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Review Article

Synaptic targeting by Aβ oligomers (ADDLS) as a basis for memory loss in early Alzheimer's disease

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Abstract Early diagnosis and treatment of Alzheimer's Disease (AD) ultimately will require identification of its pathogenic mechanism. Such a mechanism must explain the hallmark of early AD—a profound inability to form new memories. For many years, the most promising hypothesis maintained that memory failure derived from neuron death induced by insoluble deposits of amyloid fibrils. Newer findings, however, suggest that memory loss, especially in early AD, may be a failure in synaptic plasticity caused by small soluble Aβ oligomers ("ADDLs"). ADDLs are neurologically potent toxins that rapidly inhibit long-term potentiation and reversal of long-term depression, classic paradigms for learning and memory. In human samples, ADDLs show striking increases in AD brain and CSF. The ADDL hypothesis is considerably reinforced by nerve cell biology studies showing that ADDLs specifically attack synapses, essentially acting as gain-of-function pathogenic ligands. Selective damage by ADDLs to memory-linked synaptic mechanisms provides an appealing explanation for early AD memory loss and suggests that ADDLs provide a valid target for therapeutics and diagnostics.

Keywords: Plasticity; Glutamate receptors; Spines; Actin; Arc; Tau phosphorylation; ROS; Diagnostics; Therapeutics

Based on a lecture at the Alzheimer's Association International Conference on Prevention of Dementia: Early Diagnosis and Intervention

1. The new therapeutics: early diagnosis and intervention with disease-modifying agents

The first successes in Alzheimer's Disease (AD) therapeutics emerged from a strategy designed to combat disease symptoms, with its pragmatic short-term goal being to postpone rather than eliminate mental decline [1]. In this regard, available drugs such as Aricept (Eisai Co., Ltd, Teaneck, NJ) and Namenda (Forest Laboratories, Inc., New York, NY) have been of genuine help. At the molecular level, Aricept inhibits acetylcholinesterase [2], whereas the recently approved Namenda likely acts by antagonizing *N*-methyl-D-aspartate-type glutamate receptors [3]. Intervening at these targets, however, only delays inexorable disease progression. The next phase in AD therapeutics, for which positive signs are visible, will be to provide medicines that are truly disease modifying. These medicines will be selected for their ability to neutralize the very first steps in pathogenesis, a central theme of the 2005 Alzheimer's Association international conference on early diagnosis and intervention. Even now, however, there is no consensus regarding what specific mechanism to target. As alternatives are evaluated, a critical benchmark will be whether a hypothesis accounts for the cognitive hallmark of early AD—the remarkable failure to form new memories. Although no consensus exists, evidence is accumulating to support the hypothesis that memory loss is a synaptic dysfunction caused by soluble oligomers of amyloid beta (Aβ). Although the fibrillogenic Aβ also produces AD's defining amyloid plaques, it is clear that insoluble amyloid fibrils are not the only neurotoxin formed by Aβ, and probably not the most important one. Soluble subfibrillar species, the size of an average globular protein, are increasingly likely candidates as the hidden toxins of AD memory loss.

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2. The original amyloid cascade provided a powerful but faulty hypothesis for AD memory loss

2.1. The First Landmark Discovery in Amyloid Toxicology

In 1992, when studies of amyloid precursor protein (APP) molecular biology and the genetics of AD were beginning to pay off richly, the possible toxicity of amyloid beta was the hottest topic in science, and at the heart of one of its biggest controversies [4]. If Aβ were a neurotoxin, it presented a molecular target for therapeutics. The problem was that Aβ toxicity was evident in some laboratories but not in others. This controversy was resolved by the discoveries of Pike et al [5] and Lorenzo and Yankner [6], who established 2 important principles: (1) Aβ indeed is neurotoxic, but (2) Aβ is innocuous until it undergoes structural reorganization. In nerve cell culture experiments, fresh solutions of monomeric Aβ were nontoxic, but tested again 24 hours later, the aged solutions were lethal to neurons. The toxic gain-of-function was accompanied by a gain-of-structure. The most obvious new structures were large Aβ-derived fibrils, indistinguishable from the fibrillar amyloid found in Alzheimer's hallmark plaques.

2.2. Toxic Aβ: Aberrant signal transduction induces AD-type Tau phosphorylation

The original idea that Aβ fibrils might constitute the initiating pathogenic molecule in AD was reinforced by multiple studies from nerve cell biology. Not only could fibrils directly attach to surface membranes of cultured neurons, imaged by electron microscopy [7], the attack on neurons stimulated the AD-like phosphorylation of tau, suggesting that Aβ toxins act upstream from tangles in the cascade of AD pathology. Observed initially in experiments with the human SHSY5Y neuronal cell line [8] and central nervous system (CNS) cultures [9], the connection to tau has been substantiated in a triple transgene mouse model, which shows significant reduction in phospho-tau levels in response to antibodies against Aβ. Knockout of tau, moreover, blocks the degenerative impact of Aβ fibrillar preparations [10], adding further strong support for a cascade from Aβ toxins to tau.

Consistent with their stimulation of tau phosphorylation, toxic preparations of Aβ selectively alter specific signal transduction pathways. Initial studies showed stimulation of focal adhesion kinase and Fyn [11,12], 2 protein tyrosine kinases coupled to memory mechanisms, cell structure, neuron death, and tau phosphorylation [7]. Physiologically, a Fyn pathway stimulated by insulin leads through GSK-3β to transient tau phosphorylation [13], but the neurodegenerative accumulation of phospho-tau induced by toxic Aβ is sustained rather than transient. Stimulation of tau phosphorylation by Aβ toxins unified the hallmarks of AD pathology. Coupling to specific signaling pathways, moreover, raised the intriguing possibility that corrupted signal transduction in itself might underlie memory loss, a premise that motivates much of today's research.

2.3. The original amyloid cascade hypothesis: Fibrils and nerve cell death

These early findings from nerve cell biology, coupled with major progress in human genetics and pathology, provided an exceptionally strong foundation for the concept that Aβ-derived fibrillar toxins play a primary role in AD pathogenesis. This hypothesis, summarized in 1992 in a seminal review by Hardy and Higgins [14], has been extraordinary in its ability to generate experimental progress. The benchmark evidence for the amyloid cascade (coupled with more than 13,000 citations to Aβ in PubMed) indeed seems compelling:

- AD is linked by human genetics to the Aβ peptide.
- Aβ in vitro self-assembles into lethal neurotoxins.
- Aβ-derived toxins stimulate AD-like tau phosphorylation.
- Toxic Aβ preparations contain abundant, readily detectable fibrils.
- Fibrils formed in vitro mimic those found in AD plaques.
- AD is accompanied by significant nerve cell death.

