MECHANISMS OF DISEASE

Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide

Christian Haass and Dennis J. Selkoe‡*

Abstract | The distinct protein aggregates that are found in Alzheimer's, Parkinson's, Huntington's and prion diseases seem to cause these disorders. Small intermediates — soluble oligomers — in the aggregation process can confer synaptic dysfunction, whereas large, insoluble deposits might function as reservoirs of the bioactive oligomers. These emerging concepts are exemplified by Alzheimer's disease, in which amyloid β-protein oligomers adversely affect synaptic structure and plasticity. Findings in other neurodegenerative diseases indicate that a broadly similar process of neuronal dysfunction is induced by diffusible oligomers of misfolded proteins.

Amyloid
Tissue deposits of insoluble, proteinaceous fibrils that are rich in β-pleated sheet structure and therefore bind to the histochemical dye Congo red in a polarized manner.

**Adolf Butenandt Institute, Department of Biochemistry, Laboratory for Alzheimer's and Parkinson's Disease Research, Ludwig Maximilians University, 80336 Munich, Germany. ‡Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115, USA. e-mails: chaass@med.uni-muenchen.de; dselkoe@rics.bwh.harvard.edu*
doi:10.1038/nrm2101

Degenerative diseases of the human brain have long been viewed as among the most enigmatic and intractable problems in biomedicine. As research on human neurodegeneration has moved from descriptive phenomenology to mechanistic analysis, it has become increasingly apparent that the morphological lesions long used by neuropathologists to confirm a clinical diagnosis after death might provide an experimentally tractable handle to understand causative pathways. This concept came as no surprise to those pursuing a small and arcane field of medicine, the study of amyloidosis. Amyloids were defined some 150 years ago as tissue deposits of extracellular filaments (usually called fibrils) that were microscopically — and in severe cases, macroscopically — visible in various organs in several seemingly unrelated human diseases.

In the twentieth century, amyloidologists found that amyloid fibrils were proteinaceous in origin, and by the 1960s some amyloid subunits (such as the fragments of immunoglobulins that accumulate in certain immune-related amyloidoses) were identified as specific proteins. Biochemical and ultrastructural experiments established the remarkable insolubility of amyloid fibrils in aqueous buffers. X-ray-diffraction analyses on purified fibrils indicated that the constituent proteins were unusually rich in highly ordered, β-pleated sheet structure, providing an apparent explanation for their long-known property of binding to the histochemical dye Congo red in a polarized manner.

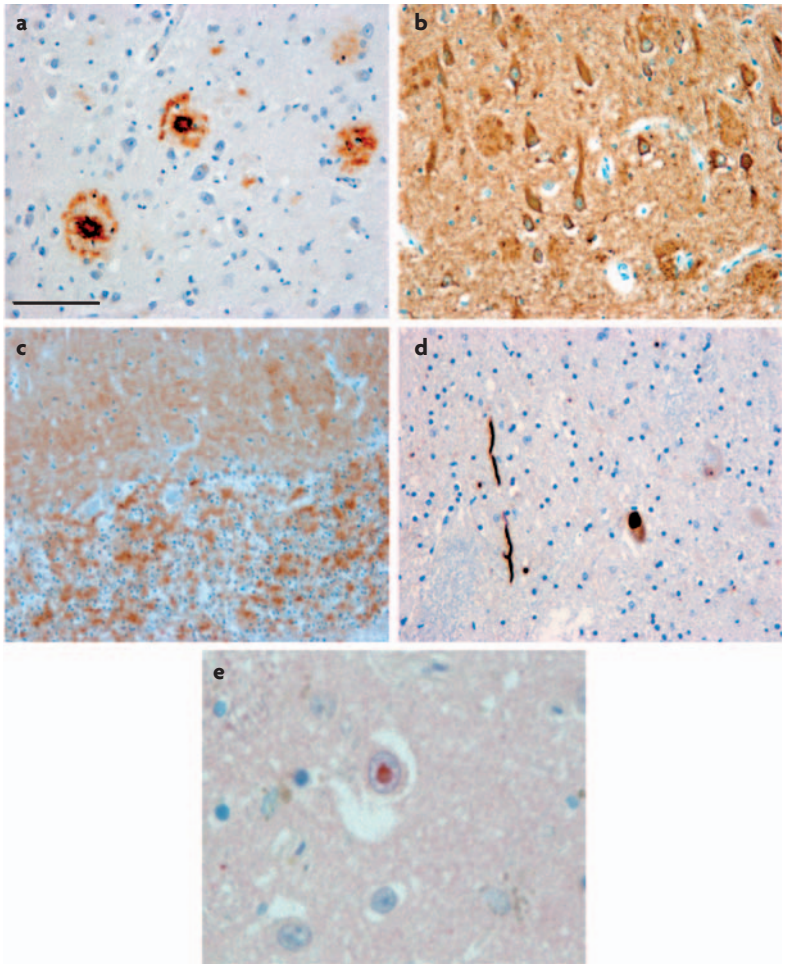
These advances in elucidating amyloid deposits in peripheral tissues encouraged amyloidologists to apply their methods to neurodegenerative disorders, most notably with the seminal studies in 1984 of Glenner on the cerebrovascular amyloid deposits of Alzheimer's disease (AD). As research on the protein deposits of AD grew increasingly intensive, partly because of the commonness of the disorder, similar concepts and methods began to be applied to other human neurodegenerative disorders.

In this review, we will illustrate the emerging trend to define several human neurodegenerative syndromes as disorders of protein folding and oligomerization through the example of AD. In AD, a small peptide, amyloid β-protein (Aβ), forms long, insoluble amyloid fibrils, which accumulate in spherical microscopic deposits known as senile plaques. However, the relevance of these plaques to AD pathogenesis was unclear and even questioned by many investigators, a contentious issue that might now be coming to a rather simple and surprising solution. Therefore, an emphasis of this review will be on an important recent development from studies that attempted to identify the toxic moiety responsible for synaptic dysfunction and neuronal cell loss in AD. The production of the apparent toxic species — soluble Aβ oligomers — and their subsequent ability to cause neuronal injury depends on the precision of an intramembranous proteolytic cleavage, which is unprecedented and will therefore be described in detail. We will also discuss how the production of Aβ and its capacity to form oligomers is affected by mutations that cause aggressive forms of familial AD. Some oligomeric species of Aβ are small and soluble enough to diffuse readily through the

Box 1 | **Pathological inclusions in neurodegenerative disorders**

Many neurodegenerative disorders are characterized by the formation of insoluble inclusions. However, recent evidence indicates that these deposits might not be responsible directly for the primary neurotoxic effects and memory loss of these disorders. They might function as a reservoir for soluble oligomers, which are able to diffuse through the brain parenchyma and into synaptic clefts. Insoluble deposits and diffusible oligomers from different diseases are composed of individual amyloidogenic proteins as different as amyloid β-protein (Aβ), tau, prion protein (PrP), α-synuclein and huntingtin. They might share common structural epitopes and common mechanisms of neurotoxicity and memory impairment[79].

