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REVIEW

The toxic Aβ oligomer and Alzheimer's disease: an emperor in need of clothes

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The 'toxic Aβ oligomer' hypothesis has attracted considerable attention among Alzheimer's disease researchers as a way of resolving the lack of correlation between deposited amyloid-β (Aβ) in amyloid plaques—in terms of both amount and location—and cognitive impairment or neurodegeneration. However, the lack of a common, agreed-upon experimental description of the toxic Aβ oligomer makes interpretation and direct comparison of data between different research groups impossible. Here we critically review the evidence supporting toxic Aβ oligomers as drivers of neurodegeneration and make some suggestions that might facilitate progress in this complex field.

Accumulation of abnormally folded proteins is a key histopathological characteristic of many neurodegenerative disorders. In Huntington's disease, the polyglutamine protein huntingtin is present in intranuclear inclusions; in prion disease, infectious prion particles bind to the membrane bound protein. In Parkinson's disease, α synuclein aggregates as Lewy bodies in the cytoplasm of neurons. In Alzheimer's disease, both intra- and extracellular amyloids—that is, neuronal tangles containing phosphorylated tau and amyloid plaques consisting of amyloid peptides (Aβ)—are observed. Thus, aggregates accumulate extracellularly or in the cytoplasm, in the nucleus and at the cell membrane in different diseases.

A plausible generic explanation for the toxicity of intracellular aggregates involves the idea that the sequestration of crucial proteins together with amyloid leads to cellular dysfunction and death. Deregulated proteostasis could initiate and certainly enhance such protein failure. In Alzheimer's disease, tau aggregates occur intracellularly and could indeed theoretically trap functional proteins, such as tau itself, possibly leading to microtubule destabilization or other cellular dysfunction and death. Alzheimer's disease is somewhat unusual, however, in that there are two aggregates—intracellular tau and extracellular Aβ—that potentially could result in neuronal dysfunction and death. A central question in the field is: by what mechanism might extracellular amyloid peptides such as Aβ harm cells? An alternative hypothesis suggests that small soluble oligomeric structures consisting of amyloid peptides can cause cellular toxicity. Metastable oligomeric structures have indeed been described in preparations of amyloid forming peptides such as α synuclein, tau, prion and Aβ. When produced intracellularly, oligomers expose flexible hydrophobic surfaces[1] that might contribute to trapping vital proteins, but they also, when produced extracellularly (as is largely the case for Aβ), cause potentially toxic alterations of cell membranes[1]. This notion of a toxic Aβ oligomer has been strongly promoted in Alzheimer's research because it provides potential explanations for the toxicity of the extracellular Aβ peptide and for the lack of a correlation between deposition of insoluble Aβ plaques and neuronal loss: Aβ oligomers might be able to mediate toxicity at some distance from established plaques. Further, they could subtly damage and predispose sensitive neurons to the formation of intracellular tau aggregates. The present avalanche of publications claiming the identification of various toxic Aβ oligomers would indeed seem to support this hypothesis. However, the exact meaning of the term 'toxic Aβ oligomer' is very confusing. This elusive soluble Aβ species is in danger of becoming a way to explain inconsistencies in existing models without applying the scientific rigor needed to make real progress. The burden of proof for this 'invisible toxin' is on the person making the claim, as with the (in)famous teapot of the British philosopher Bertrand Russell.

In this review we investigate the evidence for toxic Aβ oligomers and their role in Alzheimer's disease, the definition of toxic Aβ oligomers and the question of whether it is reasonable to consider toxic oligomers as drug targets.

The case for oligomers in Alzheimer's disease

The case for a central role of Aβ in the pathogenesis of Alzheimer's disease is very strong[2]. However, the precise role of Aβ in the disease—whether as a trigger of subsequent neuronal death processes, a toxic threshold that needs to be surmounted before neuronal death processes occur or a continual driver of neuronal death—has yet to be clarified[3]. Mutations in the genes encoding either the substrate (β-amyloid precursor protein (*APP*)) or the enzyme (presenilin (*PSEN*)) that generate Aβ are sufficient to cause the disease[4]. The most significant genetic risk factor for late-onset Alzheimer's disease, *APOE4*, affects Aβ clearance[5] or its oligomerization[6]. The alternative hypothesis[7] proposing that loss of function of PSEN interferes with synaptic function independently of Aβ does not explain the effects of *APP* mutations, *APP* duplications or *APOE4*. This failure to explain these effects, together with the fact that all studied *PSEN* mutations also affect Aβ, unequivocally points to abnormalities in Aβ generation or clearance as being a primary and seminal cause of Alzheimer's pathology. This compelling genetic evidence for Aβ's role in Alzheimer's disease does not necessarily imply strong support for

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the pathological role of Aβ plaques or provide mechanistic support for a role in neuronal death. Indeed, the number of Aβ plaques in the brain does not correlate to the severity of cognitive decrements in patients, and alterations in Aβ metabolism and even amyloid plaques are already present many years before clinical symptoms can be observed[8]. In transgenic mouse models of Alzheimer's disease that overexpress APP and/or presenilin, findings show no correlation between amyloid plaques and cognitive alterations or neurodegenerative changes. Finally, there is no description of an amyloid plaque only dementia, whereas tau only pathology can cause neuronal loss in frontotemporal lobar dementia.

How, then, can the relationship between Aβ and cognitive alterations be understood? More than 20 years ago, it was demonstrated *in vitro* that nontoxic monomeric Aβ was converted into a toxic species after incubation for a few days in buffer[9]. Toxicity was associated with the appearance of "high molecular weight species" in SDS–PAGE. Atomic force microscopy, ultracentrifugation and gel filtration techniques later demonstrated the presence of "metastable Aβ protofibrils"[10,11] in Aβ preparations generated *in vitro*. These protofibrils have a smaller diameter than amyloid fibrils and high β-sheet content, and they are likely to be on a pathway to amyloid fibril formation. *APOJ* (encoding clusterin), a genetic risk factor for Alzheimer's disease, was shown to promote such soluble Aβ structures[12]. These and still other oligomeric assemblies[13,14] disturb neurotransmission and can cause cell death *in vitro* and *in vivo*. Several investigators have demonstrated that soluble Aβ oligomeric species can be extracted with saline buffers from the brain tissue of patients with Alzheimer's disease and that the presence of such soluble species is more strongly correlated than amyloid plaques with disease symptoms[15,16]. Finally, at least two clinical mutations in APP itself in the middle of the Aβ peptide[17,18] seem to increase tendency of the mutated Aβ to oligomerize.

