetics of Aβ42 accumulation, such that Aβ42 accumulation in the apoE-deficient mice is delayed relative to that observed with the apoE4 mice. Alternatively, since the extent of Aβ42 accumulation is determined by the interplay between uptake and clearance, it is also possible that apoE-related effects on the clearance of Aβ42 contribute significantly to the observed effects at 10 days. Indeed, it has recently been shown that the clearance of Aβ is more effective in apoE3 mice than in apoE4- and apoE-deficient mice [1, 27, 28]. This suggests that the observation that the levels of Aβ42 are particularly low in the thiorphan-treated apoE3 mice at 10 days (Figure 2(a)) may be due, at least in part, to enhanced clearance of Aβ42 by apoE3. Further studies are required for unraveling the role of clearance-related mechanisms in mediating the effects of the different apoE isoforms and of apoE deficiency on the intracellular levels of Aβ42.

Examination of the effects of apoE-deficiency on the oligomerization of Aβ42 revealed that whereas the levels of Aβ42 in the apoE-deficient mice at 10 days are lower than those of the apoE4 mice (Figure 2(a)), the two mice groups have similar levels of oligomerized Aβ42 (Figures 2(b) and 2(c)). This suggests that either apoE does not play a rate-limiting role in the early stages of oligomerization of Aβ42 which are detected with I-11, or that apoE deficiency has an indirect stimulatory effect on the aggregation of Aβ42.

The levels of Aβ42 and oligomerized Aβ42 in the CA1 neurons of the different mice groups and time points reveal that they correlate positively with the corresponding levels of mitochondrial pathology (Figures 1, 2, 4). This suggests that the limiting step in the observed effects of apoE4 on the mitochondria is stimulation of the accumulation and the oligomerization of Aβ42. One implication of this conclusion is that the effects of apoE4 on the mitochondria are not mediated via direct effects of apoE4 on the mitochondria, which is consistent with our recent finding that the apoE4 which accumulates in CA1 neurons following inhibition of neprilysin does not colocalize with mitochondria [19].

The mechanisms underlying the accumulation of Aβ42 and oligomerized Aβ42 in the mitochondria are not fully understood. Since Aβ42 also accumulates in the lysosome of the CA1 neurons of the neprilysin inhibited apoE4 mice [19], it is possible that Aβ42 reaches the cytoplasm and the mitochondria via the lysosomal pathway and lysosomal leakage. Alternately since extracellularly applied Aβ42 accumulates in the mitochondria of neuronal cultures [29], it is also possible that the extracelluar Aβ42 which accumulates following inhibition of neprilysin reaches the mitochondria via this route.

In conclusion, the present findings show that the isoform-specific accumulation of Aβ42 and oligomerized Aβ42 in hippocampal neurons, following activation of the amyloid cascade *in vivo,* is mediated by a gain-of-function property of apoE4. Furthermore, since the resulting mitochondrial pathology correlates with the levels of accumulated Aβ42 and oligomerized Aβ42, this suggests that the overall pathological effects of apoE4 in this system are driven by the effects of apoE4 on the accumulation of Aβ42 and that consequently an anti-apoE4 therapeutic strategy may be effective in counteracting the synergistic pathological effects of apoE4 and Aβ42.

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Review Article

Impacts of Membrane Biophysics in Alzheimer's Disease: From Amyloid Precursor Protein Processing to Aβ Peptide-Induced Membrane Changes

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An increasing amount of evidence supports the notion that cytotoxic effects of amyloid-β peptide (Aβ), the main constituent of senile plaques in Alzheimer's disease (AD), are strongly associated with its ability to interact with membranes of neurons and other cerebral cells. Aβ is derived from amyloidogenic cleavage of amyloid precursor protein (AβPP) by β- and γ-secretase. In the nonamyloidogenic pathway, AβPP is cleaved by α-secretases. These two pathways compete with each other, and enhancing the non-amyloidogenic pathway has been suggested as a potential pharmacological approach for the treatment of AD. Since AβPP, α-, β-, and γ-secretases are membrane-associated proteins, AβPP processing and Aβ production can be affected by the membrane composition and properties. There is evidence that membrane composition and properties, in turn, play a critical role in Aβ cytotoxicity associated with its conformational changes and aggregation into oligomers and fibrils. Understanding the mechanisms leading to changes in a membrane's biophysical properties and how they affect AβPP processing and Aβ toxicity should prove to provide new therapeutic strategies for prevention and treatment of AD.

1. Introduction

The senile plaque composed of neurotoxic amyloid-β peptide (Aβ) is a pathologic characteristic of Alzheimer's disease (AD) [1–6]. Aβ is derived from the cleavage of a type I membrane protein, (AβPP), by β- and γ-secretases [7]. Alternatively, AβPP can be cleaved by α-secretase to produce a neurotrophic, neuroprotective α-secretase-cleaved soluble AβPP (sAβPP$_\alpha$) through a nonamyloidogenic pathway [8]. These two pathways compete with each other, and increasing sAβPP$_\alpha$ has been suggested as a potential therapy for AD [9]. The cleavage of AβPP by β-secretase (BACE), the primary step to produce Aβ [10, 11], occurs mainly in lipid rafts, which are highly ordered membrane microdomains enriched in cholesterol, sphingolipids and saturated phospholipids [12–17]. On the other hand, the activity of α-secretases is favored in nonraft domains [18]. Therefore, AβPP processing can be altered by manipulating membrane lipid composition, such as removal of cholesterol and sphingolipids [19–22]. Since AβPP, α-, β-, and γ-secretases are membrane proteins, AβPP processing can be also affected by the biophysical membrane properties such as membrane fluidity and molecular order [19–26].

Amyloidogenic cleavage of the AβPP leads to the production of Aβ peptides of different length [27]. An increasing amount of evidence supports the notion that cytotoxic effects of Aβ are strongly associated with its ability to interact with membranes of neurons and other cerebral cells, astrocytes, microglial, and cerebral endothelial cells. Aβ peptides in different forms can directly bind to membrane molecules and alter biophysical membrane properties [28–33]. Aβ can also indirectly affect membrane properties by binding to membrane receptors and triggering downstream signaling pathways. Moreover, there is evidence that Aβ_{1-42} oligomers can accelerate the amyloidogenic processing of AβPP by changing membrane physical properties and interacting with lipid raft-related ganglioside GM-1 [25]. Membrane properties and composition, and the presence of metal ions,

in turn, play critical roles in Aβ cytotoxicity associated with its conformational changes, aggregation into oligomers and fibrils, and ability to interact with membrane molecules [34–40].

In this paper, we summarize the effects of membrane composition and properties on AβPP processing and interactions of physiologically relevant $A\beta_{1-40}$ and $A\beta_{1-42}$ with membranes studied in membrane models, cell cultures, and *in vivo*. Understanding the mechanisms leading to changes in a membrane's biophysics and how they cause changes in AβPP processing and Aβ toxicity should provide insights into new therapeutic strategies for prevention and treatment of AD.

2. Role of Physical Membrane Properties in AβPP Processing

2.1. Cholesterol on Physical Properties of Membranes and AβPP Processing. Cholesterol is an essential component of the cellular membrane and plays a vital role in the regulation of membrane functions. Distribution of cholesterol within the plasma membrane is not even: the highest level of free cholesterol inside the plasma membrane is found in cytofacial bilayer leaflet [41, 42]. The exofacial leaflet contains substantially less cholesterol, and it is mostly condensed in lipid rafts, which are more tightly packed than nonlipid raft domains due to intermolecular hydrogen bonding involving sphingolipid and cholesterol [43]. This asymmetric distribution of cholesterol is altered by aging: it is significantly increased in exofacial leaflet with increasing age [42, 44]. Membrane cholesterol levels can also be modulated by specific inhibitors of the cellular biosynthesis such as statins (3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) inhibitors), or it can be selectively extracted from plasma membrane by methyl-β-cyclodextrin (MβCD) [41]. The percentage of cholesterol in phospholipid bilayers affects many biophysical parameters of lipid bilayers, such as thickness, thermomechanical properties, molecular packing, conformational freedom of phospholipid acyl chains and water, molecular oxygen permeability, membrane hydrophobicity, membrane excitability in neurons, internal dipolar potential, and membrane fluidity [45–51].

It has been shown that intracellular cholesterol homeostasis regulates AβPP processing [52]. A model of membrane compartmentalization has been suggested for AβPP present in two cellular pools, one associated with the cholesterol-enriched lipid rafts, where Aβ is generated, and another outside of rafts (i.e., nonraft domains), where α-cleavage occurs [13]. It has been reported that membrane cholesterol depletion decreased the content of AβPP in cholesterol and sphingolipid-enriched membrane microdomains and subsequently inhibited the amyloidogenic pathway to produce Aβ [19, 53]. In contrast, cholesterol accumulation in Niemann Pick type C (NPC) model cells has been shown to shift AβPP localization to lipid rafts [54]. Exposure of cholesterol to astrocytes, primary neurons, and glial cultures inhibited the secretion of $sAPP_\alpha$ and reduced cell viability [55–57]. It has been demonstrated that cholesterol decreased the secretion of $sAPP_\alpha$ by interfering with AβPP maturation and inhibiting glycosylation of the protein [56]. Furthermore, some studies showed that cholesterol levels in the membranes were positively correlated with β-secretase activity [58], while lovastatin enhanced the α-secretase activity [55].

The results of another study showed that the cholesterol transport inhibitor, U18666a, increased $sA\beta PP_\alpha$ secretion and intracellular $A\beta PP_\alpha$ levels and reduced secretion of $A\beta_{1-40}$ in N2aAPP "Swedish" cells [59]. Inhibition of intracellular cholesterol transport also altered presenilin localization and AβPP processing in neuronal cells [60]. Similarly, inhibition of Acyl-coenzyme A:cholesterol acyltransferase (ACAT) modulated AβPP trafficking and reversed diffuse brain amyloid pathology in aged AβPP transgenic mice [61–63]. Nevertheless, lowering cholesterol by treatment with statins was found to reduce [13, 21, 64] or enhance Aβ generation, depending on the condition of the study [65]. One possible explanation for the controversial results is that moderate reduction in cholesterol is associated with a disorganization of detergent-resistant membranes (DRMs) or lipid rafts, allowing more β-site AβPP cleaving enzyme (BACE) to contact AβPP and resulting in increased Aβ generation, whereas a strong reduction of cholesterol inhibits the activities of BACE and γ-secretase, resulting in a decrease in Aβ generation [14]. On the other hand, in the low-dose statin treatment conditions [65], enchanted Aβ production could be a result of feedback upregulation of HMG-CoA mPNA and increased cholesterol level.

Consistent with the membrane compartmentalization model, cellular cholesterol depletion results in an increase in membrane fluidity, a parameter which characterizes an average lateral motion of phospholipid molecules within the lipid bilayer [19, 66–69]. On the other hand, an increase in membrane fluidity has been demonstrated to shift AβPP processing to nonamyloidogenic cleavage by α-secretase [54–56, 65–68]. It has been reported that the removal of cholesterol with methyl-β-cyclodextrin or treatment with lovastatin increased membrane fluidity, which resulted in higher expression of the α-secretase and impaired internalization of AβPP [19]. At the same time, cholesterol enrichment has been shown to reduce membrane fluidity [70, 71]. Cholesterol enrichment that impeded membrane fluidity may lower $sA\beta PP_\alpha$ production by hindering the interaction of the substrate with its proteases [72]. Interestingly, substitution of cholesterol by the steroid 4-cholesten-3-one induces minor change in membrane fluidity and reduces $sA\beta PP_\alpha$ secretion, whereas substitution of cholesterol by lanosterol increases membrane fluidity and $sA\beta PP_\alpha$ secretion [19]. These results suggest reversible effects of cholesterol on the α-secretase activity depending on membrane fluidity.

Many studies support the notion that Aβ production occurs in endosomes [22, 73–77]. Since AβPP is a transmembrane protein, its internalization from the plasma membrane is regulated by key regulators of endocytosis, such as Rab5, and this process has been found to enhance AβPP cleavage by β-secretase leading to increased Aβ levels [78]. In contrast, AβPP, lacking its cytoplasmic internalization motif, can

accumulate at the plasma membrane and undergo cleavage by α-secretase [10, 11]. Cholesterol has been demonstrated to increase clathrin-dependent AβPP endocytosis in a dose-dependent and linear manner [79]. There were also studies demonstrating that alterations in cholesterol transport from late endocytotic organelles to the endoplasmic reticulum had important consequences for both AβPP processing and the localization of γ-secretase-associated presenilins [60]. It has been suggested that cholesterol increase in AD could be responsible for the enhanced internalization of clathrin-, dynamin2-, Eps15-, and Rab5-dependent endocytosis of AβPP and the ensuing overproduction of Aβ [79]. Alternatively, AβPP internalization could be reduced by lowering cholesterol, which leads to an increase in membrane fluidity, AβPP accumulation on the cell surface, and increased sAβPP$_\alpha$ secretion [19].

2.2. Fatty Acids on Membrane Physical Properties and AβPP Processing. Fatty acids are other essential components of the cellular membranes. They are important ingredients in various dietary sources and play a central role in the normal development and function of the brain [80–83]. For example, long-chain polyunsaturated fatty acids (PUFAs) of the ω-6 and ω-3 series, the major polyunsaturated fatty acids in the central nervous system [84], are essential for prenatal brain development and normal brain functions [83, 85, 86]. Animals specifically deficient in dietary ω-3 fatty acids have been demonstrated to have reduced visual acuity and impaired learning ability [86, 87]. Diets enriched in long-chain ω-3 PUFA (DHA) have been shown to modulate gene expression for brain function, improve synaptic and neurotransmitter functions of neurons, enhance learning and memory performances, and display neuroprotective properties [86, 88–90]. Arachidonic acid (AA), another abundant fatty acid in the brain, is a second messenger [91] and a precursor for the synthesis of eicosanoids [92]. The presence of PUFAs in neuronal cells influences cellular function both directly, through effects on membrane properties, and indirectly by acting as precursors for lipid-derived messengers [93, 94].

In fact, the disturbed metabolism of fatty acids is associated with AD [95]. For instance, lower levels of DHA have been reported in serum samples taken from AD patient [96], while greater consumption of DHA has significantly reduced the likelihood of developing AD [97]. Dietary ω-3 PUFA depletion has been shown to activate caspases and decrease NMDA receptors in the brain of a transgenic mouse model of AD [98]. DHA and curcumin have been shown to suppress Aβ-induced phosphorylation of tau tangles and the inactivation of insulin receptors in primary rat neurons [99]. Recently, reduced expression of the neuronal sortilin-related receptor SorLA/LR11 (LR11), a sorting protein that regulates APP trafficking to β- and γ-secretases, was identified as a probable genetic risk factor for late-onset Alzheimer's disease [100]. DHA, in turn, has been found to increase LR11 expression in primary rat neurons, human neuronal line, and aged nontransgenic and DHA-depleted APPsw AD transgenic mice [101]. In 15-month-old AβPP/presenilin-1 mice, DHA supplementation improved spatial memory, decreased Aβ deposition, and slightly increased relative cerebral blood volume, indicating that a DHA-enriched diet can diminish AD-like pathology [102].

Dietary fatty acids may integrate into cell membranes to change their physical properties and subsequently alter cell functions. The ability of fatty acids to modulate membrane properties and functions [90, 94, 103–109] depends both on the saturation degree of the fatty acids and the trans/cis ratio of the unsaturated fatty acids [110–113]. For example, diets enriched in unsaturated PUFAs, DHA, and AA have been shown to increase membrane fluidity of neurons and other cells [71, 89, 114, 115]. DHA has been also capable of counteracting cholesterol-induced decreases in platelet membrane fluidity and modulating platelet hyperaggregation [71]. Similarly, cis-polyunsaturated linolenic, α-linoleic, and eicosatrienoic fatty acids increased membrane fluidity [110]. In contrast, membrane incorporation of saturated acids led to decreased membrane fluidity [109, 112, 116]. PUFAs have also been shown to affect many other membrane properties, such as molecular order, compressibility, permeability, fusion, and protein activity [117, 118].

Since AβPP, α-, β-, and γ-secretases are membrane protein molecules, AβPP processing can be altered by manipulating the membrane lipid composition. It was mentioned before that an increase in membrane fluidity has resulted in an increase in nonamyloidogenic cleavage by α-secretase to produce sAβPP$_\alpha$ [19, 25]. At the same time, enrichment of cell membranes with PUFAs increases membrane fluidity and, consequently, promotes nonamyloidogenic processing of AβPP [113]. It has been shown that a typical Western diet (with 40% saturated fatty acids and 1% of cholesterol) fed to transgenic AβPP/PS1 mice increased Aβ, while diets supplemented with DHA decreased Aβ levels compared to regular diet [119]. Similarly, DHA has been shown to decrease the amount of vascular Aβ deposition [120] and reduce cortical Aβ burden [121] in the aged Alzheimer mouse model. In this model, DHA modulated AβPP processing by decreasing both α- and β-AβPP C-terminal fragment products and full-length AβPP [121]. DHA has also been shown to stimulate nonamyloidogenic AβPP processing resulting in reduced Aβ levels in cellular models of Alzheimer's disease [122]. At the same time, the study of the effects of fatty acids on cell membrane fluidity and sAβPP$_\alpha$ secretion in relation to degrees of unsaturation has suggested that not all unsaturated fatty acids, but only those with 4 or more double bonds, such as arachidonic acid (20 : 4), eicosapentaenoic acid (20 : 5), and DHA (22 : 6), increased membrane fluidity and led to an increase in sAβPP$_\alpha$ secretion, while oleic acid (18 : 1), linoleic acid (18 : 2), and α-linolenic acid (18 : 3) did not [113]. Moreover, the results of another experiment have indicated that treatment of PSwt-1 cells with oleic acid and linoleic acid increased γ-secretase activity and Aβ production [123].

2.3. Phospholipases A_2 on Membrane Physical Properties and AβPP Processing. Phospholipases A_2 (PLA$_2$s) are ubiquitously distributed in mammalian cell enzymes that catalyze

the hydrolysis of fatty acids from *sn*-2 position of phospholipids. PLA_2s are classified into three major families: calcium-dependent cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$), and calcium-independent PLA_2 ($iPLA_2$). These enzymes are responsible for maintenance of phospholipid homeostasis in the cell membrane. They are also important in production of lipid mediators, such as arachidonic acid. Activation of PLA_2s has been implicated in diverse cellular responses such as mitogenesis, differentiation, inflammation, and cytotoxicity, and changes in PLA_2s' activities occur in many neurodegenerative diseases, including AD [124–136].

It has been shown that immunoreactivity of $cPLA_2$ (group IIA and IVA) increased in reactive astrocytes in severe AD patient brains [124–126]. Upregulations of $sPLA_2$-IIA and PLA_2-IVA were reported in the hippocampus of AD patients [126, 137, 138]. The levels of activated $cPLA_2$-IVA were also increased in the hippocampus of hAβPP mice [138]. Furthermore, Aβ has been shown to activate $cPLA_2$ in primary rat and mouse brain endothelial cells, astrocytes, cortical neurons, and in PC12 cells [139–143]. Contradictory, both increased and reduced PLA_2 activity has been reported in platelets of AD patients [144, 145]. At the same time, PLA_2 activity was significantly decreased in the parietal and, to a lesser degree, in frontal cortex of AD brains. Lower PLA_2 activity correlated significantly with an earlier onset of the disease, higher counts of neurofibrillary tangles and senile plaques and an earlier age of death, indicating a relationship between abnormally low PLA_2 activity and a more severe form of the illness [146].

PLA_2s play key roles in modulation of membrane properties under pathological and physiological conditions. For instance, in immortalized rat astrocytes (DITNC cells), $cPLA_2$ mediated the Aβ-induced membrane molecular order increase (biophysical parameter which characterizes molecular packing of lipids and water in lipid bilayers) [147]. In primary rat cortical astrocytes, ROS induced by menadione, a redox active agent, also altered astrocyte's membrane molecular order through activation of $cPLA_2$ [148]. PLA_2 activation has been shown to affect lipid membrane fluidity and AβPP processing as well [149, 150]. In AD brains, there is evidence for reduced membrane fluidity coupled with decreased PLA_2 activity [146, 151, 152]. Similarly, inhibition of PLA_2 activity in rat hippocampus has been shown to reduce membrane fluidity and impair the formation of short- and long-term memory [150, 153]. Arachidonic acid (AA), PLA_2-hydrolyzed product, increased fluidity of membranes in cultured cerebral endothelial cells [154, 155] and hippocampal neurons *in vivo* [114]. Secretory $sPLA_2$-III and AA have been shown to increase membrane fluidity and $sA\beta PP_\alpha$ secretion and decrease levels of $A\beta_{1-42}$ in SH-SY5Y cells [156]. Another hydrolyzed product of PLA_2, DHA, has also been demonstrated to increase membrane fluidity and $sA\beta PP_\alpha$ secretion in HEK cells and in neuronal SH-SY5Y overexpressing AβPP cells [157]. In addition, it has been reported that nonspecific PLA_2 inhibitor partially suppressed muscarinic receptor-stimulated increases in $sA\beta PP_\alpha$ secretion in SH-SY5Y [23]. Since PLA_2 increases membrane fluidity and nonamyloidogenic cleavage of AβPP, PLA_2 activity modulation can be considered as a potential target for AD treatment.

3. Role of Membrane Physical Properties in Aβ-Membrane Interaction and Aβ Cytotoxicity

3.1. Aβ-Membrane Interactions Studied in Membrane Models and in Cell Cultures. Cleavage of AβPP leads to the production of Aβ peptides of different length, of which $A\beta_{1-40}$ is the major species and $A\beta_{1-42}$ is the most fibrillogenic and toxic component in AD plaques [27]. Numerous studies have demonstrated direct interaction of $A\beta_{1-40}$ and $A\beta_{1-42}$ with components of the plasma membrane, which consequentially disrupts the membrane properties [28–33, 158–160]. There are several suggested types of Aβ-membrane interactions. Aβ peptide can be retained in a membrane upon AβPP cleavage, thus being prevented against release and aggregation [161]. It also can be released as soluble monomers into the extracellular environment and then be removed [161, 162]. On the other hand, Aβ can reinsert into a membrane and form ion-conducting pores or bind to a membrane surface by undergoing accelerated aggregation and form nonspecific structures, which causes thinning and deformation to the membrane [161, 163–166].

$A\beta_{1-42}$ has been shown to reduce membrane fluidity and accelerate the amyloidogenic processing of AβPP [25, 33, 167–172]. *In vivo*, a decrease in membrane fluidity of synaptosomes isolated from frontal and hypothalamic neurons of 3-month-old mice, administrated with Aβ, has been demonstrated [171]. By using *in situ* atomic force microscopy and fluorescence spectroscopy, randomly structured $A\beta_{1-42}$ has been reported to decrease membrane fluidity of planar bilayers composed of total brain lipids, and this effect is cholesterol-content dependent: the most dramatic effect has been seen for cholesterol-enriched samples [168]. DPH (1,6-diphenyl-1,3,5-hexatriene) fluorescence study has shown a similar effect of $A\beta_{1-40}$ on membrane fluidity of unilamellar liposomes with a strong correlation to Aβ aggregation state and pH [170]. It has been reported that unaggregated peptides and pH 7 do not affect membrane fluidity, while aggregated Aβ at pH 6 or 7 decreased membrane fluidity in a time- and dose-dependent manner [170]. Studies of SH-SY5Y human neuroblastoma cells have shown some contradictory results. In this observation, $A\beta_{1-42}$ monomers increased fluidity of cell membranes, and Aβ-Aluminium complex promoted even a greater effect [172]. Differences in effects of Aβ on fluidity could result from the tissue source and preparation, whether Aβ is soluble or aggregated, and the age of the organism. The differences in effects of Aβ on fluidity could also be the result of differences in the location of the fluorescent probes in the membrane environment and the lifetime of the fluorescent probes.

The fluorescence microscopy of a membrane with the environmentally sensitive probe Laurdan has demonstrated the ability of $A\beta_{1-42}$ oligomers to affect the membrane molecular order [147, 173, 174]. It has been shown that an interaction of $A\beta_{1-42}$ with artificial membranes made them molecularly disordered (more water molecules were

partitioned into the membrane core) due most likely to the insertion of the peptide into the bilayer and the direct alteration of membrane lipid packing. In the same study, the effect of $A\beta_{1-42}$ oligomers on immortalized rat astrocytes (DITNC cells) membranes was opposite. DITNC membranes become more molecularly ordered upon incubation with Aβ in a time-dependent manner, and it was consistent with activation of $cPLA_2$. At the same time, in the presence of NADPH oxidase inhibitor, the membranes of the cells remained molecularly disordered. These results suggested an indirect effect of Aβ through the signaling pathway involving NADPH oxidase and $cPLA_2$ [147].

