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Small assemblies of unmodified amyloid β-protein are the proximate neurotoxin in Alzheimer's disease

W.L. Klein [a], W.B. Stine Jr. [b], D.B. Teplow [c,*]

[a] *Department of Neurobiology and Physiology, Cognitive Neurology and Alzheimer's Disease Center, Northwestern University Institute for Neuroscience, Evanston, IL, USA*

[b] *Department of Medicine, Division of Geriatrics, Evanston Northwestern Healthcare Research Institute, Evanston IL, USA*

[c] *Department of Neurology, Harvard Medical School, and Center for Neurologic Diseases, Brigham and Women's Hospital, 77 Avenue Louis Pasteur (HIM-756), Boston, MA 02115-5727, USA*

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Abstract

Pioneering work in the 1950s by Christian Anfinsen on the folding of ribonuclease has shown that the primary structure of a protein "encodes" all of the information necessary for a nascent polypeptide to fold into its native, physiologically active, three-dimensional conformation (for his classic review, see [Science 181 (1973) 223]). In Alzheimer's disease (AD), the amyloid β-protein (Aβ) appears to play a seminal role in neuronal injury and death. Recent data have suggested that the proximate effectors of neurotoxicity are oligomeric Aβ assemblies. A fundamental question, of relevance both to the development of therapeutic strategies for AD and to understanding basic laws of protein folding, is how Aβ assembly state correlates with biological activity. Evidence suggests, as argued by Anfinsen, that the formation of toxic Aβ structures is an intrinsic feature of the peptide's amino acid sequence—one requiring no post-translational modification or invocation of peptide-associated enzymatic activity.

Keywords: ADDLs; Alzheimer's disease; Amyloid; Amyloid β-protein; Oligomers; Paranucleus; Protofibril; Neurotoxicity

1. Introduction

AD is a disease of multifactorial etiology. In the last two decades, a *corpus* of genetic, cell biologic, biochemical, and animal studies has emerged supporting the hypothesis that the amyloid β-protein (Aβ) is a seminal etiologic factor in AD. The key clinical question then becomes, what is the mechanism by which Aβ causes neuronal injury and death? To answer this difficult question, structure–activity relationships are being elucidated in vitro, in cell culture systems, in transgenic animals, and in humans. Historically, the primary structure of Aβ, deduced from its cognate cDNA and from protein structure analyses of brain amyloid, has been used as a starting point for the chemical synthesis of the peptide and the subsequent in vitro study of its assembly and neurotoxic properties. The identification and characterization of physiologically active assembly intermediates in these experiments then has stimulated the search for similar assemblies in vivo. Through this combination of in vitro and in vivo approaches, fundamental new insights into the role of Aβ in AD have emerged.

The predominant Aβ peptides found in vivo are the 40- and 42-residue peptides Aβ(1–40) and Aβ(1–42), respectively. These peptides form a variety of structures, including multiple monomer conformers [67], different types of oligomers [5,31,74], Aβ-derived diffusible ligands (ADDLs) [38,50], protofibrils [23,75], fibrils [68], and spheroids [25,78]. The structural relationships among these assemblies, as well as differences in the assembly processes of Aβ(1–40) and Aβ(1–42), are areas of active investigation. In each of the following three sections, unique perspectives are provided on the biophysical and biological behavior of different subsets of Aβ assemblies. These studies demonstrate that the neurophysiologic effects linked to Aβ assembly in AD can be recapitulated by synthetic Aβ(1–40) and Aβ(1–42) in carefully controlled experiments in vitro. The primary structure of the Aβ peptides studied is native. No chemical modification of the peptides is associated with the observed physiologic effects. Thus, as proposed by Anfinsen with respect to the encoding of protein tertiary structure [2], we argue that the physiologic activities of Aβ and its assemblies are encoded in its native primary structure and

* Corresponding author. Tel.: +1-617-525-5270; fax: +1-617-525-5252.
E-mail address: teplow@cnd.bwh.harvard.edu (D.B. Teplow).

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do not depend on the ability of Aβ to catalyze chemical reactions. For a discussion of the relationship of Aβ oligomers and polymers to redox-associated stressors and chemistry in AD, the reader is referred to the excellent article by Butterfield and Bush (this volume), and references therein.

2. Aβ-derived diffusible ligands (ADDLs)

Over the last decade, an increasing body of evidence has been produced that supports a fundamental shift in our view of the pathogenic mechanism of AD. Prior to this period, the primacy of fibrils was undisputed. However, new evidence supports the hypothesis that pre-fibrillar structures, including activated monomers [67], small oligomers [5,38,74] and protofibrils [23,24,75], are the key neurotoxic effectors in AD. In this section, we focus on small Aβ oligomers, termed Aβ-derived diffusible ligands (ADDLs), that are formed by Aβ(1–42). We discuss the ability of ADDLs to inhibit synaptic plasticity, review recent findings that synaptopathic ADDLs are bona fide components of AD pathology that represent the missing link in the amyloid cascade, and consider how Aβ oligomerization produces highly specific protein ligands that target synapses and disrupt signaling events essential for long-term memory formation. We note that the conclusions reached regarding mechanisms of Aβ-mediated neurotoxicity are consistent with the experimental data; however other interpretations may also be reasonable.

2.1. Neurological dysfunction due to ADDLs provides a basis for the memory-specific nature of AD

A characteristic of AD, especially in its early phases, is its specificity for memory, a fact that must be explained for a molecular mechanism to satisfactorily account for the disease. At several levels, the properties of ADDLs fulfill this criterion. It has been known for several years that ADDLs are neurologically active. Hippocampal slices exposed briefly to ADDLs lose their capacity for long-term potentiation (LTP), a classic experimental paradigm for synaptic plasticity and memory [38]. Within 45 min, LTP is completely inhibited, even when slices are exposed to ADDLs at low doses (100 nM). Inhibition also is detectable in situ in mice stereotactically injected with ADDLs [32]. Loss of LTP is highly selective and is not part of a broad synaptic deterioration [76]. Recent experiments have shown that cell-derived oligomers also inhibit LTP [74] in slice preparations and in animal models. Moreover, a new trigenic mouse AD model exhibits age-dependent loss of LTP that precedes plaque formation and thus likely is due to oligomeric species of Aβ [51].

