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Cytotoxic Intermediates in the Fibrillation Pathway: Aβ Oligomers in Alzheimer's Disease as a Case Study

William L. Klein

1. Abstract

Multiple diseases, as diverse as diabetes and mad cow disease, exhibit accumulations of abnormal protein fibrils. Generically referred to as "amyloid," these self-assembling fibrils typically have been considered the pathogenic molecules that cause cellular degeneration (toxins, not just tombstones). A prominent example is the "amyloid cascade hypothesis" proposed for Alzheimer's disease (Hardy and Higgins, 1992). Fibrils, however, are not the only toxins generated by protein self-association, probably in some cases not even the most relevant ones. We now know of toxic subfibrillar species—soluble oligomers and protofibrils. The emerging hypothesis considered here is that these novel subfibrillar assemblies, the hidden toxins, constitute significant pathogenic molecules in diseases of fibrillogenic proteins.

Clues leading to this hypothesis have come in many instances from studies of Aβ (Klein *et al*., 2001), the fibrillogenic peptide responsible for amyloid plaques in Alzheimer's disease (AD). Alzheimer's disease is the most common form of dementia in the elderly, affecting 10% of individuals older than 65 (Hebert *et al*., 2003) and more than 25 million individuals world-wide. This chapter examines the investigation of Aβ's role in AD essentially as a case study. Its objectives are to (1) review the link between Alzheimer's dementia and fibrillogenic proteins and show that pathogenesis truly involves Aβ; (2) show how key problems in the amyloid cascade hypothesis disappear with the discovery of subfibrillar Aβ assemblies; (3) discuss cellular mechanisms of the new toxins that explain why AD is a disease of memory loss; (4) consider data that clinically substantiate a new, oligomer-initiated amyloid cascade hypothesis; (5) assess whether the impact of subfibrillar toxins can provide a broad mechanism applicable to multiple fibrillogenic proteins; (6) evaluate emerging implications for therapeutics and diagnostics.

At present, no cause for AD has been established; there are no effective therapeutics, and no clinical diagnostics exist. This situation, however, is rapidly changing. New insights into disease mechanisms and remarkable new therapeutic antibody strategies give us cause for optimism.

2. AD Is a Dementia Involving the Fibrillogenic Aβ Peptide

At its outset, AD is almost purely a disease of crippling memory loss (Selkoe, 2002). It first blocks the ability to form new memories and later blocks retrieval of stored ones. The experience of AD dementia is hard to imagine, but a sense of its devastation comes from the story of Auguste D,

a woman in her late forties whom Alois Alzheimer treated a century ago (Alzheimer, 1907). Working with this first AD patient, Alzheimer once asked Auguste D to write her name. Unable to do so, she said, "I have lost myself . . ." her nonscientific words revealing the real meaning of AD. With time, dementia grows inexorably severe, leaving patients in a vegetative state (Coyle, 1987). The disease also accelerates the end of life, with AD and its complications representing the fourth leading cause of death. Auguste D, whose early onset probably reflected the more virulent familial AD, died in her mid-fifties. Life span of patients with sporadic AD is variable and increasing. President Ronald Reagan lived 20 years following his initial diagnosis.

The neuropathology of AD is complex and includes brain inflammation, shrinkage of the hippocampus and cerebral cortex, and degeneration of specific neuronal populations (Terry, 1999). The primary pathology linked to AD, however, comprises two types of insoluble proteinaceous deposits, first codified by Alzheimer's findings with brain tissue from Auguste D. Work from the last 20 years has determined that these deposits comprise (1) extracellular polymers of the amyloid beta peptide (Aβ), which make up AD's "plaques," and (2) intraneuronal polymers of hyperphosphorylated tau, which make up AD's "tangles." Presence of "plaques and tangles" in higher cognitive brain regions of a demented patient provides the definitive diagnosis of AD (Braak and Braak, 1991).

Accumulation of fibrillar Aβ in plaques underlies the dominant theory for AD—the "amyloid cascade hypothesis." The hypothesis, formalized in its original form by Hardy and Higgins in 1992 (Hardy and Higgins, 1992), has been extraordinarily fertile, with reference to Aβ found in over 10,000 papers. Two key questions can be asked: (1) Why has the hypothesis been so compelling? And (2) Why has it not been accepted?

Strong support for the amyloid cascade hypothesis comes from pathology, human genetics, biochemistry, and cell biology. Twenty years ago, the key constituent of amyloid fibrils was identified by Glenner and Wong (1984) as a ~4 kDa peptide now called Aβ. Monomeric Aβ is a physiological peptide that derives proteolytically from amyloid precursor protein (APP), a single-span transmembrane protein. Aβ contains part of the APP transmembrane domain in tandem with a portion of the juxtamembrane domain. The extent of the membrane domain found in Aβ is variable, producing peptides of 39–43 amino acids. Most common is the 40-amino acid species. Physiological proteolysis of APP is complex and highly regulated (da Cruz e Silva EF and da Cruz e Silva OA, 2003), and mutations in APP that are highly conserved increase Aβ production. Most significantly, these APP mutations will cause AD (Chartier-Harlin *et al*., 1991; Goate *et al*., 1991).

Although APP mutations are rare, their discovery provided a breakthrough in understanding disease mechanism. APP-linked familial AD is indistinguishable from sporadic AD, the most common form of the disease; it also is identical to that caused by mutations in presenilins, which underlie a somewhat more frequent familial AD (Price *et al*., 1998). Comparison of dementia, pathology, and metabolic consequences among the various etiologies indicates the common denominator is elevated production of the 42-amino acid form of Aβ (Borchelt *et al*., 1996). The particular peptide form is relevant because Aβ42 readily makes fibrils, especially in comparison to the less hydrophobic and much more abundant Aβ40. When anomalously induced in brain by mutations or other disease-linked factors, elevated Aβ42 precipitates as insoluble deposits of amyoid fibrils. *Ex vivo* experiments have pointed to the pathogenic significance of these insoluble fibrils. First, fibrils were found to form *in vitro* from synthetic Aβ (Pike *et al*., 1991; Hilbich *et al*., 1991; Burdick *et al*., 1992), essentially mimicking disease pathology and providing a tool for toxicology. Second, fibrillar preparations applied to cultured neurons exert an impact clearly relevant to AD pathogenesis: they induce AD-like tau phosphorylation (Lambert *et al*., 1994), implicating amyloid fibrils in the generation of AD's diagnostic tangles, and, most significantly, they kill CNS neurons (Pike *et al*., 1991, 1993; Lorenzo and Yankner, 1994).

