

Molecular pathology of neurodegenerative diseases by cryo-EM of amyloids

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Abnormal assembly of tau, α -synuclein, TDP-43 and amyloid- β proteins into amyloid filaments defines most human neurodegenerative diseases. Genetics provides a direct link between filament formation and the causes of disease. Developments in cryo-electron microscopy (cryo-EM) have made it possible to determine the atomic structures of amyloids from postmortem human brains. Here we review the structures of brain-derived amyloid filaments that have been determined so far and discuss their impact on research into neurodegeneration. Whereas a given protein can adopt many different filament structures, specific amyloid folds define distinct diseases. Amyloid structures thus provide a description of neuropathology at the atomic level and a basis for studying disease. Future research should focus on model systems that replicate the structures observed in disease to better understand the molecular mechanisms of disease and develop improved diagnostics and therapies.

More than 100 years ago, abnormal brain inclusions were identified as the defining features of a number of neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease^{1–3}. On the basis of green–yellow birefringence under polarized light following staining with Congo red, the inclusions in Alzheimer's disease were shown to contain amyloids^{4,5}. Electron microscopy showed that amyloids consist of long and narrow filaments⁶. Over the past 40 years, amyloid- β ^{7,8}, tau^{9,10}, α -synuclein¹¹ and transactive response DNA-binding protein of 43 kDa (TBP-43)^{12,13} have been identified as the proteins that make up the inclusions that characterize the majority of cases of late-onset neurodegenerative disease. The pathogenic significance of filamentous inclusions became clear when rare cases of dominant inherited disease were found to be caused by mutations in the genes that encode the proteins that make up filaments, such as amyloid- β ¹⁴, tau^{15–17}, α -synuclein¹⁸ or TDP-43^{19,20}. Each individual with inherited disease was found to have abundant filamentous inclusions in their brain. It follows that the ordered assembly into filaments probably underlies all such cases of disease, even though the reasons for filament formation in sporadic disease remain to be fully elucidated.

Scientists have studied the molecular structures of amyloids from human brains for more than 50 years. X-ray fibre diffraction showed that amyloid filaments are enriched in β -sheet structure^{21,22}. Negative-stain electron microscopy of cortical tissues from individuals with Alzheimer's disease revealed the presence of paired helical filaments²³ (PHFs), which were subsequently shown to be made of two identical C-shaped subunits of tau²⁴. However, more powerful techniques were needed to study their structures at the atomic level. The inability to grow crystals of amyloid filaments mostly precluded X-ray crystallography, but micrometre-sized crystals of shorter peptides could be studied using electrons^{25,26}. Solid-state nuclear magnetic resonance (NMR) provided the first atomic structures of amyloids generated *in vitro*^{27–29}, although the need for isotope labelling prevented its application to brain-derived filaments.

In recent years, advances in cryo-EM have made it possible to determine atomic structures of amyloids from postmortem human brains. Whereas early cryo-EM images were of insufficient quality to allow reliable structure determination of amyloids^{30,31}, around 2013 the development of new electron detectors and image processing software yielded a step change in achievable resolutions³². However, it was not until new image processing approaches for helical filaments were developed³³ that *de novo* atomic modelling in cryo-EM maps also became possible for amyloids. The first structures were of tau filaments from the brain of an individual with Alzheimer's disease³⁴ (Box 1). Since then, multiple cryo-EM structures of amyloids from human brains have been reported.

Structures of amyloid filaments from human brains

In this section we review the proteins for which structures of amyloid filaments from human brain are available (Fig. 1).

Tau

Tau protein has a role in microtubule assembly and stabilization³⁵. Tau contains 4 homologous repeats (R1 to R4) of 31 or 32 amino acids that bind to microtubules. Six tau isoforms are present in the adult human brain, with lengths ranging from 352 to 441 amino acids. Three isoforms have all four microtubule-binding repeats (4R tau); in the other three isoforms R2 is absent, leaving three repeats (3R tau). Multiple neurodegenerative diseases, collectively called tauopathies, are characterized by abnormal assembly of tau³⁶. These diseases may be divided according to the isoform composition of their filaments. For example, in Alzheimer's disease and chronic traumatic encephalopathy (CTE), all six tau isoforms are incorporated in the filaments; in Pick's disease, only 3R tau assembles; and in corticobasal degeneration and progressive supranuclear palsy (PSP), only 4R tau isoforms form filaments.

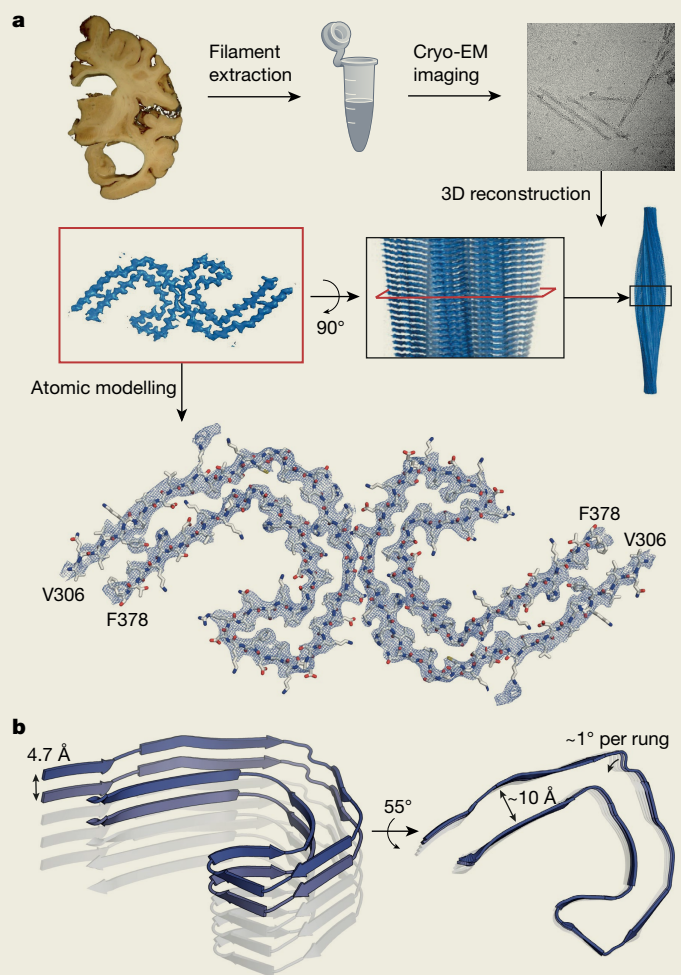
The cryo-EM structures of two types of tau filaments, PHFs and straight filaments, from the brain of an individual with Alzheimer's disease revealed that both filament types comprise two protofilaments

