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Mechanistic Studies Unravel the Complexity Inherent in Tau Aggregation Leading to Alzheimer's Disease and the Tauopathies

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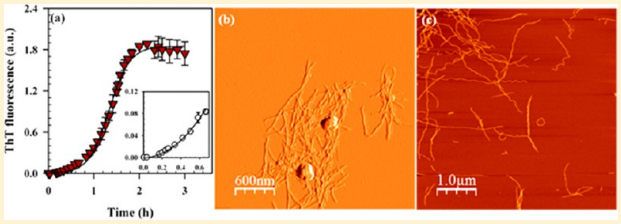
ABSTRACT: The aggregation of the protein tau into amyloid fibrils is known to be involved in the causation of the neurodegenerative tauopathies and the progression of cognitive decline in Alzheimer's disease. This review surveys the mechanism of tau aggregation with special emphasis on the information obtained from biochemical and biophysical studies. First, tau is described from a structure−function perspective. Subsequently, the connection of tau to neurodegeneration is explained, and a description of the tau amyloid fibril is provided. Lastly, studies of the mechanism of tau fibril formation are reviewed, and the physiological significance of these studies with reference to how they can clarify many aspects of disease progression is described. The aim of this review is to underscore how mechanistic studies reveal the complexity of the tau fibril formation pathway and the plethora of species populated on or off the pathway of aggregation, and how this information can be beneficial in the design of inhibitors or drugs that ameliorate neurodegeneration.



THE PROTEIN TAU

Tau was first discovered as a protein present in association with tubulin in porcine brains.[1] Named for its ability to induce tubule formation, tau belongs to the family of microtubule-associated proteins (MAPs).[2] The earliest biochemical studies performed soon after its purification showed that tau was sufficient to allow both nucleation and extension of microtubules (MTs) from purified tubulin.[3] Several of its unusual properties that arise from it being an intrinsically disordered protein (IDP) were noticed early: the protein was found to be remarkably heat- and acid-stable, and analytical ultracentrifugation coupled with sedimentation equilibrium analysis determined the protein to be highly asymmetric, with little structure as measured by circular dichroism (CD).[4]

Domain Organization of Tau. Human tau is encoded by the gene MAPT (microtubule-associated protein tau) composed of 16 exons and present on chromosome 17q21.[5,6] The protein is expressed primarily in the central nervous system (CNS), though lower levels of expression have also been detected in the peripheral nervous system as well as in the kidneys, lungs, and testes.[7,8] While the protein is abundantly expressed in the axons of the CNS, it has also been detected in the somatodendritic compartments and in oligodendrocytes.[9,10]

In the adult human brain, there are six isoforms of the protein that arise from alternative splicing of exons 2, 3, and 10 in the pre-mRNA.[11] Exons 2 and 3 encode 29-amino acid inserts each in the N-terminus (called N), and hence, tau isoforms may be 2N (both inserts), 1N (exon 2 only), or 0N (neither). Exon 10 encodes a 31-amino acid stretch in the C-terminus, which constitutes a microtubule-binding repeat (called R),[12,13] and hence, tau isoforms may be comprised of four repeats (4R, exon 10 included) or three repeats (3R, exon 10 excluded). Thus, alternative splicing results in six proteins with lengths ranging from 352 to 441 residues, which migrate as a set of six bands with apparent molecular masses of 48−67 kDa following sodium dodecyl sulfate−polyacrylamide gel electrophoresis.[14]

Domains of tau have been defined on the basis of the character of the primary sequence, on the basis of limited proteolysis and on the basis of interactions with other molecules.[15] The overall amino acid composition of tau is hydrophilic as expected for an IDP that possesses high solubility.[15] The ∼120 N-terminal residues are predominantly acidic, while the ∼40 C-terminal residues are largely neutral (the numbering of residues follows that of 2N4R, the longest tau isoform) (Figure 1a). Residues 150−243, while largely basic, are more importantly proline-rich and are hence called the P1 and P2 domains. The repeat domain region, so called because it is composed of either 3R or 4R, depending on the isoform, is predominantly basic. Because residues 369−400 are weakly homologous to the repeats, the region is termed R′ (Figure 1a).

Limited digestion with chymotrypsin *in vitro* results in tau being cleaved after Tyr 197, generating an N-terminal domain (residues 1−197) and a C-terminal domain (residues 198−441).[16] Because the N-terminal domain projects away from the MT surface, it has been called the "projection domain", while the C-terminal domain that binds to MTs and promotes their assembly has been called the "assembly domain".[16−18] A detailed analysis of the interaction of tau with MTs, assayed by means of direct binding experiments, light scattering, and video microscopy experiments, confirmed and extended this

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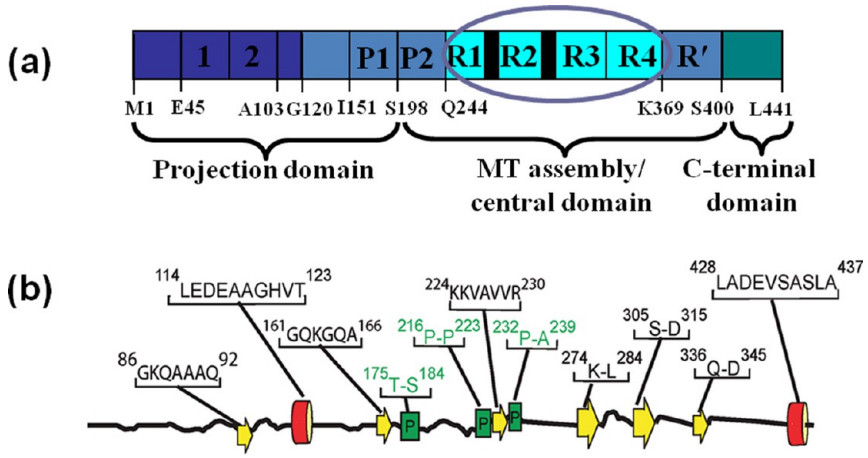

Figure 1. Domains and structural elements in tau. (a) Domain organization of the longest tau isoform (2N4R). The N-terminal domain contains two inserts labeled 1 and 2, while the central domain contains four repeat sequences (R1−R4) that constitute the fibril core and are involved in microtubule binding. P1 and P2 are proline-rich sequences; R′ is a sequence stretch that is weakly homologous to the repeats. The locations of the hexapeptide motifs (PHF6* and PHF6) at the beginning of repeats R2 and R3, which serve as important nucleating sites for tau aggregation, are labeled by black boxes. (b) Structure of tau deduced from NMR spectroscopic measurements.[20] The majority of the chain is disordered (black lines) with transient elements of secondary structure (α-helix, red; β-strand, yellow; polyproline II, green). Panel b is reproduced from ref 20. Copyright 2009 M. D. Mukrasch et al.

definition of the assembly domain.[15] The repeat domain (residues 252−376) constitutes the core of the microtubule-binding, assembly domain.[19] The strongest binding to MTs occurs, however, with the repeat domain and either one or both of the flanking regions, P1, P2 and R′.[15] More recent experiments using nuclear magnetic resonance (NMR) spectroscopy[20] have confirmed these findings and defined three functional domains of tau: (i) the projection domain (residues 1−197) comprising the N-terminus and P1, (ii) the central domain (residues 198−400) comprising the repeat domain and the flanking regions, P2 and R′, and (iii) the C-terminal domain (residues 401−441) (Figure 1a). NMR spectroscopic measurements of residual dipolar couplings and paramagnetic spin relaxation rates have revealed a correlation between the proposed domain architecture and the intrinsic flexibility of the domains. While the central domain possesses the lowest intrinsic flexibility on the nanosecond to microsecond time scale consistent with its role in MT binding, the other two domains fluctuate rapidly between different conformations.[20]

Structure of Tau. As mentioned earlier, tau is an IDP and hence does not possess any defined secondary or tertiary structure as measured by probes such as CD, Fourier transform infrared (FTIR) spectroscopy, X-ray solution scattering, or electron microscopy.[21] The earliest studies thus likened tau to a "Gaussian polymer" that behaved akin to a random coil.[21] More recently, high-resolution probes such as small-angle X-ray scattering (SAXS) and NMR spectroscopy have been applied to the study of the full-length tau isoforms and the shorter repeat domain constructs. SAXS studies revealed that both the tau isoforms and repeat domain constructs are comparable in their dimensions to random coils because they possess values for the hydrodynamic radius much larger than those expected for comparably sized structured proteins.[22] The NMR spectroscopic measurements revealed the existence of transient secondary structures (α-helix, β-strand, and polyproline II).[20] While the largest stretch of residual β-structure was found to be localized to the beginning of repeats R2−R4, two stretches of transient α-helical structure were detected in the N-terminus and in the C-terminal tail. The majority of the residues were found, however, to exist in an aperiodic, disordered conformation (Figure 1b).

It has long been known that the absence of local order in the form of secondary structures does not preclude the existence of global order in the form of long-range contacts.[23] Several studies have examined the tau protein for signs of global folding. The earliest evidence of a global structure came from the existence of antibodies with conformational epitopes; these discontinuous epitopes comprised residues near both the N-terminus and the repeat domain.[24,25] The first structural corroboration came from a fluorescence resonance energy transfer (FRET) study that proposed a "paper clip" structure for tau wherein both the N- and C-termini are folded such that they are in the neighborhood of the repeat domain, with the N-terminus lying beyond the FRET-able distance of the repeat region.[26] Subsequently, paramagnetic relaxation enhancements of NMR signals, measured over a large number of distances, confirmed the existence of a network of long-range interactions in monomeric tau.[20] More importantly, in agreement with the paper clip model, it was seen that the N-terminus interacts weakly with the C-terminus, while repeat R3 forms a transient interaction with the amphipathic helix in the C-terminal tail. A more recent study using single-molecule FRET, while supporting the existence of long-range contacts between the termini, as well as between the termini and the repeat domain, disagreed with respect to the paper clip model.[27] Quantitative analysis of their data showed that the distances between the repeat domain and the respective termini were shorter than the distance between the two termini alone, and hence, the global fold of tau is more akin to an "S shape" rather than a paper clip.

