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REVIEW

# Aβ Oligomers – a decade of discovery

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**Abstract**

Converging lines of evidence suggest that progressive accumulation of the amyloid β-protein (Aβ) plays a central role in the genesis of Alzheimer's disease, but it was long assumed that Aβ had to be assembled into extracellular amyloid fibrils to exert its cytotoxic effects. Over the past decade, data have emerged from the use of synthetic Aβ peptides, cell culture models, β-amyloid precursor protein transgenic mice and human brain to suggest that pre-fibrillar, diffusible assemblies of Aβ are also deleterious. Although the precise molecular identity of these soluble toxins remains unsettled, accumulating evidence suggests that soluble forms of Aβ are indeed the proximate effectors of synapse loss and neuronal injury. Here we review recent progress in understanding the role of soluble oligomers in Alzheimer's disease.

**Keywords**: Aggregation, Alzheimer's disease, amyloid β-protein, oligomerization, synaptic dysfunction.

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Substantial genetic, animal modeling and biochemical data have emerged to suggest that the amyloid β-protein (Aβ) plays a central role in Alzheimer's disease (AD). Aβ is derived from the β-amyloid precursor protein (APP) by the action of two aspartyl proteases referred to as β- and γ-secretases (Fig. 1) (Haass *et al.* 1992; Seubert *et al.* 1992; Shoji *et al.* 1992). APP is first cleaved by β-secretase allowing its large ectodomain to be shed into the luminal and extracellular fluid and leaving a membrane bound C-terminal stub. This 99 amino acid long stub is subsequently cleaved by γ-secretase, causing Aβ to be released (Fig. 1). Depending on the exact point of cleavage by γ-secretase, three principal forms of Aβ, comprising 38, 40 or 42 amino acid residues, respectively, are produced. The relative amount of Aβ42 formed is particularly noteworthy, because this longer form of Aβ is far more prone to oligomerize and form amyloid fibrils than is the more abundantly produced Aβ40 peptide (Burdick *et al.* 1992; Jarrett *et al.* 1993). Production of Aβ is a normal process (Haass *et al.* 1992; Seubert *et al.* 1992; Shoji *et al.* 1992), but in a small number of individuals, the over-production of all Aβ, or an increased proportion of the 42 amino acid form, appears sufficient to cause early onset AD (Citron *et al.* 1992; Cai *et al.* 1993; Suzuki *et al.* 1994; Bentahir *et al.* 2006; Kumar-Singh *et al.* 2006; Rovelet-Lecrux *et al.* 2006).

There are seven major pieces of evidence in support of a causative role for Aβ in AD. The first came from the localization of the *APP* gene to chromosome 21. AD-like neuropathology is invariably seen in Down's syndrome (trisomy of chromosome 21, Olson and Shaw 1969; Mann *et al.* 1984; Motte and Williams 1989) and results from increased APP expression and consequent higher Aβ levels life-long. This relationship was strongly supported by the detection of a rare case of Down's syndrome in which the distal location of the chromosome 21q breakpoint left the patient diploid for the *APP* gene (Prasher *et al.* 1998). This individual showed no signs of dementia, and amyloid deposition was essentially absent from the brain upon death at age 78 years. More recently, it was discovered that

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*Abbreviations used*: Aβ, amyloid β; AD, Alzheimer's disease; ADDL, Aβ-derived diffusible ligand; APP, β-amyloid precursor protein; CM, conditioned medium; LTP, long-term potentiation.