ADDLs not only inhibit the positive synaptic plasticity of LTP, they also exaggerate the negative synaptic plasticity of long-term depression (LTD). Although without impact on the onset of LTD, ADDLs block its reversal [76], and prolonged maintenance of LTD coupled with inhibition of LTP gives an overall shift in net synaptic activity toward inhibition. Whether this shift leads to decreased stability in synaptic structure, as has been hypothesized [33,76], is unknown, although decreased synapse levels in both transgenic mice and AD subjects have been correlated with increased levels of soluble Aβ species [41,47].

The discovery that ADDLs cause a rapid, non-degenerative inhibition of LTP led to the somewhat surprising prediction that AD memory-loss in its early stages might actually be reversible [33,38]. This prediction was confirmed independently by two groups investigating transgenic mouse models of early AD, those of Steven Paul [15] and Karen Hsiao-Ashe [34]. In both models, Aβ antibodies could reverse hAPP (human APP) transgene-dependent memory failure. The treatment described by Dodart et al. was remarkably efficacious—reversal of memory failure was accomplished by a single antibody injection and occurred after only 24 h. In both studies, antibody-mediated reversal of memory failure did not depend on elimination of plaques. These studies built upon earlier work by Morgan et al. [46], who showed that active vaccination with Aβ preparations blocked the age-associated onset of memory failure in hAPP transgenic mice. Cognitive benefits in Morgan's studies also were plaque-independent. Studies of memory loss in transgenic mice by three groups thus provide significant evidence for the hypothesis that memory loss is a synaptic dysfunction triggered by soluble Aβ assemblies (e.g., ADDLs).

ADDL-induced neuronal dysfunction provides a cell biological rationale for the memory-specific nature of AD, especially characteristic in the early stages of the disease. An underlying concept is that ADDLs are ligands that attack memory-relevant synapses, where they disrupt signal processing required for memory formation. This possibility has gained prominence with recent revisions to the amyloid cascade hypothesis [22,59] which posit a role for synaptic dysfunction in memory loss (no longer attributing dementia solely to neuron death) and that incorporate the idea that key synaptopathic molecules are oligomers (e.g., ADDLs or protofibrils). Amyloid fibrils are no longer considered the only active molecular pathogen, and perhaps not even the pathogen most responsible for memory loss.

2.2. Establishing the clinical relevance of ADDLs: small Aβ oligomers are AD's hidden toxins

In vitro experiments demonstrate that ADDLs interfere specifically with memory-associated phenomena. The crucial question is whether ADDLs exist in vivo and exhibit the same toxic properties. It has become possible to address this issue using conformation-specific antibodies that can discriminate between ADDLs and monomers, eliminating signal-to-noise problems found in assays of crude brain extracts. These antibodies can be produced by immunizing rabbits with ADDLs [39] or with mixtures of fibrils and ADDLs (M.P. Lambert, unpublished),

as employed in preclinical therapeutic vaccine experiments [58].

"Dot blot" assays, capable of detecting less than one femtomole of ADDLs, have revealed ADDLs in transgenic mice, and more importantly, in AD brain [10,18]. These experiments used soluble brain extracts prepared without detergents or harsh chemicals that might alter the structure of the assembly from that found in situ. Results from mice support key predictions—ADDL immunoreactivity is transgene-, age-, and region-dependent. Behaviorally, preliminary water maze experiments indicate that when ADDL levels increase, memory functions decrease [77]. The data suggest, moreover, that before memory loss becomes evident, ADDL levels must exceed a threshold. This threshold presumably is related to the amount of compensatory "synaptic or cognitive reserve [4]." Once this reserve is exhausted, damage to memory function becomes evident. The presence of ADDLs in transgenic mice with memory loss is consistent with the inference that memory recovery mediated by passive immunization with Aβ-specific antibodies is due to ADDL elimination or neutralization.

2.3. ADDLs are abundant in soluble AD brain extracts

Recent human studies have established that ADDLs are bona fide elements of AD pathology [18]. Soluble brain extracts prepared in physiological buffer (F12 culture medium) by high-speed centrifugation contained ADDLs and the ADDL concentration showed a striking AD-dependence. ADDL levels in AD subjects were increased as much as 70-fold over controls, and population averages were elevated 12-fold. Correlations between ADDL concentration and cognitive status have not yet been carried out. An elevated ADDL level in one control subject is consistent with the possibility that individuals with mild cognitive impairment (MCI) or pre-symptomatic AD might present with increased ADDL concentration as the first stage of pathology. These findings support the hypothesis that ADDLs may be the "missing link" in the amyloid cascade, accounting for the historically poor correlation between insoluble amyloid deposits and cognitive status.

Structurally, synthetic ADDLs and brain-derived ADDLs are indistinguishable in molecular weight and isoelectric point. The major soluble species is a dodecamer with a pI of 5.6. ADDLs made in vitro occur in various sizes, but the dodecamer is the characteristic species found under physiological conditions. This species also has been identified using chemical cross-linking methods (see Section 4). Additional oligomers from AD brain can be extracted with ionic detergents, suggesting they are associated with other molecules. Synthetic and brain-derived ADDLs also appear to have equivalent conformations because they are both recognized by conformation-specific antibodies produced by immunization with synthetic ADDLs. In ligand overlay assays, which are conformation-sensitive and monitor protein–protein interactions, synthetic and human ADDLs both attach specifically to the same three proteins. These results provide strong evidence that AD brains contain the same molecules found previously to disrupt memory mechanisms in cell and animal experiments.

Perhaps the most convincing evidence that human and synthetic ADDLs are identical molecules comes from experiments that reveal patterns of ADDL attachment to highly differentiated hippocampal neurons maintained in culture. ADDLs, whether obtained from brain or made in vitro, bind to nerve cell surfaces and display a characteristic staining pattern associated with "hot spots" on neuronal dendrites (Fig. 1). Importantly, these hot spots are synapses, and ADDLs in AD brain extracts are synaptic ligands, just like their synthetic counterparts. These observations are highly