The inference from these findings is simple and persuasive: AD is, in essence, the consequence of neuron death induced by insoluble deposits of large amyloid fibrils.

The original amyloid cascade has been a powerful hypothesis, but despite its compelling support, it misses the mark in explaining memory loss in early AD. In its place, newer findings indicate that the crucial molecules are not found in senile plaques and that their neurological impact significantly precedes neurodegeneration. These findings, while retaining the central role for Aβ-derived toxins, strongly support a new concept: early memory loss comes from failure of synaptic plasticity, not neuron death, and the crucial molecular pathogens are small soluble Aβ oligomers, not fibrils. How these concepts emerged and now underlie new opportunities for early-stage diagnostics and disease-modifying therapeutics is reviewed in the remainder of this article.

2.4. Eliminate fibrils, block neuron death, eliminate dementia—A flawed prediction

With respect to AD therapeutics, the original amyloid cascade hypothesis provided a straightforward recommendation: eliminate fibrils, block neuron death, eliminate dementia. Although appealingly straightforward, this recommendation has significant flaws—it targets the wrong molecule and the wrong mechanism. The flaws are epitomized by the findings of 2 important transgenic mouse vaccine studies published in 2002. Before these publica-

tions, it had been established by Schenk et al. [15] that active vaccination of transgenic hAPP mice with fibril preparations eliminates plaques, a landmark discovery with potentially therapeutic consequences. In their follow-up studies, Dodart et al. [16] and Kotilinek et al. [17] tested the therapeutic impact of passive vaccines. Consistent with the amyloid cascade hypothesis, their hAPP transgenic mouse models manifested both age-onset plaques and memory failure. However, vaccination with monoclonal antibodies against Aβ produced consequences strikingly inconsistent with the hypothesis. First, memory loss was found to be reversible; one study showed reversal after about 2 weeks [17] and the other in as little as 24 hours [16]. Second, despite reversing memory loss, the Aβ antibodies did not reduce amyloid plaque burden. These models of early AD reveal an Aβ-dependent memory loss that is plaque-independent and not the consequence of neuron death. In fact, some neuropathologists had forcefully argued that plaque burden correlates poorly with dementia [18–20], and most tg-mouse models of AD manifest little or no death of neurons (although strains have been developed that do show significant neuron death [21]. The evidence points to a conclusion that is almost an oxymoron—memory loss in a disease defined by plaques and tangles is likely to be plaque independent.

If plaques do not initiate the disease, how does one explain the impact of APP mutations, the key role of Aβ peptides, and the therapeutic efficacy of anti-Aβ monoclonals? A reasonable solution to this conundrum would be the existence of hidden Aβ-derived toxins, capable of sublethal but neurologically damaging activity.

An early clue came from experiments by Oda et al. [22,23] on the interaction of Aβ with ApoJ (clusterin), an upregulated, plaque-associated molecule in AD brain. Rather than promote plaque formation, ApoJ proved surprisingly effective at blocking Aβ from forming large aggregates. Inhibition of aggregation occurred even at substoichiometric doses of ApoJ (1 part ApoJ, 20 parts Aβ), suggesting a chaperonelike action. Toxicology experiments with the PC12 pheochromacytoma cell line provided an even bigger surprise. ApoJ-Aβ solutions, which lacked sedimentable aggregates of Aβ, remained highly effective at blocking MTT reduction, an indicator of vital mitochondrial function and vesicle trafficking. The ApoJ experiments provided the first indication that Aβ-containing structures other than large insoluble fibrils might be pathogenic.

3. The alternative to fibrillar toxins: Neurological damage by soluble Aβ oligomers

3.1. A different outcome for Aβ self-assembly: Globular rather than fibrillar proteins

The nature of Aβ structures produced by the chaperonelike impact of ApoJ was investigated by Lambert et al [24],

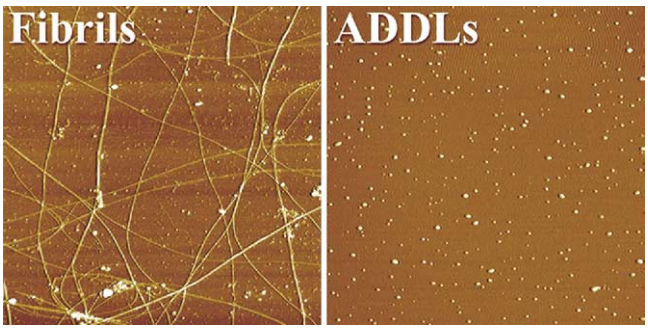

Fig. 1. Aβ self-assembly takes alternative paths. Assembled species obtained in vitro from monomeric Aβ42 are highly sensitive to conditions of incubation. As illustrated by atomic force microscopy, the same monomers can generate not only fibrils but also fibril-free solutions of small oligomers (ADDLs). Adapted and reprinted from *Trends in Neuroscience*, Vol 24, Klein WL, Krafft GA, Finch CE, Targeting small Aβ oligomers: The solution to an Alzheimer's disease conundrum? pages 219–24, copyright 2001, with permission from Elsevier.

leading to the discovery that toxin-producing Aβ self-assembly does not lead inexorably to large fibrillar aggregates or even protofibrils. An alternative outcome comprises small globular molecules, only several nanometers across (Figure 1). A powerful tool for assessing the spectrum of structures present in solution has been atomic force microscopy, earlier shown to be valuable for characterizing fibril structure [25]. Atomic force microscopy (AFM) images of ApoJ-Aβ solutions ruled out the presence of fibrils or short rodlike protofibrils, showing only small structures roughly comparable in dimensions to soluble globular proteins smaller than 100 kDa. Unlike the readily observed fibrils, such nanoscale structures would be cryptic to conventional neuropathology and easily missed in electron microscopy.

Because ApoJ is difficult to obtain, alternative methods have been developed to generate fibril-free, globular Aβ assemblies. In one approach [26,27], Aβ42 is monomerized by hexafluoroisopropanol (HFIP), dissolved in fresh dimethylsulfoxide (DMSO), diluted into cold F12 medium (as used for PC12 cultures), and briefly centrifuged. The supernatants are completely free of protofibrils [28]. Even after 24 hours at 37°, micromolar solutions exhibit no rodlike protofibril structures, establishing that the globular molecules seen in AFM are at least metastable. Most importantly, these ApoJ-free solutions are neurotoxic, proving that the active molecules are homo-oligomers of Aβ, not ApoJ-Aβ complexes.