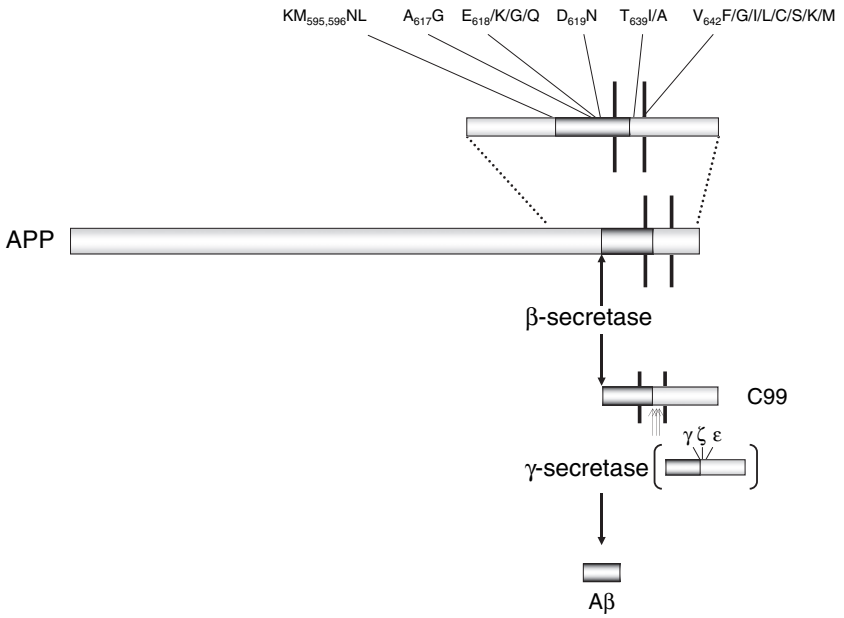


**Fig. 1** β-Amyloid precursor protein (APP) mutations and processing. The initial cleavage leading to amyloid β-protein (Aβ) production is mediated by the aspartyl protease, β-secretase (also called β-amyloid cleaving enzyme-1, BACE-1), and occurs immediately N-terminal to the Aβ domain, simultaneously generating APPsβ and the membrane-tethered C-terminal fragment, C99 (Vassar *et al.* 1999; Cai *et al.* 2001). C99 serves as a substrate for γ-secretase, a unique aspartyl protease the active site of which is provided by presenilin (Schroeter *et al.* 2003; Kopan and Ilagan 2004; Huppert *et al.* 2005). Gamma acts deep within the membrane and cleaves at at least three different positions in the transmembrane helix of APP. The first cleavage, referred to as the ε-site, occurs 9–10 residues C-terminal to the AβVal40 position and gives rise to the cytoplasmically released intracellular C-terminal domain (Gu *et al.* 2001; Weidemann *et al.* 2002). The second cleavage occurs six residues C-terminal of AβVal40 and is referred to as the ζ-site (Zhao *et al.* 2004; Kakuda *et al.* 2006). The final cut occurs at several possible peptide bonds together referred to as the γ-site and gives rise to Aβ peptides the most abundant form of which is 40 amino acids long. Point mutations in APP that are associated with familial Alzheimer's disease or cerebral amyloid angiopathy are all clustered around the α-, β- and γ-cleavage sites.

duplication of the *APP* locus on chromosome 21 caused early onset AD and/or cerebral amyloid angiopathy in five unrelated families (Rovelet-Lecrux *et al.* 2006). Second, synthetic Aβ peptides are toxic to hippocampal and cortical neurons, both in culture and *in vivo* (Pike *et al.* 1991; Busciglio *et al.* 1992; Lambert *et al.* 1998; Hartley *et al.* 1999; Hoshi *et al.* 2003; Deshpande *et al.* 2006). Third, inherited mutations in the *APP* gene that immediately flank or occur within the Aβ region alter the amount or aggregation properties of Aβ and are sufficient to precipitate early-onset AD (Levy *et al.* 1990; Chartier-Harlin *et al.* 1991; Goate *et al.* 1991) (Fig. 1). Mutations near the β-site elevate Aβ production (Citron *et al.* 1992), whereas mutations proximate to the γ-site specifically increase the amount of $A\beta_{42}$ formed (Suzuki *et al.* 1994). In the case of the five point mutations within the Aβ sequence (Fig. 1), these substitutions are clustered around the central hydrophobic core of Aβ and cause an increase in steady state levels of Aβ and/or an increased propensity of the mutant peptide to aggregate (Wisniewski *et al.* 1991; De Jonghe *et al.* 1998; Nilsberth *et al.* 2001; Van Nostrand *et al.* 2001; Betts *et al.* unpublished data). Fourth, inherited mutations within the *presenilin* 1 and 2 genes increase the $A\beta_{42}/A\beta_{40}$ ratio throughout life and cause very early and aggressive forms of AD (Bentahir *et al.* 2006; Kumar-Singh *et al.* 2006). In this regard, presenilin has been found to constitute the catalytic site of the protease [γ-secretase (Wolfe *et al.* 1999; Esler *et al.* 2000; Li *et al.* 2000)] which generates the C-terminus of Aβ (Fig. 1).

Fifth, in humans, Apo E has three common alleles, ε2, ε3 and ε4, and genetic epidemiology shows that the ε4 allele is a major risk factor for developing late-onset AD, whereas the ε2 allele appears to be protective (Corder *et al.* 1993; Rebeck *et al.* 1993; Saunders *et al.* 1993; Strittmatter *et al.* 1993). Studies of genetically manipulated mice reveal that Apo E generally facilitates Aβ fibrillogenesis, with isoform-specific effects of human Apo E expressed in mice mimicking those observed in AD (Fagan *et al.* 2002). When endogenous mouse Apo E is knocked out and then this mouse is crossed with a human APP transgenic mouse, deposits of fibrillar Aβ are virtually absent. Conversely, when human Apo E is expressed transgenically in mice genetically lacking endogenous Apo E, fibrillar deposits occur. In these studies, the appearance of fibrillar plaques occurred earliest and most robustly in mice expressing the human Apo E ε4 allele, while

mice expressing the ε3 and ε2 alleles took longer to develop senile plaques (Fagan *et al.* 2002).

Sixth, mice transgenic for mutant human APP show a time-dependent increase in extracellular Aβ and develop certain neuropathological and behavioral changes similar to those seen in AD (for overview see, Hsiao 1998; Ashe 2005). Seventh, injection of synthetic Aβ into the brains of tau transgenic mice or co-expression of mutant APP with mutant tau accelerates tau hyperphosphorylation and leads to tangle formation reminiscent of the other hallmark lesion that characterizes AD (Gotz *et al.* 2001; Lewis *et al.* 2001; Oddo *et al.* 2003; Santacruz *et al.* 2005). These approaches demonstrate that Aβ can self-associate to form several different assembly forms, from Aβ dimers all the way to aggregates of amyloid fibrils (Fig. 2).

### Aβ self-association is required for toxicity

Amyloid β-protein is a natural product and is present in the brains and cerebrospinal fluid (CSF) of normal humans throughout life (Haass *et al.* 1992; Seubert *et al.* 1992; Vigo-Pelfrey *et al.* 1993; Ida *et al.* 1996; Walsh *et al.* 2000). Thus, the mere presence of Aβ does not cause neurodegeneration; rather neuronal injury appears to ensue as a result of the ordered self-association of Aβ molecules (Pike *et al.* 1991; Busciglio *et al.* 1992; Geula *et al.* 1998). Within the amyloid plaques that characterize AD, some of the Aβ is organized into insoluble fibrils of 6–10 nm diameter, and *in vitro* synthetic Aβ can form amyloid fibrils similar to those present in human brain (Castaño *et al.* 1986; Kirschner *et al.* 1987).