Function of Tau. The first function of tau that was characterized is its role as a MAP in stabilizing MTs by binding to their surface and promoting their self-assembly from tubulin subunits.[1,3] Subsequently, several studies have focused on the measurement of affinities for MTs by the repeat regions of tau, individually and in combination, and dissociation constants that vary over an order of magnitude from 0.01 to 1 μM have been reported.[15,28−30] It appears likely that some of this discrepancy in reported affinities might arise from the formation of tau

fibrils induced by the presence of a charged MT surface, which confounds the usage of the cosedimentation assay (tau mixed with preformed MTs in various ratios) in determining the amount of tau that is distributed between the free, soluble fraction and the MT-bound, insoluble fraction.[31,32] Using tau concentrations as low as 1 μM apparently controls for this variable; under such conditions, a reasonable estimate of the tau−MT affinity appears to be ∼0.1 μM.[28,29,31] Additionally, although the precise binding site of tau on MTs remains unresolved, consensus appears to exist that the tau conformation distributed on MTs arises from intramolecular folding and domain interactions;[33] for example, while the repeat domains are the catalytic domains required for MT assembly, the flanking domains, P2 and R′, likely function as targeting domains that position tau on the MT surface.[34] Tau, however, is also known to have other roles in interactions with MTs. For example, tau is known to protect MT ends from their innate tendency to undergo stochastic growth and shrinkage,[35] although overstabilization by tau has been shown to impair cell viability.[36] The N-terminal, projection domain of tau is believed to function as an "entropic bristle",[37,38] which ensures a certain spacing between successive MTs, and between MTs and other cell components.[39] Tau is also known to inhibit MT-dependent axonal transport by motor proteins via the competition for binding sites.[40]

Independent of its interactions with MTs, tau, as befits an IDP that possesses a multiplicity of conformations, has several other interaction partners.[41,42] Tau has been shown to interact with the plasma membrane,[43] bind and bundle actin filaments,[44] and colocalize with stress-induced actin−cofilin rods.[45] The PXXP motifs in the proline-rich regions of tau allow its binding to SH3 domain-containing proteins, including the tyrosine kinases, Fyn and Src.[46] By functioning as a protein scaffold, tau is thought to regulate signaling cascades that control neurite growth.[47] There has been speculation that the interaction of tau with growth factors may underlie the reason as to why chemotherapy-resistant cancer cells are found to overexpress the protein.[41,48]

A number of studies have examined the role of tau in the cellular response to heat shock. In neurons, tau has been shown to bind DNA and protect it against heat and oxidative stress.[49] Motifs in the repeat domain that mediate the interaction of tau with chaperones such as Hsc70 or Hsp70 and the ubiquitin ligase CHIP have been identified.[50,51] There has been speculation that the cell utilizes two different types of chaperones to protect itself against the detrimental effects of tau aggregation: MTs that sequester tau when it is present in the bound state and hsp70 chaperones that bind tau present free in the cytosol.[38]

Lastly, the necessity and, hence, importance of a protein in the cellular context can often be gauged from knockout studies that remove the protein completely. The acute knockdown of tau in cultured neuronal cells does not affect the stability or dynamics of MTs.[52] To date, four independent tau knockout mice have been made.[53−56] In all cases, longevity was not significantly impaired and there were no major neurological deficits.[57,58] In only one of the knockout mice were small differences in MT organization detected in small caliber axons,[53] and motor functions and learning and memory as measured by certain behavioral tests were found to be slightly impaired.[59] In two other knockout mice, a delay in early axon formation was seen in cultured neurons obtained from the transgenic mice.[54,60] Interestingly, old but not young tau$^{-/-}$ mice have been seen to present with memory deficits and aggressive behavior.[60] These results have been largely reconciled by suggesting that compensating mechanisms for tau knockdown, such as the activity of other MAPs,[61] function at early times but fail subsequently as a function of age.[60,62]

■ ALZHEIMER'S DISEASE AND TAUOPATHIES

Alzheimer's disease (AD) is known to be histopathologically characterized by two definitive lesions: extracellular, senile plaques composed of aggregates of protein amyloid β (Aβ)[63] and intracellular, neurofibrillary tangles (NFTs) composed of aggregates of hyperphosphorylated forms of the protein tau.[64,65] While all six isoforms of the tau protein aggregate to form NFTs, there are no mutations in tau that link it with familial forms of AD. The progress of NFT pathology in AD, in terms of the number and location of NFTs, is known, however, to correlate with cognitive decline, and the degree of severity of the disease,[66,67] thereby suggesting a strong connection between tau aggregation and AD.

A more direct cause and effect relationship of the tau protein to disease was established by pioneering genetic studies that demonstrated that mutations in the MAPT gene are associated with neurodegenerative diseases called frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17).[68−70] Other examples of these diseases collectively called tauopathies include Pick's disease, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and dementia pugilistica.[62] The main pathological hallmark of all the tauopathies is the deposition of filamentous inclusions composed of hyperphosphorylated tau either in nerve cells or in both nerve cells and glial cells.

Mutations and Their Effects. Mutations in the MAPT gene that cause tauopathies can be broadly divided into two classes: mutations that cause changes at the level of the protein and mutations that influence the alternative splicing of the tau pre-mRNA.[62]

Mutations at the protein level alter or delete a single amino acid in tau and have been reported in exons 9−13 (Figure 2). The majority of these mutations hence occur within, or in the vicinity of, the microtubule-binding repeat domain.[71] Several studies have shown that these missense mutations weaken tau's ability to bind MTs and the ability to polymerize tubulin into MTs *in vitro*.[72,73] The second class of mutations that result in splicing defects account for at least half of the mutations that are associated with FTDP-17.[71] These mutations are single-base pair changes that occur within either intron 10 or exon 10 and influence alternative splicing of the tau pre-mRNA (Figure 2), primarily by the destabilization of the stem−loop structure present at the intron−exon junction. This splicing defect translates into a change in the relative amounts of the 3R versus the 4R tau isoforms in the human brain. Because under healthy conditions the 3R and 4R isoforms are present in equal amounts, it appears that an alteration of this balance could lead to disease.[62,74]

Sporadic AD and Tauopathies. Although familial forms of AD and tauopathies exist as described above, the vast majority of these diseases (∼90%) are sporadic, and the underlying cause(s), which are likely to be a complex mix of genetic susceptibility, the environment, and age, remain unresolved.[75] In sporadic AD, polymorphism of apolipoprotein E4 (ApoE4) has been predominantly associated with an increased risk of manifesting disease.[76] In addition, a genome-wide analysis study determined that there are several, weak,

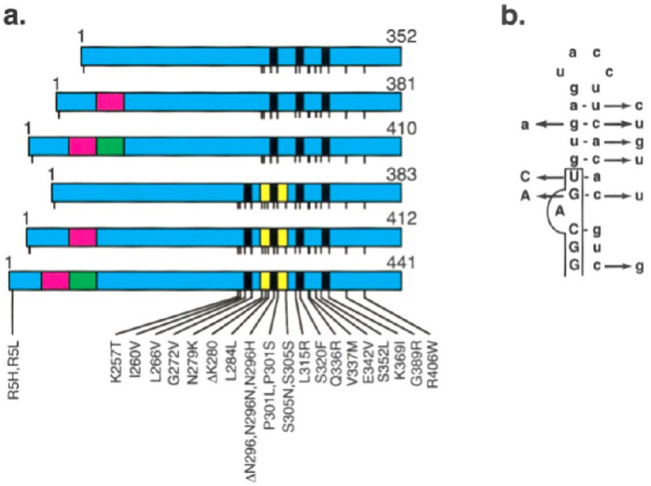

Figure 2. Mutations found in the protein tau in various tauopathies. (a) Schematic of the six tau isoforms (352−441 amino acids) that are expressed in adult human brain, with mutations in the coding region indicated (mutations are numbered according to the longest isoform). Twenty missense mutations, two deletion mutations, and three silent mutations are shown. The six tau isoforms are produced by alternative mRNA splicing from a single gene. They differ by the presence or absence of three inserts, colored red (encoded by exon 2), green (encoded by exon 3), and yellow (encoded by exon 10). (b) Stem−loop structure in the pre-mRNA at the boundary between exon 10 and the intron after exon 10. Nine mutations are shown, two of which (S305N and S305S) are located in exon 10. Exon sequences are boxed and shown in uppercase letters, with intron sequences shown in lowercase letters. Reproduced from ref 71. Copyright 2005 John Wiley and Sons.

single-nucleotide polymorphisms in the MAPT gene, resulting in a fairly complex pattern of association with AD.[77] A more definitive association of polymorphisms in the MAPT gene and disease risk has been uncovered in the context of sporadic tauopathies.[75] There are two haplotypes, H1 and H2, which characterize the MAPT gene in populations of European ancestry. Both haplotypes encode the same wild-type protein sequence, and hence, these polymorphisms exist solely at the level of the nucleotide sequence and intron size. The inheritance of the H1c haplotype has been shown recently to be a risk factor for CBD and PSP, while the H2 haplotype has a strong negative association with PSP, suggesting a neuroprotective role.[75] Studies have shown that the H1c allele increases the expression levels of total tau and enhances the inclusion of exon 10 during alternative splicing.[78] This would automatically result in an increase in the levels of 4R tau isoforms relative to 3R tau isoforms, and this presumably results in disease.

COMMON MINIMAL CONNECTION OF ALL DISEASE CONDITIONS: AGGREGATION OF TAU INTO AMYLOID FIBRILS

The common minimal connection between AD and all the tauopathies, irrespective of the mutation or modification found on the tau protein, is the aggregation state of tau.[38] Under all these diseased conditions, monomeric, disordered tau is known to be converted into polymeric, ordered fibers called amyloid fibrils.[79] From the reductionist viewpoint of a biochemist, a thorough understanding of the mechanism of tau aggregation provides a good starting point for understanding what goes wrong in disease. An understanding of the mechanism of tau aggregation necessitates the performance of careful kinetic experiments. However, before kinetics are studied, an equilibrium study of the start and end points of the reaction facilitates the interpretation of the kinetics. Our current understanding of the tau monomer was described in an earlier section. In this section, our current understanding of the tau aggregates/fibrils will be described.

In this context, it may be worthwhile to note that several of the studies that have aimed to decipher the structure of the final tau fibrils or understand the mechanism of tau aggregation utilize recombinant tau protein, purified from *Escherichia coli*. The recombinant protein does not possess any of the post-translational modifications that are often found on the tau protein in disease.[38] It still, however, serves as a good model system for studying the aggregation process because it is not clear whether some of these modifications are the cause or the consequence of protein aggregation. Hyperphosphorylated tau, for example, is found in NFTs, but an increase in the level of tau phosphorylation has been seen under normal, physiological conditions as well: the level of tau phosphorylation is elevated in normal fetal brains,[80] in hibernating animals,[81] and under conditions of anesthesia-induced hypothermia.[82]

Tau Fibrils: Paired Helical Filaments (PHFs) and Straight Filaments (SFs). The tau protein was identified as the core constituent of the NFTs found in AD brain samples by antibody reactivity studies.[64,65,83] The term "paired helical filament" (PHF) was coined, to describe these intracellular filaments long before studies had revealed their primary component to be tau.[84] The term refers to a twisted double-helical fibril with a crossover repeat of ∼80 nm and widths alternating between 8 and 20 nm. A second class of fibrils called straight filaments (SFs) is also found in NFTs. SF refers to fibrils that are ∼15 nm wide, without the pronounced modulation in width seen in PHFs.[85] An early study concluded that both the PHFs and SFs represented different assemblies of a common structural unit.[85] All these early studies, however, had visualized the PHFs and SFs using electron microscopy (EM) and its variants, and questions were hence raised about whether the two subfibers of the PHF were real or arose from sample preparation and staining artifacts.

