Impaired tau–microtubule interactions are prevalent among pathogenic tau variants arising from missense mutations

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Pathogenic mutations in the MAPT gene that encodes the microtubule (MT)‐associated protein tau promote tubulin assembly and stabilize MTs by binding longitudinally along the MT surface. Tau can aberrantly aggregate into pathological inclusions that define Alzheimer’s disease, frontotemporal dementias, and other tauopathies. A spectrum of missense mutations in the tau‐encoding gene microtubule‐associated protein tau (MAPT) can cause frontotemporal dementias. Tau aggregation is postulated to spread by a prion‐like mechanism. Using a cell‐based inclusion seeding assay, we recently reported that only a few tau variants are intrinsically prone to this type of aggregation. Here, we extended these studies to additional tau mutants and investigated their MT binding properties in mammalian cell‐based assays. A limited number of tau variants exhibited modest aggregation propensity in vivo, but most tau mutants did not aggregate. Reduced MT binding appeared to be the most common dysfunction for the majority of tau variants due to missense mutations, implying that MT‐targeting therapies could potentially be effective in the management of tauopathies.
iatrogenic spread between AD patients (42), experimental studies support the hypothesis that tau can spread in a prion-like manner along anatomical connections to other neurons. A previous study used a cell-based assay to examine prion-like seeding in 19 missense pathogenic tau mutants and revealed that only mutants at the Pro-301 position were uniquely prone to seed induced aggregation (43). Building from this unexpected finding, we investigated and characterized an extensive series of tau mutants for MT binding using a mammalian cell-based assay, and we extended the previous series of pathogenic tau mutants for prion-like seeding. These studies show that most tau mutants share a common mechanism of impaired MT binding with only heterogeneous potential for aggregation.

Results

tau variants with mutations at the Pro-301 position severely impaired MT binding compared with WT tau and several other tau mutants in the R1 and R2 repeats

Although tau–MT associations can be visualized in the cytoplasm by immunofluorescent labeling, the amount of tau that is directly bound to MTs cannot be quantified by this method (Fig. S1). A previously established cell-based MT-binding assay (44–46) was performed on diverse tau missense mutants (Fig. 1) to assess changes in MT binding associated with a spectrum of tau variants with missense tau mutations. Furthermore, most previous studies investigated tau mutants with an in vitro MT-binding assay that used recombinant tau expressed from bacteria and tubulin assembled from bovine or porcine sources (Table 1). This cell-based MT-binding assay is more physiologically relevant as it, at least partially, incorporates the effects of post-translational modifications of a proline-rich domain, an MTBD composed of four MT-binding repeats in the 4R isoforms, and a C-terminal region. Indicated are the locations of the pathogenic tau missense mutants that were investigated herein; they were numbered relative to the longest 2N4R tau isoform expressed in the human brain. Most tau missense mutants are clustered within the MTBD.

Figure 1. Schematic of 4R tau protein depicting major structural domains. Tau is an MT-associated protein that consists of an N-terminal region, a proline-rich domain, an MTBD composed of four MT-binding repeats in the 4R isoforms, and a C-terminal region. Indicated are the locations of the pathogenic tau missense mutants that were investigated herein; they were numbered relative to the longest 2N4R tau isoform expressed in the human brain. Most tau missense mutants are clustered within the MTBD.

with or without paclitaxel, a drug that hyperstabilizes and promotes the formation of MTs (47, 48). Without paclitaxel, most of the tubulin is not polymerized and soluble (Fig. 2A); hence, the majority of tau (∼87%) is also soluble. When paclitaxel is present, tubulin polymerizes into MTs and shifts into the pellet fractions. In the presence of paclitaxel-stabilized MTs, ∼41% of WT tau is found in the MT pellet fraction (Fig. 2B). Therefore, detection of tubulin in the insoluble fraction was used to confirm that paclitaxel was active in all the experiments that followed. Notably, there are also several smaller tau bands observed by immunoblot in this assay, and these are likely due to some degradation from incubation at 37 °C required for these MT-binding assays. However, the major bands are likely full-length tau with other forms of post-translational modifications (Fig. S3).

