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Modeling Tau Polymerization in Vitro: A Review and Synthesis†

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ABSTRACT: The major antigenic component of neurofibrillary pathology in a large number of neurodegenerative diseases consists of the microtubule-associated protein tau. It is currently unclear how tau protein makes the transition from an important component of the microtubule-based cytoskeleton to an insoluble polymerized state. In vitro techniques have been employed to study the polymerization of tau in an effort to understand the underlying molecular mechanisms responsible for this process. These efforts have resulted in the elucidation of roles played by the different parts of the molecule in the polymerization process. Here we discuss the advantages and disadvantages of the various techniques used to model tau polymerization and the discoveries arising from these techniques that have led to a better structural understanding of tau polymerization in relation to Alzheimer's disease and other tauopathies.

The microtubule-associated protein tau is an important component of the neuronal cytoskeleton. In the central nervous system, it exists as six differentially spliced isoforms arising from a single gene located on chromosome 17 (reviewed in ref *1*). These isoforms were originally identified by their ability to copurify with microtubule preparations (*2*) and were subsequently found to stabilize microtubules both in vitro and in vivo (*2*−*9*). Tau has attracted considerable interest since the discovery that it is a major component of the intracellular fibrillar pathology found in Alzheimer's disease (AD)[1] (*10*−*15*). The tau protein contained in the pathological structures of AD is present in highly ordered polymeric structures found in three compartments: Neuropil threads are comprised largely of straight filaments while neurofibrillary tangles and dystrophic neurites are comprised mainly of paired-helical filaments (reviewed in ref *16*). The accumulation of any tau filaments, regardless of their morphology, correlates well with the severity and regionally appropriate cognitive impairment in AD patients (*17*). Although no mutations in the tau gene have been found to be directly linked to AD, such mutations have been found to lead directly to neurodegeneration in several frontal lobar atrophies collectively termed frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) (reviewed in ref *16*). This suggests that tau dysfunction can lead directly to neurodegeneration, and the presence of pathological tau inclusions in AD suggests that tau plays a major role in that disease as well.

The molecular mechanisms directly involved in the pathogenesis of AD are not known, but it is believed that the polymerization of tau protein could be involved. As

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[1] Abbreviations: AD, Alzheimer's disease; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; MTBR, microtubule-binding repeat; FTIR, Fourier transform infrared spectroscopy; CD, circular dichroism; ThS, thioflavin S; PHF, paired-helical filament; GSK-3b glycogen synthase kinase 3b; MAPK, mitogen-activated protein kinase; MARK, microtubule affinity regulating kinase.

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mentioned above, tau protein in an abnormally aggregated state strongly correlates with dementia in AD (*17*). In addition, animal models demonstrate that the appearance of intracellular tau inclusions is concomitant with neurodegenerative changes in neurons (*18*−*20*). Compounds that inhibit tau polymerization can arrest neurodegeneration in this model system (*21*). And although tau-mediated cytotoxicity may arise from other sources than self-aggregation, such as overexpression (*22*), hyperphosphorylation (*23*), or proteolysis (*24*, *25*), the most common feature of all neurodegenerative tauopathies is the presence of abnormally aggregated tau protein. For these reasons much effort has been expended in attempts to understand tau's transition from its soluble or microtubule-bound state to the relatively insoluble polymeric (filamentous) state. The mechanisms underlying this process constitute the subject of this review and synthesis.

Inducing Tau Polymerization in Vitro

Due to its random coil nature when in soluble form, nonmodified tau does not spontaneously self-associate into filamentous structures in vitro. However, the addition of several nonproteinaceous compounds to the incubation medium has been found to enhance the polymerization of well-characterized, nonmodified tau protein, thus facilitating the study of tau polymerization in vitro. The two major classes of compounds of this sort are polyanions [heparin (*26*), polyglutamate (*27*), or RNA (*28*)] and fatty acids or fatty acid-like molecules [arachidonic acid (*29*), docosahexaenoic acid (*30*), and alkyl sulfonate detergents (*31*)]. Although the exact mechanism by which these inducer molecules promote the polymerization of tau is not known, recent evidence suggests that the clustering of the negative charges on the inducers plays an important role.

Heparin vs Arachidonic Acid. Although both polyanionic compounds and fatty acids enhance tau polymerization in vitro, there are significant differences in their practical application. Polyanionic compounds such as heparin have a greater efficacy in promoting the polymerization of tau that has been truncated such that it only includes its microtubule-binding repeats (amino acids 244−360, using the numbering of ref *32*) (*27*, *33*). The induction of tau polymerization by heparin can be further enhanced by the elimination of a cysteine under oxidizing conditions (*33*, *34*); conversely, the rate of tau polymerization is enhanced with the inclusion of MTBR2 under reducing conditions (*34*). These results have led to the conclusion that the heparin induction of tau polymerization is enhanced by the formation of tau dimers through cysteine cross-linking of tau at position 322 and is inhibited through intramolecular cysteine cross-linking at positions 291 and 322 (*33*, *34*).