Studies using atomic force microscopy (AFM) were hence performed with the aim of settling this debate. An early study advanced the concept of a twisted ribbon to describe the PHF because even though the structures seen displayed the reported periodicities of ∼80 nm, two distinct subfibers were not seen, and the maximal fibril height was determined to be only ∼12 nm.[86] Another study that imaged PHFs in solution concluded, however, that the structural model that best fit the experimental data was one that was compatible with the earliest descriptions of the PHFs: two coupled ribbons with ∼20 and ∼15 nm heights at the peak and valley of the fibril cross section, respectively, and a half-period of ∼75 nm.[87] A more recent study, which examined fibrils formed by different recombinant human tau isoforms and repeat domain constructs, concluded that while there was considerable polymorphism in the fibrils seen, there was no evidence of the PHF being composed of two subfibers.[88] Fibril morphologies were found to differ in length, bending, internal twist, periodicity, and thickness, within and across isoforms and repeat domains. A statistical analysis revealed that the fibrils could be broadly classified as thick or thin. The thick fibrils had heights of ∼18 and ∼9 nm at the peak and valley of the fibril cross section, respectively, and periodicities of ∼65−70 nm. The thin fibrils had heights of ∼14

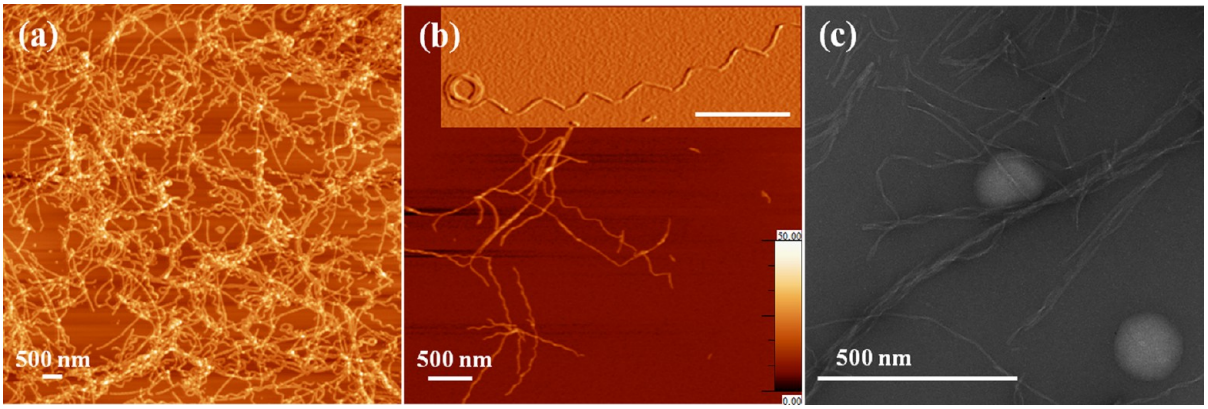

Figure 3. Morphologies of fibrils formed by the 4R domain of tau in the presence of heparin, in 25 mM Tris buffer, 50 mM NaCl, and 1 mM DTT (pH 7), are diverse. (a) AFM image of fibril clumps formed by 10 μM tauK18 (4R domain of tau) at the end of the aggregation reaction. (b) AFM image of fibrils formed by 25 μM tauK18 at the end of the aggregation reaction. The inset shows an image in the amplitude format of a coiled spring-like fibril obtained under the same condition. The *Z* height for AFM images shown in panels a and b corresponds to 50 nm as shown in the color scale. (c) TEM image of fibrils formed by 50 μM tau4RD (4R domain of tau) at the end of the aggregation reaction. The spherical structures seen are aggregates of heparin molecules in all likelihood. The scale bar for both the AFM and the TEM images corresponds to 500 nm. The heparin concentration under all conditions was 37.5 μM. Panel b of the figure is reproduced from ref 143. Copyright 2012 Elsevier.

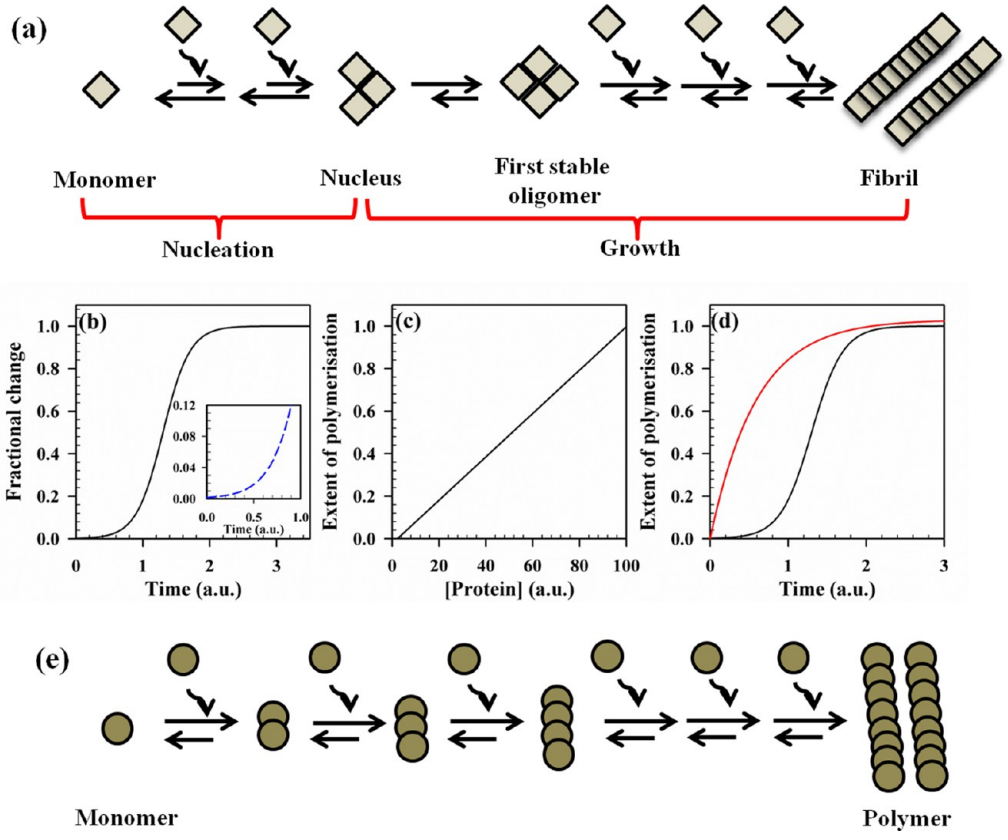

Figure 4. Mechanism of amyloid fibril formation. (a) Schematic of the NDP model showing nucleation and growth phases. The three distinct features of a NDP model are shown in panels b–d: (b) the presence of a lag phase (the inset shows the t^2 dependence of the initial 10% of the data), (c) a critical concentration, and (d) the abolition of the lag phase by seeding (red line, seeded curve; black line, unseeded curve). (e) Schematic of the isodesmic/downhill polymerization model.

and ~8 nm at the peak and valley of the fibril cross section, respectively. In addition, all tau fibrils were seen to possess a subperiodicity of ~17–19 nm along the longitudinal axis. An example of tau fibril polymorphism is shown in Figure 3.

Internal Structure of Tau Fibrils. The earliest studies of the ultrastructure of the tau fibril utilized Pronase digestion coupled with antibody labeling and EM to show that the core of the fibrils formed by full-length tau comprised the MT-binding repeat domain with the flanking N- and C-termini forming an outer fuzzy coat.[89] Subsequently, protease digestion studies coupled with CD and FTIR spectroscopy of the peptides showed that repeats R2 and R3 are the most important building parts of the fibril core.[90] This was further corroborated by fluorescence quenching experiments.[91] Conclusive electron and

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X-ray diffraction experiments then showed that both PHFs from brain samples and *in vitro* assembled tau fibrils possess the cross-β motif with the characteristic meridional diffraction pattern of 0.47 nm, thereby firmly establishing tau aggregates as bona fide amyloid fibrils.[92,93]

Several other high-resolution probes have been also applied to the study of the internal structure of the tau fibril. Scanning transmission electron microscopy (STEM) analysis of both PHFs immunopurified from AD brains and *in vitro* assembled tau fibrils revealed a packing density in the fibril core of 4−5 tau molecules/nm of fibril length, irrespective of the tau isoform.[89,94,95] Electron paramagnetic resonance (EPR) studies of tau fibrils[96] further demonstrated that the tau fibril core is composed of parallel in-register β-sheets and that two different models, namely, the β-helix (left- or right-handed) or the intersheet hairpin, arranged minimally in a β-sheet bilayer, would be compatible with the structural constraints deduced. The EPR studies also highlighted the importance of the stacking of side chains as a stabilizing force in the construction of the fibril.[97] Lastly, solid-state NMR (ssNMR) spectroscopy has been applied to the study of fibrils assembled from the truncated three-repeat domain of tau.[98,99] These studies suggest the existence of three major β-strands in repeats R1, R3, and R4, arranged in parallel and in register and connected by short kinks, which constitutes the rigid core of the fibril. In addition, these studies have also demonstrated that in addition to hydrophobic interactions,[100] the tau fibril core is stabilized by the formation of distinct salt bridges.[98]

Several studies have also focused on the identification of amyloidogenic sequences in the tau protein. The earliest protease digestion study together with a proline scanning mutagenesis study identified two main hexapeptide motifs at the beginning of repeats R2 and R3 (VQIINK and VQIVYK called PHF6* and PHF6, respectively) as being crucial for tau aggregation.[90,93] Disruption of these motifs nullifies tau's ability to aggregate *in vitro*,[93] as well as in cellular and animal models.[101,102] Other studies, utilizing X-ray fiber diffraction, have confirmed that PHF6 is sufficient to form bona fide amyloid fibrils,[103−105] besides demonstrating the existence of a dry "steric zipper" interface in the fibrils, arising from the interdigitation of a pair of parallel, in-register β-sheets.[104] In addition to these hexapeptide motifs, there are secondary amyloidogenic sequences in the tau protein that are fully capable of fibril formation.[106]