Three tau mutants (P301L, P301S, and P301T) located at the same amino acid residue within the PGGG motif of the R2 repeat (Fig. 2C) were assessed, because previous in vitro and cell culture assays for P301L tau had shown significant decrease in MT binding (49–54). Likewise, studies using brain lysate of P301S tau transgenic mice have confirmed a decreased MT binding (55). In the cell-based assay, it was revealed that all these mutants at Pro-301 reduced MT binding similarly and significantly (Fig. 2, D–G). These findings show that the change of a proline residue to a leucine, serine, or threonine have a similar effect; thus, the Pro-301 position and disruption of this PGGG motif is likely more important than the specific amino acid change.

Mutants within the R1 and R2 repeats such as G273R, ΔK280, L284R, and V287I were assessed for altered MT binding (Fig. 3). Interestingly, G273R, a mutant in another PGGG motif but within the R1 repeat, did not significantly affect MT binding compared with WT tau (Fig. 3, C and G). Likewise, the V287I mutant did not affect MT binding (Fig. 3, F and G). We observe that both ΔK280 and L284R significantly decreased MT binding (Fig. 3, D, E, and G). The effects for ΔK280 are consistent with previous in vitro studies that showed varied results but generally indicated that ΔK280 decreased MT binding (51, 52, 56). Interestingly, G273R and V287I flank the other two mutations, suggesting that only a restricted region of the R2 repeat is responsible for most of the R2 binding activity. Overall, tau mutants in the R1 and R2 repeats show mixed results for MT binding (Fig. 3G).

Most tau variants with mutations in the R3 and R4 repeats impaired MT binding

Many pathogenic tau variants with missense mutations are clustered within R3 and R4 repeats, a region that prominently contributes to tau MT binding (Fig. 1). Within the R3 repeat, tau mutations are scattered in multiple locations (Fig. 4A). K317M, a mutant near the center of the R3 repeat, significantly impaired MT binding (Fig. 4, C and G). Both G335S and G335V also decreased MT binding (Fig. 4, E–G). Surprisingly, S320F, a mutant at the center of R3, did not alter MT binding (Fig. 4, D and G). This mutant also appeared to have more degradative bands.

Similar to the majority of mutants in the R3 repeat, mutants such as S352L, S356T, V363A, V363I, and G366R in the R4 repeat decreased MT binding (Fig. 5). Thus, most mutants in

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Table 1
Summary of findings on tau aggregation and MT binding or polymerization for tau variants with pathogenic mutations investigated

| tau mutations | Cell-based MT binding assay | In vitro MT binding assay | In vitro MT assembly | In vitro tau aggregation | In vivo tau aggregation |
|---------------|-----------------------------|---------------------------|-----------------------|--------------------------|------------------------|
| RSH           | ↑*                         | NA                        | (106)                 | ↑ (106)                  | ⇓ (43)                 |
| R5L           | ↑                          | NA                        | (107, 108)            | ⇓ or ↑ (82, 107–109)     | ⇓ or * (43)            |
| G273R         | ⇓                          | NA                        | NA                    | NA                       | NA                     |
| L284R         | ⇓                          | NA                        | NA                    | NA                       | NA                     |
| V287I         | ⇓ or ⇓ (45)                | NA                        | (51, 52)              | (52, 111)                | ⇓ or (43, 45)          |
| ΔK280         | (45)                       | NA                        | (50–110)              | (52, 111)                | as reviewed in Ref. 17  |
| P301L         | (45, 53)                   | or ⇓ (49–52)              | (112, 115, 116)       | (114, 116)               | (43)                   |
| P301S         | (55)                       | NA                        | NA                    | NA                       | NA                     |
| P301T         | *                           | NA                        | NA                    | NA                       | NA                     |
| K317M         | *                           | NA                        | (82)                  | (82)                     | (43)                   |
| S280F         |                             | NA                        | (116)                 | (116, 117)               | (116, 117)             |
| G3355         |                             | NA                        | (118)                 | (82, 118)                | (82, 118)              |
| G335V         |                             | NA                        | (119)                 | (119)                    | *                      |
| S356T         |                             | NA                        | (119)                 | (119)                    | *                      |
| S352L         |                             | (118)                     | (82, 118)             | (82, 118)                | *                      |
| V363I         |                             | NA                        | (82, 118)             | (82, 118)                | *                      |
| G366R         |                             | NA                        | (82, 118)             | (82, 118)                | *                      |
| K369I         |                             | NA                        | (82, 118)             | (82, 118)                | *                      |
| R466W         | (45)                       | or ⇓ (45, 50, 53)         | (50, 108)             | (108, 113)               | (43)                   |

