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Critical Review

Soluble Protein Oligomers as Emerging Toxins in Alzheimer's and Other Amyloid Diseases

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Summary

Amyloid diseases are a group of degenerative disorders characterized by cell/tissue damage caused by toxic protein aggregates. Abnormal production, processing and/or clearance of misfolded proteins or peptides may lead to their accumulation and to the formation of amyloid aggregates. Early histopathological investigation of affected organs in different amyloid diseases revealed the ubiquitous presence of fibrillar protein aggregates forming large deposits known as amyloid plaques. Further *in vitro* biochemical and cell biology studies, as well as studies using transgenic animal models, provided strong support to what initially seemed to be a solid concept, namely that amyloid fibrils played crucial roles in amyloid pathogenesis. However, recent studies describing tissue-specific accumulation of soluble protein oligomers and their strong impact on cell function have challenged the fibril hypothesis and led to the emergence of a new view: Fibrils are not the only toxins derived from amyloidogenic proteins and, quite possibly, not the most important ones with respect to disease etiology. Here, we review some of the recent findings and concepts in this rapidly developing field, with emphasis on the involvement of soluble oligomers of the amyloid-β peptide in the pathogenesis of Alzheimer's disease. Recent studies suggesting that soluble oligomers from different proteins may share common mechanisms of cytotoxicity are also discussed. Increased understanding of the cellular toxic mechanisms triggered by protein oligomers may lead to the development of rational, effective treatments for amyloid disorders.

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Keywords Amyloid disorders; Alzheimer's disease; soluble amyloid oligomers.

Abbreviations Aβ, amyloid β-peptide; AD, Alzheimer's disease; ADDLs, Aβ-Derived Diffusible Ligands; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; APP, amyloid precursor protein; CSF, cerebrospinal fluid; FAP, familial amyloid polyneuropathy; GSK-3β, glycogen synthase kinase 3β; HEWL, hen egg-white lysozyme; IAPP, islet amyloid polypeptide; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; NMDA-R, NMDA receptor; NR1, NMDA-R subunit 1; PI3K, phosphatidylinositol 3-kinase; Src, Src family tyrosine kinase; ROS, reactive oxygen species; TTR, transthyretin; VGCC, voltage-gated calcium channels.

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INTRODUCTION

Amyloid diseases comprise an important class of human pathologies, including Alzheimer's, Parkinson's and Huntington's diseases, the transmissible spongiform encephalopathies, type II diabetes mellitus and various forms of systemic amyloidosis. Those diseases have in common the presence of characteristic lesions in affected tissues, consisting of intra- or extracellular aggregates of misfolded proteins. At present, more than 20 different proteins or peptides (some of which present in various mutant forms) are known to form amyloid deposits associated with human pathologies (*1, 2*).

The notion that amyloid formation may be a much more widespread process than previously thought has recently gained support in biology and in medicine as amyloid aggregates from different proteins have been found to form *in vitro* as well as in an increasing number of degenerative pathologies in which normally harmless proteins or peptides convert into toxic species (*2*). Early histopathological findings demonstrated the presence of fibrillar protein aggregates in intracellular inclusions or in extracelullar deposits, designated amyloid plaques (e.g., *3–5*). Amyloid plaques were found in different affected organs, including the brain in neurodegenerative diseases (e.g., Alzheimer's disease and the transmissible encephalopathies), pancreas in type II diabetes, and heart,

liver and kidney in various forms of systemic amyloidosis. Regardless of the different amino acid sequences of the proteins or peptides from which they are formed, protein amyloids share many common physical and tinctorial properties (*6*). Because of this and of their ubiquitous presence in affected tissues, the fibrils present in amyloid plaques soon became likely suspects in the pathogenesis of different amyloid diseases. Further biochemical studies demonstrated that amyloid preparations obtained *in vitro* from different proteins contained abundant and easily detectable fibrils closely resembling those found in amyloid plaques (e.g., *7–10*). Giving further support to the notion that fibrils were the 'villains' in amyloid diseases, cell biology studies showed that synthetic fibril preparations were toxic to a variety of primary cells in culture as well as to different cell lines (e.g., *11–13*). Finally, studies using transgenic animal models of amyloid diseases also demonstrated the presence of abundant amyloid plaques containing fibrillar protein aggregates in affected tissues (for early examples, see *14–18*). This large body of evidence provided support to what seemed to be a solid concept, namely that amyloid fibrils played crucial roles in the pathogenesis of amyloid diseases.