3.2. Globular toxins are built of SDS-resistant oligomers

The composition of the globular neurotoxins has been analyzed by gel chromatography and electrophoresis [27,28]. Although intramolecular crosslinking methods have been developed to stabilize and characterize the subfibrillar species present in solutions of Aβ40 [29] as well as Aβ42 [30], it is possible to observe a full ladder of Aβ42 homo-oligomers on Western blots even without crosslink-

ing [26,28]. SDS-resistant Aβ42 oligomers typically range from dimer to 24-mer. Aβ40, which physiologically is much more abundant than Aβ 42 and which does not correlate well with AD, does not generate stable oligomers [29,31]. The spectrum of oligomers resolved by electrophoresis is much more evident by Western blot than silver stain, although Western blot patterns are markedly antibody dependent. The commonly used 4G8 monoclonal, for example, is unusually sensitive to dimers, while poorly recognizing mid- to large-sized oligomers [27,28]. Other experimental conditions also influence the analysis. For example, oligomers are stable in SDS at room temperature, but they break down when subjected to boiling, a step often used in SDS-PAGE. Although it has been suggested that SDS-PAGE itself might generate small oligomers, in fact monomers sans oligomers are readily detected by SDS-PAGE using fresh preparations [28]. Oligomerization occurs quickly, however, even at low concentration. Oligomers form from 10 nmol/L solutions of monomer within minutes [32], and the process of immunoblotting will generate oligomers from concentrated monomers if SDS is not constantly present.

Not all oligomer states are equally abundant, and oligomer assembly itself is extremely sensitive to in vitro conditions, presumably reflecting the conformational dynamics of Aβ. Smaller SDS-resistant species (3- and 4-mers) are favored in solutions kept at 4°, but prominent 12-mers emerge when dilute solutions of tetramers are incubated at 37° [26]. Larger oligomers do form at low temperature, detectable by size exclusion chromatography, but these are less SDS resistant (Rong Liu and William Klein, unpublished data). Conformation differences, moreover, exist between oligomers of the same size. For example, SDS-stable oligomers have been observed that are not toxic and that do not react with antibodies that bind the toxic species [28]. Physiologically relevant factors also have been identified that affect oligomerization, including the oligomer-promoting action of divalent cations [33] and levuglandin [34]. Overall, however, the mechanism of oligomer formation, the stability of oligomers, and the relationship between oligomerization and fibrillogenesis still remain poorly characterized.

3.3. Neurological impact: Oligomers rapidly block synaptic information storage

Although metastable Aβ oligomers are structurally very interesting, the reason they have generated major interest is because of their remarkable impact on mechanisms germane to memory loss. Memory formation begins at synapses, so one might anticipate that destruction of memory formation in Alzheimer's disease itself begins at synapses. A crucial question, therefore, has been whether oligomers might compromise synaptic plasticity. The answer has been clearcut. Assayed using long-term potentiation (LTP) and long-term depression (LTD), the classic electrophysiologic models for learning and memory, Aβ oligomers are neurologically potent CNS toxins that rapidly disrupt synaptic information storage.

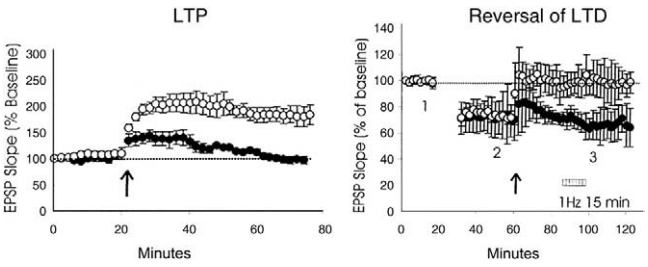

Fig. 2. ADDLs block LTP and prevent the reversal of LTD by tetanic stimulation. ADDLs are neurologically active molecules that rapidly inhibit synaptic plasticity, blocking the maintenance of LTP and the reversal of LTD. Hippocampal sections here were exposed to submicromolar doses of ADDLs (filled circles) for 60 minutes before start of recording (adapted and reprinted with permission from *Brain Research*, Vol 924, Wang HW, Pasternak JF, Kuo H, Ristic H, Lambert MP, Chromy B, et al. Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rate dentate gyrus, pages 133–40, copyright 2002, with permission from Elsevier). As can be seen from the normal EPSPs, ADDLs do not cause broad degeneration of synaptic transmission. The rapid and selective impact on synaptic plasticity led to the prediction that if early AD memory loss is ADDL-induced it potentially could be reversible [24].

Initial experiments by Lambert et al [7,24] tested stereotaxic injections of ApoJ-derived oligomers on LTP in living mice. Although neither ApoJ alone nor amyloid fibrils had any impact, the injected oligomers produced a rapid and profound inhibition. These first observations have been confirmed and extended in brain slice experiments in which oligomers prepared without ApoJ completely block LTP at sub-micromolar concentrations [24]. Inhibition occurs within an hour and is selective for particular aspects of plasticity (Figure 2). Although oligomers inhibit LTP, they do not inhibit LTD [35]. However, oligomers do block the reversal of LTD. The net neurological effect of oligomers in the hippocampus thus is to repress positive synaptic feedback. To account for these altered states of plasticity, it has been hypothesized that oligomers disrupt glutamate receptor trafficking [36,37], which is essential both for LTP maintenance and LTD reversal [38]. Recent evidence supports this possibility [39–41]. A mechanism to account for disrupted trafficking is discussed at the end of the article.

Although neurons in brain slices exposed to oligomers for longer periods ultimately degenerate and die [24,42], the initial impact of oligomers on synaptic plasticity is rapid and selective. There is no effect, for example, on evoked action potentials, indicating a nondegenerative mechanism. A prediction emerged from these findings that was somewhat iconoclastic: if oligomers were responsible for memory loss in early AD through an impact on synaptic plasticity, then early memory loss should be reversible [7,24]. As discussed earlier, this prediction was verified in the impres-

sive vaccine experiments on mouse models by Dodart et al and Kotilinek et al [16,17].