The study of the interaction of $A\beta_{1-42}$ with unilamellar lipid vesicles has demonstrated increased vesicle fusion and a thinning of the lipid bilayer and enhancing of these effects at pH 7 and at a high Aβ/lipid ratio [175]. The micropipette manipulation of giant unilamellar vesicles has shown the ability of $A\beta_{1-42}$ to affect the membrane lysis tension depending on artificial membrane lipid composition. It has been found that neither $A\beta_{1-42}$ nor $A\beta_{1-40}$ changed mechanical properties of glycerophospholipid-cholesterol vesicles, while partial substitutions of cholesterol with 7β-hydroxycholesterol that contained additional dipole of oxysterol led to a dramatic reduction of the lysis tension upon Aβ treatment. The results of this experiment strongly suggest that a negative bilayer surface charge is required for Aβ-membrane interaction [176].

The Aβ-membrane interaction may also be followed by the incorporation of Aβ into the membranes and formation of cation-selective channels, which lead to alteration of membrane permeability and electrical conductance [31, 177–186]. The study of the effects of soluble $A\beta_{1-42}$ oligomers on planar lipid and tethered lipid bilayers has indicated that Aβ oligomers were inserted into the hydrophobic core of the bilayer, affecting both membrane leaflets and significantly increasing membrane ion current [31, 178]. It has been demonstrated that Aβ-induced ion conductance was different from ion transfer through water-filled pores and depended on peptide concentration and membrane lipid composition [178]. Similarly, $A\beta_{1-40}$ caused a dose-dependent increase in the Na^+, Ca^{2+}, and K^+ influx in the lumen of liposomes formed from the acidic phospholipids but did not change cation conductance in a case of liposomes formed from the neutral phospholipids [179]. There was also evidence that Aβ induced an increase in membrane conductance, which was dependent on the area compressibility of the lipid bilayer. Membranes with a large area compressibility modulus were almost insensitive to $A\beta_{1-42}$ oligomers, while membranes formed from soft, highly compressible lipids were very sensitive to the presence of oligomers [31].

In vitro, after $A\beta_{1-42}$ treatment, the membranes of human neuronal hNT cells and neuron-like differentiated PC12 cells depolarized and exhibited enhanced membrane permeability [183, 186]. Patch-clamp studies of a cell line derived from hypothalamic gonadotrophin-releasing hormone GnRH neurons have demonstrated spontaneous formation of Zn^{2+}-sensitive channel pores upon $A\beta_{1-40}$ monomers treatment [184]. Similarly, $A\beta_{1-40}$ aggregates induced perforation of hippocampal neuronal synapses, causing an increase in membrane conductance, intracellular calcium, and ethidium bromide influx [185]. It has been suggested that Aβ-induced membrane depolarization and increased ions influx in neurons was not just due to forming of cation-selective pores but rather was a consequence of events resulting from downstream pathways with involvement of metabotropic glutamate receptor and G-proteins [186].

3.2. Membrane-Associated Conformational Modifications and Aggregation of Aβ. In a previous section, we discussed the ability of Aβ to alter biophysical properties of membranes and the dependence of these processes on membrane environment. These studies suggest that membrane property and composition, in turn, play a critical role in conformational changes and aggregation of Aβ. The aggregation of Aβ is a complicated multistep process consisting of several phases: monomers soluble oligomers (clusters of small numbers of peptide molecules without a fibrillar structure) protofibrils (aggregates of isolated or clustered spherical beads made up of 20 molecules with β-sheet structure) mature fibrils [187]. Although the mechanism which initiates Aβ aggregation is not fully understood, it has been shown to be modulated by several factors, including concentrations of monomers and their conformational transitions, sequential changes in the Aβ primary structure, and interactions with metal ions and membrane molecules [35, 37, 40, 169].

In vitro studies have demonstrated that Aβ monomers can exist in three major conformation forms: α-helix, β-sheet, or random coil depending on physical properties and chemical composition of the environment [188, 189]. Since the toxic Aβ mostly consists of β-sheets, even though the original hydrophobic component of Aβ is a part of a transmembrane α-helix of AβPP, the conformational transition of Aβ from α-helix or random coil to β-sheet is most likely the very first step in the formation of oligomers and fibrils [190]. Multidimensional NMR spectroscopy and circular dichroism (CD) studies have demonstrated that alterations in the membrane structure and biophysics can trigger the conversion of soluble α-helical monomeric $A\beta_{1-40}$ into the oligomeric β-sheet conformation [161, 189]. It has been shown that, in an ordered membrane system, Aβ adopted a single α-helical confirmation, while in disordered micelles Aβ rather adopted soluble β-sheet oligomeric conformation [189]. At the same time, study of the neutral and negatively charged bilayers showed an increase in β-sheet content as the negative charge on the lipid membrane increased [161, 191].

In situ atomic force microscopy and total internal reflection fluorescence microscopy studies have indicated that the size and the shape of $A\beta_{1-40}$ and $A\beta_{1-42}$ aggregates, as well as the kinetics of their formation, depended on the physicochemical nature of the surface [192, 193]. For example, on hydrophilic surfaces (mica) $A\beta_{1-42}$ formed particulate, pseudomicellar aggregates, while on hydrophobic surfaces (graphite) Aβ formed uniform, elongated sheets with dimensions consistent with the dimensions of β-sheets. The results of this study suggested that Aβ fibril formation may be driven by interactions at the interface of aqueous

solutions and hydrophobic substrates, which occurs in membranes and lipoprotein particles *in vivo* [192]. Similarly, $A\beta_{1-40}$ fibril growth was especially prominent on chemically modified negatively charged quartz surfaces, while no fibril formation was observed on the positively charged surfaces [193].

Numerous *in vitro* and *in vivo* studies have demonstrated that lipid bilayer composition, as well as membrane-associated proteins, can regulate Aβ aggregation in both enhancement and inhibition manner [37–40]. It has been suggested that binding of Aβ to the ganglioside-containing membranes can induce a structural transition from random coil to β-sheet in Aβ40/42 and accelerate fibril formation [194–196]. Electron microscopy studies have demonstrated that interaction of Aβ40/42 with plasma, lysosomal, and endosomal membranes accelerated fibrillogenesis of Aβ, while the presence of Golgi membranes hindered the process [169].

4. Conclusion

An increasing amount of evidence demonstrates that a lot of cellular processes in AD are intimately associated with physical properties and organization of membranes. The primary step in Aβ accumulation, the amyloidogenic cleavage of AβPP, is affected by the membrane properties such as membrane fluidity and molecular order and can be modulated by removal of cholesterol and manipulation of membrane lipid composition. Aβ-membranes interaction, in turn, affects biophysical membrane properties and accelerates the amyloidogenic processing of AβPP. Aβ has been reported to reduce membrane fluidity, affect molecular order and membrane lysis tension, induce thinning of the lipid bilayer, and increase membrane conductance. Aβ can also indirectly affect membrane properties by binding to membrane receptors and triggering downstream signaling pathways leading to oxidative stress and inflammation. On the other hand, the ability of Aβ to interact with membranes of neurons and other cerebral cells depends on physical properties and organization of membranes, such as cholesterol content, lipid composition and Aβ/lipid ratio, pH, the presence of metal ions, and bilayer surface charge. Membrane properties and composition play critical roles in Aβ cytotoxicity associated with its conformational changes and aggregation state as well. Therefore, understanding how membrane properties and organization are related to cellular pathways in AD should prove to provide insights into the mechanisms of the pathogenesis in AD.

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Review Article

Protein Kinase C-Regulated Aβ Production and Clearance

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Alzheimer's disease (AD) is the most common form of dementia among the elderly population. AD, which is characterized as a disease of cognitive deficits, is mainly associated with an increase of amyloid β-peptide (Aβ) in the brain. A growing body of recent studies suggests that protein kinase C (PKC) promotes the production of the secretory form of amyloid precursor protein (sAPPα) via the activation of α-secretase activity, which reduces the accumulation of pathogenic Aβ levels in the brain. Moreover, activation of PKCα and mitogen-activated protein kinase (MAPK) is known to increase sAPPα. A novel type of PKC, PKCε, activates the Aβ degrading activity of endothelin converting enzyme type 1 (ECE-1), which might be mediated *via* the MAPK pathway as well. Furthermore, dysregulation of PKC-MAPK signaling is known to increase Aβ levels in the brain, which results in AD phenotypes. Here, we discuss roles of PKC in Aβ production and clearance and its implication in AD.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia among the elderly population [1, 2]. A major hallmark of AD is the abnormal processing and accumulation of neurite plaques containing amyloid β-peptide (Aβ) in the brain [3, 4]. Amyloid precursor protein (APP) is mainly cleaved by the α-secretase enzyme (Figure 1), producing the secretory form of amyloid precursor protein (sAPP; β-amyloid (Aβ) 17–42), which is soluble and nontoxic [5]. However, when APP is cleaved by β- and γ-secretase enzymes [6], it leads to the formation of Aβ1–40 and Aβ1–42, which are insoluble unlike sAPP, and results in the accumulation of amyloid plaques [7]. In the production of Aβ1–42, the Aβ1–42/Aβ1–40 ratio is associated with the amount of insoluble Aβ aggregation [8]. On the other hand, the abnormal hyperphosphorylation of tau results in insoluble fibrils and neurofibillary tangels in the brain [9, 10]. Thus, an understanding of the pathological processes of APP and tau in AD is a critical therapeutic target in preventing or delaying AD in humans [11–13]. Here, we review the role of protein kinase C (PKC) in Aβ production and clearance through α-secretase or Aβ-degrading enzyme activity. Among several PKCs, we focus on the role of PKCε in Aβ levels because several recent findings have demonstrated that the activation or overexpression of PKCε promotes the Aβ degradation activity of endothelin converting enzyme type 1 (ECE-1) [14, 15].

2. PKC and Aβ Plaques

PKC is a phospholipid-dependent serine/threonine kinase and consists of at least 12 isoenzymes [18, 19]. PKCs can be classified into three subfamilies based on their protein structure and second messenger requirements: conventional (or classical), novel, and atypical. Conventional PKCs contain the α, β1, β2, and γ isoforms and require Ca^{2+}, diacylglycerol (DAG), and a phospholipid such as phosphatidylcholine for activation. Novel PKCs include the δ, ε, η, θ, and μ isoforms and require DAG or phospholipids but do not require Ca^{2+} for activation. On the other hand, atypical PKCs consisting of protein kinase ζ, ι, and λ isoforms do not require either Ca^{2+} or diacylglycerol for activation [20].

Numerous studies have suggested that phorbol 12-myristate 13-acetate (PMA), a nonspecific PKC activator, is capable of lowering secreted Aβ levels in neurons [21–24]. Based on these results, several studies have attempted to identify precisely which PKC isozyme actually regulates

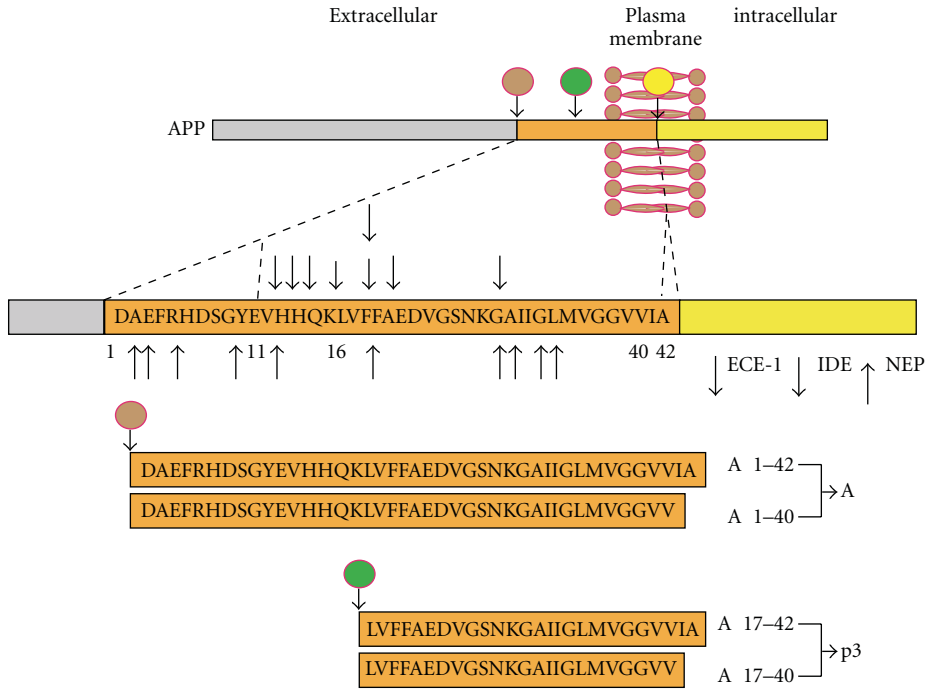

Figure 1: Amyloid metabolism by secretases and Aβ-degradation enzymes (ECE-1, IDE, NEP). Aβ-degrading proteases play an important role in regulating Aβ levels via known cleavage sites (adapted from [1, 16, 17]).

APP processing. The overexpression of PKCα or PKCε, but not PKCθ, has been shown to induce APP secretion from cells [25]. Interestingly, specific inhibition of either PKCα or PKCθ in CHO cells expressing APP695 was associated with a loss of PMA-mediated APP secretion [26]. In addition, experiments with a dominant negative fragment of PKCε reduced phorbol ester-induced secretion of sAPPα [15, 27]. However, even though intraparenchymal administration of phorbol esters reduces Aβ levels and decreases amyloid plaque density in mice expressing an amyloidogenic variant of human APP, α-secretase activity is not increased in the brain [28]. This raises the possibility that PKC reduces Aβ levels *in vivo* by another mechanism.

3. Aβ Clearance and Peptidases

The accumulation of Aβ in the brain is one of the main symptoms of AD [3]. An abnormality in the proteolytic degradation of Aβ appears to be associated with the progression of AD [29]. As shown in Figure 1, several proteases that degrade Aβ in mice include insulin-degrading enzyme (IDE), neprilysin (NEP), and endothelin-converting enzyme (ECE) 1 and 2 [16, 30]. IDE (insulysin) is a 110 KDa thiol zinc-metalloendopeptidase which is expressed in the cytosol, peroxisomes, and endosomes and on cell surfaces, and it is the major enzyme responsible for insulin degradation *in vitro* [31]. However, IDE has also been found to degrade Aβ in neuronal and microglial cells [32] and to eliminate the neurotoxic effects of Aβ [33]. Consistently, IDE-null mice showed increased levels of Aβ in the brain [34]. NEP is another key player in Aβ clearance [35]. In the brain, NEP is mainly expressed on neuronal plasma membranes [36]. NEP-null mice show defects in both the degradation of exogenously administered Aβ and in the metabolic suppression of endogenous Aβ levels in a gene dose-dependent manner [37]. The importance of these zinc-metalloendopeptidases in Aβ clearance is demonstrated by the fact that the transgenic overexpression of IDE or NEP in neurons significantly reduces Aβ levels and plaque associated with AD pathology [38]. Angiotensin-converting enzyme (ACE) is a membrane-bound zinc metalloprotease [39]. ACE mainly converts angiotensin I to angiotensin II, which is critical in the regulation of blood pressure, body fluid, and sodium homeostasis [40]. Recent studies indicate that ACE expression also promotes the degradation of Aβ [41].

Several receptor-mediated Aβ clearance mechanisms have already been examined [42]. Low-density lipoprotein receptor-related protein (LRP) and the receptor for advanced glycation end products (RAGE) regulate Aβ levels across the blood-brain barrier [43]. Both LRP and RAGE are multiligand cell surface receptors that mediate the clearance of a large number of proteins in addition to Aβ. LRP mainly removes Aβ from the brain to the periphery whereas RAGE appears to influx Aβ back to the brain from the periphery [42, 43].

4. Endothelin-Converting Enzymes (ECEs)

ECEs are a class of type II transmembrane metalloproteases, which convert pro-ET into endothelin [44]. Two different ECEs, including ECE-1 and ECE-2, are expressed in brain regions related to AD [45, 46]. Although ECE-1 is

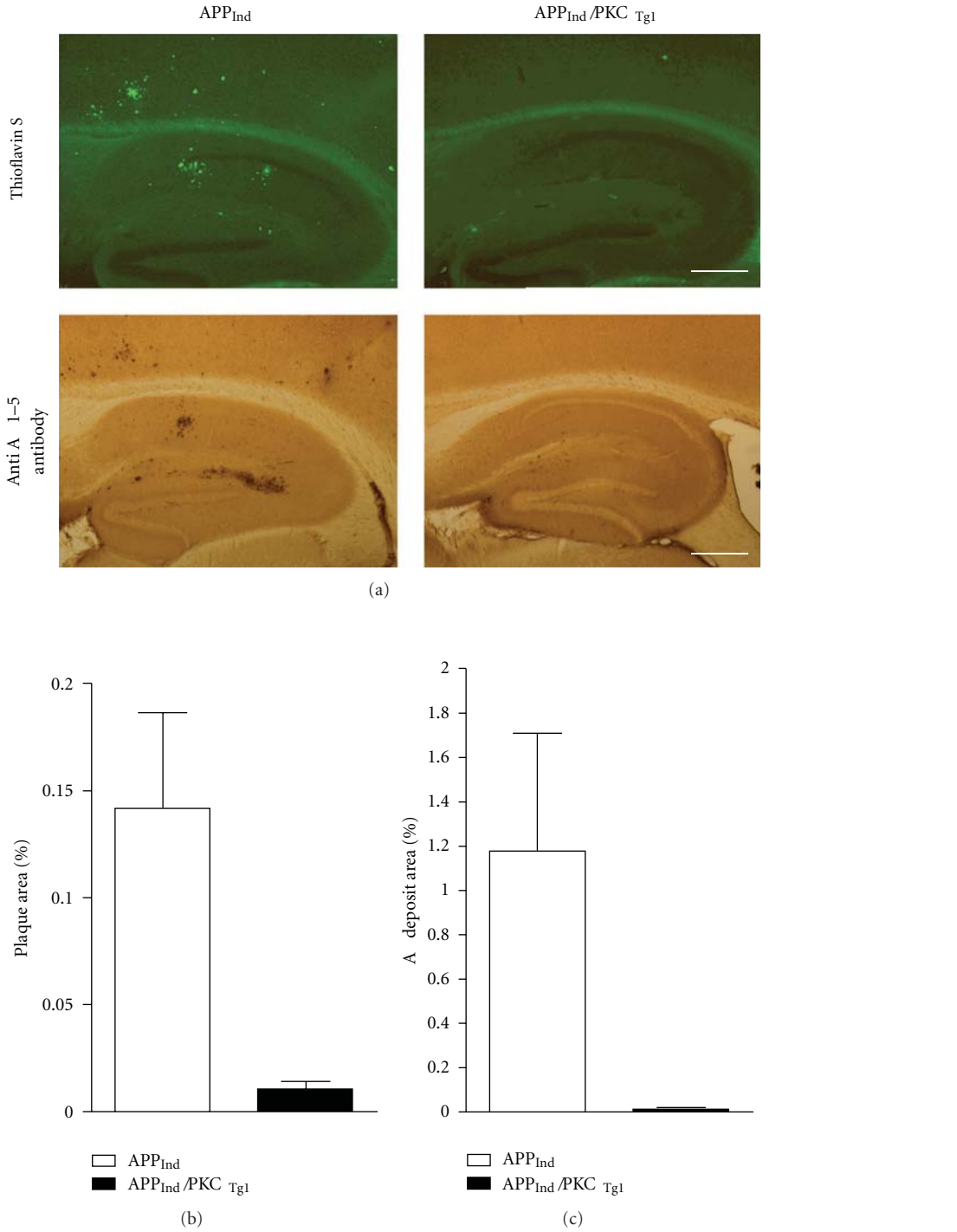

FIGURE 2: Overexpression of PKCε reduces the amyloid plaque burden and inhibits Aβ accumulation in brain parenchyma. (a) Thioflavin S staining and anti-Aβ immunostaining revealed fewer plaques and Aβ immunoreactive deposits in the hippocampus and neocortex in APP$_{Ind}$/PKCε_{Tg1} mice than in APP$_{Ind}$ mice. Scale bar: 200 μm. Quantification of (b) thioflavin S staining and (c) Aβ deposits in hippocampus and cortex sections (adapted from [14]). $P < .05$ by two-tailed t-test.

abundantly expressed in vascular endothelial cells [47], it is also expressed in nonvascular cells, including hippocampal and neocortical pyramidal neurons, cerebellar Purkinje cells, and astrocytes [48]. ECE-2 is also expressed in the brain, especially in several subpopulations of neurons in the thalamus, hypothalamus, amygdala, and hippocampus [46]. Studies have demonstrated that ECE-1 is a key enzyme for the degradation of Aβ in the brain [49]. The *in vivo* function of ECE has been examined in ECE-1 heterozygous (+/−) and ECE-2 null (−/−) mice. In both cases, levels of Aβ were

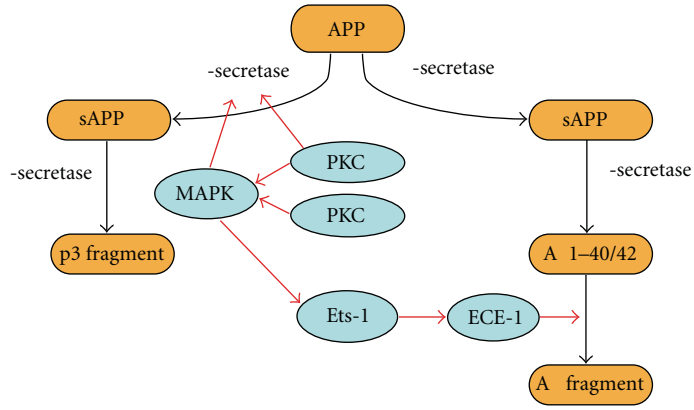

Figure 3: Schematic summary of role of PKC-MAPK-dependent Aβ production and clearance. PKCα upregulates α-secretase activity while PKCε stimulates Aβ-degrading activity of ECE-1, probably via MAPK-dependent Ets-1 pathway. MAPK is also known to activate α-secretase activity independently or through PKC activation.

increased compared with wild-type mice, suggesting that these ECEs are an important Aβ-degrading enzyme *in vivo* [50]. Another study demonstrated that NEP (−/−)/ECE-1 (+/−) or NEP (−/−)/ECE-2 (−/−) mice have increased accumulation of both Aβ1–40 and Aβ1–42 in the brain [51]. Interestingly, a genetic variant of human ECE-1 (ECE1B C-338A) with increased promoter activity was associated with a reduced risk of sporadic AD in a French Caucasian population [45]. ECE-1 degrades synthetic Aβ levels *in vitro* [50] and is the main ECE for Aβ degradation. Recently, the expression of ECE-2 has also been shown to be a relevant Aβ-degrading enzyme and is dramatically increased at both mRNA and protein levels of patients with AD [52].

Endothelin-1 (ET-1) is the major peptide formed by ECE-1, and its cellular actions are mediated via two G-protein coupled receptors, ET_A and ET_B, which are widely distributed in the brain [53]. ET-1 levels appear elevated in postmortem brains from patients with Alzheimer-type dementia [54]. A study indicates that ET-1 is increased in brain microvessels isolated from patients with AD and promotes the survival of brain neurons [55]. However, this effect might be associated with the protective actions of ET-1 *in vivo*, rather than contributing to the AD pathology [56].

5. PKCε, MAPK, and ETS Pathways

The activation of PKCs has suggested a neuroprotective function in animals [57]. PKC activators can also prevent the production of Aβ and extend the survival of AD transgenic mice [58]. However, chronic treatment of nonspecific PKC activators such as phorbol esters at high doses could increase levels of Aβ by decreasing PKC function or increasing APP synthesis [59]. These studies also suggest that the chronic application of phorbol esters may differentially regulate the function of PKC isoforms, downregulating PKCα and upregulating PKCε. There are several mechanisms by which the activation of PKCs could regulate the reduction of Aβ. Interestingly, our recent study demonstrates that overexpression of human PKCε reduces Aβ levels significantly in the brain (Figure 2). As shown in Figure 3, activation of PKCs including PKCα is known to promote α-secretase activity [25, 60], while activation or overexpression of PKCε stimulates Aβ-degrading activity of ECE-1, probably via MAPK-dependent Ets-1 pathway [14, 15]. MAPK is also known to activate α-secretase activity independently [61] or through PKC activation [62–64]. Since MAPK can activate Ets-1 and 2 [65], it is possible that PKCε-mediated MAPK could control ETS pathways and thus regulate ECE expression in the brain. Additionally, ETS transcription factors play a key role in cell growth, differentiation, and survival [66]. ETS proteins form complexes and act synergistically with other transcription factor families such as PEA3 or AP-1 [67]. Ets-1 has been known to be involved in angiogenesis [68]. However, another research indicates that upregulation of Ets-2 is closely associated with AD neurodegenerative lesions in the brain [69].