Panel **a** shows Alzheimer's disease with Aβ-positive senile plaques, and panel **b** shows tau-positive neurofibrillary tangles, neurophil threads and dystrophic neurites. Panel **c** shows PrP deposits in the cerebellum of a Creutzfeldt–Jakob case. Panel **d** shows substantia nigra of a Parkinson's disease case with an α-synuclein-positive Lewy body (right) and Lewy neurites (left). Panel **e** shows a ubiquitin-positive huntingtin inclusion in a case of Huntington's disease. The scale bar represents 100 μm.



brain parenchyma and affect synaptic structure and function and, ultimately, neuronal survival. We will also describe the emergence of highly specific treatment strategies that are based on the new molecular understanding of AD. Throughout the review we will indicate the timeliness of the topic — rapidly expanding research on other brain disorders such as Parkinson's disease (PD) and Huntington's disease (HD) has suggested common features that resemble the fundamental pathogenic process in AD.

Lewy body
(LB). A deposit of α-synuclein typically found in neuronal cell bodies of patients with Parkinson's disease or related disorders.

'Reverse genetics' enables AD gene discovery

Spectacular advances in human genetics and the availability of the DNA sequence of the entire human genome have greatly facilitated the identification of genes involved in diverse hereditary disorders, including several prominent neurodegenerative disorders (for neurodegenerative disease histopathology, see BOX 1). A recent and particularly compelling example is PD[1]. Although the cytopathological hallmarks of PD, known as Lewy bodies (LBs) and Lewy neurites (LNs) (BOX 1), have long been known, the amyloidogenic neuronal protein that is deposited in LBs and LNs remained unidentified. Until a decade ago, PD was considered by most authorities to be essentially a sporadic (that is, non-familial) disorder. Yet it was through genetics, not biochemistry, that the protein component of LBs and LNs was identified.

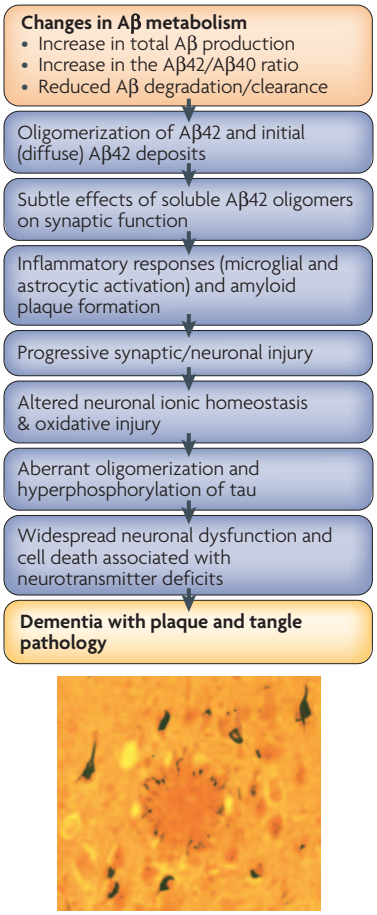
Linkage analysis and positional cloning of the first PD-associated gene in a family with autosomal dominant parkinsonism identified a missense mutation in the gene that encodes the neuronal protein α-synuclein[2]. α-synuclein is a naturally unfolded, soluble cytoplasmic protein that is highly expressed in neurons and that can bind to the membranes of synaptic, and other, vesicles[3]. After the discovery that α-synuclein was genetically implicated in familial PD, immunohistochemistry combined with biochemical purification quickly showed that α-synuclein was the principal protein component of LBs and LNs[4,5]. In this example, geneticists directly enabled biochemists to isolate the needle from the haystack of candidate proteins. In a similar manner, breakthrough discoveries of causative genes allowed the subsequent identification of aggregation-prone proteins in disorders such as amyotrophic lateral sclerosis (ALS)[6,7] and HD[8].

The discovery of the first AD causative gene. By contrast, a biochemical hypothesis preceded and guided the discovery of the first causative gene in AD. The AD causative gene encodes β-amyloid precursor protein (APP), which is a single-transmembrane, receptor-like protein that is expressed ubiquitously in neural and non-neural cells. After the Bavarian psychiatrist Alois Alzheimer presented his first clinicopathological case in Tübingen on November 3, 1906 (REF. 9), it became clear that amyloid plaques in the cerebral cortex (BOX 1) were invariably associated with the disease that now bears his name.

In the 1980s, biochemists focused on the isolation of the amyloid to identify its principal component. Glenner and Wong purified microvascular amyloid deposits from the meninges of AD brains and provided a partial sequence of an ~4-kDa subunit protein that they named amyloid β-protein (Aβ)[10]. Shortly thereafter, Masters, Beyreuther and co-workers identified the same protein as the subunit of amyloid plaque cores that were isolated from post-mortem AD cortices[11]. Around the same time, the microtubule-associated protein tau was identified as the main constituent of the hallmark neurofibrillary tangles (BOX 1) that accumulate inside many neurons and their processes in AD brains[12–14]. Tau is a highly soluble cytoplasmic protein that binds to tubulin during its polymerization into microtubules in neurons and thereby stabilizes these important cytoskeletal organelles.

Box 2 | **The amyloid (or Aβ) cascade hypothesis**

Gradual changes in the steady-state levels of amyloid β-protein (Aβ) in the brain are thought to initiate the amyloid cascade[22–24]. Aβ levels can be elevated by enhanced production and/or reduced clearance. In particular, the Aβ42/Aβ40 ratio can be augmented by mutations in three different genes (β-amyloid precursor protein (*APP*), presenilin-1 (*PS1*) and *PS2*) that cause familial forms of Alzheimer's disease. This relative increase of Aβ42 enhances oligomer formation, which causes subtle and then increasingly severe and permanent changes of synaptic function. In parallel, Aβ42 forms microscopically visible deposits in the brain parenchyma, first as relatively benign diffuse (non-fibrillar) plaques. As the diffuse plaques begin to acquire fibrils of Aβ, local inflammatory responses (microgliosis and astrocytosis) are observed. Synaptic spine loss and neuritic dystrophy also occur. Over time, these events result in oxidative stress, altered ionic (for example, calcium) homeostasis and a host of additional biochemical changes. Neurofibrillary tangles are induced by altered kinase and phosphatase activities and contribute to additional defects, including some in axonal transport. The cascade culminates in widespread synaptic/neuronal dysfunction and cell death, leading to progressive dementia associated with extensive Aβ and tau pathology.



Glenner also showed that the amyloid deposits that occur in the brain vessels of young adults with Down syndrome were composed of Aβ[15]. Since 1969, it had been known that middle-aged patients with Down syndrome develop the amyloid plaques and neurofibrillary tangles that are typical of AD[16]. On this basis, Glenner assumed that the gene ultimately found to encode Aβ might be causative of AD cases. The subsequent cloning of *APP*, which encodes a large, type 1 membrane glycoprotein, by Beyreuther and co-workers in 1987, and its localization to the long arm of chromosome 21, was consistent with this hypothesis[17].

These biochemical findings pointed strongly to the *APP* gene as a site which geneticists should search for AD-causing mutations. The first such mutation was discovered in a family with hereditary cerebrovascular amyloidosis with multiple haemorrhages[18]. Shortly thereafter, a distinct APP missense mutation was found in a family with early-onset AD[19], and then additional mutations in other families were detected[20,21].