Data from multiple disciplines thus show amyloid fibrils to be self-assembled neurotoxins generated in the AD brain by elevated levels of monomeric Aβ42, a peptide firmly linked by pathology and genetics to disease mechanism. Such lines of evidence provided a firm footing for the fundamental principles of the original amyloid cascade hypothesis: (1) the primary molecular pathogen in AD was presumed to be the insoluble amyloid fibril, and (2) AD dementia was presumed to derive from fibril-induced nerve cell death.

3. Why the Fibril-Based Cascade Hypothesis Unraveled: A Singular Illustration with a Transgenic Mouse AD Model

Despite its great theoretical and experimental appeal, the amyloid cascade hypothesis in its original formulation has proven to be a flawed concept, never attaining a consensus for its acceptance. Epitomizing the fundamental flaws in the original amyloid cascade hypothesis are results from an extraordinary experimental vaccine study by Dodart *et al.* (2002).

The Dodart protocol administered monoclonal antibodies against Aβ to transgenic (tg) mice carrying the hAPP gene. These mice provide a powerful model of early AD, manifesting age-dependent appearance of amyloid plaques along with age-dependent memory loss. Dodart's study produced two significant findings, each inconsistent with the original amyloid cascade hypothesis. First, vaccinated animals exhibited reversal of their memory loss, with recovery evident in as little as 24 hours. Reversibility cannot be reconciled with a mechanism for memory loss based on nerve cell death. Second, therapeutic benefits of the anti-Aβ antibodies accrued despite no loss of amyloid plaques. Thus, the amyloid fibrils were not the pathogenic agents. Kotilinek *et al.* (2002) independently confirmed that memory loss is reversible and plaque-independent in studies with different mice and different Aβ antibodies. Earlier, prophylactic vaccine studies also had indicated memory loss was fibril-independent (Janus *et al*., 2000; Morgan *et al*., 2000).

Results with the experimental Aβ vaccines cannot be explained by the original amyloid cascade hypothesis. They are consistent, however, with earlier concepts regarding AD pathology and plaque independence. Scheibel, for example, suggested almost 30 years ago that neuron death may be less significant for dementia than structural changes in neuronal morphology relevant to synaptic function (Scheibel and Tomiyasu, 1978). Neuropathologists, moreover, have long raised concerns over poor correlations between amyloid plaque burden and AD dementia (Katzman *et al*., 1988; Terry, 1994). With respect to plaque burden in AD models, a lack of correlation between amyloid and brain dysfunction caused many early tg hAPP strains to be rejected as inappropriate models of AD (Klein *et al*., 2001). In fact, they may have been excellent models of early AD dementia, but one caused by subfibrillar Aβ toxins, not amyloid fibrils.

4. If Not Fibrils, What? Discovery of Aβ's Hidden Toxins

Molecular-level experiments identified another crucial failing of the amyloid cascade, a finding that ultimately led to discovery of the cascade's missing link. First of all, early benchmark discoveries by Pike *et al.* (1993) and Yankner *et al.* (Lorenzo and Yankner, 1994) had established that synthetic Aβ preparations could kill central nervous system neurons, but only after Aβ had self-associated. Initially, it appeared that the structural state required for toxicity was fibrillar, a reasonable inference given the abundance of fibrils in toxic preparations along with the diagnostic presence of fibrils in AD brain. Inhibitors of fibrillogenesis thus were expected to block neurotoxicity, a prediction that

stimulated significant effort within the pharmaceutical industry. This prediction, however, proved to be untrue.

Oda and colleagues (Oda *et al*., 1994, 1995) reported in the mid-1990s that clusterin, an AD-upregulated protein also known as ApoJ, blocked the *in vitro* formation of large Aβ42 aggregates. Clusterin therefore should have blocked neurotoxicity. However, in experiments with the PC12 pheochromacytoma cell line, clusterin-treated solutions of Aβ were even more toxic than untreated solutions **(Figure 4-1; see color insert)**. Inhibition of Aβ fibrillogenesis without loss of toxicity also was observed by Butterfield and colleagues in experiments with glutamine synthetase (Aksenov *et al*., 1996). Oda *et al*. concluded that the role of fibrillar Aβ in AD pathogenesis required reevaluation (Oda *et al*., 1994).

The toxicology experiments along with the animal model studies establish an important conclusion: fibrils cannot be the only toxin produced by Aβ self-assembly. Other species, not as obvious as fibrils, must also exist. Additionally, these hidden toxins should carry sublethal activities, to account for the reversibility of memory loss in vaccination protocols.

Pursuit of Aβ assemblies formed in the presence of clusterin led to the discovery of novel subfibrillar toxins, identified as small globular Aβ oligomers (Lambert *et al*., 1998). Atomic force microscopy has shown clusterin-treated preparations to be entirely free of large or small fibrillar molecules. Toxic preparations contain only globular structures with Z-heights of roughly 4–5 nm. Alternative methods of preparation, not requiring clusterin, have verified the toxins solely comprise assemblies of Aβ (Lambert *et al*., 2001; Walsh *et al*., 2002; Klein, 2002; Chromy *et al*., 2003; Stine *et al*., 2003).

Assessed by nondenaturing electrophoresis, the globular toxins readily enter gels, confirming the absence of fibrils, while denaturing gels show SDS-stable oligomers that range from trimer to 24-mer (Lambert *et al*., 1998; Chromy *et al*., 2003). In solutions prepared at 4 degrees, the predominant SDS-stable oligomers are tetramers, but in solutions boosted to 37 degrees, larger oligomers emerge, predominantly 12-mers. After their formation, oligomers appear metastable, showing no rapid conversion to fibrils (Klein, 2002; Chromy *et al*., 2003).

5. Oligomers Have Profound Neurological Impact, Accounting for Reversibility of Memory Loss

Aβ oligomers are similar in size to an average soluble globular protein, and would be invisible to conventional neuropathology. Supposing a relevant neurological impact, the presence of such molecules in brain potentially could explain the poor correlation between fibril deposits and dementia. They would be, in essence, the missing links in the amyloid cascade. In fact, the relevant impact as well as the association of oligomers with AD pathology has been experimentally proven.

Neurologically, AD is characterized at its outset by a specificity for memory loss (Selkoe, 2002), and a valid molecular mechanism must account for this benchmark feature of the disease. A classic experimental paradigm for investigating memory mechanisms has been hippocampal long-term potentiation (LTP). In LTP, the electrophysiological synaptic output in response to a defined input grows larger after a brief burst of excitation (Bennett, 2000). The paradigm essentially is an electrophysiological training session, with a long-lasting potentiated synaptic output measurable for days or even longer. Long-term potentiation is not memory per se, rather a form of synaptic plasticity, but its unique properties suggest a close linkage to memory mechanisms (Malenka, 2003). Long-term potentiation has proven to be highly sensitive to Aβ oligomers.