These observations support but do not prove the hypothesis that ill-defined soluble Aβ species are involved upstream in the pathogenesis cascade that causes Alzheimer's disease.

Aβ: from monomer production to plaque assembly

The monomeric Aβ (molecular weight ~4 kDa) is produced mainly in neurons from APP (molecular weight ~120 kDa) via sequential scission by the enzymes β- and γ-secretase (**Fig. 1**). An alternative α-secretase pathway operates in other cell types, generating shorter fragments that are thought to be nonamyloidogenic (see ref. 4 for further discussion). For the present debate, it is important to realize that Aβ is a heterogeneous mixture of peptides having different solubility, stability and biological and toxic properties. C-terminal heterogeneity is generated by the γ-secretase itself. This protease cleaves APP at different positions, generating a variety of peptides, of which Aβ43, Aβ42, Aβ40, Aβ38 and Aβ37 variants are detected in cell culture and body fluids (Aβ numbering indicates the last C-terminal amino acid residue and the number of amino acids comprising the peptide, with residue 1 being the amino acid residue aspartyl 1 of the Aβ sequence in APP; see **Fig. 1**). Additional heterogeneity is generated by enzymatic processes mediated by aminopeptidases, glutaminyl cyclase or isomerases (reviewed in ref. 4), and by phosphorylation reactions[19], resulting in a mix of more than 20 Aβ peptides that all participate to different extents in putative Aβ functions in the normal brain and oligomerization and fibrillization in the Alzheimer's disease–afflicted brain. Aβ40 is continuously and abundantly produced in both healthy and Alzheimer's–affected brain tissue, whereas other Aβ peptides are continuously produced at lower levels.

The literature devoted to Alzheimer's disease has historically focused on the Aβ42 species. This peptide was found to be increased

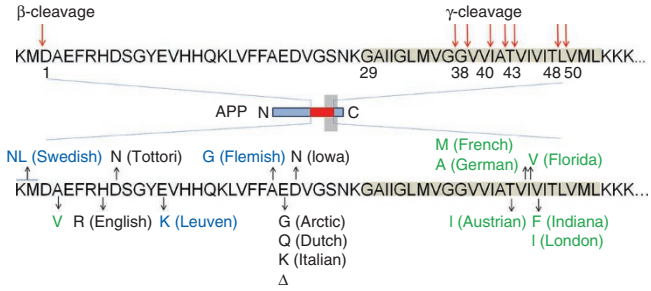

Figure 1 Generation of Aβ from amyloid precursor protein. The sites of β- and γ-secretase–mediated cleavage are indicated with arrows, and the transmembrane domain of APP is highlighted in gray. γ-cleavage produces a pool of Aβ fragments that vary in length and hydrophobicity. The mutations in Aβ region of APP either increase the total Aβ production (marked in blue), alter Aβ biophysical properties (in black) or affect the Aβ spectrum in both quantitative and qualitative ways (in green).

in cells expressing APP or presenilin harboring mutations associated with familial Alzheimer's disease (FAD). Early literature demonstrated that Aβ42 provided small "nidi" that initiated amyloid fibril formation robustly, leading to the nucleated polymerization mechanism of Aβ amyloidogenesis[20]. This view has dominated the field, but the focus on Aβ42 as the disease-causing and fibrillization-inducing agent has biased our understanding of the disease process. Indeed, aggregation and toxic properties are studied with solutions containing a single type of Aβ, but these properties are likely to be strongly influenced by the presence of other Aβ species. Aβ43, apart from Aβ42, is increased in certain patients with FAD, whereas shorter Aβ peptides such as Aβ37 or Aβ38 are decreased[21]. Finally, evidence is accumulating that polymerization of Aβ is complex and occurs via metastable intermediaries in a process called "nucleated conformational conversion"[22].

Therefore, qualitative changes in the spectrum of Aβ peptides, more than quantitative increases of individual Aβ peptides, are determining the biophysical and pathobiological properties of what we call Aβ. Minor changes in the levels of long Aβ42 or Aβ43, or pyroglutamate-modified Aβ, can drive the seeding of plaques, and, possibly independently, neurotoxicity. The complexity of these processes needs to be accommodated as we consider the transition from normal Aβ production to pathologically relevant Aβ oligomer generation in incipient Alzheimer's disease.

Amyloid plaques might exist apart from or in equilibrium with oligomeric forms of Aβ. They consist of cross-β-sheet units of Aβ peptides that are arranged to form amyloid fibrils. Plaques may be considered inert sinks of aberrantly folded proteins, lipids and free metals. But neurons in the vicinity of plaques display dystrophic neurites and synaptic loss, and elevated resting calcium levels are seen in neurons surrounding plaques in mouse models of Alzheimer's disease[23]. This suggests that plaques are not entirely innocuous. A dynamic equilibrium between toxic oligomers and inert fibrils might exist around the plaques, resulting in local 'spillover' of neurotoxic species in surrounding tissue. It is also possible that the presence of these hydrophobic deposits triggers inflammatory processes that result ultimately in neuronal damage.