6. Conclusion

In Alzheimer's disease (AD), it has long been known that activated PKCs reduce Aβ levels in the brain. PKC is also suggested to be a functional biomarker of AD [70]. The steady-state level of Aβ depends on a balance between production and clearance. In addition to Aβ production, several researchers suggest that enzyme-mediated degradation of Aβ is also critical for the regulation of Aβ levels [71]. Especially, since PKC is a key modulator in Aβ production or clearance in the brain [15, 58, 72], regulation of PKC activity could be a useful treatment target for AD [14, 73, 74]. However, the functional relevance of each PKC isoform in regulating Aβ levels in AD remains to be studied. Moreover, while α-secretase-mediated cleavage of APP via PKC isoforms reduces amyloid, detailed mechanisms of how PKC isoforms activate the enzyme-degradation system await further investigation. Therefore, PKC isoform-specific ligands or viral-mediated overexpression of PKC isoform as well as specific shRNAs approaches may unveil detailed molecular bases that underlie PKC-regulated Aβ clearance.

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Research Article

Intracellular APP Domain Regulates Serine-Palmitoyl-CoA Transferase Expression and Is Affected in Alzheimer's Disease

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Lipids play an important role as risk or protective factors in Alzheimer's disease (AD), a disease biochemically characterized by the accumulation of amyloid beta peptides (Aβ), released by proteolytic processing of the amyloid precursor protein (APP). Changes in sphingolipid metabolism have been associated to the development of AD. The key enzyme in sphingolipid *de novo* synthesis is serine-palmitoyl-CoA transferase (SPT). In the present study we identified a new physiological function of APP in sphingolipid synthesis. The APP intracellular domain (AICD) was found to decrease the expression of the SPT subunit SPTLC2, the catalytic subunit of the SPT heterodimer, resulting in that decreased SPT activity. AICD function was dependent on Fe65 and SPTLC2 levels are increased in APP knock-in mice missing a functional AICD domain. SPTLC2 levels are also increased in familial and sporadic AD *postmortem* brains, suggesting that SPT is involved in AD pathology.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the most common cause of dementia in the elderly, clinically characterized by a progressive loss of memory. Pathological hallmarks for AD are the presence of amyloid plaques, composed of amyloid beta peptides (Aβ), and neurofibrillary tangles, which consist of hyperphosphorylated tau proteins [1–3]. Aβ peptides are released by sequential processing of the amyloid precursor protein (APP), a large type-I transmembrane protein, by β- and γ-secretases. The β-secretase BACE1 generates the N-terminus of Aβ and a C-terminal stub of 99 amino acids (aa), which is further cleaved by γ-secretase to release Aβ and the intracellular domain of APP (AICD) [4–6]. The γ-secretase represents a protein complex of at least four proteins in which the presenilins constitute the active centre of the protease [7–10]. APP, BACE1 as well as Presenilin 1 (PS1), Presenilin 2 (PS2) and the other components of the γ-secretase complex are all transmembrane proteins, pointing towards a role of lipids, especially the lipid composition of cellular membranes, in the development of AD. Several lipids, including cholesterol and the sphingolipids sphingomyelin and ganglioside GM1, have been shown to influence the generation of Aβ [11–13] and a deregulation of sphingolipid metabolism was recently connected to AD

[12, 14]. The first step involved in sphingolipid *de novo* synthesis is the condensation of serine and palmitoyl-CoA to generate 3-dehydrosphinganine, catalyzed by the enzyme serine-palmitoyl transferase (SPT), which is suggested to be the rate-limiting enzyme in sphingolipid synthesis (Figure 1) [15]. 3-Dehydrosphinganine is further transformed to dihydroceramide, which is then desaturated to form ceramide, the simplest sphingolipid. Ceramide can be converted to sphingomyelin, sphingosine or various glycosphingolipids, which are ubiquitous constituents of membrane lipids and which are involved in various cellular events, including signal transduction, proliferation, differentiation, apoptosis and the maintenance of neuronal tissues and cells [16–19]. Furthermore, sphingolipids along with cholesterol have been shown to be required for the formation of detergent-resistant membrane microdomains, also called rafts, which are discussed to be the membrane microdomains where amyloidogenic processing of APP preferentially occurs [20–24].

2. Materials and Methods

2.1. Cell Culture. SH-SY5Y, MEF PS1r, MEF PS1/2−/−, MEF APPwt, MEF APP/APLP2−/− and MEF carrying PS1 familial Alzheimer's Disease mutations (E280A, A285V, T354I) cells were cultivated in DMEM (Sigma, Taufkirchen, Germany), 10% FCS (PAN Biotech, Aidenbach, Germany). For PS1 or PS-FAD/pCDNA3.1 retransfected MEF PS1/2−/− cells additional Zeocin (300 μg/mL) (Invitrogen, Karlsruhe, Germany) and for SH-SY5Y-FE65 Knock-down cells additional HygromycinB (400 μg/mL) (PAN Biotech, Aidenbach, Germany) was used.

2.2. Human and Murine Brain Material. Human FAD, SAD and corresponding control brain samples were obtained from Brain-Net (for details see Tables 1 and 2 in Supplementary Materials available online at doi:10.4061/2011/695413). Age- (+/− 3 months) and gender-matched APP−/− mice brains and APP$\triangle$CT15 mice brains and corresponding controls have been described previously and at least 3 mice brains of different mice were analysed [25].

2.3. Determination of Peptides Effects. To determine the effect of Aβ40 (10 ng/mL) and Aβ42 (1 ng/mL) (B. Penke, Szeged, Hungary) or AICD (sequence in 1-letter code: KMQQNGYENPTYKFFEQMQN) (2 μM) (Genscript Corporation, Piscatway, USA) synthetic peptides were incubated for 6 days in cell culture. Detection of intracellular Aβ was performed as described previously [26].

2.4. Knock-Down Experiments. According to the manufacturers protocol we used the SureSilencing shRNA Plasmid (SABioscience, Frederick, USA). The following insert sequences were used to generate the Fe65 knock-down: 5 -TCC CTG GAC CAC TCT AAA CTT-3 ; 5 -CAA CCC AGG GAT CAA GTG TTT-3 ; 5 -AAG GCT TTG AGG ATG GAG AAT-3 ; 5 -TGT CCA CAC GTT TGC ATT CAT-3 . As control the following sequence was used: 5 -GGA ATC TCA TTC GAT GCA TAC-3 .

2.5. Quantitative Real-Time PCR Experiments. Total RNA was extracted from cells or tissue using TRIzol reagent (Invitrogen, Karlsruhe, Germany), according to manufacturers' protocols. 2 μg total RNA were reverse-transcribed using High Capacity cDNA Reverse Transcription Kits, and quantitative real-time PCR analysis was carried out using Fast SYBR Green Master Mix on 7500 Fast Real Time PCR System (7500 Fast System SDS Software 1.3.1.; Applied Biosystems, Darmstadt, Germany). Changes in gene expression were calculated using 2-($\Delta\Delta$Ct) method [27]. Results were normalized to β-actin. The following primer sequences were used: **murine**: Sptlc1: 5 -GCA GGA GCG TTC TGA TCT TA-3 and 5 -CCG GAC ACG ATG TTG TAG TT-3 ; Sptlc2: 5 -AAG TGC CAC CAT GCA ACA GA-3 and 5 -TTG GCT CCA GGC ACA CTA CA-3 ; β-Actin: 5 -CCT AGG CAC CAG GGT GTG AT-3 and 5 -TCT CCA TGT CGT CCC AGT TG ; **human**: Sptlc2: 5 -TAT GGA GCT GGA GTG TGC AG-3 and 5 -GAA TTC GTT GCA AAT CCC AT-3 ; β-Actin: 5 -CTT CCT GGG CAT GGA GTC-3 and 5 -AGC ACT GTG TTG CGC TAC AG-3 .

2.6. Lipid Extraction. A modified Bligh and Dyer [28] method was used to extract lipids to measure SPT activity as described below. After stopping the reaction by adding 3,75 mL $CHCl_3$: MeOH : HCl (1 : 2 : 0,06), mixture was vortexed for 1 h at room temperature (RT). Then 1,25 mL $CHCl_3$ was added and vortexed again for 1 h at RT. After adding 1,25 mL $CHCl_3$ and 1,25 mL H_2O, samples were vortexed for another 10 min before centrifugation at 5000 rpm for 10 min. The phase containing lipids was transferred to another glass tube and evaporated under nitrogen-flow at 30 C. 1 mL H_2O was added to evaporated lipids before another 3,75 mL of $CHCl_3$: MeOH : HCl (1 : 2 : 0,06) was added. The extraction cycle described here was repeated one time and after final evaporation under nitrogen-flow at 30 C lipids were dissolved in 100 μL $CHCl_3$.

2.7. Protein Determination. Protein determination was carried out according to Smith et al. [29]. Briefly, we used 20 μL of bovine serum albumin (Sigma, Taufkirchen, Germany) for the standard curve in a concentration range of 0,1–1,1 μg/μL. 0,5–2 μL of each sample was loaded onto a 96-well plate (BD, Heidelberg, Germany) in triplicates. 200 μL of buffer (4% $CuSO_4$: BCA-solution (Sigma, Taufkirchen, Germany) (1 : 39)) was added to each well, and assay plate was incubated for 15 min at 37 C and for another 15 min at RT. Absorbance was determined at a wavelength of 550 nm using a MultiscanEX (Thermo Fisher Scientific, Schwerte, and Germany).

2.8. Determination of SPT Activity. For analysis of SPT enzyme activity cells are harvested into 500 μL buffer A containing 100 mM HEPES (Sigma, Taufkirchen, and Germany) and 50 μM pyridoxal phosphate (Sigma, Taufkirchen,

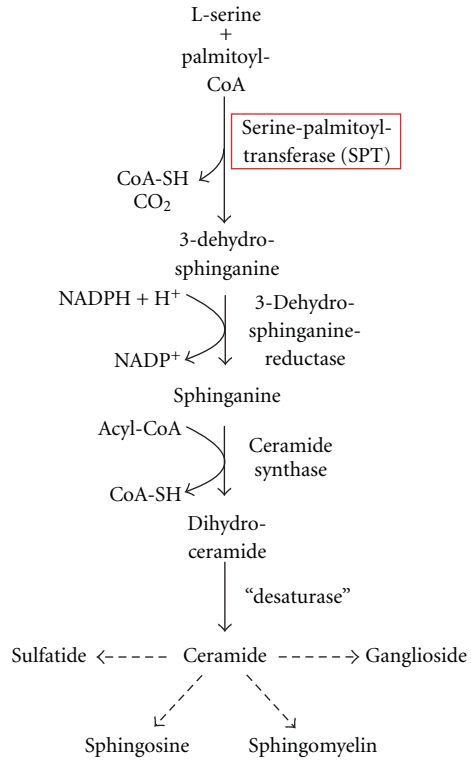

Figure 1: Biosynthetic pathway of sphingolipid *de novo* synthesis.

Germany) supplemented with complete protease inhibitor, and protein levels are adjusted to protein amount of 2,5 mg/mL. The reaction is started by adding 400 μL of buffer B containing 1 mM palmitoyl-CoA (Larodan AB, Malmö, Sweden) and 10 μCi ^{14}C-L-serine (Perkin Elmer, Rodgau-Jügesheim, Germany) at 37 C in glass tubes. The reaction is stopped after 0, 2, 4, 8, 16, 32, and 64 min by transferring 500 μl of the reaction mixture in glass tubes containing 3,75 mL $CHCl_3$: MeOH : HCl (1 : 2 : 0,06). Lipid extraction was carried out as described above. To measure the radioactivity of the individual sample, 2 mL of scintillation liquid was added to each samples and radioactivity was determined in a scintillation counter (Perkin Elmer, Rodgau-Jügesheim, Germany).

2.9. Statistical Analysis. All quantified data represent an average of at least three independent experiments. Error bars represent standard deviation of the mean. Statistical significance was determined by two-tailed Student's t-test; significance was set at P .05, P .01, and P .001, n.d. = not detectable.

3. Results

3.1. Altered SPT Activity and SPTLC2 Expression in PS1/2- and APP/APLP2-Deficient Cells. To analyze the influence of APP and APP cleavage products on sphingolipid biosynthesis, we used mouse embryonic fibroblasts (MEFs) devoid of the catalytic components of the γ-secretase complex, PS1 and PS2 (MEF PS1/2– /–) [30, 31], and MEF devoid of the PS substrate APP and the APP-like protein APLP2 (MEF APP/APLP2– /–). The common feature of both cell lines is the lack of Aβ peptides and of AICD. The analysis of the activity of the key enzyme for the regulation of sphingolipid levels in cells revealed that the SPT activity was significantly increased in MEF PS1/2– /– and MEF APP/APLP2– /– cells (Figures 2(a) and 2(b)) compared to the corresponding control cells. In order to examine if increased SPT activity is caused by an elevated SPT gene expression, we performed real-time PCR (RT-PCR) analysis of the corresponding cell lines. Mammalian SPT is a heterodimer of two subunits, the 53 kDa subunit long chain base 1 (SPTLC1 or LCB1) and the 63 kDa subunit long chain base 2 (SPTLC2 or LCB2) [32, 33]. Gene expression of the subunit SPTLC1 was not altered in PS1/2– /– cells compared to control cells, whereas gene transcription of the subunit SPTLC2 was significantly increased in PS1/2-deficient cells (Figure 2(c)). Interestingly, SPTLC2 is considered to be responsible for the catalytic activity of SPT [32, 34], indicating that the deficiency of PS1/2 influences the expression of the catalytic subunit of SPT. A similar result was obtained for APP/APLP2– /– compared to wt cells; gene expression of SPTLC1 was unchanged, whereas SPTLC2 gene transcription was significantly increased in APP/APLP2-deficient cells (Figure 2(d)), suggesting that not PS itself, but at least one of the cleavage products of APP regulates SPT gene transcription.

3.2. AICD Regulates SPTLC2 Expression. AICD is discussed to regulate gene transcription by a mechanism comparable to the function of the Notch intracellular domain, which is also released by γ-secretase activity, in gene expression [35–37]. To elucidate the effect of AICD on SPTLC2 gene transcription, we analyzed APP knock-in mouse embryonic fibroblasts deficient of full-length APP, expressing an APP construct, that lacks the last 15 aa from the C-terminus (MEF APPΔCT15) and hence a functional AICD domain [25], compared to wt cells. Importantly, the deleted 15 aa include the presumably critical YENPTY motif of APP to which adaptor proteins like Fe65 and X11 are proposed to bind through their phosphotyrosine-binding domains and which are responsible for nuclear targeting of AICD [35, 38, 39]. Indeed, RT-PCR analysis of MEF APPΔCT15 cells showed strongly increased gene expression of the SPT subunit SPTLC2 (Figure 3(a)), indicating that the presence of a functional AICD domain decreases SPTLC2 expression. In accordance with increased SPTLC2 expression, SPT activity was significantly increased in MEF APPΔCT15 cells (Figure 3(a)). To exclude that altered Aβ production, which might be caused by the truncated APP construct APPΔCT15 [40, 41], would be responsible for increased SPTLC2 expression in MEF APPΔCT15 cells, we incubated MEF APPΔCT15 cells with a synthetic AICD peptide, corresponding to the last 20 aa of the C-terminus of APP. APPΔCT15 cells, incubated with solvent control only, showed in comparison to APPΔCT15 cells incubated with the AICD peptide, increased SPTLC2 expression, emphasizing that AICD decreases SPTLC2 gene transcription (Figure 3(b)). Incubation with Aβ peptides and

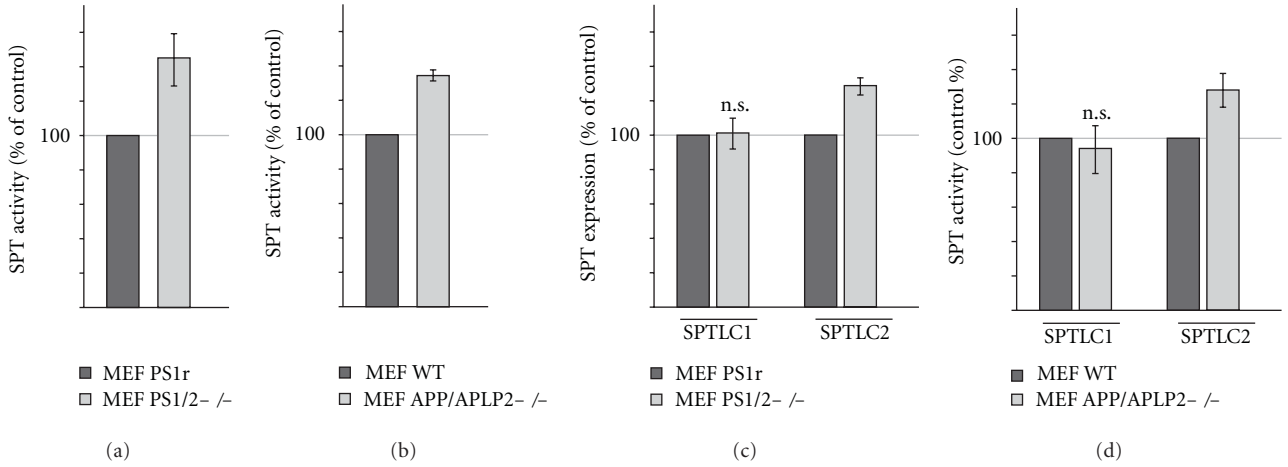

Figure 2: SPT activity and expression in PS1/2- and APP/APLP2-deficient cells. (a) SPT activity in mouse embryonic fibroblasts devoid of PS1 and PS2 (MEF PS1/2− /−) and MEF PS1/2− /− retransfected with PS1 (MEF PS1r). (b) SPT activity in mouse embryonic fibroblasts lacking APP and the APP-like protein APLP2 (MEF APP/APLP2− /−) and corresponding wild-type cells (MEF WT). (c) RT-PCR analysis of SPTLC1 and SPTLC2 expression, the two subunits of SPT, in MEF PS1/2− /− and MEF PS1r. (d) SPTLC1 and SPTLC2 expression in MEF APP/APLP2− /− and MEF WT.

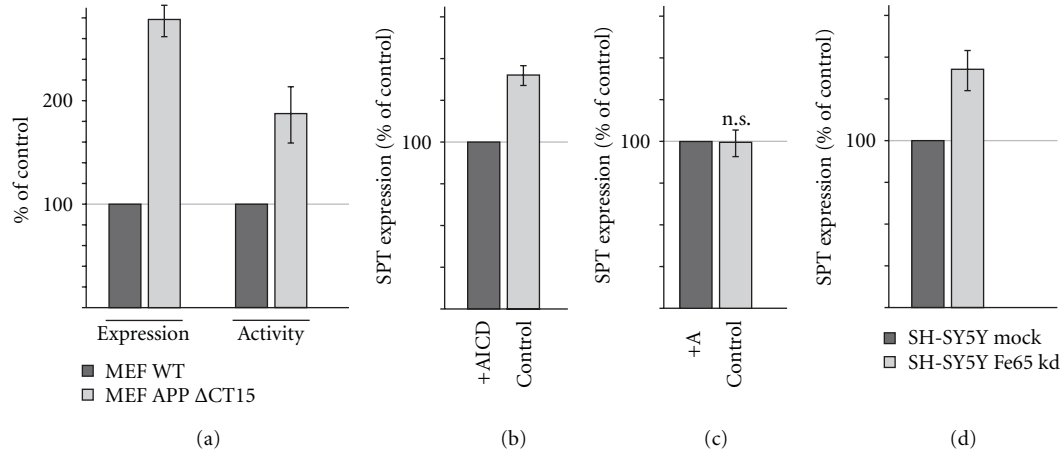

Figure 3: SPTLC2 expression is reduced in the presence of functional AICD. (a) Mouse embryonic fibroblasts expressing an APP construct lacking the last 15 amino acids (aa) and therefore a functional AICD domain (MEF APP△CT15) show increased SPT expression and activity compared to control fibroblasts (MEF WT). (b) MEF APP△CT15 cells incubated with functional AICD peptide show compared to MEF APP△CT15 cells incubated with solvent control decreased SPTLC2 expression. (c) MEF APP△CT15 cells incubated with Aβ peptides and solvent control showed no difference in SPTLC2 expression. (d) SPTLC2 expression in Fe65 knock-down SH-SY5Y cells is increased.

solvent control showed no differences in SPTLC2 expression (Figure 3(c)), demonstrating that Aβ peptides do not contribute to the regulation of SPTLC2 gene transcription. The uptake of the peptides was confirmed by incubating APP/APLP2− /− MEFs with Aβ peptide. Only in incubated cells intracellular Aβ could be detected by western blot analysis whereas the unincubated knockout cells showed no intracellular Aβ (supplemental Figure 1). To further evaluate the role of AICD in regulating gene expression of SPTLC2, we generated Fe65 knock-down human neuroblastoma SH-SY5Y cells. RT-PCR of Fe65 showed that Fe65 expression was reduced to 42%. As expected, the Fe65 knock-down cells had increased SPTLC2 expression (Figure 3(d)), further emphasizing a physiological role of AICD in the regulation of SPT expression. Supporting the *in vivo* relevance of these findings, brains of APP knock-out mice (APP− /−) as well as brains of mice expressing the APP△CT15 construct had significantly increased SPTLC2 expression (Figures 4(a) and 4(b)). Taken together these results indicate that AICD regulates cellular SPTLC2 gene transcription *in vivo* and that this regulation is dependent on adaptor proteins like Fe65.

3.3. Analysis of SPTLC2 Expression in FAD. In order to evaluate a potential role of this AICD-mediated regulation of SPT in AD, we investigated whether familial forms of Alzheimer's disease (FAD) show changes in sphingolipid

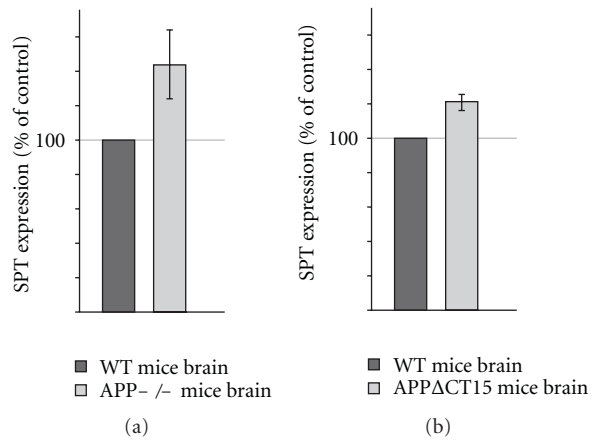

Figure 4: SPTLC2 expression in APP−/− and APP△CT15 mice brains. (a) SPTLC2 expression in APP-deficient mice brains (APP−/−) compared to wild-type (wt) mice brains. (b) SPTLC2 expression in APP△CT15 mice brains is increased compared to wt mice brains.

de novo synthesis. For this, MEF cells were generated that express familial PS1 mutations known to cause early onset Alzheimer's disease (EOAD) [42]. MEF PS1/2−/− cells were retransfected with three PS1-FAD mutations, E280A, A285V and T354I and wild-type PS1 (MEF PS1r). All FAD cell lines were PS expression level matched to the control cells. In accordance to the literature we found that analysed PS-FAD mutations result in a decreased total γ-secretase activity (data not shown) and therefore affecting AICD production [43–48]. RT-PCR analysis revealed that SPTLC2 expression was significantly increased in PS1 E280A, PS1 A285V, and PS1 T354I cells, supporting a potential role of SPT in AD (Figure 5(a)). Although the analysis of *postmortem* brain samples allows to draw only limited conclusions regarding the molecular mechanism, it should be noted that SPTLC2 expression was increased in familial AD *postmortem* brains, caused by the mutations I143T, L174R and L286V compared to age- and gender-matched controls (+/− 10 years). Confirming this result, analysis of *postmortem* brain samples of 40 sporadic AD brains compared to age- and gender-matched control brains showed that in 24 cases SPTLC2 expression was increased whereas in 16 cases SPTLC2 was decreased (Figures 5(b) and 5(c)). Although the mean difference between the sporadic AD cases compared to control brain samples reached significant levels (mean = 1,52; $P < .01$; SEM = 16%) it appears that in familial AD mutations the phenotype of increased SPTLC2 levels has a higher penetrance.

Although this finding emphasizes a potential role of SPT in AD and underlines our findings made by different cell culture and mouse models, it should be pointed out that in principal no molecular insights should be drawn by analyzing human *postmortem* material. Therefore and to avoid potential overinterpretation of these results obtained by *postmortem* brains we decided not to analyze the AICD levels.