3.4. The amyloid cascade hypothesis revisited: Memory loss in early AD is a synaptic disease caused by soluble Aβ oligomers

Long-term potentiation is not memory but it is a good experimental paradigm for the study of memory mechanisms. The rapid impact of oligomers on LTP thus is intuitively appealing in its relevance to AD, and in 1998 Lambert et al [24] proposed a new hypothesis that attributed early memory loss to oligomer-induced failure in synaptic plasticity. The experimental foundation for this concept has been substantiated in multiple investigations [43–52], with especially strong support found in a major study of LTP by Walsh et al. [53]. This group found that oligomers formed in a cell culture model (medium conditioned by hAPP-transfected CHO cells) are exceptionally potent at inhibiting LTP in vivo. By Western blots, the cell-derived oligomers comprise mostly small SDS-stable oligomers, free of structures that might be considered protofibrils, although given antibody selectivity and gel conditions it is unclear whether mid-sized oligomers also might be present (e.g., 9- to 24-mers). As predicted, controls using insulin degrading enzyme, which proteolyzes monomers but not oligomers, ruled out the possibility that monomeric Aβ contributed to inhibition, whereas drugs that blocked Aβ production also blocked accumulation of the neurologically active molecules.

The compelling verification that oligomers are neurologically significant and sufficiently stable to accumulate in a cell model, along with the vaccine results validating the prediction that memory loss is reversible, provided impetus for significant modifications in the amyloid cascade hypothesis as updated in 2002 [54], 10 years after the original Hardy-Higgins review. The new cascade includes 2 significant emendations: (1) early memory loss is attributed to synapse failure, not neuron death, and (2) synapse failure is attributed to Aβ oligomers, not amyloid fibrils.

The hypothesis that Aβ oligomers play a role in AD pathogenesis has stimulated interest in the broader possibility that toxic protein oligomers may be common to multiple diseases [55]. Aβ is one of 23 different fibrillogenic proteins that are disease linked, and, until recently, it had been assumed that the pathogenic molecules were fibrillar. However, a number of these proteins now have been found to generate subfibrillar, oligomeric cytotoxins [56–58]. In some cases, contrary to earlier dogma, oligomers exhibit cytotoxicity but fibrils do not. Extrapolation from Aβ as a specific case study thus is providing general insight into diseases of protein folding and misassembly. If these initial results hold true, strategies that target elimination of soluble oligomeric toxins could provide new therapeutics for a broad range of significant diseases.

4. Clinical substantiation that AD pathology includes neurologically active Aβ oligomers

If soluble Aβ oligomers are to be valid targets for AD therapeutics, they must be present in human brain and manifest a strong AD-dependent accumulation. All the evidence reviewed so far comes from experimental models and oligomeric toxins generated in vitro. The critical question is whether Alzheimer's-affected brains contain identical neurologically active Aβ oligomers.

Early evidence indicated that prefibrillar assemblies indeed existed in AD-affected brains [59,60]. Because fibrils were regarded as the initiators of pathogenesis, it was assumed the oligomers simply were surrogates for ongoing fibrillogenesis. The oligomers appeared to be transient intermediates en route to formation of the pathogenically relevant amyloid fibrils. Now, however, given the potential of the oligomer hypothesis to account for AD memory loss, significant efforts have sought to substantiate the AD-dependent accumulation of brain oligomers.

Because not all oligomers are toxic [28], it has been important to determine whether brain-derived oligomers are structurally equivalent to the neurologically-disruptive, laboratory-derived oligomers. The key tools for rigorous analysis have been conformation-dependent antibodies. Such antibodies are readily generated by oligomer-based vaccines [27], which are highly immunogenic and superior to short peptides for generating antibodies recognizing conformational epitopes. Polyclonal antibodies have been obtained that are at least 1,000 times more sensitive for oligomers than monomers [27,32] and are capable of detecting less than 0.1 fmol oligomerized Aβ in dot immunoblots. Monoclonal antibodies have been obtained with similar properties [61], whereas some show unique attributes. One interesting monoclonal, for example, binds oligomers but not fibrils [28], whereas another binds fibrils but not oligomers [62], suggesting that fibrils are not simple assemblies of oligomeric subunits. A second approach to antibody generation by Kayed et al. [63] has been to use oligomers coupled to gold particles. These immunogens have generated oligomer-selective antibodies with an intriguing ability to recognize oligomers of multiple fibrillogenic proteins besides Aβ (e.g., alpha synuclein and islet amyloid polypeptide); it has been proposed therefore that particular structural domains may be common to toxic protein oligomers in general [63]. The generic oligomer antibodies do not bind fibrils, consistent with concept that structures of nonfibrillar and fibrillar toxins are not related in a simple manner.

4.1. Oligomers occur in human brain and are strikingly elevated in AD

Assays based on oligomer-selective antibodies have provided compelling evidence that oligomers are bona fide

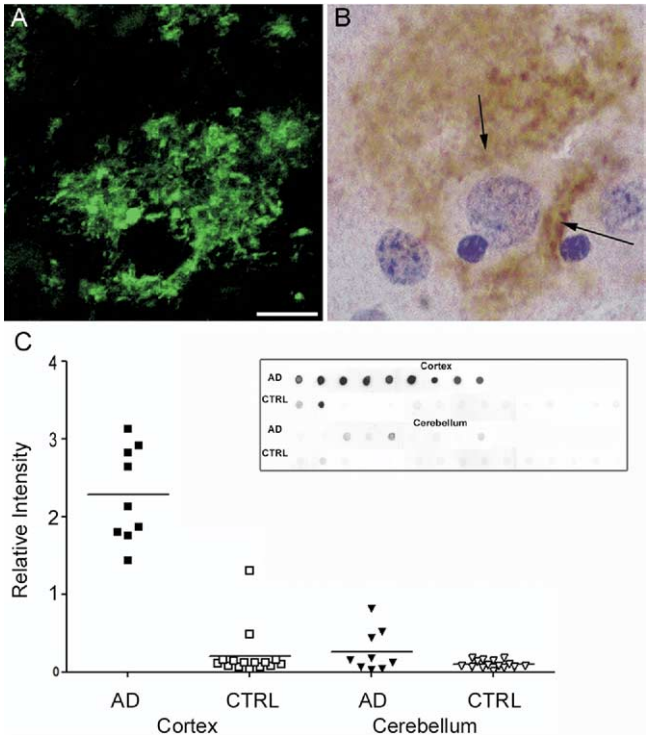

Fig. 3. Aβ oligomers are deposited extracellularly around neurons and are highly elevated in Alzheimer's-affected cortex. Clinical substantiation of ADDL involvement in AD pathology has come from immunomicroscopy and immunochemistry. Cortical sections from AD brain treated with ADDL-generated antibodies show diffuse synaptic-type labeling surrounding individual pyramidal neurons (fluorescent- or peroxidase-conjugated secondary antibodies, A and B, respectively). Similar staining is evident in mild cognitive impairment. Dot immunoblots of soluble brain extracts confirm the AD-dependent accumulation of ADDLs (C). ADDL pathology is pronounced in cortex but not cerebellum. Reprinted with permission from Lacor et al. [37] copyright 2004 by the Society for Neuroscience.

components of AD pathology: oligomers occur in human brain and are strikingly elevated in AD, oligomers from AD brain are structurally homologous to synthetic oligomers, and hAPP mouse models of AD accumulate oligomers as predicted. Oligomers found in cerebrospinal fluid (CSF), moreover, appear to provide a significant biomarker of AD, potentially of value for future clinical diagnostics.