Exposure of hippocampal slices to fibril-free oligomer preparations completely inhibits LTP (Lambert *et al*., 1998; Wang *et al*., 2002). Inhibition occurs within minutes, and stems from disrup-

tion of signal transduction rather than synaptic degeneration—electrophysiological activity in axons and synapses remains normal. Originally observed through *ex vivo* experiments, oligomer-induced loss of LTP has been confirmed in animal studies employing cerebral microinjections (Walsh *et al*., 2002). The possibility that contaminating monomers of Aβ might be responsible has been ruled out through application of insulin degrading enzyme, a protease that degrades monomers but not oligomers (Walsh *et al*., 2002).

Long-term potentiation experiments have been critical in establishing that subfibrillar assemblies of Aβ are neither inert nor neurologically irrelevant. Inhibition of this classic paradigm for learning and memory is profoundly relevant to the neurological dysfunction of AD. The impact, which is rapid and highly selective, also suggested an iconoclastic prediction: memory loss in early AD might prove to be reversible (Lambert *et al*., 1998; Klein *et al*., 2001). As discussed above, such reversibility was indeed found in tg mouse models by Dodart *et al*. (2002) and Kotilinek *et al*. (2002).

The globular Aβ oligomers that inhibit LTP have been referred to as ADDLs (for Aβ-Derived Diffusible Ligands with a dementing activity), based on their neurological impact coupled with their diffusible nature and capacity for specific targeting of cell surface proteins (next section).

6. How Oligomers Attack Neurons—A Molecular Mechanism for Why AD Is Specific for Memory Loss

The impact of oligomers on LTP is in harmony with the concept that synapse failure, prior to neuron death, underlies AD memory loss (Klein *et al*., 2001; Selkoe, 2002). Additional strong support for this concept comes from recent cell biology experiments that reveal how oligomers associate with neurons.

It has been suggested that oligomers might insert directly into membranes (Arispe *et al*., 1994), perhaps after partially unfolding. This suggestion is consistent with the fact that Aβ contains a significant hydrophobic domain. Atomic force microscopy (AFM) has shown, moreover, that Aβ can form structures with pore-like appearance (Chromy *et al*., 2003; Lashuel *et al*., 2003), while ion flux data indicate Aβ can generate functional pores in model membranes (Arispe *et al*., 1993; Rhee *et al*., 1998). Because of the ensuing ion flow, such pores in cell membranes potentially would be cytotoxic.

On the other hand, oligomers are readily water soluble and SDS stable, presumably because their hydrophobic domains are sequestered within the globular structure. This reduces the likelihood they insert readily into plasma membrane lipids. Also at odds with lipid insertion, oligomers do not attach to cells that have been trypsinized (Lambert *et al*., 1998). Perhaps most cogently, simple insertion predicts that oligomers should attach to cells nonspecifically. This prediction has not borne out. Instead, the pattern of binding is strikingly specific and most significant.

In experiments with differentiated cell cultures, oligomers clearly bind nonrandomly (Lambert *et al*., 2001; Gong *et al*., 2003). Oligomers added to hippocampal cultures attach to particular neurons, not to all, while in cerebellar cultures they bind to almost no cells. This neuron-selective ligand activity presumably derives from the quaternary structure of oligomers. Supporting this idea, size-exclusion chromatography indicates ligand activity fractionates with larger oligomers (12–24 mers), not with monomers or very small oligomers (Chromy *et al*., 2003). Most significantly, specificity at the subcellular level is striking: binding sites localize in dendritic arbors where they comprise discrete punctate clusters (Gong *et al*., 2003). This pattern is exactly as would be predicted for synaptic localization.

Very recent experiments have validated the prediction that oligomers specifically target synapses (Lacor *et al*., 2003). Oligomer binding sites in hippocampal neuron cultures, analyzed for colocalization with the synaptic marker PSD-95 (Allison *et al*., 2000), occur at synapses more than 90% of the time. Not all synapses are targeted, only about 50%, further validating the predicted ligand-like binding. Specific binding implies a receptor target, but as yet no receptor for oligomers has been identified. The discovery that oligomers are ligands that attach to particular synapses provides strong support for the hypothesis that memory loss in AD is a synapse failure caused by synapto-toxic Aβ oligomers.

7. Immediate Consequences of Oligomer Binding: Signal Transduction Targets

What happens at synapses immediately after oligomers attach? Three broad alternatives, originally proposed for the toxicity of fibrillar Aβ (Yankner, 1996), apply equally well to oligomers and are under investigation. First, oligomers could generate localized ion flux via transmembrane pores (Caughey and Lansbury, 2003), as observed in model membranes (Arispe *et al*., 1994). Second, oligomers might generate synaptically localized oxidative damage. A large number of studies have shown that oligomers as well as fibrillar forms of Aβ generate reactive oxygen species (Hensley *et al*., 1995; Longo *et al*., 2000; Mattson, 2004). Third, binding to specific toxin receptors could lead indirectly to a downstream impact on signaling pathways (Lambert *et al*., 1998).

No matter what the trigger, it has become clear that oligomers do cause particular changes in molecular signaling pathways germane to synaptic plasticity and memory mechanisms. Early findings of Cotman and colleagues with cultures of cortical neurons showed oligomers at low doses blocked the ability of the neurotransmitter glutamate to induce CREB phosphorylation (Tong *et al*., 2001), a signaling event implicated in gene regulation relevant to memory formation. A detailed investigation germane to the mechanism of LTP inhibition has come from Wang *et al*. (2004a), who recently undertook a pharmacological strategy to investigate the role of specific protein kinases and receptors. The ability of particular agents to abolish oligomer-induced inhibition of LTP has implicated c-Jun-terminal kinase, p38 MAP kinase, cyclin-dependent kinase 5, and metabotropic glutamate receptors in the mechanism of synapse failure. In harmony with these observations, hAPP tg mice show activation of c-Jun-terminal kinase and p38 pathways, although the impact localizes to neurons near deposits of amyloid fibrils (Savage *et al*., 2002). A possible relationship between oligomer binding and plaque development is discussed later.