Soluble Aβ oligomers: constant change

As Heraclitus observed around 2,500 years ago, "change alone is unchanging." Thus, between the monomeric Aβ and the amyloid Aβ fibrils in the plaques, intermediary soluble oligomers must exist and they might explain the neurotoxicity ascribed to soluble fractions in various experiments. However, the literature provides no clarity as to which of the bewildering list of Aβ oligomer species identified

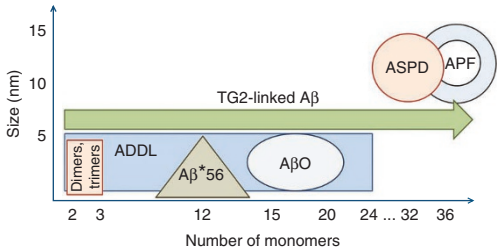

Figure 2 Size overlap in major natural and *in vitro* generated Aβ oligomers. Note that dimers and amylospheroids—both Alzheimer's brain–derived species—are located at opposite ends of the spectrum. The Aβ preparation called ADDL (Aβ-derived diffusible ligands), may encompass the Aβ species known as low-*n* mers, Aβ*56 and AβO, all having comparable dimensions. Transglutaminase TG2 generates a broad range of Aβ oligomers, but, to date, their structure and size distribution has not been characterized extensively. The annular protofibrils (APF) are the largest species in this spectrum; however, they are composed of smaller building blocks (more details are given in **Table 1** and **Fig. 3**).

to date is pathologically relevant in the human disease (summarized in **Figs. 2** and **3**). The multiplicity of Aβ species is derived not only from native biological variation, as discussed, but also from the different techniques used to generate such species *in vitro* or to isolate them from brain tissue. It is also likely that several of the identified species have similar or overlapping properties (**Fig. 2**), but different laboratories working in the field do not extensively share and compare their Aβ oligomer samples (see **Table 1** and **Fig. 3** for an overview of species described in the literature).

A couple of important issues should be addressed before we discuss these different Aβ oligomeric species in more detail. One is the 'SDS resistance' of the oligomers, which is used as a defining characteristic in most of the relevant literature. Bitan *et al.*[24] have demonstrated that SDS can actually induce artificially oligomerization of Aβ, and Hepler *et al.* applied Aβ oligomers, Aβ fibrils and Aβ monomers to SDS-PAGE and obtained monomer, trimer and tetramers as major bands in each preparation[25]. Therefore, SDS-PAGE is not a reliable method to analyze Aβ oligomers or to make comparisons between different laboratories. Given that every technique has its own limitations, only the combination of SDS-PAGE with other analytical tools (gel filtration, dynamic light scattering, atomic force microscopy) can provide a more precise definition of the species under study. For instance, a 65–80-kDa peak is seen in size-exclusion chromatography of some Aβ oligomer preparations. This peak suggests Aβ species with the mass of a dodecamer, but this fraction actually contains structures of 150–1,000 kDa as detected with multi-angle laser light scattering[25]. Aβ oligomers consist of amphiphilic peptides that might adopt very heterogeneous morphologies explaining their unusual behavior in many analytical methods[25].

If analytic approaches do indeed affect the conformation and aggregation state of the Aβ oligomers, this brings into question the real identity of 'stable' Aβ oligomers. Any given isolation procedure could select a certain population of oligomers that might not reflect the spectrum of Aβ oligomers that are used in bioactivity experiments. An alternative to the idea of static and specific stable oligomers is the possibility that these noncovalently bound Aβ assemblies are in a continuous dynamic equilibrium with monomers and other Aβ aggregates (**Fig. 3**) that varies with different peptides as well as with concentration, temperature, pH, salt, other proteins and lipids. Adding a specific Aβ oligomer preparation to an *in vivo* assay will immediately affect this equilibrium, making it very difficult to know exactly which component of the preparation causes the biological activity observed. Careful monitoring of the stability of the Aβ oligomer over time in different biophysical conditions, recovery of the putative toxic species from the cell culture medium at the end of the experiment and covalent stabilization of the oligomer are only partial remedies to this problem.

Apart from different preparations, many different 'toxicity' assays are used to demonstrate biological activity of the Aβ oligomers. Several laboratories use the mitochondrial MTT (tetrazole) test despite serious concerns regarding its validity in view of the nonspecific interference of Aβ peptide with MTT dye reduction[26]. Other cytotoxicity tests[26,27] are probably more reliable when assessing Aβ toxicity. Some groups also measure induction of apoptosis or other markers of cell death. Interestingly, almost all Aβ oligomers show synaptotoxicity as measured by changes in dendritic spine morphology, altered long-term potentiation (LTP) and long-term depression (LTD) in hippocampal slices; effects on neurotransmission in cell culture; or defects in memory and cognition in rodents[13,14,28–33] (see **Table 1** and **Figs. 4** and **5**). These rather subtle synaptic alterations triggered by Aβ in model systems are thought to reflect the early cognition problems seen in people with Alzheimer's disease, although other investigators consider them a 'far cry' from the massive cell loss seen in affected human brain tissue[33]. Acute, widespread cell loss by Aβ would in fact not be expected, as neuronal death manifests only in the advanced form of the disease, which evolves from the incipient pathology over many decades. The Aβ toxicity initiating the early changes in brain tissue must therefore be subtle, and it is probably transient until the damage caused by the Aβ oligomer stress, combined with accumulating tau pathology, results in irreversible neurodegeneration. An alternative view is that the alterations in synaptic processing that can be induced *in vivo* in disease models do not have a human correlate and that the onset of cognitive decline in Alzheimer's disease occurs when tau-mediated neuronal loss and dysfunction essentially overwhelm 'cognitive reserve'.

Brain-derived oligomeric species

With regard to oligomer identification and characterization, the literature can be divided into two schools of thought: '*in vitro*' and '*ex vivo*'. Each has its own advantages and pitfalls, not all of which are always acknowledged in the literature.

The strongest correlative support for the potential role of soluble Aβ species in Alzheimer's disease comes from work on brain tissue from patients with Alzheimer's. Several investigators have demonstrated that such species can be extracted from affected brain tissue using saline buffers and without detergents[15,16].

