Intracellular amyloid-β in Alzheimer's disease

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Abstract | The primal role that the amyloid-β (Aβ) peptide has in the development of Alzheimer's disease is now almost universally accepted. It is also well recognized that Aβ exists in multiple assembly states, which have different physiological or pathophysiological effects. Although the classical view is that Aβ is deposited extracellularly, emerging evidence from transgenic mice and human patients indicates that this peptide can also accumulate intraneuronally, which may contribute to disease progression.

Declarative memory
Aspect of human memory that stores facts and experiences. It can be further subdivided into episodic memory and semantic memory.

Pathognomic
A lesion or sign, the occurrence of which provides evidence that a particular disease is present.

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Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder, and one of the most devastating diagnoses that patients and their families can receive. The clinical symptoms result from the deterioration of selective cognitive domains, particularly those related to memory. Memory decline initially manifests as a loss of episodic memory, which is considered a subcategory of declarative memory. The dysfunction in episodic memory impedes recollection of recent events including autobiographical activities. Elucidating the underlying molecular determinants that trigger the disruption of recent episodic memory, and eventually the decline in the other cognitive domains, is among the most crucial unanswered questions in the AD field.

In 1907, Alois Alzheimer described two pathological alterations in the brain of a female patient suffering from dementia[1]. These two lesions represent the hallmark pathognomic features of the disease, and their observation during postmortem examination is still required for a diagnosis of AD. Alzheimer described a 'peculiar substance' occurring as extracellular deposits in specific brain regions, which are now referred to as amyloid plaques. It was not until the mid-1980s that it was discovered that the plaques consist of aggregates of a small peptide called amyloid-β (Aβ)[2,3]. The second lesion described by Alzheimer, neurofibrillary tangles (NFTs), occurs intraneuronally. In the late 1980s, it was discovered that NFTs are composed of aggregates of the tau protein, which becomes abnormally hyperphosphorylated[4–7].

Although plaques and NFTs are pathognomic, it would be misleading to create the impression that these are the only significant pathological changes occurring in the AD brain. In fact, numerous other structural and functional alterations ensue, including inflammatory responses and oxidative stress[8–11]. The combined consequences of all the pathological changes, including the effects of the Aβ and tau pathologies, is severe neuronal and synaptic dysfunction and loss; at the time of death, the brain of a patient with AD may weigh one-third less than the brain of an age-matched, non-demented individual.

Understanding the molecular pathways by which the various pathological alterations compromise neuronal function and integrity and lead to clinical symptoms has been a long-standing goal of AD research. Success in developing mouse models that mimic various facets of the disease process has greatly facilitated this effort. One recurring theme that has emerged from the study of some, albeit not all, AD mouse models is the occurrence of the Aβ peptide within neurons. Although intracellular Aβ is firmly associated with the human muscle disease inclusion body myositis (IBM; BOX 1), its role in AD has been more controversial. The key question is what role does intracellular Aβ have under physiological and pathological conditions in the human and mouse brain? In this Review, we consider recent evidence implicating intracellular Aβ in the pathogenesis of AD, including its relationship to extracellular amyloid plaques as well as its effects on synaptic function and learning and memory.

APP processing and Aβ generation

Aβ is produced by endoproteolysis of the parental amyloid precursor protein (APP), which is achieved by the sequential cleavage of APP by groups of enzymes or enzyme complexes termed α-, β- and γ-secretases (FIG. 1). Three enzymes with α-secretase activity have been identified, all belonging to the ADAM family (a disintegrin- and metalloproteinase-family enzyme): ADAM9, ADAM10 and ADAM17 (also known as tumour necrosis factor converting enzyme)[12]. Several

Multivesicular body
(MVB). MVBs have a role in transporting cargo to the lysosome system for degradation, and throughout the neuron for signalling purposes, and are important in regulating and degrading transmembrane proteins.

Neoepitope
Antibody recognition site of a terminal sequence in which the same sequence of amino acids are not recognized when located internally as part of the intact protein.

groups identified β-site APP-cleaving enzyme 1 (BACE1), which is a type I integral membrane protein belonging to the pepsin family of aspartyl proteases, as the β-secretase[13–15]. The γ-secretase has been identified as a complex of enzymes composed of presenilin 1 or 2, (PS1 and PS2), nicastrin, anterior pharynx defective and presenilin enhancer 2 (REFS 16–20).

The cleavage and processing of APP can be divided into a non-amyloidogenic pathway and an amyloidogenic pathway. In the prevalent non-amyloidogenic pathway, APP is cleaved by the α-secretase at a position 83 amino acids from the carboxy (C) terminus, producing a large amino (N)-terminal ectodomain (sAPPα) which is secreted into the extracellular medium[21]. The resulting 83-amino-acid C-terminal fragment (C83) is retained in the membrane and subsequently cleaved by the γ-secretase, producing a short fragment termed p3 (REF. 22). Importantly, cleavage by the α-secretase occurs within the Aβ region, thereby precluding formation of Aβ.

The amyloidogenic pathway is an alternative cleavage pathway for APP which leads to Aβ generation. The initial proteolysis is mediated by the β-secretase at a position located 99 amino acids from the C terminus. This cut results in the release of sAPPβ into the extracellular space, and leaves the 99-amino-acid C-terminal stub (known as C99) within the membrane, with the newly generated N terminus corresponding to the first amino acid of Aβ. Subsequent cleavage of this fragment (between residues 38 and 43) by the γ-secretase liberates an intact Aβ peptide. Most of the full-length Aβ peptide produced is 40 residues in length ($Aβ_{40}$), whereas a small proportion (approximately 10%) is the 42 residue variant ($Aβ_{42}$). The $Aβ_{42}$ variant is more hydrophobic and more prone to fibril formation than $Aβ_{40}$ (REF. 23), and it is this longer form that is also the predominant isoform found in cerebral plaques[24].