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Box 1

Cryo-EM structure determination of brain-derived amyloids

a, Amyloid filaments are extracted from human brains and imaged using cryo-EM. Helical reconstruction methods are used to generate a 3D density map for the filaments, in which an atomic model is built. The example shown is for tau PHFs from the brain of an individual with Alzheimer's disease. Only amino acids V306–F378 (in the numbering of the 441-amino acid tau isoform) are ordered and form the filament core (weaker density is also visible for amino acids 273–274, 304–305 and 379–380). The remaining amino- and carboxy-terminal amino acids probably adopt random conformations and make up the fuzzy coat of the filament. The ordered core of tau PHFs comprises two symmetrically packed, C-shaped protofilaments. **b**, Individual protofilaments are made of stacked copies of tau molecules in identical conformations, forming long β -sheets along the helical axis. Individual β -strands within the β -sheets, and thus individual tau molecules, are 4.7 Å apart. The filaments are twisted, because each tau molecule is rotated by approximately 1° (around the helical axis) compared with its neighbour. In the plane perpendicular to the helical axis, β -sheets pack against each other with a typical distance of approximately 10 Å. The conformation of the molecules in a single protofilament is called the protofilament fold. Specific folds define different diseases, as shown in Fig. 1.



with a common, C-shaped ordered core—the Alzheimer tau fold. Different inter-protofilament packing arrangements distinguish PHFs and straight filaments. The ordered core, which forms a β -sheet-rich structure that is characteristic of amyloids, comprises amino acids 306–378, in the numbering of the 441-amino-acid tau isoform (Box 1). Because tau is an intrinsically disordered protein, the remaining amino acids at the amino and carboxy termini likely adopt random conformations. Their disordered nature leads to less well-defined densities in electron microscopy images, which have been termed the fuzzy coat³⁷. Additional cases of Alzheimer's disease showed the same tau filament structures³⁸. This was also true of different brain regions from individual cases of Alzheimer's disease. Because valine 306 at the start of the ordered core is the first amino acid of R3, tau monomers can be incorporated into the filaments irrespective of whether they contain R2 (4R tau) or not (3R tau), explaining the presence of all 6 tau isoforms in Alzheimer's disease inclusions. This model also enabled a prediction: if amino acids comprising R1 followed by R3 or R2 followed by R3 were to be part of the ordered core, then such structures could select for 3R or 4R tau isoforms, respectively.

This prediction turned out to be correct. In the following years, structures were determined for tau filaments from Pick's disease³⁹, CTE⁴⁰, corticobasal degeneration^{41,42}, PSP and other tauopathies⁴³.

The presence of most of R1 without R2 in the ordered core of filaments from Pick's disease explained their selectivity for 3R tau; the presence of all of R2 in the ordered cores of filaments from corticobasal degeneration, PSP and other diseases explained why they incorporate only 4R tau. Tau filaments from the brains of multiple individuals with the same disease showed identical folds. In most diseases, two or more filament types were observed, and different filament types in a given disease were always composed of protofilaments with a common fold. The folds were markedly different for the different diseases, enabling a structure-based classification of tauopathies⁴³ (Fig. 1, blue). The 4R tauopathies are the most structurally diverse, including a subdivision into corticobasal degeneration-like and PSP-like folds. The discovery of a different fold in one of seven individuals with PSP, combined with a re-evaluation of their neuropathology and the observation that specific folds define distinct tauopathies, suggested that this individual suffered from a previously unknown disease, which was termed limbic-predominant neuronal inclusion body 4R tauopathy⁴³.

Although specific tau folds define distinct diseases, several conditions share a tau fold. Commonalities in structure suggest that common mechanisms may give rise to filamentous inclusions. Familial British and Danish dementias and primary age-related tauopathy share the Alzheimer fold. The Alzheimer tau fold was also observed

