

**SECTION I. CNS AGENTS**

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**Chapter 4. Emerging Themes in Alzheimer's Disease Research: Paradigm Shift in Drug Discovery**

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Introduction - This year marks the 95<sup>th</sup> anniversary of the first published case of Alzheimer's disease (AD), a disease currently afflicting more than 12 million people worldwide. Over the past 15 years, the pace of AD research has accelerated dramatically, and the discovery focus has moved from cholinergic enhancement medicines to drugs that interfere with amyloid. Recent results have emerged to challenge the core dogma of the prevailing "amyloid cascade" hypothesis, which invokes amyloid plaque deposition as the cause of AD. The present report examines this central hypothesis, highlighting recent results that implicate non-fibrillar A $\beta$  oligomers rather than fibrils as the molecular pathogens in AD, and evaluating those drug discovery approaches that are poised to capitalize on these new findings.

HISTORICAL BACKGROUND

In 1907, Bavarian psychiatrist Alois Alzheimer described pathology and symptoms of a 51 year-old woman, Auguste D., who had suffered progressive cognitive decline, learning and memory deficits, and paranoid and delusional behavior (1). Alzheimer used a silver stain on cortical tissue samples to reveal prevalent and highly unusual lesions that he called "neurofibrillary tangles" and "senile plaques". These lesions remain the basis for definitive post-mortem diagnosis of Alzheimer's disease (AD). Alzheimer's initial report did not discuss any definitive cause for AD, but he did observe (his italics): "**Plaques are not the cause of senile dementia, but only an accompanying feature of senile involution of the central nervous system**" (2).

Alzheimer's early exoneration of amyloid plaques apparently escaped most AD researchers, including Glenner and Wong, who in 1984 identified the 39-43 residue amyloid  $\beta$  peptide as the major plaque component (3). Shortly thereafter, synthetic A $\beta$  peptides were shown to assemble into fibrils with staining properties identical to senile plaques (4), and in 1990, synthetic A $\beta$  was shown to be neurotoxic (5). With the A $\beta$  peptide sequence in hand, several groups identified the gene encoding the A $\beta$  precursor protein (APP) in 1987, setting the stage for discovery of APP mutations responsible for certain familial AD cases (e.g., 6-9). Based on these findings, the "amyloid cascade" hypothesis was articulated in 1992, implicating accumulated amyloid plaques and fibrils as the cause of AD (10).

A close corollary to the amyloid cascade hypothesis is inflammation mediated by glial activation (11). Activated glial cells are often closely associated with amyloid plaque deposition, while inflammatory mediators such as IL-1 can increase A $\beta$  production (12-15). This can lead to more plaques, leading to more activated glia, setting up an accelerating cascade. A number of studies suggest that glial activation exacerbates fibril toxicity, and one study even demonstrated that A $\beta$  fibril toxicity requires glial activation, arguing for an essential link between amyloid plaques, inflammation and AD (16,17).

The 20<sup>th</sup> century closed with amyloid deposition and inflammation dominating the AD mechanistic landscape, but as the 21<sup>st</sup> century began, a major challenge to these two widely-held beliefs has emerged. Soluble neurotoxic oligomers of A $\beta$  1-42 known as A $\beta$ -derived diffusible ligands (ADDLs), first described in 1998, recently have been identified in impaired transgenic AD mice and in AD brain, and shown to interfere directly with neuronal long-term potentiation (LTP) and to exert region-specific neurotoxicity in CA1 (18-24). These results establish ADDLs as the likely molecular pathogens in AD and provide a unifying explanation for many confounding observations previously not reconciled by the amyloid cascade hypothesis.