MECHANISM OF AMYLOID FIBRIL FORMATION

The mechanism of amyloid fibril formation studied *in vitro* under conditions that mimic physiological environments has been largely described as nucleation-dependent polymerization (NDP).[107] In general, a NDP mechanism for fibril assembly shows three defining characteristics: (i) a sigmoidal growth curve with three distinct phases (lag, growth, and steady-state/plateau), (ii) a critical concentration, equivalent to the dissociation constant for the monomer from fibril, below which fibrils do not assemble, and (iii) the abolition of the lag phase by the addition of preformed fibrils called "seeds"[107] (Figure 4a−d). An aggregation mechanism is generally not considered NDP until all three criteria are met.[108]

Several mathematical models have been developed for the description of the NDP mechanism, and these have been compared elsewhere.[109,110] All these models, however, subscribe to a few basic tenets of the NDP mechanism, which are described below. The NDP mechanism rests primarily on the description of a "nucleus", a species whose formation represents the bottleneck in fibril formation and is thus rate-limiting.[111] The nucleus is an oligomer present in rapid pre-equilibrium with the monomers. From a thermodynamic viewpoint, the formation of the nucleus is unfavorable because of the competition between enthalpic gain that arises from the formation of new contacts and entropic loss that arises from the immobilization of monomers in a polymer.[112] In kinetic terms, this implies that the nucleus is the first species following which the rate constants for monomer addition exceed those for monomer loss (Figure 4a).[113] The growth/elongation process that follows nucleation hence occurs without an energetic cost, and an assumption of most NDP models is that it occurs by monomer addition. Lastly, as expected, another feature of the NDP mechanism is the strong protein concentration dependence of the kinetics of fibril formation.

The features described above are especially important in distinguishing the NDP mechanism from an alternate mechanism for fibril assembly, the isodesmic or downhill polymerization mechanism[108,114] (Figure 4e). In this simpler model, no nucleus exists and the rate constants for monomer addition and loss are identical for all polymers, independent of polymer size. Although the isodesmic mechanism presumes that all oligomeric species formed are productive, it is possible to see a lag in fibril assembly if the rate constants are such that the formation of oligomeric species is disfavored.[108] The converse, however, is equally true; the absence of a lag phase does not rule out the NDP mechanism because the observation of a distinct lag ultimately depends on both the resolution of the experimental probe and the relative rates of nucleation versus growth.[114] In this context, it is important to note that the end of the lag phase in a NDP mechanism does not correspond to the cessation of nucleation, as commonly assumed.[111] It hence becomes imperative while determining a mechanism for fibril formation to examine all aspects of the data and then either to conduct simulations to test a proposed mechanism for its ability to describe the data or to use analytical solutions from defined mathematical models to fit the data and then evaluate the goodness of fit.

The earliest mathematical model proposed for protein aggregation is that of Oosawa and Kasai, who were concerned with the description of actin assembly.[115] The closed form solutions they obtained from their homogeneous nucleation model showed that the initial kinetics of monomer loss during aggregation followed a t^2 dependence (Figure 4b, inset). Subsequently, Ferrone used a linear perturbation approach to show that the initial monomer loss kinetics (for approximately the first 10% of the reaction) was better described by a $\cos(t)$ dependence for homogeneous nucleation[111] and a $\cosh(t)$ dependence if secondary pathways for fibril growth were included.[113] Secondary pathways are parallel pathways for the growth of fibrils that exist alongside the primary pathway for fibril growth and can be of three kinds: fibril fragmentation, lateral growth/branching, and heterogeneous nucleation on the fibril surface.[111,113] In this context, it is worthwhile to clarify the difference between homogeneous and heterogeneous nucleation: while the former refers to the formation of the oligomeric nucleus that represents the initial bottleneck to fibril formation, the latter refers to the formation of a nucleation site on an elongated fibril that allows for subsequent lateral fibril growth from that site.

MECHANISM OF TAU FIBRIL FORMATION

Mechanistic studies of tau fibril formation *in vitro* utilize recombinant tau preparations because reproducible and robust kinetic studies require defined and consistent preparations of protein[116] and recombinant fibrils have been shown to recapitulate structural properties of PHFs isolated from diseased brains (see the section on tau fibrils). In addition, several studies utilize the MT-binding repeat domain (4R/3R) of tau as a model system to study aggregation because the repeat domain constitutes the core of the amyloid fibril[89] and hence serves as a minimal model system for understanding the mechanism of aggregation.

Need for Inducers/Ligands for Studies of Tau Fibril Formation. All mechanistic studies of tau fibrillization *in vitro* utilize an inducer to initiate aggregation.[117] This is because, unlike other amyloid fibril-forming proteins, including other IDPs such as α-synuclein, tau does not spontaneously form fibrils at physiological protein concentrations (1−10 μM), even under buffer conditions of low or high pH and/or low or high temperature.[100] Supraphysiological protein concentrations of >200 μM coupled with extremes of buffer conditions are very often required to ensure spontaneous fibrillization.[118−120] It has been suggested that extremes of pH or temperature do not result in spontaneous fibril formation at physiological protein concentrations, because unlike α-synuclein, tau does not possess sufficient mean hydrophobicity to form a collapsed species under these buffer conditions.[100] One study showed that spontaneous fibril formation by full-length tau at a protein concentration of ∼8 μM took 8 months in a 10 mM Hepes, 100 mM NaCl (pH 7) buffer.[121] On the other hand, in the presence of an inducer, tau has been found to aggregate within the more reasonable time scale of days and at physiological concentrations, thereby allowing for robust, mechanistic studies of the kinetics of aggregation.[122,123]

Various compounds have been used as inducers for tau aggregation, including sulfated glycosaminoglycans such as heparin,[124] RNA,[125] fatty acids,[123] detergents and vesicles,[126] lipids,[127] and compounds such as quinones,[128] taurine,[129] hexafluoro-2-propanol,[130] and urea.[131] The vast majority of these inducers are polyanions: heparin and RNA are negatively charged polymers, while fatty acids and detergents have been shown to function as inducers above the critical micellar concentration where they form negatively charged micelles.[132,133] Indeed, it has been hypothesized that the inducers provide a negatively charged surface that stabilizes the aggregation-competent conformations of tau and increase the local concentration of the protein, thereby allowing the energy barrier for aggregation to be overcome.[117,134] A recent single-molecule FRET study that directly measured the change in tau conformation in the presence of heparin showed that the binding of heparin to full-length tau resulted in a compaction of the MT-binding repeat domain while the N- and C-termini were found to move farther from the repeat domain.[27] Such a structural transition is consistent with that expected for an assembly-competent conformation because the amyloidogenic sequences that possess the nascent β-strand-like structures required for tau aggregation are located in the repeat domain.[27] Further, the importance of a negatively charged surface in providing a modicum of charge compensation and a scaffold for stabilizing tau conformations has been demonstrated by the equivalence of anionic microspheres made from impermeable polystyrene and permeable fatty acid micelles, in inducing tau fibril formation.[135] The *in vivo* relevance of an inducer for tau aggregation arises from the observation that tau PHFs in AD brains are frequently found anchored to organelle membranes.[136] Hence, membranes in general, or lipid byproducts formed as a result of a disturbance in lipid metabolism, may potentially induce the cascade of events that lead to tau aggregation.[117]

Given the relevance of inducers for tau aggregation, an important aim of mechanistic studies is to delineate the kinetic role of these inducers. Initial studies concluded that the influence of inducers such as heparin and RNA was obviously kinetic because the presence of these ligands accelerated the kinetics of aggregation.[122] An increase in the concentration of the ligand further shortened the time required for aggregation, and hence, it appeared likely that these inducers were modulating nucleation. A direct quantitative measurement of the kinetic role of the inducer was required, however, to prove this conjecture. Two such studies used a direct measurement of aggregation rates in the presence of different concentrations of inducer (arachidonic acid micelles and heparin) to demonstrate that the binding of ligand to tau protein molecules was an obligate step that preceded nucleation,[137,138] and that aggregation hence occurs through a ligand-dependent NDP mechanism (see below). Indeed, NMR spectroscopic and calorimetric studies have also been performed to identify binding sites and determine the dissociation constant for tau−heparin binding.[139,140] While the calorimetric study concluded that under reducing conditions, low-molecular weight heparin binds to full-length tau at the repeat domain with micromolar affinity and at a second site with much weaker (millimolar) affinity, the NMR study identified multiple binding sites of micromolar to millimolar affinities for small heparin fragments bound to full-length tau. More recently, a single-molecule FRET study that examined titrations of full-length tau with low-molecular weight heparin determined that tau has one very high-affinity binding site for heparin with a nanomolar dissociation constant, which this study alone was able to resolve because of the picomolar protein concentrations utilized in the assay.[27]

NDP Model versus Isodesmic Model. As discussed in the preceding section, the description of an aggregation mechanism as NDP has relied on the demonstration of three features, namely, a sigmoidal progress curve, the existence of a critical concentration, and the effects of seeding. Tau aggregation in the presence of inducers such as heparin and fatty acid or anionic micelles conforms to the majority of these features and has hence been described as ligand-induced NDP by several studies,[137,138,141] though it must be noted that in all these studies, the manifestation of the seeding phenomenon (the addition of preformed fibrils as seeds to bypass the lag phase) required the additional presence of the inducer. Thus, although tau aggregation is well described by a modified NDP mechanism, it does not qualify for the classical description of NDP. In only one instance, namely, tau aggregation in the presence of the small-molecule dye inducer thiazine red (ThR), the mechanism has been described as classical homogeneous nucleation.[95] This was because thiazine red did not function as a ligand and the aggregation rates were thus found not to depend on the molar ratio of protein to dye. Because the effects of the inducer were not at the level of binding, the aggregation condition was concluded to have approximated classical equilibrium nucleation.