To assess soluble oligomers, brain extracts have been prepared using physiological buffers to avoid chemical perturbation. Soluble fractions, obtained after ultracentrifugation and analyzed by dot immunoblots, show a remarkable, AD-dependent increase in oligomers. AD subjects show levels up to 70-fold higher than found in controls [36]; (verified with 3 different oligomer-selective antibodies [37,63]). The large increase in extractable oligomers is regionally selective, robust in frontal cortex, but marginally detectable in cerebellum [37] (Figure 3C), a pattern consistent with other aspects of AD pathology.

With respect to structure, 3 types of data indicate the soluble oligomers in brain extracts are equivalent to the toxic oligomers prepared in vitro. First, brain oligomers react with conformation-dependent antibodies raised against synthetic oligomers. These conformation-dependent antibodies not only distinguish oligomers from monomers, they also distinguish toxic oligomers from nontoxic species; neither capacity is typical of antibodies generated against short peptides [27]. Second, synthetic and AD brain-derived preparations show equivalence by 2-dimensional gel analysis. Each contains a prominent oligomer with isoelectric point of ~5.6 and SDS-stable mass of ~55 kDa, i.e., 12-mers [36]. Additional species have been obtained by extracting brain with SDS, indicating some oligomers are trapped or tightly bound. Third, in cell culture experiments, both synthetic and soluble AD brain-derived oligomers show attachment to nerve cell surfaces in identical, ligandlike patterns ([36,37] discussed below). No binding occurs with extracts of control brain. Overall, the data indicate that neurologically active oligomers formed in vitro are structural homologs of oligomers that accumulate in AD brain.

Future experiments will need to evaluate the toxicology of oligomers obtained from AD brain in neurological and cell biological assays. Preliminary results strongly indicate, however, that synthetic and brain-derived oligomers also are equivalent in this respect. For example, added to mature hippocampal neuron cultures, each stimulate AD-type tau phosphorylation (Fernanda De Felice and William Klein, unpublished data). Coupling to tau phosphorylation further substantiates the relevance of oligomers to AD pathology and indicates a role in early stages of the disease.

4.2. TG-mice models of AD accumulate soluble oligomers

Animal models of AD are essential for studies of mechanism and drug discovery, so it has been important to determine whether they also accumulate oligomers. Early evidence suggested indirectly that oligomers should be present. For example, some hAPP transgenic mouse strains manifest brain damage (synapse loss, deficiencies in LTP, poor memory performance) that does not correlate with plaque burden, in some cases showing brain damage in the complete absence of amyloid deposits. These animals essentially recapitulate the poor correlation between plaques and dementia in humans but in an extreme manner. Although originally rejected as inadequate models because they lacked amyloid plaques, some of these strains in fact may be excellent models for early AD caused by toxic oligomers.

In harmony with human data, occurrence of oligomers recently has been confirmed in multiple strains of tg-mice. Even when the commonly used 4G8 antibody shows no increase, assays with oligomer-selective antibodies detect increases in immunoreactivity that reach several hundred–fold over controls [32]. These robust

increases in oligomers are regionally selective, indicating broad diffusion does not occur. In the highly regarded triple transgene model of Oddo et al. [64], which produces AD-like tau phosphorylation as well as plaques, 2 different oligomer-selective antibodies show the same pattern of developmental increase, with a transient peak in oligomers at 6 months and a sustained elevation after 15 months [65]. Most importantly, oligomer neutralization by passive vaccination results in decreased phospho-tau, consistent with the unifying hypothesis that pathologic changes in tau constitute a downstream consequence of oligomer activity.

Quantitative correlation of oligomer levels with memory dysfunction first was evident in experiments by Westerman et al. [66]. Their preliminary data indicated that memory loss occurred after oligomer levels increased past certain threshold levels, a possibility consistent with the concept of cognitive reserve [67]. More recently, this group has reported that memory loss correlates with particular SDS-stable oligomers (9- to 12-mers), mirroring the species found in vitro and in AD brain. Another mouse model, developed by Ohno et al [68], also shows highly upregulated soluble oligomers; in these animals, knockout of BACE eliminates oligomers and blocks deficiencies in a fear-linked memory task.

The abundance of oligomers resulting from transgene expression in these animals likely explains why brain deficiencies correlate poorly with amyloid plaques, and it substantiates their value as models for early AD pathology and memory loss.

4.3. Human neuropathology: Distribution of oligomers in situ is consistent with links to disease onset

Immunohistochemistry with oligomer-selective antibodies readily distinguishes AD brain sections from controls [62,69]. Besides confirming the presence of oligomers, results reveal an unexpected specificity to oligomer distribution in situ. In lightly stained AD brain sections, oligomers show a perineuronal localization [37], indicating the predominant localization is not cytoplasmic (Figure 3A & B). Although oligomers accumulate in the cytoplasm of certain tg-mice strains [70], this could be the consequence of very high oligomer production in mice. Oligomers also do not colocalize with compact amyloid fibril deposits [63]. Perineuronal distribution likely corresponds to so-called diffuse deposits, a nomenclature that may be misleading with respect to cellular mechanisms. "Diffuse deposit" implies a nonselective extracellular precipitate, but the distribution surrounding individual neurons suggests oligomers may associate with particular sites in dendritic arbors. Perineuronal distribution is readily observed for isolated, individual neurons, suggesting the source of oligomers may be that same neuron.

Perineuronal/dendritic staining around isolated neurons appears to be the first sign of pathology in preclinical brain sections [69]. This fact, along with observations that soluble oligomers in control brain extracts sometimes show minor elevations [36], indicates that oligomer accumulation begins preclinically. The relationship between accumulation and dementia is unlikely to be straightforward, with current results indicating that dementia would manifest only after oligomer levels exceed some critical threshold. Recently developed plaque imaging agents [71] probably will be inadequate to detect oligomers at threshold levels because oligomer distribution is distinctly segregated from thioflavin-positive plaques [63]. However, alternative imaging agents that specifically target oligomers are under development [72] and might provide a means for early AD detection.