#### THE ROLE OF A $\beta$ AND THE AMYLOID CASCADE HYPOTHESIS

The definitive molecular etiology of AD has been elusive. Strong evidence suggests the main constituent of the amyloid plaque, A $\beta$ , plays a prominent role in disease progression. This evidence comes from multiple lines of investigation. For example, A $\beta$  levels are modulated by all four identified genetic components associated with AD: mutations in APP, presenilin-1 (PS-1) & PS-2 and the inheritance of the epsilon 4 allele of apolipoprotein E (apoE4) (reviewed in 9). Early onset familial AD (FAD) mutations in PS and some FAD APP mutations result specifically in increases of A $\beta$  1-42 production in cell culture and in transgenic mouse models (25-27). Although apoE4 does not increase A $\beta$  production, it is less efficient in clearing A $\beta$  1-42 from the brain, due to its lower A $\beta$  1-42 binding affinity (28). APP, the precursor to the A $\beta$  peptide, is localized on the 21<sup>st</sup> chromosome and Down's syndrome (trisomy 21) patients develop AD-like symptoms and pathologies at an early age (29). Evidence also comes from *in vitro* and *in vivo* experiments demonstrating that A $\beta$  is neurotoxic (5, 30). Thus, the A $\beta$  1-42 peptide plays a critical role in the pathogenesis of AD.

A connection between A $\beta$  and neurofibrillary tangles has not been fully established, however, several studies have demonstrated that A $\beta$  specifically up-regulates paired helical filaments (PHF) specific tau phosphorylation (31,32). Recent data on cyclin-dependent kinase 5 (Cdk5) supports such a connection (33,34). When Cdk5 associates with its regulatory subunit, p35, Cdk5/p35 kinase activity is initiated, a requirement for neurite growth. p25, a truncated fragment of p35, causes Cdk5 to be constitutively activated and mislocalized *in vivo*. Cdk5/p25 kinase phosphorylates tau and causes morphological degeneration (including the appearance of PHF) and profound apoptotic cell death of primary neurons. p25 is produced and accumulates in brains of patients with AD (33). Moreover, A $\beta$  1-42 induces conversion of p35 to p25 in primary cortical neurons (34).

A $\beta$  is derived by proteolytic cleavage of  $\beta$ -APP, an integral membrane protein of unknown function. It is highly expressed in the brain and APP knockout mice show age-related cognitive deficits and neuropathology (35). Recent data suggest that APP may normally serve as a membrane cargo receptor for kinesin-1. Kinesin-1 is responsible for ATP-dependent movement of vesicular cargoes within neurons (36,37). Whether this, or other yet to be identified functions, require proteolytic processing of APP is not known. However, A $\beta$  is detected in cognitively normal individuals, suggesting APP processing occurs in the absence of disease (38-40).

For generation of A $\beta$ , proteolytic processing of APP is essential. APP is cleaved by sequential actions of three unique proteases,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretases (reviewed in 41). Each secretase cleaves at a unique site to generate several different APP derivatives. The A $\beta$  peptide is produced by sequential activity of  $\beta$ - then  $\gamma$ -secretase, which produce peptides ranging from 40-43 amino acids.  $\beta$ -secretase (also referred to as  $\beta$ -site APP-cleaving enzyme, BACE) has been identified and BACE knockout mice produce reduced A $\beta$  levels (42). On the other hand,

the exact identity of  $\gamma$ -secretase remains unclear. The  $\gamma$ -secretase cleavage sites lie within the transmembrane region of APP. This unusual transmembrane proteolytic activity has recently been shown to require the presenilins (PS). However, PS are also involved in cleavage of the developmental protein, Notch (43-46). Thus, the phenotype of the PS-1 knockout is late embryonic lethal, making this transgenic ineffective for examining the *in vivo* role of PS-1 in  $A\beta$  generation (47,48). Recently, postnatal, neuron-specific PS-1 deficient mice have been shown to possess reduced  $A\beta$  levels, which further validates the essential role of PS-1 in  $\gamma$ -secretase activity (49,50).  $\alpha$ - and  $\gamma$ -secretases are potential therapeutic targets because they are essential for  $A\beta$  generation. However, since APP is not the only substrate for these enzymatic activities, side effects of inhibiting either activity may prevent their ultimate therapeutic use. Moreover, any potential therapy that modifies APP processing may affect the normal functioning of APP and its metabolic derivatives (*vide infra*).