Early studies clearly demonstrated that aggregation of Aβ was essential for toxicity, but characterization of the assemblies that formed *in vitro* was limited, and it was assumed that since amyloid fibrils were detectable, these assemblies mediated the observed toxicity. Yet, this ignored the concern that in patients dying with AD, there is a relatively weak correlation between the severity of dementia and the density of fibrillar amyloid plaques (Katzman 1986; Terry *et al.* 1991; Dickson *et al.* 1995). Evidence for the involvement of soluble, non-fibrillar Aβ in AD has been gleaned through four distinct experimental approaches that utilize (i) synthetic Aβ peptides; (ii) cell culture systems in which APP is over-expressed; (iii) APP transgenic mice; and (iv) human CSF and postmortem brain.

### Pre-fibrillar Aβ assemblies are present in human brain and brains of APP transgenic mice

In the case of human brain, it has long been recognized that amyloid plaque number does not correlate well with severity of dementia (Katzman 1986; Terry *et al.* 1991; Dickson *et al.* 1995); indeed this has been frequently cited as a critical flaw in the amyloid cascade hypothesis. However, recent studies have shown a robust correlation between soluble Aβ levels and the extent of synaptic loss and severity of cognitive impairment (Lue *et al.* 1999; McLean *et al.* 1999; Wang *et al.* 1999). Here, the term 'soluble Aβ' loosely describes any form of Aβ that is soluble in aqueous buffer and remains in solution following high speed centrifugation. Typically, measurement of soluble Aβ has been achieved using assays that cannot identify the aggregation state of the species detected (Funato *et al.* 1998; Morishima-Kawashima and Ihara 1998; Stenh *et al.* 2005). Thus, although the assembly states of these Aβ species are unknown, their failure to pellet following ultracentrifugation indicates that they are not fibrillar in nature.

While a huge amount of data has been gathered concerning the primary sequence of Aβ found in human brain, only limited attempts have been made to assess the assembly forms of cerebral Aβ. Using aqueous buffer free of detergents or chaotropes, Kuo *et al.* (1990) isolated a range of non-fibrillar forms of Aβ from both AD and control human brain. These species were defined by their solubility following a 220 000 *g* spin and by their ability to pass through filtration devices with three distinct molecular weight cut-offs (MWCO). This procedure identified four resolvable pools: Aβ that could not pass through a 100 kDa MWCO filter; Aβ that passed through a 100 kDa MWCO filter, but could not pass through a 30 kDa MWCO filter; Aβ that passed through a 30 kDa MWCO filter, but could not pass through a 10 kDa MWCO filter, and monomeric Aβ that passed through the 10 kDa filter. Both control and AD brain contained a continuous distribution of Aβ species from monomer up to oligomers in excess of 100 kDa, with the major contribution coming from low-n oligomers ranging from dimers to octamers. However, given that Aβ can also bind to other proteins, the molecular weight distribution determined by ultrafiltration cannot be definitively ascribed to homo-oligomers of Aβ.

In a complementary study, McLean and colleagues extracted samples of frontal cortex and putamen in PBS and centrifuged these at 175 000 *g* for 30 min. Western blot analysis of the supernates from AD brain revealed the presence of variable proportions of monomeric, dimeric and trimeric Aβ species (McLean *et al.* 1999). Such sodium dodecyl sulfate (SDS)-stable low-n oligomers have also been detected in human CSF by LC-MS (Vigo-Pelfrey *et al.* 1993) and appear to represent highly stable non-covalently associated dimers of $A\beta_{1-40}$ and trimers of either $A\beta_{6-42}$ or $A\beta_{1-35}$. Higher molecular weight SDS-stable homo-Aβ assemblies have not been reported in human CSF or soluble extracts of human brain. The presence of similar SDS-stable dimers and trimers in the soluble fraction of human brain and in extracts of amyloid plaques (Roher *et al.* 1996; Enya *et al.* 1999; Funato *et al.* 1999; McLean *et al.* 1999) suggest that SDS-stable low-n oligomers of Aβ are the fundamental building blocks of insoluble amyloid deposits and could be the earliest mediators of neuronal dysfunction.

 

Just as amyloid plaque density in the human brain does not correlate with severity of dementia (Katzman 1986; Terry *et al.* 1991; Dickson *et al.* 1995), memory impairment and changes in neuron form and function observed in APP transgenic mice can occur well before the first signs of amyloid deposition (Moechars *et al.* 1999, Hsia *et al.* 1999, Chapman *et al.* 1999, Mucke *et al.* 2000, Westerman *et al.* 2002, Dineley *et al.* 2002, Wu *et al.* 2004). Further evidence supporting soluble forms of Aβ as the principal mediators of neuronal compromise comes from a report using PDAPP mice in which Aβ-mediated deficits of memory were reversed by a single intraperitoneal injection of an anti-Aβ antibody (Dodart *et al.* 2002). In these acute (<24 h) experiments, brain amyloid burden was not decreased, suggesting that the antibody was acting on soluble, diffusible species of Aβ and that neutralization or clearing of these small intermediates allowed an overnight return to near-normal object recognition performance.