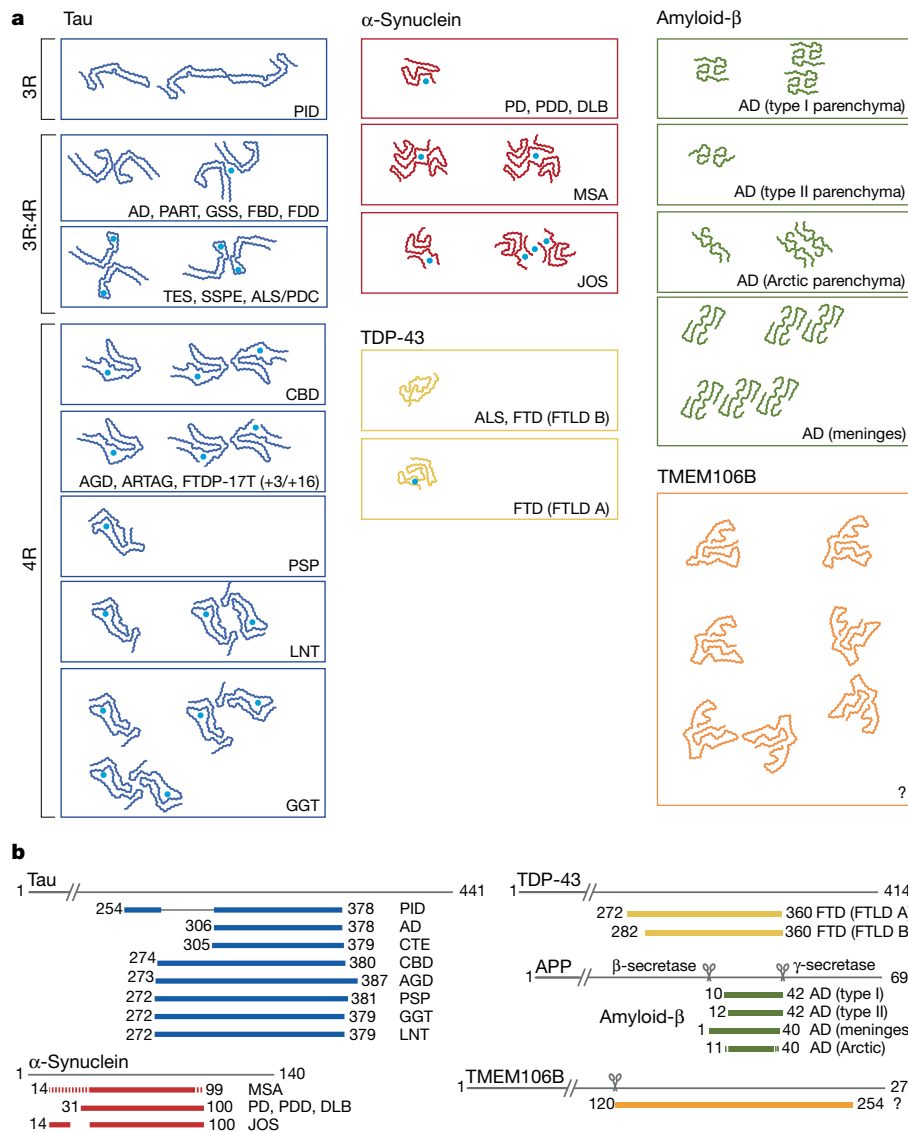


Fig. 1 | Cryo-EM structures of brain-derived amyloids. a, Main-chain traces of tau (blue), α -synuclein (red), amyloid- β (green), TDP-43 (yellow) and transmembrane protein 106B (TMEM106B; orange) proteins, with positions of non-proteinaceous cofactors (cyan dots). The diagnoses associated with each filament fold are indicated. AD, Alzheimer's disease; AGD, argyrophilic grain disease; ALS/PDC, ALS-parkinsonism dementia complex; ARTAG, ageing-related tau astrogliopathy; CBD, corticobasal degeneration; DLB, dementia with Lewy bodies; FBD, familial British dementia; FDD, familial Danish dementia; FTD, frontotemporal dementia; FTLD, frontotemporal lobar degeneration and TDP-43 pathology; FTDP-17T (+3/+16), frontotemporal dementia and parkinsonism linked to chromosome 17 caused by mutations in the tau gene (mutations +3 and +16 in intron 10); GGT, globular glial tauopathy;

GSS, Gerstmann-Sträussler-Scheinker disease; LNT, limbic-predominant neuronal inclusion body 4R tauopathy; PART, primary age-related tauopathy; PD, Parkinson's disease; PDD, Parkinson's disease dementia; PID, Pick's disease; SSPE, subacute sclerosing panencephalitis; TES, traumatic encephalopathy syndrome (the clinical syndrome associated with CTE). **b**, The amino acids that span the ordered cores of the structures in **a** are shown with thick lines in the same colours as in **a**. Dashed lines indicate variations among the protofilaments. For APP, cleavage sites for β -secretase and γ -secretase are highlighted; for TMEM106B, cleavage at Ser120 is highlighted. For APP, the amino acid numbering relates to the amyloid- β peptides and not APP; for JOS, only the amino acid numbering of the wild-type sequence is indicated.

in inherited forms of Alzheimer's disease caused by mutations in the gene encoding amyloid protein (*APP*)³⁸ and in cases of Gerstmann-Sträussler-Scheinker disease caused by mutations in the gene encoding prion protein⁴⁴. Similarly⁴⁵, tau filaments from argyrophilic grain disease, ageing-related tau astrogliopathy and mutations in intron 10 of the gene encoding tau (+3/+16) share a common fold. The latter indicates that the relative overproduction of 4R tau is sufficient to give rise to the argyrophilic grain disease fold. It has also been shown that tau filaments from the amyotrophic lateral sclerosis (ALS)-parkinsonism dementia complex of Guam and the Kii peninsula of as well as tau filaments from subacute sclerosing panencephalitis⁴⁶ are identical to those from CTE, suggesting that shared environmental

mechanisms, perhaps related to neuroinflammation, may underlie amyloid formation.

A characteristic shared by all postmortem tau filament structures is the presence of additional densities that cannot be attributed to tau. Many of the additional densities face outwards into the surrounding solvent or fuzzy coat; others (indicated with cyan circles in Fig. 1) are buried in the ordered core of the filaments. These densities may be caused by post-translational modifications of tau or by non-covalently bound cofactors that may have a role in the formation of the specific folds in the different diseases. Thus, their identification is important. The observation that the additional densities tend to be disconnected from tau densities favours the cofactor hypothesis. However, tau is also

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heavily modified in disease³⁶, and distinct post-translational modification patterns have been observed for tau filaments from different diseases^{42,47}. So far, the limited quality of the additional densities in the cryo-EM maps means that their identity remains ambiguous.