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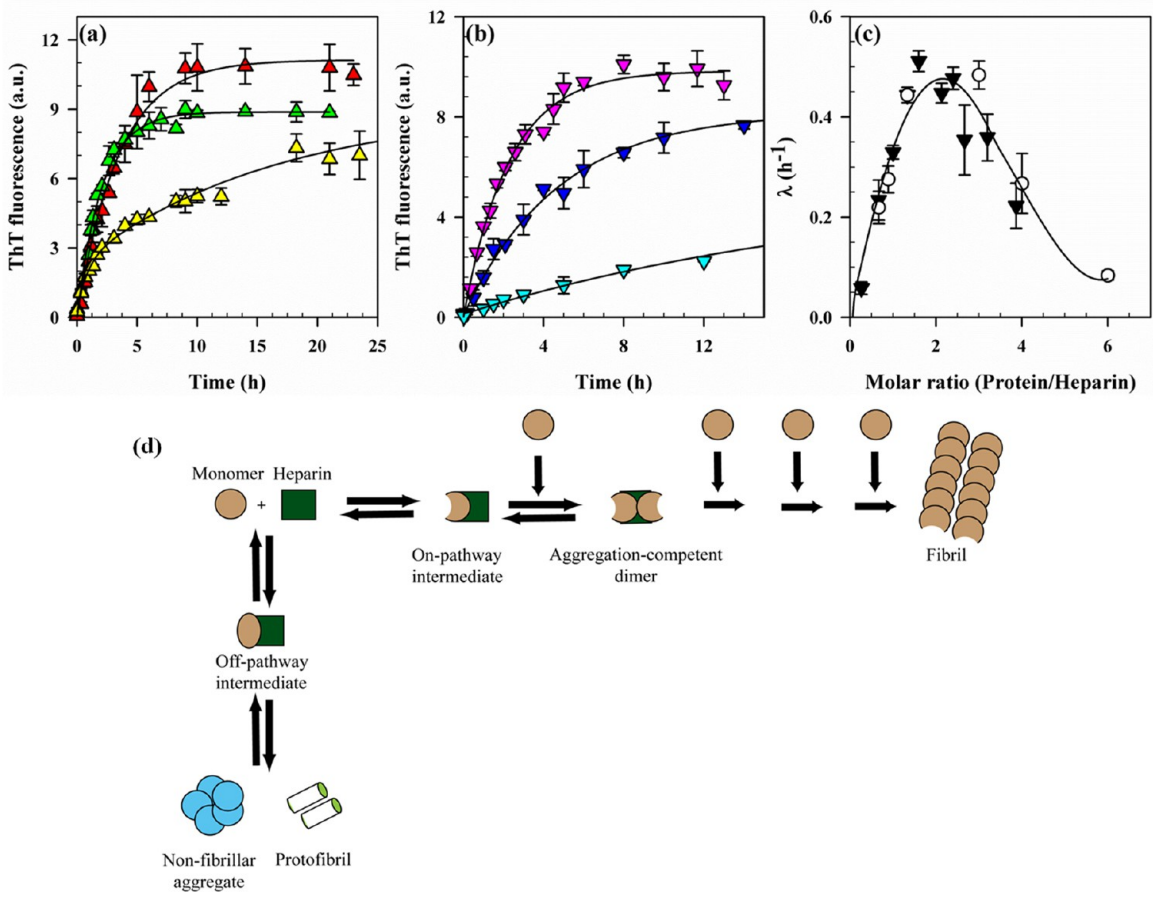

Figure 5. Mechanism of tau fibril formation in the presence of heparin. (a) Monophasic kinetics of fibril formation by 50 μM tau4RD (4R domain of tau) in the presence of 8.3 μM (yellow triangles), 16.6 μM (green triangles), and 56.2 μM heparin (red triangles). (b) Monophasic kinetics of fibril formation by 10 μM (light blue triangles), 25 μM (dark blue triangles), and 50 μM tau4RD (magenta triangles) in the presence of 37.5 μM heparin. (c) Bell-shaped dependence of the apparent rate constant of ThT fluorescence-monitored fibrillization kinetics on the protein:heparin molar ratio, when the heparin concentration is varied (▼) or when the protein concentration is varied (○). (d) Minimal model for the formation of fibrils by the 4R domain of tau in the presence of heparin verified by means of kinetic simulations. Protein binds heparin and undergoes a conformational change to form an on-pathway intermediate, PH. When a second protein molecule binds the same heparin molecule, an aggregation-competent dimer (PHP) is formed. Elongation leading to fibril formation occurs by the process of addition of the monomer to this building block. An off-pathway intermediate in the form of a tight-binding, P*H complex also forms and modulates the aggregation kinetics. It appears that this off-pathway intermediate can aggregate to form the rodlike protofibrils and nonfibrillar aggregates that are observed to accumulate transiently. Reproduced from ref 138. Copyright 2011 American Society for Biochemistry and Molecular Biology.

An early study that examined the aggregation mechanism of a 3R domain tau construct in the presence of heparin utilized disulfide-linked tau dimers as the starting species for aggregation and concluded that the nucleus consists of 8−14 tau monomers.[141] This nucleus size is, however, likely to be an overestimate because the data used to estimate nucleus size comprised the concentration dependence of the maximal rate of self-assembly, a parameter that contains coupled contributions from both nucleation and growth.[142] Two more recent studies that examined the aggregation mechanism of the 4R domain tau constructs in the presence of heparin used a minimal model derived from kinetic simulations,[138] and fits of the initial ∼10% of the kinetic data to analytical solutions derived for an extended NDP mechanism,[143] to conclude that the nucleus consists of two or three monomers. This nucleus size is likely to be a more reasonable estimate of the size of the equilibrium nucleus.

One study alone has proposed an isodesmic mechanism for tau aggregation in the presence of both heparin and arachidonic acid micelles.[144] This study determined that the kinetic role of the inducers was limited to allosteric regulation; the binding of the inducer resulted in a conformational change in the tau monomer that made it aggregation-competent. In addition, the aggregation kinetics were monophasic and not sigmoidal. Further, the inducers appeared to inhibit aggregation at high inducer:protein ratios, with aggregation kinetics being modulated primarily by the relative amounts of protein and inducer present. A recent study of heparin-induced tau four-repeat domain aggregation that possessed several of these similar features demonstrated, however, that even in the absence of a visible lag phase (Figure 5a,b), the aggregation mechanism is minimally described by a ligand-induced NDP model and not by the isodesmic model.[138] In this study, the aggregation rate displayed a bell-shaped dependence on both protein and heparin concentrations that translated into a single bell-shaped dependence of the aggregation rate on the protein:heparin molar ratio (Figure 5c). The aggregation rate was maximal at a molar ratio of two protein molecules to one heparin molecule and was reduced at both lower and higher molar ratios (Figure 5c). A quantitative analysis of the extent of polymerization as

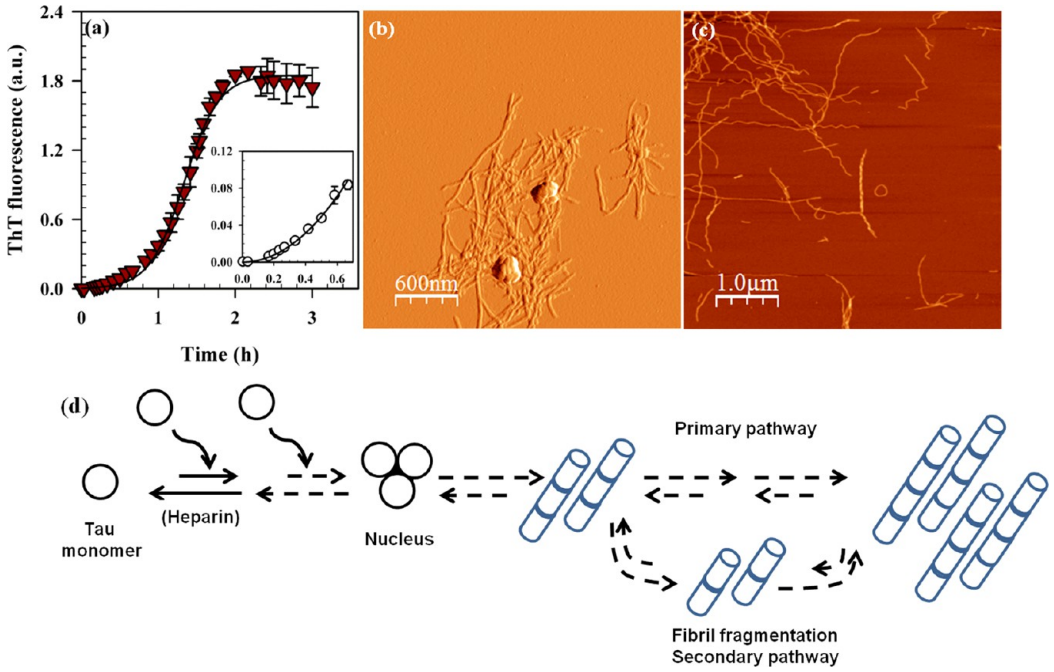

Figure 6. Evidence of fibril fragmentation as the dominant secondary pathway for fibril growth during the aggregation of the 4R domain of tau (tauK18) in the presence of heparin. (a) ThT fluorescence-monitored kinetics of aggregation of 25 μM tauK18 in the presence of heparin. The inset shows a fit of the initial 10% of the data to a cosh(t) equation. (b) Clumps of fibril fragments are visible during the lag phase (10 min) of aggregation of 25 μM tauK18. (c) Fibril morphologies seen at the end (4 h) of 25 μM tauK18 aggregation include annular fibrils. (d) Schematic of fibril fragmentation as a secondary pathway for tau fibril growth. Reproduced from ref 143. Copyright 2012 Elsevier.

measured by thioflavin T (ThT) fluorescence, as a function of both heparin and protein concentrations, further revealed that the rate-limiting step of nucleation involved the formation of an aggregation-competent dimer where the same heparin molecule had bound two successive protein molecules. The inhibition of tau aggregation at higher concentrations of protein arose from the formation of an off-pathway intermediate that led to the formation of both rodlike protofibrils and amorphous, non-fibrillar aggregates. The inhibition of tau aggregation at higher concentrations of heparin arose from a decrease in the relative stability of the aggregation-competent dimer at heparin concentrations that exceeded the dissociation constant for the formation of the on-pathway intermediate (Figure 5d).