These and other findings led to the formal proposal of a hypothesis of disease in which excessive Aβ accumulation and deposition could trigger a complex downstream cascade that resulted in the symptoms of AD[22,23]. In its most recent iteration[24], the amyloid (or Aβ) hypothesis states that the gradual accumulation and aggregation of this small hydrophobic peptide initiates a slow but deadly cascade that leads to synaptic alterations, microglial and astrocytic activation, the modification of the normally soluble tau protein into oligomers and then into insoluble paired helical filaments, and progressive neuronal loss associated with multiple neurotransmitter deficiencies and cognitive failure (BOX 2).

Regulated intramembrane proteolysis of APP

Initially, the mechanism by which the partially intramembrane Aβ region (FIG. 1a) could be liberated as a free peptide from its precursor was enigmatic and was considered to require some pre-existing membrane injury. It was assumed that the hydrophobic interior of the membrane bilayer would need to be damaged to allow access of a protease and water to effect cleavage. But this concept was disproven when Aβ was unexpectedly discovered to be produced normally by the intramembranous proteolysis of APP throughout life and APP was found to circulate in extracellular fluids, including cerebrospinal fluid (CSF) and plasma[25–28]. This discovery opened up the dynamic study of Aβ, which heretofore had only been obtained through painstaking isolation from post-mortem human brain. As predicted by the amyloid hypothesis, all AD-causing APP mutations that have been identified so far occur either within or flanking the Aβ region of this large polypeptide. Accordingly, the mutations that flank the Aβ region increase the production of the highly amyloidogenic Aβ42 isoform, whereas the mutations within the Aβ region enhance the oligomerization of the peptide[29] (BOX 3).

Intramembrane proteolysis liberates Aβ. Because the generation of Aβ species with variably hydrophobic C termini — and therefore different propensities to oligomerize — is directly relevant to the development of AD, we will review the current understanding of the biochemical mechanisms that underlie Aβ production, with a focus on intramembrane processing.

Aβ generation has turned out to be just one example of a general physiological mechanism now known as regulated intramembrane proteolysis (RIP)[29–31]. In a principal version of this process, membrane proteins first undergo a regulated shedding of their ectodomains by membrane-anchored proteases known as secretases or sheddases, releasing the large luminal domains into extracellular fluids. The membrane-retained stubs can then be cleaved within their transmembrane domains (TMDs) to release small hydrophobic peptides (for example, Aβ in the case of APP) into the extracellular space and intracellular domains (ICDs) into the cytoplasm (FIG. 1a). The free ICDs might have specific functions, including the activation of nuclear signalling pathways, as in the case of the Notch ICD, which is liberated by γ-secretase from membrane-bound Notch[32].

In the case of APP and certain other RIP substrates, ectodomain shedding can be mediated by either of two distinct membrane-anchored proteases, α-secretase (believed to consist of one or more members of the ADAM family of metalloproteases) or β-secretase (also called β-site APP-cleaving enzyme (BACE))[29].

Lewy neurite
(LN). A deposit of α-synuclein typically found in swollen neuronal processes of patients with Parkinson's disease or related disorders.

Amyloid plaques
Spherical extracellular deposits predominantly composed of the amyloid β-peptide and found in the brains of all cases of Alzheimer's disease.

Neurofibrillary tangle
An intraneuronal filamentous aggregate composed of abnormally phosphorylated tau protein found in several human neurodegenerative disorders, including Alzheimer's disease.

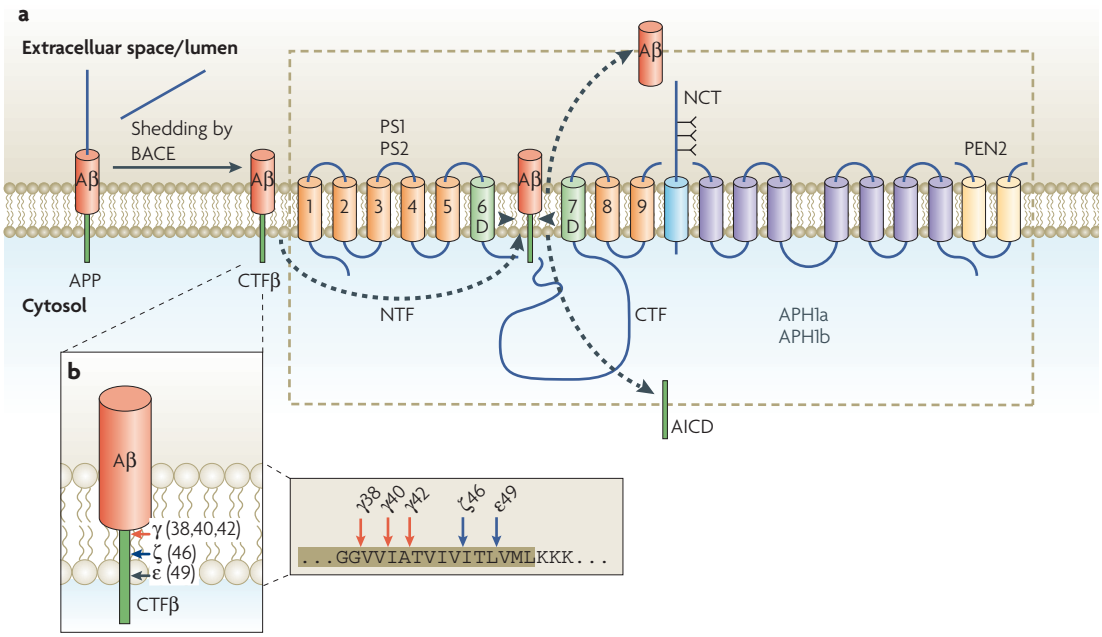


Figure 1 | **Amyloid β-protein generation by normal proteolytic processing of β-amyloid precursor protein.** **a** | Amyloidogenic processing of β-amyloid precursor protein (APP) by β-site APP-cleaving enzyme (BACE) and the γ-secretase complex. In this pathway, full-length APP (left) is first processed by BACE, and the large ectodomain is secreted. The remaining membrane retained stub (CTFβ) binds to a docking site on the surface of the γ-secretase complex and is then transferred to the active site that includes transmembrane domains 6 and 7 of presenilin-1 (PS1) or PS2. PS1 and PS2 are both activated by presumed autoproteolytic cleavages, which create their N-, and C-terminal fragments (NTF and CTF). These bind to each other and also to 3 other essential γ-secretase components, APH1a (or APH1b), PEN2 and nicastrin (NCT). All four proteins form the core complex required for γ-secretase activity (shown in dashed box). The two intramembrane aspartate residues in the NTF and CTF of presenilins (marked with a D) are a crucial part of the unusual catalytic site of the protease. The γ-secretase cleavage occurs in the middle of the membrane and liberates amyloid β-protein (Aβ) and the APP intracellular domain (AICD). The function of the AICD is unclear. **b** | Various proposed sites of intramembrane proteolysis by γ-secretase. The amino-acid sequence around the cleavage sites of APP is shown (numbers refer to the sequence of Aβ; shaded amino acids are in the transmembrane domain). γ-secretase cuts its substrates several times. The cleavage sites are referred to as ε, ζ and γ (from the C- to N-terminal). The γ-site is variable and can occur at least after amino acids 38, 40 and 42. This cleavage is highly relevant for the subsequent aggregation propensity of Aβ. Some γ-secretase-modifying drugs shift the cleavage at Aβ42 to amino acid 38, and the resultant peptide aggregates much less readily.