Figure 2. tau mutants at the Pro-301 residue significantly impaired MT binding. A and B, cell-based MT-binding assay performed with HEK293T cells transfected to express WT tau with or without the presence of paclitaxel as described under “Experimental procedures.” Antibody specific for β-tubulin (clone TUB 2.1) was used to assay the polymerization of tubulins. 3026 is a polyclonal antibody against total tau. 3026 and 3016 (anti-tau) were used to assay the aggregation of tau. C, P301L, P301S, and P301T tau mutants are in the PGGG motif of the R2 repeat. In the presence of paclitaxel, P301L (D), P301S (E), and P301T (F) all demonstrate significantly decreased MT binding when compared with WT tau. The relative molecular masses of protein markers are indicated on the left. On the right, FL is for full-length tau, and the brace indicates degradative tau bands. G, one-way ANOVA with Dunnett’s test was performed with n = 18 for WT tau and n = 3 for each of these tau mutants. ****, p < 0.0001.

the R3 and R4 repeats impaired MT binding, indicating that impairment of binding in R3 and R4 repeats is not well-compensated by the other repeats.

tau variants within the N- and C-terminal regions display varying effects on MT binding

In addition to mutants within the MTBD, we also tested several tau mutants clustered around the N- and C-terminal regions of tau (Fig. 6A). Surprisingly, R5L and R5H were the only mutants that depicted increased MT binding (Fig. 6, C and D), suggesting that these N-terminal tau mutations likely cause long-range conformational changes promoting the tau–MT interactions, at least in a proportion of tau molecules.

Both C-terminal region mutants K369I and R466W displayed decreased MT binding (Fig. 6, E and F). Although these tau mutations are not within the MTBD, they are both close to this region. This finding suggests that the area adjacent to the repeats also can contribute to MT binding or that these mutations induce conformational changes in the adjacent MTBD.
tau mutants in the PGGG motifs and the R4 repeat have varied aggregation propensities

We previously studied the aggregation properties of 19 FTDP-17 tau missense mutants with or without exogenous K18 seeds and found that only S320F moderately self-aggregated, whereas all three mutants (P301L, P301S, and P301T) at Pro-301 within the R2 PGGG motif were robustly permissive to seed-induced aggregation with conformational templating (43). These studies were extended to include new tau mutants used for MT binding studies. First, additional mutants within the PGGG sequences were assessed for their propensity to aggregate: G273R in the R1 repeat; G335S and G335V in the R3 repeat; and G366R in the R4 repeat (Fig. 7). As a baseline, WT tau did not aggregate with or without K18 seeding (Fig. 7, B and F). In contrast, P301L tau was used as a positive control that normally does not intrinsically aggregate but will readily aggregate when seeded with preformed exogenous K18 fibrils (Fig. 7, C and H). The G273R and G335V tau mutants self-aggregated at modest levels with or without seeding (Fig. 7, D, F, and H). G366R within the R4 repeat negligibly aggregated, but this was not statistically significant (Fig. 7, G and H). None of these mutants within the PGGG motif aggregated more when seeded with K18 preformed fibrils further highlighting the uniqueness of Pro-301 tau mutants (Fig. 7 and Fig. S4).

The tau mutants (S356T, V363A, and V363I) close to the R4 PGGG motifs within the R4 repeat were assessed for aggregation (Fig. 8A). S356T and V363I tau modestly aggregated even without seeding, but this did not increase with the presence of K18 seeds (Fig. 8, C and E). The V363A mutant in the same location as V363I might modestly aggregate but was not statistically significant (Fig. 7, G and H).
tically significant (Fig. 8, D and F). Other tau variants with mutations in various repeats (L284R, V287I, K317M, S352L and K369I) did not aggregate with or without K18 seeding (Fig. 9).