α -Synuclein

α -Synuclein is a 140-amino-acid protein that binds to lipids through its amino-terminal repeats^{48,49}. It assembles into filaments in a number of neurodegenerative diseases, of which Parkinson's disease, Parkinson's disease with dementia, dementia with Lewy bodies and multiple system atrophy (MSA) are the most common³⁶. Lewy pathology (in Parkinson's disease, Parkinson's disease with dementia and dementia with Lewy bodies) comprises Lewy bodies in nerve cell perikarya and processes, together with Lewy neurites in nerve cell processes, both of which are made of abundant α -synuclein filaments. MSA is associated with abundant filamentous α -synuclein inclusions in both nerve cells and glial cells, mainly in oligodendrocytes (Papp–Lantos inclusions). Heterozygous gene dosage and missense mutations in the gene encoding α -synuclein cause Parkinson's disease and dementia with Lewy bodies, but not MSA.

Cryo-EM structures of α -synuclein filaments from human brains (Fig. 1, red) revealed that, as for tau⁴³, specific filament folds of assembled α -synuclein define different diseases. In MSA, two types of filaments are each made of two different protofilaments⁵⁰. Their proportions vary between brain regions and individuals. This was the first example of multiple protofilament folds in a human disease. Structure determination of α -synuclein filaments from Lewy pathology disorders was complicated by a lack of helical twist in many filaments. Nevertheless, helical reconstruction from a minority of twisting filaments revealed a single protofilament with the Lewy fold, which is markedly different from the MSA folds. Two-dimensional averaging indicated that also the non-twisting filaments adopt the Lewy fold⁵¹. The cryo-EM structure of α -synuclein filaments from the brain of an individual with juvenile-onset synucleinopathy (JOS) uncovered a third fold of assembled α -synuclein from human brain⁵². JOS is probably caused by a mutation in the gene encoding α -synuclein, which gives rise to the insertion of seven amino acids in α -synuclein. The JOS fold differs from the Lewy fold, but it resembles a substructure that is common to the MSA protofilament folds.

Similar to tau, additional densities for unidentified non-proteinaceous cofactors are also present in α -synuclein filaments from human brains. For MSA filaments, the extra densities are located at the protofilament interfaces. The extra densities in the Lewy and JOS folds are in a similar location. All α -synuclein filaments from human brains have a fuzzy coat that encompasses the amino- and carboxy-terminal regions of α -synuclein.

TDP-43

TDP-43 is a 414-amino-acid protein involved in mRNA processing⁵³. The abnormal assembly of TDP-43 is characteristic of the vast majority of cases of ALS and around half of cases of frontotemporal dementia^{12,13}. Missense mutations in *TARDBP*, the gene encoding TDP-43, can cause both diseases^{19,20}. Frontotemporal dementia with TDP-43 pathology comprises at least four types of frontotemporal lobar degeneration (FTLD A to D)⁵⁴. FTLD A exhibits inclusions in nerve cell perikarya, nuclei and processes, whereas inclusions are mainly restricted to nerve cell perikarya in FTLD B, processes in FTLD C and nuclei in FTLD D. Inclusions are found throughout the cortical layers in FTLD B and D, whereas they are mainly limited to the superficial layers in FTLD A and C. Inclusions comprise full-length TDP-43, together with truncated carboxy-terminal fragments^{12,13}.

As for tau and α -synuclein, cryo-EM has shown that distinct TDP-43 filament folds define different neurodegenerative conditions (Fig. 1, yellow). In ALS and FTLD B, a single filament type comprises one protofilament with a double spiral-shaped fold⁵⁵. The same fold was

present in the frontal and motor cortices of one individual and in the frontal cortex of a second individual, supporting the view that ALS and FTLD B are part of a spectrum of diseases. The structures of TDP-43 filaments from FTLD A, including those from individuals with mutations in the gene encoding progargulin protein, revealed a distinct, chevron-shaped fold⁵⁶. Their ordered core contains the same amino acids as ALS and FTLD type B filaments, with an additional ten amino acids towards the amino terminus, which may explain the presence of distinct carboxy-terminal fragments in this disease⁵⁷. The presence of a buried arginine without charge compensation in the FTLD A fold led to the identification of arginine citrullination. The structures of TDP-43 filaments in FTLD C and FTLD D remain to be determined.

Regions at the amino and carboxy termini form the fuzzy coat of TDP-43 filaments. Two RNA recognition motifs and a DIX domain probably adopt folded domain structures in the amino-terminal fuzzy coat. Because these domains adopt random orientations relative to the filament, they are not visible in the cryo-EM structure. As for tau and α -synuclein, additional densities that cannot be attributed to TDP-43 are present in the ordered cores of brain-derived filaments.

Amyloid- β

Amyloid- β , the bulk of which comprises peptides 40 (A β 40) or 42 (A β 42) amino acids long, is generated through proteolytic cleavage of APP by β - and γ -secretases^{7,58–60}. In Alzheimer's disease, filaments of amyloid- β are found in brain parenchyma and cerebral blood vessels, with parenchymal deposits being enriched in A β 42 and blood vessel deposits being enriched in A β 40. Most disease-causing mutations in *APP* flank the amyloid- β region and the secreted peptide is wild type⁶¹. Others, such as the Arctic mutation (E693G)⁶², are within amyloid- β . Unlike tau, α -synuclein and TDP-43, which form intracellular inclusions, amyloid- β deposits are extracellular.