The membrane-associated stub, which is created by BACE cleavage, can then undergo an intramembrane scission that is mediated by the γ-secretase complex, a special type of aspartyl protease with a unique active site and cleavage mechanism[33,34] (FIG. 1a). This complex is composed of presenilin-1 (PS1) or PS2, nicastrin, APH1 and PEN2 (FIG. 1a)[29,35–37]. All four proteins are both necessary and sufficient to reconstitute γ-secretase activity in yeast, which lacks this enzyme[37].

At least in the case of its two most well studied substrates, APP and Notch, γ-secretase can carry out multiple intramembrane cleavages. Current evidence indicates that the PS–γ-secretase complex can cleave at different sites (referred to as γ, ε and ζ) in the TMD (FIG. 1b). The ε-cleavage close to the cytoplasmic border of the TMD releases the free ICD into the cytosol[38–40]. It seems that the remaining membrane-anchored fragment undergoes an intermediate scission ~3 residues N-terminal to the ε-cut at the so-called ζ-site[41,42]. Thereafter, Aβ is released into biological fluids by the final cuts at the γ-site. The γ-cut is variable and occurs after Aβ amino acids 38, 40 or 42. The precise sites of these γ-cleavages have an important influence on the self-aggregating potential and resulting pathogenicity of Aβ, as only the Aβ42 peptide has a strong propensity to oligomerize *in vivo*.

Aβ oligomers induce synaptic dysfunction

Research on AD seeks to answer a central question: what causes the onset of a subtle, intermittent impairment of hippocampal neuronal function and, therefore, episodic memory? Substantial evidence indicates that Aβ might be the central player in AD pathology. However, perhaps the most persistent argument against the amyloid hypothesis as summarized earlier is that many apparently healthy older humans have substantial amounts of amyloid in their limbic and association cortices upon post-mortem examination. These Aβ deposits are overwhelmingly of the diffuse type — they are not composed of amyloid fibrils and they have little or none of the surrounding neuritic and glial cytopathology found in mature neuritic plaques[43]. Furthermore, reports of weak

Regulated intramembrane proteolysis
(RIP). Regulated cleavage of the luminal domain of certain membrane proteins is followed by a constitutive cleavage in the transmembrane domain. At least in some cases this process is involved in signalling pathways.

Intracellular domain
(ICD). One of the cleavage products of intramembrane proteolysis in the RIP pathway, it is liberated into the cytosol and in some cases targeted to the nucleus.

Box 3 | **Familial Alzheimer's disease and the role of amyloid β-protein oligomerization**

A strong clue to the importance of amyloid β-protein (Aβ) oligomerization in Alzheimer's disease (AD) pathogenesis came from the observation that some AD-causing mutations in β-amyloid precursor protein (APP) and all AD-causing mutations in presenilin-1 (PS1) or PS2 enhance the production of the Aβ42 peptide[117,118]. The enhanced production of the Aβ42 peptide results in an increase in absolute Aβ42 levels, or at least in an increase in the Aβ42/Aβ40 ratio (that is, Aβ42 can increase at the expense of Aβ40). The more hydrophobic Aβ42 peptide, with its C-terminal alanine and isoleucine residues, aggregates more rapidly, therefore forming stable Aβ oligomers at an earlier time point[58,119–121]. Moreover, Aβ42 tends to form stable trimeric and/or tetrameric oligomers, whereas Aβ40 does not[121]. Inherited missense mutations directly in the Aβ region of APP increase the propensity of the peptide to aggregate; for example, the E693G (Arctic) APP mutation strongly enhances oligomerization[122]. The aggregation process is reflected in the initial microscopic deposition of Aβ42 in the form of early (diffuse) plaques in AD brains[123].

In three genetic conditions, the increased production of all forms of Aβ precipitates AD. These conditions are Down syndrome, the K595M and N596L(Swedish) double APP mutation at the β-site APP-cleaving enzyme (BACE) cleavage site[124,125], and duplication of a small region of chromosome 21q containing the *APP* gene[126]. Importantly, the recent discovery of the families with a duplication at chromosome 21q has revealed an exciting analogy to inherited Parkinson's disease, where rare families carry a duplication or even triplication of the α-synuclein gene[127].

On the basis of these and other findings, one might conclude that a relative increase in Aβ42 versus Aβ40 levels seems to be sufficient to trigger the AD phenotype. The above results in familial AD fit nicely with the recent discovery in transgenic mice that a marked elevation of only Aβ40 does not lead to plaque formation but can actually serve to retard the deposition of Aβ42 in the brain[128]. In accordance, expression of various AD-causing presenilin mutations in cell lines derived from *PS1 PS2* double-knockout mice indicates that at least some mutations do not result in increased levels of Aβ42 but rather result in decreased levels of Aβ40 (REF. 129), thereby reducing the levels of this putative 'anti-aggregation factor'. Furthermore, the deletion of the PS1 loop *in vivo* results in reduced Aβ40, but not Aβ42, generation and thereby triggers amyloid plaque pathology in mice[130].

quantitative correlations between manual microscopic counts of amyloid plaques in post-mortem brain sections and the extent of cognitive symptoms measured pre-mortem are fraught with methodological challenges. Counting spherical plaques in two-dimensional cross sections provides an imprecise measure of Aβ amounts and misses small and heterogeneous Aβ-assembly forms. Last, the cognitive testing done before the patient's death has often been done with simple, insensitive mental status screens.

The advent of specific Aβ enzyme-linked-immunosorbent assays (ELISAs) coupled with western blotting and mass spectrometry has now enabled a more precise and comprehensive assessment of Aβ quality and quantity. Such studies indicate that biochemically-measured levels of soluble Aβ, including soluble oligomers, correlate much better with the presence and degree of cognitive deficits than do simple plaque counts[44–47]. This evidence, coupled with the fact that large (~20–120-μm diameter) fibrillar plaques present much less Aβ surface area to neuronal membranes than do a multitude of small oligomers that can diffuse into synaptic clefts, indicates that such soluble assembly forms are better candidates for inducing neuronal and/or synaptic dysfunction than plaques, *per se*.

Importantly, the idea that large aggregates of a disease-causing protein can actually be inert or even protective to neurons has been supported by work on other protein-folding disorders. For example, in cell-culture studies of HD, less cell death has been observed when large aggregates of polyglutamine-rich huntingtin protein are present in the cells than when only soluble huntingtin is present without these inclusions[48,49]. Analogous findings have been reported in a mouse model of spinocerebellar ataxia in which the polyglutamine-rich forms of the ataxin-1 protein are expressed[50].