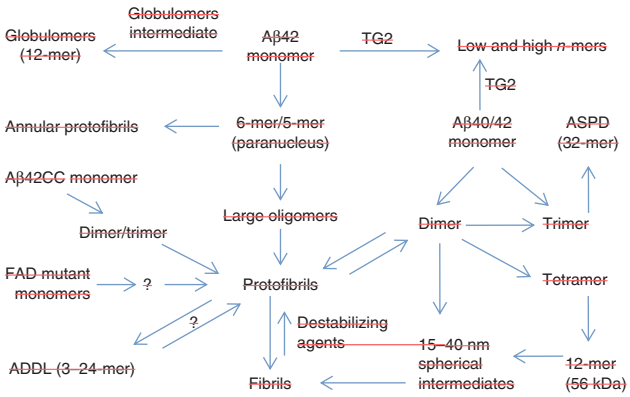

Figure 3 Scheme of interconversion between different natural and synthetic Aβ assemblies based on pathways described in the literature. Aβ monomers, oligomers and fibrils exist in a complex equilibrium, sensitive to numerous external factors. Coexistence of several oligomeric populations that do or do not propagate into fibrils is possible. Despite the differences in structure, stability and concentration, all oligomers may contribute to Aβ toxicity.

Table 1 Major soluble Aβ preparations and deleterious effects associated with them

Species	Structure	Biological effects
Aβ-derived diffusible ligands (ADDL)[13,90,93]	3–24-mer Major component, 17 kDa (tetramer) Globular structures 2–5-nm height Off-pathway A11-positive	LTP impairment (500 nM) Tau mis-sorting into dendrites and tau phosphorylation (5 μM) Abnormal mitochondrial distribution in neurites caused by impaired tau-dependent axonal transport (800 nM) Cytotoxicity in primary neurons (micromolar concentration) Cognitive impairment in mice
Aβ*56 (refs. 39,48)	12-mer Globulomers 56 kDa A11-positive	NMDAR-dependent synaptotoxicity Memory deficiency in middle-aged mice Tau phosphorylation: data not available No cytotoxicity Cognitive impairment in rats (micromolar concentration)
AβO[69,94,95]	15–20-mer Spherical vesicles 2–5 nm A11-positive	NMDAR-dependent synaptotoxicity Tau phosphorylation: data not available Cytotoxicity in human neuronal cultures (5 μM)
Aβ42 and Aβ42:40 (3:7) synthetic oligomers[27]	β-sheet enriched Transient on-pathway species A11-positive	Impairment of synaptic transmission (1 μM) Tau phosphorylation (our unpublished data) Cytotoxicity in primary neurons (micromolar concentration) Memory deficiency in mice
Transglutaminase 2–induced oligomers[40]	Apparently no secondary structure Off-pathway A11-positive (our unpublished data)	LTP impairment (100 nM) Tau phosphorylation: data not available No cytotoxicity in primary neurons (our unpublished data)
Aβ42CC protofibrils[88]	Intramolecular disulfide bond stabilized antiparallel β-sheet–based structure that does not propagate into fibrils Weak reactivity with A11	Synaptotoxicity (1 μM) (our unpublished data) Tau phosphorylation: data not available Caspase-3 activation in human neuroblastoma cells (1–10 μM)
Annular protofibrils (APF)[96]	Off-pathway Form pores, 11–14-nm outer diameter; 2.5–4-nm inner diameter Composed of spherical A11-positive Aβ42 oligomers assembled in 6 hexamers, (36-mer), 155 kDa	Hypothesized to cause Ca^{2+} overload and cytotoxicity (micromolar concentration)
SDS-stable dimers and trimers isolated from WT or FAD *hAPP*- and WT or FAD *PSEN1*- and *PSEN2*-transfected CHO cell culture medium[14,28,30,48,97,98]	6-, 8- and 12-kDa assemblies Enriched in N-end (Arg5) truncated species Contain Aβ40 and Aβ42	LTP impairment NMDAR and mGluR-dependent LTD facilitation Tau phosphorylation No cytotoxicity Cognitive impairment in mice (low-nanomolar concentrations)
SDS-stable brain-derived dimers[34,49,99] and synthetic dimers ($Aβ_{1–40}$Ser26Cys)[a]	8–12-kDa assemblies 3–4-nm height No detectable secondary structure	LTP impairment (100 nM)[b] Tau phosphorylation in primary neurons (0.5 nM) No cytotoxicity to primary neurons (micromolar concentration) but toxic to neurons cultured with microglia
SDS-stable amylospheroids (ASPD; synthetic or brain-derived)[36,37,100, a]	10–15-nm spherical Aβ assemblies 32–150-mers A11-negative	Synaptic impairment: data not available Tau protein kinase 1/GSK-3β activation (210 nM); NMDAR-independent cytotoxicity in neurons (high-picomolar to low-micromolar concentration). The highest toxicity is ascribed to either 32-mer (7.2 nm) or to ASPD >10 nm

[a]Isolated from brains of humans with Alzheimer's disease. [b]LTP alteration by dimers[34] was later attributed to the dimer-derived β-sheet–containing protofibrils[35].

Further fractionation of these brain extracts by means of nondenaturing size-exclusion chromatography was performed, and the highest synaptotoxicity in the late-eluting fractions was found correlating with the presence of soluble Aβ[34]. Aβ ran as a dimer in SDS-PAGE and interfered with synaptic function in different paradigms. The authors therefore defined Aβ dimers as the minimal toxic species *in vivo*, but whether the synaptotoxicity observed in their preparations was indeed directly caused by the dimers present in their purified fractions remains unproven. Furthermore, the exact amino acid composition of the putative 'dimeric' species has not been definitively clarified. Additional contamination by unknown protein or lipid, covalent modification of Aβ or the rapid aggregation of the putative dimers into larger structures during assays might provide alternative explanations. The authors confirmed that Aβ dimers made by cross-linking synthetic Aβ were synaptotoxic, although no direct quantitative comparison with the brain-derived dimers was made. Moreover, in a later study, the authors found that these synthetic dimers rapidly aggregate in metastable protofibrils[35], suggesting that a more complicated interpretation of the available evidence is needed.