However, despite its compelling support, the 'fibril hypothesis' fails to explain crucial clinical and pathological aspects of several degenerative diseases. For example, there is a lack of direct correlation between amyloid plaque burden and neurodegeneration in prion diseases (reviewed in *19*). In Alzheimer's disease (AD), it has been pointed out that there is poor or absence of correlation between plaque burden and dementia (*20–22*). Moreover, two interesting studies, carried out using different transgenic mouse models of AD presenting amyloid plaques and elevated brain levels of the amyloid-β peptide (Aβ) – the main constituent of amyloid plaques – showed that passive immunization using anti-Aβ antibodies led to the reversal of memory loss without reduction in amyloid plaque burden (*23, 24*). In one of those studies, memory recovery was observed as early as 24 hours after antibody administration, well before any changes in amyloid burden could be detected (*24*). These findings led to two major conclusions: (i) The transgenic mice investigated in those studies manifest a reversible Aβ-dependent memory loss that is not caused by neuronal death (at least not in its early stages), and (ii) memory loss is not instigated by fibrils present in amyloid plaques.

Pancreatic islet amyloid deposits containing islet amyloid polypeptide (IAPP, also known as amylin) are a characteristic pathological feature of type II diabetes mellitus. Working with transgenic mice expressing human IAPP in pancreatic beta cells, Verchere and co-workers found that mice older than 13 months developed extensive amyloid deposits in the pancreatic islets (*25*). Ultrastructural analysis revealed that those deposits were composed of human IAPP-immunoreactive fibrils that accumulated between beta cells and islet capillaries. Interestingly, however, less than half of the mice with islet amyloid deposits were hyperglycemic, revealing poor correlation between amyloid deposits and disease manifestation. On the other hand, in younger (6- to 9-month old) transgenic mice, islet amyloid deposits were less commonly observed but were always associated with severe hyperglycemia. A subsequent study suggested that the mechanism of IAPP toxicity involved membrane disruption by early IAPP aggregates, described as 'intermediate-sized toxic amyloid particles' (*26*). A more recent study showed that human IAPP transgenic rats, which present apoptosis of beta-cells and develop diabetes between 5 and 10 months of age, develop islet amyloid (*27*). Importantly, however, the extent of amyloid deposits was not related to the frequency of beta-cell apoptosis.

Another interesting example comes from studies of familial amyloid polyneuropathy (FAP), which is associated with aggregation of mutant transthyretin (TTR) and deposition of TTR amyloid fibrils throughout the connective tissue, particularly affecting the peripheral nervous system. Nerve biopsies from FAP patients showed that nonfibrillar TTR aggregates, negative for Congo Red birefringence which is typical of amyloid fibrils, were present at early disease stages (*28*). Non-fibrillar, non-Congophilic TTR aggregates have also been demonstrated in transgenic mouse models of this disease (*29*). Moreover, in human FAP nerves, increased axonal expression of markers of inflammation and oxidative stress can be identified by immunohistochemistry before fibril deposition (*28, 30*). These observations suggest that FAP pathology may also be related, at least in part, to non-fibrillar amyloid aggregates of TTR.

As illustrated by the examples presented above, the fibril hypothesis of pathogenesis does not fully account for degeneration and for some of the clinical/pathological features in patients and in animal models of amyloid diseases. Therefore, much as in the plot of a good mystery book, amyloid fibrils – which for many years had been regarded as the main suspect responsible for degeneration – no longer seemed to be the real 'serial killers'. A possible alternative emerged: Soluble amyloid oligomers, hitherto unsuspected and much smaller than the large, conspicuous insoluble fibrils found in amyloid plaques, now appear to be the hidden toxins that cause early cell dysfunction in degenerative amyloid diseases. Indeed, recent studies have shown that, for an increasing number of human amyloid diseases, soluble oligomers of the corresponding proteins/peptides are found in association with pathology, while the correlation between amyloid fibril burden and pathology is not necessarily evident (see Table 1 for selected examples). A comprehensive review of the roles of soluble protein oligomers in human pathology would be beyond the scope of the present review. Thus, we focus our discussion on recent evidence describing the presence and possible roles of soluble oligomers of the Aβ peptide in AD. As discussed below, however, there are reasons to believe that soluble oligomers from different proteins may share common structural features and biological actions.

Table 1
Emerging roles of soluble protein oligomers in human amyloid diseases[1]

Protein or peptide	Disease	Affected tissue	*In vitro* formation of oligomers (refs)	Presence of oligomers in affected tissue or poor/lack of correlation of amyloid deposits with disease manifestation (refs)
Aβ peptide	Alzheimer's disease	Brain	*50*, *52–64*, *68–70*, *78–80*, *85*, *87*, *88*, *102*, *103*	*40–49*, *103*, *108*
Prion protein	Creutzfeldt-Jakob disease, Kuru, fatal familial insomnia, Gerstmann-Straussler-Scheinker disease, transmissible spongiform encephalopathies	Brain	*114–128*	*19*, *129–132*
α-synuclein	Parkinson's disease	Brain	*133–140*	*141–143*
Huntingtin	Huntington's disease	Brain	*144*	*145–148*
Stefin (cystatin) B	Myoclonus epilepsy type 1	Brain	*149*	N/A
Islet amyloid polypeptide (amylin)	Type 2 diabetes mellitus	Pancreas	*150–152*	*25*, *153*
Human lysozyme	Familial or hereditary amyloidosis	Liver, kidney	*154*	N/A
Transthyretin	Familial amyloid polyneuropathy	Peripheral nerve	*155*	*28*, *29*
Immunoglobulin light chain	Primary amyloidosis	Kidney, lung, heart	*156*, *157*	*158*
Apolipoprotein A1	Familial or hereditary amyloidosis	Heart	N/A	*159*
Desmin	Desmin-related cardiac amyloidosis	Heart	N/A	*160*, *161*
β_2-microglobulin	Dialysis-related amyloidosis	Joint spaces	*162*, *163*	N/A

[1]Selected references are provided for each condition. N/A: not available.