However, it must also be pointed out that large plaques of fibrillar Aβ in AD brains typically show surrounding dystrophic neurites, indicating that insoluble aggregates might contribute to neuronal injury. Indeed, fibrillar Aβ deposits have been associated with local synaptic abnormalities and even with the breakage of neuronal processes[51]. The problem is that large, insoluble protein aggregates are likely to be intimately surrounded by a number of smaller, more diffusible, assemblies (for example, oligomers). So, it becomes difficult to ascertain whether the large aggregates are directly inducing local neuronal injury and dysfunction. At the current stage of research, one should not conclude that either large, insoluble deposits or small, soluble oligomers represent the sole neurotoxic entity; indeed, a continuous dynamic exchange between these forms might well be detrimental. Nevertheless, we hypothesize that diffusible oligomers have the principal role, particularly during the earliest, even pre-symptomatic, stages of the AD process.

Different types of synthetic and natural Aβ oligomers. A large and confusing body of literature describes many types of assembly forms of synthetic Aβ, including protofibrils (PFs), annular structures, paranuclei, Aβ-derived diffusible ligands (ADDLs), globulomers and amyloid fibrils (for reviews, see REFS 52,53; the different types of recognized Aβ oligomers are summarized in TABLE 1). In general, soluble oligomers are defined as Aβ assemblies that are not pelleted from physiological fluids by high-speed centrifugation, and not all of the aforementioned synthetic assembly forms fulfil this definition. Moreover, soluble oligomers can bind to other macromolecules or to cell membranes and can therefore become insoluble. PFs are intermediates that were observed in the course of studying the fibrillization of synthetic Aβ[54–57]. They are flexible structures that can continue to polymerize

Table 1 | **Oligomeric assemblies of Aβ**

Oligomeric assembly	Characteristics	References
Protofibril (PF)	Intermediates of synthetic Aβ fibrillization; up to 150 nm in length and ~5 nm in width; β-sheet structure: bind Congo red and Thioflavin T	54–57
Annular assemblies	Doughnut-like structures of synthetic Aβ; outer diameter of ~8–12 nm; inner diameter of ~2.0–2.5 nm	58,59
Aβ-derived diffusible ligands (ADDLs)	Synthetic Aβ oligomers smaller than annuli; might affect neural signal-transduction pathways	60,61
Aβ*56	Apparent dodecamer of endogenous brain Aβ; detected in the brains of an APP transgenic mouse line and might correlate with memory loss	62
Secreted soluble Aβ dimers and trimers	Produced by cultured cells; resistant to SDS; resistant to the Aβ-degrading protease IDE; alter synaptic structure and function	63,64,69

Aβ, amyloid β-protein; APP, β-amyloid precursor protein; IDE, insulin-degrading enzyme.

in vitro to form amyloid fibrils or can depolymerize to lower-order species. PFs are narrower than *bona fide* amyloid fibrils (~5 nm versus ~10 nm). Ultrastructural analyses of synthetic PF preparations by electron microscopy and atomic force microscopy have revealed both straight and curved assemblies of up to 150 nm in length. Synthetic Aβ PFs have been shown to contain substantial β-sheet structure, as they can bind to Congo red or Thioflavin T in an ordered manner.

Annular assemblies of synthetic Aβ are doughnut-like structures, with an outer diameter of 8–12 nm and an inner diameter of 2.0–2.5 nm, that can be distinguished from PFs by atomic force microscopy and electron microscopy[58,59]. Some laboratories have observed smaller oligomeric species of synthetic Aβ than annuli and have designated these ADDLs[60]. Apparent ADDL-like oligomeric assemblies have been isolated from post-mortem AD brains, and their presence correlated with memory loss[61]. Chemical stabilization of synthetic Aβ42 assembly intermediates has revealed an apparent hexamer periodicity, with hexamer, dodecamer and octadecamer structures observed[58]. Whether the recently described Aβ*56, an apparent dodecamer of natural Aβ detected in the brains of an APP transgenic mouse line[62], might represent an *in vivo* analogue of synthetic ADDLs remains unclear, as direct structural comparisons have not been possible.

Whereas most of the Aβ assembly intermediates described above have only been observed upon *in vitro* incubation of synthetic Aβ, small oligomeric Aβ forms do occur *in vivo* and might therefore be relevant to disease pathogenesis. Intracellular and secreted soluble dimeric and trimeric oligomers have been described in cultured cells[63,64] (FIG. 2a), and SDS-stable oligomers of varying sizes have also been detected by western blotting in APP transgenic mouse brain and human brain[62,65–68]. Such natural (that is, non-synthetic) Aβ oligomers can be resistant not only to SDS but also to the Aβ-degrading protease insulin-degrading enzyme (IDE), which can only digest monomeric Aβ[69]. Naturally secreted monomeric and oligomeric Aβ species are being characterized in experiments *in vivo* to decipher their effects on synaptic structure and function[69,70] (see below). Aβ oligomers produced by cultured cells could be related to the aforementioned Aβ*56 (REF. 62), which seems to represent a brain-derived soluble dodecamer that has amnestic activity. Like the Aβ oligomers produced from cultured cells[69], Aβ*56 might disrupt synaptic function and therefore affect memory[62]. Whether Aβ*56 and species that are similar to it are stable assemblies of only Aβ under native conditions, or whether smaller oligomeric assemblies can associate with another protein is currently unknown. However, Aβ*56 and Aβ trimers secreted by cultured cells could turn out to share common synaptotoxic properties.

How does Aβ induce memory loss?

An intensively studied electrophysiological correlate of learning and memory is long-term potentiation (LTP). Repetitive, high frequency electrical stimulation of certain synaptic circuits, for example the CA3–Schaefer collateral–CA1 pathway in the mammalian hippocampus, can induce a prolonged potentiation of synapse firing (LTP) that is referred to as inducing synaptic plasticity.

There is now considerable evidence that ADDLs of synthetic human Aβ[60] and soluble, low-number (low-n) oligomers of naturally secreted human Aβ[69,70] (FIG. 2) can all inhibit the maintenance of hippocampal LTP. In the case of the cell-derived oligomers, this inhibition occurs at low- to sub-nanomolar concentrations that are similar to those that can be found in human CSF. This effect has been shown by both *in vivo* microinjection in living rats[69] and by treatment of hippocampal slices[71]. The effects of the natural oligomers on LTP are specifically neutralized by anti-Aβ antibodies *in vivo*, either through active vaccination or passive infusion[72] (see below). The same oligomers have been shown to interfere rapidly and reversibly with the memory of a learned behaviour in wake, behaving rats[73]. Taken together, these various results provide compelling evidence that decreased hippocampal LTP and altered memory function can be directly attributed to an isolated, biochemically defined, assembly form of human Aβ (with low-n soluble oligomers probably ranging from dimers to dodecamers), in the absence of amyloid fibrils or PFs. Whether Aβ*56 (REF. 62), an apparent dodecameric Aβ assembly, can also inhibit hippocampal LTP has not yet been investigated. It might turn out that a range of low-n oligomeric assemblies of Aβ can affect synaptic structure and function. Therefore, the identification of a single cytopathological Aβ species is unlikely.