4. Discussion

Sphingolipids play important roles in biological processes like regulation of cell growth and signal transduction and represent ubiquitous constituents of membrane lipids in eukaryotes [18, 49–51]. Serine-palmitoyl transferase (SPT) is the rate limiting enzyme that catalyzes the first step of *de novo* biosynthesis of sphingolipids, finally resulting in the synthesis of the three main types of complex sphingolipids: sphingomyelins, glycosphingolipids, and gangliosides [15]. Alterations in sphingolipid metabolism are discussed to contribute to the development of AD. Brains of AD patients show altered ganglioside level [52], elevated ceramide and sphingosine levels and reduced sphingomyelin levels [14]. Furthermore, gangliosides and sphingomyelin have been shown to influence Aβ generation [12, 13]. Sphingolipids along with cholesterol have been shown to be enriched in detergent-resistent membrane microdomains, also called rafts [53–55]. Interestingly, β- and γ-secretases are discussed to be present in rafts [20–24]. Ganglioside GM1 is present in rafts, increases Aβ generation and has been found to bind to Aβ [13, 56, 57]. Notably GM1-Aβ is favourably generated in the ganglioside-enriched, raft-like microdomains and exerts neurotoxic effects and might act as a seed for Aβ aggregation in amyloid plaques [56, 58].

Although it is well established that a deregulation of sphingolipid metabolism is present in AD, the underlying cellular mechanism that causes changes in sphingolipid metabolism is poorly understood. It is known that Aβ increases neutral and acidic sphingomyelinase activity [12, 14] and that expression of acidic sphingomyelinase is elevated in brains of AD patients [14]. In the present study we identified SPT, the rate limiting enzyme in sphingolipid biosynthesis, to be regulated by APP processing and to be affected in AD. The first indication of increased SPT activity in AD was obtained by the use of PS1/2- and APP/APLP2-deficient cell lines, which showed increased SPT activity. The elevated SPT activity is caused by increased expression of the SPT subunit SPTLC2, which represents the catalytic subunit of the SPT heterodimer [32, 34]. Because PS- and APP/APLP2-deficient cells are both devoid of Aβ and AICD peptides, we analyzed whether these peptides are responsible for altered SPTLC2 expression. Analysis of mouse embryonic fibroblasts expressing an APP construct that lacks a functional AICD domain identified AICD as the molecular mediator of decreased SPTLC2 gene transcription. This result was further substantiated by the incubation of MEF APP△CT15 cells with AICD, resulting in decreased SPTLC2 expression in presence of AICD. By partially rescuing the altered SPTLC2 expression with an AICD peptide incubation, potential artefacts which could be caused by clonal heterogeneity of MEFs could be ruled out. Fe65 is an important protein that binds to the YENPTY motif in the APP C-terminus and is essential for nuclear transport of AICD [35, 38, 39]. Indeed, Fe65 knock-down increased SPTLC2 expression, which taken together with the above results clearly identifies AICD as a regulator of SPT transcription. AICD was controversially discussed to be involved in the regulation of gene transcription [35, 38, 39].

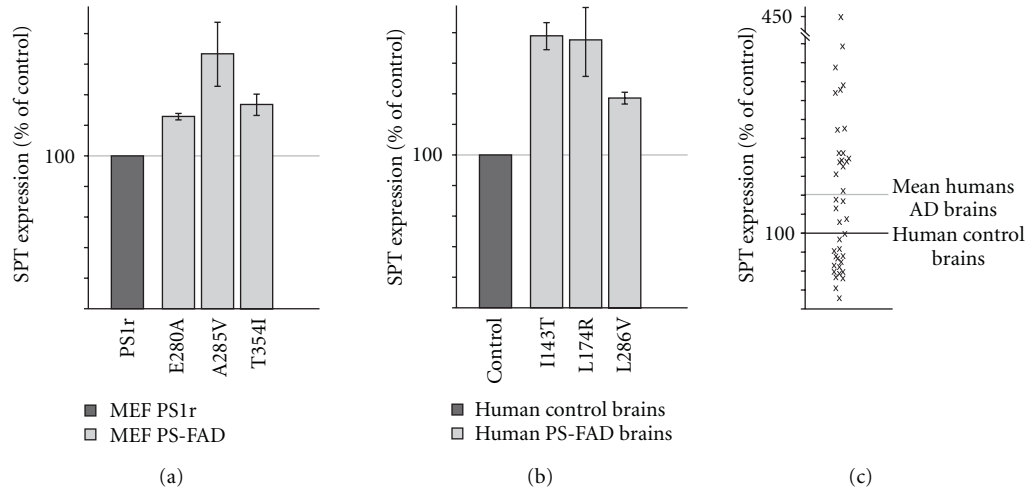

Figure 5: SPT expression in Alzheimer's disease. (a) Mouse embryonic fibroblasts devoid of PS1 and PS2 were retransfected with the familial PS1 mutations PS1 E280A, PS1 A285V, PS1 T354I (MEF PS-FAD), and PS1 wild-type (MEF PS1r), respectively. All PS1 mutations are known to cause early onset AD and show increased expression of SPTLC2. (b) Human *postmortem* PS-FAD brains, caused by the mutations I143T, L174R and L286V show increased SPTLC2 expression compared to age- and gender-matched control brains (+/− 10 years). (c) Analysis of SPTLC2 expression in 40 sporadic AD human *postmortem* brains compared to age- and gender-matched control brains (+/− 10 years). Pairwise normalization with the respective age- and gender-matched controls.

However, increasing evidence exists that AICD regulates the expression of multiple genes similar to the function of the Notch intracellular domain. For example, expression of APP, β-secretase BACE1, neprilysin, EGF-receptor, LRP1 and glycogen-synthase-kinase-3β (GSK-3β) has been shown to be regulated by AICD [35, 59–62]. Recently, two further genes were identified, patched homolog 1 (PTCH1) and transient receptor potential cation channel subfamily C member 5 (TRPC5) [63]. The identification of SPTLC2 expression to be regulated by AICD also contributes to our understanding of altered sphingolipid levels in AD. SPTLC2 expression was increased in cells expressing PS mutations known to cause EOAD and in human PS-FAD *postmortem* brains, supporting the relevance of altered SPT expression and activity in the development of AD. Taking into consideration that elevated SPT expression results in increased *de novo* synthesis of sphingolipids, major components of lipid rafts, one might speculate that increased SPTLC2 expression exerts its toxic effect by increased Aβ generation in lipid raft microdomains of the membrane, known to be involved in the amyloidogenic processing of APP. Nevertheless further experiments have to be done to clarify the question whether the observed change in SPTLC2 levels in the human sporadic and familiar AD brains are cause or consequence of Alzheimer's disease.

5. Conclusions

In conclusion, our results demonstrate that APP processing downregulates SPT expression, the rate limiting enzyme in sphingolipid *de novo* synthesis by an AICD/Fe65-mediated mechanism and that SPT expression is affected in AD.

Acknowledgments

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Research Article

Ceramide and Related-Sphingolipid Levels Are Not Altered in Disease-Associated Brain Regions of APP^{SL} and $APP^{SL}/PS1^{M146L}$ Mouse Models of Alzheimer's Disease: Relationship with the Lack of Neurodegeneration?

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There is evidence linking sphingolipid abnormalities, APP processing, and neuronal death in Alzheimer's disease (AD). We previously reported a strong elevation of ceramide levels in the brain of the APP^{SL}/PS1Ki mouse model of AD, preceding the neuronal death. To extend these findings, we analyzed ceramide and related-sphingolipid contents in brain from two other mouse models (i.e., APP^{SL} and $APP^{SL}/PS1^{M146L}$) in which the time-course of pathology is closer to that seen in most currently available models. Conversely to our previous work, ceramides did not accumulate in disease-associated brain regions (cortex and hippocampus) from both models. However, the APP^{SL}/PS1Ki model is unique for its drastic neuronal loss coinciding with strong accumulation of neurotoxic Aβ isoforms, not observed in other animal models of AD. Since there are neither neuronal loss nor toxic Aβ species accumulation in APP^{SL} mice, we hypothesized that it might explain the lack of ceramide accumulation, at least in this model.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in adults. Pathologically, the hallmarks of AD are amyloid plaques and neurofibrillary tangles, associated with widespread neuronal loss. Its fundamental causes and the pathological cascades leading to symptoms, however, remain poorly understood. Lipids and lipid peroxidation products have important roles in central nervous system homeostasis. Extensive evidence supports an important role of cholesterol in the development and possibly progression of AD [1–3]. The role of other lipids, such as ceramides and related-sphingolipids, (sphingomyelins, sulfatides, and glycosphingolipids) is also emerging. Ceramides are the core constituent of most sphingolipids. They can be produced by hydrolysis of sphingomyelin (SM) via sphingomyelinases (SMases) or synthesized de novo from fatty acyl CoA and sphingosine. Conversely, in the Golgi, ceramides may be transformed into SM by the addition of phosphorylcholine. Additionally, glycosyltransferases can attach sugar to ceramide, turning it into glucosylceramide or galactosylceramide (Galcer), a key step in the generation of complex glycosphingolipids [4, 5].

Ceramides are important second messenger molecules that regulate diverse cellular processes including cell growth, differentiation, and apoptosis. Ceramide levels also increase in response to aging and various age-related stress factors and are directly involved in apoptotic signaling in various cell types, including neurons [6–8].

There is evidence linking sphingolipid abnormalities, APP processing, and neuronal death in AD. In vitro, it has been shown that β-amyloid added to cultured neurons [9, 10] or oligodendrocytes [11] increase SMase activity, leading to an increase in ceramide levels [12]. Additional reports have found that ceramide levels increase β-amyloid synthesis [12, 13] and favor gamma secretase processing of APP [14–16] so that inhibition of ceramide synthesis

confers protection against β-amyloid [9]. Thus, it has been suggested that ceramides and β-amyloid may synergize to induce neuronal death.

Several studies have examined the sphingolipid alterations in human AD brain. For example, the total phospholipid and sulfatide content in AD was decreased as compared to normal [17–19], while the ceramide and galactosylceramide levels were elevated [9, 18]. Enhanced ceramide levels have been reported in the cerebrospinal fluid (CSF) of patients with AD [20] and changes in the activity of several key enzymes of ceramide metabolism, in gene expression of pathways associated to sphingolipid metabolism have been found in brains of AD patients [21, 22].

During the last years, numerous mouse transgenic lines have been created and have been screened for various aspects of AD pathology [23, 24]. Unfortunately, very little work has been done on determining if sphingolipid content is likewise perturbed in these rodent models of AD. In one study, long-chain ceramides were shown to be elevated in presenilin 1 (PS1M146V) mouse brain and to induce apoptosis in PS1 astrocytes [25]. In a second study, sulfatides, a class of sulfated galactocerebrosides, were found to be decreased in brain tissues from two APP transgenic mice (i.e., APPV717F and APPsw), whereas no significant changes in the content of other sphingolipid classes including SM, Galcer, and ceramides were noted [26]. By contrast, using the new mouse mutant APP^{SL}/PS1Knock-in line, we have recently found an early and significant increase of ceramides in the cortex of these mice, without significant changes in other sphingolipid levels [27]. However, the APP^{SL}/PS1Ki model is unique for its drastic neuronal loss, not observed in other animal models of AD [28]. The discrepancy in the few data available and the lack of knowledge of sphingolipid levels in the brain of other rodent models of AD prompted us to investigate whether the sphingolipid composition is altered in the brain of two other mouse models of AD: single APP^{SL} and double $APP^{SL}/PS1^{M146L}$ transgenics. Concentrations of ceramides, SM, Galcer, and sulfatides were determined in three brain regions: the cortex and the hippocampus, the two brain regions typically associated with the disease, and the cerebellum, a nonvulnerable region with no Aβ plaques. For all analyses, age-matched $PS1^{M146L}$ (amyloid free) mice as well as nontransgenic wild-type mice (WT) were used.

2. Materials and Methods

2.1. Chemicals. All organic solvents were of analytical grade and came from VWR International (Strasbourg, France). HPTLC-plates Silicagel 60, 10 × 10- or 10 × 20 cm were purchased from Merck (VWR International). Lipid standards (nonhydroxy fatty acid (NFA) containing ceramides, ceramides containing 2-hydroxy fatty acids (2-HFA), sphingomyelins, cerebroside sulfate (sulfatides), and galactosylceramides (a mixture containing 2-hydroxy fatty acids and nonhydroxy fatty acids) were purchased from Sigma-Aldrich (France). Aminopropyl-bonded (LC-NH_2) silica gel cartridges (100 mg matrix) were from Supelco (Saint Quentin Fallavier, France).

2.2. Transgenic Mice. Generation and detailed neuropathological analyses of the APP^{SL} and the $APP^{SL}/PS1^{M146L}$ transgenic mice have been described earlier [29, 30]. In brief, these mice express human APP751 with Swedish and London mutations (Thy1 promoter) either alone or in combination with human presenilin-1 (M146L, HMG-CoA promoter). In this study, 12-month-old ($n = 5$) $APP^{SL}/PS1^{M146L}$ double transgenic, 12-month-old ($n = 5$) $PS1^{M146L}$ single-transgenic littermates, and 12-month-old ($n = 6$) nontransgenic mice as well as 24 month-old ($n = 5$) APP^{SL} single transgenic and 24-months ($n = 5$) nontransgenic littermates were used (generous gift of Sanofi-Aventis, Vitry sur Seine, France). APP^{SL} mice were analyzed at 24 months of age and $APP^{SL}/PS1^{M146L}$ mice were assessed at 12 months of age because they revealed comparable levels of amyloid plaques in the brain at these respective ages. The genetic background of the mice was mixed CBA/C57BL/6. All the mice used in this study were female, because a gender effect with female mice displaying more severe pathology than male has been mentioned in several studies [27, 31, 32].

All experiments were performed in compliance and following approval of the Sanofi-Aventis Animal Care and Use Committee and in accordance with standards of the guide for the care and use of laboratory animals (CNRS ILAR) and with respect to French and European Community rules.

2.3. Brain Tissue Preparation. The mice were anesthetized with pentobarbital (40 mg/kg, IP) and sacrificed. Brains were removed immediately, and cortex, hippocampus, and cerebellum were dissected. These cerebral regions were homogenized by 10 up-and-down strokes of a prechilled Teflon-glass homogenizer in 20 volumes of buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) and supplemented with 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, protease, and phosphatase inhibitor cocktails (50 μL/g tissue and 10 μL/mL lysis buffer, resp.). The homogenates were centrifuged at 15,000 g for 15 min at 4 C. The resulting pellet was resuspended in 3 volumes of ice-cold water, altogether 1.5 mL, homogenized at 4 C (10–15 strokes) and then sonicated for 20 s using a sonifier (Branson Ultrasonics, sonifier 450, Danbury, CT). Samples from each mouse brain were analyzed separately and in duplicate.

2.4. Lipid Extraction from Tissues and Thin-Layer Chromatography. Total lipids from brain homogenates were prepared according to previously described procedures [27, 33]. Briefly, the homogenates were added to 8 vol. of rapidly stirring methanol, then 4 vol. of chloroform were added. The mixture was stirred overnight, and then centrifuged at 1,000 g for 10 min. The pellet was re-extracted with chloroform-methanol-water (4 : 8 : 3, v/v/v) and the two supernatants combined, evaporated to dryness. Partitioning was carried out using diisopropyl ether/1-butanol/50 mM aqueous NaCl (6 : 4 : 5, v/v/v) according to the method of Ladish and Gillard [34]. The total upper phase was evaporated to dryness and taken up in chloroform. The lipids were fractionated using solid-phase extraction on

100 mg LC-NH_2 cartridges (Supelco, L'Isle d'Abeau, France) as previously described [27]. The eluted fractions containing, respectively, free ceramides, galactosylceramides (Galcer), alkali-stable phospholipids (SM), and sulfatides were applied to HPTLC plates with a Linomat 5 (Camag, Switzerland). Prior to SM analysis, the phospholipid-containing fraction was subjected to mild alkaline methanolysis (treatment with chloroform: 0.6 N NaOH in methanol 1 : 1 (v/v) at room temperature for 1 h) to remove glycerophospholipids. To quantify each lipid species (ceramides, SM, Galcer, and sulfatides), calibration curves were obtained by running in parallel known amounts of purified lipid standards. For free ceramides, 15–20 mg of brain tissue (wet weight) was spotted per lane. The plates were developed with chloroform-methanol 50 : 3 (v/v) and visualized by charring for 10 min at 180 C with 3% cupric acetate in 8% phosphoric acid solution. For SM analysis, 6–9 mg of tissue was spotted per lane. The plates were developed with chloroform-methanol-water 60 : 35 : 8 (v/v/v). SM was visualized with sulfuric acid-$CuSO_4$-ammonium molybdate spray reagent followed by heating at 110 C for 15 min. Galcer and sulfatides (about 0.4 mg and 2 mg of tissue per lane, resp.) were developed in chloroform-methanol-water 65 : 25 : 4 (v/v/v), sprayed with orcinol-H_2SO_4 reagent, and then heated at 150 C for 2 min.

Each sphingolipid species was quantified by scanning densitometry of the plates at 396 nm for ceramides, 540 nm for Galcer and sulfatides, and 750 nm for SM with the Camag TLC Scanner 3/WinCats software system.

2.5. Statistical Analysis. The results were presented as mean ± S.D. values. Owing to the small number of animals per group, statistical analysis of the data was performed using a nonparametric Kruskall-Wallis test followed by a posthoc test of Dunn for multiple comparisons. For the comparison of two means, the Mann-Whitney test was used. All calculations were performed using GraphPad Prism software 3.02 (GraphPad Software, Inc.). P values less than .05 were considered as significant.

3. Results

In single APP^{SL} mice (Figure 1(a)), a moderate but not significant elevation of NFA-ceramides was seen in the cortex (+22%), with no changes of the 2-HFA-ceramide level. Conversely, in the hippocampus (Figure 1(b)), the NFA-ceramide level did not differ between WT and APP^{SL} mice, whereas a tendency towards an increase of 2-HFA-ceramides was noted (+19%), although it was not significant.

Surprisingly, as shown in Figure 1(c), the level of 2-HFA-ceramides in the cerebellum of APP^{SL} mice was significantly increased in comparison to the counterpart of their WT littermates (+50%, $P < .05$). Conversely, the NFA-ceramides showed a slight but not significant decrease compared to WT control values. However, no difference in the total ceramide content was noticed in the cerebellum of both WT (51.35 ± 1.45 nmol/mg tissue) and APP^{SL} mice (50.28 ± 3.31 nmol/mg tissue).

Because the double-transgenic mouse model $APP^{SL}/PS1^{M146L}$ develops neuropathological features of AD earlier than the single APP^{SL} mice [29], the sphingolipid analysis was performed in the brain regions of this model in younger animals (12 months of age). As shown in Figures 2(a) and 2(b), concentrations of NFA-ceramides as well as those of 2-HFA-ceramides did not differ between wild-type, $PS1^{M146L}$, and $APP^{SL}/PS1^{M146L}$ mice in the two disease-associated brain regions (cortex and hippocampus). In contrast to the changes of ceramide content in cerebellum of APP^{SL} mice, the NFA-ceramide as well as the 2-HFA-ceramide levels were unchanged in this brain region in $APP^{SL}/PS1^{M146L}$ mice relative to their WT controls and PS1 littermates (Figure 2(c)). However, NFA-ceramide content showed a tendency towards a decrease, while 2-HFA ceramides tended to slightly increase in the cerebellum of the $APP^{SL}/PS1^{M146L}$ mice.

It should be noted that, although cortex and hippocampus of WT mice displayed almost identical ceramide content (Figures 1(a), 1(b), 2(a), and 2(b)), a relatively lower NFA-ceramide content was manifest in the cerebellum (Figures 1(c) and 2(c)). In normal human brain, the ceramide levels were also reported much higher in the cortex versus the cerebellum [18].

Typical ceramide profiles from either cortex, hippocampus, or cerebellum of the different transgenic mice and wild-type controls were shown in Figure 3. Since 2-HFA-ceramides are present in extremely low levels leading to a weak staining on the HPTLC plate, densitometric scanning of the plate was shown to visualize the peak corresponding to the 2-HFA-ceramide species (Figure 3).

To test whether other related-sphingolipids could be altered in the brain of transgenic mice, the content of SM, Galcer, and sulfatides in lipid extracts of the three examined brain regions was assessed by HPTLC analysis. Figures 1(d)–1(f) show that sphingolipids (SM, Galcer, and sulfatides) did not display significant changes in both cortex, hippocampus, and cerebellum of APP^{SL} mice compared to the WT littermates. Similarly, the levels of SM, Galcer, and sulfatides did not differ among nontransgenic, $PS1^{M146L}$ and $APP^{SL}/PS1^{M146L}$ mice in all brain region examined (Figures 2(d)–2(f)). It should be noted that there are differences of SM, Galcer, and sulfatide concentrations between cerebral tissues (cortex and hippocampus) and cerebellar tissues. In both models, cerebellum displayed higher levels of Galcer and sulfatides than cerebral tissues. This is in accordance with Cheng et al. [26] who also reported a 2-fold higher level of sulfatides in the cerebellum compared to the cortex, in two other transgenic mouse models of AD. In contrast, SM levels were almost identical in hippocampus and cerebellum, but higher than those of cerebral cortex (Figures 1(d)–1(f) and 2(d)–2(f)). Examples of HPTLC profiles of sulfatides, Galcer, and SM and from either cortex, hippocampus, or cerebellum were represented in Figures 4(a), 4(b), and 4(c), respectively. It should be mentioned that the HPTLC profiles of each sphingolipid class were similar in all examined brain regions from both mouse models.

4. Discussion

Using the same methodology as in our previous work [27], we herein analysed the sphingolipid composition of two additional models (i.e., single APP^{SL} and double $APP^{SL}/PS1^{M146L}$ mice) in which the time-course of pathology is much closer to that seen in the majority of currently available models. The main results of this study are (1) a moderate but not significant increase of NFA-ceramides and 2-HFA-ceramides in the cortex and the hippocampus respectively, of the APP^{SL} mice, (2) unaltered ceramide levels in the two disease-associated brain regions from $APP^{SL}/PS1^{M146L}$ mice, (3) unexpected alterations of the ceramide profile in the cerebellum of APP^{SL} mice, a region with no Aβ pathology, and (4) no significant changes in the other related-sphingolipids in all brain structures examined of both transgenic models. Based on our results and those from the literature, we will first discuss the possibility of a relationship between neurodegeneration, toxic Aβ accumulation, and ceramide content. For the second time, why an amyloid-free brain region such as cerebellum showed ceramide alterations is discussed.

Ceramides were shown to accumulate in AD human brain regions and their levels vary by disease severity suggesting that they could be indicators of AD progression [9, 18, 35]. Similarly to what happens in human AD, we previously found that ceramides increase very early in the cortex of the APP^{SL}/PS1Ki mouse model, preceding the neuronal loss [27]. By contrast, our present results reveal that in single APP^{SL} mice, ceramide levels were not significantly altered in disease-associated brain regions (e.g., hippocampus or cortex). This is consistent with the findings of Cheng et al. [26] who reported no changes in ceramide content in any brain region from APPsw and APP^{V717F} transgenic mice. An important difference between these single APP mouse lines and the APP^{SL}/PS1Ki model is that the latter develops an early and massive neuronal loss which correlates with strong accumulation of intracellular Aβ42, Aβ aggregates, and Aβ42 oligomers [28, 36]. Although intraneuronal Aβ accumulation has also been documented in various APP models [29, 37, 38], a striking difference between the models used in the present study and the APP^{SL}/PS1Ki mice is the nature of the Aβ peptides which accumulate. Indeed, in APP^{SL}/PS1Ki mouse brain, extremely high levels of N-truncated Aβx-42 variants and abundant oligomers were detected, which closely resembles that found in AD brain. By contrast, in APP^{SL} mouse brain, with the same total Aβ levels as in APP^{SL}/PS1Ki mice, only very limited levels of Aβ42 N-terminal truncated isovariants were detected [28]. In the APP^{SL}/PS1Ki mice, Aβx-42 is the major form accumulated with a ratio Aβx-42/total Aβ close to 1. In comparison, this ratio is approximately of only 0.2-0.3 in the APP^{SL} mice, and 0.3-0.4 in the double transgenic $APP^{SL}/PS1^{M146L}$ mice, similar to the range of values reported for a large number of other APP-based transgenics [28, 29].