Therefore, at least in part, the notions and concepts discussed in relation to Aβ oligomers may be also applicable to oligomers from other amyloid proteins/peptides. A deeper understanding of the toxic mechanisms triggered by oligomers at the cellular level may allow the development of rational, effective treatments for different amyloid-related disorders.

AMYLOID-β, THE PATHOLOGICAL AGENT IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common form of dementia of the elderly, and current estimates suggest it may affect more than 12 million individuals worldwide (*31*). AD is neuropathologically characterized by extracellular accumulation of fibrillar deposits of the amyloid-β peptide (Aβ) in senile plaques, intraneuronal neurofibrillary tangles consisting of abnormally hyperphosphorylated tau protein, oxidative neuronal damage, synaptic loss and neuronal degeneration. Given its high prevalence, lack of effective treatments and the uncertainty as to the mechanisms of pathogenesis, AD is one of the most extensively studied amyloid-related disorders.

Aβ, the main constituent of amyloid deposits in AD, is generated by cleavage of the amyloid precursor protein (APP), a single-transmembrane domain protein, by the β-amyloid precursor cleaving enzyme (BACE, also known as β-secretase; *32, 33*) and subsequent cleavage of the remaining membrane-bound C-terminal fragment of APP by gamma-secretase (*34*). Gamma-secretase is an aspartyl protease complex consisting of at least four components: nicastrin, APH-1, PEN-2, and presenilin (for a recent review, see *35*), with the latter

containing the proteolytic active site that carries out cleavage of APP within its transmembrane domain (*36*). Mutations in the APP and presenilin genes are associated with early onset, familial forms of AD and lead to increased Aβ production (reviewed in *37*), strongly suggesting a casual relationship between Aβ generation and pathogenesis in AD. This conclusion is further supported by a wealth of biochemical, cell biological and *in vivo* studies that have conclusively established that aggregated forms of Aβ are highly toxic to neurons (see below).

IN VITRO FORMATION AND *IN VIVO* DETECTION OF Aβ OLIGOMERS

Both *in vitro* and *in vivo,* Aβ assembles into different types of supramolecular structures ranging from small soluble oligomers to insoluble amyloid fibrils. Monomeric Aβ, which is physiologically produced by normal proteolytic procesing of APP, is not neurotoxic, and aggregation is required for toxic gain-of-function (*11*). Aβ fibrils were first identified and attracted significant attention because they are conspicuously present in the most prominent lesions in AD brains – the senile plaques. Retrospectively, it seems natural, therefore, that fibrils were initially viewed as the toxic species responsible for memory impairment and neuronal loss in AD (reviewed in *38*).

In recent years, however, the build-up of soluble Aβ oligomers in the brains of affected individuals has been recognized as an additional neuropathological hallmark of AD (reviewed in *39*). Early evidence indicated elevated levels of soluble Aβ and presence of pre-fibrillar Aβ assemblies in AD-affected brains (*40–43*). AD patients show brain levels of soluble Aβ oligomers up to 70-fold higher than control, age matched non-demented subjects (*44*), and cerebrospinal fluid (CSF) levels of Aβ oligomers are also higher in AD than in control subjects (*45*). The large increase in extractable soluble oligomers appears to be specific of brain regions responsible for cognitive functions that are impaired in AD patients, being present in the frontal cortex and not detectable in cerebellum (*46*). Elevated brain levels of soluble Aβ oligomers have also been found in different transgenic mouse models of AD (*47–49*), giving further support to the notion that Aβ oligomers play crucial roles in AD pathogenesis.

In a pioneering study, Lambert and co-workers showed that soluble Aβ oligomers formed spontaneously *in vitro* under conditions in which fibril formation was inhibited (*50*). They also found that such oligomers – which they named ADDLs (for Aβ-Derived Diffusible Ligands) – were neurotoxins that killed neurons in organotypic cultures. Significantly, oligomers prepared *in vitro* from synthetic Aβ were shown to inhibit long-term potentiation (LTP) in hippocampal slices (*50*). Because LTP is a measure of synaptic plasticity and is extensively used as an experimental paradigm to study memory-related processes at a cellular level, those early observations suggested that soluble Aβ oligomers could be directly involved in the impairment of synaptic plasticity and memory formation in AD (see *39* and *51* for reviews). Studies from different groups have also demonstrated that synthetic Aβ monomers aggregate *in vitro* in a time-dependent fashion to form oligomers, which eventually may form fibrils (e.g., *52–55*). In addition, secreted Aβ oligomers are found in culture media from cells expressing human APP (*56–58*).