The basic aspects of the aggregation kinetics were further recapitulated by a minimal set of kinetic simulations, built on the fundamental premise of NDP reactions that the steps leading to the heparin-bound noncovalent dimer were an order of magnitude slower than the subsequent fibril growth steps[138] (Figure 5d). The formation of an off-pathway intermediate, in the form of a protein−heparin complex that had bound heparin more tightly and at a different site than the on-pathway intermediate (Figure 5d), was an additional species whose formation was included in the kinetic simulations. These assumptions together with the intuitive fact about polymerization reactions that while association reactions are bimolecular and hence dependent on both protein and inducer concentrations, dissociation reactions are unimolecular and hence independent of the concentration of aggregating species were sufficient to describe the entire kinetic data set.[138] As a footnote to this discussion, it must be noted that despite the initial description of tau aggregation in terms of the isodesmic model,[144] in subsequent studies of tau aggregation by the same group, the aggregation data are now fit to a two-step, minimal Finke−Watzky aggregation model that is based on NDP.[145,146]

Secondary Pathways for Tau Fibril Growth. Several studies of the kinetics of amyloid fibril formation by numerous proteins have demonstrated that the kinetic profile of fibrillization is typified by a steep exponential increase in the magnitude of the signal after the lag phase,[147−150] rather than the gentle quadratic (t^2) increase predicted by Oosawa for classical homogeneous nucleation.[115] This steep increase in the magnitude of the signal, described as an autocatalytic phenomenon, can be fully ascribed to the existence of secondary pathways for fibril growth, wherein existing fibrils catalyze the growth of new fibrils.[151] Although tau aggregation in the presence of inducers has been predominantly described by a ligand-induced NDP model, a recent study described for the first time the existence of fibril fragmentation as a bona fide secondary pathway for tau fibril growth[143] (Figure 6). This study examined the kinetic profile of aggregation of the 4R domain tau construct in the presence of heparin, at different concentrations of protein, using ThT fluorescence as the probe (Figure 6a). Analysis of the initial portion of the kinetic data using the cosh(t) solution derived by Bishop and Ferrone, for a mathematical model that describes an NDP mechanism augmented by secondary polymer growth pathways,[113] led to the conclusion that the dominant secondary pathway during tau fibrillization is fibril fragmentation. This conclusion was further corroborated by seeding experiments and AFM imaging of the lag phase of aggregation (Figure 6b). The demonstration of fibril fragmentation as a secondary pathway for tau fibril growth has implications for the understanding of the mechanisms of toxicity in AD and tauopathies because fibril fragments, in addition to soluble oligomers and protofibrils, have been shown to be toxic, in cell viability assays.[152,153]

Effect of FTDP-17 Missense Mutations on Tau Aggregation *in Vitro*. Several studies have focused on understanding the effect of the FTDP-17 missense mutations

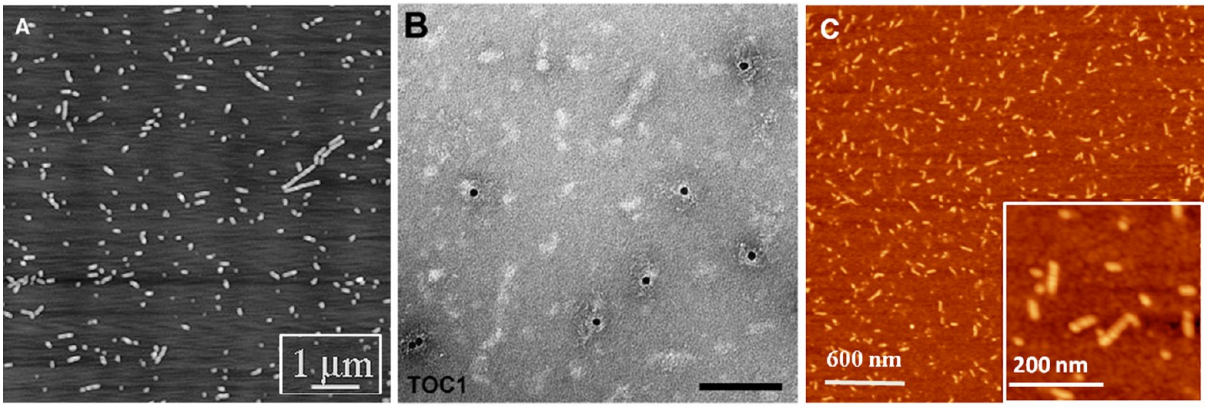

Figure 7. Oligomeric and protofibrillar species formed by tau *in vitro*. (a) AFM image of oligomers (mean height of 18−21 nm) called spherical nucleation units (SNUs) and short fibers that are linear aggregates of SNUs, formed by full-length 2N4R tau in the presence of heparin with agitation at 1000 rpm. (b) TEM image of oligomers (<50 nm length) formed by full-length 2N4R tau in the presence of arachidonic acid, immunogold labeled with a TOC-1 (tau oligomeric complex-1) monoclonal antibody; the scale bar is 100 nm. (c) AFM image of short, rodlike protofibrils (mean height, 2.6 ± 0.5 nm; mean length, 51 ± 16 nm) formed by the 4R domain of tau in the presence of heparin, with the inset showing the beaded appearance of the protofibrils. Panel a is reproduced from ref 168. Copyright 2010 The Alzheimer's Association with permission from Elsevier. Panel b is reproduced from ref 170. Copyright 2011 American Society for Biochemistry and Molecular Biology. Panel c is reproduced from ref 138. Copyright 2011 American Society for Biochemistry and Molecular Biology.

on tau's intrinsic aggregation propensity and the acceleration of *in vitro* aggregation relative to that of the wild type.[93,145,154−158] The main conclusion appears to be that the quantitation of the effect of a particular mutation varies depending on the experimental technique, tau construct, and/or inducer utilized[145,154,155,158] and that different mutations appear to differentially modulate nucleation and/or growth.[145,158] While studies have shown that the FTDP-17 missense mutations do not significantly alter secondary structure as measured by probes such as CD and FTIR spectroscopy,[93,154,156] no study has yet examined the effects of these mutations on the global fold of tau. In this context, it may be worthwhile to illustrate the mechanistic effects of FTDP-17 mutations by considering the example of P301L because P301L tau shows the most dramatic increase in its level of aggregation relative to that of wild-type tau, irrespective of measurement technique and of whether aggregation is induced by fatty acid,[145] ThR,[158] or heparin.[154,156] Also, several mouse models of tauopathy use P301L.[159,160] In the case of the fatty acid inducer, the faster aggregation rate appears to arise from a slower nucleation rate coupled to a faster elongation rate, resulting in the formation of longer fibrils, when compared with those of wild-type tau.[145] In the case of the ThR inducer, the faster rate appears to arise from a lower critical concentration for aggregation combined with an increase in the nucleation rate and an increased efficiency of addition of monomer to the elongating fibrils.[158] Similar careful dissection of the P301L aggregation mechanism in the presence of heparin has not yet been conducted. It should be noted, however, that only the study that used ThR specifically demonstrated that an increased level of aggregation by P301L did not arise from a change in the interaction of mutant protein with ThR.[158] Tau aggregation in the presence of both fatty acid and heparin is known to be affected by the ratio of protein to inducer present, and published studies have not demonstrated that this remains unchanged in the presence of the mutation.[145,154−156] This would be important to establish for a robust assessment of the mechanistic consequences of the mutation. It is also possible that FTDP-17 mutations may modulate the contribution of the secondary pathway for tau fibril growth. This remains an important avenue for future research.

Prefibrillar Tau Oligomers and Protofibrils and Their Kinetic Identities. The identification of soluble oligomers and protofibrils as intermediates during protein aggregation has become an important goal of mechanistic studies of amyloid fibril formation ever since it became clear that these intermediates appear to be the toxic species responsible for neurodegeneration.[152] With reference to AD, for example, several studies have demonstrated the existence of toxic prefibrillar oligomeric species formed by the Aβ protein.[161] More recently, the formation of oligomeric forms by the tau protein, with their concentration being elevated during the early stages of neurodegeneration in AD, has also been demonstrated.[162] Further, studies from cellular and transgenic mouse models have observed that cell death and memory deficits precede or occur in the absence of NFT accumulation, thereby strengthening the case for the existence of tau oligomers or early fibrillization intermediates that are responsible for toxicity.[163,164] A study that directly measured the toxicity of tau oligomers versus fibrils demonstrated that the oligomers cause mitochondrial and synaptic dysfunction and impair memory.[165]

An important goal of *in vitro* studies is to identify these aggregation intermediates, either oligomeric and/or protofibrillar species, and clarify their mechanistic roles, as to whether they lie on or off the pathway of fibril formation. A few *in vitro* studies have identified oligomeric and protofibrillar species formed by the tau protein. The earliest *in vitro* study identified species called granular tau oligomers formed by the full-length 2N4R tau isoform in the presence of heparin.[162] These oligomers were composed of ∼40 tau monomers, were 15−25 nm in height as measured by AFM, and were also found to be elevated in the frontal cortex in the brains of early stage AD patients who had not yet developed NFT pathology.[166] Another study showed that the levels of these oligomeric species were inversely correlated with the levels of heat shock proteins in brain samples.[167] Thus, the authors concluded that the granular tau oligomers were prefibrillar intermediates on the pathway of tau fibril formation, but no kinetic studies were

performed to deduce if the oligomers were on or off the pathway. Another study that used AFM to examine the formation of oligomers by the same full-length tau isoform in the presence of heparin observed the formation of oligomers 18−21 nm in height, which were christened "spherical nucleation units" (SNUs) (Figure 7a).[168] The SNUs were found to assemble linearly into tau fibrils, and the extent of the decrease in abundance was found to correlate with the extent of the increase in fibril lengths; the study therefore implied that the oligomers formed were on-pathway intermediates to fibril formation.

A multidimensional NMR study of oligomers formed by a C-terminal fragment of human tau (residues 255−441) in the presence of heparin and TMAO identified the existence of a heterogeneous oligomeric population that arose due to specific intermolecular interactions at the two hexapeptide motifs of tau (PHF6 and PHF6*).[169] The study could not distinguish, however, if the oligomers were on- or off-pathway intermediates and could remark, at most, that the oligomers were likely to be composed of two or more tau monomers. Finally, a relatively recent study employed a photochemical cross-linking technique to stabilize tau oligomers formed in the presence of the inducer, arachidonic acid.[170] The oligomers identified were tau dimers that formed even in the absence of inducer but were required to be stabilized by the cross-linker, to be detectable. The amount of dimer formed was found to increase in the presence of arachidonic acid. Aggregation of the purified dimer *in vitro* was found to result in oligomers as assayed by electron microscopy, as opposed to fibrils that were formed from monomers as the starting species. A monoclonal antibody that was generated against the purified dimer was found to specifically label tau oligomers *in vitro* and not tau fibrils (Figure 7b). Immunostaining of AD brains showed, subsequently, that the size of a subpopulation of oligomeric aggregates that were reactive to this antibody was elevated at early stages of neurodegeneration, when NFT pathology was not yet visible. However, although these prefibrillar tau oligomers were identified as putatively toxic forms, no kinetic experiments were performed to elucidate their role on the pathway of fibril formation.