Using another well-characterized APP transgenic mouse model, Tg2576, Lesne and colleagues searched for the appearance of an Aβ species that coincided with the first observed changes in spatial memory (Lesne *et al.* 2006). Starting at 6 months, the age when changes in performance on the Morris-water maze are first apparent in Tg2576 mice, Aβ species that migrated on SDS-PAGE as nonamers and dodecamers were detected. Aβ monomer, trimer and hexamer were seen at earlier time points and hence were not considered to be associated with a deleterious effect on cognition. Indeed, comparison of spatial memory and the levels of Aβ monomer, trimer, hexamer, nonamer and dodecamer revealed that only nonamer and dodecamer levels correlated with impairment of spatial memory. The authenticity of these various Aβ species as discrete assemblies was confirmed using gel filtration; this method combined with immunoaffinity chromatography was used to achieve purification of the dodecamer. Injection of purified dodecamer into the ventricle of normal pre-trained wild-type rats caused a dramatic fall-off in spatial memory performance, thus demonstrating that a soluble, brain-derived form of Aβ can directly mediate brain dysfunction. However, that nonamer and dodecamer alone are the only Aβ assemblies capable of altering brain function appears highly unlikely. It has been previously documented that the same Tg2576 mice show impaired performance in a hippocampal-dependent contextual fear conditioning assay, decreased spine density in the dentate gyrus, and impairment of long-term potentiation (LTP) at ages long before the first apparent detection of Aβ dodecamer (Dineley *et al.* 2002; Jacobsen *et al.* 2006; Lesne *et al.* 2006). Thus, while the appearance of dodecamer correlates with the impairment of spatial memory in Tg2576 mice, it does not correlate with changes in other forms of memory, nor does it correlate with changes in synaptic form and function. Therefore, it seems likely that other lower-n oligomers may be responsible for the observed effects.

## Cell-derived low-n oligomers of human Aβ are potent synaptotoxins

Sodium dodecyl sulfate-stable low-n oligomers (dimers, trimers and tetramers) reminiscent of those detected in human and mouse brain have been detected in the conditioned medium (CM) and/or lysates of a variety of cell lines (Podlisny *et al.* 1995; Xia *et al.* 1997; Morishima-Kawashima and Ihara 1998; Walsh *et al.* 2000; Townsend *et al.* 2006a). Chinese hamster ovarian (CHO) cells that express mutant (V717F) human APP (referred to as 7PA2 cells) produce and secrete low nanomolar amounts of SDS-stable low-n Aβ oligomers (Podlisny *et al.* 1995) that migrate in denaturing gels as dimers, trimers and occasionally tetramers (Walsh *et al.* 2002b). The species detected in 7PA2 CM have been confirmed as *bona fide* Aβ oligomers by both N-terminal radiosequencing and precipitation with $A\beta_{40}$- and $A\beta_{42}$-specific C-terminal antibodies (Podlisny *et al.* 1995; Walsh *et al.* 2000). Because of the easy maintenance and fast growth rate of 7PA2 cells, 7PA2 CM has been our media of choice to investigate the biological activities of natural, cell-derived Aβ oligomers. Microinjection of small volumes (*c.* 1.5 μL) of 7PA2 CM into the lateral ventricle of the brain of an anesthetized wild-type rat inhibited hippocampal LTP (Walsh *et al.* 2002a). Evidence that the block of LTP was mediated by Aβ oligomers emerged from biochemical manipulation of the sample. Immunodepletion of the CM with an anti-Aβ antibody prevented the block of LTP, whereas immunodepletion of the abundant soluble APPsα derivative had no effect. Most importantly, pre-incubation of the CM with insulin degrading enzyme, a protease that efficiently degrades Aβ monomer but not oligomers, did not alter the LTP effect (Walsh *et al.* 2002a). In addition, we employed size exclusion chromatography (SEC) to fractionate 7PA2 and CHO- (control) CM (using non-denaturing, non-disaggregating buffers) and showed that the block of LTP was specifically mediated by low-n oligomers, not by Aβ monomers or any larger aggregates (Walsh *et al.* 2005). Taken together, these results showed for the first time that a biochemically defined, oligomeric assembly of naturally secreted human Aβ alters hippocampal synaptic plasticity both *in vivo* and *in vitro*.

Whether LTP is a valid electrophysiological surrogate of learning and memory is still contentious (Dudai 2002). Therefore, we proceeded to assess whether an intermittent impairment of short-term memory, the earliest symptom of AD, could actually be induced by soluble low-n oligomers of Aβ. For these experiments, we utilized the Alternating Lever Cyclic Ratio (ALCR) test, a procedure proven to be highly sensitive for measuring drug effects on cognitive function in rats (O'Hare *et al.* 1996; Richardson *et al.* 2002). In this procedure, wild-type rats learn a complex sequence of lever-pressing requirements. The animals must alternate between two levers, switching to the second lever after pressing the

first lever enough times to get a food pellet. Errors are scored when the rat perseverates on a lever after reward (a 'perseveration error'), or when an animal switches levers before completing the required number of presses on that lever (a 'switching error').

Rats microinjected with Aβ-containing CM showed a marked increase in both switching and perseveration errors when tested 2 h after injection, but recovered to baseline when retested 24 h later (Cleary *et al.* 2005). Evidence that this transient interruption of a learned behavior was attributable to Aβ oligomers came from the findings that immunodepleting the CM of Aβ rendered the CM inactive, and that SEC fractions containing oligomers induced the deficits, whereas monomer-containing fractions had no effect (Cleary *et al.* 2005).