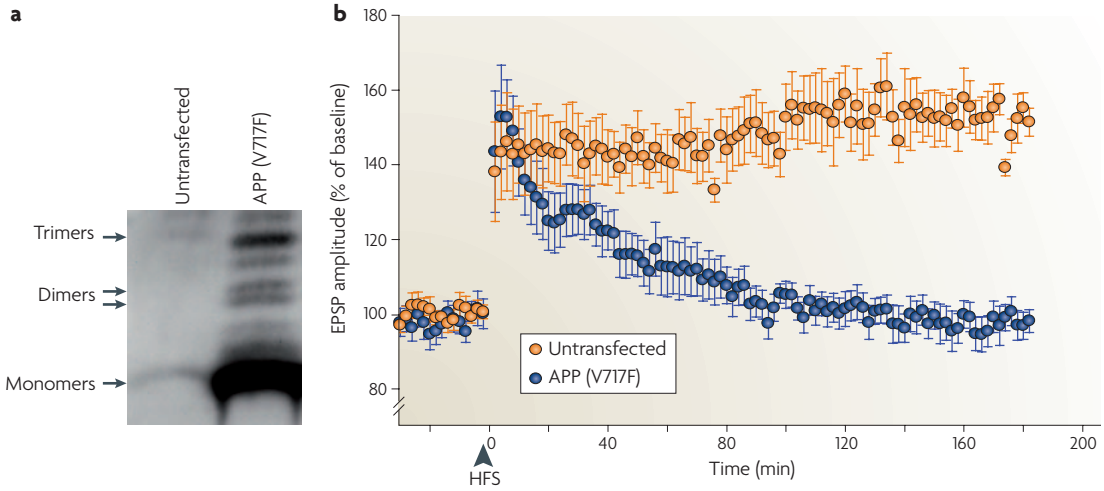


Figure 2 | **Naturally secreted SDS-stable amyloid β-protein oligomers block hippocampal long-term potentiation *in vivo*. a** | Small oligomeric assemblies of amyloid β-protein (Aβ) are secreted from CHO cells that express the familial Alzheimer's disease causing β-amyloid precursor protein (APP) V717F mutation but are not present in untransfected CHO cells. Monomers and SDS-stable dimers and trimers are indicated. All Aβ species were immunoprecipitated from conditioned media with an Aβ polyclonal antibody, separated by SDS–PAGE and western blotted with an Aβ monoclonal antibody. **b** | The SDS-stable oligomers of human Aβ shown in part **a** block hippocampal long-term potentiation (LTP) in rats *in vivo*. Just 1.5 μl of conditioned medium from CHO cells secreting Aβ oligomers (blue) or from untransfected CHO cells (orange) were injected intracerebroventricularly into wild-type adult rats. LTP of excitatory synaptic transmission in the CA1 area of the hippocampus was induced by high frequency stimulation (HFS). The conditioned medium that contained SDS-stable oligomers of human Aβ abrogated LTP, whereas the medium from control cells had no effect. EPSP, excitatory postsynaptic potential.

Binding of oligomers to synaptic plasma membranes. The biochemical mechanism by which soluble oligomers bind to synaptic plasma membranes and interfere with the complex system of receptor and/or channel proteins and signalling pathways that are required for synaptic plasticity is under intensive study. Intriguingly, Kamenetz and colleagues[70] showed that neuronal electrical activity stimulated BACE and therefore increased Aβ generation, and the resulting increased levels of Aβ then depressed synaptic transmission. Moreover, Cirrito and colleagues used *in vivo* microdialysis probes to demonstrate that interstitial fluid Aβ concentrations correlate with the synaptic activity in APP transgenic mice[74].

It is possible that soluble Aβ oligomers interfere with signalling pathways downstream of certain NMDA (*N*-methyl-D-aspartate) or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) receptors at synaptic plasma membranes in a manner that allows an initial LTP response but not its persistence. In this regard, it is interesting to note that the application of Aβ to cortical slices has been reported to promote the endocytosis of some NMDA receptors through a mechanism that involves initial binding of the Aβ to α7 nicotinic receptors[75]. Consistent with this model, Aβ treatment lowered NMDA-evoked currents. It has also been shown that oligomeric Aβ can interfere indirectly with LTP through an inhibition of a ubiquitin C-terminal hydrolase (UCH). This enzyme enhances recycling of ubiquitin, which is required to label unfolded proteins destined for proteasome degradation. Aβ treatment inhibited UCH, which in turn blocked LTP[76]. However, it is currently unclear how the extracellular Aβ can reach and affect the cytoplasmic ubiquitin system. Again, selective receptors might be involved.

Future challenges. It is now important to confirm and extend the above findings using other experimental systems in which natural Aβ oligomers are harvested and purified — preferably from APP transgenic mouse brains or even AD brain tissue — and then systematically characterized on hippocampal slices or transgenic animals. The recently described experimental paradigm of exogenous induction of cerebral β-amyloidogenesis by injection of amyloid-plaque-containing brain extracts[77] might provide an *in vivo* animal model to identify neurotoxic properties of naturally produced and purified oligomeric assemblies of Aβ.

Unfortunately, we are just at the beginning of deciphering the cellular and molecular mechanisms that underlie neurotoxicity caused by soluble oligomers. It is clear that human neurodegeneration in general and AD in particular are strongly age dependent, so that a host of age-related biochemical alterations can make neurons and their processes more vulnerable to the effects of soluble oligomers. We do not yet know whether soluble oligomers bind to specific receptors, causing selective malfunctions in a subset of neurons, or whether oligomers adsorb nonspecifically to various receptors and channel proteins and cause a range of adverse signalling effects. In addition, there is evidence that amyloidogenic proteins (at least in their synthetic form) can physically intercollate into and penetrate membranes, leading to permeabilization[78]. In this scenario, pore-forming assemblies might arise from low-n oligomers.

Such diverse mechanisms of oligomer-induced neuronal, and also glial, membrane alteration can presumably lead to numerous downstream biochemical changes, including altered signalling pathways, perturbed calcium homeostasis, production of reactive oxygen species, triggering of inflammatory cascades and mitochondrial dysfunction. However, many of our current concepts about the molecular mechanisms involved in Aβ-mediated neuronal injury have arisen from *in vitro* experiments and tissue-culture studies with synthetic Aβ fibrils at supraphysiological concentrations and should therefore be considered with care until they are validated *in vivo*. Recent *in vivo* experiments such as those mentioned above have supported the new concept that memory loss can be caused by bioactive soluble oligomers that directly disrupt synaptic integrity in the hippocampus.

Soluble oligomers in other disorders

Importantly, small oligomeric assemblies of misfolded proteins have been identified in other neurodegenerative disorders. This has been facilitated by the generation of an antibody to synthetic Aβ oligomers that specifically identifies a common structure that is present in several different amyloid-prone synthetic proteins[79]. The smallest soluble Aβ oligomers that are recognized by this antibody are probably octamers. Strikingly, this conformation-specific antibody not only detected oligomeric assemblies of Aβ but also detected soluble oligomers derived from the following recombinant disease proteins: α-synuclein (involved in PD), islet amyloid polypeptide (IAPP; involved in type II diabetes), huntingtin with extended polyglutamine stretches (involved in HD) and the prion protein (PrP; involved in transmissible and inherited spongiform encephalopathies) (see BOX 1 for neurodegenerative disease histopathologies). It seems that different aggregation-prone proteins that characterize neurodegenerative diseases such as AD, PD, HD and prion disorders have some common structural features. Therefore, soluble proteins of entirely different sequences can fold into β-sheet-rich structures that contain one or more shared conformational epitopes. This realization not only indicates that assemblies produced by different disease-causing amyloid proteins might initiate similar cytotoxic mechanisms, but also raises the possibility of targeting their common structures for therapeutic treatment (see below).