Altered synaptic signaling may be closely associated with altered synaptic structure. Oligomers bound at synapses cause abnormal induction of Arc (Lacor *et al*., 2004), a synaptic immediate early gene normally induced by synaptically activated kinases (Ying *et al*., 2002). Arc protein in synaptic spines appears to affect f-actin organization (Lyford *et al*., 1995), and it influences trafficking of glutamate receptors (Rial Verde *et al*., 2003). Arc is of special interest because long-term memory formation requires its proper, transient expression. It has been proposed that sustained Arc expression blocks memory formation (Guzowski, 2002), and, in fact, its overexpression in Arc tg mice produces poor learners (Kelly and Deadwyler, 2003). In oligomer-treated cultures, Arc is induced at synapses within minutes; its expression, however, remains elevated for hours, and Arc, in affected neurons, spreads ectopically throughout dendritic arbors (Lacor *et al*., 2004). An appealing hypothesis is that sustained Arc expression caused by oligomers produces synapse failures that underlie AD memory loss. This hypothesis suggests two testable predictions: In an Arc-dependent manner, oligomers should (1) inhibit glutamate receptor upregulation, which is involved in synaptic plasticity, and (2)

generate abnormalities in synaptic spine structure. Current evidence links abnormal spine structure with mental deficiencies and possibly with AD (Fiala *et a*l., 2002).

8. Cascading Consequences—Can Oligomer-Induced Synapse Dysfunction Lead to Synapse Destruction and Neuron Death?

Alzheimer's disease is a progressive dementia, and its pathology extends beyond synapse failure to encompass synapse destruction and neuron death. Experiments with tg animal models and brain slice cultures indicate these degenerative phenomena could derive from oligomer toxicity.

Theoretically, oligomer-induced synapse dysfunction itself might engender synapse loss. Because oligomers inhibit LTP, but prolong LTD (long-term depression), they profoundly shift synaptic activity from higher to lower levels (Wang *et al*., 2002). Activity levels profoundly influence synapse formation during brain development (Constantine-Paton and Cline, 1998), and it is conceivable that lowered activity due to oligomers might inhibit synaptogenesis in mature brain (adult synaptogenesis is required to replace synapses due to turnover). Alternatively, synapse turnover theoretically might be accelerated directly, triggered by oligomer-induced anomalies in spine structure, perhaps coupled to Arc overexpression. In fact, synapse loss does occur in certain hAPP tg mouse AD models (Hsia *et al*., 1999), including an AD-like degeneration of the cholinergic phenotype (Buttini *et al*., 2002). Synapse loss correlates with levels of the total soluble Aβ, not with fibrillar amyloid (Mucke *et al*., 2000), and the mechanism involves signal transduction through a Fyn kinase pathway (Chin *et al*., 2004). Fyn, which localizes to postsynaptic densities and regulates glutamate receptor activity (Tezuka *et al*., 1999), has been linked to synaptic plasticity, memory formation, Aβ toxicity, and AD pathology (for review, see Klein, 2000).

Ultimately, oligomers destroy neuronal viability. Mature brain slice cultures exposed to oligomers for extended periods (days rather than minutes) exhibit neuron death that is regionally specific (Lambert *et al*., 1998). Parts of the hippocampus are particularly vulnerable, while the cerebellum is spared (Kim *et al*., 2003), as in AD. As seen with synapse loss, the oligomer-induced death also is Fyn-mediated. Whether loss of synapses might lead to death of neurons is speculative, but knockout of a postsynaptic density scaffolding protein has been reported to induce neuronal apoptosis (Gardoni *et al*., 2002). Neuron death might be caused analogously by damage to postsynaptic density proteins by synaptically bound oligomers, perhaps by localized oxidative damage. The case for this hypothesis is weakened, however, by the absence of evidence for neuron death in tg mice models.

9. In Vivo Experimental Support for Synaptotoxic Oligomers: Data from Mouse Models of Early AD

Synthetic Aβ oligomers are synaptotoxins with an experimental impact germane to AD memory loss, so a critical question is whether such oligomers can form in brain. An indication that oligomers can form spontaneously *in vivo* first came from findings that oligomers accumulate in culture media of cells transfected with hAPP (Podlisny *et al*., 1995). These oligomers later were found to be synaptotoxic (Walsh *et al*., 2002). The fact that oligomers can be maintained in solution for days indicates, moreover, a stability sufficient for accumulation in tissue (Chromy *et al*., 2003). Neurological consequences that are plaque-independent in hAPP tg mice support this possibility (Klein *et al*., 2001).

Recent analysis of brain extracts has provided direct confirmation of oligomer accumulation in hAPP tg mice. Dot-blot immunoassay shows the presence of soluble oligomers that are age-,

region-, and transgene-dependent (Chang *et al*., 2003). To minimize extraction artifacts, analysis was performed on soluble fractions obtained without detergents or chemicals that might disrupt fibrillar assemblies. Interestingly, a widely used monoclonal (4G8) that does not discriminate between monomers and oligomers (Lambert *et al*., 2001) does not detect transgene-dependent oligomers that are revealed by oligomer-selective antibodies (L. Chang, unpublished). Measurement of total pools thus provides an insensitive indicator for pathogenic forms of soluble Aβ.

Preliminary evidence from tg mice indicates that when oligomer levels go up, performance on a water-maze memory task goes down [memory evaluated as described by Kotilinek *et al.* (2002); oligomers measured according to Chang *et al.* (2003); data provided by Chang, Kotilinek, and Ashe]. However, overall levels of soluble oligomers are less relevant to memory loss than the extent of compromised synapses. It might be anticipated, moreover, that synaptic dysfunction must exceed some threshold level before memory loss occurs. For example, even though oligomers are present, memory would be normal with subthreshold occupancy of toxin receptors, or even with complete occupancy at a subthreshold fraction of the relevant synapses. This would be analogous to Parkinson's disease, in which motor dysfunction manifests only after 60% of dopamine-producing neurons die. The threshold concept is consistent with the idea that susceptibility to AD memory loss is influenced by an individual's "synaptic reserve" (Mesulam, 1999).

10. Clinical Validation—Oligomers in Human Brain, Elevated up to 70-Fold in AD

The crucial test of the oligomer hypothesis is whether it can be substantiated by clinical data. Are oligomers present in human brain, and do they accumulate in AD? Strong support has been obtained by Kayed *et al*. (2003), who found that diffuse, early-stage plaques in AD brain are stained by an antibody against Aβ oligomers, essentially as predicted by Hardy and Selkoe (2002). Thioflavin-positive dense-core plaques, which contain fibrillar Aβ, do not crossreact. (An intriguing aspect of the Kayed study is that their antibody appears to interact generically with oligomers made from several species of proteins, considered again later.) Another oligomer-selective antibody gives diffuse stain that surrounds neuronal cell bodies, apparently binding oligomers within dendritic arbors (Bigio, Lacor, Lambert, and Viola, unpublished).