'Aβ oligomers' is a general denomination commonly used to define non-fibrillar (or pre-fibrillar) soluble Aβ aggregates. However, 'Aβ oligomer' preparations reported in the literature usually comprise a highly heterogeneous mixture ranging from dimers/trimers to 12/24-mers (or even higher order assemblies) of Aβ (for examples, see *50, 59, 60*). The identity of the specific oligomer species that accounts for neurotoxicity is still controversial. For example, Townsend and co-workers recently showed that Aβ oligomers that are naturally secreted from cultured cells inhibit certain forms of hippocampal LTP (*61*). Through fractionation of the different oligomer species secreted by the cells, they showed that this effect was mostly attributable to Aβ trimers. On the other hand, Lesne and co-workers found that the levels of a 56 kDa Aβ species (which they called Aβ*56) showed the best correlation with memory deficits in a transgenic mouse model of AD (*49*). Furthermore, they showed that the 56 kDa oligomers purified from mice brains disrupt memory when injected into the brains of young rats. Deshpande and co-workers (*62*) recently showed that two different preparations differing in the composition of soluble Aβ oligomers bind rapidly and with high affinity to synaptic contacts and cell membranes of human fetal cortical neurons in culture. However, the time course of the toxic effects was dependent on the oligomer composition of each preparation: Aβ toxicity was five-fold faster with a preparation that contained a higher proportion of larger oligomers (12–24mers) (*62*). As illustrated by these examples, further studies are required to determine whether a single specific Aβ oligomer form or a combination of multiple species accounts for neuronal toxicity.

NEURONAL IMPACT OF SOLUBLE Aβ OLIGOMERS

Aβ oligomers bind to specific excitatory post-synaptic sites in neurons, induce aberrant expression of the memory-linked protein Arc, and interfere with synaptic plasticity by blocking both long-term potentiation (LTP) and the reversal of long-term depression (LTD) (*46, 50, 63*). Furthermore, very recent studies have shown that Aβ oligomers disrupt neuronal calcium homeostasis, induce oxidative stress and instigate tau hyperphosphorylation at AD-specific sites (*60, 64, 65*). Collectively, these data imply Aβ oligomerization as an upstream phenomenon leading to neuronal dysfunction and, ultimately, to dementia in AD. However, the precise mechanisms by which Aβ oligomers exert their toxic action in neurons are still largely unknown and are a matter of intense investigation.

In the following sections, we discuss recent studies addressing the neuronal impact of Aβ oligomers and some of the mechanisms that have been proposed to explain those effects. Where detailed information is available in this regard, we also describe the specific oligomer preparations used in those studies.

Deregulation of Calcium Homeostasis and Oxidative Stress

Sustained increases in cytosolic calcium levels trigger several intracellular events detrimental to neuronal function and survival, including exacerbated neurotransmitter release, endoplasmic reticulum stress, mitochondrial damage, production of reactive oxygen species (ROS) and activation of apoptotic pathways (*66*). Calcium homeostasis is altered by presenilin mutations and neuronal calcium dysregulation appears to play key roles in Aβ-induced neuronal dysfunction (reviewed in *67, 68*). Aβ oligomers disrupt calcium homeostasis in both SH-SY5Y neuroblastoma cells (*59*) and in primary hippocampal neurons in culture (*60, 65*). As discussed below, different mechanisms have been proposed to explain Aβ oligomer-induced neuronal calcium dysregulation, including membrane permeabilization, activation of voltage-gated calcium channels and activation of NMDA receptors.

Bobich and co-workers (*69*) showed that pre-incubation of rat cortical nerve endings with physiological concentrations (10 nM) of Aβ promotes a discrete but significant increase in K^+-induced release of glutamate and noradrenalin, a phenomenon known to be dependent on the increase in calcium level at axonal terminals. In their Aβ_{1-42} preparations, they detected two discrete oligomer species with apparent molecular weights of 16 and 20 kDa accounting for $\sim$5 and $\sim$8% of the total Aβ, respectively, while the remaining 87% of Aβ appeared as monomers in SDS-PAGE analysis. The authors concluded that oligomers were responsible for the increase in neurotransmitter release since this could be prevented by Congo red, which inhibits Aβ oligomerization by stabilizing monomers (*70*). Aβ-induced neurotransmitter release was inhibited by blockers of N-type voltage gated calcium channels (VGCC), suggesting that Aβ promotes neurotransmitter release and causes excitotoxic neuronal damage via modulation of N-type VGCC.