Mutations in three genes — *APP*, *PS1* and *PS2* — are known to cause autosomal dominant AD, which generally manifests with an early-onset pathogenesis[25]. One commonality of the disease-causing mutations in these genes is that they all affect the metabolism or stability of Aβ. These genetic mutations have been used to generate transgenic mouse models of the disease. One common mutation in APP is known as the Swedish mutation (APP_{Swe}), in which a double amino-acid change leads to increased cleavage of APP by the β-secretase[26]. Other mutations, such as the Arctic mutation (APP_{Arc}), increase the aggregation of Aβ, leading to early onset, aggressive forms of the disease[27]. Mutations in the presenilins, such as the PS1M146V mutation, increase levels of $Aβ_{42}$ (REFS 28,29), which aggregates more readily than $Aβ_{40}$. Increased dosage of the *APP* gene also results in AD[30,31]. Similarly, Down syndrome, in which triplication of chromosome 21 (on which APP resides) occurs, leads to Aβ accumulation early in life[32, 33].

Intracellular Aβ

The Aβ peptide was first identified as a component of extracellular amyloid plaques in the mid-1980s. Not long thereafter, reports describing the existence of intracellular Aβ began to appear in the literature. In the first study to report the presence of intraneuronal Aβ, an antibody against residues 17–24 of Aβ was used, and Aβ-immunoreactive material was observed in neurons in the cerebellum, cerebrum and spinal cord of individuals with or without AD neuropathology[34]. As the participants in this study ranged in age from 38 to 83 years, these findings suggested that the occurrence of intracellular Aβ might not be an age-dependent event. Curiously, however, the authors also reported that the Aβ-immunoreactive material was frequently present in NFT-containing neurons[34], perhaps a prescient indicator that these two pathologies might be linked (for a review, see REF. 35).

Since this original report, there have been a large number of studies on post-mortem AD, Down syndrome and transgenic mouse brains which have provided evidence for the presence of intracellular Aβ within neurons (TABLE 1). Careful studies using C-terminal-specific antibodies against $Aβ_{40}$ and $Aβ_{42}$ have established that most of the intraneuronal Aβ ends at residue 42, and not at residue 40 (REF. 36). Furthermore, immunogold electron microscopy has been carried out to demonstrate that $Aβ_{42}$ can be found in multivesicular bodies (MVBs) of neurons in the human brain, where it is associated with synaptic pathology[37].

Despite the publication of numerous reports in a range of animal species indicating that Aβ may accumulate intracellularly, the acceptance of this concept has been slow and controversial, mainly for technical reasons. One understandable objection relates to the extent of antibody crossreactivity, as it is plausible that Aβ-specific antibodies may also recognize full-length APP or its other derivatives. Antibodies against the C-terminal region of Aβ, which recognize the neoepitope generated by proteolysis, have helped to address this criticism. Indeed, to unambiguously confirm the presence of Aβ in cells, multiple antibodies, including antibodies to N- and C-terminal neoepitopes, must be used. Moreover, mouse neurons derived from APP-null mice have been used as vital controls to demonstrate the specificity of antibody staining, thereby increasing confidence that the immunoreactive material is indeed Aβ[37]. Another issue relates to pretreatment steps in immunohistochemical protocols: two groups have demonstrated

Box 1 | **Intracellular Aβ in muscle: inclusion body myositis**

Inclusion body myositis (IBM) is the most common cause of muscle degeneration among the elderly. The average age of disease onset is around 50 years, and the prevalence of this disorder is about 4–9 cases per 1,000,000 (REF. 145), although it is believed to be under-diagnosed. The clinical features of IBM are progressive muscle weakness and atrophy, and both proximal and distal muscles can be affected, particularly the arms and quadriceps. In 1991, Congo-red-positive inclusions in vacuolated muscle fibres from patients with IBM were described[128]. Follow up work showed that these inclusions exhibited amyloid-β (Aβ)-immunoreactivity[129,130]. Unlike in Alzheimer's disease, however, Aβ appears to only accumulate intracellularly in IBM. Other dementia-related proteins also amass in the affected muscle fibres, including tau, apolipoprotein E (APOE), α-synuclein and prion protein[146]. Several groups including our own have developed transgenic mouse models of IBM based on amyloid precursor protein overexpression, all of which point to intracellular Aβ as having a central and early role in the muscle degeneration and motor phenotype[141–143].

Mild cognitive impairment (MCI). A transition stage between the cognitive changes of normal aging and Alzheimer's disease.

that a heating protocol (microwave antigen retrieval or hydrated autoclaving) markedly enhances intraneuronal Aβ immunoreactivity, whereas formic acid exposure, a common pretreatment step in Aβ immunostaining, is not optimal for visualization of intracellular Aβ[38,39].

Recent studies suggest that the buildup of intracellular Aβ may be an early event in the pathogenesis of AD and Down syndrome. In patients with mild cognitive impairment (MCI), intraneuronal Aβ immunoreactivity has been reported in brain regions that are more prone to the development of early AD pathology, such as the hippocampus and the entorhinal cortex[36]. Similarly, it has been shown that the accumulation of intracellular Aβ precedes extracellular plaque formation in patients with Down syndrome[32]. These results suggest that the accumulation of intraneuronal Aβ is an early event in the progression of AD, preceding the formation of extracellular Aβ deposits. Indeed, it has been demonstrated that intraneuronal Aβ levels decrease as extracellular plaques accumulate[33]. These conclusions are also consistent with results from transgenic mouse models, in which intracellular Aβ accumulation appears as an early event in the progression of the neuropathological phenotype, preceding the accumulation of extracellular Aβ plaques[40–47]. In a well-utilized model of AD, the 3xTg-AD mouse (which overexpresses APP_{Swe}, and tauP301L, as well as carrying a PS1M146V knock-in mutation), intraneuronal Aβ levels decrease as extracellular plaques start to build up[48], consistent with studies of the brains of patients with Down syndrome[32,33].