4.4. Nanotechnology based assays of cerebrospinal fluid confirm the extracellular presence of oligomers

Occurrence of oligomers in soluble brain extracts, along with their perineuronal distribution, provides indirect evidence that oligomers occur extracellularly and thus are potentially accessible for clinical assays. If extracellular localization were in fact true, then oligomers should occur in CSF, but neither enzyme-linked immunosorbent assays nor dot immunoblots verify this prediction, even using the most sensitive oligomer-generated antibodies. However, in a unique approach using nanotechnology, it has been possible to increase sensitivity by orders of magnitude while still retaining immunospecificity [73]. The assay used a sandwich complex in which oligomers are immunocoupled to magnetic particles on one side and to DNA-coated gold nanoparticles on the other. After separation of analyte, amplification is achieved by ultrasensitive methods for DNA quantitation. The assay, dubbed a *BioBarcode* because of its potential to quantify multiple analytes in a single sample, has established that immunoreactive oligomers are indeed present in CSF. Most significantly, CSF oligomer levels show marked AD dependence, with median levels for AD subjects elevated 10-fold over controls ($p < 0.0001$ for 30 samples). Overlap between populations is virtually nonexistent. Although a much larger sample is needed to validate these results, and the current assay itself is still being enhanced, the use of oligomers as biomarkers in AD diagnostics shows unique promise.

5. Cellular mechanism—Oligomers specifically target synapses and disrupt the molecular cell biology of memory

Oligomers block synaptic information storage in electrophysiological experiments. They trigger AD-type pathology in transgenic models and likely instigate memory loss. And, in human brain, oligomers show a striking accumulation around neurons that begins in very early stages of AD.

There clearly is strong need to understand how oligomers act in terms of molecular mechanisms. How is it that oligomers attack neurons, and what memory-relevant molecular changes are triggered? An appealing hypothesis is suggested by the putative association of oligomers with dendritic arbors in situ: perhaps oligomers attack and disrupt signaling pathways specifically at sites critical to memory formation, in essence acting as pathogenic ligands.

5.1. Patterns in culture recapitulate patterns in vivo: Oligomers are "Aβ-derived diffusible ligands" (ADDLs) that bind to hot-spots on dendritic surfaces

One would predict, if the ligand hypothesis were true, that incubation of cultured neurons with soluble oligomers from AD brains should generate a perineuronal pattern analogous to that observed in brain sections. Experiments with highly differentiated monolayers of rat hippocampal neurons, using oligomer-selective antibodies to localize distribution, have confirmed this prediction [36,37]. Oligomers in crude extracts from AD brains show remarkably patterned attachment to neuron surfaces, localizing at small hotspots that are especially rich in mature dendritic arbors. CSF from AD subjects also gives this pattern, although binding is faint, consistent with very dilute oligomer levels. Patterns of identical perineuronal hotspots are generated when neurons are incubated with synthetic oligomers. Extracts from control brain, however, show no binding. Although it has been argued that assemblies of Aβ interact with neuron membranes by creating porelike structures in the lipid bilayer, the hotspot pattern of oligomer binding is not consistent with random insertion into plasma membrane lipids. The selective binding of Aβ oligomers, whether from AD brain or prepared in vitro, substantiates their ligand nature. This property, coupled with their neurological impact and their solubility, has led to the acronym "ADDL" (pronounced "addle"), for a pathogenic "Aβ-Derived Diffusible Ligand."

The pattern of binding to cultured neurons supports the hypothesis that diffuse deposits in AD originate with oligomers bound to particular sites in dendritic arbors. If oligomers are gain-of-function pathogenic ligands (ADDLs), a key question is how specific is their target? Distribution in culture at first looks somewhat random, but binding actually shows remarkable specificity. In any given hippocampal culture, only a subset of neurons is attacked by oligomers, typically between 25% and 50% of neurons present. Neurons side by side in culture can be ADDL-positive and ADDL-negative [37], even with similar morphology and expression of memory-relevant molecules such as CaMKII. What distinguishes them is not yet known. Binding sites, however, are sensitive to low doses of trypsin [24], indicating the existence of particular proteins that serve adventitiously as oligomer receptors.

5.2. A key to memory loss—Oligomers (ADDLs) specifically target synapses

The identity of the hotspots themselves may be the key to memory loss mechanisms. More than 90% of ADDL hotspots colocalize with puncta of PSD-95 [37], a major component of postsynaptic densities, which in mature hippocampal cultures is a reliable marker for postsynaptic terminals [74]. Colocalization of ADDLs with PSD-95 establishes that ADDLs are ligands that specifically target synapses. Roughly 50% of the synapses do not bind ADDLs, so specificity extends also to the nature of the synapses. The fact that ADDLs are ligands that directly and specifically attack synapses provides substantial strength to the hypothesis that memory loss could be an oligomer-induced failure of synaptic plasticity.

Synaptic binding is evident for ADDLs obtained from AD brain as well as prepared in vitro, but it is not certain precisely which oligomeric states can act as ligands. Simple fractionation of brain extracts or synthetic ADDLs by cutoff filters indicates an abundance of high-affinity ligands between 10 and 100 kDa [37], consistent with the 12-mers identified by 2D gel analysis. Some binding activity does not pass the 100-kDa filter, consistent with observations of larger oligomers in vitro and in SDS extracts of brain tissue (up to 48-mers; 216 kDa; [31,36]). Although it is difficult to rule out that very small oligomers simply are cryptic to antibodies, biotinylated Aβ monomers and small oligomers obtained by HPLC-SEC show no binding when assayed by fluorescein-streptavidin [37]. The apparent disparity in size between synaptic ligands and the very small LTP-inhibiting oligomers in conditioned medium [53] is an issue still to be resolved.

5.3. Subsynaptic targeting: ADDLs bind to synaptic spines, altering structure and composition

High-resolution confocal immunofluorescence microscopy shows that the synaptic targeting of ADDLs frequently localizes to dendritic spines. This is illustrated here by a neuron double labeled for ADDLs and CaMKII (Figure 4). Although other sites also may be targeted, spines are particularly interesting because they contain the postsynaptic signal transduction components of excitatory synapses. Signaling events within spines orchestrate the molecular plasticity essential for initiating memory formation. CaMKII, for example, plays a critical role in synaptic information storage and is enriched in synaptic spines.