The first cryo-EM structures of A β 40 filaments (Fig. 1, green) were from the meninges of individuals with sporadic Alzheimer's disease⁶³. The A β 40 filaments were comprised of two identical protofilaments in which all 40 amino acids are ordered, indicating the absence of a fuzzy coat. Filaments made of four and six protofilaments, with the same protofilament fold, were also present.

Even though abundant parenchymal amyloid- β deposits are the defining feature of only a single disease (Alzheimer's disease), cryo-EM structures have revealed the existence of several different amyloid folds. Cryo-EM of parenchymal A β 42 filaments extracted from the brains of five individuals with Alzheimer's disease revealed two different filament types⁶⁴. For individuals with sporadic Alzheimer's disease, a predominance of type I A β 42 filaments was observed; for individuals with familial Alzheimer's disease and other conditions with co-pathology of parenchymal A β 42, a predominance of type II A β 42 filaments was observed. Type I and type II filaments, which both consist of two identical, S-shaped protofilaments, differ mainly in their protofilament packing, with protofilament folds differing in the orientations of a few amino acids. It remains unclear what determines whether type I or type II filaments form in the brains of individuals with Alzheimer's disease. Cryo-EM structures of A β 42 filaments with the E693G Arctic mutation showed that most filaments consist of two pairs of non-identical protofilaments⁶⁵. Differences with the type I and type II filaments of wild-type A β 42 may arise from the lack of a side chain at glycine 693. Changes in the amino acid sequence of A β 42 thus give rise to distinct protofilament folds.

These observations suggest that parenchymal amyloid- β deposits are fundamentally different from inclusions of tau, α -synuclein and TDP-43. Whereas specific folds of tau, α -synuclein and TDP-43 define different diseases, distinct folds of parenchymal amyloid- β , including wild-type amyloid- β and amyloid- β with the Arctic mutation all lead to Alzheimer's disease. This is reflected by the presence of different types of parenchymal amyloid- β plaques⁶⁶.

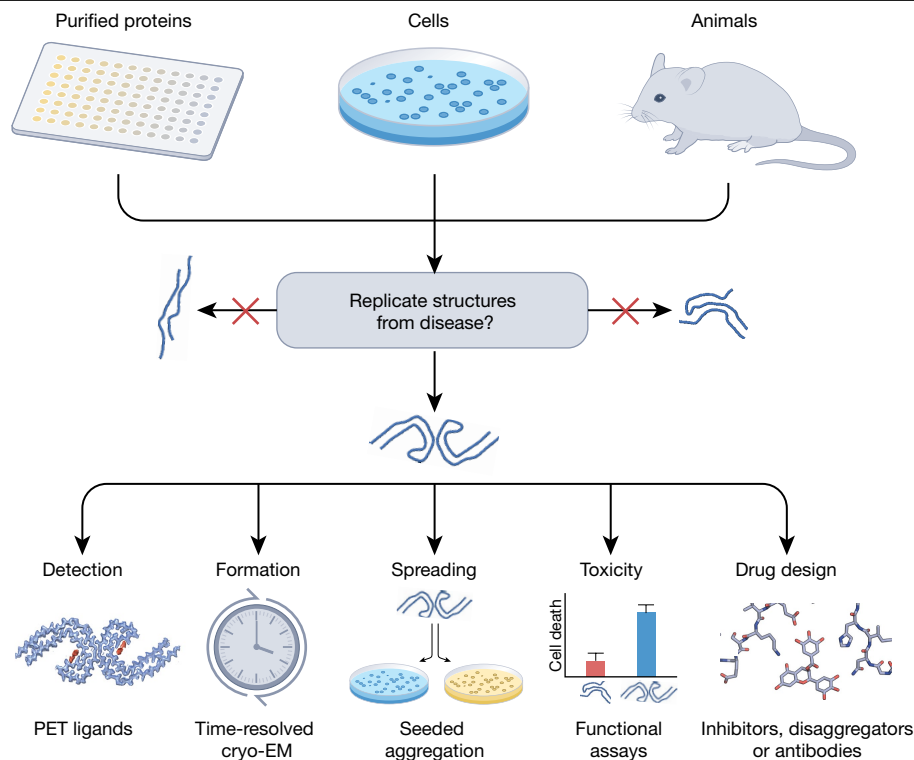


Fig. 2 | The impact of amyloid structures on research into neurodegenerative diseases. With the characterization of specific amyloid structures that define different diseases, a key priority of the field should be the development of model systems that replicate the same structures as observed in disease by

in vitro assembly with purified proteins, in cells and in animals. This process may identify the causes underlying the structural specificity of amyloids in disease, and the models may be used to gain insights into amyloid detection, formation, spreading, toxicity and the development of new therapies.

TMEM106B

Cryo-EM led to the discovery that a carboxy-terminal fragment of the lysosomal TMEM106B forms amyloid filaments in human brains^{67–69}. Three filament folds were described (Fig. 1, orange), with only a single fold being present in a given brain. Filament formation requires cleavage at serine 120, which is buried inside the core of the filaments. It remains to be determined whether the filaments have a carboxy-terminal fuzzy coat, whether cleavage at serine 120 is pathological, and what the relevant proteases are.

Abundant filaments of TMEM106B were found in neurologically normal individuals and those with Alzheimer’s disease, other tauopathies, synucleinopathies and TDP-43 proteinopathies. Unlike for tau, α -synuclein and TDP-43, no relationship was observed between the TMEM106B filament fold and disease. Immunoblotting of filaments extracted from individuals of different ages demonstrated that they accumulate in an age-dependent manner^{69–71}. Immunohistochemistry showed the presence of TMEM106B inclusions in neurons, astrocytes, oligodendrocytes and microglia, with a broad distribution in the brain^{67,70}. Lack of staining by Congo red, silver and antibodies against the amino-terminal region of TMEM106B, ubiquitin or p62, may explain why these inclusions were not detected previously.