Polyanion induction of tau polymerization has several advantages. First, the filaments resulting from this paradigm have the morphological characteristics of paired-helical filaments (PHFs), twisting with a regular periodicity of approximately 80 nm (*26*, *35*). In addition, heparin has been successfully used to measure structural changes in tau upon polymerization (*36*−*38*). However, heparin induction of truncated forms of tau routinely requires unphysiologically high protein concentrations and incubation periods of 24−48 h to reach steady-state levels. This time frame increases to as much as 2 weeks when using full-length tau protein. Thus kinetic studies are infeasible, and studies investigating the effects of changes to the tau protein outside the microtubule-binding repeats become unwieldy using this methodology.

The use of arachidonic acid to induce tau polymerization has the advantage of having a high efficacy for promoting the polymerization of full-length tau at physiological protein concentrations much more rapidly than heparin (*34*). In addition, changes to the tau molecule outside its microtubule-binding repeat regions can be easily modeled since these areas are not deleted (*39*−*44*). However, the presence of the entire molecule makes the quantitation of secondary structures difficult due to their relative paucity, although some encouraging structural results have been obtained with the full-length protein and with paired-helical filaments (*38*, *45*). Although the filaments induced by arachidonic acid have the morphological characteristics of straight filaments (*29*), they appear to contain a high degree of structural similarity to paired-helical filaments in that they are recognized by antibodies and fluorescent dyes that react with autopsy-derived paired-helical filaments and paired-helical filaments induced by heparin (*46*). In fact, arachidonic acid-induced filaments will nucleate from paired-helical filaments from human AD brain, suggesting that the two filament types have a strong structural similarity (*46*).

It is not clear why the addition of polyanionic compounds or free fatty acids to solutions of pure tau protein results in the self-aggregation of tau into filamentous structures. It has been proposed that the negative charges of the polyanionic compounds could provide a substrate similar to the surface of the microtubule, with which tau has strong interactions (*27*, *47*, *48*). The interaction of tau with the negative charges could then confer an "induced-fit" conformation to the microtubule-binding repeat regions similar to the one that has been proposed for tau binding to microtubules (*49*). In fact, the addition of polyanionic compounds to peptides corresponding to the microtubule-binding repeats induces a shift from random coil to β structure (*36*). This is accompanied by the self-association of the peptides, reinforcing the importance of the microtubule-binding repeats in the polymerization of tau.

The great structural differences between the polyanionic compounds and the fatty acids could lead one to hypothesize that their modes of induction of polymerization are quite different. However, a potential common mechanism of action exists since free fatty acids are likely to be in micellar form when they induce the polymerization of tau (*31*, *34*). If so, the negatively charged surface of a micelle would constitute a polyanionic cluster. Although the hypothesized micelles cannot be detected by laser light scattering or electron microscopy, it is interesting to note that tau filaments in Alzheimer's disease often originate or terminate in membranes (*50*). The interaction of tau with clusters of negative charges, either by polyanions or by fatty acids in micellar form, could induce a conformational change that leads to its polymerization.

Measuring Tau Polymerization in Vitro

To successfully model tau polymerization in vitro, a reliable method for the quantification of the amount of polymer formed is required. Four different techniques have

been used to quantify tau polymerization: fluorescence through the reporter dye thioflavin S (ThS), electron microscopy, centrifugation, and laser light scattering.

Thioflavin S is a dye that exhibits an increase in fluorescence at 480−520 nm in the presence of compact β-sheet structures, such as is found in PHFs. It is commonly employed in the histological examination of AD brains and has been successfully used to monitor the polymerization of tau in vitro (*27*). This technique is advantageous because of the relative rapidity with which measurements can be made, although the minimal unit of tau polymer required for ThS binding is not known, making it difficult to quantitate. In addition, ThS demonstrates a large increase in fluorescence in the presence of tau and arachidonic acid even when no filaments can be observed by electron microscopy (*34*). Thus its use in kinetic studies remains questionable.

Negative stain transmission electron microscopy allows the investigator to unequivocally demonstrate bona fide tau polymerization in vitro, although the technique is somewhat cumbersome and unsuited to detailed kinetic studies. In addition, a lack of detailed information about the structure of tau filaments prevents the measurement of the actual mass of polymer formed in an in vitro polymerization reaction.

Centrifugation allows the direct quantitation of the amounts of free and polymerized tau, although the centrifugal force required to sediment bona fide tau filaments in solution is not known. Moreover, the concentration of tau protein, especially insoluble tau filaments, is difficult to measure and can differ greatly depending upon the technique employed (*9*, *39*).

Right-angle laser light scattering employs a laser to illuminate tau polymerization reactions in a fluorometer cuvette. The amount of scattered light is directly proportional to the mass of tau filaments in suspension (*40*), although this relationship only applies to filaments that lie within a certain length distribution (*51*, *52*). An additional caveat is that any particle, whether derived from tau or not, that falls within this length distribution will exhibit these same properties. These considerations require that the morphology of the filaments be ascertained by electron microscopy.

Important Aspects of Tau Polymerization in Vitro

The techniques for inducing and monitoring tau polymerization described above have provided valuable insights into the mechanisms governing tau−tau interactions (illustrated in Figure 1).