Demuro et al. (*59*) found that amyloid oligomers from different proteins, including Aβ, induce a rapid and sustained increase in cytosolic calcium in SH-SY5Y cells. The calcium increase was insensitive to the calcium channel blocker, cobalt, and both intracellular and extracellular calcium sources appeared to contribute to oligomer-induced cytosolic calcium overload. Amyloid oligomers also caused a rapid cellular leakage of anionic fluorescent dyes, suggesting a general increase in membrane permeability. Permeabilization of biological membranes by Aβ was proposed several years ago by Rojas and co-workers. In a series of studies, they showed that Aβ forms cation-selective membrane pores permeable to calcium and to some monovalent cations, including cesium, lithium, sodium and potassium, in planar lipid bi-layers (see *71*). Based on those studies, they proposed a molecular model for the oligomeric assembly of Aβ monomers to form a membrane pore (*72*).

Excessive levels of reactive oxygen species (ROS) have been implicated in the molecular etiology of AD (*73–76*). A number of studies of AD brain have shown elevated markers of oxidative stress, including oxidized forms of lipids, proteins and DNA (reviewed in *74–76*). Low levels of ROS are physiologically required for memory-related processes such as LTP (*77*). However, memory mechanisms may be disrupted by elevated ROS levels, supporting a connection between oxidative stress and memory impairment in AD. This underscores the importance of establishing whether ROS are coupled to the elevation in levels of soluble Aβ oligomers, another major aspect of AD pathology. Both Aβ oligomers and a soluble preparation of Aβ_{1-40} have been shown to stimulate ROS production and lipid peroxidation and to decrease the levels of reduced glutathione in primary cortical neurons (*78, 79*). In addition, pre-fribrillar Aβ_{1-40} induces lipid peroxidation in neuronal progenitor cells (*80*). Recent results have shown that Aβ_{1-42} oligomers stimulate excessive production of ROS in rat hippocampal primary neurons (*65*). ROS generation was inhibited by an intracellular calcium chelator, suggesting a role for calcium in oligomer-induced oxidative stress. Accordingly, we found that Aβ oligomers induce an increase in cytosolic calcium levels. Interestingly, calcium increase could be blocked by memantine, a blocker of N-methyl-D-asparte (NMDA)-type glutamate receptors, and by antibodies against the extracellular domain of the NMDA receptor (NMDA-R), suggesting that NMDA-R activation is required for this effect.

Interactions with Cell Surface Receptors

Overstimulation of excitatory NMDA-Rs leads to excitotoxic neuronal death through the activation of a series of Ca^{2+}-dependent mechanisms, including mitochondrial dysfunction and ROS generation. The involvement of excitotoxicity in Aβ-induced neuronal death has been demonstrated in a number of studies (*73*). For example, NMDA-R antagonists protect cultured neurons against Aβ_{1-42} toxicity (e.g., *81* and references therein). Because activation of glutamate receptors causes neuronal depolarization, we have proposed that pharmacological hyperpolarization of neurons might constitute an approach to block excitotoxicity induced by excess glutamate (or glutamate receptor agonists) or by Aβ (*82*). Indeed, recent studies from our group have shown that activation of inhibitory gamma-amino butyric acid $(GABA)_A$ receptors protects hippocampal and cortical neurons against Aβ_{1-42} toxicity (*83, 84*). However, few recent studies have directly addressed the participation of NMDA-Rs in Aβ toxicity using well-characterized oligomer preparations of the peptide.

Roselli et al. (*85*) found that oligomers of $A\beta_{1-40}$ peptide (0.1 – 10 μM) induce degradation of postsynaptic-density 95 (PSD95), a scaffolding protein that plays a pivotal role in organizing the architecture of the post-synaptic density, including anchoring of glutamate receptors. This effect was dependent on extracellular calcium influx, and could be blocked by NMDA-R antagonists, but not by antagonists of α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA) or metabotropic glutamate receptors. The predominant aggregates in the preparations used in that study were reported to be low N-oligomers (mainly monomers to tetramers). Another recent study showed that $A\beta_{1-40}$ oligomers (2 μM) induced degradation of dynamin 1, a protein involved in synaptic vesicle recycling, via calpain activation evoked by a sustained calcium influx (*60*). Both calcium influx and dynamin 1 degradation were inhibited by the NMDA-R blockers, memantine and MK-801, but not by the L-type calcium channel blocker, nimodipine. Furthermore, an intracellular calcium chelator, BAPTA, also blocked Aβ-induced dynamin 1 degradation. The oligomer preparation used in that study was obtained by pre-aggregating $A\beta_{1-40}$ followed by sedimentation and removal of fibrils by centrifugation, and comprised mostly high molecular weight oligomers ($\sim$50 – 75 kDa).

In line with results described above, we recently found that blockade of the NMDA-R channel by memantine or MK-801 blocks the increase in cytosolic calcium concentration and the stimulation of ROS production induced by $A\beta_{1-42}$ oligomers (500 nM) in hippocampal neurons (*65*). The preparation used in that study comprised oligomers ranging from trimers/tetramers up to 12/24-mers. Antibodies directed to the extracellular N-terminal domain of the NMDA-R subunit 1 (NR1) completely blocked ROS formation and reduced Aβ oligomer binding to neuronal synapses by $\sim$60%. Importantly, the N-terminal antibody to the NR1 subunit of NMDA-Rs did not cause functional impairment of the receptor, as shown by the fact that ROS formation induced by NMDA was unaffected by the antibody. This indicates that it is possible to block pathological ROS formation induced by Aβ oligomers without blocking normal function of NMDA-Rs, a possibility that may be of considerable interest from the point of view of developing new therapeutics for use in AD.