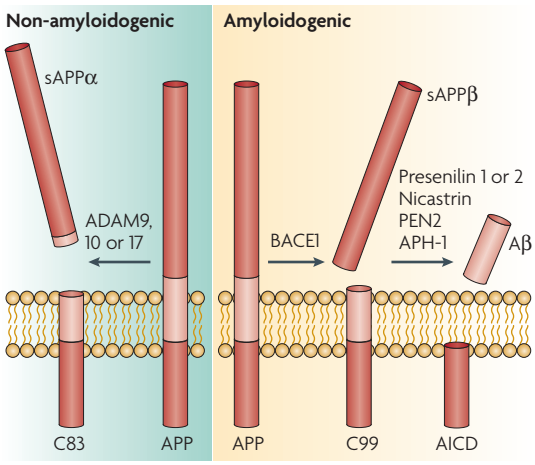


Figure 1 | **APP proteolysis.** The amyloid-β (Aβ) peptide is derived via proteolysis from a larger precursor molecule called the amyloid precursor protein (APP), a type 1 transmembrane protein consisting of 695–770 amino acids. APP can undergo proteolytic processing by one of two pathways. Most is processed through the non-amyloidogenic pathway, which precludes Aβ formation. The first enzymatic cleavage is mediated by α-secretase, of which three putative candidates belonging to the family of a disintegrin and metalloprotease (ADAM) have been identified: ADAM9, ADAM10 and ADAM17. Cleavage by α-secretase occurs within the Aβ domain, thereby preventing the generation and release of the Aβ peptide. Two fragments are released, the larger ectodomain (sAPPα) and the smaller carboxy-terminal fragment (C83). Furthermore, C83 can also undergo an additional cleavage mediated by γ-secretase to generate P3 (not shown). APP molecules that are not cleaved by the non-amyloidogenic pathway become a substrate for β-secretase (β-site APP-cleaving enzyme 1; BACE1), releasing an ectodomain (sAPPβ), and retaining the last 99 amino acids of APP (known as C99) within the membrane. The first amino acid of C99 is the first amino acid of Aβ. C99 is subsequently cleaved 38–43 amino acids from the amino terminus to release Aβ, by the γ-secretase complex, which is made up of presenilin 1 or 2, nicastrin, anterior pharynx defective and presenilin enhancer 2. This cleavage predominantly produces $Aβ_{1–40}$, and the more amyloidogenic $Aβ_{1–42}$ at a ratio of 10:1. AICD, APP intracellular domain; APH-1, anterior pharynx defective; PEN2, presenilin enhancer2.

The notion of whether Aβ accumulates intraneuronally no longer seems in dispute, as a large number of studies from diverse laboratories have firmly documented its existence in the human brain (TABLE 1). In a comprehensive study, 99 brains were analysed from controls and from patients with AD and Down syndrome[49]. In agreement with previous findings[37], this study also found that most of the intraneuronal Aβ ends at residue 42, although truncation at the N terminus of Aβ was reported at a higher frequency compared with other studies. The authors also noted that the product of α- and γ-secretase cleavage of APP, p3, was more readily abundant within cells than intracellular Aβ. Based on these studies, it seems that intraneuronal Aβ immunoreactivity appears in the first year of life, increases in childhood, and stabilizes in the second decade of life, remaining high through adulthood even in healthy brains. Curiously, these authors reported that intraneuronal Aβ was not predictive of brain amyloidosis or NFT degeneration. However, as we will discuss below, it may be a requirement that intraneuronal Aβ exist in a certain assembly state to induce these pathological alterations, and thus far no studies have systemically examined this issue in the human brain.

Intracellular sites of Aβ production

Although there is a large body of evidence to demonstrate that Aβ accumulates intracellularly, a key question that remains to be addressed is whether the intracellular Aβ builds up because a portion of the generated Aβ is not secreted and consequently remains intracellular, or alternatively, whether secreted Aβ is taken back up by the cell to form these intracellular pools. To address these important issues, it is vital to understand how and where Aβ is cleaved and released from its parent protein, APP (FIG. 2). APP localizes to the plasma membrane[50] and has been postulated to have roles in cell adhesion[51] and cell movement[52], but APP has also been localized to the trans-Golgi network[53], endoplasmic reticulum (ER), and endosomal, lysosomal[50] and mitochondrial membranes[54]. The liberation of Aβ could potentially occur wherever APP and the β- and γ-secretases are localized, and it is likely that this occurs in several cellular compartments. Should Aβ cleavage occur within the confines of the cell, then that Aβ would be intracellular; if liberation of Aβ occurs at the plasma membrane or in the secretory pathway, then