Role of the Microtubule-Binding Repeats. Each microtubule-binding repeat (MTBR) contains a highly conserved 18 amino acid repeat region that is known to interact with tubulin and microtubules (*53*, *54*). In addition, the less well conserved 13−14 amino acid interrepeat (IR) regions have also been shown to interact with microtubules and greatly influence tau binding (*49*). This is especially true of the IR sequence between MTBR1 and MTBR2. This region contains two lysines vital for the enhancement of four repeat isoforms of tau binding to microtubules (Lys 273 and Lys 280). If this region forms an amphipathic α helix similar to the one measured in MTBR3 (*55*), the vital lysines would be placed on the same face of the helix, optimally positioning them for interactions with the negatively charged surface of the microtubule.

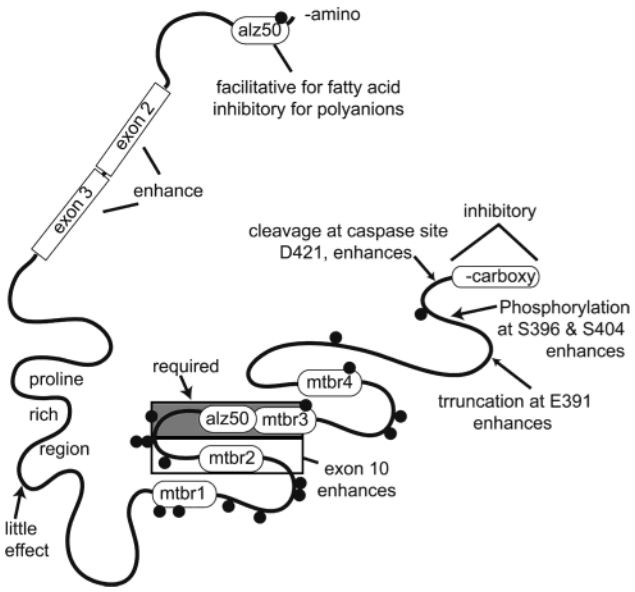

FIGURE 1: Schematic representation of the tau protein and the regions important for its polymerization. The natively unfolded tau molecule is illustrated here with the major domains of the protein labeled along with a brief description of their effects on polymerization: the amino acid terminal projection domain (amino acids 1−155), the relatively basic proline-rich region (amino acids 155−243), the microtubule-binding repeats (amino acids 244−360), and the carboxy-terminal domain (amino acids 361−441). The alternatively spliced exons 2, 3, and 10 are also highlighted. In addition, modifications of tau that are thought to influence polymerization, such as the Alz50 conformation, C-terminal truncation events at E391 and D421, and phosphorylation at S396 and S404, are also labeled. The sites of FTDP-17 mutations (see Table 1) are denoted by dots.

Alternative splicing of the tau gene transcript can result in the inclusion or exclusion of exon 10, which corresponds to MTBR2 and the interrepeat region between MTBR1 and MTBR2. Therefore, tau isoforms contain either three MTBR (3R tau) or four MTBR (4R tau) (*32*). The inclusion of exon 10 in 4R tau results in an increased affinity for microtubules, and 4R tau stabilizes microtubules significantly more strongly than the 3R form (*8*, *9*, *56*).

The microtubule-binding repeats have been shown to be vital to the polymerization of the tau molecule. It has been demonstrated that residues 306-VQIVYK-311 in the third microtubule-binding repeat (second repeat of 3R tau) are required for the induction of tau polymerization by polyanions (*36*). This stretch of amino acids has a propensity for forming β-strand structure, especially in the presence of inducer (*36*). This β strand likely represents the minimum unit required for tau−tau interactions, since a peptide comprised of these amino acids was demonstrated to be capable of self-polymerization (*36*). The ability to polymerize was completely abolished by substituting a proline for any of the amino acids in the region, strengthening the argument for the importance of β structure in tau polymerization.

An adjacent stretch of residues in the third microtubule-binding repeat has been shown to be very important for the induction of polymerization by fatty acids. Tau polymerization in the presence of arachidonic acid was greatly diminished by removing amino acids 314-DLSKVTS-320 (*41*). This region has a high predicted propensity for forming α-helical structures in solution and has been demonstrated to do so in hydrophobic environments in vitro (*55*). More-

over, analysis of this region predicts that such a helix would be amphipathic (*43*, *55*). The β-strand-forming region and the predicted amphipathic helix are separated by a proline, providing a predicted strand−turn−helix motif that could serve as the structural unit required for tau polymerization. Although the available experimental evidence is restricted to the third repeat, the other three are predicted to have similar motifs, and it is conceivable that this repeat could provide a "seed" for secondary structure formation in the others. The presence of β strands (*38*, *57*, *58*) and α-helical structures (*45*) in PHFs suggests that the transformation of the microtubule-binding repeats of tau from natively unfolded regions into a strand−turn−helix through the interactions with polyanions or fatty acids provides the backbone for the polymerization of tau in disease.

Although the tau−tau association sites comprising the core of the filament involve the MTBRs, other regions of the tau molecule clearly play a role in the regulation of its polymerization. Disclosure of their involvement in the process has required polymerization studies utilizing the intact tau molecule. Those studies indicate that modifications in certain extra-MTBR regions are involved in the conversion of the intact tau molecule into a structured conformation capable of polymerization.