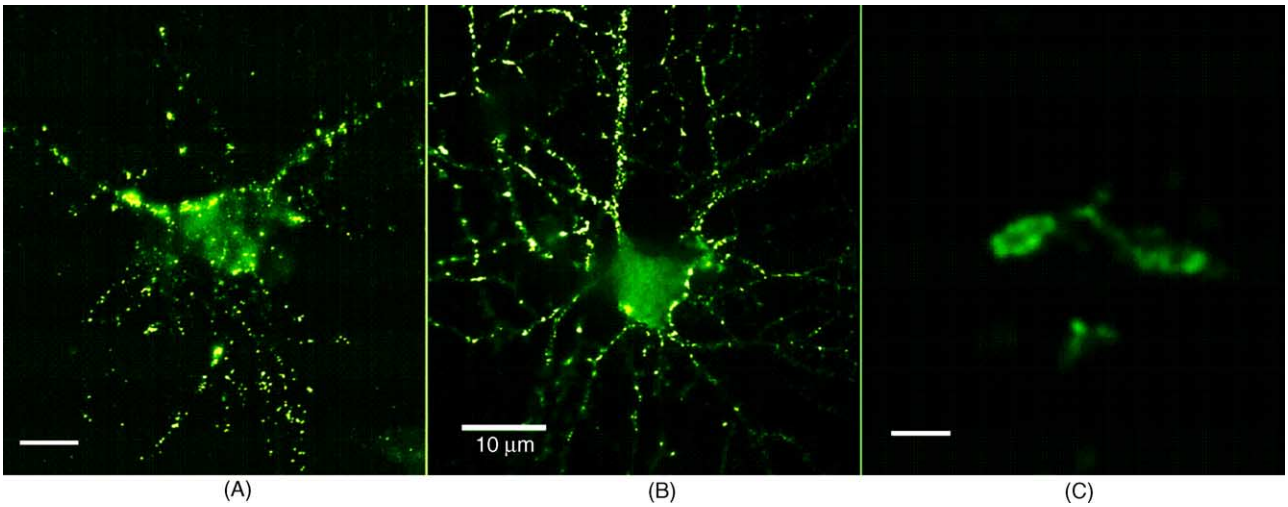

Fig. 1. Human and synthetic ADDLs are synaptic ligands. Mature hippocampal cultures were incubated with brain extracts of synthetic ADDLs, washed, and imaged by immunofluorescence microscopy using oligomer-dependent antibodies. (A) AD-brain soluble extract, (B) synthetic ADDLs, (C) control-brain soluble extract. Bar = 10 μm. Synaptic binding is evident after 5 min incubation and occurs at cell surfaces.

relevant with respect to the hypothesis that AD memory loss is a synapse failure caused by ADDLs.

2.4. ADDLs target synapses and interfere with memory-relevant immediate early gene expression

Earlier work with flow cytometry strongly suggested that ADDLs were ligands for particular proteins on neuronal cell surfaces [38]. To study in greater detail the specificity and cell biology of ADDL attachment to neurons, recent experiments have used long-term cultures (>21 days) of dissociated rat hippocampal neurons [37]. These neurons undergo remarkable structural differentiation in culture, including robust synaptogenesis, which makes the model ideal for investigating synaptic cell biology. The basic paradigm is to incubate mature cultures of hippocampal neurons with synthetic ADDLs and then probe with ADDL-specific antibodies. ADDLs bind to these neurons with topological specificity, attaching mostly to dendrites and exhibiting a punctate pattern reminiscent of rafts, focal contacts, or synaptic terminals. Binding occurs within minutes and is localized to the cell surface (being detected without permeabilization and using living cells). Hot spots are seen in hippocampal and cortical, but not cerebellar, cultures, a specificity of binding consistent with AD vulnerability. Binding is eliminated by prior incubation of ADDLs with antibodies, suggesting binding is to receptors selective for ADDLs.

Observed at high-resolution, binding sites coincide with dendritic spines, and double-label experiments show that ADDL hot spots co-localize to a remarkable degree (93%) with clusters of PSD-95. PSD-95 is a scaffolding protein of postsynaptic densities found at excitatory CNS synapses. In well-differentiated hippocampal neurons, clusters of PSD-95 occur only at synapses [1]. Hot spots of ADDL binding thus are specific for synaptic terminals, or put another way, ADDLs act as synapse-specific ligands.

Ligand specificity is evident even between different synapse populations, as only half the PSD-95-labeled synapses bind ADDLs. Thus, although the majority of ADDL hot spots are associated with PSD-95, not all sites of PSD-95 display bound ADDLs. Preliminary experiments indicate the synapses are the sort implicated in LTP and memory formation. For example, double-label confocal immunofluorescence microscopy shows that CaMKII-positive neurons are targets of ADDLs, and higher magnification shows ADDLs at CaMKII-synaptic spines, consistent with PSD-95 co-localization. Specificity of synaptic targeting by ADDLs does not correlate, however, with the CaMKII phenotype, as images have been obtained which show two adjacent CaMKII-positive cells, only one of which having ADDL-positive synapses.

Human brain ADDLs, like their synthetic counterparts, bind to dendritic hot spots that co-localize with clustered PSD-95. Identical patterns have been found in preliminary experiments using CSF, indicating ADDLs in AD can be found in extracellular compartments. Binding patterns observed in culture are mirrored by ADDL distributions in situ. Fixed sections from AD frontal cortex examined using ADDL-specific antibodies show extracellular, not intracellular, staining, consistent with the postulated cell surface-specific activity of ADDLs as synaptic ligands. Binding at neuronal cell surfaces is indicated by perimeter staining that outlines cellular boundaries. Kayed, Glabe, Cotman and colleagues also have reported that Aβ oligomer staining in AD tissue is separate from congophilic plaques [29]. These data support the hypothesis that diffuse stain in AD pathology derives from synaptic attachment of ADDLs, an extension of the hypothesis framed by Hardy and Selkoe [22].

A new finding [37] of relevance to the molecular basis of memory loss is that ADDL attachment to spines induces expression of Arc (activity-regulated cytoskeletal-associated protein), a synaptic immediate early gene whose properly controlled expression is essential for long-term memory formation [20]. Normal memory function depends on pulsate synaptic induction of Arc. ADDLs, however, ectopically generate an increase in Arc protein that is rapid, large, and sustained. Expression begins at synapses, then spreads throughout entire dendritic arbors. Ectopic Arc expression has been hypothesized to inhibit long-term memory formation, in principle by creating noise that overloads synaptic information processing [20]. Consistent with this prediction, overexpression of Arc in transgenic mice produces “slow-learners,” a dysfunction attributed to aberrant spine structure [30]. Additionally, in an action relevant to dysfunctional synaptic plasticity, Arc overexpression disrupts AMPA receptor trafficking [55], predicted to be a consequence of ADDL action [18]. Blocking insertion of AMPA receptors into synaptic membranes has been proposed to account for ADDLs inhibition of LTP and prolongation of LTD. The impact of ADDL-induced Arc thus could provide a mechanism that leads to synapse failure and memory loss.