P301L/S320F tau uniquely self-aggregated compared with other tau double mutants

We previously discovered that the combined double tau mutant P301L/S320F robustly self-aggregates without exogenous seeding (43). From our additional screening, tau mutants with some baseline ability to self-aggregate were combined to P301L to create similar double mutants. Surprisingly, other double mutants did not exhibit the enhanced aggregation effect. P301L/G273R tau aggregated modestly compared with P301L/S320F tau (Fig. 10, A, B, and E). Other double mutants like P301L/G335V tau and P301L/S356T tau did not aggregate (Fig. 10, C, D, and E). In fact, the addition of P301L to these mutants did not change their ability to aggregate. This suggests that P301L/S320F tau may form a unique conformation and represent a different tau strain.

Discussion

Aggregation of tau to form pathological inclusions is a hallmark of tauopathies and is universally observed in postmortem brains of patients with MAPT mutations, although variable brain regional distribution can be observed even between patients with the same MAPT mutation (57). In recent years, prion-like conformational templating of aggregated tau has gained significant attention as a mechanism in the progressive spread of tauopathies in many experimental models (31, 58–61). Recently, we investigated the aggregation propensity of 19 FTDP-17 tau mutants in a cell-based tau-seeding assay and demonstrated that except for three mutants at Pro-301 (i.e. P301L, P301S and P301T), WT and the other assayed tau mutants displayed relatively limited to no aggregation (43).
Herein, we extend these studies to an additional 12 tau mutants and demonstrated that none of them were prone to seeded aggregation, although four mutants (G273R, G335V, S356T or V363I) were identified that modestly and intrinsically aggregated. Similarly, we had previously found that the S320F mutant had a modest tendency to aggregate but was not enhanced with exogenous K18 seeding (43). Combining the P301L and S320F mutants resulted in tau that robustly aggregated even without seeding. However, combining the P301L mutant with either G273R, G335V, or S356T did not have a synergistic effect of tau aggregation (Fig. 10). These findings further support the notion that tau aggregation is tightly regulated by global folding, such as the proposed paperclip-like structure that is not intrinsically permissive to aggregation (62), as well as key local molecule structures supported by recent cryo-EM studies (63, 64). The synergistic aggregation of the combined P301L/S320F tau double mutant is likely due to the unique relative distance and locations of these two mutations initiating and potentiating an amyloid-permissive stretch (43). In this structure, S320F enhances the hydrophobicity of an amyloid pocket, whereas the Pro-301 mutations disrupt a fold that would otherwise suppress amyloid formation (43, 63). The increased propensity of S320F tau to promote aggregation with or without seeding is consistent with the notion that it has greater property to aggregate with a lower nucleation threshold and a moderate ability to elongate as amyloid structures.

Figure 5. Most of the tau variants mutants in the R4 repeat significantly reduced MT binding. A, tau mutants S352L, S356T, V363I, V363A, and G366R are located within the R4 repeat. B–G, MT-binding assay was performed in the presence of paclitaxel as described under “Experimental procedures.” HEK293T cells were transfected with WT or the indicated tau mutations. S352L, S356T, V363I, V363A, and G366R tau mutants significantly decreased MT binding. Immunoblots were probed with anti-β-tubulin antibody TUB 2.1 or total tau antibody 3026 as indicated. S = supernatants; P = pellet fractions. The relative molecular masses of protein markers are indicated on the left. On the right, FL is for full-length tau, and the brace indicates degradative tau bands. H, one-way ANOVA with Dunnett’s test was performed with n = 18 for WT tau and n = 3 for each tau mutant. *** p < 0.001; ****, p < 0.0001.
tau mutants impair microtubule interaction

![Diagram of tau mutants](image)

**Figure 6.** tau mutants in the N and C termini had differential effects on MT binding. A, diagram shows N- and C-terminal tau mutants. B–F, MT-binding assay was performed in the presence of paclitaxel as described under “Experimental procedures.” HEK293T cells were transfected with WT or the indicated tau mutations. Both the RSH and RSL tau mutants in the N terminus significantly increased MT binding, whereas the mutants near the C terminus, K369I and R406W, decreased MT binding. Immunoblots were probed with anti-β-tubulin antibody TUB 2.1 or total tau antibody 3026 as indicated. S = supernatants; P = pellet fractions. The relative molecular masses of protein markers are indicated on the left. On the right, FL is for full-length tau, and the brace indicates degradative tau bands. G, one-way ANOVA with Dunnett’s test was performed with n = 18 for WT and n = 3 for each tau mutant. ***, p < 0.01; ****, p < 0.0001.

tau displays a greater potential for amyloid elongation but is limited by the lack of significant nucleation to initiate aggregation. Thus, the combination of both S320F and P301L yields an aggressive tau strain that can self-aggregate and is highly permissive to amyloid aggregation. Similar to this double mutant, other structural alterations such as post-translational modifications can result in unique tau strains permissive to aggregation as suggested by some experimental models (38, 65, 66).