The cellular source and site of generation of  $A\beta$  are not known, although proteolytic processing of APP can occur during and after APP intracellular trafficking so its metabolic derivatives (including  $A\beta$ ) can be released into vesicle lumens and the extracellular space. In AD brains extracellular  $A\beta$  appears as compact, fibrillar deposits and amorphous, non-fibrillar, diffuse deposits (reviewed in 9). Compact amyloid plaques, also known as neuritic amyloid plaques, are localized to the limbic and association cortices and are associated with dystrophic neurites. The limbic and association cortices are regions severely damaged in AD. On the other hand, diffuse amyloid plaques are prevalent throughout the brain, and are not associated with neuritic elements. In addition, diffuse amyloid plaques appear before neuritic amyloid plaques in brains from Downs syndrome patients and APP transgenic mice (e.g., 29,51). These observations may support the view that diffuse amyloid plaques are precursors of the neuritic amyloid plaque.

Based on these observations, and because fibrillar  $A\beta$  preparations were neurotoxic to cultured neurons, it was postulated the fibrillization of  $A\beta$  into these amyloid plaques caused AD-related dementia (the amyloid cascade hypothesis). However, careful AD neuropathology studies have not been able to correlate the density of neuritic amyloid plaques and neurodegeneration or clinical dementia (52,53). Recent studies demonstrate that the severity of neurodegeneration correlates with small, stable oligomers of  $A\beta$  (54,55). In addition, recent studies in APP transgenic mice suggest that amyloid plaque deposition is not required for synaptic loss or learning/memory deficits (20,56). Thus, the classical amyloid cascade hypothesis needs to be re-evaluated.

#### OLIGOMERIC $A\beta$ CAUSES NEUROTOXICITY

The amyloid cascade hypothesis was first challenged in a 1995 report that demonstrated the neurotoxicity of a non-fibrillar  $A\beta$  preparation (57). Soon, numerous laboratories reported identification and neurotoxicity of small, non-fibrillar  $A\beta$  assemblies (19,58-61). One such preparation consisting of ADDLs killed mature neurons in organotypic hippocampal slice cultures at nanomolar concentrations (19). Fyn, a protein tyrosine kinase in the *src* family, was required for this toxicity implicating a specific signaling pathway underlying ADDL toxicity. ADDL toxicity may involve generation of reactive oxygen species, superoxide or peroxynitrite through a specific signaling pathway, because ADDLs reversibly inactivate the free radical-sensitive enzyme, aconitase, prior to initiating neurotoxicity (62). In addition, ADDLs inhibit LTP, a classical model of synaptic plasticity and memory (19,22).

More recent studies have demonstrated that cell-derived A $\beta$  oligomers have biological activity *in vivo*. Intracerebroventricular microinjection of conditioned medium from APP overexpressing cells completely blocked LTP in the CA1 hippocampal region. A $\beta$  oligomers similar to ADDLs were the culprit because conditioned medium devoid of monomeric A $\beta$  was active. Monomeric A $\beta$  was removed with an insulin-degrading enzyme. Plus, conditioned medium devoid of oligomeric A $\beta$  was inactive. Here, oligomerization was prevented with a selective  $\beta$ -secretase inhibitor, which selectively blocked A $\beta$  dimer and trimer formation at doses that allow appreciable monomer production (23).

Several transgenic mouse models of AD support the concept that soluble A $\beta$  oligomers may be the primary source of synaptic dysfunction. A comparison of multiple lines of transgenic mice that overexpress wild-type or mutated human APP reveals a dissociation between A $\beta$  plaque deposition and synaptic dysfunction and loss (20,63). A $\beta$  plaque deposition only occurred in the mouse lines possessing the mutated APP transgene, while loss of synaptophysin immunoreactivity occurred in both wild type (no plaques) and mutant lines (plaques). Synaptophysin is a presynaptic axonal terminal marker. Importantly, there is good correlation between cognitive decline and loss of synaptophysin-immunoreactive presynaptic terminals in AD-vulnerable regions of the brain (e.g., 64-66).