TMEM106B had previously come to the fore when a genome-wide association study of patients with FTLD–TDP identified variation in *TMEM106B* as a risk factor for disease⁷². This was particularly true of cases of frontotemporal dementia caused by mutations in the gene encoding progranulin. However, the mechanisms by which variation in *TMEM106B* contributes to neurodegenerative disease remain unknown. TMEM106B inclusions do not co-localize with inclusions of tau, α -synuclein or TDP-43. The TMEM106B risk haplotype has been reported to correlate with the presence of TMEM106B inclusions, although its effects on filament structure are unknown⁷¹. It remains to

be determined whether and how the formation of TMEM106B filaments influences neurodegenerative diseases, and why abundant amyloid filaments of TMEM106B are apparently less detrimental than amyloid- β , tau, α -synuclein and TDP-43 filaments. It is possible that the absence of a fuzzy coat has a role in this effect.

Insights into neurodegeneration

Here we describe how the structures of amyloid filaments from human brains advance the understanding of the mechanisms of neurodegeneration (Fig. 2).

Amyloid detection

Being able to detect different types of filaments within living individuals will aid diagnosis, the recruitment of participants for clinical trials and the monitoring of drug efficacy. Pittsburgh compound B (PiB), an analogue of the amyloid-binding compound thioflavin T, made it possible to image amyloid- β plaques using positron emission tomography (PET)⁷³. PiB and related compounds such as florbetapir have been used in tens of thousands of patients. Their interaction with amyloid- β plaques may be structure-dependent, since PiB does not appear to bind to the amyloid- β plaques in patients with Alzheimer’s disease and the Arctic mutation^{65,66}. A number of PET ligands for assembled tau in Alzheimer’s disease have also been identified, the most widely used of which is flortaucipir⁷⁴. These ligands have been characterized using autoradiography on brain tissue sections from individuals with Alzheimer’s disease and have been used to visualize amyloid aggregates in the brains of living patients⁷⁵.

PET ligands specific for tau filaments in 3R and 4R tauopathies, assembled α -synuclein and TDP-43 filaments are still needed and amyloid structures may inform their development^{76,77}. Three studies have recently explored how PET ligands bind to tau filaments. The first of

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these identified multiple binding sites for the compound PM-PBB3 to tau PHFs and straight filaments⁷⁸. This study illustrated some of the difficulties of using cryo-EM to study small molecule compounds bound to amyloids. Because PM-PBB3 does not bind in a 1:1 stoichiometry with tau, the cryo-EM density for PM-PBB3 was of insufficient quality to reveal atomic interactions of the compound with tau. Two subsequent studies using the compounds GPT-1 and flortaucipir revealed more details, as 1:1 stoichiometric binding enabled descriptions of their molecular interactions with tau^{79,80}. Similar studies with α -synuclein and TDP-43 filaments are anticipated.

Amyloid formation

A better understanding of how brain amyloids form is crucial for their prevention. Postmortem brain samples will be of limited use, since they represent the end stage of disease and provide limited scope for experimental perturbation. In vitro assembly of amyloids with purified recombinant proteins has been used extensively as a minimal model system for amyloid formation.

In recent years, cryo-EM has yielded multiple structures of in vitro assembled filaments of recombinant or synthetic amyloid- β ^{81–83}, tau^{84,85}, α -synuclein^{86–98} and TDP-43^{99–101}. Notably, none of the in vitro assembled amyloid structures are identical to those of filaments extracted from postmortem human brains.

On the basis of these differences, it has been argued that the presence of the detergent sarkosyl, which is often used to extract amyloids from brain samples, may alter their structures. However—at least for brains from individuals with Alzheimer's disease—immunopurification of tau without sarkosyl has led to the same PHF and straight filament structures as observed with sarkosyl⁷⁸. In addition, extraction of amyloids from Alzheimer's disease brain slices soaked in phosphate-buffered saline led to the same amyloid- β and tau filament structures as observed with sarkosyl extraction¹⁰². It is therefore more likely that the different structures resulting from in vitro assembly provide a further illustration of the polymorphism of amyloids—that is, that a given protein can adopt many different amyloid structures.

Earlier observations of structural polymorphisms of in vitro assembled amyloids led to the hypothesis that amyloids in diseased tissues might also comprise a pool of multiple conformations¹⁰³. However, although it is possible that not all filament types are extracted from brain tissues with the methods used, such conformational clouds have not been observed. Although cryo-EM image classification methods are not guaranteed to detect all structures present, they do describe the majority. Using two-dimensional image classification, minority structures of only a few per cent of all filaments present in cryo-EM data sets have been identified, demonstrating the sensitivity of the technique^{40,43}. Despite this, only one or two distinct filament types, often with a single protofilament fold, have been detected by cryo-EM for the diseases examined so far.

The structural homogeneity of amyloids within each disease contrasts with the wide variety of structures formed in vitro, suggesting that specific factors determine amyloid structures in human brains. For example, the cellular environment of distinct brain cells and/or post-translational modifications might be conducive to the formation of different amyloid structures. It is also possible that as yet unknown cofactors co-assemble with the filaments to form specific structures. Identification of the factors that drive formation of the different structures may thus yield mechanistic insights into disease.