Molecular confirmation that oligomers constitute bona fide components of AD pathology comes from recent immunoanalysis of soluble brain extracts (Gong *et al*., 2003). Quantitatively, AD brains contain strikingly elevated levels of soluble oligomers, up to 70-fold more than in controls. These large increases occur in frontal cortex but not cerebellum (L. Chang, unpublished), consistent with the specificity of AD for memory and cognition. Structural analysis confirms that the immunoreactive species are Aβ oligomers, detectable by conformation-sensitive antibodies raised against synthetic oligomers and indistinguishable from synthetic molecules in 2D gels (Gong *et al*., 2003). The most prevalent soluble oligomers are 12 mers, with an isoelectric point of 5.6, although extraction in the presence of SDS indicates the presence of additional species.

Perhaps most importantly, the soluble oligomers in AD brain extracts behave as synaptic ligands (Gong *et al*., 2003; Lacor *et al*., 2003). As with oligomers made *in vitro*, those obtained from brain tissue bind to mature hippocampal neurons at dendritic hot spots **(Figure 4-2; see color insert)** and colocalize with synaptic markers by confocal microscopy. The oligomeric synaptic ligands from AD brain collect in crude fractions between 10 and 100 kDa, consistent with the 2D gel analysis. Water-soluble oligomers in this molecular weight range were observed earlier but were regarded as intermediates en route to fibril formation and neurologically insignificant (Kuo *et al*., 1996). We now know that oligomers are neurologically active, and the fact that oligomers from AD brain can target

hippocampal synapses strongly supports the hypothesis that memory loss in AD is an oligomer-mediated synapse failure.

11. New "Oligomer-Driven" Amyloid Hypothesis

In the seminal 1992 version of the amyloid cascade hypothesis (Hardy and Higgins, 1992), memory loss was considered the consequence of fibril-induced nerve cell death. Like any great theory, the amyloid cascade has evolved with new discoveries. We now know that fibrils are not the only Aβ-derived neurotoxins—Aβ42 readily forms soluble, globular oligomers that target synapses and rapidly alter the molecular machinery of memory. These oligomers, the missing links in the original hypothesis, have been incorporated into an updated cascade (Klein *et a*l., 2001; Hardy and Selkoe, 2002) that features two novel concepts: (1) AD memory loss can be caused by synapse failure, independent of nerve cell death; and (2) the pathogenic agents of synapse failure are small diffusible oligomers, not fibrils. An adaptation of the cascade presented recently by Hardy and Selkoe (2002) is shown in **Figure 4-3; see color insert**.

The new cascade hypothesis accounts well for key features of the disease. Most importantly, it explains why early AD is memory-specific: oligomers are toxic ligands that specifically attack synapses critical for long-term memory formation. It suggests the possibility, moreover, that mild cognitive impairment, a plaque-independent memory loss, could have the same molecular basis; also, were oligomers to stimulate abnormal tau phosphorylation, as found for fibrillar preparations (Lambert *et al*., 1994; Busciglio *et al*., 1995), they could play a role in frontal lobe dementia, another plaque-independent dysfunction associated with tangles (Zhukareva *et al*., 2004). With respect to diffuse plaques, their formation presumably originates in attachment of oligomers to synapses. A threshold for memory loss would be determined by the degree to which synapses can remain unoccupied and functional, giving a cellular basis for the concept of cognitive reserve. Extent of memory loss would reflect the fractional occupancy of toxin receptors at particular synapses as well as fractional occupancy of all at-risk synapses. Fluctuations in occupancy, whether due to ligand disassociation or receptor replacement or synaptic turnover, would account for day-to-day fluctuations in cognitive performance, common in early AD. Finally, given the key role played by diffusible subfibrillar toxins, the new cascade explains why AD correlates so poorly with amyloid plaque burden.

Like the original amyloid cascade hypothesis, the emended cascade presumably will continue to evolve. Three issues for the future will be considered briefly in the remaining sections: (1) what is the relationship between oligomers and the process of fibrillogenesis? (2) Do toxic oligomers of Aβ provide precedent for mechanisms common to multiple fibrillogenic proteins? (3) Does the new amyloid cascade provide a rational basis for effective AD therapeutics and diagnostics?

12. Mechanisms of Aβ Oligomerization and Fibrillogenesis

In multiple milieus (AD brain; mouse models; *in vitro*), Aβ1-42 assembles to form soluble oligomers as well as insoluble amyloid fibrils. How these disparate outcomes are achieved is not fully understood, and whether smaller implies precursor to the larger remains speculative. At issue is whether oligomers and fibrils form via alternative mechanisms. Are there distinct pathways for assembly, or are oligomers simply metastable intermediates that eventually cobble together to create fibrils?

Insight into precursor–product relationships comes from early studies of Aβ fibrillogenesis. Two groups (Harper *et al*., 1997; Walsh *et al*., 1997) independently established the existence of sub-

fibrillar intermediates called protofibrils (PFs), which are structurally distinct from the small oligomers discussed so far. Protofibrils are linear rather than globular, they extend in length up to 400 nm, and they develop masses up to one million daltons. Kinetic experiments indicate that protofibrils are the immediate precursors to full-fledged fibrils (Walsh *et al*., 1997). The pathogenic protofibril-to-fibril pathway, which includes the concept of a critical concentration for monomer to drive assembly, in many respects resembles the physiological process of cytoskeletal protein assembly.

Initially, protofibrils were thought to be relevant only as structural intermediates, existing transiently en route to production of toxic amyloid fibrils. Evidence now indicates that PFs themselves are neurologically active. In neuronal cultures, PFs trigger action potentials (Hartley *et al*., 1999), and they ultimately induce neuronal death (Walsh *et al*., 1999). It has not been reported whether PFs block synaptic plasticity. Unlike small oligomers, however, PFs appear to attach nonselectively to neurons, broadly coating entire cell surfaces (Hartley *et al*., 1999). The presence of PFs in AD brain has not been established, although they reportedly occur in cerebral spinal fluid of AD patients (Pitschke *et al*., 1998). Whether or not PFs are clinically relevant, the current molecular and cellular data indicate that Aβ self-assembles into two different subfibrillar Aβ species, each with cytotoxic activity.