An important feature of spines is their dynamic cytoskeleton [75]. Spines are rich in actin and actin-associated proteins, and this molecular machinery regulates spine geometry and also the trafficking of glutamate receptors, essentially the underpinnings of synaptic plasticity. Attachment of ADDLs to spines alters these fun-

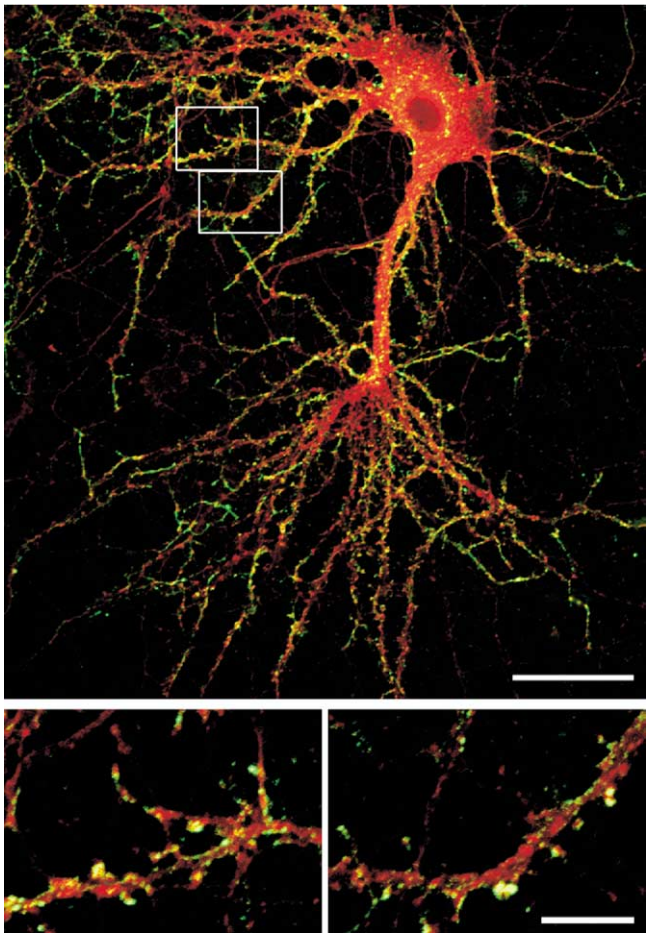

Fig. 4. Localization of ADDL binding sites to dendritic spines. ADDLs act as pathogenic gain-of-function ligands that target synapses. Here ADDLs have been added to cultures of highly differentiated hippocampal cells (21 DIV), which then were immunolabeled for ADDLs (green) and αCaMKII (red). In overlaid images, ADDLs show a highly patterned distribution seen to coincide (yellow) with dendritic spines. More than 90% of the ADDL puncta occur at synapses. Scale bars: Top, 40 μm; Bottom insets, 8 μm. Adapted and reprinted with permission from Lacor et al. [37] copyright 2004 by the Society for Neuroscience.

damental mechanisms. After several hours of exposure to ADDLs, levels of glutamate receptors in the plasma membrane drop significantly [39,40]. Moreover, spine geometry undergoes significant restructuring, with the short clublike structure of controls supplanted by long, spindly spines. This type of aberrant spine geometry is known to exhibit low receptor levels [76], consistent with the impact of ADDLs. Most significantly, spines that are aberrantly long and spindly have been linked to mental retardation [77]. The basis for the ADDL-induced shift in spine geometry is not known, but it is accompanied by rearrangements in drebrin (Pascale Lacor and William Klein, unpublished data), an actin-regulating protein. With continued ADDL exposure, spines eventually collapse, with a near-complete elimination of drebrin. Abnormalities in drebrin similarly manifest in hAPP transgenic mice [78]. The abnormalities in mice are blocked by the nutritional supplement DHA, an omega-3 fatty acid reported to have cognitive benefits [79,80]. A possibility thus emerges that DHA might prove useful in early AD, potentially by ameliorating the deleterious impact of ADDLs.

5.4. Attack on synapses disrupts translation of arc, an immediate early gene essential for memory

Another actin-associated protein in synaptic spines also has been linked to memory loss mechanisms and may prove especially salient. This protein is Arc (an acronym for "Activity-Regulated Cytoskeletal-associated" protein), and it is ectopically induced in response to ADDLs. Molecular consequences of this aberrant expression of Arc have the potential to drive memory loss.

Arc is the product of an immediate early gene required for long-term memory formation [81]. A portion of Arc mRNA traffics specifically to spines, where its translation is induced by synaptic transmission [82]. In its role in memory formation, Arc normally is induced only transiently, and chronic overexpression has been predicted to impair long-term memory, essentially by increasing noise in information processing [83]. Experiments with Arc tg-mice have verified this prediction [84]. Chronically high levels of Arc in these animals correlate with poor performance in learning tasks. Because of the influence Arc exerts on f-actin, the learning dysfunction has been attributed to loss of spine structural plasticity. Arc overexpression, however, also results in removal of synaptic membrane glutamate receptors [85], another possible basis for failure of synaptic plasticity and memory loss.

These observations are germane to AD because ectopic Arc overexpression is induced by ADDLs, which, as summarized earlier, also alter spine geometry and cause loss of cell surface glutamate receptors. Neurons exposed to ADDLs for 5 minutes show induction of small Arc puncta that coincide with ADDL-labeled spines [37]. This response, however, is not transient. After 6 hours, Arc immunoreactivity remains markedly elevated and spreads ectopically throughout the dendritic arbor (Figure 5). ADDLs thus provoke chronic Arc overexpression, a specific molecular anomaly associated with dysfunctional learning. Whether ADDL-induced Arc causes the observed restructuring in spine geometry and loss of cell surface glutamate receptors is a prediction yet to be tested. With even longer exposure to ADDLs (e.g., in tg-mice), Arc levels decrease [86], consistent with degeneration of synaptic terminals [87]. Although the Arc response is very intriguing, oligomers also affect other important signaling pathways germane to synaptic plasticity [44,49,50,88], including pathways that elevate tau phosphorylation [65] and ROS [89]. Overall, a comprehen-