Since loss of synaptic terminals strongly correlates with severity of dementia we searched for a link between oligomers and synaptic loss. For this, organotypic hippocampal sections were prepared from P5-7 Sprague-Dawley rats and biolistically transfected with enhanced green fluorescent protein so that the fine architecture of pyramidal neurons could be observed. Using two photon laser scanning microscopy dendritic spines were readily detected along the projection pathway of apical dendrites. Strikingly, the number of spines is dramatically decreased when neurons were grown in the presence of sub-nanomolar concentrations of cell-derived Aβ oligomers (Shankar *et al.* 2007). This effect is Aβ- and oligomer-specific since the decrease in spine density could be rescued by addition of anti-Aβ monoclonal antibodies, and neurons incubated in the presence of Aβ monomer alone showed a normal distribution of spines. As with the oligomer-mediated change in ALCR performance the effect of oligomers on dendritic spines is also reversible. In experiments were neurons are incubated in oligomer-containing medium for 10 days and then transferred back into normal culture medium for a further 5 days spine density rebounded back to near normal levels (Shankar *et al.* 2007). Together these data clearly demonstrate that cell-derived low-n Aβ oligomers can trigger hippocampal synapse loss and may be important effectors of synaptic dysfunction in AD.

## Detection and toxicity of non-fibrillar assemblies of synthetic Aβ

Since the elucidation of the Aβ sequence in the 1980s (Glenner and Wong 1984; Masters *et al.* 1985; Selkoe *et al.* 1986; Weidemann *et al.* 1989) investigators have used synthetic Aβ to examine its toxicity properties. While pioneering work from the laboratories of Cotman and Yankner demonstrated that Aβ had to undergo 'aggregation' to impart toxicity (Pike *et al.* 1991; Busciglio *et al.* 1992), it took many more years before the role of non-fibrillar assemblies was investigated. The first hint that Aβ peptides may form assemblies other than fibrils came from a study using solution hydrodynamics, in which two distinct Aβ assemblies were detected (Snyder *et al.* 1994). Analytical ultracentrifugation revealed large fibrillar aggregates which could be readily sedimented at speeds similar to those employed in a microfuge and smaller aggregates invisible to the naked eye which could not be removed by microfuge centrifugation. The microscopic appearance of these structures and whether they represented a single discrete species was not investigated. However, work from the same group demonstrated that addition of Apo J (also known as Clusterin) inhibited formation of mature fibrils and lead to the formation of a heterogeneous mixture of short-flexible fibrils (Oda *et al.* 1995). Surprisingly, such $A\beta_{1–42}$: Apo J mixtures were apparently toxic causing substantial inhibition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by PC-12 cells. However, it was not clear if the structures produced in the presence of Apo J were homo-oligomers of Aβ and if such structures could be formed in the absence of Apo J.

Using a combination of SEC, quasi-elastic light scattering and electron microscopy we defined two distinct assemblies formed by both $A\beta_{1–40}$ and $A\beta_{1–42}$ (Walsh *et al.* 1997). The larger assemblies were typical amyloid fibrils and the smaller assemblies were a heterogeneous mix of structures ranging in size from spheres *c.* 5 nm in diameter to curvilinear structures up to 200 nm in length. Because these structures share some physical similarities with amyloid fibrils but appeared before the detection of fibrils, we referred to them as protofibrils (PFs) (Walsh *et al.* 1997). An independent and simultaneous study using atomic force microscopy identified PFs as metastable intermediates formed during Aβ fibrillogenesis (Harper *et al.* 1997a,b). Subsequent studies demonstrated that the formation of PFs *in vitro* is dependent on concentration, pH and ionic strength (Harper *et al.* 1999). PFs appear to behave as true fibril intermediates in that they can both form fibrils and dissociate to low molecular weight species of Aβ (Harper *et al.* 1999; Walsh *et al.* 1999). Annular PFs with external and internal diameters of *c.* 8 and *c.* 2 nm have been detected and seem to be particularly well-populated in preparations of Aβ peptide bearing the Arctic mutation (E22G) (Lashuel *et al.* 2002). Under conditions where there appeared to be little conversion of PFs to fibrils, addition of PFs to rat cortical cultures caused a time-dependent decrease in neuronal viability as measured by LDH release or an increase in Hoechst staining (Hartley *et al.* 1999; Isaacs *et al.* 2006). Moreover, PFs caused a dose-dependent inhibition of MTT reduction by primary neuronal cultures that was detectable after only 2 h of incubation with cells (Walsh *et al.* 1999) and an almost immediate enhancement of electrical activity of neurons (Hartley *et al.* 1999). Using whole-cell patch-clamp analysis on primary neocortical neurons, applications of synthetic $A\beta_{1–40}$ PFs induced an instantaneous increase in excitatory

 

post-synaptic currents, an increase in action potentials and a large membrane depolarization (Hartley *et al.* 1999). Fibril preparations also enhanced EPSC activity, whereas monomeric Aβ preparations had no such effects. This excitability was reversible and concentration dependent, with activity starting in the low micromolar concentrations (Hartley *et al.* 1999). Importantly, PFs and fibrils have distinct biological activities. For instance, the addition of the specific NMDA receptor antagonist D-2-amino-5-phosphonovalerate attenuated PF-stimulated activity by *c.* 72%; whereas the same dose reduced fibril-induced activity by only 38% (Ye *et al.* 2003). In contrast, the application of the AMPA glutamate receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione, produced only a 23% decline in PF-induced activity, but reduced fibril-induced activity by some 50%. These data suggest that glutamate receptor/channels are involved in PF-induced neuronal excitability and that PFs have inherent electrophysiological activities distinct from fibrils.