PFs generate fibrils, but do globular oligomers generate PFs? If so, it would place oligomers on the pathway to fibrillogenesis. Under some conditions oligomers and PFs are evident in the same preparation. Molecular imaging by AFM, for example, shows the coexistence of oligomer-sized particles and PFs; PFs, moreover, exhibit a "bead-on-a-string" morphology, suggesting arrays of oligomers that have associated (Walsh *et al*., 1999; Chromy *et al*., 2003) **(Figure 4-4; see color insert)**. Protofibrils subjected to denaturing gel electrophoresis comprise monomers and SDS-stable oligomers. Support for a precursor–product relationship also comes from a new approach to oligomerization and fibrillogenesis using photo-induced crosslinking, which stabilizes all species for subsequent electrophoresis (Bitan *et al*., 2003a). Aβ42 generates three classes of SDS-stable oligomers, and the largest, which comprise 12 mers and 18 mers, appear to give rise to molecules the size of PFs, detected by light scattering. The kinetics, however, have not yet been verified. Significantly, Aβ40, which is much more abundant in brain than Aβ42 and less germane to AD, does not give rise to the larger oligomers. Bitan *et al.* suggest that differences in ability to populate the higher order oligomer states would account for the difference in biological activity between Aβ40 and Aβ42.

Immunoanalysis of oligomers and PFs suggests that if a precursor–product relationship does exist, it is less simple than beads-on-a-string. Antibodies have been generated that recognize oligomers but not PFs or fibrils (Lambert *et al*., 2001; Chromy *et al*., 2003); this implies that if oligomers were to generate PFs, the antibody-sensitive domains either become masked or undergo conformation changes. Precedent for this idea is found in studies of fibrillogenesis of a multiple myeloma immunoglobulin light chain, in which fibrils from off-pathway oligomers are generated only after structural rearrangement of metastable oligomers to a disordered species more prone to fibrillogenesis (Souillac *et al*., 2003).

Relevant to assembly *in vivo*, conditions have been established that permit oligomers to form *in vitro* at low nanomolar Aβ concentrations (Yu *et al*., 2002). Even when prepared at micromolar Aβ, oligomers can be maintained for days without formation of protofibrils (Chromy *et al*., 2003), in harmony with the accumulation of oligomers as molecular entities in AD brain. The basis for the relative stability of oligomers is unknown, although it may derive from the unusual charge structure of Aβ, with its two lengthy hydrophilic/hydrophobic domains. The hydrophobic C-terminal is clearly influential given the much greater stability of Aβ42 oligomers over those of Aβ40 (Stine *et al*., 2003). Photo-induced crosslinking experiments indicate, moreover, that alanine at the 42 position is essential for generating quantum-like jumps in formation of 12 mers and 18 mers (Bitan *et al*., 2003b). Covalent changes also may be relevant. For example, acceleration of oligomerization has been found to be cata-

lyzed by prostaglandin H2, suggested to link pathogenic oligomerization to cyclooxygenase (Boutaud *et al*., 2002), a possible target for AD therapeutics. Small oligomers also are susceptible to interpeptide crosslinking through metal ion-catalyzed oxidative reactions (Atwood *et al*., 2004; Butterfield and Bush, 2004), a finding that has engendered development of copper-selective chelators as possible therapeutic drugs (Cherny *et al*., 2001). Oxidative crosslinking of oligomers may not be required for their SDS stability, because replacement of the redox-relevant methionine at position 35 does not preclude oligomerization (Klein *et al*., 2004), but crosslinking could potentially influence oligomer conformation. Evidence that oligomers of the same size can develop different structures comes from immunoblot experiments with conformation-sensitive antibodies (Chromy *et al*., 2003).

States of initial conformation are likely to be especially relevant to the outcome of self-assembly. Monomeric Aβ, as all proteins, will exist at least transiently in multiple conformation states (Ferreira and De Felice, 2001). Hypothetically, given sufficiently deep energy wells, alternative monomer conformations could be sufficiently long-lived to generate alternative structural outcomes for self-assembly. One metastable conformer, for example, could generate oligomers while another generates protofibrils and fibrils. Conformational shifts may account for the chaperone-like action of ApoJ (clusterin) in promoting oligomer accumulation while retarding fibril formation. Well-recognized inconsistencies in experiments with Aβ suggest the peptide indeed has significant conformational sensitivity to micromilieu. Because experimental conditions influence Aβ and the nature of its self-assembly, considerable potential exists for *in vitro* artifacts. This underscores the value of verifying that experimentally produced oligomers mimic the properties of AD brain-derived oligomers, as recently reported (Gong *et al*., 2003).

Unfortunately, while mechanistic understanding of oligomer and fibril assembly *in vitro* is limited, even less is known regarding assembly *in vivo*. The list of unanswered or partially answered questions is lengthy. What is the origin of oligomers in AD—do they form inside cells or out? Is oligomerization *in vivo* favored by mis-folding, or is it driven simply by mass action, because Aβ has such a strong propensity for self-assembly? Does synaptic binding imply oligomers have physiological functions? Aβ, after all, is a product of remarkably complex processing that involves multiple cell compartments, proteases, trafficking, and signal-dependent regulation. Perhaps oligomers normally control positive synaptic feedback or influence synaptic turnover? Why is oligomer accumulation age dependent? Why, for example, do oligomers not form during development or in mature adults despite the same levels of APP? Are there chaperones present to prevent oligomer formation, perhaps part of the regulatory process? Do these chaperones become less active in AD? What is the relationship between the synaptic binding of oligomers and the formation of amyloid fibrils? Do oligomers at synapses act as seeds for fibril formation? Might binding to toxin receptors alter the conformation of oligomers, engendering formation of bead-on-a-string protofibrils and the subsequent generation of fibrils? Would elimination of oligomers keep new fibrils from forming and accelerate the removal of old ones?

13. Pathogenic Aβ Oligomers—First of Many? All Proteins Likely Have the Capacity to Oligomerize

For AD, the amyloid cascade has been a fertile and adaptive hypothesis, evolving both with respect to toxin structure and the underlying cellular mechanisms. A pertinent question is whether the concepts developed for Aβ might apply to other diseases. Might other fibrillogenic proteins, which have been linked to a multitude of diseases, similarly produce subfibrillar pathogenic assemblies? For at least several cases, the answer appears to be "yes," although the evidence is far from conclusive.