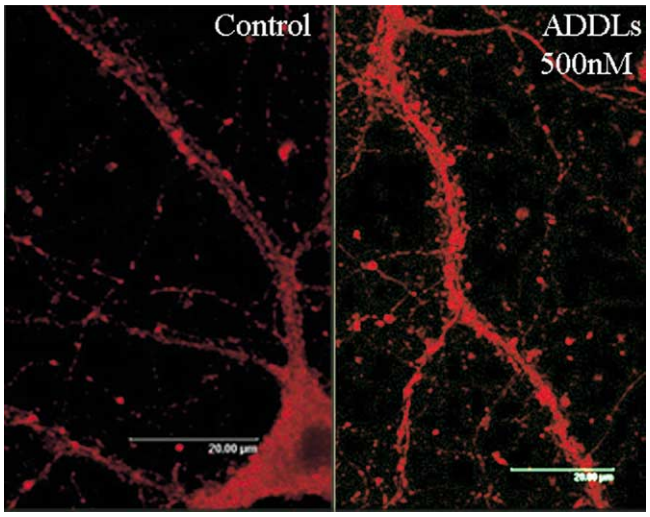

Fig. 5. ADDLs attack on synapses disrupts Arc, an immediate early gene essential for memory. ADDLs induce the rapid and sustained expression of Arc, a protein of which proper transient expression is required for long-term memory formation. Here mature hippocampal cultures were treated for 6 hours with vehicle or ADDLs. Vehicle-treated samples show Arc largely restricted to cell bodies, with few spine heads showing higher-than-basal levels. ADDL-treated samples exhibit large increases of Arc in a subset of neurons. Intense Arc-IR occurs in dendritic spine heads, which show abnormal protrusion, and ectopically throughout dendritic shafts. Scale bars: 20 μm. Adapted and reprinted with permission from Lacor et al. [37] copyright 2004 by the Society for Neuroscience.

sive understanding of the relationships between ADDLs and signaling remains an important but distant goal.

5.5. ADDLs at synapses—A hypothetical basis for early AD, its diagnosis, and treatment

This article began with the premise that an acceptable hypothesis for AD must explain why its earliest stages selectively target memory formation. Findings reviewed here strongly support the hypothesis that specific loss of memory function in early AD is the consequence of targeting and functional disruption of particular synapses by ADDLs. In the ADDL hypothesis (Figure 6), local oligomerization of Aβ42 provides gain-of-function pathogenic ligands that bind specifically to surface membrane proteins localized in synaptic spines. The affected synapses are those critically placed in memory circuits, and they are targeted because of localized ADDL production as well as adventitious ADDL capture by synaptically localized toxin receptors. As pathogenic agonists, ADDLs disrupt proper regulation of the spine cytoskeleton and thereby disrupt spine geometry and receptor trafficking. These spine deficiencies impair synaptic information storage. When the net impact of deficiencies in spine cell biology passes a critical threshold, the result is failed memory formation.

With respect to implications for diagnostics and therapeutics, the large elevation of ADDLs in AD-CSF suggests that prescriptions for drug treatments of early AD may some day rely on an ADDL-based diagnostic assay. Until specific vaccines and drugs are developed, nutritional and lifestyle approaches may provide helpful stop-gaps. Nutritional DHA supplements, for example, may provide benefit through mechanisms that likely are ADDL-coupled. Cognitive benefits also come from exercise, which is known to boost production of brain-derived neurotrophic factor (BDNF) [90], a physiological regulator of Arc [91], and insulin signaling [92], a physiological regulator of tau phosphorylation [13]. DHA, BDNF, and insulin all in some way may countermand or interact with ADDL signaling.

Long-range strategic approaches to ADDL therapeutics are similar to those generated to attack amyloid fibrils [93]. Overall, the most appealing approach is to target the pathogenic ADDLs themselves, not the cellular molecules that generate them. Although secretase inhibitors are effective in lowering Aβ, they appear likely to have unacceptable side effects [94]. Given the soluble nature of the target, passive vaccines that specifically neutralize ADDLs are especially

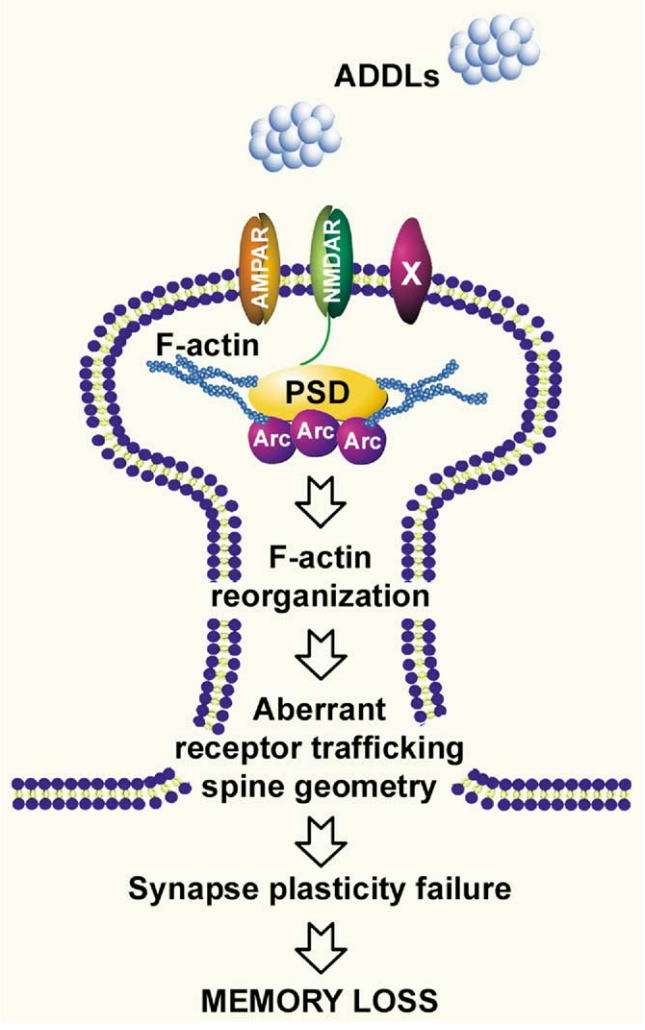

Fig. 6. Attack on synapses by ADDLs—A basis for early AD memory loss?

appealing and may obviate the side effects and variable responses to active fibril-oriented vaccines observed in clinical trials [95]. Neutralizing ADDLs also may be germane to longer-term degenerative mechanisms, because, with persistent ADDL exposure, targeted synapses lose structural stability and, ultimately, targeted neurons lose viability [96]. Precedents from several studies also suggest it may be feasible to discover small molecules that could act as lead compounds for anti-oligomerization drugs [28,46,97,98]. Given that ADDLs at least indirectly affect NMDA receptor signaling pathways and that the AD-drug Namenda is an NMDA receptor antagonist [3], prospects seem likely for new and more effective receptor-directed drugs to emerge from ADDL-based screens. Finally, because ADDLs are pathogenic ligands that attack specific synapses, the search for specific ADDL receptors as potential drug targets is actively underway.

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