The Role of the Carboxy Terminus. The carboxy-terminal region of the tau molecule is often included with the microtubule-binding domain (*1*) and has been shown to influence the binding of 3R tau isoforms to microtubules (*49*). In addition, this region has also been hypothesized to be involved in the spacing of microtubules in conjunction with the amino terminus (*59*).

While intact tau protein is capable of polymerizing into filamentous structures in the presence of inducer molecules in vitro, the removal of its carboxy terminus enhances the process for both the polyanionic (*28*) and arachidonic acid (*41*, *43*, *44*) induction of polymerization. Therefore, it has been concluded that the carboxy terminus of tau acts to inhibit its polymerization. Since peptides corresponding to the carboxy terminus (residues 422−441) have been shown to assume an α-helical structure that would be predicted to be amphipathic by sequence analysis, a potential mechanism for this inhibition would be the interaction of this helix with either the predicted amphipathic helix in MTBR3 or another such predicted helix in MTBR4 (*43*). This interaction could be either inter- or intramolecular since peptides containing these residues are capable of inhibiting the polymerization of either full-length or carboxy-truncated tau proteins (*43*). The hypothesis further proposes that this interaction interferes with tau−tau interactions via the microtubule-binding repeats. It is possible that such a mechanism exists to prevent the self-association of free tau in the cytoplasm when it is not bound to microtubules, where such interactions could be advantageous (*48*). However, if the carboxy terminus is modified by phosphorylation (see below) or removed through truncation, then the tau molecules would be much more likely to adopt a conformation favorable for polymerization.

The inhibition of tau polymerization by its carboxy terminus suggests that truncation events known to occur in AD may have mechanistic consequences. Both of the truncations observed in tau in AD [at E391 by an as yet unidentified protease(s) (*60*) and at D421 by members of the caspase family (*44*)] have been shown to enhance the polymerization of tau in vitro (*41*, *43*, *44*) and could implicate modification of the carboxy terminus as a major event in the polymerization of tau in AD. The caspase-mediated truncation of tau at D421 observed in AD can be induced in cultured primary neurons through the addition of β-amyloid, providing a potential link between the two pathologies observed in AD while strengthening the hypothesis for the role of the carboxy terminus in inhibiting tau polymerization (*44*).

The Role of the Amino Terminus. The amino-terminal region projects away from the surface of the microtubule upon tau binding and is sometimes referred to as the projection domain (*1*). It is believed by some that this region is involved in the spacing of microtubules since the knockout of the tau gene results in altered microtubule organization in small caliber axons in mice (*61*). Another hypothesis is that the projection domain interacts with nonmicrotubule cytoskeletal elements, such as motor proteins (*7*, *62*), and other cellular components, including the plasma membrane (*63*). The alternative splicing of exons 2 and 3 to generate isoforms of tau with differently sized projection domains could influence these hypothesized interactions. Isoforms either contain neither exon2 nor exon3 (0N), only exon2 (1N), or both exon2 and exon3 (2N). These combinations can exist in either 3R or 4R tau isoforms, giving rise to the following six isoforms: 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R.

The role that the amino terminus of tau plays in its polymerization is not as well defined as that of the carboxy terminus. As mentioned above, removal of both the amino and carboxy termini facilitates the polyanionic induction of tau polymerization by heparin (*28*). By contrast, if the amino terminus is removed and the carboxy terminus is kept intact, the fatty acid induction of tau polymerization is greatly reduced, suggesting a facilitative role for the amino terminus in the polymerization process (*42*). This result echoes immunohistochemical observations using Alz50, a conformationally sensitive antibody that recognizes the specific interaction of tau's amino terminus with its microtubule-binding repeats (*64*−*66*). Alz50-reactive neurofibrillary tangles can be detected before the appearance of many other markers of tau pathology in AD (*67*). This naturally leads to the supposition that the association of the amino terminus with the MTBR region is an early and perhaps seminal conformational change required for the polymerization of full-length tau.

The interaction between the amino terminus and the MTBR region appears to be quite different from the interaction of the carboxy terminus with this domain. First, the amino terminus does not appear to have a strong tendency to form secondary structure, even in a hydrophobic environment (*42*). Sequence comparisons suggest that the interaction between these two regions could be electrostatic in nature, with the amino terminus having a net negative charge and MTBR 3 having a net positive charge. The functional significance of this interaction is not clear but further argues in favor of tau being required to adopt a specific conformation or conformations in order to polymerize. Unlike the carboxy terminus, the amino terminus must be tethered to the rest of the molecule in order to impart its effect on the polymerization of tau (*42*). In fact, the removal of exons 2 and 3, which lie in the intervening region between the amino

terminus and the MTBR, has a large negative effect on polymerization (*39*).

The Role of the Proline-Rich Region. The relatively basic proline-rich region of tau is predicted to be highly flexible due to its high proline and glycine content. Although very little is known about the function of this region, its flexibility could allow a dynamic range in the spacing of microtubules such that as cargo travels through an axon, this region could expand and contract to allow the microtubule cytoskeleton to be stretched apart or compacted to allow easier passage. Relatively little is known about the role of tau's proline-rich region in the polymerization process, but it appears as if it has only a small influence on polymerization. If this region (amino acids 155−244) is deleted while keeping the rest of the molecule intact, the rate and extent of tau polymerization are not significantly changed in the presence of arachidonic acid (*41*). However, a more detailed analysis of the role of this region has not been performed.