Data from Parkinson's disease (PD), the second most common neurodegenerative disorder in aging, are the most developed. Three mutations have been found that implicate the fibrillogenic protein α-synuclein in loss of dopamine-producing neurons (Caughey and Lansbury, 2003). In PD pathology, fibrils of α-synuclein accumulate intracellularly, in structures called Lewy bodies (Norris *et al*., 2004), while overexpression of α -synuclein in cultured cells causes death of dopaminergic neurons (Xu *et al*., 2002). *In vitro* studies of fibrillogenesis by Conway, Lansbury, and colleagues (Caughey and Lansbury, 2003) show sequential appearance of structures analogous to those described for Aβ: small globular structures, bead-on-a-string protofibrils, and large fibrils. Self-association is promoted by two PD-coupled point mutations in α-synuclein. However, although both mutations accelerate assembly of the globular intermediates, one of the mutations actually inhibits fibrillogenesis (Conway *et al*., 2000), which led to the hypothesis that subfibrillar species constitute the pathogenic agents. Consistent with this idea, strains of tg mice have been developed that lose dopaminergic synapses and develop abnormalities in motor behavior even though their α-synuclein inclusions are nonfibrillar (Masliah *et al*., 2000). Similarly, lentiviral transfection has produced rats whose α-synuclein inclusions are nonfibrillar and that suffer dopaminergic neuron death (Lo Bianco *et al*., 2002); in contrast, transfected rats with fibrillar inclusions show no cell death. A corresponding situation is evident in the human brain: individuals without disease symptoms often present fibril-containing Lewy bodies. These cases (asymptomatic incidental Lewy body disease) are 10 times more frequent than PD (Goldberg and Lansbury, 2000). It has been proposed that incidental Lewy body disease may result when α-synuclein rapidly produces fibrils in a manner that excludes accumulation of pathogenic, subfibrillar species (Caughey and Lansbury, 2003). Similarly, with respect to Aβ, it is not uncommon for individuals to present high levels of amyloid plaques without showing symptoms of Alzheimer's disease (Katzman *et al*., 1988). Mechanistically, the subfibrillar forms of α-synuclein attach and insert into membranes with higher affinity than fibrils (Ding *et al*., 2002), potentially creating cytotoxic pores. Pore-like structures generated *in vitro* by α-synuclein are evident by atomic force microscopy, and PD mutations foster production of the pore-like structures (Volles and Lansbury, 2002). More recently, it also has been reported that α-synuclein oligomers, as well as fibrils, can bind to proteasomes and inhibit protease activity (Lindersson *et al*., 2004).

Unlike PD and AD, the human spongiform encephalopathies are rare, but interest is very high given their ability to be transmitted between individuals and species (e.g., "mad cow disease"). Research largely has focused on verifying that disease transmission is based, surprisingly, on proteins (prions) rather than nucleic acids. Prions in their abnormal, disease-state conformation are fibrillogenic, and they accumulate in diseased brain as ordered aggregates and amyloid plaques (Fournier and Grigoriev, 2001). In disease transmission, pathogenic prions convert normal cellular prions to the abnormal conformation. Conversion may involve disease-state prions in aggregated form, with a stabilizing energy-well provided by aggregation. It has been noted that small oligomers of prions also can be formed, and that their assembly is off-pathway with respect to fibrillogenesis (Baskakov *et al*., 2002). Whether the oligomers are neurotoxic is unknown, although a recent study of the yeast prion system suggests that small oligomeric species are important for transmission (Narayanan *et al*., 2003). At present, the relationship between states of prion aggregation, the structures required for transmission, and those essential for neurotoxicity all remain to be clarified.

A brief overview of other studies further indicates that production of cytotoxic, subfibrillar oligomers is an aspect of pathogenesis common to multiple diseases of fibrillogenic proteins. These oligomers are implicated not only in neurological diseases but in various other organ failures. *(1) ADan and ABri*. Mutations in the BRI gene cause AD-like dementia with additional neurological dysfunctions. The mutations promote release of two fibrillogenic peptides, ADan and ABri, in the brain (Austen *et al*., 2002). The peptides are found, respectively, in familial Danish dementia and familial British dementia. Culture experiments show toxicity associated with formation of SDS-

stable, nonfibrillar, low-molecular-mass oligomers. The small oligomers are more potent in inducing neuronal apoptosis than PFs or fibrils (El Agnaf *et al*., 2001). Recently, deposits of nonfibrillar ADan aggregates have been found in the brain (Gibson *et al*., 2004). *(2) Transthyretin*. Mutations in transthyretin (TTr) produce amyloidoses associated with familiar amyloid polyneuropathy and with familial amyloid cardiomyopathy. Cell culture experiments indicate that small soluble aggregates are cytotoxic species, with no toxicity found associated with TTR amyloid fibrils or soluble aggregates greater than 100 kDa (Reixach *et al*., 2004). *(3) IAPP*. Type II diabetes is associated with islet amyloid polypeptide (IAPP), a fibrillogenic protein cosecreted with insulin. Insulin resistance leads to excessive release of IAPP. Results with a murine model of type 2 diabetes indicate that the formation of islet amyloid, rather than the amyloid per se, is related to increased β-cell apoptosis. These findings, suggesting that pathogenesis, might be coupled to subfibrillar IAPP oligomers rather than islet amyloid (Butler *et al*., 2003), are recently substantiated by findings that IAPP can make toxic oligomers that induce apoptosis in replicating β-cells (Butler *et al*., 2004). IAPP toxins in cell culture are aggregated but not necessarily fibrillar. Protofibrillar species have been observed that exhibit pore-like channel activity, like the PFs of α-synuclein (Anguiano *et al*., 2002). *(4) Immunoglobulin light chain*. Immunoglobulin light-chain amyloidosis is associated with organ damage. As with IAPP, clinical findings suggest the process of amyloid fibril formation (i.e., putatively, oligomer formation) itself exerts toxic effects, independently of the amount of amyloid deposited (Bellotti *et al*., 2000). As mentioned earlier, some light chains generate off-pathway oligomers that must become disordered before fibrils can form (Souillac *et al*., 2003). By *in situ* AFM, immunoglobulin light chain structures can be seen that are similar to the annular or taurus-shaped morphologies found to be toxic with α-synuclein PFs (Zhu *et al*., 2004). *(5) Beta-2-microglobulin*. Serum levels of fibrillogenic β-2 microglobulin rise during renal failure, and dialysis-associated amyloidosis shows deposits in joints, bones, and organs. Evidence from single-channel conductance experiments suggest oligomers of β-2-microglobulin form channel structures, again supporting the oligomer-pore hypothesis for pathogenesis (Hirakura and Kagan, 2001).