From studies on PD, there is evidence for pathogenic activity of oligomeric assemblies of α-synuclein. Like synthetic Aβ, recombinant α-synuclein can form pore-like annular structures *in vitro*[80]. Moreover, *in vitro* PF formation is accelerated by α-synuclein mutations that cause early onset PD; the A30P α-synuclein mutation promotes the formation of spherical PFs but actually slows the conversion to insoluble amyloid fibrils[59,81–83]. Similar oligomeric assemblies have also been associated with other neurodegenerative diseases. These include annular structures that are composed of polyglutamine (for example, the aggregation-causing repetitive sequence in huntingtin[84]) and ring-like protofibrillar structures in the rare neurodegenerative disorder known as familial British dementia[85].

Yet another example comes from the study of tau, the amyloidogenic protein of familial frontotemporal dementia and AD (BOX 1), which can form soluble oligomers. Dimeric intermediates of recombinant tau can further assemble into 8–14-unit aggregates *in vitro*[86]. Cytotoxicity has been associated with such oligomeric structures that seem to arise prior to the formation of classical paired helical filaments[87].

More recently, TDP43 was found to be deposited in tau- and synuclein-negative inclusions in a disorder designated as frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) as well as in ALS[88]. TDP43 is a ubiquitously expressed, highly conserved nuclear protein that can bind to nucleic acids. Pathological TDP43 in FTLD-U becomes hyperphosphorylated, ubiquitylated and cleaved to generate an insoluble, disease-associated 25-kDa C-terminal fragment. Currently, it is not known whether the 25-kDa fragment forms any type of oligomer. However, its apparent lack of affinity for Congo red might indicate that at least large β-sheet-rich fibres are not formed.

Clearly, more work is needed to connect *in vitro* findings that used high concentrations of recombinant and synthetic proteins to what occurs endogenously in biological tissues. Structural data on different amyloidogenic proteins that were affinity purified with, for example, the antibody described above[79] are required to better understand potentially shared mechanisms of neuronal dysfunction in distinct neurodegenerative disorders.

Targeting Aβ oligomers

In the case of AD, compounds that are currently in preclinical and early clinical development that lower the production of Aβ monomers, such as β- or γ-secretase inhibitors (FIG. 3), also decrease the formation of soluble, bioactive oligomers[69]. But the findings on the specific pathogenicity of small, soluble oligomers (see above) has raised the question of attempting to target the oligomeric forms directly without perturbing the enzymatic activities of the secretases, which process many important substrates[32,89] (FIG. 3).

One way this might be accomplished is through the new and promising approach of immunotherapy, through either active Aβ-peptide vaccination[90] or passive infusion of anti-Aβ monoclonal antibodies[91] (FIG. 3). A Phase 2 trial of an Aβ1–42 vaccine in patients with AD was associated with the development of a T-cell-mediated, autoimmune meningoencephalitis in 6% of patients, leading to cessation of dosing[92]. Despite this self-limited reaction, all patients were followed, and a portion of the patients, who subsequently developed anti-Aβ antibodies, seemed to have some slowing of their cognitive decline, as measured by a general cognitive-status test[93]. A later report, which included all of the vaccine-trial subjects, could not confirm an overall benefit on such general cognitive tests[94]. However, when responders were grouped on the basis of their antibody titre, a titre-dependent stabilization of certain tests of declarative memory was found to be statistically significant[94]. Clearly, additional studies of Aβ immunotherapy with higher numbers of patients and without interruption

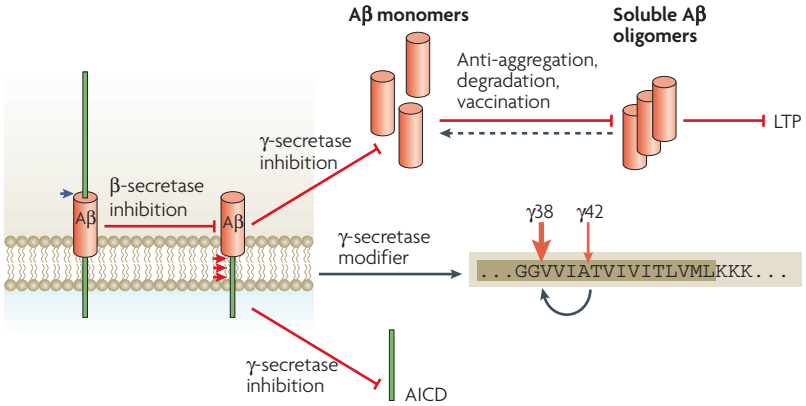


Figure 3 | **Therapeutic approaches targeting amyloid β-protein production and oligomerization.** Several therapeutic strategies, some of them already in early clinical trials, have emerged from the growing knowledge of amyloid β-protein (Aβ) generation and the effects of soluble Aβ oligomers on synaptic function. Inhibition of either β- or γ-secretase should lower Aβ generation. However, this might also block the biological function of these enzymes in cell-fate decisions (γ-secretase[32]) or in myelination (β-secretase[89]), potentially causing unwanted side effects. γ-secretase modulators such as certain non-steroidal anti-inflammatory drugs (NSAIDs) and their derivatives, on the other hand, will not block the γ-secretase cleavage but rather shift its cleavage site from the rapidly aggregating 42-residue variant to the far less amyloidogenic 38-residue form (shaded amino acids are in the transmembrane domain). This shift will not affect potential signalling functions of γ-secretase substrates (Fig. 1a). Other therapeutic strategies have emerged from the concept of selectively lowering the levels of soluble Aβ oligomers. All secretase inhibitors will also decrease oligomer formation; however, this could be accompanied by the side effects mentioned above. Anti-aggregation drugs, which prevent or even disrupt oligomers, will shift the pool of oligomeric Aβ to the benign Aβ monomers (dashed arrow). Aβ immunotherapy might also allow the selective neutralization of Aβ oligomers. Antibodies directed selectively against these oligomers might prevent their formation and/or disrupt pre-existing oligomers (dashed arrow). Any Aβ-related treatment strategy will lower the amyloid plaque load as well. AICD, β-amyloid precursor protein intracellular domain; LTP, long-term potentiation.

by adverse events are required to fully confirm this apparent slowing of decline in memory. An alternative immunotherapeutic approach to circumvent brain inflammation from active vaccination uses passive infusions of a monoclonal antibody to the Aβ N terminus, and this is currently in Phase 2 clinical testing.

Brain examination of a few patients who died of natural causes after receiving this active vaccine has revealed areas of the cerebral cortex with apparent decreases in amyloid plaque burden, accompanied by local Aβ-containing microglia[95,96]. As a note of caution, one needs to consider recently reported neuropathological findings in two patients from the interrupted vaccine trial; although cored amyloid plaque levels decreased, soluble Aβ levels increased sharply[96]. This might be due to a lack of full clearance of all Aβ species under the conditions of the interrupted trial. Whether the increased levels of soluble Aβ also result in enhanced amounts of soluble Aβ oligomers in these cases is currently unknown. Ongoing trials of passive immunotherapy use antibodies that can bind not only to amyloid plaques but also to soluble monomers and oligomers.