Table 1 | **Intracellular Aβ in human diseases and animal models**

Species	Findings	References
Human	Families with *APP* gene duplication show prominent intraneuronal $Aβ_{x-40}$	30,31
	Studies documenting intraneuronal Aβ, including $Aβ_{42}$, staining in AD	36,70,96, 124–127
	Intraneuronal Aβ appears early in life; strong $Aβ_{17-42}$ staining mainly in structures with low susceptibility of developing amyloid plaques or NFTs	49
	Intraneuronal Aβ reported to be an early event in Down syndrome hippocampal and cortical neurons, preceding extracellular Aβ and NFTs	32,33
	Intracellular Aβ occurs in vacuolated muscle fibres in inclusion body myositis	128–130
Monkey	Intracellular Aβ localized within cortical neurons; no clear association was found between the presence of intracellular Aβ and senile plaques. Intracellular Aβ generation changed with age: $Aβ_{42}$ significantly increased in brains from older monkeys (>30 years of age), whereas $Aβ_{40}$ remained the same regardless of age	131,132
Dog	Canines naturally develop Aβ pathology including intracellular Aβ in select neurons in the entorhinal cortex	133,134
Mouse	AD mouse models showing intraneuronal Aβ	40–45,47, 103,135–140
	3xTg-AD mice show intraneuronal Aβ that correlates with synaptic and LTP dysfunction and early memory impairments	46,112
	Mouse models of inclusion body myositis point to a central role for intracellular Aβ in the muscle degeneration and motor deficits	141–143
Rat	Transgenic rats harbouring intraneuronal Aβ in the hippocampus and the cerebrum develop mild spatial memory deficits	144

Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; LTP, long-term potentiation; NFT, neurofibrillary tangle.

it would be released into the extracellular fluid. It is likely that both occur, but it seems that the vast majority of Aβ is secreted, suggesting that Aβ is predominantly produced at the plasma membrane, or as part of the secretory pathway, so that it is rapidly expelled from the cell.

The first evidence that Aβ may be generated intracellularly as well as at the plasma membrane was provided in 1993. The NT2 cell line is a human cell line that is capable of generating a pure, reproducible neuronal population (NT2N neurons) upon differentiation with retinoic acid. Using NT2N cells, it was shown that Aβ is formed intracellularly under constitutive conditions[55]. Furthermore, cells harbouring wild-type APP and APP_{Swe} were found to process APP differently: cells expressing APP_{Swe} form Aβ intracellularly, whereas cells expressing wild-type APP do not[56]. Similarly, a recently described duplication in the *APP* gene in humans is also associated with higher levels of intracellular Aβ formation[30,31]. As mouse models of AD are typically based on *APP* overexpression, particularly of mutant alleles such as APP_{Swe} and APP_{Arc}, these latter two studies may help explain why intracellular Aβ staining is more prominent in transgenic mouse brains than in human brains.

Of current interest is a new genetic variant of the sortilin-related receptor 1 (SORL1) gene that is linked to late-onset AD[57]. SORL1 regulates trafficking of APP from the plasma membrane into retromer recycling endosomes so that APP holoprotein can be recovered. APP holoprotein that is not cleaved at the plasma membrane and not diverted into recycling endosomes by SORL1 is internalized into the early/late endosome system[58]. The endosomes are a likely site of Aβ generation owing to their acidic nature — BACE1, the β-secretase, has optimal activity at acidic pH, and APP and BACE1 interactions have been observed by FRET (fluorescence resonance energy transfer) microscopy within the endosomes[50]. Genetic variants in SORL1 increase APP in these Aβ-producing endosomes, which corresponds to an increased risk for late-onset AD[57]. This study shows how Aβ produced intracellularly in the endosome compartments is linked to the development of AD. It was shown that blocking APP internalization, either by removing its cytoplasmic domains or by removing potassium from the medium, significantly reduced Aβ levels, demonstrating that the internalization of APP by endocytosis is an important pathway for the generation of Aβ[59]. In addition, it has been demonstrated that reducing APP internalization by site-directed mutagenesis correlates with a reduction in $Aβ_{42}$ levels[60]. Furthermore, low-density lipoprotein (LDL) receptor-related protein 1B (LRP1B), an LDL family member which has high homology with LRP, binds APP holoprotein at the plasma membrane, preventing Aβ internalization and leading to decreased Aβ production and increased sAPPα secretion[61].

In addition to the endosome system, strong evidence suggests that Aβ is generated intracellularly along the secretory pathway[62]. It has been shown that retention of APP in the ER blocks production of $Aβ_{40}$ but not $Aβ_{42}$, suggesting that $Aβ_{42}$ can be produced in the ER[63–66]. It was further shown that $Aβ_{40}$ could be produced in the trans-Golgi network[67]. Interestingly, these sites of Aβ production were limited to neurons, as in non-neuronal cells both $Aβ_{40}$ and $Aβ_{42}$ were produced at the cell surface rather than intracellularly[67].

Retromer recycling endosomes
The retromer complex has been shown to be important in recycling transmembrane receptors from endosomes to the trans-Golgi network.

Holoprotein
The full-length, native polypeptide, before proteolytic cleavage events that might occur during maturation.

Minigene
A fragment of a genomic sequence that includes the exons and introns and is cloned into a eukaryotic expression vector.

Lipid raft
A specialized membrane domain that is enriched in cholesterol.

Nucleation seed
A molecule that facilitates the assembly of a polymeric structure.

Synaptosome
Isolated synapse of a neuron obtained via homogenization of nerve tissue.

Reuptake of extracellular Aβ

In addition to Aβ being produced intracellularly, it is also possible that previously secreted Aβ, which forms the extracellular Aβ pool, could be taken up by cells and internalized into intracellular pools. Aβ can bind to various biomolecules, including lipids, proteins and proteoglycans. The binding of the various forms of Aβ to the plasma membrane has been studied, and a number of putative Aβ transporters have been identified[68–71]. Consequently, it is likely that some intracellular Aβ is derived from extracellular Aβ pools, and is taken up into the cells through receptors or transporters. Recent work showed that, in mice with a toxin-induced compromise of the blood–brain barrier, fluorescently labelled Aβ that is injected into the tail vein can accumulate intracellularly in pyramidal neurons in the cerebral cortex[72]. This study provides direct evidence that neurons can take up extracellular Aβ, and that Aβ produced in the periphery may contribute to brain Aβ load.