Previous studies have shown that more than 90% of the immunoreactivity of $A\beta_{1-42}$ oligomers bound to mature hippocampal cultures coincides with PSD-95 (*44*), suggesting that oligomers bind specifically to dendritic spines. In harmony with this notion, we found that the NR1 subunit of NMDA-Rs co-immunoprecipitates with $A\beta_{1-42}$ oligomers from oligomer-treated detergent-extracted rat synaptosomal membrane preparations. Thus, although there may be additional types of oligomer receptors in neurons (as suggested by the fact that the anti-NR1 antibody does not completely abolish oligomer binding), these observations suggest that the NMDA-R complex is a post-synaptic target of Aβ oligomers.

Decreased surface expression of NMDA-Rs induced by $A\beta_{1-42}$ ($\leq$1 μM) has been recently demonstrated (*86 – 88*), suggesting another mechanism by which oligomers (explicitly used in *88*) may disrupt activity-dependent synaptic plasticity. In this regard, decreased expression of AMPA receptors induced by Aβ has also been recently reported (*85, 89*). In addition to a possible interaction with or regulation of glutamate receptors, other neuronal surface proteins may be involved in Aβ toxicity. For example, direct binding (*90*) and inhibition of nicotinic acethylcholine receptors by submicromolar concentrations of Aβ has recently been reported by different groups (e.g., *91 – 93*).

Tau as a Downstream Target of Aß Oligomers

Neurofibrillary tangles (NFTs) are AD-characteristic neuropathological lesions consisting of intraneuronal aggregates of hyperphosphorylated tau protein. Under physiological conditions, tau plays important roles in microtubule stabilization and dynamics. On the other hand, tau has also been implicated in the pathogenesis of neurodegenerative diseases including AD and other so-called tauopathies (*94*). Of relevance to the mechanisms of pathogenesis in AD, various *in vivo* and *in vitro* studies have shown that Aβ deposits are associated with neuronal tau hyperphosphorylation (*95 – 101*). These studies establish a causal relationship between elevated levels of Aβ, tau hyperphosphorylation and formation of NFTs in AD.

Increased brain levels of soluble Aβ directly correlate with NFT density in AD patients (*43*) and Aβ oligomers activate glycogen synthase kinase-3β (*102*), one of the kinases that appear to be involved in pathological hyperphosphorylation of tau. Moreover, Aβ oligomers accumulate in the brains of Tg2576 APP transgenic mice and of triple transgenic mice harboring presenilin, APP and tau mutations, and appear to be causally related to tau phosphorylation in those animals (*47, 103*). These observations suggest that Aβ oligomers trigger the activation of intracellular pathways leading to tau hyperphosphorylation.

Synthetic $A\beta_{1-42}$ oligomers and soluble extracts containing Aβ oligomers obtained from AD brains have recently been found to instigate tau hyperphosphorylation at AD-specific epitopes in mature neuronal hippocampal cultures (*64*). An anti-oligomer antibody prevented binding of Aβ oligomers to neurons and blocked tau hyperphosphorylation. Immunocytochemical analysis revealed that P-tau levels were selectively increased in a subpopulation of neurons that specifically exhibited Aβ oligomer binding, but not in neurons that were free from oligomer binding (*64*).

Another recent study demonstrated that tau confers a marked sensitivity of neuronal microtubules to destabilization induced by $A\beta_{1-42}$ oligomers (*104*), providing a direct connection between the toxic impact of Aβ and neuronal dysfunction triggered by cytoskeletal changes. In this regard, it is interesting to note that hippocampal neurons obtained from

tau knockout mice were found to be resistant to Aβ-induced neurite degeneration (*105*). Collectively, the results described above suggest that tau hyperphosphorylation triggered by Aβ oligomers is a crucial step in neurodegeneration in AD.

Intracellular Aβ Oligomers

Most of the studies described in the preceding sections used extracellularly added Aβ to investigate the impact of oligomer preparations, either by adding oligomers to neuronal cultures or by injecting them into the brains of animals. However, several lines of evidence indicate that intracellular accumulation of Aβ is also an important feature of AD neuropathology (reviewed in *106*). $A\beta_{42}$ accumulates early inside neurons in AD brain and in transgenic AD models. In the latter, marked accumulation of $A\beta_{42}$ in multivesicular bodies was found to be associated with morphological alterations in processes and synapses (*107*). It is likely, therefore, that Aβ may also trigger toxic events by acting intracellularly. Recent work demonstrates that Aβ oligomers accumulate within primary neurons from Tg2576 mice in culture as well as in Tg2576 and AD brains (*108*). By using an antibody that specifically recognizes Aβ oligomers, but not monomers, those authors found by immunoelectron microscopy that intracellular Aβ oligomers are localized within endosomal vesicles and along microtubules of neuronal processes. Oligomer accumulation was associated with pathological alterations within processes and synaptic compartments of Tg2576 and AD brains. Another recent study using a triple transgenic mouse model also detected age-dependent brain accumulation of intraneuronal Aβ oligomers (*47*). Furthermore, that study showed that a single intrahippocampal injection of an anti-oligomer antibody was sufficient to clear both Aβ and tau pathologies. Though antibody therapy is likely to block mainly extracellular oligomers, these experiments do not rule out a possible direct role for intracellular Aβ oligomers in tau pathology.