One way to identify these factors is to form the same structures as observed in disease using in vitro assembly of recombinant proteins. So far, this has only been possible for tau¹⁰⁴. In this case, amino- and carboxy-terminal truncations^{105,106} were essential to obtain PHFs. Moreover, the presence of sodium chloride led to the formation of tau filaments with the CTE fold. Similar in vitro assembly reactions with full-length tau did not yield filaments, suggesting that its amino and carboxy termini may inhibit assembly. However, in disease, most filaments are made of full-length tau¹⁰⁷. The in vitro assembly conditions

for full-length recombinant tau remain to be established. Experiments with phosphomimetic mutants in the carboxy-terminal domain of tau suggest that post-translational modifications in the fuzzy coat may have a role in reversing its inhibitory role in filament assembly¹⁰⁴.

Reconstituting amyloids from other tauopathies, as well as determining the conditions for reconstituting disease-related amyloids of amyloid- β , α -synuclein and TDP-43, will provide important avenues for future research. As was done for tau PHFs, screens that sample many parameters, including post-translational modifications and cofactors, may be necessary. Biochemistry and mass spectrometry may guide the design of these screens. High-throughput cryo-EM structure determination pipelines are in place¹⁰⁸. Besides potentially identifying the molecular determinants of disease-specific structures, the ability to produce large amounts of disease-specific amyloids from recombinant proteins will facilitate high-throughput screens for specific binders that could be developed into new PET ligands or approaches to inhibit or disassemble filaments. Conformational antibodies developed in this way could be used for neuropathological diagnosis and therapy. Comparing how different structures of the same recombinant protein behave in disease models will reveal which aspects of disease are affected by amyloid structure. Moreover, once assembly conditions that replicate structures of a given disease have been identified, cryo-EM studies at different time points during the assembly process, possibly complemented with NMR experiments, may provide further insights into the molecular mechanisms of their formation.

Amyloid seeding

Observations that amyloid pathology in Alzheimer's disease, Parkinson's disease, frontotemporal dementia and ALS sequentially affects connected brain regions^{109–112} and that this does not occur following surgical denervation¹¹³, together with experimental studies in cells and animals^{114,115}, have led to the hypothesis that pathogenic spreading of amyloids through templated seeding underlies disease progression, with mechanisms similar to those proposed for prion diseases¹¹⁶. Two assumptions are central to the templated seeding hypothesis. The first is that the presence of a small amount of amyloids—the seeds—amplifies amyloid formation beyond the levels that would have happened spontaneously. The second assumption is that the newly formed amyloids adopt the same structures as the seeds. The observations that specific amyloid structures characterize different diseases and that filament structures are the same in distinct regions of a given brain^{38,43,51,55,64,78} support the hypothesis of templated seeding. Moreover, seeds with different structures behave differently in model systems of amyloid propagation. For example, α -synuclein filaments extracted from the brains of individuals with MSA were more potent at inducing synucleinopathy than extracts from individuals with Lewy pathology when injected peripherally or intracerebrally in mice¹¹⁷.

Kinetic models for seeded aggregation with recombinant or synthetic proteins have existed for many years¹¹⁸. Over the past ten years, cell-based fluorescent sensors have been developed for the robust and sensitive detection of disease seeds¹¹⁹. Moreover, amyloid- β , tau, α -synuclein and TDP-43 have been observed to assemble and spread in the brains of animals following the administration of small amounts of seeds^{120–124}. However, limited data have been available to assess whether seeded aggregation replicates the structures of the seeds.

Cryo-EM of brain-derived amyloids has raised doubts about the structures formed in some models of templated seeding. For example, tau filament structures⁴³ extend beyond the carboxy terminus of the tau construct (amino acids 244–372) that is used in biosensor cell lines for tau aggregation¹¹⁹, indicating that the structures of tau seeds cannot be replicated in these cell lines. Moreover, when seeds from brains or cerebrospinal fluid were used to template the in vitro assembly of recombinant α -synuclein^{125,126} or amyloid- β ¹²⁷, cryo-EM structures of the resulting filaments were different from those extracted from brains^{51,64}. The same was true when the structures of seeded aggregates were

determined by solid-state NMR^{128,129}. In the only study reporting the structures of both the seeds and the products of seeded aggregation, for recombinant α -synuclein with MSA seeds, the seed structures were not replicated¹³⁰. Similar structures were obtained in a study that used seeds from the cerebrospinal fluid of individuals at different stages of Parkinson's disease¹²⁶, suggesting that in vitro conditions rather than the seeds determined which structures were formed. Recent observations suggest that the same may also hold for seeded aggregation in cultured cells¹³¹. This does not mean that seed amplification assays should always be avoided. For instance, they are useful for distinguishing between diseases, such as between Parkinson's disease and MSA^{132,133}. However, when interpreting the amyloid filament structures formed during amplification, the fact that they may be different from those of the seeds must be considered.

The models for templated seeding may need reconsidering. Replication of structure would be easiest to explain by primary nucleation, in which filaments grow through incorporation of new monomers at their ends. However, kinetic findings suggest that secondary nucleation—in which filaments provide a catalytic surface along their entire lengths—has a major role¹³⁴. It remains unclear how the side surface of a filament would be able to act as a template for its cross-sectional structure. Further studies are thus needed to reveal the molecular mechanisms of seeding.

Amyloid toxicity

Although the presence of amyloid filaments defines many neurodegenerative diseases and genetics provides evidence for a gain of toxic function, the underlying molecular mechanisms of how amyloid formation leads to neurodegeneration remain largely unknown.