Shortly after the isolation and identification of PFs, Lambert and colleagues reported the detection of small (5–6 nm in diameter) globular structures of synthetic $A\beta_{1-42}$, which they referred to as Aβ-derived diffusible ligands (ADDLs) (Lambert *et al.* 1998). These structures appeared similar to the smallest PF species (Harper *et al.* 1997a,b, Walsh *et al.* 1997, Nybo 1999) but were free of fibrils or large PFs (Lambert *et al.* 1998). Moreover $A\beta_{1-42}$ populates these ADDL assemblies to a higher degree than does $A\beta_{1-40}$ (Lambert *et al.* 1998), and they appear relatively stable in Hams-F12 media (Lambert *et al.* 1998; Dahlgren *et al.* 2002). The occurrence of spheres 4–5 nm in diameter immediately before the detection of PFs suggests that ADDLs are the earliest macromolecular assembly of synthetic Aβ detectable by current imaging techniques (Goldsbury *et al.* 2000; Dahlgren *et al.* 2002). Indeed, recent results suggest that ADDLs, like PFs may not represent a discrete Aβ assembly, but rather include a mixture of species (Hepler *et al.* 2006). The solubility of PFs and ADDLs following ultracentrifugation at speeds similar to that used to identify soluble Aβ species in human brain has not yet been addressed, so how these assemblies formed from synthetic Aβ relate to the soluble forms of Aβ present in brain is unclear. ADDLs have been shown to cause neuronal death, block LTP (Lambert *et al.* 1998; Wang *et al.* 2002) and inhibit reduction of MTT by neural cell lines (Dahlgren *et al.* 2002; Wang *et al.* 2002; Kim *et al.* 2003). When incubated with organotypic mouse brain slice cultures for a 24 h period low concentrations of ADDLs (5 nmol/L) caused *c.* 20% loss in cell number (Wang *et al.* 2002), whereas at higher concentrations (500 nmol/L) and brief incubation periods (45–60 min) cell loss was not evident but a near complete abrogation of LTP was observed (Lambert *et al.* 1998; Wang *et al.* 2002). Consistent with their synaptotoxic activity, ADDLs have been shown to avidly bind and decorate dendritic arbors of certain cultured neurons (Gong *et al.* 2003; Lacor *et al.* 2004). The *in vivo* relevance of ADDLs is suggested by the finding that antibodies raised to solutions of $A\beta_{1-42}$ that contained monomer and ADDLs produced antibodies (M93 and M94) that prevented Aβ-induced toxicity and showed that both synthetic $A\beta_{1-42}$ and some forms of brain-derived Aβ bound specifically to the surface of cultured hippocampal neurons (Lambert *et al.* 2001; Gong *et al.* 2003). These antibodies only weakly reacted with monomer and preferentially recognized assembled forms of Aβ (Lambert *et al.* 2001), and dot blot analysis of soluble extracts from human AD brain revealed a dramatic increase in M93 immunoreactivity compared with control brain (Gong *et al.* 2003). Similarly, soluble brain extracts from old Tg2576 mice displayed an increase in M93 immunoreactivity compared with non-transgenic controls (Chang *et al.* 2003). However, how truly specific these antibodies are for ADDLs versus other soluble Aβ assemblies is unclear, and thus it remains difficult to attribute M93 immunodetection to a single specific Aβ assembly form (Hepler *et al.* 2006).

In recent years there has been a number of studies suggesting that non-fibrillar, soluble forms of Aβ other than ADDLs and PFs are also toxic to cultured neurons (Hoshi *et al.* 2003; Kayed *et al.* 2003; Barghorn *et al.* 2005; Maloney *et al.* 2005; Whalen *et al.* 2005; Kelly and Ferreira 2006). For example, when Deshpande and colleagues examined the effect of three distinct assembly forms of synthetic Aβ – high molecular weight oligomers (formed as described by Demuro *et al.* 2005), ADDLs and fibrillar Aβ – they found that all three preparations were toxic to primary human cortical neurons, but that the extent and mechanism of toxicity differed (Deshpande *et al.* 2006). Low micromolar concentrations (5 μmol/L) of high molecular weight synthetic oligomers caused wide-spread death within 24 h, whereas similar concentrations of ADDLs took five-times longer to cause cell loss, and fourfold higher concentrations of fibrillar Aβ took 10 days to induce only modest cell death. Both high molecular weight oligomers and ADDLs bound rapidly and avidly to synaptic contacts. High molecular weight oligomers caused activation of the mitochondrial death pathway, but activation of this pathway also occurred when *sub-lethal* levels of the same oligomers were used, suggesting that such changes may underlie defective synaptic activity in neurons that are still viable. Other studies have reported that the application of sub-lethal concentrations of various non-fibrillar Aβ assemblies can alter neuronal architecture, cause perturbations in axonal transport and reduced cell surface levels of NMDA receptors (White *et al.* 1999; Maloney *et al.* 2005; Snyder *et al.* 2005; Kelly and Ferreira 2006; Tamagno *et al.* 2006; Zhao *et al.* 2006). Studies using synthetic Aβ peptide, Aβ-containing cell culture medium, APP transgenic mouse and human brain demonstrate that Aβ toxicity is a complex and multifaceted phenomenon that may be induced by multiple assembly

 

forms of Aβ and which can result in a variety of effects ranging from reversible changes in synaptic form and function all the way to frank neuronal loss.

### Translating knowledge of non-fibrillar assemblies into effective therapies

As the human population continues to age and the numbers affected by AD soar the need for therapies that alter the disease progression become ever more urgent. Although incomplete, our current understanding of the disease process is sufficient to facilitate the development of rational therapeutic strategies. The studies reviewed above suggest that in the human brain it is likely that multiple Aβ assemblies that are in dynamic equilibrium almost simultaneously alter brain cell function and that different toxic effects may occur virtually concurrently in various regions of the cerebrum. Thus, preventing the formation of cytotoxic oligomers should prove an effective means for treating AD. A number of different anti-amyloid therapies that will dis-assemble, neutralize or prevent the formation of Aβ oligomers are under development. While information is lacking regarding the range of Aβ assemblies present in human brain, therapeutic intervention should target the earliest stages of oligomerization, thus removing all potential Aβ toxins rather than fixating on a single Aβ assembly.