One limitation of our seeding studies is that we used in vitro polymerized K18 tau as preformed fibrils to induce intracellular tau aggregation. Some studies have shown that tau aggregates isolated from brain lysates have higher-seeding potencies than recombinant tau fibrils (67). In addition, our in vitro generated tau fibrils likely do not comprise all of the misfolded tau strains that can occur in vivo (68, 69). Therefore, seeding of WT and other tau mutant could occur with more virulent seeds. Nevertheless, our data further highlight the uniqueness of Pro-301 tau mutants.

tau is an “intrinsically disordered” protein that can assume a variety of different conformations (6, 70, 71). When tau associates with MTs, it adopts a more stable conformation than transiently binds to MT (8, 9, 72, 73). The tau–MT interaction is very flexible and allows tau to readily move between the MT-bound and -unbound states (73, 74) and can be regulated by tau phosphorylation (29, 30, 75–78). This interaction allows tau to maintain MT homeostasis by stabilizing MTs, regulating their dynamic instability, and promoting tubulin assembly (6, 7, 10, 79). Different tau mutants also impair physiological dynamic instability and tubulin assembly (17, 49, 80–82), both of which are likely regulated by the MT-binding affinity of tau. Compared with the small subset of tau mutants that presented enhanced prion-like seeded aggregation, most tau mutants investigated so far display reduced activity of tubulin polymerization activity or MT binding (Table 1) (17). However, most studies investigating the impact of tau mutants on MT binding used in vitro MT binding with recombinant tau expressed from bacteria that lack the folding and post-translational modifications associated with mammalian expression. Herein, we conducted a survey of FTDP-17 tau mutants present in various regions of tau for effects on MT binding using a cell-based assay. When compared with previous in vitro studies of MT binding or tubulin polymerization assays, our cell-based findings for ΔK280 and P301L, P301S, G335S, G335V, S352L, V363A, G366R, K369I, and R406W consistently demonstrate reduced MT interactions (Table 1). Furthermore, we showed that L284R, P301T, K317M, and S356T also reduce MT binding, supporting the overarching notion that impaired MT binding is the most common altered property of pathogenic tau mutants due to missense mutations. Surprisingly, and at odds with in vitro MT assembly assays, both the R5H and R5L mutants increased MT interaction in the cell-based assay. The reasons for these differences are not clear, but mammalian expression of these tau mutations must confer long-range conformational differences allowing potentiated MT interaction.

A few missense mutants such as G273R and V287I did not appreciably affect tau MT binding. It is likely that these could primarily influence exon 10 splicing due to their close proximity to other splicing mutations that impact key RNA sequence elements that regulate splicing (83, 84). Many intronic, silent, and even missense MAPT mutations affect the splicing of exon 10, altering the normal ratio of 3R to 4R tau isoforms (6, 57, 83, 84). How this altered ratio of WT tau isoforms results in neurodegeneration is still unclear, but altered isoform-specific cellular localization and MT–binding properties could be involved. Generally, 3R tau isoforms bind MTs with lower affinity and promote MT polymerization less than 4R tau isoforms (14, 85,
As a result, 3R tau isoforms are less potent regulators of MT dynamics (80, 87). Furthermore, 3R and 4R tau appear to have different binding sites and interactions with MTs (81, 88). It is possible for some missense tau mutants to affect both MT binding and tau exon 10 splicing, resulting in a change in the 3R/4R ratio that causes dysregulation of MT dynamics (81).