The mechanism by which ADDLs specifically target synapses is not understood. Current evidence suggests that ADDLs target a transmembrane synaptic protein that facilitates disruptive interactions between ADDLs and the post-synaptic density. Specific ADDL-binding proteins are enriched in synaptosomes (>10-fold; D. Richardson, unpublished) and a subpopulation of synaptosomes with bound ADDLs can be isolated by magnetic immunobeads (A. San Clemente, unpublished). Structure–function analysis indicates that ligand activity is not associated with all oligomeric species. Size exclusion chromatography of ADDL preparations produces peaks of ∼15 and 50–70 kDa. Only the larger oligomers exhibit binding. This separation into biologically active/inactive forms is evident whether assays reveal binding to synapses in culture or to particular proteins in overlay assays [11]. Because these assemblies arise from the same peptide, i.e., from a single primary structure, the distinct biological activities of the assemblies

must be due to differences in higher order structure (secondary, tertiary, and quaternary).

3. Assembly-dependence of Aβ function

As discussed in Section 2, the "amyloid cascade" hypothesis maintains that aggregation of Aβ into amyloid deposits, a pathologic hallmark of AD, induces a toxic gain of function. However, these plaques appear "at the wrong time and in the wrong places" to explain the clinical progression of dementia in humans [48] or the neuronal dysfunction and loss in transgenic mouse models of AD [12]. Thus, recent debate within the AD community has focused on whether fibrils (amyloid) or monomers, oligomers, and protofibrils, are the active species of the peptide that ultimately cause the synaptic loss and dementia associated with the disease [21,22,33,62]. In vivo, the concentration of small, stable oligomers of Aβ(1–42) in brain, plasma, and cerebrospinal fluid (CSF) [36,57] correlate with the severity of neurodegeneration in AD [41,45]. In vitro data demonstrate that soluble Aβ oligomers and protofibrils are neurotoxic [24,38,50,73,74]. Although "oligomeric Aβ" has been incorporated in an updated version of the amyloid cascade hypothesis [22], the role of fibrillar and oligomeric Aβ in the pathogenesis of AD remains unresolved.

A complicated, and often controversial, literature exists with respect to Aβ structure–activity correlations in vitro. Early experiments demonstrated that fibril formation, detectable in preparations of Aβ following lengthy incubation, was necessary for neurotoxicity [53]. Within these preparations, fibrils were readily identifiable either electron microscopically or through binding of thioflavin T, a widely used amyloidophilic histochemical. However, in vitro activity did not always correlate with the presence of fibrils and many groups have described significant variability in activity between different Aβ peptide lots. These differences in biological activity do not appear to be the result of differences in chemical purity, primary structure modifications, or the ability of the peptide to form fibrils [26,64].

Part of the controversy surrounding the Aβ structure–function debate results from a lack of methods to effectively study Aβ assembly and characterize conditions that influence this process. The ability to chemically synthesize Aβ for in vitro studies provides a useful approach to address the complex relationship between peptide structure and function. However, understanding the structural biology of Aβ has been hampered by the inability to use traditional methods for determining protein structure. These include X-ray crystallography and solution NMR, techniques that so far have not been able to produce high-resolution structures of peptide oligomers or higher-order assemblies of full-length native Aβ. Despite this technical challenge, lower resolution approaches applied in vitro have yielded important clues as to how Aβ structure correlates with biological activities relevant to AD. One of the first clues that Aβ structure–function relationships might be significantly more complex than first realized was the description of biologically active, non-fibrillar assemblies detected in a soluble Aβ fraction formed in the presence of apolipoprotein J [50]. This early structure–function study benefited from the novel characterization of Aβ assemblies by atomic force microscopy (AFM). AFM is a particularly powerful approach capable of revealing a wide range of Aβ structures [66] and structures of other amyloidogenic proteins, including α-synuclein [13], lysozyme [9], and prion protein [60].

To better understand the relationship between Aβ structure and function, we determined the conditions necessary to produce stable preparations of several distinct conformational species of Aβ(1–42) [65]. AFM analysis of synthetic Aβ(1–42) directly resuspended in aqueous solutions revealed a heterogeneous mixture of oligomers, globular aggregates, and fibrils [65]. To provide better control over peptide assembly, steps were taken to consistently produce an unaggregated starting material. This was accomplished by suspension of Aβ(1–42) in hexafluoroisopropanol (HFIP), a solvent that disrupts peptide–peptide interactions and facilitates the formation of α-helical structure. Following HFIP treatment, the peptide was resuspended in DMSO, a solvent previously shown to produce monomeric Aβ solutions [63]. These unaggregated preparations were characterized by AFM and found to contain homogeneous populations of spheres with diameters of ∼1 nm, thought to be peptide monomers (Fig. 2A). Starting with this unaggregated Aβ(1–42) preparation, we developed two aggregation protocols that consistently produce extensively oligomeric (∼2–5 nm in height, Fig. 2A) or fibrillar (∼4 nm in height and several microns in length, Fig. 2A) assemblies of Aβ(1–42) [14,65].

We next characterized several additional variables affecting Aβ(1–42) assembly, including peptide concentration, incubation time, temperature, pH, ionic strength, and solubility [65]. All of the Aβ structural conformations were readily discriminated by AFM, whereas Western blot analysis provided only limited information for uncross-linked assemblies. Fibril formation was favored at acidic pH (10 mM HCl, no added salt). Examination of side-chain pK_a values suggests that Coulombic interactions resulting from the protonation of Asp, Glu, or His residues may be involved, consistent with results of prior studies of the formation of Aβ assembly intermediates [31]. Oligomer formation is favored at physiologic pH (7.4) and ionic strength (150 mM), where hydrophobic interactions in the C-terminal region may predominate. Unaggregated, oligomeric, and to some extent fibrillar, Aβ(1–42) preparations remain in the supernate following centrifugation, emphasizing the importance of differentiating between preparations that are "kinetically soluble [56]," i.e., do not sediment during experimental observation but would do so if incubated long enough, and those that are monomeric, oligomeric, or fibril-free. Soluble fractions cannot be considered "fibril-free" unless direct morphologic examination is done. Despite the dramatic