These interesting findings raise important questions to be addressed in future studies: First, are the extracellular oligomers detected in conditioned media of Aβ overproducing cells or in the CSF of transgenic AD models and AD patients already secreted in oligomer form, or is Aβ secreted as monomers that aggregate extracellularly? If they are secreted as oligomers, therapies aimed to interfere with the oligomerization process should act intracellularly. Second, do extracellular Aβ oligomers enter neurons, and is internalization required for toxicity? Understanding the process of oligomerization of Aβ inside and/or outside neurons, as well as the dynamic exchange between those two pools, may shed light on Aβ toxic mechanisms and provide novel alternatives to modulate Aβ toxicity.

GENERAL TOXICITY OF AMYLOID OLIGOMERS

As mentioned above, soluble oligomers formed from several unrelated proteins appear to be causally implicated in a number of human degenerative diseases. A growing body of evidence indicates that cytotoxicity is an inherent property of such oligomers. It has been shown that even proteins that are not related to amyloid diseases can form oligomers and fibrils *in vitro* under partially denaturing conditions (*2*). Interestingly, oligomers produced *in vitro* from such non disease-related proteins are also cytotoxic to cells in culture (for an early example, see *109*). Similar to the actions of Aβ oligomers, the cytotoxicity of non disease-related amyloid oligomers involves disruption of calcium homeostasis and oxidative stress (*110*). These findings led to the proposal that amyloid oligomers possess generic structural motifs and/or physicochemical properties that impart toxicity, independent of the amino acid sequence of the protein that gives rise to them (*109*). Further support to this hypothesis was provided by the finding that a conformational antibody raised against a micellar, oligomer-mimicking Aβ preparation recognizes a generic epitope present in oligomers formed from different proteins (*55*). Remarkably, blocking that epitope with the antibody led to neutralization of oligomer toxicity.

We recently found that soluble oligomers from hen egg-white lysozyme (HEWL) – a normally harmless, non disease-related protein – are toxic to rat primary cortical neurons in culture and induce widespread neurodegeneration *in vivo* when injected into the parietal cortex of adult rats, suggesting that accumulation of generic amyloid oligomers in the brain is sufficient to induce neuronal death as observed in amyloid-related neurodegenerative diseases (*111*).

As previously discussed, tau hyperphosphorylation is a characteristic feature of AD, and it is readily induced by Aβ oligomers. Increased levels of hyperphosphorylated tau have also been found in animal models of Parkinson's disease (*112*) and transmissible spongiform encephalopathy (*113*), pathological conditions in which oligomers from α-synuclein and the prion protein, respectively, appear to play important roles. These observations suggest that tau phosphorylation may be a common consequence of the accumulation of amyloid oligomers in affected brain areas in different neurodegenerative diseases. In our study (*111*), we found that HEWL oligomers induce tau hyperphosphorylation at AD-specific sites in primary cortical neurons in culture, showing that oligomers from a non disease-related protein are capable of triggering a specific type of cellular response that is characteristically observed in AD. These results support the hypothesis of a generic toxic oligomer conformation and further suggest that some of the specific pathological features observed in different amyloid diseases may depend on the characteristics of the affected tissue/cell type rather than on the identity of the amyloidogenic protein.

CONCLUDING REMARKS

The studies reviewed here point to soluble protein oligomers as key targets for the development of novel

therapeutic strategies aimed to treat or prevent amyloid diseases. Oligomers formed from different amyloidogenic proteins appear to share common structural properties and mechanisms of cytotoxicity, suggesting that it may be possible to utilize common principles to develop oligomer-directed therapies against different amyloidotic degenerative diseases. For many years, progress in this area was hampered by the fact that most research groups were chasing amyloid fibrils and plaques in their attempts to develop novel targets and therapeutics. Therefore, recognition that protein oligomers are the proximal toxins in amyloidosis represents a milestone in understanding the etiology of those diseases.

In the case of AD, significant progress has been made following the seminal discoveries that led to the concept that soluble oligomers of Aβ are the proximal neurotoxins that cause early synaptic dysfunction and memory loss. Several mechanisms of synaptic attack by Aβ oligomers have been discovered in recent years (Fig. 1), revealing novel molecular targets for strategies aimed at blocking neuronal dysfunction. Because Aβ oligomers interfere with pathways and processes that are vital for normal synaptic function, stability and plasticity, hence memory-related processes, a major challenge will be to design strategies to selectively block pathological interference by oligomers without blocking normal physiological function.