Aβ binds to the α7 nicotinic acetylcholine receptor (α7nAChR) with high affinity[73], and it has been shown that this binding results in receptor internalization and accumulation of Aβ intracellularly[70]. Using bungarotoxin binding studies, it was shown that 3xTg-AD mice show a loss of the α7nAChRs restricted to brain regions that accumulate intraneuronal Aβ[74]. This finding is consistent with the idea that Aβ binding to α7nAChRs causes the internalization of the receptors (which manifests as loss of bungarotoxin binding in the 3xTg-AD mice) and intracellular accumulation of the peptide[70]. The theme of Aβ binding to cell surface receptors which are then internalized is common to a number of receptors. Recent studies have shown that apolipoprotein E (APOE) receptors, members of the low-density lipoprotein receptor (LDLR) family, modulate Aβ production and Aβ cellular uptake[68]. Another member of this family, LRP, binds to Aβ directly, or through ligands such as APOE, and undergoes rapid endocytosis, facilitating Aβ cellular uptake[68]. This effect is well documented *in vivo* — overexpression of an LRP minigene results in increased membrane-generated and intracellular-generated $Aβ_{42}$, whereas *APOE* knockout dramatically reduces intracellular Aβ in PDAPP mice (which overexpress human *APP V717F*, under the control of the *PDGFβ* promoter, leading to age-related brain Aβ pathology)[75]. Thus, it seems that a large portion of intracellular Aβ accumulates because of the interaction between Aβ, APOE and LRP. APOE*ε4 is the major genetic risk factor for AD, and it is notable that one of its functions appears to be to directly mediate the accumulation of intracellular Aβ.

In addition to LRP and nicotinic receptors, Aβ internalization has been reported through the scavenger receptor for advanced glycation end products (RAGE), in neurons and microglia[69,76,77]. Binding of Aβ to RAGE in neurons sets off a cascade of events that result in oxidative stress and NF-κB activation. This leads to increased production of macrophage-colony stimulating factor[78] and an enhanced microglial response. In addition to these downstream effects, it has been demonstrated that RAGE–Aβ complexes are internalized and that they co-localize with the lysosomal pathway in astrocytes in the brain of patients with AD[77]. The formyl peptide receptor-like 1 (FPRL1) is a G-protein-coupled receptor associated with inflammatory cells, including astrocytes and microglia, that binds to Aβ and mediates the chemotactic response to $Aβ_{42}$. Thus, receptor binding again results in Aβ internalization[79]. Internalization is rapid and results in cytoplasmic Aβ aggregates that stain with Congo red in macrophages[71].

It is plausible that intracellular Aβ has different roles in different cell types and that internalization in glial cells may be part of the regulatory system that seeks to control rising extracellular Aβ levels by taking the peptides up and degrading them. In neurons, the effects of intracellular Aβ are likely to be different. Neuronal Aβ uptake has also been shown to be mediated through NMDA (*N*-methyl-D-aspartate) receptors[80]. Blocking this NMDA receptor–Aβ internalization prevents pathogenicity, including increased microglial activation and cathepsin D levels[81]. This is consistent with the NMDA antagonist memantine being protective against Aβ-mediated cognitive decline in AD[82] and mouse models[83]. This highlights again the importance of the intracellular pool of Aβ for cognitive impairment in AD.

Assembly state of intracellular Aβ

Aβ is produced as a monomer, but readily aggregates to form multimeric complexes. These complexes range from low molecular weight dimers and trimers to higher molecular weight protofibrils and fibrils (BOX 2). The oligomeric species of Aβ have been found to be the most pathological, from dimers disrupting learning and memory, synaptic function and long term potentiation (LTP)[84,85], to dodecamers affecting cognition and memory in transgenic mouse models[86].

Supporting a crucial role for the formation of Aβ oligomers intracellularly, it has been shown that in tissue derived from human brain, Aβ oligomerization initiates within cells rather than in the extracellular space[87]. Work from animal models has supported this view: using two different oligomer-specific antibodies, it was shown that in the 3xTg-AD mice, Aβ oligomerization begins intracellularly[88]. Additionally, others have reported that Aβ oligomerization occurs in the endosomal compartments[89].

Aβ oligomerization has also been shown to occur during interactions with lipid bilayers, in particular cholesterol- and glycosphingolipid-rich microdomains known as lipid rafts[90,91]. As biological lipid membranes can modulate both protein folding dynamics and rates of protein aggregation, different lipid compositions in different subcellular compartment membranes may have a role in Aβ aggregation. It was found that Aβ fibrillogenesis was accelerated in the presence of plasma and endosomal and lysosomal membranes[92]. Furthermore, Aβ oligomerization is accelerated through the interaction of Aβ and clusters of monosialogangliosides in lipid rafts, resulting in the generation of Aβ bound to the GM1 ganglioside, which can then act as a nucleation seed for further Aβ aggregation[93,94]. GM1 levels in lipid rafts of synaptosomes have been shown to increase with age, perhaps explaining the increase in Aβ aggregates with

age[95]. These results suggest that lipid rafts containing ganglioside clusters can serve as a conformational catalyst or a chaperone, generating a membrane-bound form of Aβ with seeding ability, and that plasma and intracellular membranes are vital for the generation of toxic species of Aβ. It may be that the relatively low levels of intracellular Aβ in the brain of patients with AD (compared with relatively high extracellular Aβ levels), are vital for the seeding of toxic oligomers that give rise to pathological events and further seed extracellular plaque formation — for example, by secreting these oligomeric species into the extracellular space. Secreted oligomers may also facilitate other pathological events, such as disruption of synaptic transmission[85]. This means that intracellular Aβ could be sufficient to initiate and propagate the AD pathology.