The only definitive evidence of an off-pathway role for tau protofibrils, until now, was from a study that examined the kinetics of fibril formation by the 4R domain of tau in the presence of heparin.[138] This study identified short, thin, rodlike protofibrils, 2.6 nm in height and 50 nm in length, on average, which were populated transiently, early during the time course of fibril formation (Figure 7c). The data on the protein concentration dependence of the kinetics of fibril formation revealed the existence of an off-pathway intermediate. As described earlier, this off-pathway intermediate was known to be a protein−heparin complex that had bound heparin more tightly than the on-pathway intermediate (Figure 5d). The same off-pathway intermediate was deduced to lead to the formation of both rodlike protofibrils and nonfibrillar aggregates. This conclusion was further affirmed by the fact that the removal of the 14 C-terminal residues from this 4R domain construct resulted in a tau construct that aggregated without the formation of the protofibrils or the amorphous aggregates.[143] Clearly, the tight binding site for heparin that led to the formation of the off-pathway intermediate lies within the deleted 14-amino acid stretch; 6 of the 14 amino acids in the aforementioned stretch are a part of the native sequence of the tau protein. An independent study has already identified 4 residues in this 6-residue stretch as a secondary amyloidogenic sequence for tau aggregation that forms noncanonical fibrils (similar to the protofibrils) that are ∼3−5 nm in height.[106] Hence, it appears that small changes in sequence composition, even within the MT-binding repeat domain, can dramatically alter the type of aggregation intermediates that are populated. This study also helps emphasize the primacy of kinetic experiments in unraveling the on- or off-pathway roles of aggregation intermediates.

STRUCTURAL HETEROGENEITY DURING TAU FIBRIL FORMATION AND ITS KINETIC ORIGINS

Several studies have reported on the existence of structural heterogeneity in amyloid fibrils wherein fibrils with differences in external morphology and/or internal structure form under different or even the same aggregation condition.[171] For example, STEM measurements of the mass per unit length of two different fibril polymorphs formed by Aβ1−40 have demonstrated that the main difference between the polymorphs arises from a difference in the packing of the protofilaments that comprise the fibril.[172] X-ray crystal structure data on fibrils formed by short amyloidogenic peptides have also provided insights into variations in the steric zipper structure of the fibril cross-β core.[104] These variations may be at the level of the orientation of the β-strands within the β-sheet (parallel or antiparallel), at the level of the orientations of the β-sheets (parallel or antiparallel), or at the level of the packing of the β-sheets (face to face or face to back). These observations have led to the proposition that structural heterogeneity in fibrils arises from two main kinds of polymorphism, packing and segmental polymorphism, or a combination of the two.[173] While the former refers to fibril polymorphs that arise from the difference in the packing of the β-sheets in the cross-β core, the latter refers to polymorphs that arise due to the presence of multiple amyloidogenic segments in the same protein, each segment forming a different type of fibril. Indeed, understanding the structural heterogeneity in amyloid fibrils is important from the perspective of therapeutic interventions because a small molecule or drug compound that is designed to inhibit further growth of one fibril polymorph may not work as well against others. In this context, it is important to understand the kinetic origins of amyloid fibril polymorphism, and the current hypothesis is that the different fibril polymorphs possibly arise from the utilization of distinct aggregation pathways.[172,174] This hypothesis implies that the same structural heterogeneity should also manifest in the prefibrillar intermediates found on the pathway of fibril formation, and this has been shown to be the case for oligomers and protofibrils formed by proteins such as β_2-microglobulin, α-synuclein, prion, and barstar.[171]

With reference to tau, some evidence exists for structural heterogeneity in tau fibrils, as measured by EPR spectroscopy and ssNMR spectroscopy. EPR spectroscopy coupled with site-directed spin labeling indicated that in the case of fibrils formed by the 2N4R full-length tau isoform in the presence of heparin, an 18-residue stretch in repeat R2 (G272−S289) could exist either in a disordered conformation or in the form of a β-strand conformation that contributes to the parallel in-register β-sheet core.[97] The ssNMR spectroscopic data of fibrils formed by the 3R domain tau construct in the presence of heparin indicated the existence of two sets of resonances for a four-residue stretch in repeat R3 (K321−S324), as a result of polymorphism in the formation of the intermolecular disulfide bridge at the C322

residue.[99] It appears likely that the fibril polymorphs in both instances described above arise from the utilization of distinct kinetic pathways for aggregation, but no direct evidence exists for this hypothesis. Understanding the kinetic origins of structural heterogeneity in tau fibrils is thus a challenging but important focus of mechanistic studies that are geared toward the intelligent design of inhibitors or drugs.[175]

PHYSIOLOGICAL RELEVANCE OF THE ENHANCED UNDERSTANDING OF THE TAU AGGREGATION MECHANISM

The primary physiological relevance of a rigorous biochemical study that delineates the mechanism of tau aggregation *in vitro* is an enhanced understanding of the behavior of the protein under carefully controlled, minimal conditions. This understanding is likely to lead to a more confident ability to predict how the same biochemical system will behave *in vivo* under more complex conditions of macromolecular crowding and under conditions of deregulation during disease. Further, as discussed below, a robust biochemical assay that allows the quantitative screening of candidate drug molecules and compounds has implications for the successful development of drugs that ameliorate neurodegeneration.

Tau Aggregation Mechanism *in Vivo*. Because the aggregation mechanism of tau *in vitro* was described in an earlier section, it is worthwhile to examine what is known about the aggregation mechanism of tau *in vivo* and how the current status of knowledge from *in vitro* studies can inform our understanding of disease progression *in vivo*. The bulk of our knowledge about disease progression in AD cases comes from descriptive immunohistochemical studies of NFTs found in cell bodies of neurons. Three stages in disease progression may be identified on the basis of reactivity, to antibodies against both nonphosphorylated and phosphorylated tau epitopes, to fluorescent dyes that label the cross-β motif of fibrils, and to silver stains that label NFTs.[142] The first stage involves the appearance of tau dissociated from MTs in the form of structures called pretangles that occur free in the cytoplasm as well as associated with the membranes of cell organelles.[176,177] These pretangles are characterized by reactivity to antibodies specific for phosphorylated tau but not to dyes such as ThS, Congo red, or thiazine red.[178] Thus, the pretangles appear to be nonfibrillar aggregates of tau that do not yet possess the cross-β motif. This stage is also accompanied by the mis-sorting of tau from the axon to the somatodendritic compartment.[179] The second stage involves the appearance of dye reactivity and, hence, signals the occurrence of a conformational change; several of these dye-positive structures have been found associated with membranes.[177,178] The third and final stage involves the appearance of distinct, mature, silver-positive NFTs composed of tau fibrils, 300−600 nm in length on an average, with several post-translational modifications, including hyperphosphorylation, covalent cross-linking, and proteolysis.[135,136,178−180] While several studies in cellular and animal models recapitulate at least some of these basic stages, an additional observation has been that symptoms of cell death and neuronal loss are seen prior to the appearance of NFTs.[163,164,181] In the light of recent *in vitro* studies, it appears likely that these toxicity effects might arise from the formation of oligomers, protofibrils, and/or fibril fragments prior to the formation of mature fibrils. The actual detection of these short, nanometer-scale toxic structures within cells will require the usage of super-resolution microscopic techniques in the future;[182] currently, the best evidence for these structures *in vivo* still relies on reactivity with antibodies that are known to be specific to the pretangles.

Prion-like Behavior of Tau in Cellular and Animal Models. The aggregation of the prion protein results in fatal neurodegenerative diseases called the transmissible spongiform encephalopathies, the most famous of which is the well-known mad cow disease.[183] Prion aggregation has, however, always remained distinct from the aggregation of all other neurodegenerative proteins because of the infectious nature of the prion aggregates that lead to the transmission of disease from one individual to another.[183] Nevertheless, recently, several studies have begun to notice similarities between the behavior of amyloidogenic proteins like tau, Aβ, and α-synuclein and the behavior of the prion.[184] Studies have shown, for example, that the addition of extracellular tau aggregates, but not monomer, to cultured cells caused full-length tau protein present within the cells to misfold and aggregate.[185] Similarly, the intracerebral injection of aggregates composed of mutant P301S tau into transgenic mice expressing wild-type human tau that does not form aggregates on its own resulted in the formation of NFTs that spread from the site of injection to adjoining regions of the mouse brain.[186]

More definitive evidence of the trans-synaptic spread of tau aggregates through the mouse brain was provided by two very recent studies[159,187] that relied on the controlled expression of the P301L mutant human tau transgene in specific cells of the entorhinal cortex of the mice. As the mice aged, symptoms of progressive NFT pathology were observed starting at the entorhinal cortex and moving to the dentate gyrus and the hippocampus, regions that are neuro-anatomically connected to the specific cellular layer of the entorhinal cortex in which human tau was expressed. Most importantly, there was no detectable expression of human tau mRNA in brain regions outside of the entorhinal cortex. Another recent study in cells used FRET as a tool, together with the transfection of donor and acceptor fluorophore-tagged tau proteins into two different cell populations that were subsequently cocultured, to demonstrate that intracellular tau fibrils formed by one of the cells were taken up by the other cells and induced aggregation.[188] Bulk, fluid phase endocytosis appears to be the mechanism by which recombinant tau aggregates are internalized by both cells and primary neurons.[189] With regard to the internalization process, it appears that low-molecular weight tau aggregates (spherical oligomers on TEM, with diameters of ∼10−30 nm) and short fibril fragments (40−250 nm in length) are endocytosed rather than tau monomers or long, intact fibrils.

As mentioned earlier, this process of the trans-synaptic spread of aggregates in mouse and cellular models of neurodegeneration has also been demonstrated for α-synuclein and Aβ.[184] This process of self-perpetuating, seeded aggregation is being increasingly regarded as a common mechanism underlying neurodegeneration.[184] This common mechanism of spreading *in vivo* implies that the biochemical mechanism of aggregation of proteins such as tau and prion should also share basic similarities. The recent demonstration of fibril fragmentation as a dominant secondary pathway for fibril growth by tau[143] unifies the basic mechanistic features of tau and prion aggregation, because mammalian and yeast prion aggregation *in vitro* are also characterized by fibril fragmentation as a secondary pathway for elongation.[149] In fact, the discovery of prefibrillar oligomers of tau and of fibril fragmentation as a

secondary pathway for tau fibril growth is biochemical proof for the prion-like behavior of tau, especially because it appears that oligomers and fibril fragments are the aggregates that undergo endocytosis and cell to cell transfer (see above). Further, it has also been shown that wild-type tau can adopt multiple, distinct fibrillar conformations *in vitro* that can be maintained by a process of template-assisted conformational change, akin to what has been suggested for prion strains.[190] Taken together, the behavior of tau as a prion-like agent or a prionoid[183] now appears to be beyond dispute. The physiological consequences of this behavior are only now slowly being uncovered.