Based on our results and recent studies on tau prion-like seeding and propagation (17, 31, 43, 58–61), we propose an integrated paradigm of tau pathogenesis to highlight different but not mutually exclusive mechanisms that can lead to tauopathies (Fig. 11). The majority of tau missense mutants can affect MT binding and cause MT dysfunction by impairing MT sta-
bilization, tubulin assembly, and regulation of dynamic instability (Fig. 11A). These changes in MT associations may also result in altered cellular localization. Although tau is mainly an axonal protein (11), tau pathology in AD occurs predominantly in somatodendritic compartments such as neurofibrillary tangles and neuropil threads, with a paucity of white matter pathology (6, 32, 91), likely a result of improper cellular trafficking of pathogenic tau. For many forms of mutant tau, decreased MT binding leads to an increased pool of soluble tau, although tau phosphorylation at specific residues can also contribute to reduced MT binding, possibly in sporadic cases of AD and other tauopathies (29, 30, 75–78). Furthermore, in AD Aβ oligomers or aggregates may be the primary driver of tau toxicity by promoting hyperphosphorylation of different tau isoforms (92–94). For WT tau and tau mutants that are not intrinsically prone to aggregation, unbound tau may slowly convert into new conformers that aggregate over time. The combined events of impaired MT binding, MT dysfunction, soluble tau conformers, and eventual tau aggregates can all contribute to toxicity and cell death. Consistent with this model, several MT-based drugs have been shown to have some benefits in animal models of tauopathies (95–97).

For tau mutants such as those involving the Pro-301 residue that have robust impacts on aggregation, the best approach is likely to be a combination of MT-based and aggregation inhibitors therapies (Fig. 11B). However, due to the unique robust aggregation-driven nature of models based on Pro-301 tau mutants, it would be prudent to cautiously assess the universality of experimental studies and translational therapies based solely on these models for AD and other tauopathies. Nevertheless, it is possible that some of the many post-translational modifications linked to pathogenic tau (6, 91, 98, 99) might mimic the impact of these mutations on tau aggregation propensity and prion-like conformational templating.

Although familial tau mutations are responsible for only a minority of cases with tauopathies, the wide spectrum of tau mutants provide information on the diverse pathomechanisms that can provide invaluable insight to understanding sporadic...
**Figure 9.** Additional tau mutants did not show any propensity to aggregate. A, diagram depicts tau mutations L284R, V287I, K317M, S352L, and K369I within R2, R3, and R4 repeats. HEK293T cells were transfected with WT or tau mutants and were assessed for aggregation with or without exogenous K18 fibrillar seeds. Compared with WT tau (B), L284R (C), V287I (D), K317M (E), S352L (F), and K369I (G) did not aggregate with or without exogenous K18 seeds. Immunoblots were probed with total tau antibody 3026. The relative molecular masses of protein markers are indicated on the left.

**Figure 10.** P301L/S320F tau was uniquely capable of robust self-aggregation compared with other double tau mutants. HEK293T cells were transfected with different tau double mutations and were fractionated into Triton-soluble and Triton-insoluble fractions as described under “Experimental procedures.” A, P301L/S320F tau double mutant presented extensive intrinsic aggregation. B, P301L/G273R tau modestly aggregated but did not show an enhanced effect from the addition of P301L. P301L/G335V tau (C) and P301L/S356T tau (D) did not significantly aggregate. Immunoblots were probed with total tau antibody 3026. The relative molecular masses of protein markers are indicated on the left. E, one-way ANOVA with Dunnett’s test was performed with n = 9 for WT tau and n = 3 for each tau mutant. ****, p < 0.0001; and ns, statistical significance.
cases of tauopathies. Since numerous diverse tau missense mutants lead to a similar outcome of impaired MT interaction, other tau modifications in patients with sporadic tauopathies might result in a similar aberrant outcome that leads to neurodegeneration. Our findings presented here in addition to the observed MT changes in AD (18–21) underscore that a greater emphasis on understanding and targeting MT changes in tauopathies should be considered. MT function is associated with numerous cellular functions that can result in many modes of cell injury and toxicity. It is also important to gain further insights into the relative involvement of MT dysfunctions versus tau aggregation in the pathobiology of tauopathies. Even if several pathogenic mechanisms are involved, MT-based therapy will likely be important in treating Alzheimer’s disease and other tauopathies.

**Experimental procedures**

**tau protein purification**

The tau protein fragment K18, which consists of the MT-binding repeats from Gln-244 to Glu-372 relative to the 2N4R human tau with an added methionine at the N terminus, was expressed from the bacterial plasmid pRK172 in BL21 (DE3)/RIL *Escherichia coli* (Agilent Technologies, Santa Clara, CA). K18 tau protein and K18 with mutations were purified as described previously (43, 100). Protein concentration was measured by the bicinchoninic acid assay with albumin as a standard.

