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

Permalink
https://escholarship.org/uc/item/2pg4858t

Journal
Neurobiology of Aging, 25(5)

ISSN
0197-4580

Authors
Klein, WL
Stine, WB
Teplow, DB

Publication Date
2004-05-01

DOI
10.1016/j.neurobiolaging.2004.02.010

Peer reviewed
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

Received 1 December 2003; received in revised form 4 February 2004; accepted 12 February 2004

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.

© 2004 Elsevier Inc. All rights reserved.

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
do not depend on the ability of Aβ to catalyze chemical re-
actions. 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
prioncy of fibrils was undisputed. However, new evidence
supports the hypothesis that pre-fibrillar structures, includ-
ing 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 synap-
tropic 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 signal-
ing events essential for long-term memory formation. We
note that the conclusions reached regarding mechanisms
of Aβ-mediated neurotoxicity are consistent with the ex-
perimental 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 tri-
genome 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 pro-
longed maintenance of LTD coupled with inhibition of LTP
gives an overall shift in net synaptic activity toward inhibi-
tion. Whether this shift leads to decreased stability in synap-
tic 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-degenera-
tive inhibition of LTP led to the somewhat surprising predic-
tion that AD memory loss in its early stages might actually
be reversible [33,38]. This prediction was confirmed inde-
pendently 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 mem-
ory 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 trans-
genomic mice by three groups thus provide significant evidence
for the hypothesis that memory loss is a synaptic dysfunc-
tion triggered by soluble Aβ assemblies (e.g., ADDLs).
ADDL-induced neuronal dysfunction provides a cell bi-
ological rationale for the memory-specific nature of AD,
epecially characteristic in the early stages of the disease.
An underlying concept is that ADDLs are ligands that at-
tack 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 demen-
tia 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 inter-
fer with memory-associated phenomena. The crucial
question is whether ADDLs exist in vivo and ex-
hibit the same toxic properties. It has become possible
to address this issue using conformation-specific antibod-
ies 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 mix-
tures 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 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.

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 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.
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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation 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 dysregulation of function. However, these plaques appear “at the wrong time and in the wrong places” to explain the clinical progression of dementia in human...
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.

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 La Du, 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 apolipoprotein 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 modification to Aβ. These studies of the formation of peptide-specific, neurotoxic, pre-fibrillar assemblies provide 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 ≤1/600 and Cu existed at a ratio ≤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 photolysysis 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, 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 Met35 → Met(O) (lanes 3 and 4), Met35 → Met(O2) (lanes 5 and 6), Met35 → Nle (lanes 7 and 8), and Met35 → 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–H group) or Ala (C–CH3 group) at position 41 largely abrogated paranucleus formation, whereas Val (C–CH(CH3)2 group), Leu (C–CH2CH(CH3)2 group), and ile (C–CH(CH(CH3)2)CH3 group) formed paranuclei normally. Residue 42 was critical for paranucleus self-association. Aβ(1–41) and [Gly42]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. Bio-physical studies of the mutant Aβ allotypes 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 Met35 in controlling Aβ assembly

It has been suggested that Aβ redox reactions involving Met35 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 Met35 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(O2)35]Aβ(1–40), [Met(O)35]Aβ(1–42), and [Met(O2)35]Aβ(1–42). In addition, norectine (Nle) and homoleucine (Hle), amino acids in which the CH3 side chain of Met is replaced with CH3CH2– or (CH3)2CH–, respectively, were studied. Oxidation of Met35 either to the sulfoxide (O) or sulfone (O2) had no effect on Aβ(1–40) oligomerization. Surprisingly,
either oxidation product totally blocked paraneuronal for-
mation by Aβ(1–42). In fact, the [Met(O)35]Aβ(1–42) and [Met(O)35]Aβ(1–42) peptides oligomerized indistinguish-
ably from wild type Aβ(1–40), producing a dynamic equilib-
rium 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 C35 substituent, as opposed to its van der
Waals volume, was the key factor controlling paraneuronal
(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 biophys-
ical state. This state determines peptide conformation, as-
sembly properties, and phase behavior (partitioning among
membrane and aqueous phases), characteristics of direct rel-
evance to the neurotoxic potential of Aβ.[2]

4. Conclusions

In this brief review, evidence has been presented that AD
is, in part, a synapse failure which is a direct result of the for-
mation of toxic, synapse-specific, oligomeric, Aβ ligands.
The assembly of these toxic oligomers is intrinsic to the pri-
mary structure of Aβ and does not require chemical modifi-
cation of the peptide or the invocation of peptide-associated
enzymatic activity. The native Aβ assemblies discussed here
all have identical primary structure—including Met35
and the histidines involved in metal coordination. How-
ever, 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.

5. Conflict of interest statement

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

Acknowledgments

We gratefully acknowledge Dr. Mary Jo LaDu for her
significant contributions to this article and her generous
support. Funding for this work was provided by the National
Institutes of Health (NS38332, AG18921, and NS44147
(D.B.T.), AG11385, AG18877, and AG022547 (W.L.K.),
the National Science Foundation (EEC0118025 (W.L.K.),
the Foundation for Neurologic Diseases (D.B.T.), the Illi-
nois Department of Public Health Alzheimer’s Disease Re-
search Fund (33280010) (W.L.K.), the Boothroyd, Feiger,
and French Foundations (W.L.K.), the Institute for the Study
of Aging (W.L.K.), a benefactor of Northwestern Univer-
sity (W.L.K.), and Acumen Pharmaceuticals, Inc. (W.L.K.).
W.B.S. was supported by the Charles Walgreen Jr. Fund,
and by NIH grants AG19121, AG021184, and AG13854,
and Alzheimer’s Association grant IIRG-01-3073 (to M.J.
LaDu).

References

[1] Allison DW, Chevin AS, Gelland VI, Craig AM. Postsynaptic
scaffolds of excitatory and inhibitory synapses in hippocampal
neurons: maintenance of core components independent of actin
Williamson NA, et al. Neurotoxic, redox-competent Alzheimer’s
β-amyloid is released from lipid membrane by methionine oxidation.
loss is greater in presenile than senile onset Alzheimer disease:
implications for the cognitive reserve hypothesis. Neuropathol Appl
Teplow DB. Amyloid β-protein (Aβ) assembly. Aβ40 and Aβ42
oligomerize through distinct pathways. Proc Natl Acad Sci USA
2003;100:130–5.
erization—permutation interactions revealed by photo-induced
84.
al. A molecular switch in amyloid assembly: Met35 and amyloid
elements controlling early amyloid β-protein oligomerization. J Biol
HAO, Dobson CM, et al. Ultrastructural organization of amyloid
immunodetection of synthetic and endogenous amyloid β oligomers
and its application to Alzheimer’s disease drug candidate screening.