The Role of Phosphorylation. Tau phosphorylation is of great interest since it is known to be a primary mechanism for regulating tau's interaction with microtubules and it is clear that tau is highly phosphorylated in the fibrillar pathologies found in AD (reviewed in ref *1*). Tau phosphorylation can be detected in early stages of neurodegeneration, and it has been hypothesized that the phosphorylation state of tau can be directly correlated with the severity of pathology in AD (*68*). Despite this evidence, the role of tau phosphorylation in neurodegeneration is still very poorly understood.

It is possible that an increase in tau phosphorylation can lead directly to an increased ability to polymerize. Tau protein purified in a phosphorylated state is capable of self-polymerization in vitro in the absence of inducer molecules whereas nonphosphorylated recombinant tau protein is not (*69*). In addition, "hyperphosphorylated" tau derived from AD tissue has been shown to bind to and sequester nonphosphorylated tau protein from microtubules, with a preference for 2N4R tau, suggesting that a relatively minor degree of tau phosphorylation could trigger the polymerization process (*70*). In addition, the potential involvement of a proline-directed phosphorylation site in tau was seen in Pin1 (prolyl isomerase) knock-out mice. These mice accumulate tau phosphorylated at T231 that is polymerized into twisted or straight filaments reminiscent of those found in AD (*71*). This work demonstrates the potential importance of Pin1 and site-specific tau phosphorylation in neurodegenerative disease.

However, proline-directed phosphorylation sites targeted by GSK-3β and MAPK in the regions of 0N3R tau flanking the microtubule-binding repeats have only a very mild effect on the polyanion induction of tau polymerization, whereas sites in the microtubule-binding repeat regions targeted by MARK and PKA inhibited polyanion-induced polymerization of tau (*72*). In addition, a cluster of five phosphate-mimicking glutamate residues, or pseudo-phosphorylation sites (pPO$_4$), in the carboxy terminus of 0N3R tau had little effect on its polymerization or its ability to stabilize microtubules, while a cluster of five pPO$_4$ sites in the proline-rich region inhibited its ability to stabilize microtubules and to polymerize in the presence of heparin in vitro (*73*). Although this study stands in marked contrast to an earlier report indicating that proline-directed phosphorylation sites in the proline-rich region of tau had little effect on the polymerization (*72*), it suggests that hyperphosphorylation may counter the assembly process.

In a narrower context, mutation of Ser 396 and Ser 404 into pPO$_4$ sites in order to mimic the proline-directed phosphorylation sites that correspond to epitopes recognized by the AD2 and PHF-1 antibodies that stain AD pathology (*74*) resulted in the enhanced polymerization of 2N4R tau in the presence of arachidonic acid (*41*). Since these pPO$_4$ sites fall in a region between the microtubule-binding repeats and the inhibitory carboxy terminus that is predicted to be highly flexible, it was hypothesized that these changes could decrease the inhibitory effect of the carboxy terminus and therefore enhance tau polymerization (*41*).

In summary, site-specific phosphorylation may well impact tau's ability to form filaments in a positive manner. Hyperphosphorylation, by contrast, may be the cell's attempt to inhibit this process.

The Role of FTDP-17 Mutations. Mutations in the tau gene lead directly to neurodegeneration in several familial disorders, demonstrating that abnormalities in tau can be an important part of the neurodegenerative process. The mutations can lead to a single amino acid change in the tau molecule, an alteration of the splicing of the tau mRNA transcript, or both (reviewed in ref *16*). The major categories of functional defects in tau are decreased microtubule binding, increased propensity for self-polymerization, increased hydrophobicity, structural modification, and altered isoform ratios.

Decreased Microtubule Binding. Many of the tau mutations, including R5L (*75*), K257T (*76*), L266V (*77*), G272V (*78*, *79*), N279K (*79*), ΔK280 (*79*), ΔN296 (*80*), N296H (*80*), P301L (*78*, *79*), P301S (*81*), S320F (*82*), K369I (*83*), G389R (*84*), and R406W (*78*, *79*), show a slight to moderate decrease in their ability to bind to and stabilize microtubules. This is not surprising for the mutations that lie within the microtubule-binding repeat regions or interrepeat regions of tau. The mutations outside the MTBRs are very close to this region (K369I), lie in flexible regions of tau (G389R and R406W), or lie in regions known to interact with the microtubule-binding repeats (R5L). Also, several tau mutations are intronic and result in the altered isoform expression of 3R versus 4R tau (reviewed in ref *16*). Since 3R tau isoforms exhibit decreased microtubule binding and stabilization efficacies compared to 4R tau (*8*, *9*, *49*), these mutations could readily influence microtubule stability. A dramatic change in microtubule-binding activity would result in a microtubule cytoskeleton with either enhanced or diminished dynamicity, conditions which have been hypothesized to result in neuronal dysfunction (*9*). Another important consideration is that the loss of microtubule-binding activity would increase the intracellular concentration of tau, potentially increasing the likelihood of polymerization via mass action.