N-truncated Aβ peptides are known to aggregate more readily and are considered to be very toxic species. Thus they might play a key role in the neurotoxicity observed in the APP^{SL}/PS1Ki model. In particular, the pyroglutamate modified N-terminal truncated form of Aβ at position 3 ($A\beta_{3(pE)-42}$), which represents a major species in the brain of AD patients [39], was recently shown to induce a severe neuron loss in the TBA2 mouse model, a new model expressing only N-truncated $A\beta_{3(pE)-42}$ in neurons [40]. Interestingly, there is also a coincidence of considerable amounts of $A\beta_{3(pE)-42}$ and massive neuron loss in the APP^{SL}/PS1Ki mouse model [41]. On the basis of these findings, it is attractive to speculate that in APP^{SL}/PS1Ki mice, the strong accumulation of intracellular toxic forms of Aβ induces early elevation of ceramides. Extensive increase of ceramides could therefore activate proapoptotic pathways, leading to neuronal death. Conversely, other APP transgenic mouse models including the APP^{SL} mice have been reported to show no [42] or very low $A\beta_{3(pE)-42}$ levels [43]. This is due to the lack of posttranslational modifications such as N-terminal degradation and pyroglutamyl formation in these mice. Because APP^{SL} mice display neither neuronal loss nor accumulation of highly toxic Aβ 42 isoforms, this may at least in part explain why no significant accumulation of ceramides occurred in the disease-associated brain regions of these mice.

Thus, despite numerous neuropathological, biochemical, and even behavioral changes representative of AD developed by these APP mice [23, 28–30, 33, 44], they may not constitute a relevant model to further explore the role of ceramides in AD pathology. However, answering the question of the relationship between neurodegeneration, toxic Aβ accumulation, and ceramide elevation could be facilitated using restricted models either lacking (i.e., single APP mice) or mimicking only some specific AD-related neuropathological alterations (i.e., TBA2 mice mentioned above). Indeed, owing to the simultaneous occurrence of numerous pathological features of AD, the connection between them is often difficult to unravel.

We next determined the ceramide content in the double transgenic mouse model $APP^{SL}/PS1^{M146L}$, but in younger animals because the $APP^{SL}/PS1^{M146L}$ mice develop neuropathological features of AD earlier than single APP^{SL} mice [29]. At 12 months of age, these double transgenics revealed comparable levels of amyloid deposits than 24-month-old APP^{SL} mice. Additionally, they displayed similar ganglioside alterations [33]. Our results showed that at 12 months of age, ceramide levels were unaltered in both cortex and hippocampus of $APP^{SL}/PS1^{M146L}$ mice in comparison to age-matched $PS1^{M146L}$ mice and wild-type controls. It should be noted that there is no neuronal loss in these mice as old as 17 months, but unfortunately, older $APP^{SL}/PS1^{M146L}$ mice were not available for this study. However, in our previous work, we demonstrated that elevation of ceramides occurred very early (3 months of age) in the cortex of the APP^{SL}/PS1Ki model, preceding by far the neuronal loss detectable at 6 months of age. Why the APP^{SL}/PS1Ki mice showed an increase of ceramides several months before the appearance of neuronal death while the $APP^{SL}/PS1^{M146L}$ mice did not is currently unknown. One possible explanation is that the neuronal loss reported in the $APP^{SL}/PS1^{M146L}$ mice is moderate and more restricted than in the APP^{SL}/PS1Ki model. Indeed, the loss of neurons in the former involves

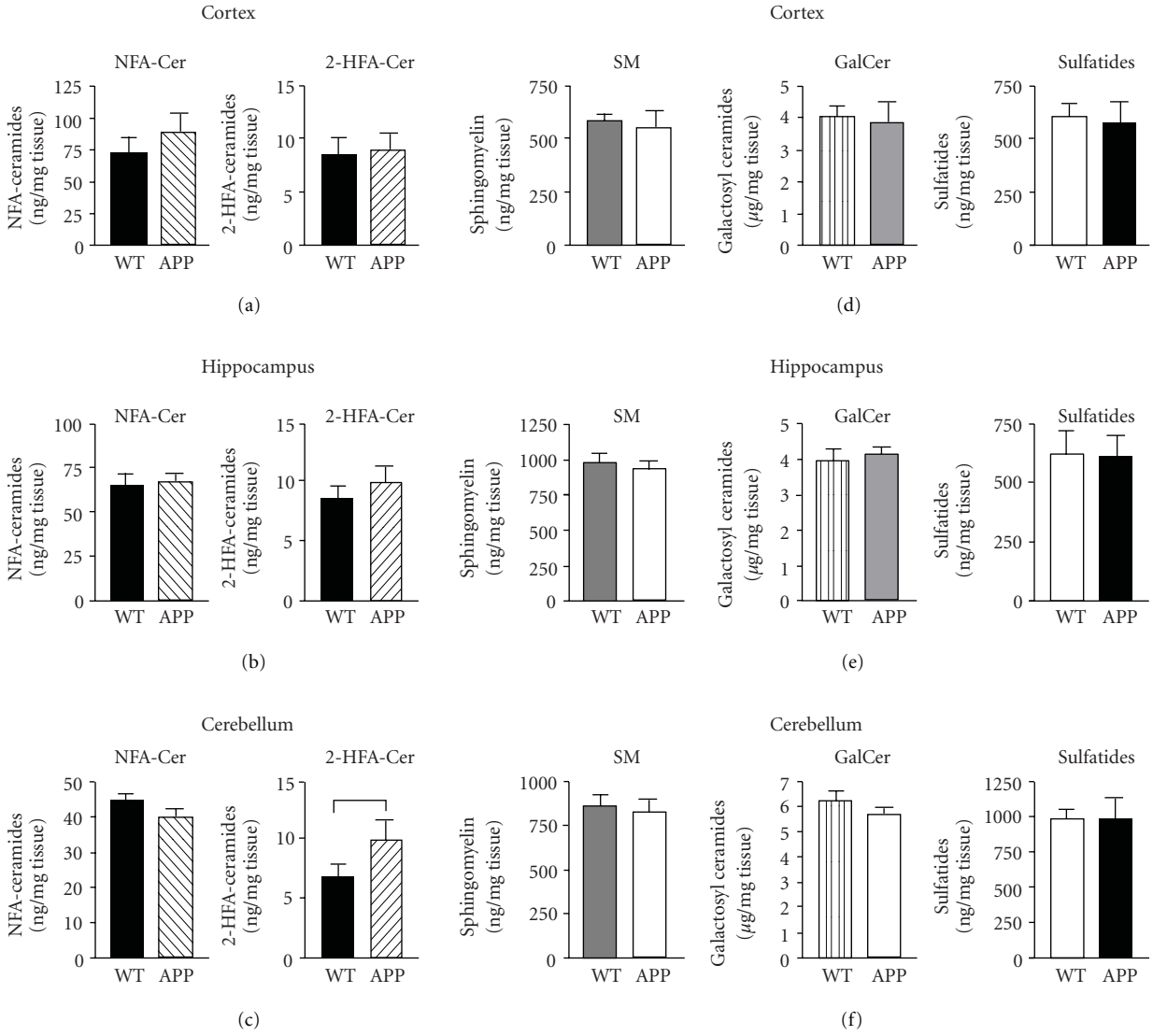

Figure 1: Levels of NFA-ceramides and 2-HFA-ceramides (a–c) and other related-sphingolipids (SM, GalCer, and sulfatides) (d–f) in the cortex, hippocampus, and cerebellum of 24-month-old APP^{SL} mice and age-matched nontransgenic mice. WT: wild-type control mice. Each sphingolipid class was analyzed by HPTLC and their respective concentrations were calculated from standard curves after densitometric scanning of the plates as described under Section 2. Values are expressed as the means ± S.D. and statistically analyzed by a Mann-Whitney test. $P < .05$ compared to the respective wild-type mice.

only the hippocampal pyramidal cell layer (loss of 30% in 17-month-old animals). This may in some way account for the lack of ceramide accumulation in the cortex of these mice. By comparison, in the APP^{SL}/PS1Ki model, the cell loss is greater (50% at 10 months of age) and has been shown to extend to other brain areas such as frontal cortex [36] and cholinergic system [45]. Moreover, as discussed above, accumulation of N-truncated Aβ peptides should be lesser in $APP^{SL}/PS1^{M146L}$ mice, since the ratio Aβx-42/total Aβ is of 0.3 only, versus 1 in the APP^{SL}/PS1Ki mice. In this context, it would seem likely that the level of highly toxic $A\beta_{3(pE)-42}$ form, which is thought to be involved in neuronal death, is reduced in the $APP^{SL}/PS1^{M146L}$ model. It is also possible that, at 12 months of age, it is too early to visualize an elevation of ceramides, owing to the slowest progression of AD lesions in these mice than in the APP^{SL}/PS1Ki model. Since we did not have older $APP^{SL}/PS1^{M146L}$ mice, we should therefore be cautious to interpret the results of ceramide composition in these mice, because we cannot exclude the possibility that ceramides increase at a later age. Further investigations on this topic are warranted.

The most unexpected finding of the present study was alteration of the ceramide composition in the cerebellum of APP^{SL} mice, a brain region lacking Aβ deposits and regarded as nonvulnerable to the disease. Intriguingly, we noted a significant increase of 2-HFA-ceramides (+50%) which was concomitant with a slight decrease in NFA-ceramides. However, considering the total ceramide content,

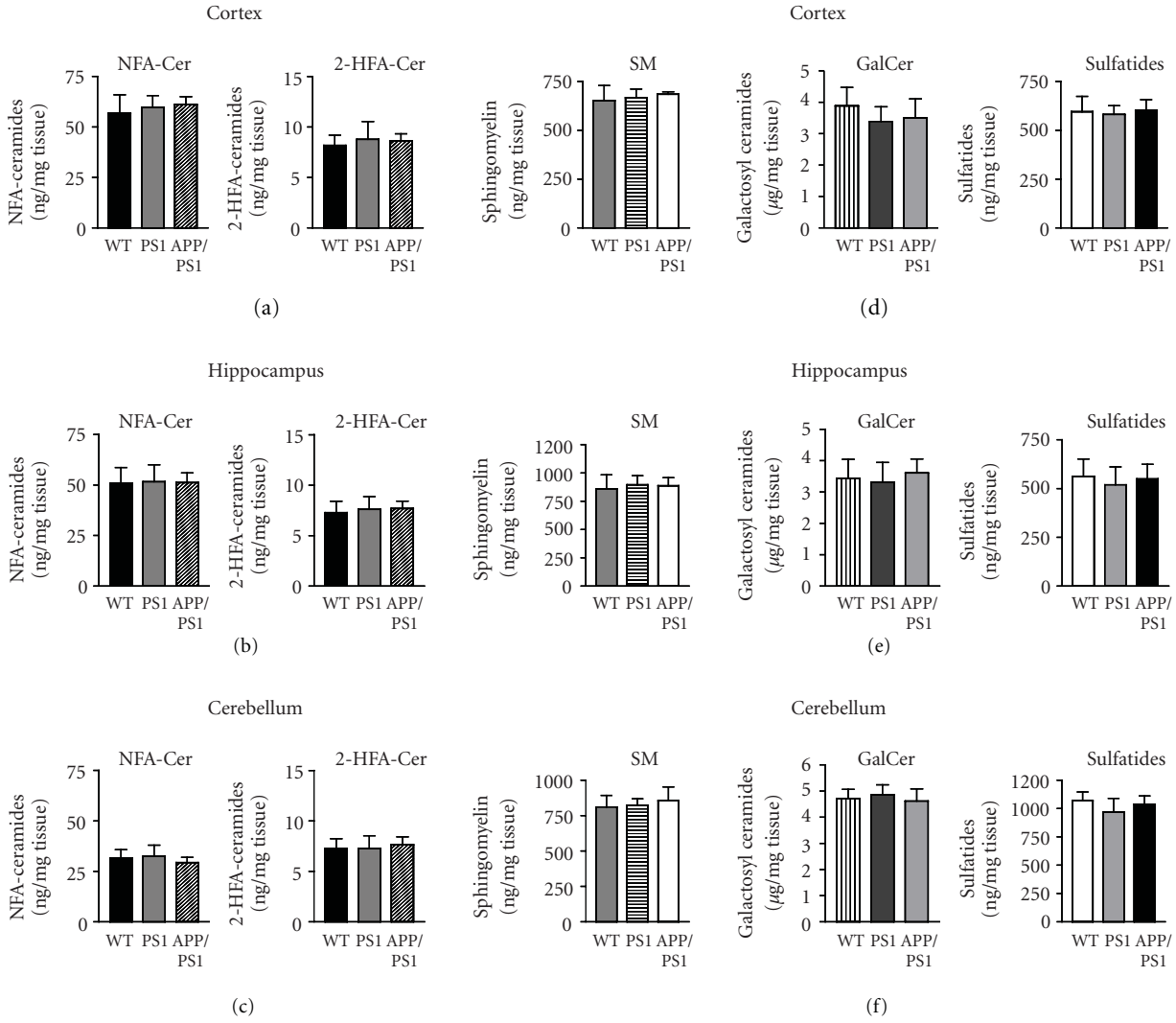

Figure 2: Levels of NFA-ceramides and 2-HFA-ceramides (a–c) and other related-sphingolipids (SM, GalCer, and sulfatides) (d–f) in the cortex, hippocampus, and cerebellum of 12-month-old $APP^{SL}/PS1^{M146L}$, $PS1^{M146L}$ mice, and age-matched nontransgenic mice. WT: wild-type control mice. Each sphingolipid class was analyzed by HPTLC and their respective concentrations were calculated from standard curves after densitometric scanning of the plates as described under Section 2. Values are expressed as the means ± S.D. There was no significant difference in all sphingolipid levels between wild-type (WT), $PS1^{M146L}$, and $APP^{SL}/PS1^{M146L}$ mice in either cortex, hippocampus, or cerebellum (Dunn's multiple comparison test following a Kruskall-Wallis test).

it was almost similar between wild-type and APP^{SL} mice. Previously, we found a more dramatic 161% increase of 2-HFA-ceramides in the cortex of $APP^{SL}/PS1Ki$ mice but contrary to the present results, it was accompanied by an elevation of NFA-ceramides.

Hydroxy FA-ceramides are relatively minor species of membrane lipids. As evident from the literature, the current knowledge about the metabolism and physiological function of 2-HFA-ceramides is very limited. In particular in AD studies, no attention was paid to 2-HFA-ceramides, rendering it very difficult to draw conclusions about the role of these ceramide species in AD physiopathology. Nevertheless, a couple of interesting facts suggest that these HFA species may participate to AD pathology: (i) it was recently found that Aβ selectively bound to sphingolipids that contained a 2-OH group on the ceramide backbone and did not effectively interact with sphingolipids that contained a nonhydroxylated fatty acid, favoring a conformational shift that disrupts membrane stability and promotes peptide-peptide interactions and oligomer formation [46]; (ii) the enzyme UDP-galactose: ceramide galactosyltransferase (CGT), which transfers galactose to both NFA- and 2-HFA-ceramides, was found to be upregulated in human AD brain [21]. Interestingly, overexpression of CGT in transgenic mice led to a reversal NFA : HFA-Galcer ratio which resulted in both decrease in HFA-Galcer and increase in NFA-Galcer levels [47]. Reducing the HFA-Galcer level would lead to an accumulation of their immediate precursors, 2-HFA-ceramides; (iii) there is some evidence for enhanced apoptosis-inducing activity of 2-HFA-ceramides compared

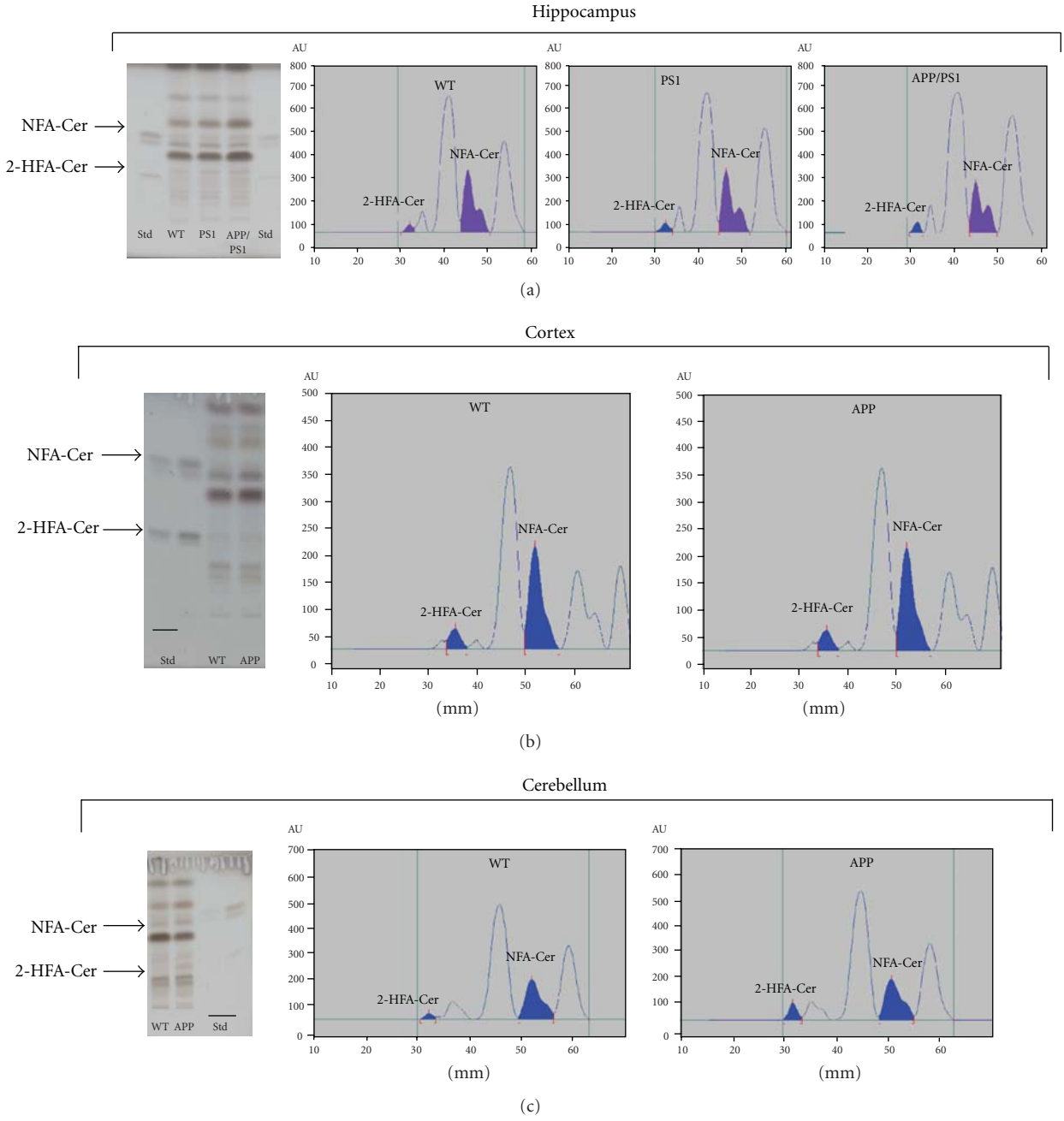

Figure 3: Typical HPTLC profiles of ceramides from hippocampus of 12-month-old $APP^{SL}/PS1^{M146L}$, $PS1^{M146L}$, and their age-matched wild-type (WT) control mice (a), and from cerebral cortex and cerebellum of 24-month-old APP^{SL} mice and age-matched WT control ((b) and (c), resp.). The densitometric analyses of the HPTLC plates were shown in parallel, respectively. Std: a mixture of standard NFA-ceramides and 2-HFA-ceramides. Ceramides were developed on HPTLC plates with chloroform-methanol (50 : 3) and located by spraying the plate with copper acetate 3% in H_3PO_4 8% reagent, followed by heating. Both 2-HFA-Cer and NFA-Cer appear as two bands corresponding to long-chain ceramides (22C; upper band) and short-chain ceramides (18C; lower band). Quantitative evaluation of ceramides was performed by scanning the plate at 396 nm with the Camag TLC Scanner 3/WinCats software system.

to NFA-ceramides [48], and this effect seems to be cell type specific. In this sight, mouse mutants with defective saposin D dramatically accumulate HFA-ceramides in cerebellum, resulting in a selective loss of cerebellar Purkinje cells [49]; (iv) we reported a gender-specific expression of HFA- versus NFA-ceramides in the APP^{SL}/PS1Ki mouse model of AD, and this biochemical feature could be related to the increase propensity of females to develop earlier neuronal loss [27].

Although the degree of 2-HFA-ceramide accumulation in the cerebellum of APP^{SL} mice is much lesser than that seen in the cortex of the APP^{SL}/PS1Ki mice, the reasons for these biochemical changes in this amyloid-free brain area

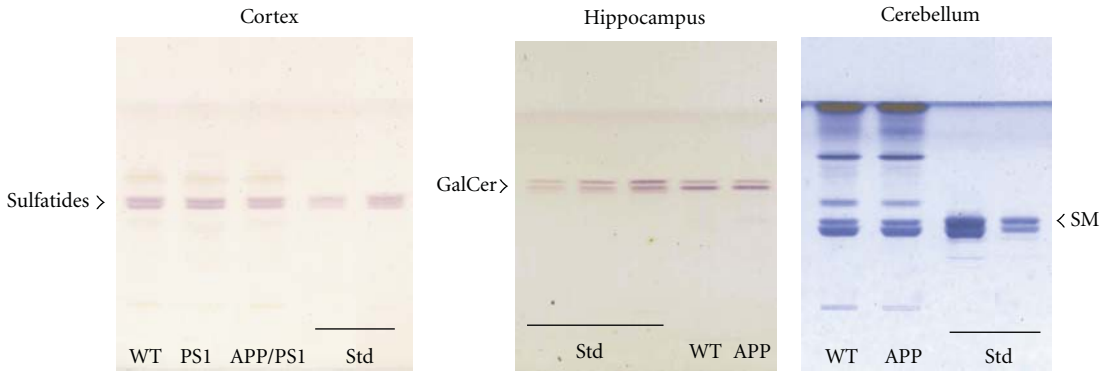

Figure 4: Examples of HPTLC profiles of sphingolipids from different brain regions of nontransgenic and transgenic mice. (a) Sulfatides from cerebral cortex of 12-month-old $APP^{SL}/PS1^{M146L}$, $PS1^{M146L}$, and their age-matched wild-type (WT) control mice. (b) Galactosylceramides (GalCer) from hippocampus of 24-month-old APP^{SL} mice and age-matched WT control mice. Galcer and sulfatides were developed in chloroform-methanol-water (65 : 25 : 4), sprayed with orcinol-H_2SO_4 reagent, and then heated. Both Galcer and sulfatides appear as two bands corresponding to the 2-HFA- (lower band) and NFA-containing galactolipids (upper band), respectively. Standard sulfatides from bovine brain resolved in three bands, as a result of different fatty acid chain length. (c) Sphingomyelins (SM) from cerebellum of 24-month-old APP^{SL} mice and age-matched WT control mice; SMs were developed with chloroform-methanol-water (60 : 35 : 8) (v/v/v) and visualized with sulfuric acid –$CuSO_4$-ammonium molybdate spray reagent followed by heating. SMs appear as two bands which result of the differences in the fatty acid chain length. Std: standard sulfatides, GalCer, and SM, respectively.

are currently unknown. Possibly, it might reflect alterations of ceramide metabolism, since there is evidence that other metabolic pathways are perturbed in the cerebellum of these mice [50]. Hydroxy FA sphingolipids are synthesized by the same set of enzymes as nonhydroxy sphingolipids, except for fatty acid 2-hydroxylase (FA2H). The expression of FA2H is highly variable among cell types and is inducible by certain stimuli [48]. One may speculate that, upon unknown signal, 2-HFA-ceramides may be preferentially synthesized instead of NFA-species. Abnormal degradation of HFA sphingolipids may be also possible. It has been shown that galactosylceramidase, which forms 2-HFA-ceramides from Galcer is up-regulated, whereas acid ceramidase which hydrolyzed 2-HFA-ceramides into HFA and sphingosine is down-regulated in human AD brain [21]. Thus, with combinatorial up- and down-regulation of enzymes involved in sphingolipid metabolism, the cell could modify the levels of 2-HFA-ceramides in response to the changing cellular environment. However, this is highly speculative and further investigation is warranted to determine whether these factors or other unknown factors contribute to such changes of the ceramide profile in the APP^{SL} cerebellum. It should be noted that also intriguing is the substantial elevation of ceramide reported by Han et al. [18] in the cerebellum of AD patients; this point also remains to be clarified.