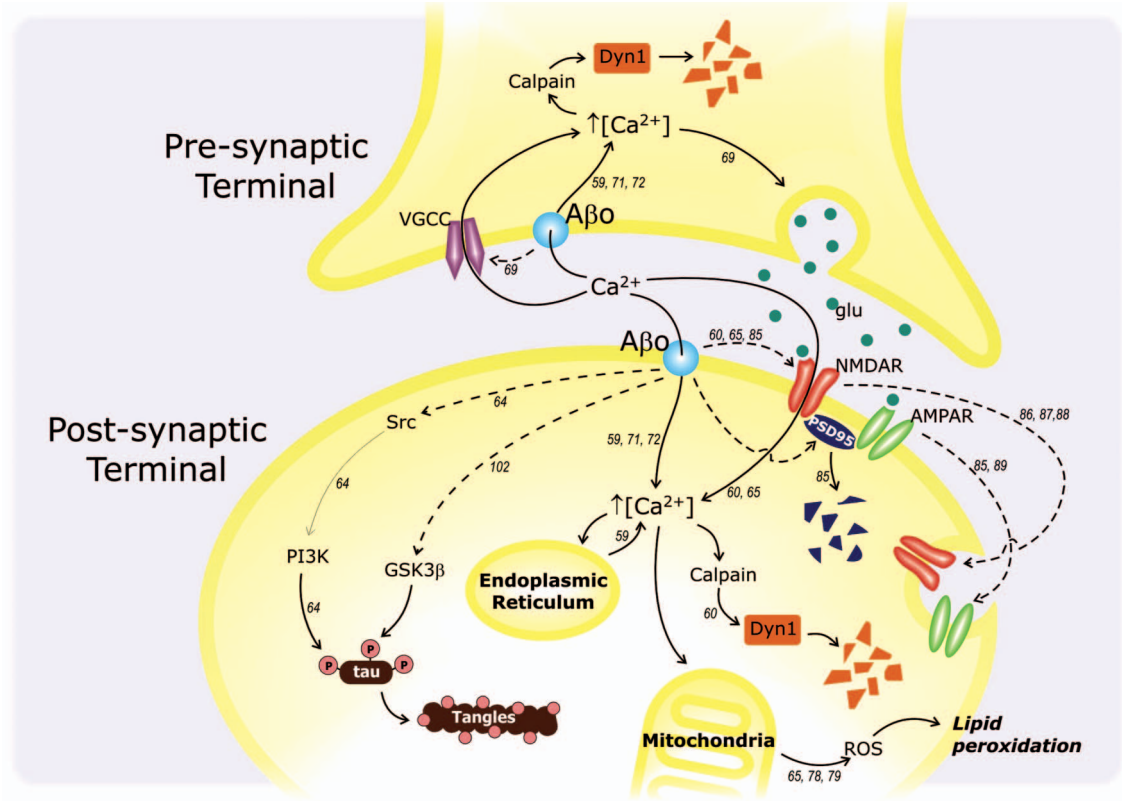

Figure 1. Neuronal impact of soluble Aβ oligomers. Pre-and post-synaptic pathways and processes suggested to be affected by Aβ oligomers (Aβo) are represented. Numbers indicate references that provide support to each specific action of Aβ oligomers. Pre-synaptically, Aβ oligomers (presumably through an increase in membrane permeability or through activation of VGCC) cause an increase in calcium levels, which may lead to activation of calpain and degradation of dynamin-1 (Dyn1), a protein involved in synaptic vesicle recycling. Elevated pre-synaptic calcium levels also cause increased neurotransmitter release. Higher concentrations of released glutamate (glu) may activate post-synaptic AMPA and NMDA receptors, leading to calcium influx. Post-synaptically, Aβ oligomers cause increased calcium influx by deregulation of NMDA receptor function. Calcium influx may also result from increased permeability of the plasma membrane induced by Aβ oligomers. Further elevation in intracellular calcium levels may be caused by mobilization of intracellular stores (e.g., calcium-induced calcium release or endoplasmic reticulum stress). Sustained elevation in calcium levels leads to calpain-mediated degradation of dynamin and to mitochondrial dysfunction and excessive production of reactive oxygen species (ROS). Aβ oligomers lead to proteasomal degradation of PSD-95, a major scaffolding protein that tethers AMPA and NMDA receptors, among other proteins, to the post-synaptic density. Reduced surface expression of AMPA and NMDA receptors is another consequence of the synaptic attack by Aβ oligomers. Activation (by mechanisms that are not yet fully elucidated) of Src/PI3K and GSK-3β by Aβ oligomers instigates hyperphosphorylation of tau.

Nonetheless, it is hoped that knowledge of the true identity of the toxic species and of their cellular partners will pave the way for rapid developments in this field.

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