Indeed, apart from the Aβ dimers and trimers[34], larger Aβ oligomers and structures such as the amylospheroids (ASPD), which are spherical Aβ assemblies of 10–15 nm[36,37], have been isolated from Alzheimer's-affected brain tissue and suggested by investigators to be the elusive toxic Aβ oligomeric species postulated above.

Experiments using brain tissue from transgenic mice have yielded very controversial results. In the J20 mouse model of Alzheimer's disease, which expresses human APP containing two familial mutations, no clear correlation between memory deficits and the presence of dimers, trimers or other Aβ species could be established[38]. The authors concluded: "These data demonstrate the presence of multiple assembly

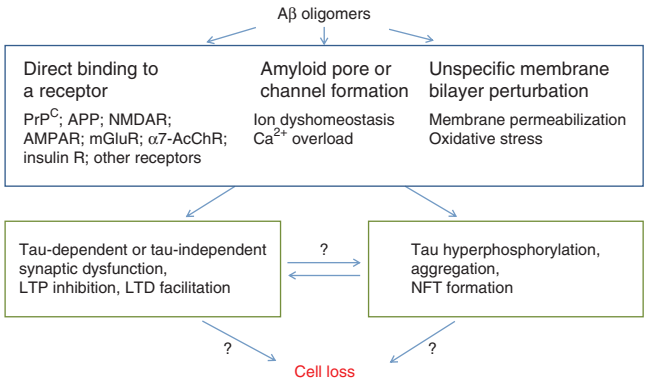

Figure 4 Key steps of Aβ-initiated neurodegenerative cascade according to the amyloid hypothesis. The diagram illustrates the main points of attack that have been described in the literature. It is clear that no consensus exists.

forms of Aβ throughout the life of J20 mice and highlight the difficulty in attributing synaptotoxicity to a single Aβ species". In Tg2576 transgenic mice that overexpress Swedish-mutated human APP, a 56-kDa species called Aβ*56 was correlated with cognitive deficits[39]. Purified Aβ*56 caused memory deficits when injected in brains of young rats. An important concern with Aβ*56 is the presence of 0.1% SDS in the initial extraction buffer[39] because, as discussed, SDS might artificially promote the aggregation of Aβ[24] and could also be a matter of concern when assessing toxicity in cellular or *in vivo* assays.

Overall, the experiments present a very mixed picture: they generally support the existence of a mixture of water-soluble Aβ species that exert synaptotoxicity, but it remains to be clarified whether this toxicity can be specifically associated with dimers, trimers or other assemblies, and whether these assemblies can mediate neurotoxicity in Alzheimer's disease. Probably the most crucial question is the nature and biological relevance of the SDS resistance of the identified dimers, trimers and larger oligomers. If it is a property induced by the presence of SDS, then it is unlikely to be meaningful with respect to the putative pathophysiological role of toxic oligomers. If it can be demonstrated, however, that additional covalent modification is responsible for the conversion of Aβ monomers to these *in vivo* dimers, and if this could be unequivocally correlated with toxicity, then their relevance would be indisputable. For instance, it is possible that Aβ monomers in brain become progressively cross-linked by transglutaminase[40] or other enzymes, or by oxidation-mediated dityrosine and dihistidine bridge formation[41,42]. A correlation of Aβ neurotoxicity with formation of His and Tyr bridges has, however, been shown only in *in vitro* preparations[41].

In vitro–generated Aβ oligomers

The advantage of *in vitro* generation of Aβ oligomers lies in the more precisely controlled starting materials: peptides are pure and buffer conditions are defined, which makes them ideal for establishing the conditions that induce the toxic conformation of Aβ. However, this paradigm still faces multiple challenges. In aqueous solutions, Aβ monomer is mostly unstructured, representing a mixture of interconverting conformations rather than a unique fold, and alterations in this mixture may determine the ultimate aggregation pathway[43]. It is, therefore, not surprising that different aggregate morphologies with different biological potencies have been generated from the same peptide[1,37,44]. Aβ oligomers may be metastable intermediates on the pathway to an insoluble, cross–β-sheet–based fiber. Authors have suggested that certain oligomers are off-pathway species that have different secondary structures and do not further aggregate to amyloid fibers (**Table 1** and **Fig. 3**), but, as discussed, it remains to be seen how stable such aggregates are *in vivo*.

The *in vitro*–generated Aβ oligomeric species meet, to a greater or lesser extent, the general criteria for a pathogenic protein species[45]: (i) their stability and/or rate of formation are sensitive to disease-associated mutations; (ii) they can also be generated from the wild-type protein (because Alzheimer's disease is predominantly sporadic); and (iii) they can be linked to a potential pathogenic mechanism. However, it is not clear whether these *in vitro*–generated species occur *in vivo* or which species are the more relevant ones for the understanding of Alzheimer's disease.

A major criticism that can be leveled at many of the experiments using *in vitro*–reconstituted toxic Aβ oligomers is that high concentrations (micromolar) of Aβ monomers are needed to see the transition from monomer to (toxic) oligomer. Although it has been argued that these 'nonphysiological' concentrations are unlikely to occur *in vivo*, cell culture experiments have shown that Aβ can become concentrated by more than two orders of magnitude to reach micromolar concentrations in acidic vesicular compartments[46].