In this study, we also examined the content of other ceramide-related sphingolipid classes including SM, Galcer, and sulfatides. Similarly to what we observed in the $APP^{SL}/PS1Ki$ model, we did not found any changes of SM and galactolipid levels in all brain regions from the two transgenic lines investigated. Similar findings were seen in APPsw and APPV717F transgenic mice, respectively, except for sulfatides [26]. Indeed, by contrast to our results, a loss of sulfatide content was observed in multiple brain regions of these animals. The reasons for such discrepancies are still unclear, but it may be ascribed to the different genetic background of mouse lines and/or the genetic constructs based on different APP mutation and different promoters. Supporting this, it has been shown for example, that APP^{SL} transgenic and wildtype mice on C57BL/6 background have lower basal cholesterol levels than the Ki mouse lines which are on C57BL/6 50%-CBA 25%-129SV 25% background [44]. Another example is the difference of lipid composition reported by Sawamura and coworkers [15] between mouse lines with C57BL/6J and FVB/N backgrounds, respectively. Moreover, these authors found that PS2 transgenic mice with C57BL/6J background displayed significant phospholipid alterations, particularly of SM, as compared to their wild-type controls, while PS2 transgenic mice with FVB/N background did not. These few examples point out the difficulties to compare the results from various mouse lines and reinforce the idea that additional studies in this field are required.

5. Conclusion

In summary, this study extends previous observations on sphingolipid alterations in animal models of AD. Despite several limitations, in particular the lack of old double transgenics, the present results demonstrated that, in the absence of neurodegeneration, no elevation of ceramides occurred in disease-affected brain regions from single APP^{SL} mice, thus corroborating recent findings in two other single APP mice [26]. Moreover, since both neuronal loss and accumulation of toxic N-truncated Aβ peptides are lacking in the APP^{SL} model, this might suggest that Aβ-induced

ceramide production is an important event leading to neuronal death. In the future, to support this hypothesis, it will be interesting to analyse the sphingolipid composition of the TBA2 mice, the new model expressing only N-truncated $A\beta_{3(pE)-42}$ and which develops a severe neuronal loss [40], to evaluate whether or not ceramides, especially the 2-OH species, accumulate in the brain of these mice.

Accumulating and crossing the information obtained from various animal models will help to better understand the exact mechanism by which these sphingolipids contribute to AD pathogenesis.

Abbreviations

AD: Alzheimer's disease
APP: Amyloid precursor protein
2 HFA: 2-hydroxy fatty acid
NFA: Nonhydroxy fatty acid.

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Research Article

Conjugated Quantum Dots Inhibit the Amyloid β (1–42) Fibrillation Process

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Nanoparticles have enormous potential in diagnostic and therapeutic studies. We have demonstrated that the amyloid beta mixed with and conjugated to dihydrolipoic acid- (DHLA) capped CdSe/ZnS quantum dots (QDs) of size approximately 2.5 nm can be used to reduce the fibrillation process. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) were used as tools for analysis of fibrillation. There is a significant change in morphology of fibrils when amyloid β (1–42) (Aβ(1–42)) is mixed or conjugated to the QDs. The length and the width of the fibrils vary under modified conditions. Thioflavin T (ThT) fluorescence supports the decrease in fibril formation in presence of DHLA-capped QDs.

1. Introduction

Nanochemistry is predominating in major fields of science and technology, specifically in biotechnology and information technology. In the near future, nanochemistry will direct and guide towards nanomedicine and nanodiagnostics [1]. However, obtaining suitable nanoparticles that can be used for diagnostic and medicinal purposes remains a significant challenge. Moreover, the effect of these nanoparticles on biological entities such as proteins is considerably significant when it comes to AD.

Oligomeric aggregates Aβ and tau protein or the protofibrils are considered as precursors for amyloid fibrillation in Alzheimer's disease [2]. A few articles have been published on the effect of nanoparticles on fibrillation process. Recently, the effect of fluorinated magnetic core-shell nanoparticles with the size range of 15.0 ± 2.1 nm has been observed on amyloid model protein insulin; these fluorinated nanoparticles show inhibition of insulin fibrils [3, 4]. Furthermore, the effect of various nanoparticles within the dimensions of 6–200 nm on another model protein, β_2 microglobulin, has been investigated [5]. Previously published studies have demonstrated that nanoparticles can act as catalysts for protein fibrillation [1, 5]. Very recently, Li and coworkers have showed an inhibition effect of N-acetyl cysteine-capped CdTe QDs of size of 3–5 nm on Aβ (1–40) fibrillation [6]. In another case, dual effect of commercial polystyrene nanoparticles with amino modification having various sizes (57, 120, and 180 nm) was observed on Aβ (1–40) and recombinant Aβ (1–40) and Aβ (1–42) proteins [7]. Furthermore, there is only one recent publication on the effect of nanoparticles on Aβ (1–42) fibrils. In this case, it was observed an increase in rate of amyloid fibrillation in presence of TiO_2 nanoparticles with size of approximately 20 nm [8].

The interaction of nanoparticles with different proteins depends upon various factors such as surface coating of nanoparticles with ligands, surface properties, size, and composition of nanoparticles [1, 5]. From the previous studies, [1, 3–8] we cannot generalize the concept that different nanoparticles can promote or inhibit the fibril formation for various amyloid model proteins. Specifically, the only investigation that explains the effect of TiO_2 on Aβ (1–42) shows that nanoparticles promote the fibrillation process by becoming nucleation centers [8].

We report for the first time in our knowledge that CdSe/ZnS QDs of size of 2.5 ± 1.3 nm can inhibit fibrillation of Aβ (1–42). In the present study, we have investigated the effect of the presence of DHLA-capped CdSe/ZnS QDs either mixed with or conjugated to Aβ (1–42) on fibrillation process of Aβ (1–42) in aqueous phase. TEM and AFM studies show that the QDs behave uniquely when they are conjugated to Aβ (1–42) in comparison to a mixed sample of Aβ (1–42) and QDs. Our study illustrates a considerable difference in morphology of the fibrils and the inhibition of fibrillation process when Aβ (1–42) is conjugated to QDs versus the mixed system Aβ (1–42) and QDs. These results are further supported by Thioflavin T (ThT) assay using fluorescence spectroscopy.

2. Experimental Section

2.1. Materials and Methods. All Chemicals were commercially purchased and used without further purification. Cadmium oxide (CdO), selenium (Se), trioctylphosphine oxide (TOPO), trioctylphosphine (TOP), and hexamethyldisilathiane [$(TMS)_2S$] were purchased from Sigma-Aldrich (Milwaukee, WI). The tetradecylphosphonic acid (TDPA) was obtained from Alfa Aesar (Ward Hill, MA). The diethylzinc ($ZnEt_2$, 15 wt% solution in hexane) was obtained from Acros Organics (MorrisPlains, New Jersey). DL-α-lipoic acid, Aβ (1–42), and ThT were purchased from MP Biomedicals (Solon, OH).

2.2. Synthesis of DHLA-Capped QDs. CdSe/ZnS QDs were synthesized using an already given protocol [9]. Briefly, cadmium oxide was reacted with a selenium reagent in the presence of a phosphine oxide surfactant at high temperature under argon flow. After the formation of the CdSe core, the diethyl zinc and hexamethyldisilathiane in TOP was added dropwise at 130 C. After the synthesis of TOPO-capped hydrophobic QDs, modification to hydrophilic DHLA-capped QDs was carried out [10]. Briefly, first DL-α-lipoic acid (1 g) was reduced using sodium borohydride (2 g) in methanol/water (v/v, 1:1) solution. After workup product was isolated in chloroform and characterized using ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.3 (*d*, 1H), 1.35 (*t*, 1H), 1.4–1.8 (*m*, 6H), 1.9 (*m*, 2H), 2.4 (*t*, 2H), 2.6–2.8 (*m*, 2H), 2.9 (*m*, 1H), and 11 (*s*, 1H).

DHLA was used for ligand exchange with TOPO; excess of DHLA (0.5 g) was added in 5 mL of TOPO-capped QDs in methanol and heated at 60 C–70 C for 4 h. Once a homogeneous QDs solution was obtained, solution was basified using potassium tert-butoxide and centrifuged to get the precipitates. The precipitates were suspended in water to obtain the hydrophilic QDs. The water-soluble QDs were filtered through 0.2 μm filter to get a clear solution.

2.3. Preparation of Aβ (1–42) Mixed and Conjugated with DHLA-Capped QDs. Aβ (1–42) was chemically conjugated to QDs by the formation of an amide bond between Asp-NH_2 end of the polypeptide chain and the –COOH end of the DHLA ligand using the protocol to conjugate proteins [11]. Freshly prepared DHLA-capped QDs (1.51×10^{-5} M, 100 μL) were taken in a clean borosilicate glass vial, and 500 μL PBS buffer (pH 7.4) was added to the QDs. To lyophilize the peptide, 0.5 mg Aβ (1–42) was dissolved in hexafluoroisopropanol (HFIP) to bring the peptide in monomer form and evaporated under gentle flow of N_2. The dried protein was then dissolved in 500 μL PBS buffer (pH 7.4) to get the final concentration of 1 mg/mL. Freshly prepared Aβ (1–42) solution was then mixed in QDs solution. 57 μL of freshly prepared 10 mg/mL EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) solution in deionized water was then added to the mixture of peptide and QDs, total volume of solution prepared was 1157 μL. The solution was stirred for 4 h at the speed of 200 rpm. There was no precipitate observed after the reaction. Aβ (1–42) mixed with DHLA-capped QDs was prepared according to the above-mentioned protocol except for the addition of EDC. Total volume of the solution was kept at 1157 μL. For the induction of fibrillation the solutions were incubated at 37 C.

2.4. Preparation of Pure Aβ (1–42) Sample for Fibrillation. Pure Aβ (1–42) solution was prepared similarly, first by lyophilizing the peptide in HFIP and evaporating the solvent under gentle nitrogen flow and then redissolving the dried peptide in 1157 μL of PBS buffer (pH 7.4). The solution was then incubated at 37 C to induce the fibril formation.

2.5. Gel Electrophoresis. Electrophoresis of QDs was performed using a MiniCuve 8.10 Electrophoresis Unit (MP Biomedicals, Solon, OH). Hand cast gels were composed of 1% agarose in 1× TBE (0.089 M Tris base, 0.089 M boric acid, and 0.002 M ethylenediaminetetraaceticacid, pH 8.3). 10 μL of each sample was loaded into wells on the agarose gel using a micropipet. The samples were run in 1× TBE buffer on the 1% agarose gel at 84 V for 75 min. For visualization, the gel was placed on a UV transilluminator, and an image was captured with a Gel Doc XR system (Bio-Rad, Hercules, CA).

2.6. TEM Measurements. TEM measurements were performed at the Center of Advanced Microscopy, Scripps Research Institute (La Jolla, CA) and at the Center of Advanced Microscopy, (CMA), Trinity College Dublin (Ireland). For the images containing amyloid, negative staining was performed using 2% uranyl acetate. Briefly, copper grids (carbon and Formvar coated 400 mesh: Electron Microscopy Sciences, Hatfield, PA) were glow discharged and inverted on an 5 μL aliquot of sample for 3 min. Excess sample

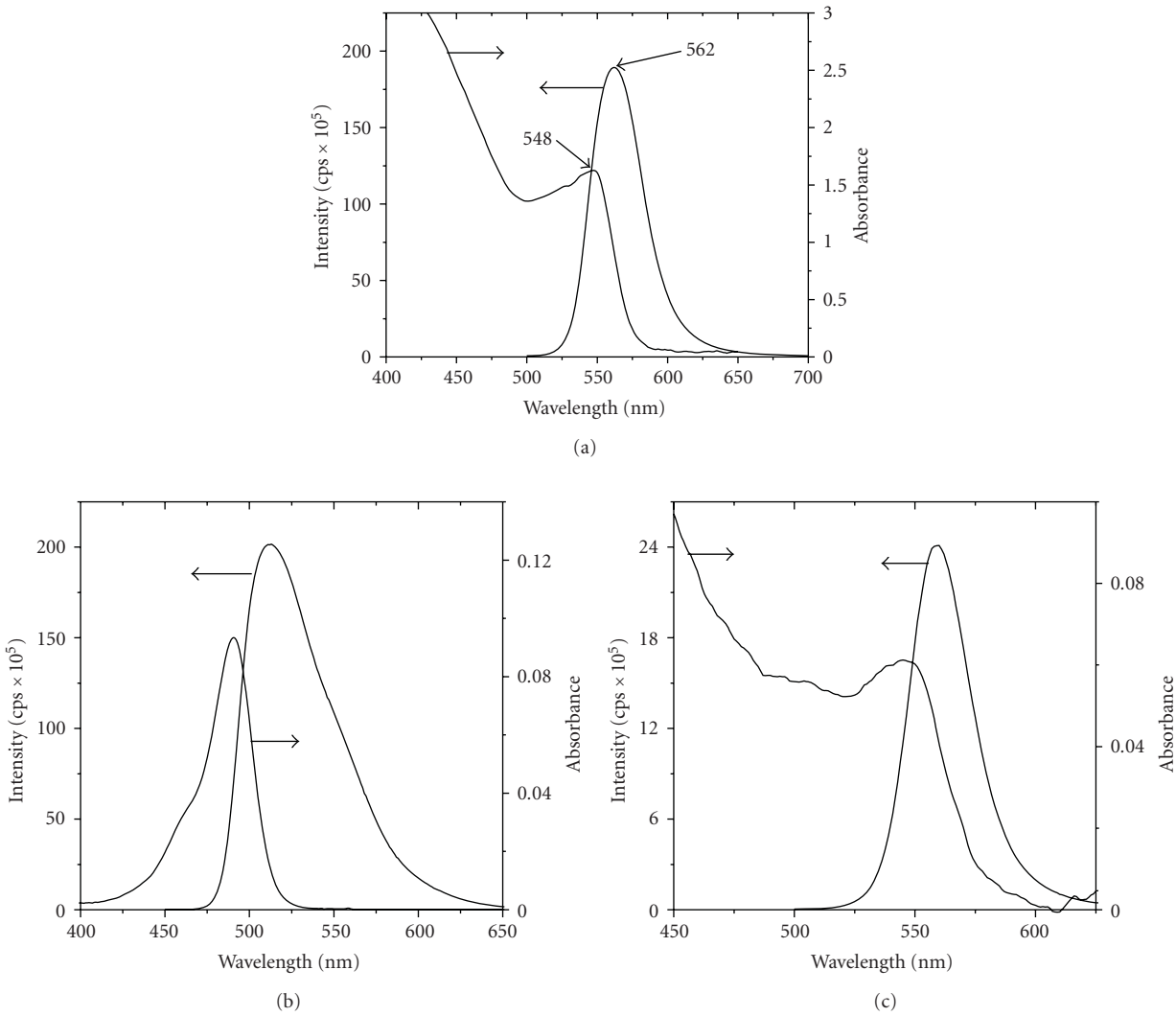


Figure 1: (a) Absorption and emission spectra of DHLA-capped QDs. Fluorescence spectrum was obtained using a quartz cell with an optical path length of 1 cm, excitation wavelength at 467 nm, and 2, 2 nm slit width at the excitation and emission, respectively; (b) UV-vis and fluorescence spectra for fluorescein; (c) DHLA-capped QDs used for quantum yield measurements.

was removed and the grids immediately placed briefly on a droplet of double-distilled water. Grids requiring the negative stain were then placed on droplets of 2% uranyl acetate solution for 2 min. Excess stain was removed and the grid was allowed to dry thoroughly. For unstained grids, the excess water was removed, and the dried grids were examined on a Philips CM100 electron microscope (FEI, Hillsbrough, OR) at 80 kV and images collected using a Megaview III CCD camera (Olympus Soft Imaging Solutions, Lakewood, CO). Grids at Ireland were examined on a Jeol 2100 electron microscope (Zeiss) operating at 200 kV and images collected using a CCD camera. Analysis of TEM images was performed using Image J software from NIH (http://rsbweb.nih.gov/ij/).

2.7. AFM Measurements. Briefly, 4 μL aliquots of Aβ solutions were deposited on freshly cleaned and dried silicon wafers (approximately 1 mm thick). After waiting for 10 min, nonadsorbed portions of the samples were washed with deionized water (400 μL). The wet surface of the silicon wafer was then dried using gentle flow of air. The samples were analyzed by atomic force microscopy (AFM, βA multimode SPM, Model no. 920-006-101, Veeco, Fremont, CA). Tapping mode approach was used to acquire the images, which allows intermittent contact of the tip with the sample and minimizes the chances of deformation of the peptide samples. The cantilever and the tip were made up of silicon. The cantilever force constant was approximately 20–100 N/m with the resonance frequency between 200 and 400 kHz. The scan rate was between 1.0 and 1.2 Hz. The software used for the analysis of fibrils was the NanoScope Control, version 5.30 and the histogram analysis was performed using the postanalysis pico image software (pico view version 1.6.2).

2.8. UV-Vis and Fluorescence Spectroscopy. UV-vis spectra of solutions were recorded on a Perkin-Elmer Lambda 900 UV/vis/NIR spectrometer (Norwalk, CT). Fluorescence

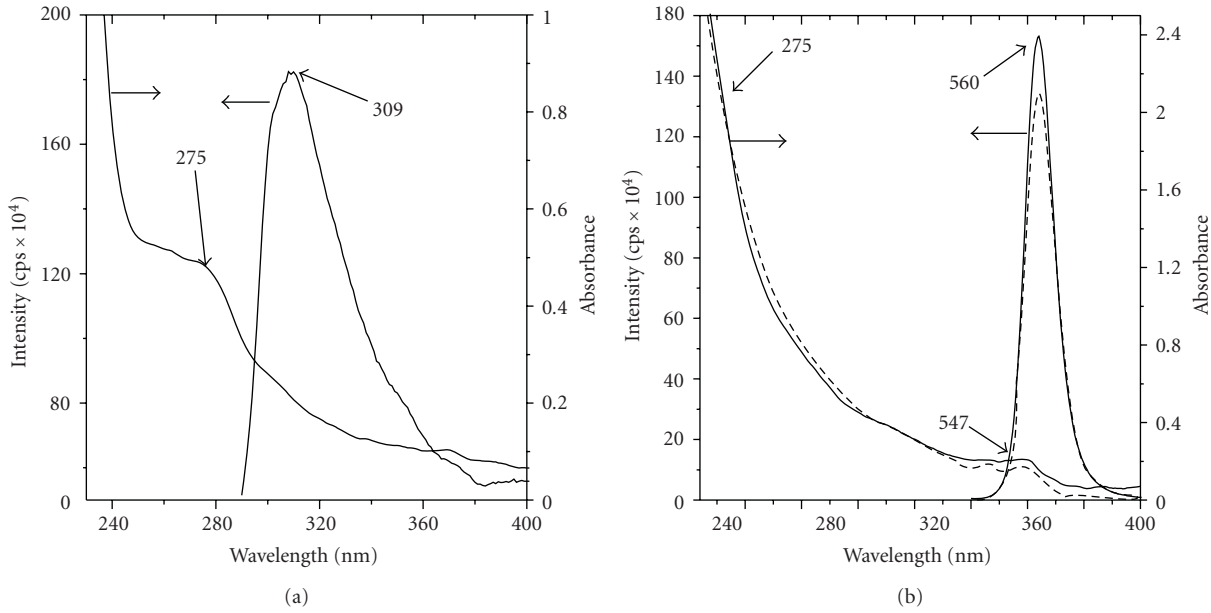


Figure 2: (a) UV-vis and fluorescence spectra of Aβ (1–42). Fluorescence spectrum was obtained using a quartz cell with an optical path length of 1 cm, excitation wavelength at 280 nm, and 5, 5 nm slit width at the excitation and emission, respectively; (b) UV-vis and fluorescence spectra for Aβ (1–42) mixed (—) and conjugated (- - -) to QDs. Fluorescence spectrum was obtained using a quartz cell with an optical path length of 1 cm, excitation wavelength at 467 nm, and 2, 2 nm slit width at the excitation and emission, respectively.

spectra were obtained using Spex FluoroLog Fluorospectrometer (Horiba Jobin Yvon, Edison, NJ). Both UV-vis and fluorescence measurements were obtained using quartz cuvette with1 cm optical path length.

2.9. ThT Fluorescence. 10 μM solution of ThT was prepared in 1xPBS buffer at pH 7.4. 30 μL of Aβ (1–42) aliquots were extracted at different time periods, and 300 μL of ThT (10 μM) was added to the samples. The ThT fluorescence was measured at 482 nm at an excitation wavelength of 440 nm in a semimicro quartz cuvette of an optical pathlength of 1 cm. Slit widths at excitation and emission were set at 5 nm.

3. Results and Discussion

3.1. Characterization of QDs and QDs Mixed and Conjugated with Aβ (1–42). Characterization of DHLA-capped QDs was carried out using UV-vis and fluorescence spectroscopy (Figure 1(a)). Quantum yield (QY) has been calculated for the QDs investigated. The fluorescein (QY is 0.94 in 0.1 M NaOH) was employed as reference. The QY was calculated by using the following equation:

$$\mathrm{QY}_q = \frac{\mathrm{QY}_f \left[A_f n_q^2 \int I_q(\lambda) d\lambda \right]}{\left[A_q n_f^2 \int I_f(\lambda) d\lambda \right]}. \quad (1)$$

A is the absorbance at the excitation wavelength, n is the refractive index of the solvent used, I is the emission wavelength-dependent emission intensity, and λ is the emission wavelength. Subscripts q and f represent the QDs and the fluorescein, respectively. QY was calculated from the intensity of luminescence and the absorbance in Figures 1(b) and 1(c) for fluorescein (0.1 M NaOH as solvent) and QDs (water as solvent), respectively. The QY of the DHLA-capped CdSe/ZnS QDs was around 25%.

3.1.1. Estimation of Aβ/QDs Ratio. To characterize the QDs conjugated to Aβ (1–42), it is important to estimate the number of Aβ monomers bound to QDs after conjugation. Firstly, characterization of samples of pure Aβ (1–42), Aβ (1–42) mixed and conjugated to QDs was performed using UV-vis and fluorescence spectroscopy (Figure 2). The emission band for the tyrosine moiety in Aβ (1–42) was observed at 309 nm at the excitation wavelength of 280 nm, slit width at emission and excitation was set at 5 nm. The absorption band for Aβ (1–42) was observed at 275 nm (Figure 2(a)), whereas the emission band for the Aβ (1–42) mixed and conjugated to QDs was observed at 560 nm at the excitation wavelength of 467 nm. The absorption band for the QDs was observed at 547 nm and for the Aβ (1–42) a little hump was observed at 275 nm as shown in Figure 2(b). Molar concentration of the QDs and Aβ (1–42) was calculated from UV-vis spectrum of the solution [12, 13], the optical path length used was 1 cm. The ratio of these concentration values gave the average number of Aβ per quantum dot nanoparticles. The molar extinction coefficient of QDs at 547 nm is $105.8 \times 10^3\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ and at 275 nm is $2.6 \times 10^3\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$. Extinction coefficient for Aβ (1–42) [14] at 275 nm is $1.4 \times 10^3\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$. The calculations to determine the ratio of Aβ (1–42) and QDs are shown below.

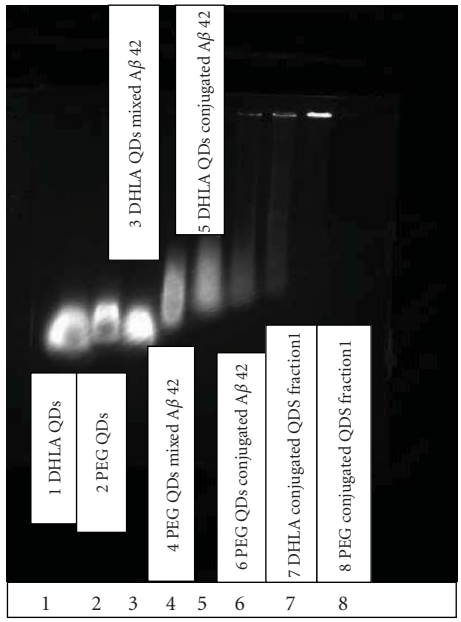


Figure 3: Gel electrophoresis image of DHLA-capped QDs (1) in comparison to DHLA-capped QDs mixed (3) and conjugated (5) to Aβ (1–42). PEG-capped QDs (2) in comparison to PEG-capped mixed (4) and conjugated (6) to Aβ (1–42). The purified fractions using gel chromatography for DHLA- and PEG-capped QDs conjugated to Aβ (1–42) are shown in wells 7 and 8, respectively.