Normal soluble proteins, not just those that are disease associated, have a capacity for fibrillogenesis, and they generate fibrils that are indistinguishable from those in pathlogical amyloid deposits (Dobson, 1999). Like their disease-associated counterparts, the normal proteins also produce subfibrillar aggregates. These are sufficiently stable to be isolated, and in two recently studied examples, the subfibrillar species, but not the full-fledged fibrils, were found to be cytotoxic (Bucciantini *et al*., 2002). Bucciantini *et al*. have suggested that inherent cytotoxicity of subfibrillar aggregates might provide a common mechanism for protein misfolding diseases. They further proposed that toxicity derives from the common structural nature of the subfibrillar aggregates, not their specific amino acid sequences. Supporting this, certain antibodies generated against subfibrillar aggregates of Aβ recognize disparate oligomer species regardless of monomer sequence, suggestive of a common conformation-dependent structure (Kayed *et al*., 2003). On the other hand, the highly specific targeting of particular synapses by ADDLs would seem less consistent with completely generic mechanisms of pathogenesis. Conceivably, different aspects of oligomer cytotoxicity may derive from different structural determinants.

14. Therapeutics and Diagnostics—New Strategies

Insights gleaned from examining Aβ oligomers as a case study extend to therapeutics and diagnostics. In a remarkable new strategy that shows increasing promise, AD researchers have been seeking to develop active vaccines and therapeutic antibodies that can neutralize Aβ-derived neurotoxins. This strategy derives from the benchmark discovery by Schenk *et al*. (1999) that amyloid

plaques can be removed from brains of tg mice models by vaccination with preparations of Aβ. These preparations prior to vaccination are aged to promote formation of fibrils, although such preparations are heterogeneous and also contain subfibrillar Aβ species (Chromy *et al*., 2003), which themselves are highly immunogenic (Lambert *et al*., 2001). Two conclusions from the Schenk study were almost completely unexpected: (1) fibrillar plaque molecules, which are resistant to many disrupting agents, can be eliminated biologically; and (2) therapeutic levels of antibodies can actually reach the brain, typically considered to be immuno-privileged. Cognitive benefits of active vaccination subsequently were reported by two other groups in studies that suggested the relevant targets might be subfibrillar (Janus *et al*., 2000; Morgan *et al*., 2000).

Clinical trials ensued shortly after the successful experiments with mouse vaccines, and, although these trials failed, they nonetheless provided a significant proof of concept. Vaccine trials with AD patients were terminated because of high incidence of brain inflammation and occurrence of patient death (Ferrer *et al*., 2004). It is uncertain whether inflammation derived from aberrant T-cell activation by the vaccine or from localized responses induced by antibodies attached to plaques. However, despite its great failings, the AD vaccine trial provided a truly exciting result: in individuals who mounted a robust immune response, the progression of Alzheimer's disease over a 12-month period was stopped in its tracks (Hock *et al*., 2003).

The potential to capture the vaccine's success while avoiding its failings has motivated vigorous efforts to develop therapeutic monoclonal antibodies by Elan, Lilly, and Merck. Unlike active vaccines, such antibodies would be unaffected by problems such as T-cell-mediated inflammation or the impaired immune response of elderly individuals. Precedent for success is evident in the memory-recovery experiments with tg mice models (Dodart *et al*., 2002; Kotilinek *et al*., 2002), which also indicated that elimination of subfibrillar species is more relevant than elimination of fibrils. Antibodies already have been described that neutralize oligomers without binding Aβ monomers or fibrils (Chromy *et al*., 2003). Such specificity would lower antibody dosage by avoiding monomers and further reduce risks of inflammation by not binding plaques. Soluble oligomers thus appear to present an optimal target for therapeutic antibodies.

Oligomers also may be important targets for therapeutic drugs. Assembly of oligomers from monomer can be blocked *in vitro* by small organic molecules (De Felice *et al*., 2004), some with efficacy at doses under 100 nanomolar (Wang *et al*., 2004b). Certain natural products also are effective. Extracts of Gingko biloba, anecdotally considered to have cognitive benefits, are surprisingly effective at blocking ADDL assembly (Yao *et al*., 2001; Chromy *et al*., 2003). Also, considered earlier, copper chelators and cyclooxygenase inhibitors may be useful in blocking aspects of oligomerization (Cherny *et al*., 2001; Boutaud *et al*., 2002). ADDL toxin receptors, as yet undiscovered, may provide targets for development of ADDL antagonists, while intervention in pertinent signal transduction pathways also might overcome ADDL synaptotoxicity. Molecules such as memantine or nonsteroidal anti-inflammatory drugs hypothetically might act at such levels.

Development of AD therapeutics, whether vaccines or drugs, will almost certainly be accelerated by invention of a valid molecular diagnostic. Although promise has emerged for detection of amyloid plaques by imaging (Klunk *et al*., 1994, 2003), it would be valuable to diagnose the disease at earlier stages. Whether oligomers might provide a biomarker is not yet clear. Striking differences exist between oligomer levels in AD and normal brain extracts (Gong *et al*., 2003), but no data are available concerning early stages of the disease or mild cognitive impairment (MCI). An appealing property of oligomers, however, is their solubility, which suggests they could diffuse from brain into cerebral spinal fluid or blood. Detection of extremely low levels of peripheral oligomers now appears feasible given advances in nanotechnology, which provide limits of detection that are orders of magnitude better than currently possible (Haes *et al*., 2004). One method, which uses a combination of immunomagnetic beads and nanoparticle-based polymerase chain reaction (PCR) (Nam *et al*., 2003),

can detect as few as 50 molecules of ADDLs; applied to 30 patients, the method has found ADDLs in cerebral spinal fluid at a level 1000% of that in age-matched controls (Georganopolou *et al*., 2005). These early results suggest that oligomer-based diagnostics might become possible in the near future.

Ultrasensitive diagnostics developed for AD potentially can be adapted to other diseases of fibrillogenic proteins such as human spongiform encephalopathy. Similarly with respect to therapeutics, successful precedents with experimental antibodies against Aβ oligomers suggest adaptation to other diseases merits investigation. Ultimately, lessons learned from a case study of AD's subfibrillar toxins, besides providing new ways to think about fibrillogenic proteins and disease mechanisms, may establish a broad platform for new approaches to therapeutics and diagnostics.

15. Abbreviations

Aβ	Amyloid β peptide
AD	Alzheimer's disease
ADDL	Aβ-derived diffusible ligands
AFM	Atomic force microscopy
APP	Amyloid precursor protein
CNS	Central neural system
LTD	Long-term depression
LTP	Long-term potentiation
PD	Parkinson's disease
PFs	Protofibrils
tg mice	Transgenic mice
TTr	Transthyretin

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Potential Conflicts

Dr. Klein is a cofounder of Acumen Pharmaceuticals, Inc., which has licensed exclusive rights to ADDLs for therapeutics and diagnostic from Northwestern University and the University of Southern California.

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