These observations were extended by another research group using a different AD transgenic mouse model (56). This AD transgenic mouse exhibits early histopathological features of AD and forms A $\beta$  deposits but not plaques. Here, spatial learning abilities were tested using the Morris water maze (MWM) model, which is sensitive to hippocampal damage. The MWM model tests the ability of the animal to spatially learn and remember the location of an escape platform using environmental cues. Transgenic AD mice showed significant deficits that increased in severity with age as compared to wild-type mice.

There is convincing evidence that oligomeric A $\beta$  species exist *in vivo*. Water soluble A $\beta$  oligomers have been detected in human brain and are elevated 12-fold in AD brain (21). Using antibodies that preferentially bind oligomeric A $\beta$  species, Lambert and colleagues demonstrated the presence of ADDLs in temporal and frontal cortices from AD brain, but not in control brains or AD cerebellum, a region not susceptible to AD-related damage (61). Oligomeric A $\beta$  species were detected in CSF samples (59). Chinese hamster ovary cells stably transfected with APP cDNA express SDS-stable A $\beta$  species including monomer, dimer, and trimer, which were identified by sequencing and affinity for specific A $\beta$  antibodies. These A $\beta$  oligomeric species may assemble shortly after A $\beta$  synthesis in intracellular vesicles as they are detected in cell lysates and isolated microsomes and they co-migrate with proteins associated with subcellular vesicles (59).

These observations strongly implicate oligomeric A $\beta$  assemblies (e.g., ADDLs) as the neurotoxins underlying the pathophysiology of AD. If true, therapies aimed at inhibiting their assembly or activity, or at elevating their clearance from the brain would be particularly effective. Specific cyclodextrin derivatives, but not cyclodextrin itself, were shown to be effective at inhibiting ADDL formation (61). Moreover, complete immuno-neutralization of ADDLs was demonstrated using antibodies raised specifically against ADDL immunogens. The possibility of effective immune intervention targeting A $\beta$  and ADDLs is stimulating another remarkable shift in AD research directions, as discussed in the following sections.

### IMMUNIZATION AND AD

AD research took a dramatic leap forward when it was shown that immunization of APP transgenic mice with aggregated A $\beta$  resulted in attenuated AD-like pathology (68). This particular vaccination regimen was effective in preventing A $\beta$ -plaque formation in young animals and reducing the progression of plaque deposition in older mice. Numerous labs have confirmed and extended this observation using different mouse models and immunization paradigms (69-72). A $\beta$  inoculations caused microglial activation (73), and antibody-mediated phagocytosis by microglia led to A $\beta$  degradation (69). Removal of existing A $\beta$  deposits by microglia after plaque decoration by anti-A $\beta$  antibodies also has been demonstrated using a novel *in vivo* multi-photon imaging technology (74), suggesting that activated microglia may enable A $\beta$  plaque removal.

A $\beta$  immunization was also shown to have beneficial functional consequences (70-72,75). For example, passive immunization with the mouse monoclonal A $\beta$  antibody m266 resulted in a rapid reversal of memory deficits in APP transgenic mice. Within 24 hours of administration, improved performance in an object recognition memory test and a holeboard learning task was observed. This reversal of memory deficits was not associated with reduced A $\beta$  deposition. An A $\beta$ -antibody complex was detectable in plasma and CSF, however, the beneficial memory effects occurred well before any meaningful reduction of total A $\beta$  (72).

Recall that nanomolar concentrations of oligomeric A $\beta$  assemblies (e.g., ADDLs) are capable of rapid inhibition of information storage mechanisms (LTP, *vide supra*, 19), and that complete *in vitro* protection from ADDL neurotoxicity was mediated by anti-ADDL antibodies (61). It is possible that the beneficial effects of A $\beta$  immunization in various rodent memory models may result from removal or direct immuno-neutralization of these oligomeric A $\beta$  species. This supports the argument for development of immunotherapies against oligomeric A $\beta$  species (61).