Increased Propensity for Polymerization. Many of the tau mutations have been shown to increase the efficacy of tau polymerization in the presence of inducer molecules. Among the mutations that increase tau polymerization are R5L (*42*), L266V (*77*), G272V (*40*, *79*), N279K (*79*), ΔK280 (*79*), P301L (*40*, *79*, *85*, *86*), P301S (*85*), V337M (*40*, *79*, *86*), K369I (*83*), and R406W (*40*, *79*, *86*). Once again, most of the tau mutations lie within the microtubule-binding repeat regions of the molecule (Figure 1). Since this region has been

Table 1: Changes in Tau as a Result of FTDP-17 Mutations[a]

mutation	$\Delta P(\alpha)$[b]	$\Delta P(\beta)$[b]	$\Delta P(t)$[b]	outcome[c]	Δ hydropathy[d]	Δ charge[e]
R5L	0.16	0.05	−0.05	enhance α	1.2	−0.2
R5H	0.10	−0.01	0	enhance α	0.2	−0.2
K257T	−0.04	0.07	−0.01	enhance β	0.4	−0.14
L266V	−0.05	0.05	−0.01	α to β	0.1	0
G272V	0.07	0.13	−0.15	reduce t	0.7	0
N279K	0.07	−0.02	−0.08	β to α	0	0.14
ΔK280	0.04	0	0.01	enhance α	0.5	−0.14
ΔN296	0.11	0.15	−0.23	t to α	1.0	0.03
N296H	0.06	−0.01	−0.09	reduce t	0	0
P301L	0.12	0.11	−0.14	t to β	0.8	0
P301S	0.03	0.03	−0.02	reduce t	0.1	0
S320F	0.05	0.09	−0.11	t to β	1.3	0
V337M	0.05	−0.09	0.01	reduce β	−0.3	0
E342V	−0.06	0.08	−0.03	α to β	1.1	0.14
K369I	−0.01	0.12	−0.08	t to β	1.2	−0.14
G389R	0.06	0	−0.09	enhance α	−0.6	0.2
R406W	0	0.06	0	no change	0.5	−0.14

[a] The values for $P(\alpha)$, $P(\beta)$, $P(t)$, hydropathy, and charge were calculated using a seven amino acid window with either the wild-type or mutant residue in the center of the window. [b] $\Delta P(\alpha)$, $\Delta P(\beta)$, and $\Delta P(t)$ refer to the difference in predicted secondary structure after the mutation based on the predictive values of Chou and Fasman scaled by an addition of 1 (*88*). $P(\alpha)$ refers to the propensity for the α helix, $P(\beta)$ refers to the propensity for the β strand, and $P(t)$ refers to the propensity for turns, or random coil. [c] Outcome is a summary of the predicted structural change as a result of the mutation. Enhance indicates that the mutation increases the propensity for structural elements that are predicted in the wild-type protein. Reduce indicates that the mutation decreases the propensity for a structure but that structure is still the predominant predicted structure. Several mutations result in the change of one predicted structure to another (i.e., β to α), and one mutant results in no change. [d] Δ hydropathy refers to the difference in average hydropathy scores after the mutation based on the values given to the amino acids by the original Kyte-Doolittle report (*89*). [e] Δ charge refers the average charge of the region containing the mutation, based on assigning glutamic acid and aspartic acid a charge of −1 and lysine and arginine a charge of +1; all other amino acids were uncharged (0).

shown to be sufficient for the oligomerization of tau, it is not surprising that mutations in this region would influence tau's polymerization. In addition, the intronic mutations in tau that lead to increased expression of four repeat isoforms could increase the propensity of tau to polymerize since four repeat isoforms assemble more readily than three repeat isoforms in the presence of arachidonic acid (*39*).

While it is not immediately obvious why missense mutations would enhance the polymerization of tau, the mutations fall into general categories that are predicted to influence the charge, the hydrophobicity, or the secondary structure of tau (Table 1). All three of these parameters have been shown to be predictive of the rate of aggregation of natively unfolded proteins (*87*). All but three of the missense mutations in tau lead to decreased flexibility in the immediate region around the mutation as predicted using the method of Chou and Fasman (*88*). All but four of the mutations increase the hydrophobicity of their immediate region as determined using the values of Kyte and Doolittle (*89*), which would also favor conformational changes in order to reduce the exposure of hydrophobic residues to water. In keeping with their ability to decrease flexibility, most of the mutations are predicted to increase the formation of structural elements (Table 1). Although we cannot currently determine the significance of these changes for tau polymerization, it becomes clear that most of the tau mutations that result in