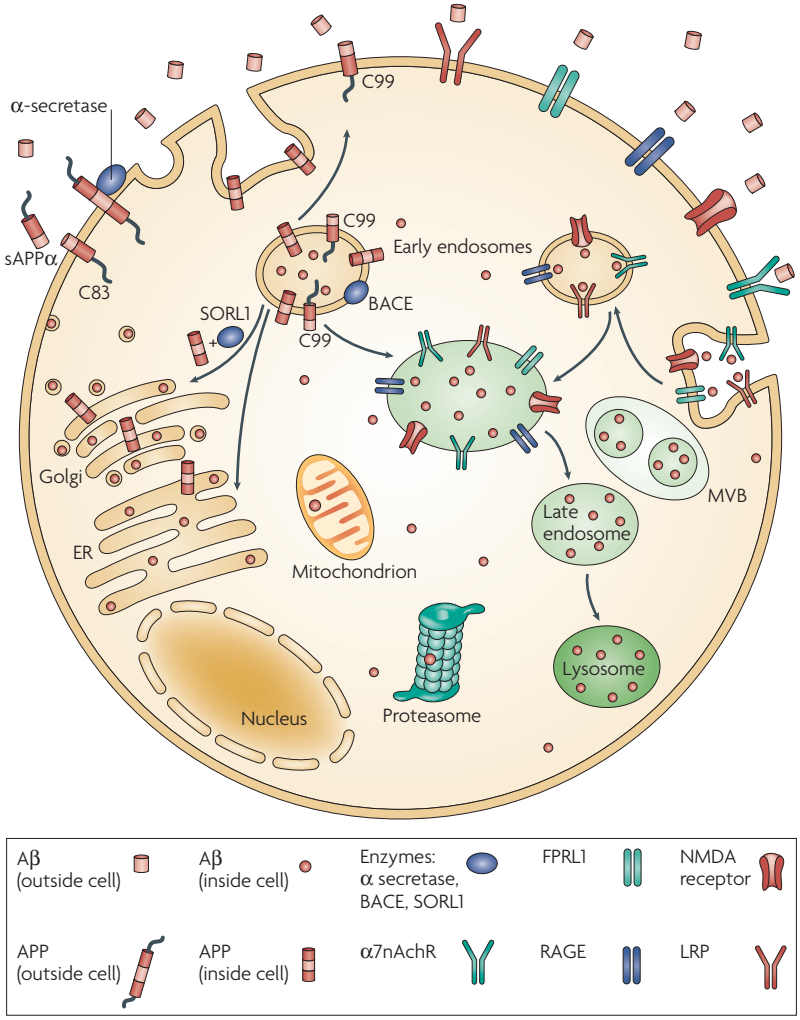


Figure 2 | **Sites of cellular Aβ production.** Amyloid-β (Aβ) is produced within the endoplasmic reticulum (ER) and Golgi system and secreted as part of the constitutive secretory pathway. Amyloid precursor protein (APP) is directed to the plasma membrane, where it is predominantly cleaved by α-secretase, releasing soluble APPα (sAPPα) into the extracellular space and leaving an 83 amino-acid fragment, known as C83, within the membrane. Unprocessed APP can be internalized into early endosomes. In the presence of the sortilin related receptor SORL1, APP is recycled back to the Golgi in retromer endosomes. Early endosomes contain β-site APP- cleaving enzyme 1 (BACE1) and have optimal pH for BACE cleavage of APP. BACE1 cleavage of APP results in a 99 amino-acid fragment, known as C99, being retained within the membrane.C99 can be shuttled back to the ER to be processed into Aβ by ER γ-secretase, shuttled back to the plasma membrane where the γ-secretase complex is also found, or processed to Aβ within the endosome/lysosome system. Extracellular Aβ (that is, previously secreted Aβ) can bind to cell surface receptors (for example, LRP, RAGE, FPRL1, NMDA receptors and α7nAChR), and this receptor–Aβ complex be internalized into early endosomes. Intracellular accumulation of Aβ is seen predominantly in the multivesicular body and lysosomes, but also in the mitochondria, ER, Golgi and the cytosol, where it is known to affect proteasome function. α7nAChR, α7 nicotinic acetylcholine receptor; FPRL1, FMLP-receptor-like protein 1; LRP, LDL receptor related protein; MVB, multivesicular body; NMDA, *N*-methyl-D-aspartate; RAGE, receptor for advanced glycation end products.

Linking intracellular and extracellular Aβ

Another key question regarding the significance of intracellular Aβ is whether the intracellular and extracellular pools of Aβ are distinct or related. Findings from human studies have been contradictory. Using immunohistochemistry and digital image analysis, it was demonstrated that brain regions with abundant intracellular Aβ accumulation showed evidence of neuronal lysis, which resulted in dispersion of the intracellular Aβ in the surrounding extracellular space[96,97]. Furthermore, using brains from patients with Down syndrome, an inverse relationship between intracellular and extracellular Aβ was demonstrated[33]. Taken together, these results suggest that extracellular Aβ may be at least partially dependent on the buildup of intraneuronal Aβ. By contrast, a recent report found that intracellular Aβ is not a predictor of extracellular Aβ deposition[49]. However, it remains to be determined whether the conclusion will be the same if the study is further extended to consider different assembly states of intracellular Aβ.