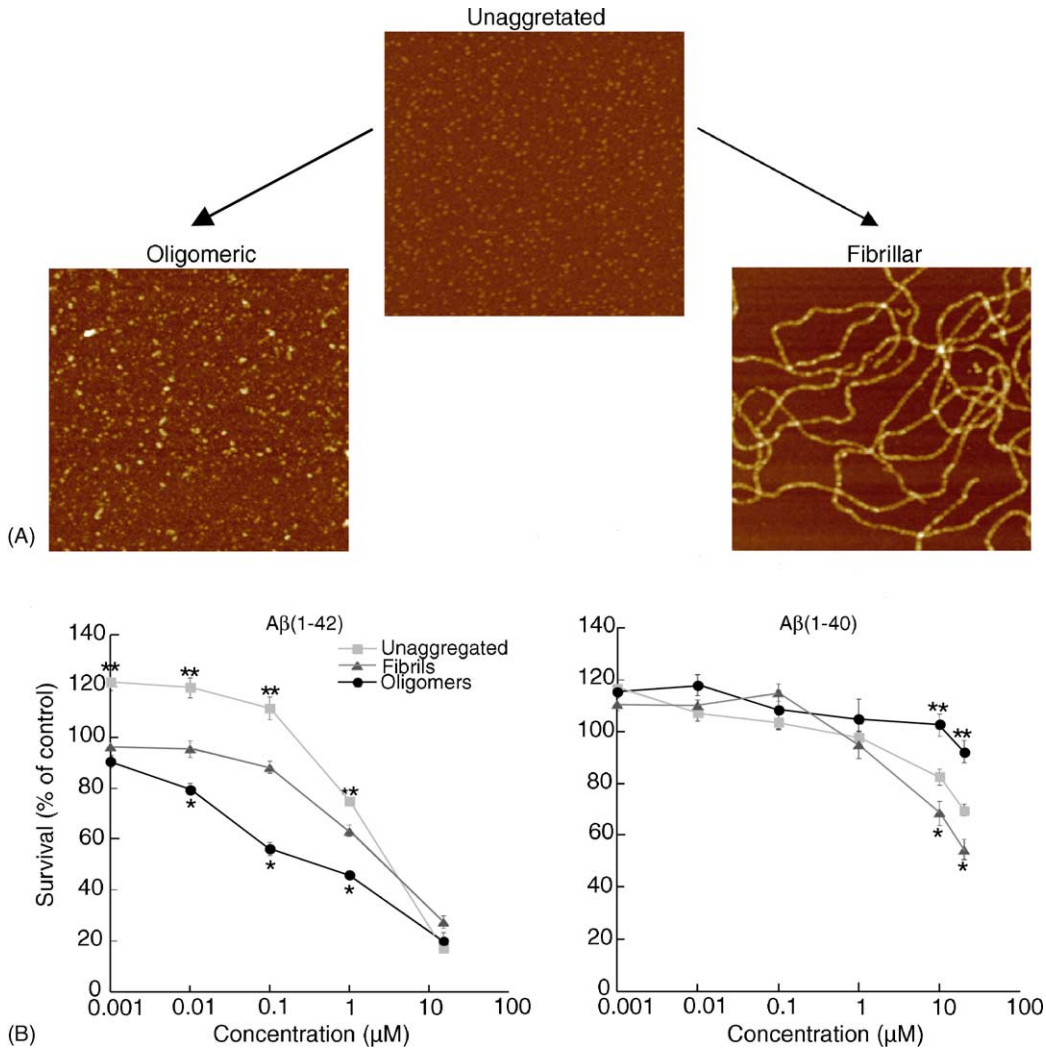

Fig. 2. Morphology and toxicity of Aβ assemblies. (A) AFM analysis of HFIP-treated, lyophilized Aβ(1–42) resuspended at a concentration of 5 mM in DMSO ("unaggregated") or incubated at 100 μM concentration for 24 h in either culture medium at 4 °C ("oligomeric") or 10 mM HCl at 37 °C ("fibrillar"). Samples were diluted to 10 μM concentration for AFM analysis. Representative 1 × 1 μm *x–y*, 10 nm total *z*-range AFM images. (B) Effect on neuronal viability of Aβ(1–42) and Aβ(1–40) prepared under oligomer- and fibril-forming conditions. Aβ peptides were incubated with Neuro-2A cells for 20 h. The MTT assay was used as an indicator of cell viability. Graph represents the mean ± S.E.M. for $n = 8$ from triplicate wells from at least two separate experiments using different Aβ preparations. *: Significant ($P < 0.01$) difference between oligomers and fibrils. **: Significant ($P < 0.01$) difference between unaggregated and both oligomers and fibrils.

differences in Aβ structure resulting from these procedures, no covalent modifications of the Aβ peptide were detected by MALDI mass spectrometry (W.B. Stine, unpublished).

Because the distinct assemblies we produced were derived from chemically unmodified, structurally homogeneous, starting preparations, they could be used for comparative structure–function studies to reveal assembly-dependent differences in biological activity. For example, the solubility of oligomer and fibril preparations were characterized in vivo by stereotaxic injection into the rat hippocampus [43]. Sections were immunostained for Aβ to assess injection location and peptide diffusion. Oligomeric Aβ(1–42) diffused rapidly (30 min post-injection) from the site of injection within the hippocampus to the corpus callosum, cortex, and multiple adjacent sections. Fibrillar Aβ(1–42) was localized primarily within the injection track. No Aβ immunoreactivity was seen in the vehicle-injected animals. These studies provide evidence that ex vivo oligomeric Aβ(1–42) assemblies remain soluble and diffuse readily in vivo.

Unmodified assemblies of oligomeric and fibrillar Aβ(1–42) are also being used as immunogens [65] to produce conformation-specific monoclonal antibodies for use in characterizing oligomeric Aβ in vivo. For example, a unique antigen/antibody screening array identified an antibody, MOAB-1, that detected oligomeric but not fibrillar Aβ(1–42). This screen also yielded several high-titer Aβ monoclonal antibodies that were not conformation-specific, including MOAB-2. Initial characterization of MOAB-1

using Western blotting, ELISA, and competitive dot-blot analysis with unaggregated, oligomeric, and fibrillar Aβ(1–42) and Aβ(1–40) demonstrated that MOAB-1 preferentially recognized oligomeric Aβ(1–42). MOAB-2, and the commercial Aβ-specific antibody 6E10, did not distinguish Aβ(1–40) and Aβ(1–42) oligomers and fibrils. The intended uses of MOAB-1 include immunohistochemistry to assay for oligomeric Aβ species in vivo, as well as the development of an ELISA capture assay to facilitate measurement of soluble Aβ oligomers in extracts of brain, CSF, and potentially serum. Detection of oligomeric Aβ allows further experimental assessments of the correlation between oligomeric Aβ and synaptic loss, tau-related neuritic dystrophy, Aβ histopathology, and memory loss and dementia. Identification of such a toxic species in situ provides a tractable target that may lead to the successful early diagnosis and treatment of AD.