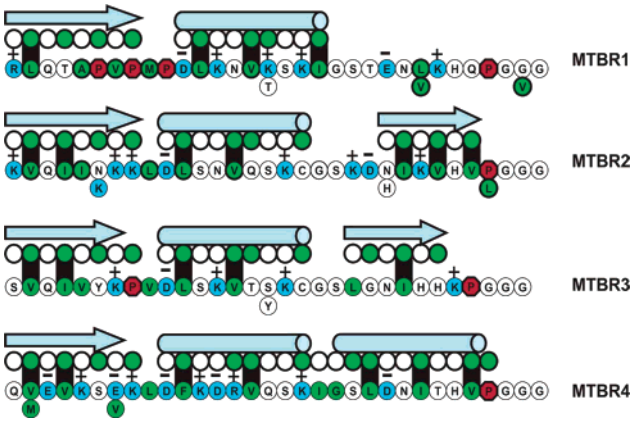

FIGURE 2: Potential amyloid-like structural elements in the microtubule-binding repeats of tau. The entire tau sequence from amino acids 244−360 is represented here. Nonpolar residues are in green, charged residues are in blue, polar residues are in white, and structure-breaking proline residues are represented as red octagons. Idealized amyloid-forming structures are drawn above the primary sequence of tau using the designation in ref *94*, where green circles denote hydrophobic residues and white circles are polar residues. β strand amyloid structures are denoted by arrows while α-helix amyloid structures are denoted by cylinders. These structures were aligned with the primary tau sequence manually. Residues that match the idealized amyloid-forming structures are aligned with rectangles. FTDP-17 mutations are listed below the primary sequence.

neurodegeneration would decrease the natively unfolded state of tau and favor the adoption of more rigid structures that is predicted to lead to the increased formation of polymers (*87*).

A Model for Tau Polymerization

Tau is normally a highly soluble natively unfolded protein (*3*, *79*, *85*, *90*). Yet in AD and many other neurodegenerative disorders it assumes an insoluble polymerized state (reviewed in ref *16*). This transition is likely accompanied by a large change in conformation and the adoption of secondary structures (Figure 2). However, until recently, identification of any structure in tau using conventional methods has proved elusive (*3*, *38*, *45*, *90*, *91*), and predictive methods reveal only a small propensity for secondary structure (*91*). Using two separate algorithms [Chou and Fasman (*88*) and GOR (*92*)], only 17.5% of the tau sequence is predicted to have a high propensity for assuming secondary structure; this level of secondary structure is at or below the detection limits of conventional biophysical methods. However, since the tau protein is able to bind to microtubules in a sequence-specific fashion and impart stability to these structures, it is likely that tau adopts an induced-fit conformation (*49*) on the surface of the microtubule (Figure 3). In this way, the positive lysine residues present in the microtubule-binding repeats of tau would be optimized to interact with the relatively negative carboxy-terminal regions of tubulin. Disruption of this sequence-specific interaction between tau and microtubules either through FTDP-17 mutations or through phosphorylation of serine or threonine residues residing in the microtubule-binding repeat regions would result in the release of tau from the microtubule and loss of its conformation in solution (Figure 3). However, if tau were to be induced to readopt its microtubule-bound conformation in solution, the amphipathic nature of the structural elements in this conformation could allow for tau molecules to self-associate as

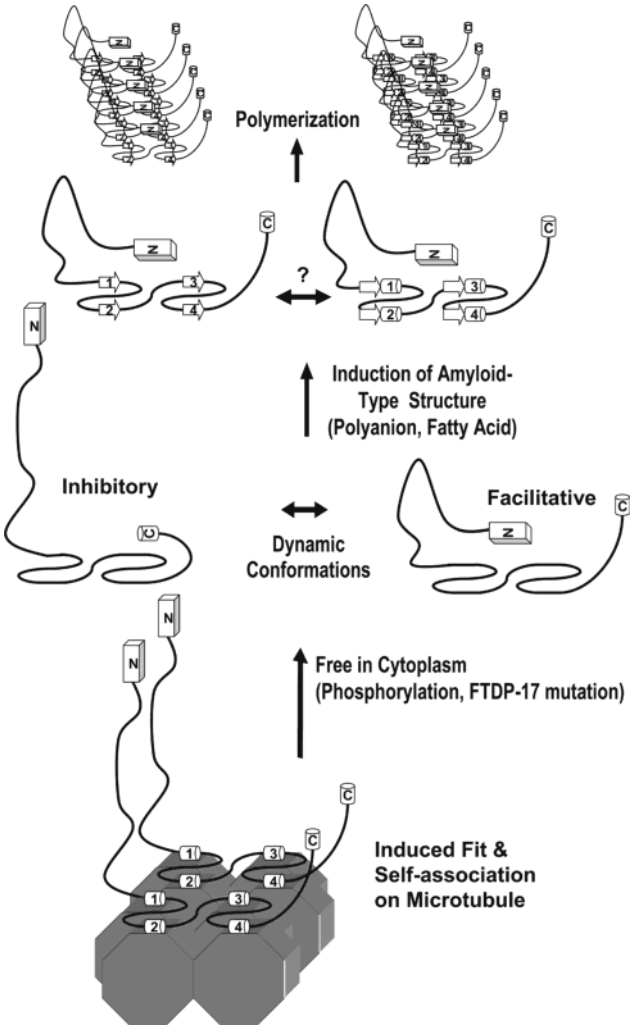

FIGURE 3: Possible conformations of tau in the cell and in disease. The tau molecule is drawn as a long flexible molecule with the amino (box N) and carboxy (cylinder C) termini projecting away from its tubulin (octagons) substrate. In this induced-fit state, it is possible that the MTBR (cylinders 1−4) are helical. When tau is not bound to microtubules and is free in the cytoplasm, perhaps as a result of phosphorylation or FTDP-17 mutations, its flexibility allows for a wide range of conformations. Experimental data suggest that conformations that bring the carboxy terminus in close proximity with the MTBR protect tau from polymerizing, whereas conformations that bring the amino terminus in close proximity to the MTBR facilitate polymerization. If the amyloid-type structure resembling the induced-fit on microtubles is generated by polyanions, fatty acids, or other unknown factors, then the tau polymerization process is greatly enhanced, leading to the accumulation of tau pathology. Two potential conformations that could lead to polymerization are drawn due to conflicting reports about the prevalence of α helices and β strands in tau polymers in disease (*38*, *45*, *57*, *58*).