### THERAPEUTIC APPROACHES: INTERSECTING THE A $\beta$ OLIGOMER AXIS

Indictment of A $\beta$  oligomeric assemblies (e.g., ADDLs) as the molecular pathogens in AD establishes a new conceptual framework for devising effective therapeutic and preventative approaches. In the broadest sense, intervention breaks down into three categories: 1) block A $\beta$  1-42 production to minimize ADDL formation; 2) enhance clearance of A $\beta$  1-42 and/or ADDLs; 3) design blockers of ADDL assembly or activity. The following sections provide an overview of current and potential approaches that intersect these A $\beta$  oligomer possibilities.

Blocking A $\beta$  Production - Many current drug discovery approaches focus on blocking A $\beta$  production, some of which may ultimately be effective in AD therapy. Lowering A $\beta$  1-42 monomer concentration significantly will reduce the rate of ADDL formation, establishing a different equilibrium that disfavors oligomeric assemblies. Lowering ADDL levels will result in direct reduction of aberrant neuronal signaling (21,23). Additional advantages may accrue for inhibitors that specifically block A $\beta$  1-42, because A $\beta$  1-42 forms oligomers more favorably than A $\beta$  1-40 (76). A $\beta$  1-40 does not assemble into stable oligomers, yet at high concentrations, A $\beta$  1-40 oligomers form and they can be chemically crosslinked to generate stable structures (77).

Secretase Inhibitors - The most direct approach to blocking A $\beta$  production involves inhibition of the secretase enzymes. Good progress has been made towards discovery of BACE inhibitors (78) and  $\gamma$ -secretase inhibitors (79), a number of which are reported to be in pre-clinical or Phase I human clinical trials. Blocking BACE should lead to reduction of both A $\beta$  1-42 and 1-40,

and the major challenge for medicinal chemists is generating sufficient potency in a molecule that can penetrate the CNS to generate inhibitory concentrations of drug. Studies of transgenic mice engineered with a disrupted BACE gene appear to develop without any detectable defects (42), suggesting chronic inhibition of BACE activity may be an acceptable therapeutic approach. For BACE inhibitors, there is the question relating to potential consequences of blocking all A $\beta$  production, because it is possible that A $\beta$  1-40 has important normal functions related to adhesion and neuronal plasticity (80,81).

Blockage of  $\gamma$ -secretase on a chronic basis may also result in unacceptable side effects, because this enzyme appears to be involved in processing of other important proteins such as Notch (43-46). Perhaps PS-1 and the protein complex with which it is associated, is not the only enzyme responsible for the C-terminal A $\beta$  cleavage. This possibility may enable design of effective inhibitors that lack limiting side effects (82,83). A recent report described  $\gamma$ -secretase inhibitors that lowered A $\beta$  production without cleaving Notch, although questions have been raised regarding the actual target of these inhibitors (84,85). Another recent report demonstrated that the NSAIDs, ibuprofen, indomethacin, and sulindac sulphide, specifically lowered A $\beta$  1-42 and increased A $\beta$  1-38, apparently by modulating  $\gamma$ -secretase, and without any effect on APP. This result has generated intense interest, because this profile of activity is highly attractive for an A $\beta$  1-42 lowering agent (86).

Modulating APP - Several approaches to modulating APP could result in effective lowering of A $\beta$ , such as blocking or reducing the synthesis of APP (87). This might be accomplished at the transcriptional level or at some post-transcriptional step. The drug phenserine, originally of interest as a cholinesterase inhibitor, has been shown to reduce the synthesis of APP, and consequently levels of A $\beta$ . It has been reported that phenserine interacts with APP mRNA at an IL-1 response element, and this element may provide the mechanism by which IL-1 increases A $\beta$  (88,89). There is continuing medicinal chemistry activity to generate phenserine analogues with optimized properties (90). A recent report has described a ribozyme capable of reducing APP mRNA, but no data were reported on reduction of actual A $\beta$  levels (91). There is evidence suggesting that various neurotransmitters, cyclooxygenase inhibitors and neuroimmunophilins also may modulate APP synthesis (92). The cyclooxygenase inhibitor effect may occur by blocking prostaglandin E<sub>2</sub>, which has been shown to elevate APP synthesis (93).