In conjunction with development of methods to characterize conformation-dependent activities in vivo, studies in vitro also have addressed the relationship between Aβ structure and function. The C-terminal region of Aβ has been shown to strongly influence the rate of amyloid fibril formation [27]. Consistent with this early observation, Aβ(1–40) incubated under conditions that produce either oligomeric or fibrillar Aβ(1–42) in 24 h did not yield observable changes in the assembly of Aβ(1–40) [65]. Oligomeric and fibrillar Aβ(1–40) assemblies were detected when the incubation time was extended to 1–6 weeks, indicating differences in assembly kinetics between Aβ(1–40) and Aβ(1–42). Based on these assembly differences, both peptides were tested in an in vitro neurotoxicity assay (Fig. 2B). Cultures of Neuro-2A cells were exposed to unaggregated, oligomeric, or fibrillar Aβ(1–42) and Aβ(1–40) incubated under matched conditions. Dose-dependent differences in cell viability (as measured by the MTT metabolism assay) were observed among the three structural forms of Aβ(1–42). The observed effect for Aβ(1–42) oligomers was 10-fold greater than that of fibrils and 40-fold greater than that of unaggregated Aβ, with 10 nM oligomer treatment inducing a significant reduction in cell viability. Aβ(1–40) incubated under Aβ(1–42) oligomer- and fibril-forming conditions existed predominantly as unassembled monomer and had significantly less effect on neuronal viability than preparations of Aβ(1–42) (Fig. 2B). These results demonstrate that the intrinsic toxic potential of Aβ depends on both its sequence and assembly state, and not solely on assembly conditions.

Two other naturally occurring alloforms of Aβ(1–42), Glu22Gln (Dutch variant) and Glu22Gly (Arctic variant) were also characterized under the aggregation protocols developed for wild type (WT) Aβ(1–42) oligomer- and fibril-formation. Both Glu22Gln and Glu22Gly alloforms incubated under WT oligomer-forming conditions exhibited increased protofibril and short fibril formation, respectively, but were not consistently different from WT Aβ(1–42) in terms of inhibition of neuronal viability. However, when incubated under WT fibril-forming conditions, Glu22Gln and Glu22Gly fibrils were larger (∼5–6 nm diameter), appeared more rigid, and decreased neuronal viability significantly more than WT Aβ(1–42) fibril preparations [14]. These data further support the primary role of Aβ sequence and assembly state in determining the biological activity of the peptide.

Another in vitro measure of Aβ activity associated with AD is the induction of an inflammatory response. Utilizing WT primary rat glial cultures, oligomeric Aβ(1–42) induces significantly greater glial activation compared to fibrils [42] (White and LaDu, unpublished observations). These changes were assessed by changes in glial morphology and expression of a number of inflammatory markers. Oligomer-treated cultures released significantly more nitric oxide (NO) than fibril-treated cultures in a dose- and time-dependent manner. Expression of interleukin-1β, tumor necrosis factor-α, and inducible nitric oxide synthase (iNOS), the enzyme that catalyzes the reaction producing NO, also demonstrated a dose- and time-dependent increase that was significantly greater for oligomer-treated cultures compared to fibril-treated cultures. These results provide additional evidence that Aβ biological activity is directly influenced by Aβ structure.

Abnormalities in the processing of AβPP to Aβ are *causal* factors, and the ε4 allele of apolipoprotein E (apoE) is the primary *risk* factor, for AD. Based in part on these genetics, the structural and functional interactions between Aβ and apoE have been the focus of considerable research (for a recent review, see [54]). To determine the effect of apoE on Aβ(1–42) oligomer- and fibril-induced neurotoxicity, primary WT neurons were cultured in the presence of glia isolated from WT and human apoE3 and apoE4 targeted replacement (apoE-TR) mice [44]. In the presence of glia from WT, apoE3-TR, and apoE4-TR mice, neurotoxicity was significantly greater with oligomeric versus fibrillar Aβ(1–42), an effect that was both dose- and time-dependent. Significant fibril-induced neurotoxicity was observed only in the cultures with apoE4-TR glia. Oligomeric and fibrillar Aβ(1–42)-induced neurotoxicity was significantly greater in the presence of glia from apoE4-TR mice compared to glia from WT and apoE3-TR mice. These observations using a neuron-glia co-culture model were consistent with neuroplasticity data in which LTP was significantly suppressed in hippocampal slice cultures from apoE4-TR mice as compared to apoE3-TR and WT mice [61]. Hippocampal slice cultures from apoE4-TR mice were significantly more susceptible to oligomeric Aβ(1–42)-induced inhibition of LTP than cultures from apoE3-TR or apoE-knockout mice [17]. Comparable doses of unaggregated Aβ(1–42) had no effect in this model of neuroplasticity, suggesting that inhibition of LTP depended on Aβ assembly. The ε4 allele thus appeared to produce a gain of negative function. Taken together, these results suggest a compromised function of fundamental neuroplasticity mechanisms, providing a direct link between ε4, oligomeric Aβ(1–42), and the memory loss that defines AD.

In summary, these findings suggest that specific assemblies of Aβ(1–42) are neurotoxic, that neurotoxicity can be exacerbated via induction of glial-mediated

neuroinflammation, and that both neurotoxicity and inhibition of LTP measured in WT and different apoE transgenic backgrounds vary with respect to Aβ assembly state. Functional differences observed in these comparative studies were linked to conformational differences resulting from the folding and assembly of native Aβ peptides, not from post-translational modifications to Aβ. These studies of the formation of peptide-specific, neurotoxic, pre-fibrillar assemblies provides an explanation for the strong genetic linkage between Aβ and AD.