they do on the surface of the microtubule (*48*) (Figure 3). Such interactions are a general feature of the fibrillogenesis of amyloid-type proteins (*93*). However, these structures would be predicted to be poor amyloid-forming proteins due to the presence of charged amino acids at the end of the predicted β sheets (Figure 2). These positively charged residues should be sufficient to block the interaction of adjacent β sheets (*94*), perhaps explaining the poor amyloid-forming propensity of tau in solution. Polyanionic compounds used to induce tau polymerization could overcome this deficit and stabilize the conformational change by mimicking the charges present on the surface of microtubules. Fatty acids such as arachidonic acid could do the same through hydrophobic interactions with the amphipathic structures. The positive charges in this region would then be neutralized by the acidic headgroup of the fatty acids; this would provide an explanation for the failure of cationic or uncharged amphipathic molecules to induce tau polymerization (*31*).

Some evidence for the proposition that the self-association of tau molecules occurs through the interaction of induced β strands in adjacent molecules comes from the fact that tau fibrils are reactive with β-strand intercalating dyes such as Congo red, thioflavins S and T, and thiazine red. In addition, PHFs purified from Alzheimer's disease brains have been reported to contain cross-β-strand structures (*38*). Finally, in support of the above hypothesis, protein−protein interactions through amphipathic β strands are a feature common to amyloidogenic proteins (*94*).

One possible scenario is that tau polymerizes via a mechanism similar to one that is characteristic of many amyloid proteins known as "domain swapping" (*95*). In this paradigm, structural elements within a given protein molecule that normally associate with one another swap with corresponding elements in a second molecule of the protein to form mixed dimers. In this way, polymerization occurs through the propagation of the domain swap from monomer to monomer. Often, one element serves as a cross-bridge between the monomeric units in a domain swap. We propose that MTBR3 could serve in this capacity for tau polymerization, since it has such a high propensity for self-association (*36*) and exists in both 3R and 4R tau.

We also propose that other interactions beyond the simple association of the β strands contribute to the polymerization of tau by stabilizing the elongating tau fibrils. Candidate structures for this proposed stabilization are the predicted amphipathic α helices also located in the MTBR region. In support of this proposition, the deletion of one of these predicted amphipathic α helices, residues 314-DLSKVTS-320, greatly inhibits the polymerization of tau (*41*). As PHFs demonstrate a large amount of α-helical structure in addition to cross-β strands (*45*), we propose that tau polymerization proceeds via amphipathic β strands in the MTBR domain forming the core of the fibril with adjacent amphipathic α helices from adjoining molecules providing the necessary stabilization to propagate the process. By this hypothesis, the amphipathic carboxy terminus could interact with the amphipathic regions of the microtubule-binding repeat regions that stabilize the fibril, thereby blocking the propagation of polymerization. The amino terminus could help to stabilize the amphipathic β strands that form the core of the fibril through electrostatic interactions with the polar face of the MTBR β strands.

Future Directions

Specific Sites of Tau−Tau Interactions. To understand tau polymerization and to determine whether it proceeds through domain swapping or a similar mechanism, it is important that the precise regions of tau that interact with one another be delineated. Since the microtubule-binding repeat regions are so similar, it is important to discover how the vital regions of microtubule-binding repeat 3 interact with other parts of

the tau molecule or with other tau molecules to form the fibrils that could contribute to the neurodegenerative process.

The Detailed Structure of Tau Filaments. Very little is known about the secondary, tertiary, and potential quaternary structures of tau fibrils. This information obviously will be very important for understanding how tau molecules come together to form filamentous structures. Since the filaments formed in different neurodegenerative disorders can have different morphologies (straight filaments, paired-helical filaments, and twisted ribbons), it will be important to understand the molecular differences that lead to the formation of different types of tau pathology. Depending on these differences, it is possible that tau filaments present in non-AD neurodegenerative diseases would not respond to identical therapeutic strategies.

Dissection of the Reaction into Its Component Parts. It will also be very important to determine how changes in the tau molecule affect kinetic/energetic parameters to result in increased polymerization. Although we can suggest mechanistic reasons for the effects of FTDP-17 mutations, we are still unable to determine whether these effects are due to increased nucleation, increased rates of polymerization, decreased rates of depolymerization, or some combination of these. In addition, it will be important to determine which parts of the molecule, or even which specific residues, are most important for the different component parts of the reaction. For example, which residues have specific interactions with inducer molecules, which domains are required for efficient nucleation, which parts are important for elongation, and which amino acids of the molecule are important in orthogonal directions in elongation? Answers to these questions will facilitate rational approaches to intervention in tau deposition in AD and other tauopathies.

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