In a recent study conducted in our laboratory, we used Aβ immunotherapy to determine whether the intracellular and extracellular Aβ pools are related. Aβ immunotherapy has been used in various mouse models and quickly and effectively leads to clearance of the extracellular plaque load[98] and improved cognition[99]. In the 3xTg-AD model, removal of extracellular Aβ plaques is shortly followed by the clearance of intraneuronal Aβ[100]. Notably, as the pathology re-emerges, intraneuronal Aβ appears first, followed by the extracellular plaques[48]. These observations show that clearance of extracellular Aβ with immunotherapy also leads to the indirect reduction of intraneuronal stores. This finding indicates that extracellular Aβ may originate from intraneuronal pools and that a dynamic equilibrium exists between the two pools, such that when extracellular pools are removed, intraneuronal pools are sequestered out of the cell.

Pathological role of intracellular Aβ

Many articles have considered the physiological and pathophysiological consequences that intracellular Aβ brings about *in vitro*[101,102]; hence, we will focus on recent evidence showing the effects of intracellular Aβ *in vivo* (FIG. 3). A recent study characterizing intracellular accumulation of Aβ in humans, including patients with AD, concluded that intracellular Aβ was abundantly present, but did not correlate with plaque load or NFT formation[49]. Notably, the aggregation state of Aβ was not investigated; thus, it is plausible that more disease-relevant forms of intracellular Aβ may still exert

Ubiquitin–proteasome system
Proteasomes are large protein complexes that degrade damaged or superfluous proteins that are tagged by a small protein called ubiquitin.

pathological effects and better correlate with extracellular Aβ and/or NFTs. The finding also does not mean that intracellular Aβ in AD does not exert pathological effects through other pathways, such as synaptic dysfunction or degeneration. The investigation of the functional consequences of intracellular Aβ in the human brain is limited to correlational studies in post-mortem brains. To better address the consequences of intracellular Aβ, animal models provide significant advantages that extend beyond mere correlations.

Within neurons, $A\beta_{42}$ appears to be localized to MVBs, which are considered late endosomes and are formed from the early endosome system. Immunogold electron microscopy shows that $A\beta_{42}$ is localized to the outer membrane of the MVBs in brains of patients with AD[37]. The accumulation of non-fibrillar Aβ within neuronal MVBs has since been shown in *APPxPS1* transgenic mice[103], and Aβ-containing MVBs were most often located in the perinuclear region. Neurons in these mice also displayed Aβ-positive granules within the peri-nuclear region of the cell body, which were double labelled mainly with lamp 2, a lysosomal membrane protein, cathepsin D, another lysosomal hydrolase, and MG160, a Golgi apparatus marker[103].

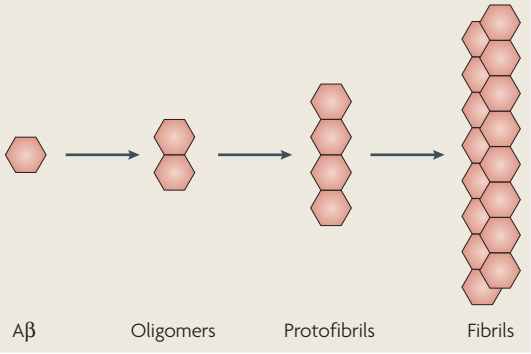
It has recently been shown that Aβ accumulation within MVBs is pathological, leading to disrupted MVB sorting via inhibition of the ubiquitin–proteasome system[104]. As the proteasome is primarily located within the cytosol, and as Aβ has been shown to inhibit the proteasome directly[105], this observation suggests that intracellular Aβ within the MVBs is mechanistically linked to cytosolic proteasome inhibition. Inhibition of the proteasome by Aβ has been shown in animal models and cell lines[104,106], and our own recent work has shown proteasome inhibition in the 3xTg-AD mice at ages at which oligomeric Aβ accumulation is seen within neuronal cell bodies[88,107]. These findings show that oligomeric Aβ accumulation within neuronal cell bodies has pathological consequences, as proteasome impairment led to the buildup of tau protein. Furthermore, we showed that proteasome inhibition, both *in vivo* and *in vitro*, leads to higher Aβ levels, suggesting that the proteasome degrades Aβ, and that Aβ must be within the cytosolic compartment for this degradation to occur.

In Tg2576 mice, accumulation of Aβ has also been observed in mitochondria[108], organelles in which all subunits of the γ-secretase have been located[109]. Progressive accumulation of intracellular Aβ in mitochondria is associated with diminished enzymatic activity of respiratory chain complexes III and IV, and a reduced rate of oxygen consumption[110]. These observations may help to explain the multitude of mitochondrial defects described in AD and mouse models of the disease[111].

There is evidence for a role for intraneuronal Aβ in synaptic dysfunction, which could underlie cognitive deficits. The 3xTg-AD mouse model of AD develops intraneuronal accumulation of Aβ at 4 months of age, which is when cognitive deficits are first detected[112]. These mice have no plaque formation, little somatodendritic tau and no hyperphosphorylated tau species, and the removal of intraneuronal Aβ with immunotherapy restores cognition to control non-transgenic levels[112]. Furthermore, the electrophysiological responses were recorded, and it was found that the appearance of intraneuronal Aβ led to a profound deficit in LTP[46], a form of synaptic transmission thought to underlie memory[113]. How intraneuronal Aβ does this is unclear, but a recent study found that Aβ oligomers induced LTP deficits in hippocampal slice cultures, and that these deficits could be rescued by increased ubiquitin C-terminal hydrolase L1 (UCHL1)[114]. This hydrolase is an integral component of the ubiquitin–proteasome system, which has been found to be impaired as a result of the accumulation of Aβ within MVBs in neurons. Furthermore, we have found decreased UCHL1 activity in the 3xTg-AD mice at time points corresponding with LTP deficits and intraneuronal accumulation of Aβ oligomers (B. Tseng, F.M.L. and K.N.G., unpublished observations). Evidence for intracellular Aβ having detrimental roles in animal models is compelling, but further evidence in human AD tissue will be required to cement intracellular Aβ as a central component of AD pathology, particularly with regards to oligomeric forms of Aβ.