4. Aβ oligomerization and AD

Initial efforts to develop targets for AD therapy focused on elucidating the mechanism by which monomeric Aβ assembled into fibrils. This approach was a natural outgrowth of the observation, made originally in the 19th century, that amyloid formation was a prominent feature of the dementia later to be termed "Alzheimer's disease" by Kraepelin [35]. In 1997, two groups reported the discovery of a pre-amyloid intermediate, the protofibril [23,75]. This filamentous assembly was narrower than classical amyloid fibrils (5 nm versus 10 nm), rarely exceeded 150 nm in length, often had a beaded appearance, and frequently was curved, suggesting flexibility. Importantly, cell biological studies showed that protofibrils were potent neurotoxins [73]. A convincing structure–activity correlation was provided by determining the effects of protofibrils on the electrical activity of cultured primary neurons [24]. The addition of protofibrils to these cells caused immediate increases in excitatory post-synaptic currents and the frequency and amplitude of action potentials. Longer term measures of toxicity, including cellular redox activity and cell death, also showed that protofibrils were highly toxic. Primary structure analyses of the Aβ peptide used in these studies, including amino acid analysis, Edman sequencing, and mass spectrometry, demonstrated that no chemical modifications were observable in the starting material. Because metals have been hypothesized to be involved in Aβ assembly, atomic absorption spectroscopy was performed on the buffers used for these and other (see below) in vitro studies. This analysis showed that levels of Zn and Cu, the two metals thought to be most important in AD, in the buffers were at or below the detection limits (Cu = 400 nM; Zn = 100 nM) of the technique (S. Maji and D.B. Teplow, unpublished observations). Thus, if present, Zn existed at a metal/Aβ molar ratio $\leq$1/600 and Cu existed at a ratio $\leq$1/150. These data support the conclusion that protofibril assembly and toxicity are derived from intrinsic characteristics of the native peptide and do not depend on stoichiometric levels of metals.

4.1. Pre-protofibrillar assemblies—the "paranucleus"

Might other biologically active intermediates exist? Having determined that protofibrils, an immediate precursor to fibrils, existed and were neurotoxic, efforts were made to determine whether pre-protofibrillar intermediates formed. To study the earliest phases of Aβ assembly, the initial oligomerization of Aβ monomer, a method was sought that could stabilize metastable structures and allow their characterization and quantitation. This was accomplished using a novel chemical cross-linking method, photo-induced cross-linking of unmodified proteins (PICUP) [16]. PICUP is a powerful method for forming covalent bonds between polypeptides utilizing photolysis of a light-harvesting catalyst (RuII) in the presence of an electron acceptor (e.g., ammonium persulfate). The cross-linking reaction is initiated with visible light (preventing the UV-induced damage associated with standard photo-cross-linking chemistries), occurs within milliseconds, requires no pre facto peptide modification, and is highly efficient (often producing >80% yields). Prior studies have demonstrated that PICUP allows quantitative analysis of the Aβ oligomer size distribution [6].

When PICUP was used to study aggregate-free Aβ preparations within minutes of their preparation, no stable single Aβ species (e.g., monomer or dimer) was found to exist [5,6]. Instead, Aβ(1–40) formed an equilibrium mixture comprising primarily monomer, dimer, trimer, and tetramer (Fig. 3, lane 1). This mixture was observable immediately upon preparation of low molecular weight (LMW; [73]) Aβ, either by filtration through 10 kDa molecular weight cut-off membranes or by size exclusion chromatography. The time necessary for the formation of this oligomer population thus did not exceed minutes (the amount of time required to perform the PICUP chemistry). Aβ(1–42) had a distinct oligomer distribution (Fig. 3, lane 2). Maxima in this distribution occurred at monomer, pentamer/hexamer, and in the M_r range 30–60 kDa. Within this range, bands of nonamer through dodecamer could be resolved and intensity maxima could be observed at dodecamer and octadecamer. Electron microscopy revealed that the smallest Aβ(1–42) oligomers were globular and had diameters ranging from ~2.5 to 5 nm. Taken together with measurements of the diffusion coefficients of the oligomers, done using quasielastic light scattering spectroscopy [40], the data suggested that "nascent" Aβ(1–42) monomer rapidly (with a time constant of minutes or less) forms pentamer/hexamer units which then self-associate to form larger assemblies. Because the initial pentamer/hexamer oligomer appears to be a building block of larger oligomers, the pentamer/hexamer unit has been termed a "paranucleus." Electron microscopy of Aβ(1–42) has shown that these paranuclei can self-associate to form the classic beaded strings that have been described previously in studies of amyloid protein assembly. These findings offer a biophysical explanation for the distinct physiologic activities of Aβ(1–40) and Aβ(1–42), e.g., the apparent increased toxicity of Aβ(1–42) (see Section 3) and the known linkage of elevated Aβ(1–42) concentration with familial AD. By definition, the differences in oligomerization between Aβ(1–40) and Aβ(1–42), and

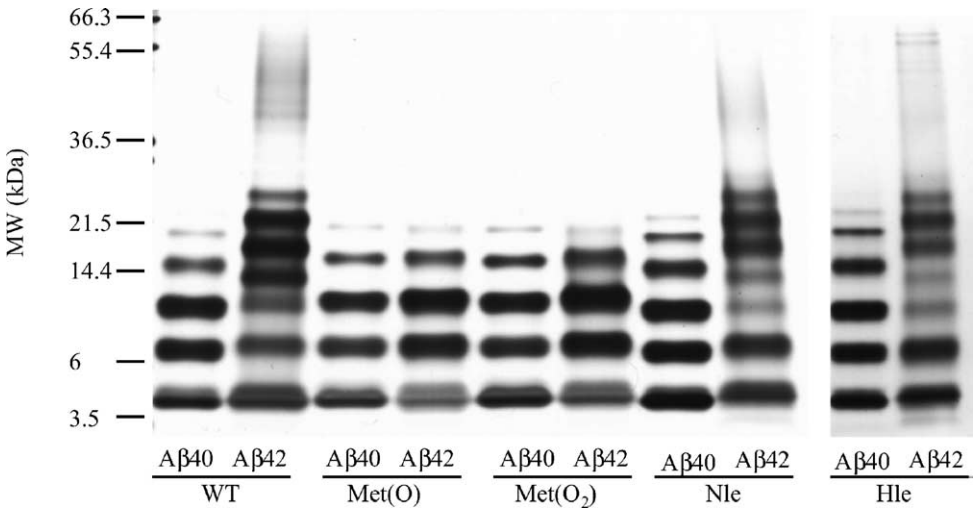

Fig. 3. Effects of residue 35 side-chain structure on Aβ oligomerization. PICUP was performed on SEC-isolated LMW Aβ(1–40) (lane 1), Aβ(1–42) (lane 2), and analogues containing the modifications $Met^{35} \rightarrow Met(O)$ (lanes 3 and 4), $Met^{35} \rightarrow Met(O_2)$ (lanes 5 and 6), $Met^{35} \rightarrow Nle$ (lanes 7 and 8), and $Met^{35} \rightarrow Hle$ (lanes 9 and 10). Following cross-linking, the products were analyzed by SDS–PAGE and silver staining. Positions of molecular weight markers are shown on the left. The gel is representative of each of three independent experiments.

the resulting differences in biologic activity between the two peptides, are due to the addition of the C-terminal Ile–Ala dipeptide. (The intriguing fact that paranucleus self-association can produce dodecamers, an oligomer size seen with ADDLs, has not escaped our notice. Whether and how these structures are related is an area of active investigation.)