3.1.2. Aβ Conjugated to DHLA-Capped QDs. QDs concentration (conjugated sample) is

$$\frac{\text{Abs}_{547}}{\varepsilon_{547} l} = \frac{0.156}{1.05 \times 10^5\,\text{M}^{-1}\,\text{cm}^{-1} \times 1\,\text{cm}} = 1.5 \times 10^{-6}\,\text{M}. \quad (2)$$

Absorption at 275 nm from QDs is

$$\text{Abs}_{547} \times \frac{\varepsilon_{275}}{\varepsilon_{547}} = 0.156 \times \frac{2637.2}{1.05 \times 10^5} = 3.9 \times 10^{-3}. \quad (3)$$

Absorption at 275 nm from Aβ (1–42) is

$$\text{Abs}_{275} - \left[\text{Abs}_{547} \times \frac{\varepsilon_{275}}{\varepsilon_{547}}\right] = 2.2 - 3.9 \times 10^{-3} = 2.2. \quad (4)$$

Aβ (1–42) concentration in the sample conjugated to QDs is

$$\frac{\text{Abs for A}\beta \text{ at } 275}{\varepsilon \text{ for A}\beta \text{ at } 275 \times l} = \frac{2.2}{1390\,\text{M}^{-1}\text{cm}^{-1} \times 1\,\text{cm}} = 1.6 \times 10^{-3}\,\text{M},$$

$$\text{Loading} = \frac{\text{A}\beta \text{ concentration}}{\text{QDs concentration}} = \frac{1.6 \times 10^{-3}\,\text{M}}{1.5 \times 10^{-6}\,\text{M}} = 1.1 \times 10^3. \quad (5)$$

Theoretically, the total number of molecules/particle of Aβ (1–42) (molecular weight 4514.1 g) present in 0.5 mg of the 1157 μL of sample can be calculated by multiplying the number of moles with N_A (Avogadro's number) number of molecules. Hence, the total number of molecules of Aβ (1–42) present in the sample is 5.8×10^{21}. Similarly, the total number of QDs particles present in the solution is 9.0×10^{17}. Therefore, the ratio of Aβ (1–42) molecules per QD particle is 6400. Subtracting the experimental value (1100) from the theoretical value (6400), it can be inferred that there are 5300 molecules of Aβ (1–42) that are free in the solution per QD particle that is conjugated to Aβ (1–42).

3.1.3. Gel Electrophoresis. To confirm that the QDs were indeed conjugated to Aβ (1–42), we have used the agarose gel electrophoresis for the control DHLA-capped CdSe/ZnS QDs along with Aβ (1–42) mixed and conjugated to the QDs (Figure 3). The gel was run in TAE buffer (pH 7.4) at 84 V for 75 min, and the volume of the samples in each well was 10 μL. Figure 3 shows the distance moved by three different samples: DHLA-capped QDs (1), Aβ (1–42) mixed with DHLA-capped QDs (3), and Aβ (1–42) conjugated with DHLA-capped QDs (5). From the gel electrophoresis, we can clearly distinguish that the distance moved by the QDs mixed with Aβ (1–42) is the same as for the pure DHLA-capped QDs. Whereas when the QDs are conjugated to Aβ (1–42), the distance moved is lower. This shows that when the QDs are chemically conjugated to Aβ (1–42), the distance moved is reduced due to the higher molecular weight. Moreover, comparison of polyethylene glycol (PEG)-capped QDs (2), Aβ (1–42) mixed PEG-capped QDs (4), and Aβ (1–42) conjugated PEG-capped QDs (6) shows that PEG-capped QDs when conjugated travel the least distance. Purified fractions of DHLA- (7) and PEG-capped (8) QDs conjugated to peptide show that free Aβ (1–42) can be separated from the Aβ (1–42) conjugated QDs. Different fractions of 1 mL each obtained from gel chromatography were checked for the presence of free Aβ (1–42) using UV-vis absorption and fluorescence spectroscopy.

4. Imaging and Analysis

4.1. TEM. First evidence on the inhibition in fibrillation comes from TEM images (Figure 4) taken on the 7th day of incubation at 37 C. TEM images containing Aβ (1–42) are negatively stained using 2% uranyl acetate solution. Three different samples, namely, pure Aβ (1–42), Aβ (1–42) mixed with DHLA-capped CdSe/ZnS QDs, and Aβ (1–42) conjugated to DHLA-capped CdSe/ZnS QDs, are shown in Figures 4(a), 4(c), and 4(d), respectively. In all the three samples, the concentration of Aβ (1–42) is 0.96×10^{-4} M and the concentration of QDs is 1.4 μM. Figure 4(a) shows a TEM image of pure Aβ (1–42). Analysis of this image indicates that the length of the fibrils varies from 30 to 1730 nm. The width of the shorter fibrils is 4.0 ± 0.7 nm whereas for the longer fibrils it is 7.5 ± 0.5 nm. Figure 4(b) presents the pure DHLA-capped CdSe/ZnS QDs, the average particle size is 2.5 ± 1.3 nm (Figure 5(a)). The size of QDs is an important parameter for the biodiagnostic studies, since smaller size QDs are capable of passing through the blood-brain barrier

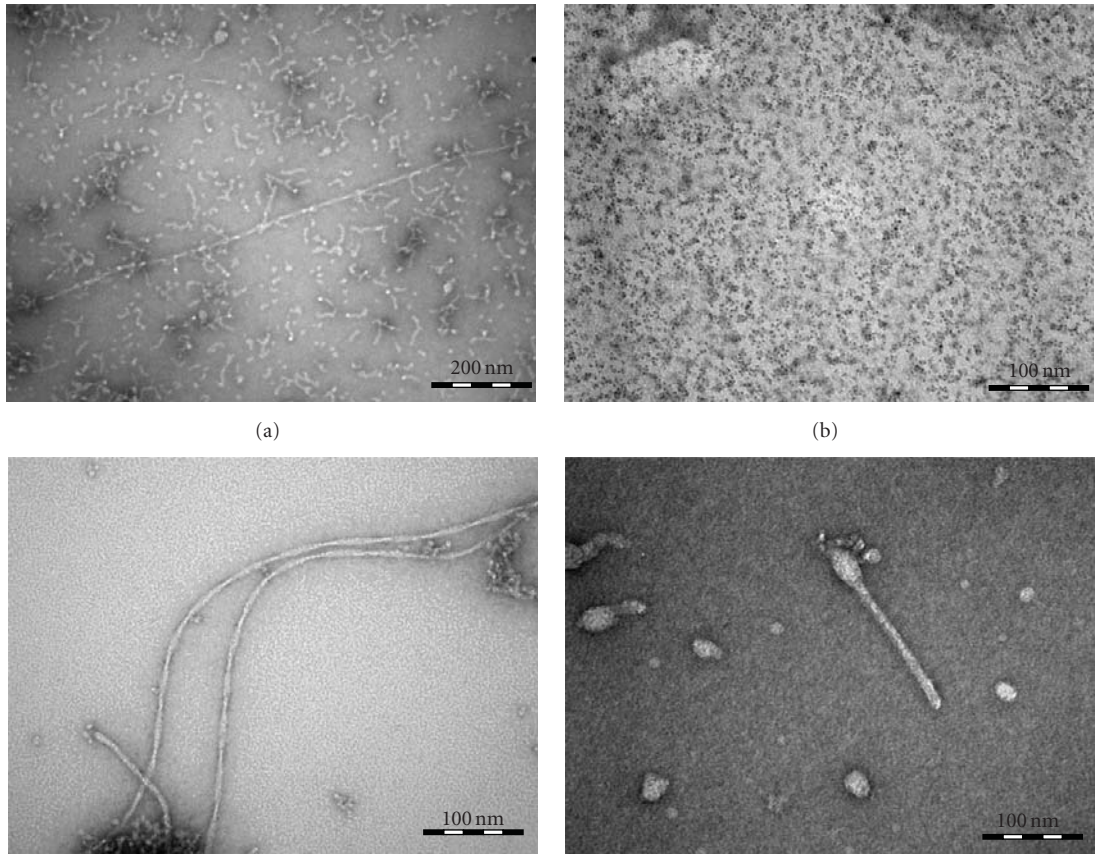


Figure 4: TEM images: (a) Aβ (1–42) in PBS buffer (pH 7.4); (b) DHLA-capped QDs; (c) Aβ (1–42) mixed with DHLA-capped QDs in PBS buffer (pH 7.4); (d) Aβ (1–42) conjugated to DHLA-capped QDs in PBS buffer (pH 7.4).

[15, 16]. Figure 4(c) shows the incubated sample of Aβ (1–42) in the presence of QDs whereas Figure 4(d) shows Aβ (1–42) conjugated QDs. Comparison of the results in the three images containing Aβ (1–42) illustrates very interesting pattern. Sample containing pure Aβ (1–42), Figure 4(a), has a large number of fibrils ranging from short fibrils around 30 nm to long fibrils around 2 micron whereas images of samples in presence of QDs are very different. Figure 4(c) is the image of Aβ (1–42) mixed with QDs; it shows long-length fibrils of around 1 micron. The short-length fibrils are not very significant in this case. The width of the fibrils for the sample having mixed QDs is 7.7 ± 0.7 nm. Figure 4(d) for Aβ (1–42) conjugated to QDs shows short-length fibrils ranging from 30 to 80 nm. For this sample, the thickness or width of the fibrils is 10 ± 3 nm. The variation in the thickness of fibrils is significant in this case as compared to the other two. One can see a distinguishable inhibition of the fibrillation when Aβ (1–42) is conjugated to QDs.

Histogram showing the size of DHLA-capped QDs is shown in Figure 5(a). The average size analyzed from the histogram obtained from TEM images is 2.5 ± 1.3 nm. Furthermore, to consolidate the results, we have performed the statistical analysis on the TEM images (13–18 images per sample). The number of short-length fibrils (80–150 nm) observed in the sample containing pure Aβ (1–42) was extremely high as compared to samples containing QDs. Statistical analysis showed (Figure 5(b)) that the number of fibrils having length 50–100 nm dropped to 90% in the case of Aβ (1–42) mixed to QDs. There were only 26% of short-length fibrils observed for Aβ (1–42) conjugated to QDs. The total number of fibrils in the samples containing QDs mixed or conjugated to Aβ (1–42) was similar (35 fibrils approximately). These results confirm that elongation of fibrils is inhibited by the presence of the QDs. Figures 6(a) and 6(b) show the TEM images of unstained samples of Aβ (1–42) mixed with QDs. Contrary to the TEM images of stained samples where we can observe only fibrils and no QDs, in this case, we were able to observe the QDs, as well as fibrils. It could be discerned from the results that QDs are enveloping the fibrils and more number of QDs are observed at the ends of the fibrils. For the samples containing Aβ (1–42) conjugated to QDs (Figures 6(c) and 6(d)), we could observe less number of QDs and short-length fibrils. The QDs observed in this case are segregated at one end of the fibrils as seen in Figure 6(d). It could be infered from the results obtained using unstained samples (Figure 6) that QDs envelop the fibrils and could block the ends to elongate. Importance of the C terminus of the Aβ (1–42) in controlling

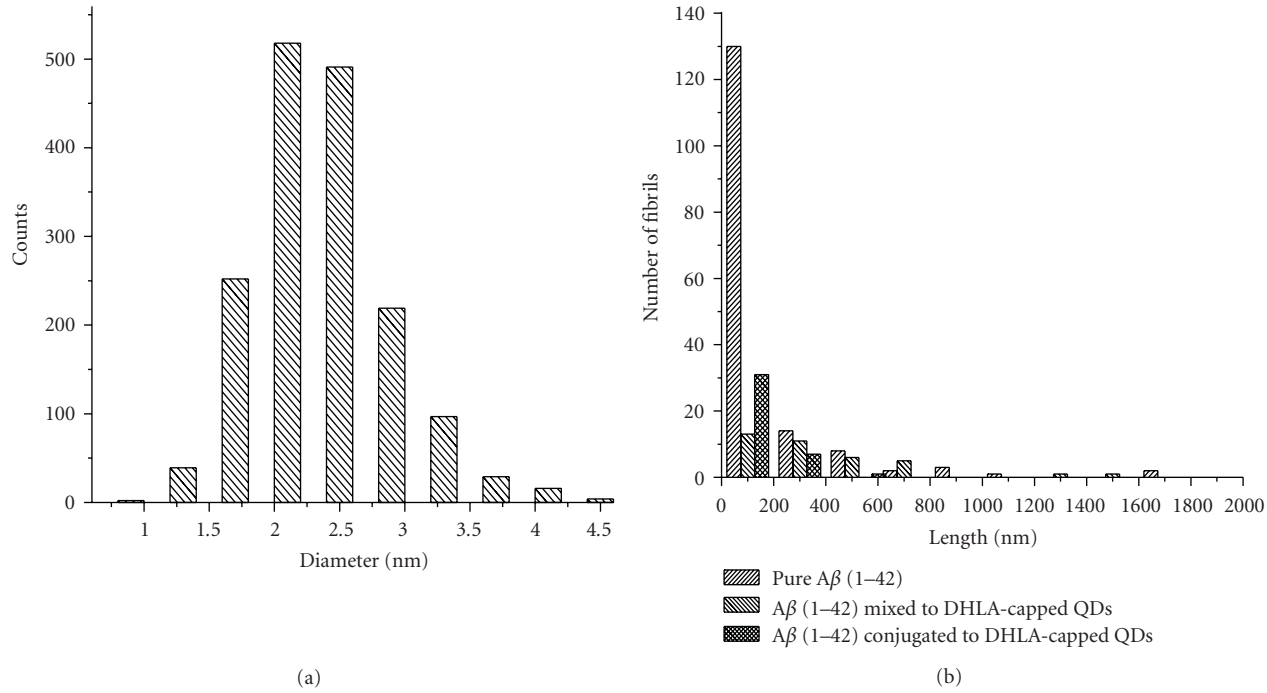


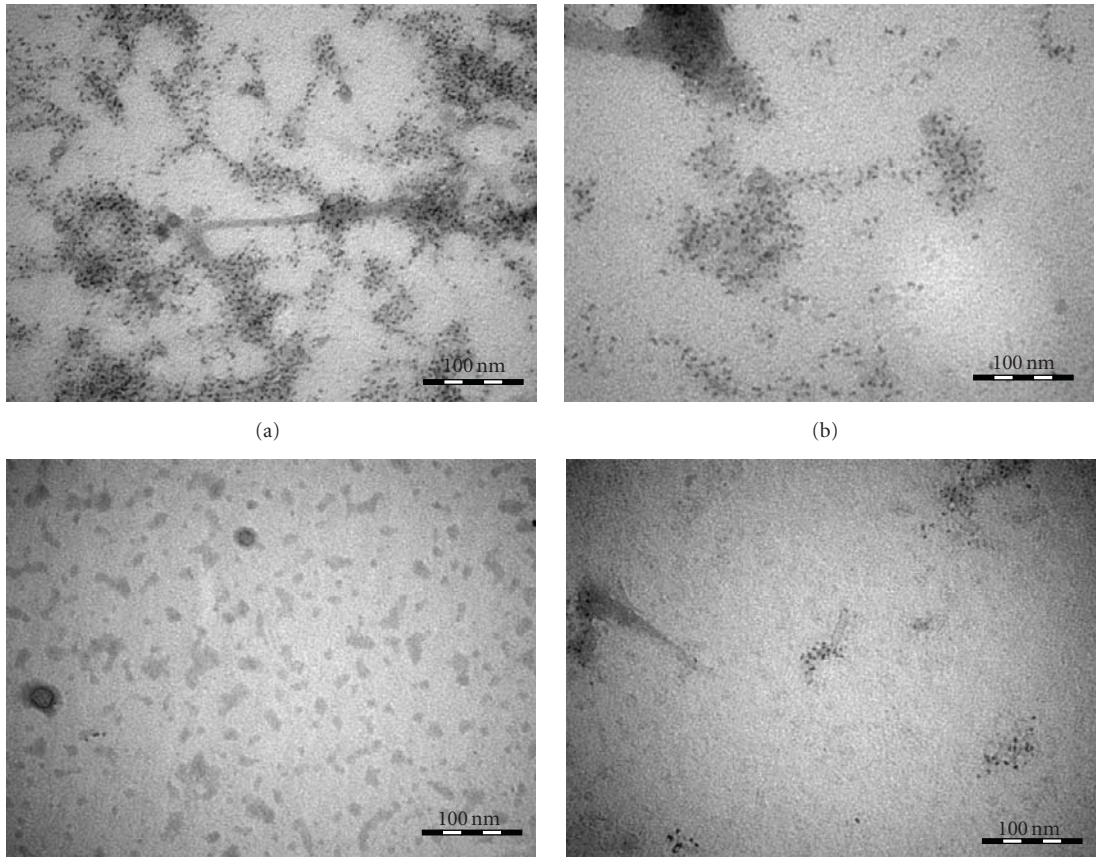
Figure 5: (a) Histogram for the size distribution of DHLA-capped CdSe/ZnS QDs; (b) statistical analysis of number of fibrils versus length of fibrils for three different samples containing Aβ (1–42) mixed to DHLA-capped QDs, Aβ (1–42) conjugated to DHLA-capped QDs, and pure Aβ (1–42).

the self-assembly of fibrillation was revealed before [17]. The reason for the inhibition of fibrillation in presence of QDs could be that the small size of particles could block the C-terminal end of the fibrils (10 nm) or the protofibrils (5 nm in diameter), which is considered as the terminus with lower degree of freedom and accessibility for elongation mechanism [18]. Furthermore, binding between the QDs and the Aβ (1–42) could block the active sites leading to low local protein concentration, hence increasing the lag time for nucleation or disrupting the nucleation process leading to inhibition of the fibrillation process [19]. Besides, presence of Aβ (1–42) conjugated to QDs in the sample containing free Aβ (1–42) may perturb the nucleation mechanism with decrease in localised concentration of the Aβ (1–42) as thereby inhibiting fibrillation process [19, 20]. However, mixed sample of Aβ (1–42) and the QDs might increase the localised concentration of the polypeptide, thereby increasing the length of fibrils but the number of fibrils still remains low suggesting that there is a perturbation in the mechanism of the fibrillation process [20].

4.2. AFM. The TEM results are supported by the AFM images as shown in Figure 7 for the 7th day of incubation at 37 C. Analysis of AFM image for Aβ (1–42) in absence of QDs (Figure 7(a)) shows that the length of the longest fibril is 523 nm and the shortest fibril is 30 nm. A bigger number of short length fibrils (30–80 nm) are observed as witnessed by the TEM images whereas in the sample containing Aβ (1–42) mixed to QDs (Figure 7(c)) the length of the longest fibrils is 849 nm, comparable to the length of fibrils (1 μm) found in the TEM images. However, when we compare the length of fibrils (Figure 7(d)) in the sample containing Aβ (1–42) conjugated to QDs, very few long fibrils were observed, which correspond to the TEM images of the same sample. The length of the longest fibrils found is 468 nm and the shortest fibril is 58 nm, while in the case of the TEM images the length of fibrils is between 30 and 80 nm. The height analysis of QDs (Figure 7(b)) shows that the root mean square height of QDs is 2.3 nm, which is comparable to the average height of QDs found in TEM images (2.5 ± 1.3 nm).

The measurement of Z-height of the Aβ aggregates shows that when Aβ (1–42) is conjugated to QDs the height distribution histogram changes significantly. It is known for the Aβ oligomers that the average height is between 3 and 5 nm, and for the fibrils it varies from 3 to 9 nm [21, 22]. From the height distribution curves (Figure 8) one can see that in the case of amyloid fibrils in absence of QDs 40% of aggregates have height greater than 8 nm whereas 60% of aggregates have height between 6 and 8 nm. For the sample of Aβ (1–42) mixed with QDs, 30% of the aggregates have height greater than 7 nm, and 70% of the aggregates have height between 1 and 3 nm. Interestingly, when we analyze the height distribution for Aβ (1–42) conjugated to QDs only 14% of the aggregates have height greater than 7 nm. Almost 90% of the aggregates have height between 2 and 4 nm.

To further examine the fibrillation process and to support the image analysis, we have performed the ThT assay for the three incubated samples. It is known that the fluorescence intensity of the ThT dye grows with increasing concentration of fibrils. It has to be pointed out that previous



(a) (b)

(c) (d)

Figure 6: TEM images of the unstained samples of Aβ (1–42) mixed to DHLA-capped QDs (a), (b) and Aβ (1–42) conjugated to DHLA-capped QDs (c), (d).

studies confirmed that the fluorescence enhancement of ThT depends upon the structure of the aggregated state of the amyloid peptides [23, 24]. Figure 9(a) shows the ThT assay on the 7th day for the samples incubated at 37 C. When the pure solution of 10 μM ThT in PBS buffer (pH 7.4) is excited at 440 nm, the emission band at 482 nm is observed with a very low intensity. In the presence of amyloid fibrils, the ThT emission band at 482 nm is enhanced significantly. For the sample containing QDs mixed to the fibrils, the intensity of the emission band at 482 nm is decreased by 66% as compared to the band for pure amyloid fibrils. Whereas the amyloid fibrils, conjugated to QDs show a decrease in intensity for the emission band by 40% as compared to the QDs mixed to the fibrils. Time course of fibrillation process using ThT can be seen in Figure 9(b). The variation of ThT intensity yields information regarding the extent of fibrillation. For the sample containing pure Aβ (1–42), a sigmoidal curve is observed, lag phase is between 0 and 24 h, and rapid progress in fibrillation is observed after 50 h of incubation. However, for the sample containing DHLA-capped QDs mixed to Aβ (1–42), lag time is increased to 48 h and it can be observed with decrease in intensity of fluorescence that the QDs are inhibiting the fibril formation. Similarly, in the case of sample containing Aβ (1–42) conjugated to the DHLA-capped QDs, a decrease in intensity of fluorescence and completion of saturation in fibrillation are observed at 72 h. These results show, in the presence of QDs, that the self-assembly of Aβ (1–42) is perturbed.

A remarkable diminution in fibrillation process in the presence of QDs and a significant change in morphology are observed. Contrary to the results that have been published previously on the nanoparticles such as TiO_2, copolymer particles, cerium oxide, QDs, and carbon nanotubes behaving as catalyst for fibrillation [5, 8], we did not observe the same behavior for the DHLA-capped CdSe/ZnS QDs. Moreover, our results are in line with a very recent publication showing the inhibition of Aβ (1–40) by CdTe nanoparticles which have similar diameter size (3–5 nm) [6]. Major difference from other set of nanoparticles being used could be the composition and the size of the particles. The size range for the particles that have been used for the previous studies varies from 16 to 200 nm whereas the QDs used in our work have an average size of 2.5 ± 1.3 nm.

To investigate the effect on the tyrosine residue, which is an intrinsic probe of Aβ (1–42), we have examined the tyrosine fluorescence spectra for the three samples on the 7th day of incubation at 37 C. There is a notable quenching

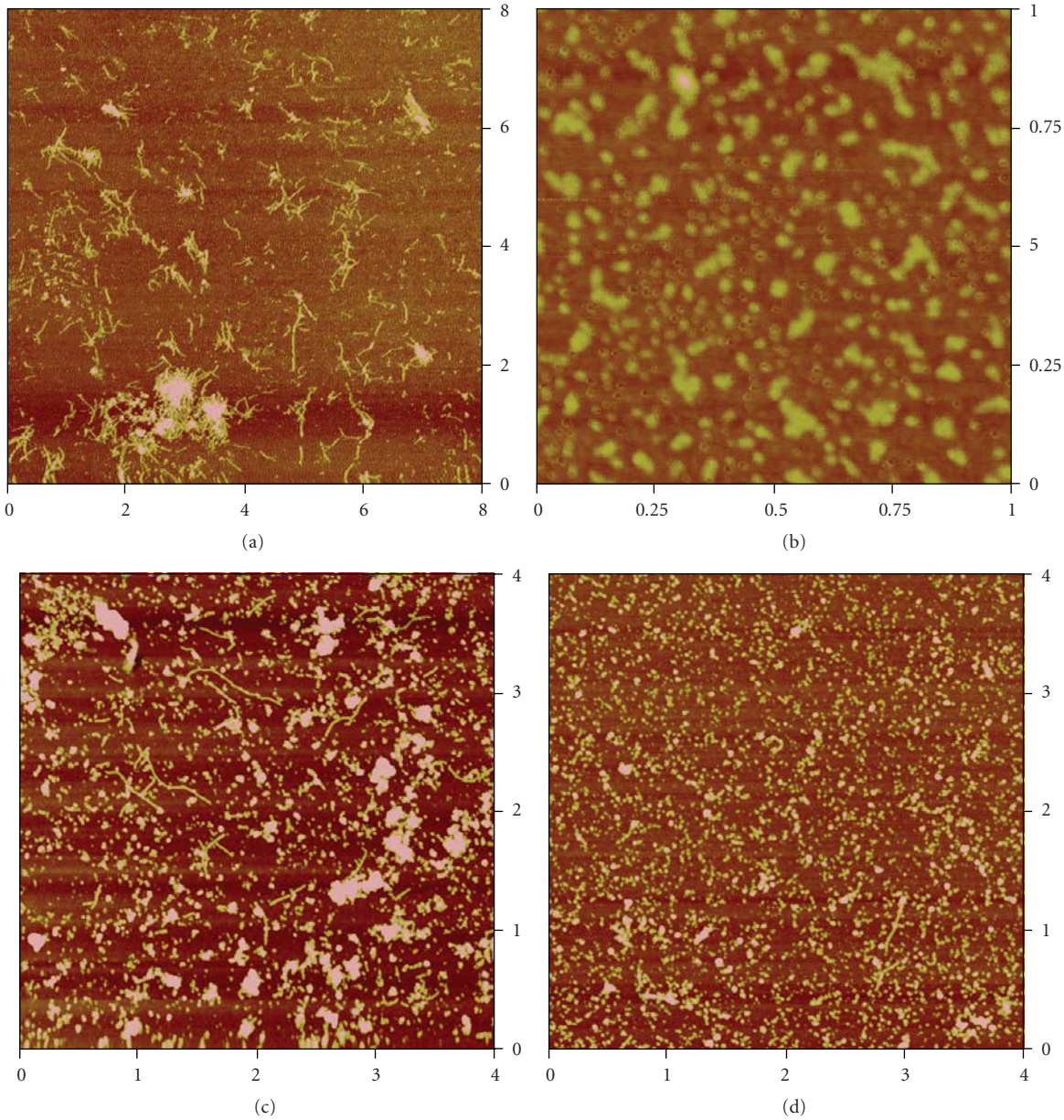


Figure 7: AFM images: (a) Aβ (1–42) in PBS buffer (pH 7.4), 8 μm × 8 μm; (b) DHLA-capped QDs, 1 μm × 1 μm; (c) Aβ (1–42) mixed with DHLA-capped QDs in PBS buffer (pH 7.4), 4 μm × 4 μm; (d) Aβ (1–42) conjugated to DHLA-capped QDs in PBS buffer (pH 7.4), 4 μm × 4 μm.

of the tyrosine fluorescence intensity at 309 nm (Figure 9(c)) in the presence of mixed or conjugated QDs. This effect could be due to the fact that the tyrosine moiety (Tyr^{10}) interacts with the QDs. For example, the three histidine residues (His^6, His^{13}, and His^{14}) in the vicinity of the tyrosine may interact or form coordination bond with the surface of QDs [25]. This phenomenon happens due to the presence of overcoated ZnS shell offering Zn ions [26], hence rendering tyrosine to interact with QDs and consequently decreasing significantly the fluorescence intensity of tyrosine band. Another explanation could be the FRET mechanism between the donor (tyrosine moiety) and the acceptor (QDs), since there is an overlap between the absorption spectrum of the acceptor (QDs) and the emission spectrum of the donor (tyrosine). FRET efficiency in case of Aβ (1–42) conjugated to QDs was 0.84 whereas for Aβ (1–42) mixed with QDs was 0.94. It could be interpreted that the Forster distance (R_o) between the Aβ (1–42) and QDs in aqueous solution was less than 60 Å which is the critical distance for energy transfer [27]. It means that in both the samples, Aβ (1–42) mixed with or conjugated to QDs, Aβ (1–42) is present very near to the QDs. This is an indirect evidence that QDs are present very near to fibrils; that is also observed in the TEM images of the unstained samples (Figure 6).