Strikingly, immunotherapy does not only affect Aβ oligomerization and plaque formation, but it also seems to lead to a clearance of hyperphosphorylated tau in neurons, at least in a mouse model of AD[97]. This finding supports the idea that Aβ oligomers are at the top of the amyloid cascade (BOX 2) and can induce tau aggregation. Proposed mechanisms of action for anti-Aβ immunotherapy include local microglia-mediated plaque phagocytosis[91], systemic sequestration of Aβ in plasma that results in its efflux from the brain[98] and/or the inhibition of Aβ oligomerization and cytotoxicity[99,100]. We will focus here on the neutralization of Aβ oligomers; refer to recent reviews[101,102] for the other mechanisms.

Neutralization of oligomeric Aβ. An early hint that Aβ immunotherapy might neutralize a subset of soluble Aβ assembly forms (FIG. 3) came from the finding that antibody-mediated reversal of cognitive dysfunction in APP transgenic mice did not necessarily require a decrease in total brain Aβ levels[99,100]. This result is consistent with *in vitro* studies showing that Aβ antibodies can inhibit synthetic Aβ fibrillogenesis and can even disrupt pre-existing fibrils[103,104]. The disassembly of fibrils is accompanied by strongly reduced neurotoxicity in culture[103]. However, such *in vitro* cytotoxicity assays are rather crude, as ill-defined mixtures of synthetic monomers, oligomers, PFs and fibrils are added to the media of cultured cells at supraphysiological (1 μM to 1 mM) concentrations. Therefore, *in vivo* analysis of the synaptic effects of natural oligomers was required to determine whether active or passive Aβ immunotherapy can specifically prevent oligomer-mediated synaptic dysfunction.

As reviewed earlier, naturally secreted soluble oligomers of human Aβ injected intraventricularly into wild-type rats potently inhibited hippocampal LTP[69] (FIG. 2), providing one model system for studying potential therapies (FIG. 3). In this regard, co-injection of an Aβ monoclonal antibody completely prevented the inhibition of LTP[72]. Importantly, endogenous antibodies that were raised by active Aβ vaccination also protected rats from oligomer-mediated LTP inhibition; the degree of protection correlated roughly with the level of circulating antibodies that recognized oligomers[72].

Selective degradation of oligomers and fibrils. Another therapeutic approach could be to stimulate the selective degradation of oligomers and fibrils (FIG. 3). However, most Aβ fibrils can be largely resistant to proteolytic degradation, as reflected by their progressive accumulation in AD brains. It is unlikely that the catalytic centres of most enzymes that have been shown to degrade Aβ monomers can accommodate larger oligomers and fibrils. In fact, one of the main Aβ proteases, IDE, catabolizes natural Aβ monomers but not soluble dimers and trimers[105], and incubation of these secreted Aβ species with IDE does not rescue LTP inhibition[69].

Among the proteases tested so far, only plasmin (a protease with a central role in fibrinolysis) and cathepsin B are capable of directly degrading Aβ oligomers and higher aggregates[106,107]. But even if only Aβ monomers could be degraded efficiently, oligomerization and consequent neurotoxicity should be reduced. This concept is supported by the beneficial effects of overexpressing either

IDE or neprilysin (another important Aβ-degrading protease[108]) in APP transgenic mice[109]. Moreover, Aβ oligomers accumulated early in life in an APP transgenic mouse line that lacked neprilysin, and these led to altered hippocampal synaptic plasticity[110].

Destabilization of Aβ oligomers. The search is on for small molecules that can specifically inhibit the formation of Aβ oligomers and/or prevent their binding to and stabilization on neuronal membranes (FIG. 3). Proteoglycans and their constituent glycosaminoglycans are associated with amyloid plaques in AD brain tissue and might stabilize the aggregates and make them more resistant to proteolysis. Studies of this interaction have indicated that they specifically enhance the nucleation phase of Aβ aggregation[111]. On the basis of these findings, one compound (Alzhemed) that is designed to prevent Aβ from interacting with glycosaminoglycans and proteoglycans is now in a Phase 3 AD trial (see Alzforum in Further information).

A related approach is derived from evidence that the initiation and propagation of synthetic Aβ aggregates can be mediated in part by membrane glycolipids. Certain glycolipid-derived sugars, such as scyllo-inositol (AZD-103), have been shown to stabilize synthetic Aβ assemblies at the low-n oligomer stage[112]. Oral administration of scyllo-inositol to APP transgenic mice resulted in significant decreases in plaque burden, surrounding cytopathology and associated deficits in water-maze performance[113].

Last, certain non-steroidal anti-inflammatory drugs (NSAIDs) indirectly reduce oligomer formation by the modulation of γ-secretase activity[114]. Such drugs decrease the formation of the aggregation-prone Aβ42 species by shifting the γ-secretase cut by 4 amino acids N-terminal to this site to produce the smaller, non-aggregating Aβ38 (FIGS 1b,3). Because these drugs do not block γ-secretase activity *per se*, they do not show deleterious side effects on the cell-fate decisions that require proper cleavage of Notch by the PS–γ-secretase complex (these deleterious effects are observed with classical inhibition of this enzyme)[115,116]. An orally bioavailable compound, R-flurbiprofen (Flurizan), which is derived from an NSAID but that lacks cyclo-oxygenase inhibitory activity, is currently in a Phase 3 human trial (see Alzforum in Further information).

Conclusions and perspectives

The identification of soluble oligomers of Aβ as diffusible assemblies that are capable of interfering with synaptic function and integrity provides an important opening for understanding the basis of memory loss in AD. Importantly, similar findings in other disorders might indicate convergent mechanisms of synaptic failure in several neurodegenerative diseases. These and other findings that are not discussed owing to space limitations indicate that amyloid-based therapeutic strategies in AD are beginning to specifically target the generation of soluble Aβ oligomers and their downstream consequences. The current hope is that one or more of the approaches described above can slow the progression of AD and its harbinger, mild cognitive impairment, even as numerous questions about the precise roles of oligomers of misfolded proteins such as Aβ remain to be answered. For AD, these unresolved questions include: is there a specific neuronal (or glial) receptor for Aβ oligomers, or do they indiscriminately perturb diverse receptor or channel proteins on synaptic membranes? Is the induction of tau hyperphosphorylation and its aggregation into paired helical filaments in AD a direct consequence of the accumulation of intra- or extraneuronal Aβ oligomers or are these much later downstream events? Can soluble Aβ oligomers trigger a pro-inflammatory cascade by directly or indirectly activating local microglia and astrocytes, and is this an important contributor to synaptic failure? Is there a common structure and cytotoxic mechanism of oligomeric assemblies derived from different neurodegenerative diseases? Let us hope that the development of safe and efficacious therapeutics for AD will not require the full resolution of these and other burning questions.

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Acknowledgements
We thank D. Teplow, G. Multhaup, E. Mandelkow and D. Walsh as well as the members of our laboratories for helpful discussions. We also thank M. Neumann for providing the figures that are included in Box 1.

Competing interests statement
The authors declare competing financial interests: see Web version for details.

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