Decreasing the production of soluble Aβ monomer is particularly attractive because it may be possible to titrate Aβ down to concentrations that will not support oligomerization (Fig. 2, point 1). It is anticipated that cell-penetrant agents that reduce intracellular and/or extracellular monomer levels below the critical concentration needed for oligomerization would thus prevent Aβ from forming toxic assemblies. The development of potent highly selective inhibitors of β- and γ-secretase that can readily enter the brain and lower Aβ production (Fig. 2, point 1) is being actively pursued. For instance, Eli Lilly and Company is currently involved in phase II trials testing the efficacy of a functional γ-secretase inhibitor, LY450139 dihydrate. This compound has already been shown to reduce the rate of formation of Aβ *in vitro* and *in vivo* (Siemers *et al.* 2006). Another active-site directed γ-secretase inhibitor studied by Wyeth Research has been shown to attenuate plaque-independent memory impairments in Tg2576 providing *in vivo* evidence that reduction of Aβ monomer levels can prevent oligomer formation and subsequent synaptotxicity (Comery *et al.* 2005).

Efforts to develop small molecules that can up-regulate enzymes involved in Aβ catabolism (Fig. 2, point 2) are at an early stage, but are justified on the bases that (1) overexpression of neprylsin (NEP), insulin degrading enzyme or Cathepsin B in APP transgenic mice decreases Aβ levels and plaque burden and thus lowers the attendant AD-like cytopathology (Leissring *et al.* 2003; Mueller-Steiner *et al.* 2006); and (2) genetic deletion of NEP elevates cerebral Aβ levels including those of oligomers in APP transgenic mice (Farris *et al.* 2007; Huang *et al.* 2006) and leads to impaired hippocampal synaptic plasticity and cognitive function (Huang *et al.* 2006). Up-regulation of NEP seems partic-

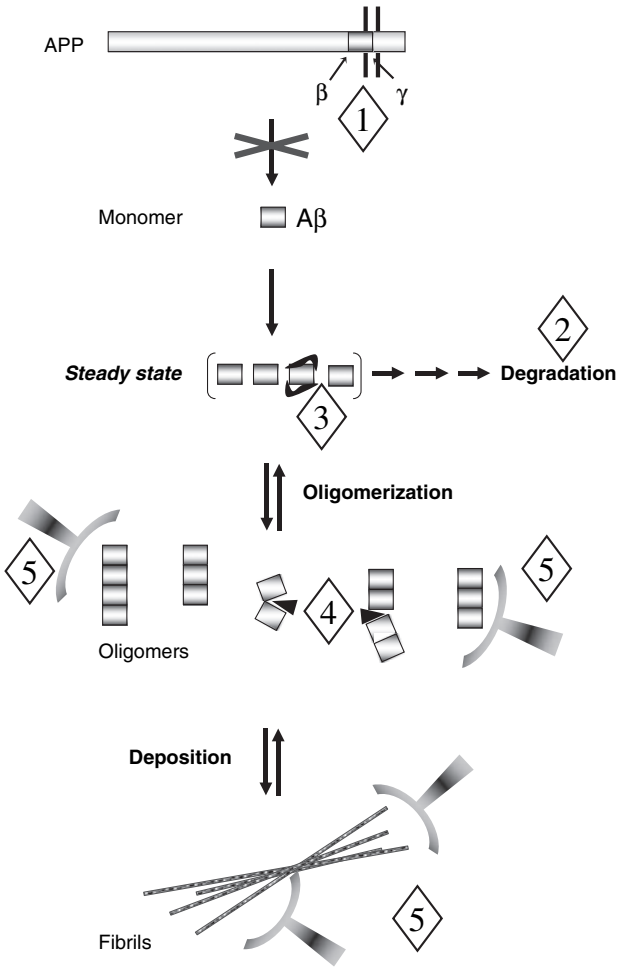


**Fig. 2** Strategies for targeting neurotoxic amyloid β-protein (Aβ) oligomers. Steady state levels of Aβ monomer are controlled by its rates of production and degradation. Above a certain critical concentration, Aβ monomers can self-associate to form dimers, trimers and larger oligomers. Consequently, inhibition of Aβ production (1) or stimulation of Aβ degradation; (2) should decrease or prevent formation of oligomers and then amyloid fibrils. Certain proteases can degrade both Aβ monomers and polymers, thus stimulation of such enzymes would serve to lower Aβ monomer levels and hence reduce *de novo* formation of oligomers while also facilitating removal of pre-existing Aβ polymers. Small molecules or biologics that bind to and stabilize Aβ monomers; (3) should prevent oligomerization and allow for the natural removal of monomers by the brain's degradative machinery. Similarly, agents capable of disrupting pre-formed oligomers; (4) should reduce the concentration of toxic oligomers. Small molecules or antibodies capable of binding to a variety of Aβ assemblies; (5) could neutralize both small and large Aβ assemblies and facilitate their elimination. In the case of antibodies, removal may occur via Fc-mediated uptake by microglia or their transport out of the brain and subsequent degradation.

 

ularly attractive, since both voluntary exercise and environmental enrichment have been reported to stimulate NEP expression and reduce amyloid pathology in APP mouse models (Adlard *et al.* 2005; Lazarov *et al.* 2005). Although, not formally tested one would anticipate that up-regulation of NEP would also decrease levels of Aβ oligomers and attenuate Aβ-mediated behavioral changes.