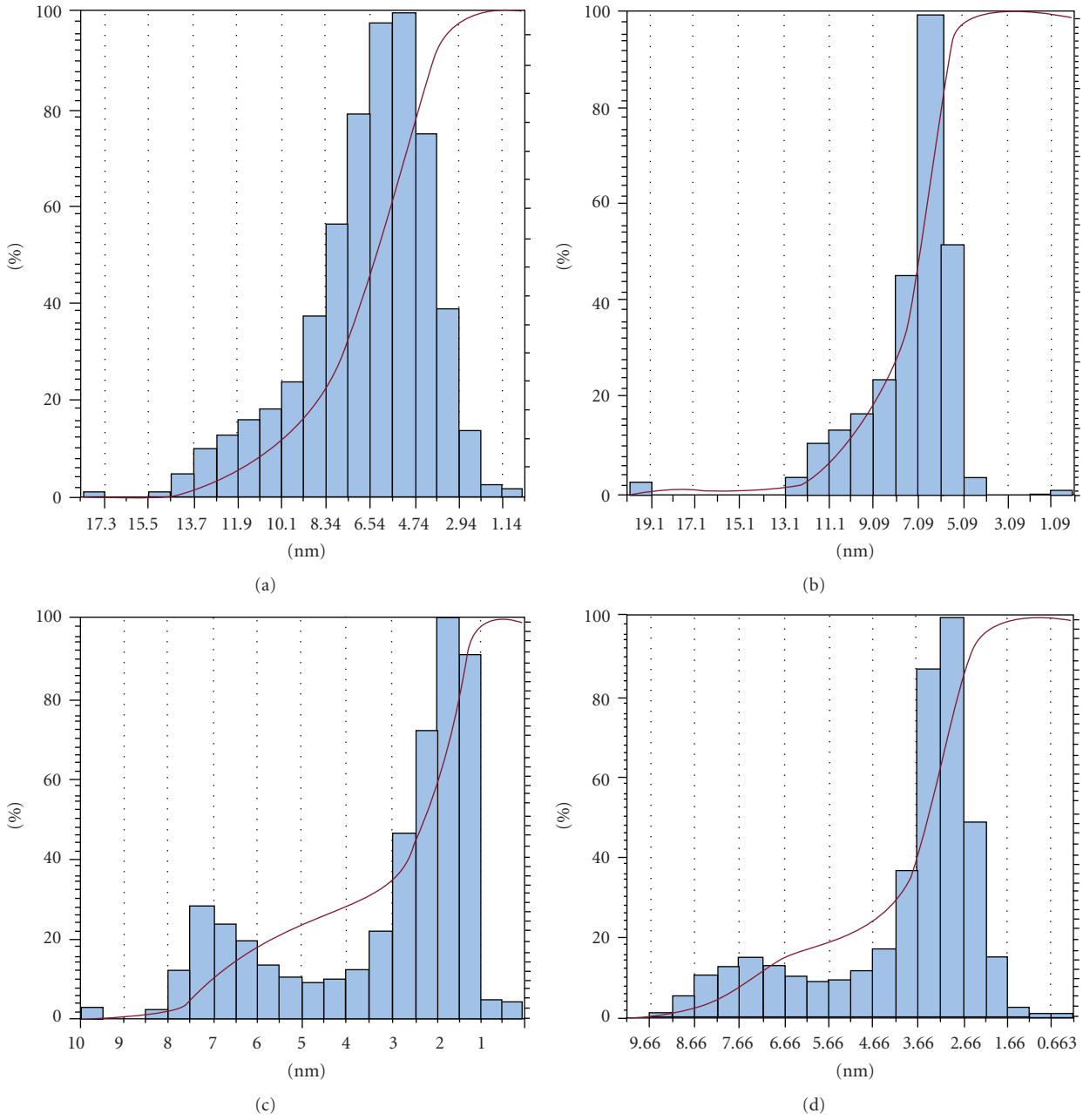


FIGURE 8: Histogram representing height of fibrils or particles versus percentage of fibrils or particles for (a) pure Aβ (1–42), (b) DHLA-capped QDs, (c) Aβ (1–42) mixed with DHLA-capped QDs, and (d) Aβ (1–42) conjugated to DHLA-capped QDs.

Furthermore, to examine the inhibition effect of CdSe/ZnS QDs, we performed the same set of experiments using polyethylene glycol (PEG) (MW 400)-capped CdSe/ZnS QDs. No inhibition on fibrillation process is observed when PEG-capped QDs are mixed or conjugated to Aβ (1–42). Figures 10(a) and 10(b) show the AFM images of PEG-capped QDs conjugated and mixed to Aβ (1–42), respectively, after 2 days. The length of the fibrils is between 700 nm and 3 μm and the height of oligomers is observed between 2.5 and 5.9 nm. Increase in intensity of fluorescence at 482 nm for the PEG-capped QDs mixed or conjugated with Aβ (1–42) shows that there is increase in fibrillation in presence of PEG-capped QDs. Experiments were also designed where DHLA- or PEG-capped QDs were purified using gel chromatography to check the inhibition effect. Similar results are obtained over a period of 72 h with the purified fractions of DHLA-or PEG-capped QDs, that is, inhibition and absence of inhibition on fibrillation process, respectively. It shows that if we change the ligand of the QDs, it changes its behavior towards the fibrillation process. The emission band for the PEG-capped QDs was observed at 472 nm, when the excitation wavelength was

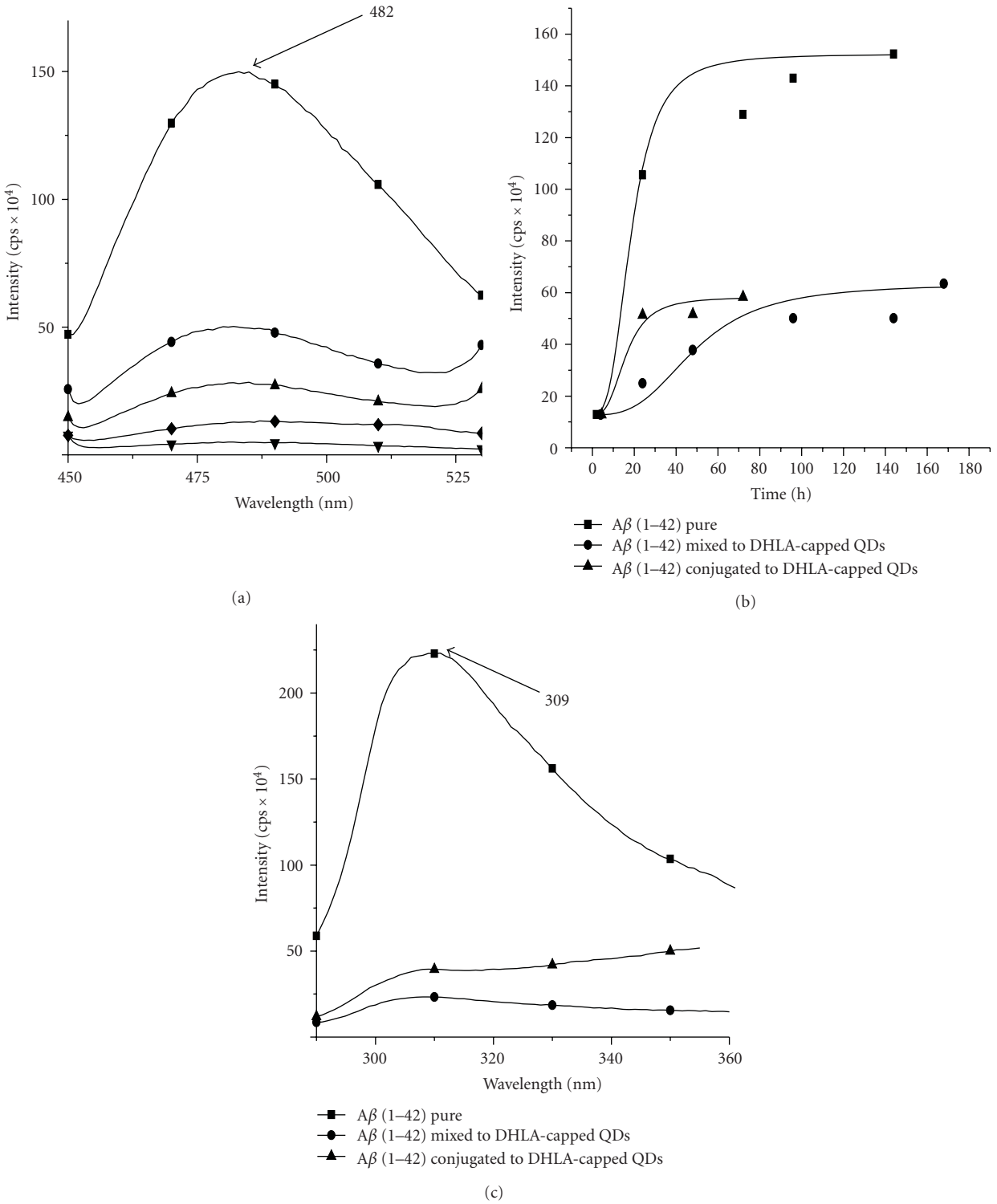


Figure 9: (a) ThT fluorescence after 7 days of incubation at 37 C for Aβ (1–42) (■); ThT assay after 7 days of incubation at 37 C for Aβ (1–42) mixed DHLA-capped QDs (•); ThT assay after 7 days of incubation at 37 C for Aβ (1–42) conjugated DHLA-capped QDs (▲);10 μM Thioflavin T (♦); 0.96 μM Aβ (1–42) (▼); (b) thioflavin T fluorescence monitored over a period of 7 days for incubated samples of pure Aβ (1–42) in comparison to Aβ (1–42) mixed and conjugated to QDs; (c) tyrosine emission intensity of the Aβ (1–42) at excitation wavelength 280 nm, quenched in the presence of QDs.

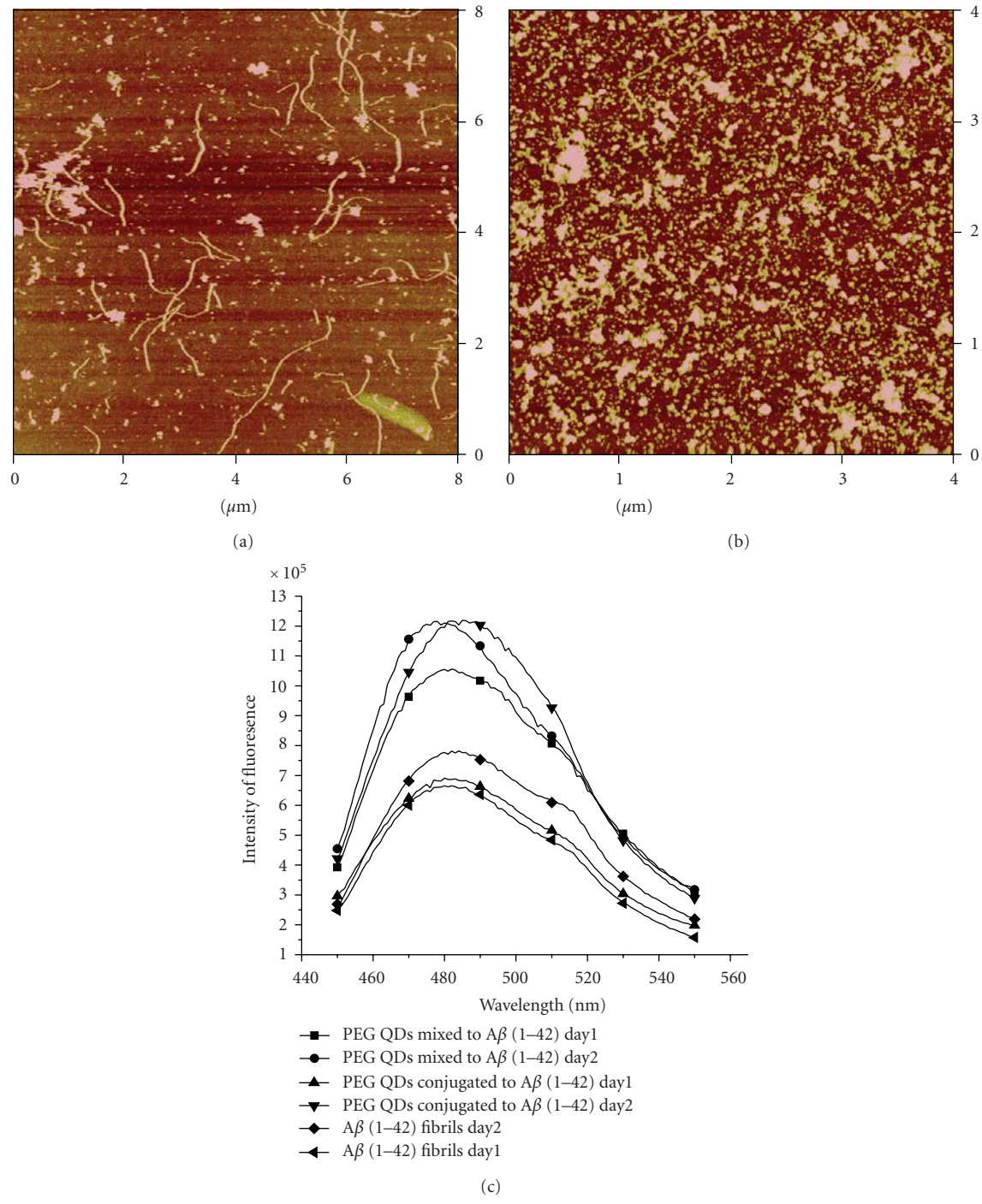


Figure 10: AFM image of (a) PEG-capped QDs conjugated with Aβ (1–42) in PBS buffer (pH 7.4), 8 μm × 8 μm; (b) PEG-capped QDs mixed with Aβ (1–42) in PBS buffer (pH 7.4), 4 μm × 4 μm; (c) comparison of ThT fluorescence for 2 days for PEG-capped QDs mixed and conjugated with Aβ (1–42) and pure Aβ (1–42) fibrils.

set at 467 nm. The absorption maxima was observed at 456 nm. The average size for the PEG-capped QDs obtained using TEM analysis was 19.4 ± 4.7 nm (Figure 10). This change in behavior might be due to the fact that PEG-capped QDs tend to aggregate in buffer solutions, and PEG polymer increases the size of the QDs [28]. These two factors make the QDs nanoparticles less dynamic in solution and less accessible for the Aβ (1–42) monomers, where the nanoparticles can block the active sites for extended fibrillation.

Figures 11(a) and 11(b) show the TEM images of PEG-capped QDs. The average size for the PEG-capped QDs obtained using TEM analysis was 19.4 ± 4.7 nm as illustrated in the histogram (Figure 11(c)).

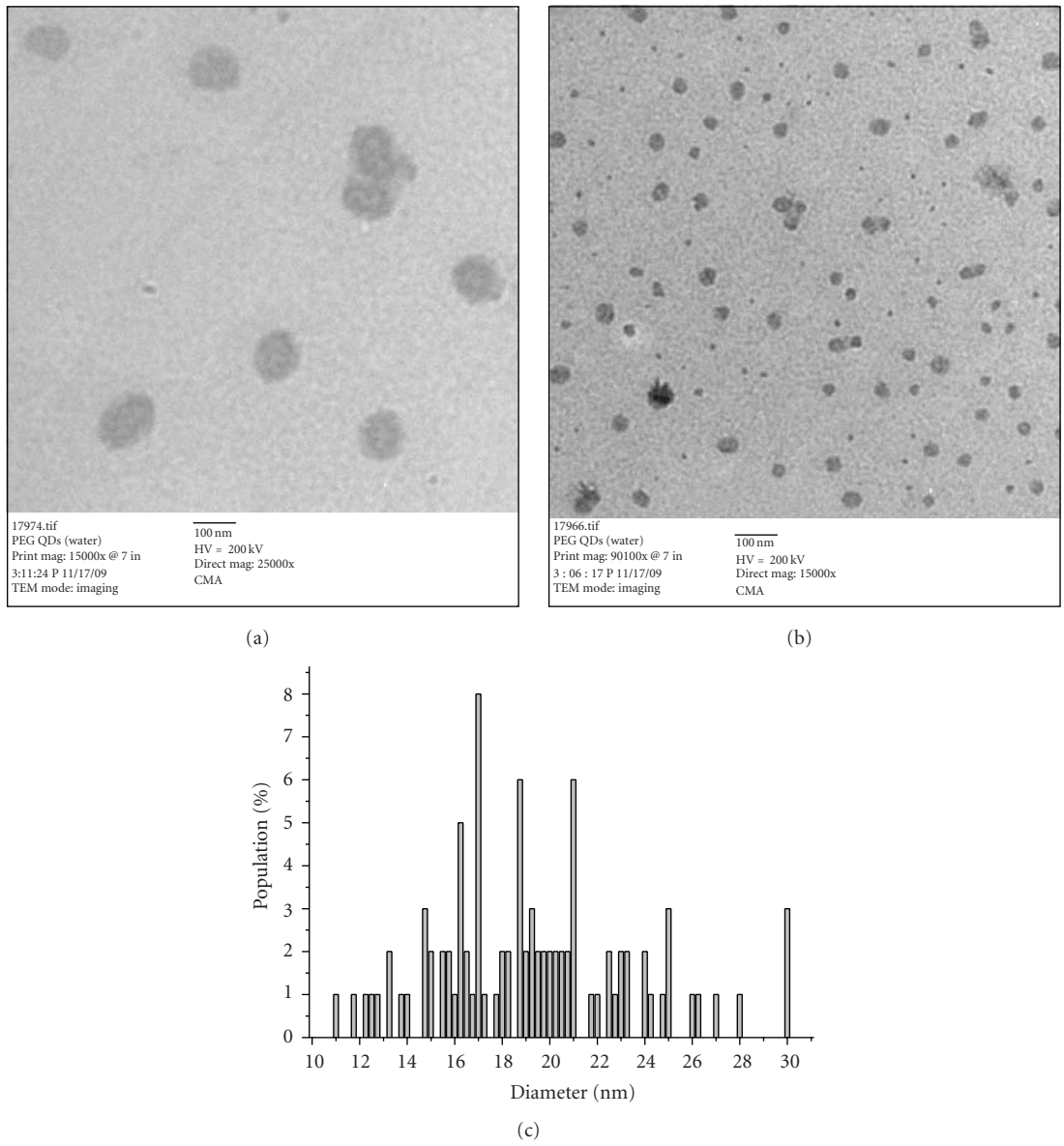


Figure 11: ((a), (b))TEM images of PEG-capped QDs (scale bar 100 nm). (c) Diameter distribution of PEG-capped QDs.

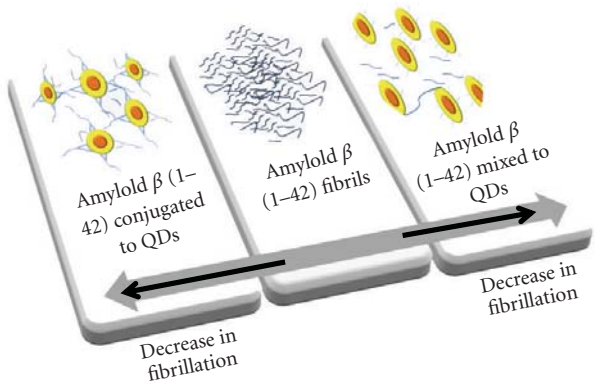


Figure 12: Diminution of fibrillation process in presence of DHLA-capped QDs.

5. Conclusion

QDs mixed or conjugated to Aβ (1–42) show a decrease in the fibrillation as compared to pure Aβ (1–42), when incubated at 37 C for 7 days. TEM images show difference in morphology and length of the fibrils. Longer fibrils (2 micron) are observed in the sample containing Aβ (1–42) mixed with QDs. Pure Aβ (1–42) sample contained large number of short- and long-length fibrils (30–1730 nm). Thicker and shortest length fibrils (30–80 nm) are observed in the case of Aβ (1–42) conjugated to QDs. The height analysis of AFM images shows significant decrease in height of aggregates greater than 7 nm (only 14%) when QDs are conjugated to Aβ (1–42) and 30% when QDs are mixed to Aβ (1–42) as compared to pure Aβ (1–42) solution. ThT assay

for the samples confirmed the inhibition of the fibrillation process when Aβ (1–42) is mixed or conjugated to QDs. Moreover, quenching of tyrosine signal is observed in the presence of QDs, which indicates an interaction of the QDs with the Tyr residue in Aβ (1–42). However, in presence of PEG-capped QDs mixed or conjugated to Aβ (1–42), an absence of inhibition on fibrillation is observed as revealed by AFM images and ThT fluorescence. The conclusion of this work is presented in Figure 12 that shows diminution in fibrillation in presence of DHLA-capped QDs, either mixed with or conjugated to Aβ (1–42).

To investigate the use of QDs *in vivo* studies is very important part of biomedical applications, there is a recent investigation showing the use of QDs for imaging and delivery purposes, where QDs carrying SNARE-tagged Rbd were delivered at the synaptic contacts in the cultures from hippocampal neurons obtained from mice [29]. Moreover, QDs doped with SiO_2 nanoparticles showed imaging and gene carrier capabilities, it was demonstrated that these QDs were internalized by primary cortical neural cells without inducing cell death *in vitro* and *in vivo* [30]. Point to be noted is CdSe quantum dots are toxic and might not be used for medicinal purposes. However, some toxicology studies have shown that the toxicity of QDs is size and concentration dependent [19]. For example, cytotoxicity studies of CdSe QDs on B16 F10 melanoma cells, and C57/BL6 mice showed no detectable toxicity [31]. Early studies have shown high toxicity of CdSe QDs due to the release of toxic Cd^{2+} ions [32]; however, coating of ZnS has shown to reduce the toxicity in cell culture to a great extent [33]. Nevertheless, extensive studies are required in the field of toxicology. In the light of these studies, it would be important to test Aβ (1–42) mixed with or conjugated to QDs in the cultures from neurons of mice to investigate the effect of QDs in *in vivo* systems.

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