Subsequent experiments have examined systematically the effects of changes in amino acid sequence at the C-terminus of Aβ(1–42) [5,8]. These studies revealed that amino acid 41 was critical for formation of paranuclei. Substitution of Gly ($C_\alpha H$ group) or Ala ($C_\alpha CH_3$ group) at position 41 largely abrogated paranucleus formation, whereas Val ($C_\alpha CH(CH_3)_2$ group), Leu ($C_\alpha CH_2CH(CH_3)_2$ group), and Ile ($C_\alpha CH(CH_3)CH_2CH_3$ group) formed paranuclei normally. Residue 42 was critical for paranucleus self-association. Aβ(1–41) and [Gly^{42}]Aβ(1–42) formed paranuclei, but no higher order oligomers. Ala42Val paranuclei did self-associate. Increasing the hydrophobicity of the C-terminus by amidation also increased paranucleus self-association.

4.2. Familial AD (FAD) and Aβ assembly

The etiology of FAD provides additional evidence that nascent, unmodified Aβ peptides are the proximate effectors of neurotoxicity. In FAD kindreds, mutations in the APP gene cause over-production of Aβ, increased synthesis of Aβ(1–42) relative to Aβ(1–40), or synthesis of peptides containing single amino acid substitutions. Biophysical studies of the mutant Aβ alloforms have provided clues as to the mechanism by which these mutations act. A Glu22Gly substitution has been found to cause FAD in a Swedish kindred [49]. In vitro studies have shown that this substitution causes accelerated protofibril formation, an observation linking enhanced formation of neurotoxic assemblies with disease etiology. Mutations causing Asp7Asn (the Tottori mutation; [72]) and Asp23Asn (the Iowa mutation; [19]) changes recently have been identified. These mutations produce familial AD, and in the Iowa case, an associated cerebral amyloid angiopathy. Interestingly, these substitutions occur at sites shown in earlier in vitro studies to control the formation of an oligomeric precursor of protofibrils [31]. One of the earliest identified mutations within the Aβ region of AβPP, the Dutch mutation (Glu22Gln), profoundly increases both the fibril nucleation rate and the fibril elongation rate [69]. In each of these cases, changes in assembly kinetics are linked to disease.

4.3. The role of Met^{35} in controlling Aβ assembly

It has been suggested that Aβ redox reactions involving Met^{35} are involved in the pathogenesis of AD (see Butterfield and Bush, this volume, and [70,71]). To explore the question of how redox chemistry at Met^{35} might affect the biophysical properties of Aβ, recent studies have examined the assembly behavior of Aβ peptide containing $Met(O)^{35}$ [28,52]. We have used the PICUP approach to study the oligomerization of wild type and oxidized forms of Aβ (Fig. 3 and [7]). The oxidized forms included [$Met(O)^{35}$]Aβ(1–40), [$Met(O_2)^{35}$]Aβ(1–40), [$Met(O)^{35}$]Aβ(1–42), and [$Met(O_2)^{35}$]Aβ(1–42). In addition, norleucine (Nle) and homoleucine (Hle), amino acids in which the CH_3S– side chain of Met is replaced with CH_3CH_2– or $(CH_3)_2CH$–, respectively, were studied. Oxidation of Met^{35} either to the sulfoxide (O) or sulfone (O_2) had no effect on Aβ(1–40) oligomerization. Surprisingly,

either oxidation product totally blocked paranucleus formation by Aβ(1–42). In fact, the [Met(O)^{35}]Aβ(1–42) and [Met(O_2)^{35}]Aβ(1–42) peptides oligomerized indistinguishably from wild type Aβ(1–40), producing a dynamic equilibrium among monomer, dimer, trimer, and tetramer. The Nle and Hle alloforms of Aβ(1–40) and Aβ(1–42) behaved very similarly to the corresponding wild type peptides. Analysis of the physical chemistry of the substitutions suggested that the polarity of the C_{γ}^{35} substituent, as opposed to its van der Waals volume, was the key factor controlling paranucleus (Aβ(1–42)-like) oligomerization. Interestingly, subsequent studies of the membrane association of Aβ have shown that Met oxidation results in the release of Aβ from the plasma membrane [3]. This effect also was postulated to be related to the increased polarity of the oxidized side-chain [3]. Taken together, these data emphasize the fact that the key factor controlling the biological activity of Aβ is its biophysical state. This state determines peptide conformation, assembly properties, and phase behavior (partitioning among membrane and aqueous phases), characteristics of direct relevance to the neurotoxic potential of Aβ. Viewed from this perspective, Met oxidation affects Aβ behavior by driving its assembly down pathways normally traversed by Aβ(1–40), resulting in Aβ(1–40)-like behavior—a type of behavior thought to be less pathogenic than that of Aβ(1–42).

5. Conclusions

In this brief review, evidence has been presented that AD is, in part, a synapse failure which is a direct result of the formation of toxic, synapse-specific, oligomeric, Aβ ligands. The assembly of these toxic oligomers is intrinsic to the primary structure of Aβ and does not require chemical modification of the peptide or the invocation of peptide-associated enzymatic activity. The native Aβ assemblies discussed here all have identical primary structure—including Met^{35} and the histidines involved in metal coordination. However, despite this identity, the biological functions of the assemblies differ. Therefore, the mechanistic basis for these differences must depend on conformational effects, and as discussed by Anfinsen [2], it is the primary structure of Aβ which controls the population of different conformational states. We note that the disease mechanisms promulgated here are not exclusionary. As discussed by Butterfield and Bush (this volume), substantial evidence from both in vitro and in vivo studies supports a role for metals and redox reactions in the etiology of AD. What remains unknown is to what degree these various mechanisms contribute to the clinical course of the disease.

6. Conflict of interest statement

WL Klein is co-founder of Acumen Pharmaceuticals, Inc., which has an exclusive license from Northwestern University and the University of Southern California to develop Aβ-Derived Diffusible Ligands (ADDLs) for Alzheimer's disease diagnostics and therapeutics.

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