Another potentially viable approach involves upregulating the  $\gamma$ -secretase enzyme activity that cleaves APP within the A $\beta$  sequence, potentially lowering overall A $\beta$  production (94). Three muscarinic receptor subtypes, M<sub>1</sub>, M<sub>3</sub>, and M<sub>5</sub>, are coupled to upregulated shedding of sAPP, suggesting that muscarinic agonists may elevate  $\gamma$ -secretase activity (95,96). Several metalloprotease enzymes have been shown to carry out this cleavage, and recent work suggests that two or more enzymes in the ADAM family have this activity (97). It appears that activation of PKC epsilon results in upregulation of  $\gamma$ -secretase activity, although which particular enzyme is involved was not established (98). The utility of this approach has not been established in any of the transgenic AD models to this point, and verification in a clinical setting may first emerge from data on one of the muscarinic compounds currently being studied (99).

Enhancing ADDL or A $\beta$  1-42 Clearance - One of the most direct intervention approaches would involve activating specific clearance of ADDLs or the A $\beta$  1-42 that forms them. Clearance of A $\beta$  was one of the motivations for the initial A $\beta$  immunization approaches discussed above, although the vaccination with fibrils clearly also targeted removal or reduction of plaques. It is likely that targeting plaques via anti-fibril antibodies played a role in generating the severe CNS inflammation suffered by human subjects receiving AN-1792, which resulted in recent

termination of that clinical study (100). Anti-A $\beta$  antibodies were expected to bind and clear A $\beta$  in the peripheral circulation, accelerating A $\beta$  efflux from the CNS and ultimately reducing brain A $\beta$  levels. Reduction of A $\beta$  was a consequence of some A $\beta$  immunization protocols in transgenic AD mouse models (68-71), but several immunization experiments led to cognitive protection with no reduction in brain A $\beta$  levels (e.g., 72). As suggested above, this raises the clear likelihood that A $\beta$  oligomers (e.g., ADDLs) are the targets for some of the generated antibodies. The recent description of rapid deficit reversal by injection of the m266 antibody indicated that "a soluble brain A $\beta$  species" was its target, and it is likely only binding to soluble A $\beta$  oligomers (e.g., ADDLs) would result in such rapid cognitive improvement. This is supported by the demonstrated neuroprotective ability of ADDL-directed antibodies (61).

Blocking ADDL Assembly or Activity - Direct interference with the assembly or activity of ADDLs represents another highly attractive strategy. The recent results that certain cyclodextrin compounds interfere with ADDL assembly and toxicity suggest that small molecules with ADDL-blocking ability and the ability to penetrate the CNS may be promising candidates for human clinical trials (61). It is possible that certain compounds designed to be blockers of fibril assembly also will have the ability to block ADDLs. However, compounds that convert plaque deposits back to A $\beta$  monomer, without interfering with oligomer assembly, could exacerbate brain damage (101). The ability to interfere directly with aberrant neuronal processes activated by ADDLs is also potentially attractive. Because one of the earliest ADDL activities is disruption of neuronal LTP (19,22), compounds that compensate for this blockage, such as nicotinic agonists may be effective therapeutics (102). Other neuron selective signaling pathways are also clearly involved in ADDL neurotoxicity, such as the Fyn kinase pathway, and compounds that are able to interfere with processes downstream of Fyn activity also may hold promise (18,19,22).

Conclusion - The paradigm shift from blocking A $\beta$  plaques and fibrils to blocking small soluble A $\beta$  toxins such as ADDLs represents a reversal in dogma and an opportunity for generating highly effective AD therapeutics. It has taken more than 90 years to accumulate compelling scientific evidence to support Alzheimer's original supposition that plaques are not the cause of AD. Clear evidence implicating ADDLs as the molecular pathogens in AD sets the stage for therapeutics that can block AD progression, prevent AD onset, and most importantly, potentially reverse existing AD deficits.

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