Small molecules or biologics which bind to and stabilize Aβ monomer should prevent oligomerization and allow for the natural removal of monomer by the brains degradative machinery (Fig. 2, point 3), however, this area of research is not yet well-developed and to our knowledge only one such agent has come to clinical trials. Neurochem. Inc. has the compound, 3-amino-1-propanesulfonic acid Tramiprosate (Alzhemed™), in phase III clinical trials at present. This molecule is a sulfated glycosaminoglycan mimetic which was designed to prevent Aβ binding to GAGs. It appears to preferentially bind to soluble Aβ, is effective in reducing aggregation of synthetic Aβ *in vitro* and can inhibit amyloid plaque formation in APP transgenic mice (Gervais *et al.* 2006)

Although many inhibitors of *in vitro* Aβ aggregation have been identified (for a review see, Walsh *et al.* 2003), molecules capable of disrupting pre-formed oligomers (Fig. 2, point 4) have not yet come to clinical trials. But recent animal studies using a small molecule inhibitor of *in vitro* fibrillogenesis, scyllo-cyclohexanehexol (AZD-103), suggest that such studies should be considered. Oral administration of this compound to APP transgenic mice caused an improvement in spatial reference memory that was coincident with a dramatic decrease of an Aβ species that migrated on SDS-PAGE with a molecular weight greater than 120 kDa (McLaurin *et al.* 2006). The decrease in this >120 kDa species occurred under conditions when dot blot analysis of brain homogenates revealed a decrease in species detected by the oligomer-specific antibody A11 (Kayed *et al.* 2003). Similarly, scyllo-cyclohexanehexol prevented the block of hippocampal LTP and the impairment of ALCR performance induced by cell-derived Aβ oligomers in rats, and this appears to involve the binding – but not the deploymerization of the cell-derived dimers and trimers (Townsend *et al.* 2006b). Together, these results suggest that scyllo-cyclohexanehexol can both disassemble large Aβ oligomers and bind to and neutralize Aβ dimers and trimers.

It has also been shown that antibodies to Aβ can effectively bind to and neutralize the effects of neurotoxic Aβ oligomers (Dodart *et al.* 2002; Klyubin *et al.* 2005). Most anti-Aβ antibodies recognize multiple different toxic Aβ assemblies. In addition to direct neutralization, antibodies could prevent cytotoxicity by facilitating the removal of soluble and deposited Aβ by promoting microglial clearance and/or by redistributing Aβ from the brain to the systemic circulation. This approach has already been shown to reduce cerebral Aβ levels, decrease amyloid-associated gliosis and neuritic dystrophy, and alleviate memory impairment in transgenic mouse models of AD (for reviews see Selkoe and Schenk 2003; Lemere *et al.* 2006). More importantly, AD patients that were immunized in a phase IIa trial with a vaccine of aggregated $A\beta_{1-42}$ peptide showed diminished cognitive decline compared with patients that received placebo (Hock *et al.* 2003; Gilman *et al.* 2005; Schenk *et al.* 2005). Unfortunately, this trial had to be stopped after only 2–3 immunizations because 18 of the 298 patients who had been immunized developed meningoencephalitis (Gilman *et al.* 2005). Notably, in at least four vaccinated AD patients that subsequently died of various unrelated causes and came to autopsy (two affected with encephalitis and two not) all showed apparent partial clearance of amyloid from the cortex (Nicoll *et al.* 2003; Masliah *et al.* 2005). Thus, in the first direct clinical test of the Aβ hypothesis, apparently (as in many preclinical studies of mouse models) targeted removal of cortical Aβ can beneficially modify AD progression. Efforts to develop an effective immunization protocol that avoids induction of encephalitis are underway. Elan Pharmaceuticals and Wyeth Research are currently conducting two separate trials to test the usefulness of a humanized monoclonal antibody to Aβ and the safety of a new active Aβ vaccine.

## Future directions

After more than a decade of intensive research on Aβ oligomerization there is good reason to believe that therapies directed at preventing the generation of toxic Aβ assemblies will soon come to the clinic. Unlike current therapies that merely treat the symptoms of AD, these new therapies have the potential to slow or even halt further deterioration. But concomitant with these exciting clinical trials, there is a necessity to further develop our understanding of the assemblies and mechanisms that underlie Aβ neurotoxicity. For instance, while there are significant data to indicate that soluble non-fibrillar forms of Aβ are proximate effectors of synaptotoxicity and that strategies directed at preventing the formation of Aβ oligomers (i.e. targeting dimers and higher) should prove efficacious, there is also a great need to fully characterize the soluble, prefibrillar Aβ species actually present in human brain. By better characterizing the synaptotoxic soluble forms of brain Aβ, we should be able to develop more effective and safe therapeutics as well as novel diagnostic assays.

To this end isolation of brain-derived Aβ using non-denaturing procedures such as flow field-flow fractionation, SEC and/or zonal centrifugation through preformed density gradients should allow us to gauge the physical dimensions and properties of these assemblies. Mass spectrometry should facilitate determination of the molecular identity of oligomers extracted from human brain and will provide clues about any non-Aβ moieties with which oligomers associate. One long-term goal of such biochemical studies would be to

sufficiently characterize natural brain-derived Aβ oligomers so that we can make synthetic mimics that are biologically relevant. Such tools should facilitate the unambiguous identification of the cellular and molecular targets with which soluble oligomers interact and consequently should illuminate the mechanisms by which these species exert their neurotoxic effects.

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