Another important point of criticism is that many publications claim that *in vitro*–generated oligomers seem less neurotoxic than the dimers and trimers or oligomers isolated from brain tissue or cell

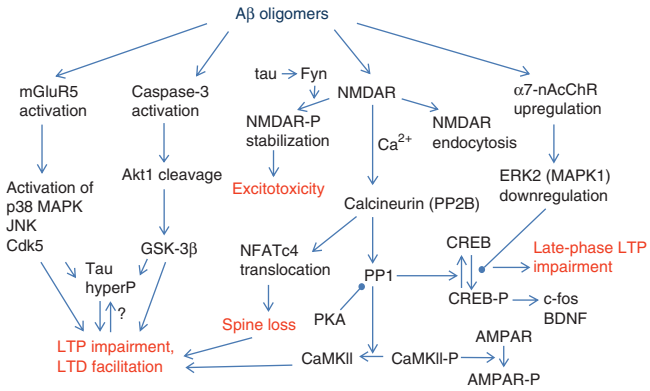

Figure 5 Molecular mechanisms of Aβ synaptotoxicity proposed in the literature. The diagram illustrates some pathways that have been invoked in different experimental paradigms to explain synaptotoxicity. It is clear that there is no agreement about what mechanism, if any, is most relevant to Alzheimer's disease. Aβ directly or indirectly alters glutamate receptor–dependent cascades, thereby leading to LTP impairment and LTD facilitation. Aberrant activation of calcineurin and NFATc4 leads to the dystrophic changes in neurites, and calcineurin-dependent dephoshorylation of CaMKII impairs the induction of AMPAR-based LTP in hippocampus. Chronic exposure to Aβ upregulates α7-nAcChR, causing hyperactivation and then downregulation of the ERK2 (MAPK1) cascade leading to an impairment of the gene transcription–dependent phase of LTP through decreased phosphorylation of CREB factor. Some protein kinases mediate pathological phosphorylation of tau and are required for Aβ-mediated synaptotoxicity. The caspase-3–mediated pathway might have a role in the Aβ-mediated LTP impairment upstream of GSK-3β. mGluR5, metabotropic glutamate receptor 5; ERK2, extracellular signal-regulated kinase 2; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; Cdk5, cyclin-dependent kinase 5; GSK-3β, glycogen synthase kinase-3β; Akt1, serine-threonine protein kinase 1; NMDAR, *N*-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptor; CaMKII, calmodulin kinase II; PKA, protein kinase A; PP1, protein phosphatase 1; NFATc4, nuclear factor of activated T-cells; α7-nAcChR, α7 nicotinic acetylcholine receptor; BDNF, brain-derived neurotrophic factor; CREB, cAMP-regulatory element binding protein.

culture. For instance, cell-derived Aβ oligomers can inhibit synaptic plasticity at low-nanomolar concentrations, whereas concentrations of synthetic Aβ must be between two and four orders of magnitude higher to produce the same effects[47]. Another study shows that synthetic Aβ oligomers impair complex learned behavior in rats at the same low-micromolar range as the Aβ*56 oligomers extracted from Tg2576 mouse brain, but that the synthetic oligomers remain less potent than low-*n* Aβ oligomers secreted by cells[48]. A recent work[49] shows that concentrations of synthetic dimers must be two to three orders of magnitude higher than those of human brain–derived dimers to induce the same cytoskeletal impairments in primary neurons. Similar results were found for human brain–derived ASPD versus synthetic ASPD generated *in vitro*[36].

The increased toxicity of these naturally derived Aβ assemblies is explained by their "biologically active conformation"[47], the nature of which remains, as we have discussed, elusive. It is therefore also not clear what exactly is measured when *in vitro* and *in vivo* oligomers are compared for their toxic activity. Quantitative dose-response curves and determination of specific activities (units of toxicity per unit oligomer) are needed, but the present status of the field does not allow this type of analysis.

Eventually, toxicity might be related to the heterogeneous character of natural aggregates or to unknown post-translational modifications absent in synthetic Aβ preparations.

In conclusion, it is clear that as long as *in vitro* and *in vivo* approaches remain incongruent, any claim of a specific toxic Aβ oligomer causing Alzheimer's disease is difficult to replicate in other systems. This debate about the exact identity of the postulated toxic species might actually be very misleading, as it suggests implicitly that precise and specific biological mechanisms—for example, interactions of specific oligomers with specific receptors—are responsible for neurodegeneration (summarized in **Figs. 4** and **5**). An alternative interpretation of the available evidence is that no specific toxic oligomer exists, but that, instead, a mixture of various oligomers and aggregates (an 'Aβ soup') is capable of interacting with cellular membranes and proteins in rather nonspecific ways (**Fig. 4**).

An alternative interpretation of Aβ oligomer toxicity

Many experiments investigating the mechanisms of Aβ oligomer–induced toxicity indeed start from the assumption that the oligomers are stable structures that interact specifically with receptors. Further study of the synaptotoxic effects of Aβ (LTP inhibition in the CA1 region of the hippocampus, dendritic spine shrinkage, LTD facilitation) has shown disturbances in the functioning of postsynaptic NMDA receptors, affecting calcium influx, a number of downstream cascades and postsynaptic AMPA receptors[50] (**Fig. 5**). Whether or not Aβ oligomers directly modulate the NMDA receptor is a matter of controversy. Alternative pathways of Aβ-induced synaptic failure would require an activation of the apoptotic machinery[32] or the upregulation of the nicotinic acetylcholine receptor α7-nAcChR[51] (**Fig. 5**). Interactions with the insulin receptor[52], effects on hypoxia-induced factor HIF-1 (ref. 53), clustering of the G protein–coupled angiotensin type 2 receptor and other mechanisms have also been described[54]. Recently, the cellular prion protein PrP^c emerged as a specific receptor for toxic Aβ oligomers[55], which also has resulted in considerable controversy.

This brief summary of the vast literature on this topic already makes clear that no single mechanism explains all aspects of Aβ oligomer toxicity. Amphiphilicity, aggregation state, covalent modifications and/or the organization of hydrophobic residues within an oligomeric assembly affect toxic effects of such oligomers. A recent work[1] studied a small bacterial peptide, HypF-N, which readily forms spherical oligomers, protofibrils and amyloid-like fibrils. Two types of Hyp-F oligomers appeared very similar in atomic force microscopy and thioflavin assays, indicating similar β-sheet content. Nevertheless, one type of oligomer was not toxic, but the other was highly toxic. The latter interacted strongly with cellular membranes and was even able to penetrate into cells. Site-directed labeling of the oligomers using pyrene demonstrated that the toxic oligomer had a lower degree of hydrophobic packing. The authors suggested that the toxicity of the oligomers was a consequence of their structural flexibility and their hydrophobic exposure. Toxicity therefore seems a generic consequence of the amphiphilic nature of these peptides. Thus, instead of specific receptors existing for Aβ toxic oligomers, it is more likely that Aβ peptides will exert multiple effects by binding to membrane proteins, targeting membrane lipids, causing oxidative stress and changing membrane dielectric properties and ion permeability.