Possible Mechanisms of Tau-Mediated Toxicity in AD and Tauopathies. Two main mechanisms of toxicity have been proposed from the perspective of tau function in both AD and the tauopathies: loss of function and gain of function.[41,62] Loss of function refers to toxicity effects that arise from the inability of tau to perform its standard function of MT stabilization when it undergoes hyperphosphorylation and dendritic mis-sorting during disease. This theory has received, however, less support in the recent past because of the discovery that the acute knockdown of tau does not result in major behavioral or longevity deficits, except in aged mice.[41,60] Further, in more than one study, it has been shown that the knockout of tau has beneficial effects on health as evidenced by reduced neuronal excitability and the greater resistance of tau knockout mice to seizures.[57,191,192] The alternative theory, namely that toxicity arises from an abnormal gain of function, is largely based on the observation that both AD and the tauopathies are associated with the formation of hyperphosphorylated NFTs, and these are therefore likely mediators of toxicity.[41,62] Although the appearance of NFTs has been shown to correlate with some measures of cognitive decline in disease, there is substantial recent evidence that posits that early intermediates are likely to be the more toxic species, and mature NFTs may even be neuroprotective in comparison.[193] Incidentally, the two main processes that have been shown to be affected by tau overexpression and subsequent aggregation are axonal transport and mitochondrial trafficking.[193]

Evidence of the toxicity of soluble forms of tau comes from several studies in transgenic mouse models and cells. An early study in a transgenic, repressible mouse model of tauopathy demonstrated that repression of transgene expression at ages corresponding to ≥4 months reversed memory deficits, although NFTs continued to be present, thereby arguing that an early intermediate was required for toxicity.[163] A subsequent study in the same model as well as a different mouse model of tauopathy showed that memory loss was correlated with the presence of 140 and 170 kDa, SDS-stable, soluble tau oligomers and not with NFTs.[194] Multimeric species of the same size were also detected in brain tissues obtained post-mortem from AD and FTDP-17 patients.[194] Other studies have shown that deficits in memory formation as measured by long-term potentiation and synaptic deficits as measured by a disturbance in the trafficking of NMDA and AMPA receptors precede neuronal loss and correlate with the mislocalization of soluble, hyperphosphorylated forms of tau to dendritic spines.[195] There is also evidence of the appearance of markers of cell death such as caspase activation, prior to the accumulation of NFT in mouse models.[196] Finally, more direct evidence of soluble forms of tau being responsible for neuronal dysfunction comes from studies in inducible cellular and mouse models that have compared the consequences of expression of pro-aggregant (ΔK280) and anti-aggregant (ΔK280/I277P/I308P) tau repeat domain constructs.[101,102] While the former species are tau constructs that accelerate tau aggregation because of their stronger propensity for β-structure, the latter species are tau constructs that contain β-strand-breaking proline mutations in the hexapeptide motifs that prevent aggregation. NFTs and various toxicity phenotypes are seen only on expression of the pro-aggregant form of tau; further, repression of transgene expression rescues cognitive deficits and synapse loss, despite the persistence of NFTs that switch their composition from a mixture of exogenous human and endogenous mouse tau to purely endogenous mouse tau.[197]

Interaction of Tau and Aβ in AD. In the context of AD where both tau and Aβ aggregate, it is interesting to examine what is known about the interplay of the two proteins in causing neurodegeneration. The earliest prevalent hypothesis regarding the cause of AD is the amyloid cascade hypothesis in which the formation of Aβ by the aberrant cleavage of the amyloid precursor protein (APP) is the crucial step driving pathogenesis.[198] The placement of tau in the amyloid cascade is a matter of much debate, but there has been a surge of recent evidence that indicates that tau's role in causing toxicity may be direct and considerable.[60] The bulk of this evidence has emerged from studies that have shown that the knockout of tau in APP transgenic mice prevented memory deficits and premature death[57,191] and that tau$^{-/-}$ cultured hippocampal neurons are protected from degeneration when they are treated with fibrillar Aβ.[199] The mechanism by which tau mediates neurotoxicity has been identified in at least one of these studies with an AD mouse model, in which it has been shown that tau is required for the trafficking of Fyn kinase into postsynaptic sites in the dendrites, where Fyn phosphorylates the NMDA receptor thereby mediating Aβ-induced excitotoxicity defects.[191] This new discovery has thus led to a "tau axis hypothesis" that couples Aβ and tau pathology in the dendrite as an important effector of neurodegeneration.[60] First, the excitotoxicity effects of Aβ are tau-dependent. Second, the exposure of neurons to Aβ triggers hyperphosphorylation of tau, and the enrichment of Tau−Fyn complexes in lipid rafts in neurons.[200] Phosphorylated tau has a higher affinity for Fyn; incidentally, mutant forms of tau do also.[201] This results in higher levels of dendritic Fyn and greater toxicity effects, thereby establishing an especially vicious feedback loop. This new hypothesis awaits exhaustive testing.[60]

Tau Aggregation Inhibitors as Candidate Drug Molecules against Neurodegeneration. Promising candidate drug molecules that have emerged from the usage of robust, high-throughput, biochemical and biophysical assays to measure tau aggregation inhibition *in vitro* include anthraquinones,[202] aminothienopyridazines (ATPZs),[203] phenothiazines,[204] and polyphenols and porphyrins.[205] In addition, these compounds have been shown not to interfere with the stability of MTs. Among these compounds, the mechanism of aggregation inhibition has been determined for the phenothiazine methylene blue (MB)[206,207] and for the ATPZs,[207] as the oxidation of Cys residues and, hence, the conversion of 4R tau (which contains two Cys residues) into a compact monomer that cannot form fibrils. With reference to 3R tau (which contains one Cys residue), the inhibitory effects of MB on fibrillization manifest only at high concentrations (>100 μM) with no significant effect at lower concentrations, while the ATPZs promote fibrillization at lower concentrations by promoting dimerization.[206,207] These results are consistent with the observation that the formation of intermolecular

dimers enhances aggregation of 3R tau *in vitro*, while the formation of a compact monomer (intramolecular dimer) inhibits aggregation of 4R tau.[132] Incidentally, MB as a drug candidate has shown substantial promise in slowing the progression of the disease in a one-year, multinational, phase II clinical trial conducted on human AD patients,[208] and a phase III clinical trial is currently underway. Studies in tau transgenic mice have shown that although low concentrations of MB reversed neuronal loss, cognitive improvements required higher dosages (>470 μM), and this was correlated with a decrease in soluble tau levels.[209] However, given the nature of its selective inhibition of 4R tau aggregation *in vitro*, the efficacy of MB might be as much because of its effect on multiple targets,[210] including the enhancement of autophagic[211] and proteosomal degradation of tau,[212] rather than purely fibrillization inhibition. Nevertheless, it is heartening to note that these drug candidates were originally identified through biochemical and biophysical assays that measured aggregation inhibition.

■ CONCLUSION

The aggregation of the intrinsically disordered protein tau into ordered amyloid fibrils is a feature of neurodegenerative disorders such as Alzheimer's disease and the tauopathies. Understanding this process of aggregation is important from the perspective of designing drugs that will abrogate neurodegeneration. Biochemical studies that utilize well-defined, minimal conditions to decipher the mechanism of aggregation from kinetic studies provide a potential entrée toward understanding this puzzle of how aggregation contributes to neuronal death and also provide the basis for high-throughput drug screens. Mechanistic studies have shown, for example, that aggregation intermediates such as soluble oligomers and protofibrils form during the aggregation of tau *in vitro*,[138,170] and that tau aggregation is characterized by fibril fragmentation as a secondary pathway for fibril growth.[143] These three categories of structures (oligomers, protofibrils, and fibril fragments) are in general known to be the structures responsible for neurotoxicity during protein aggregation in disease.[152,153] Thus, the detection of these structures *in vitro* may help explain counterintuitive results from cellular and mouse models of tauopathies that show that cell death and neuronal loss occur even in the absence of tau fibrils or PHFs.[193] Although the direct detection of neurotoxic soluble oligomers, protofibrils, and fibril fragments *in vivo* is a challenge for the future, the results from *in vitro* studies allow the rationalization of results from *in vivo* models.

Another important goal of *in vitro* studies is to understand the structural heterogeneity underlying the complex energy landscape of tau aggregation. This necessitates the usage of high-resolution biophysical probes such as EPR, NMR, and UVRR spectroscopy to structurally characterize the aggregation pathway of tau. Studies using EPR and NMR spectroscopy have mapped the core of the tau fibril and determined that akin to most other amyloidogenic proteins, the β-strands that compose the tau fibril cross-β core are arranged in an in-parallel and in-register fashion; in addition, these studies have provided evidence of the existence of distinct tau fibril polymorphs.[97,99] Dissecting the pathway(s) of tau aggregation leading to the formation of specific tau fibril polymorphs is again important from the perspective of rational drug design, geared toward preventing fibrillization.

Lastly, mechanistic studies of tau aggregation *in vitro* have informed our understanding of how neurodegenerative disease symptoms appear progressively in brain regions that are trans-synaptically connected. There is a growing body of evidence of the prion-like behavior of tau when it spreads from cell to cell in both cell culture and in transgenic mouse models of tauopathy.[184] It appears likely that this spread of neurodegeneration is connected with the "seeding" of aggregation in naive brain areas; results from biochemical studies have led to the hypothesis that the "seeds" are fibril fragments and/or soluble oligomers and protofibrils.[143,189] In the future, it is expected that the dissection of the aggregation mechanism of tau both *in vitro* and *in vivo* with higher-resolution spectroscopic and microscopic probes, respectively, will provide a fuller picture of how tau aggregation leads to neurotoxicity.

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Notes

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■ ABBREVIATIONS

MAP, microtubule-associated protein; MAPT, MAP tau; MT, microtubule; IDP, intrinsically disordered protein; CNS, central nervous system; CD, circular dichroism; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; FRET, fluorescence resonance energy transfer; AD, Alzheimer's disease; Aβ, amyloid β; NFT, neurofibrillary tangle; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; CBD, corticobasal degeneration; PSP, progressive supranuclear palsy; PHF, paired helical filament; SF, straight filament; AFM, atomic force microscopy; TEM, transmission electron microscopy; FTIR, Fourier transform infrared; EPR, electron paramagnetic resonance; ssNMR, solid-state NMR; NDP, nucleation-dependent polymerization; ThT, thioflavin T; ThR, thiazine red; ATPZ, aminothienopyridazines; MB, methylene blue; UVRR, ultraviolet resonance Raman.

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