Some researchers believe that oligomers—small, soluble and non-fibrillar assemblies—rather than mature filaments are the principal drivers of cytotoxicity and spreading of pathology¹³⁵, but little is known about their structures. Intermediate oligomeric species that lack the β -sheet structure that is typical for amyloid filaments have been observed during in vitro formation of amyloid filaments with purified recombinant proteins¹³⁶. Such intermediates have not been identified in postmortem brains, and oligomers have been defined as aggregates found in supernatants following ultracentrifugation of aqueous extracts. Surprisingly, abundant A β 42 filaments with type I and type II structures, as well as tau PHFs, were found in high-speed supernatants from Alzheimer's disease brains that were extracted by soaking in aqueous buffers¹⁰². This raises the possibility that oligomers may in fact be short filaments. Supporting this idea, recombinant tau filaments shortened by sonication are not pelleted by common ultracentrifugation procedures¹³⁷ and short tau filaments are the most seed-competent in the brains of transgenic mice¹³⁸. Similar to prions¹³⁹, a negative correlation between structural stability and seeding activity has been demonstrated for tau filaments^{140,141}.

Experimental model systems that reflect the molecular events in disease will be crucial for uncovering the molecular details of how amyloid filaments cause neurodegeneration. Such studies will be able to determine whether amyloid filaments in available model systems have the same structures as those observed in disease and whether this matters.

So far, there are no reported structures of tau, α -synuclein or TDP-43 filaments extracted from animal models. The only known structures are for amyloid- β filaments. A β 42 filaments extracted from the brains of 18-month-old *App*^{NL-F} knock-in mice were identical to type II filaments from human brains⁶⁴. These mice express humanized amyloid- β with the Swedish double mutation (K670N/M671L) and the Beyreuther/Iberian mutation (I716F)¹⁴². *App*^{NL-G-F} knock-in mice additionally have the Arctic (E693G) mutation. They develop A β 42 deposits faster than *App*^{NL-F} mice do¹⁴². However, filaments extracted from the brains of homozygous 22-month-old *App*^{NL-G-F} knock-in mice were different in structure from those observed in human brains, including a human brain with the Arctic mutation⁶⁵. Of note, filaments from homozygous

11- to 13-month-old *App*^{NL-G-F} knock-in mice¹⁴³ were the same as those from 22-month-old mice, indicating that the filament structure did not change between these ages. The latter study also provided the first insights into the 3D organization of amyloid- β deposits in the mammalian brain by cryo-electron tomography of ultra-thin tissue sections. A recent study has also found type II amyloid- β filaments and filaments that resemble type I amyloid- β filaments in mouse models that over-express human APP¹⁴⁴.

The relevance of model systems that form amyloids with structures that are distinct from those observed in disease remains unclear. It is possible that amyloid structure does not matter for some aspects of disease and thus different amyloid- β filaments all lead to Alzheimer's disease. PHFs are also characteristic of familial British and Danish dementias⁴³ as well as some cases of Gerstmann-Sträussler-Scheinker disease⁴⁴, suggesting that extracellular amyloid filaments of a certain conformation^{64,145} promote PHF formation. However, PHFs are also characteristic of primary age-related tauopathy⁴³, which lacks extracellular deposits. Conversely, distinct filament folds for tau and α -synuclein characterize different human diseases, suggesting that amyloid structures may have a role in disease. The development of model systems (cells and animals) that replicate the structures of amyloid filaments from human brains will help to uncover the mechanisms underlying neurodegenerative diseases.

Structure-based drug design

Atomic structures of amyloid filaments are expected to have key roles in the development of new therapies. Structures of amyloids and a better understanding of their formation and spreading may inform the design of inhibitors of amyloid formation^{146,147}. Cryo-EM may also provide mechanistic insights into drug actions, as exemplified by a study on epigallocatechin gallate, a component of green tea that can disassemble amyloids in vitro. The structure of this compound bound to brain-derived tau PHFs revealed how it may destabilize filaments and informed the computational selection of similar compounds with superior drug-like properties¹⁴⁸.

Structures of amyloids may also inform the development of more specific therapies. Lecanemab has been shown to produce a reduction in cognitive decline when administered to patients with early Alzheimer's disease⁷⁵. Lecanemab is a humanized monoclonal antibody raised against what was thought to be protofibrils of synthetic A β 40 peptides with the Arctic mutation¹⁴⁹. Observations by cryo-EM now suggest that protofibrils in Alzheimer's disease may in fact be short, mature A β 42 filaments¹⁰². Since the effects of lecanemab on cognitive decline have so far been relatively modest and the antibody treatment has been associated with adverse reactions, there remains ample room for improvement. More information is needed about the epitopes of lecanemab and other anti-amyloid- β antibodies, as they may not distinguish between structurally different parenchymal and blood vessel deposits of amyloid- β peptides. This could be related to some of the side effects. Knowledge of the atomic structures of brain-derived amyloids and how to produce them experimentally could thus lead to the development of more specific and safer treatments.

Outlook

Under normal conditions, the amino acid sequence of a protein determines its three-dimensional structure, which forms the basis of its function. By contrast, cryo-EM of filaments from postmortem human brains has shown that a given protein can adopt many different amyloid structures and that specific structures define distinct diseases. Although genetics indicates a direct link between filament formation and neurodegeneration, it remains unclear how filaments cause brain cell death in the various diseases. It is possible that initial filament formation occurs in distinct cellular environments, which may give rise to a specific fold. The amyloid structures themselves may directly

influence disease, as distinct structures will have different biophysical properties that may affect their formation and spreading, their interactions with other cellular components and ultimately, their toxicity in the brain. Alternatively, the mere presence of amyloid filaments, irrespective of their structures, may cause brain cells to dysfunction and die. In both scenarios, structures of amyloid filaments provide a basis for studying the molecular mechanisms of disease. For example, determining what drives the formation of specific amyloid structures observed at the end stage of disease may provide insights into the cellular and subcellular origins of filament formation during the initial stages of pathology. Moreover, new experimental models that replicate the specific structures of disease will be invaluable for gaining a better understanding of disease, and thus for developing safe and effective mechanism-based therapies.

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