Evidence for a more generic type of toxicity is also reported by other groups[26]. It was found that the ongoing self-assembly of Aβ to fibrils starting from soluble monomers and oligomeric seeding blocks was by itself sufficient to cause cell impairment. This finding implies that toxicity cannot be attributed to any particular Aβ species but is rather caused by the nucleation-dependent dynamic fibrillogenesis process itself. Thus, aggregation-dependent Aβ neurotoxicity will eventually be abrogated upon depletion of seeds or free monomers. The toxicity mechanism was suggested to be associated with the exposure of "toxic surfaces" derived from the abnormally folded peptide and their subsequent interaction with other molecules[56]. An analogy to prion toxicity could be made, as the kinetics of prion propagation are known to be tightly associated with prion neurotoxicity. However, according to one of the models[57], the prion toxicity is attributed to an elusive "lethal" species, PrP^L, possibly generated from PrP^c as a toxic intermediary during rapid PrP^{Sc} proliferation. One may speculate that this "lethal species" may be analogous to the elusive "toxic surfaces" formed by Aβ during polymerization. Thus, although the hypothesis of a dynamic fibrillogenesis-associated toxicity is compatible with many of the available observations in the literature with regard to the Aβ assembly process, the mechanism of toxicity remains unclear in this model as well.

The difference between seeding, infection and toxicity

A series of elegant studies[58,59] demonstrates that brain extracts from people with Alzheimer's disease or from transgenic mouse models of the disease can initiate amyloid plaque formation in the brains of transgenic APP23 mice overexpressing the Swedish-mutated human APP. The soluble extracts were made using a saline buffer, thus avoiding the potential for detergent-mediated artifacts. The extracts were injected either directly into the brain or into the peritoneal cavity. Both routes were able to induce Aβ plaque deposition: thus, some mechanism must exist that enables the transference of amyloid nuclei or seeds into the brain[59].

This effect can be blocked by removing Aβ from the brain extracts by immunoprecipitation. These studies are remarkably congruent with the studies discussed above that used brain extracts to identify neurotoxic Aβ oligomeric species, in the sense that synthetic Aβ does not efficiently induce Aβ amyloid formation *in vivo*[58]. This is, perhaps, the best experimental evidence that soluble Aβ species are able to diffuse or be transported within the brain parenchyma, where they can subsequently mediate a readily measurable effect.

The observations are interesting in many ways, but further research is needed to clarify outstanding issues. For instance, the analogy with prion diseases is intriguing, but it is still unclear whether the amyloid-seeding species in the brain extracts are also able to induce Aβ amyloidosis in wild-type animals. This criticism is partially addressed by a recent experiment that showed that injection of brain extract from people with Alzheimer's disease could induce amyloid plaques in transgenic

mice overexpressing human wild-type APP that normally do not develop plaques[60]. There are also a few examples in the literature of monkeys injected with Alzheimer's-derived brain extracts that later developed amyloid pathology[61], but at present, the number of animals is too small to make any firm conclusions about the infectivity of amyloid in Alzheimer's disease. These experiments are in fact seeding experiments, as there is no evidence of direct transmission from patient to patient or from mouse to mouse in those experiments; therefore, 'infection' should not be used to describe this phenomenon. Further work is obviously needed to address this issue more definitively, among other reasons because of the potentially enormous implications for our healthcare system (and our experimental laboratories studying Alzheimer's disease) if such transmissibility can be demonstrated.

For the present discussion, the major question to answer is what, if any, the relationship is between the soluble Aβ fraction used in the seeding experiments[58,59] and the neurotoxic Aβ oligomers isolated by other groups, as discussed above. At this moment, no link has been established between the seeded amyloidosis and possible accelerated neurodegeneration or manifestation of cognition deficits. It is likely that the presence of visible amyloid in these experiments and neurodegeneration do not entirely overlap, as is the case with prions[62]. Actually, if plaques are less toxic than oligomers, then accelerating amyloid formation in these experiments should improve cognitive phenotypes in the mice.

There is evidence that different morphologies of the induced amyloid fibrils and plaques exist and reflect those of the inocula used to induce them. This suggests that the amyloid seeds are polymorphic, a situation not unlike the heterogeneity of the toxic Aβ oligomers discussed above. The concept of 'Aβ strains' (in analogy with 'prion strains') is supported by *in vitro* experiments demonstrating that morphology and structure of Aβ amyloid fibrils are self-propagating[63].

In conclusion, it seems that the 'seeding Aβ extracts' and the 'toxic Aβ oligomer extracts' are both biochemically ill-defined mixtures of Aβ peptides, and it remains unclear whether specific species are responsible for these two aspects of the disease process. It is likely that the seeding Aβ species isolated from brain are in complex equilibrium with the postulated toxic Aβ oligomer conformations. The structural explanation for both properties will remain, however, elusive but nevertheless important.

Challenges for the future

For the time being, we will continue struggling with the concept of toxic Aβ oligomers, and it might therefore be extremely helpful if all publications would heed recent advice regarding the communication of findings and define very precisely the methods and quality controls used in developing toxic oligomer preparations[64]. More comparative work and exchange of materials between laboratories would also be very helpful, as we will probably find out that many of the preparations generate very similar 'oligomer soups' (**Fig. 3**).

The most logical way forward, however, is to try to define more precisely the biophysical and structural properties of the toxic Aβ-oligomeric fractions isolated from human brain. These 'natural' Aβ preparations will probably consist of a mixture of species, and it will be virtually impossible to rule out the possibility that the toxicity ascribed to native oligomers is caused by a very small subset of toxic species, undetectable in the pool of Aβ. Although this hypothesis is perfectly plausible, we should, for the time being, avoid invoking an invisible toxin, as it does not assist experimental science.