Box 2 | **Aβ assembly states**

Amyloid-β (Aβ) can exist in multiple assembly states — monomers, oligomers, protofibrils and fibrils — and it is the ability of this peptide to form fibrils and other intermediate states that impart the unique pathophysiological characteristics that define Alzheimer's disease pathology. Fibril formation is a complex, nucleation-dependent process. The mechanism driving this process, particularly in the elderly brain, is not yet known, but it appears to be closely related to protein misfolding. In its monomeric state, Aβ does not appear to be neurotoxic. By contrast, oligomeric and protofibrillar species are considered potent blockers of long-term potentiation, a form of synaptic plasticity[85].



Factors affecting intracellular Aβ

A number of factors have been shown to modulate intraneuronal Aβ in animal models of AD. One of the most interesting observations is the effect of aging. For example, young 3xTg-AD mice accumulate both soluble and oligomeric Aβ within neuronal cell bodies, but the intraneuronal pool decreases at ages in which extracellular plaques manifest[48]. This finding also parallels studies in human brain tissue, including that from patients with AD and Down syndrome[32,33,49]. These

studies suggest that the brain of patients with early stage AD might have more abundant intraneuronal Aβ, which then becomes extracellular as the disease progresses and neuronal death and lysis occur. Therefore, AD brains coming to autopsy are usually advanced end-stage brains, in which intraneuronal Aβ has relocated to the extracellular pool.

Other environmental and pharmacological factors can modulate the intraneuronal Aβ pool. We recently showed that dietary treatment of 3xTg-AD mice with docosahexaenoic acid, an n-3 polyunsaturated fatty acid, significantly reduces the soluble Aβ pool and intraneuronal Aβ immunoreactivity[115]. This same treatment has been shown to improve behaviour and pathology in other mouse models of AD[116,117]. Insulin signalling also reduces intraneuronal Aβ, by increasing trafficking to the plasma membrane where it is secreted[118]. Whether or not reducing intraneuronal Aβ without affecting or increasing the extracellular pool is beneficial for AD remains to be determined. However, it is likely that both pools contribute to cognitive decline, and there is a complex relationship between the two pools and the various aggregation states of the peptide. For example, 3xTg-AD mice that repeatedly learned to locate a hidden platform in the Morris water maze show improved cognition compared to animals that were not trained[119]. More significantly, this learning alters the dynamics between intraneuronal Aβ, extracellular plaques and Aβ oligomerization. Learning increased intraneuronal and soluble Aβ, but decreased extracellular and oligomeric Aβ, with the net effect being improved cognition. Thus, the reduction in extracellular and oligomeric Aβ was highly beneficial, despite increases in intraneuronal Aβ in aged mice with established extracellular Aβ pathology. Other factors have been shown to raise intraneuronal Aβ, and are associated with increased risk of AD. These include stress hormones[120], increased dietary cholesterol[121], oxidative stress[122], homocysteic acid[123] and the presence of the APOE*ε4 allele[75], whereas knocking out ApoE decreased intraneuronal Aβ.

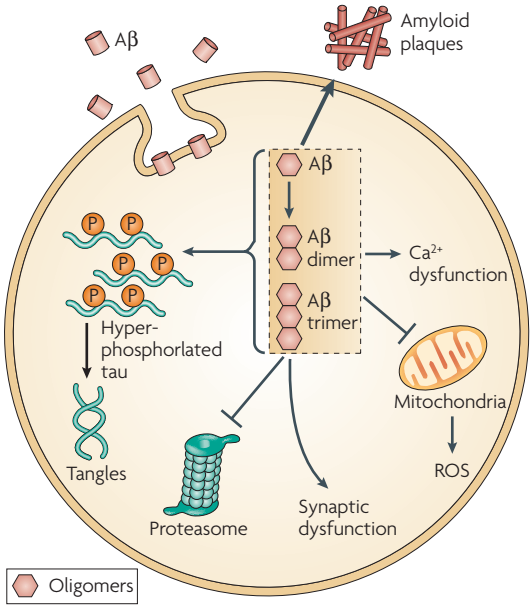


Figure 3 | **Pathological effects of intraneuronal Aβ.** Amyloid-β (Aβ), produced intracellularly or taken up from extracellular sources, has various pathological effects on cell and organelle function. Intracellular Aβ can exist as a monomeric form that further aggregates into oligomers, and it may be any of these species that mediate pathological events *in vivo*, particularly within a dysfunctional neuron. Evidence suggests that intracellular Aβ may contribute to pathology by facilitating tau hyperphosphorylation, disrupting proteasome and mitochondria function, and triggering calcium and synaptic dysfunction. ROS, reactive oxygen species.

Conclusions

There is now abundant evidence from many laboratories to document the occurrence of intraneuronal Aβ in the normal and diseased human brain. Aβ accumulation within neurons wreaks havoc on a range of cellular properties, and evidence from transgenic mouse studies suggests that it can disrupt synaptic activity, lead to proteasome dysfunction, cause calcium dyshomeostasis, and even facilitate hyperphosphorylation of tau (FIG. 3). Nevertheless, many important questions remain to be addressed, including which assembly state of Aβ predominates intraneuronally, and which state exerts the most potent effects on synaptic plasticity, learning and memory, and other cellular functions. It will be crucial to determine whether cells from younger individuals are also better able to neutralize the potential adverse effects of intracellular Aβ, perhaps through more efficient clearance or through a chaperone-mediated process. Moreover, other future experiments will need to determine the biophysical assembly states of the intraneuronal species, particularly with regards to which state best correlates with pathology and cognitive decline.

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Competing interests statement
The authors declare no competing financial interests.

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