Thus, it will be necessary to define more precisely what makes an Aβ oligomer preparation toxic. Some groups stabilized Aβ40 oligomers using photo–cross-linking and fractionated the different oligomers to obtain pure, stable dimers, trimers and tetramers[65]. On the basis of the assumption that neurotoxic activity (t) of an Aβ assembly of order i (t_i) is conserved between fractionated and crude populations of stabilized oligomers, a total toxic effect T of an oligomer mixture was found to depend on the frequency (f_i) of occurrence of each particular assembly

$$T = \sum_{i=1}^{n} f_i t_i$$

Furthermore, a nonlinear dependence of the overall toxic effect t on oligomer order n (expressed as EC_{50}, μM) was observed. The Aβ40 dimers, trimers and tetramers seemed to be 3-, 8- and 13-fold more cytotoxic, respectively, than Aβ40 monomers[65]. Thus, by knowing the toxicity of each assembly and the average number of oligomers in a mixed population, one should be able to predict the toxicity of a given mixture. Interestingly, toxicity in these experiments was independent of the presence of monomers, but it is still possible that the cross-linked oligomers further fibrillized into higher-order aggregates in the cellular assays. The oligomers were also initially fractionated using SDS-PAGE, and, although several purification steps were applied, the possibility of contamination by SDS complicates the interpretation of the toxicity assays. A very interesting alternative is the use of macrocyclic peptides to stabilize amyloidogenic sequences, which restrains them into β-strand structures but avoids further growth into fibrils[66]. Analysis shows that the resulting β-sheet dimers interact in a much more heterogeneous fashion than the parallel or antiparallel orientation observed in amyloid fibrils, providing a glimmer of insight into the heterogeneity of oligomers and a possibility of defining more precisely the nature of the oligomers tested in various biological assays[66].

Further work *in vitro* will need to take into account the complexity of the Aβ peptide mixture *in vivo*. Recent evidence confirms that small changes in the relative amounts of Aβ42 versus Aβ40, for instance, dramatically affect aggregation and neurotoxic properties of Aβ mixtures[27]. Similarly, the presence of pyroglutamate Aβ[67] or phosphorylated Aβ[19] influences these properties. Therefore, studies using complex mixtures of the various Aβ peptides mimicking the situation in the brain might be revealing. In parallel, new technologies allowing the interconversion of oligomers of different sizes to be monitored[68] need to be developed further.

Ideally, Aβ oligomeric species that can be extracted from brain tissue should be characterized, and analytical tools that permit their identification in cerebrospinal fluid (CSF) from Alzheimer's-affected brain tissue should be developed. This would obviate the criticism that the procedure for fractionation generates, rather than extracts, the oligomeric species in question. Once fully characterized, it should be possible to synthesize the species *de novo* and show comparable biological effects.

One potentially fruitful approach to dissecting the structural heterogeneity of the toxic oligomers is the generation of conformation-specific antibodies. A wide panel of antibodies is now available[44,69–87] that react more specifically with oligomeric Aβ than with monomeric Aβ, and some do not react with Aβ fibrils (**Supplementary Table 1**), indicating that Aβ oligomers do indeed have different structural epitopes. We are, however, still far from being able to use those antibodies to distinguish specific toxic conformations. As an example, the widely used polyclonal antibody A11 recognizes a generic epitope in synthetic cytotoxic Aβ42 oligomers[69] and can distinguish between nontoxic 'fibrillar' and toxic 'pre-fibrillar' oligomeric assemblies[44], but it does not bind β-sheet oligomers or protofibrils of dicysteine AßCC that bind monoclonal antibody mAb158 (ref. 88) and does not react with the neurotoxic brain-derived ASPD (**Table 1** and **Supplementary Table 1**)[36]. A further complication is that although some antibodies may recognize conformations of Aβ oligomers with greater affinity than they recognize Aβ monomers, the relative concentrations of

oligomers and monomers may still make such tools unsuitable for detecting oligomeric species from fluids such as CSF from patients with Alzheimer's disease. It is probable that a combination of technologies, such as immune capture with mass spectrometry, will be needed to obtain the resolution needed to perform these studies[89].

Finally, the field might like to establish a set of criteria that would disprove, rather than support, the relevance of Aβ oligomeric species in the Alzheimer's disease process. Given that one of the defining features of these species is that they are soluble, one should be able to detect them in the CSF of patients with Alzheimer's disease but not in controls. How can their role in Alzheimer's disease be reconciled with the effects of *APOE4* alleles and FAD mutations? Many groups demonstrate that human-derived Aβ preparations can mediate various types of neuronal damage when applied to rodent neurons in culture, yet when this situation is recapitulated *in vivo*, as it is in many transgenic mice harboring human FAD *APP* genes and producing robust plaque deposition, frank neuronal loss is not demonstrated. If Aβ oligomers are in equilibrium with deposited Aβ plaque, one might anticipate that any neurotoxic effects would also be reflected by the topographical distribution of plaques. Thus, we have discussed some of the problems and complexity of this field, but a more fundamental question remains: do the various types of oligomeric Aβ species described thus far have any role at all in the cognitive alterations and, ultimately, in the neuronal death that underpins the dementia of Alzheimer's disease?

The most promising recent avenue of research in the field is the link between Aβ oligomers and tau pathology. Several groups have demonstrated that the neurotoxicity seen with different oligomer preparations is associated with tau phosphorylation and can be attenuated when the tau gene is silenced or knocked out[49,90–92]. However, this type of experiment, using tau abnormalities as read-outs for Aβ oligomer toxicity, is at present largely descriptive. It is noteworthy that, in addition to Aβ oligomers, other nonspecific cell stressors—for example, hydrogen peroxide, glutamate or serum deprivation[90]—could to a certain extent induce similar tau alterations. This line of research is extremely interesting, as it might eventually result in the understanding of the link between the extracellular and the intracellular amyloidosis in Alzheimer's disease.

Note: Supplementary information is available on the Nature Neuroscience website.

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