**Preparation of tau amyloid seeds**

K18 tau protein or K18 with mutations were diluted in sterile PBS at 1 mg/ml with 50 μM heparin and shaking at 1050 rpm at 37 °C for at least 2 days. As described previously, amyloid formation was confirmed by K114 or thioflavin T assays (101). K18 fibrils were centrifuged and resuspended in sterile PBS to remove heparin. The purified K18 fibrils were placed in bath sonication for 60 min to produce short fibrils for seeding experiments, as described previously (43, 102).

**Plasmids for mammalian expression and site-directed mutagenesis**

The 0N4R human tau cDNA isoform was cloned into mammalian expression vector pcDNA3.1(+). The different missense MAPT mutations were created with QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) using mutation-specific oligonucleotides. All mutations and the entire cDNA tau sequence were confirmed by Sanger sequencing performed as a service by Genewiz (South Plainfield, NJ).

**Cell culture and transfection**

HEK293T cells were maintained in Dulbecco’s modified Eagle’s media with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) at 37 °C and 5% CO2. HEK293T cells were transfected by calcium phosphate precipitation. Cells were plated at 20–40% confluence in 12-well plates. For each well, 1.5 μg of DNA was added to 18.75 μl of 0.25 M CaCl2. This DNA/CaCl2 mix was further added to an equal amount of 2× BES buffer (50 mM BES, 280 mM NaCl, 1.5 mM Na2HPO4, pH 6.96) and left at room temperature for 15–20 min. The final solution was added dropwise to each well. For cell-seeding studies, 1 μM K18 was added 1 h after transfection as described previously (102). At 16 h after transfection, cells were washed with PBS, and fresh Dulbecco’s modified Eagle’s medium in 3% FBS was added. Cells were harvested 48 h after media change or at a final time of 64 h.

**Biochemical cellular aggregation assay**

Cells were lysed in 200 μl of Triton Lysis Buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM NaF) with a mix of protease inhibitors. Cell lysates were centrifuged at 100,000 × g and 4 °C for 30 min (36, 43, 103). The soluble fractions were collected. The insoluble fraction was washed with additional Triton Lysis Buffer and centrifuged again at 100,000 × g and 4 °C for 30 min. After the wash step, the pellet was resuspended in Triton Lysis Buffer. Sample buffer (10 mM Tris, pH 6.8, 1 mM EDTA, 40 mM DTT, 0.005% bro-
mphenol blue, 0.0025% pyronin yellow, 1% SDS, 10% sucrose) was added to both soluble and insoluble fractions and were boiled for 10 min. The insoluble fraction was probe-sonicated and boiled again for 10 min to ensure homogeneity.

**MT-binding assay**

Cells were lysed in 200 μl of PEM buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM MgCl2) supplemented with 0.1% Triton X-100, 2 mM GTP, 20 μM paclitaxel, and a mix of protease inhibitors as described previously (44–46). Cell lysates were incubated in a 37 °C water bath for 30 min and then centrifuged at 100,000 × g for 30 min to pellet MTs. Supernatant was transferred to a new tube, and the pellet (MT fraction with bound proteins) was resuspended in PEM buffer. The pellet fraction was homogenized, and SDS gel loading buffer was added to both fractions. Equivalent amounts of supernatant and pellet were loaded on SDS-polyacrylamide gels for Western blot analysis. Percent MT bound tau was calculated as pellet/(supernatant + pellet) × 100.

**Antibodies**

Anti-β-tubulin (clone TUB 2.1) is a mouse mAb (Sigma). tau 3026 is a rabbit polyclonal antibody against recombinant full-length 0N3R human tau (90). AT8 is an antibody specific for tau phosphorylated at Ser-202/Thr-205 (Invitrogen), and PHF-1 antibody is specific for tau phosphorylated at Ser-396/Ser-404 (generously provided by Peter Davies, Albert Einstein University, New York). T14 is an antibody against the N-terminal part of tau (83–120 amino acids), and T46 is an antibody against the C-terminal part of tau (404–441 amino acids) (104).

**Western blot analysis**

Equal proportions of each sample were loaded on 10% polyacrylamide gels and resolved by SDS-PAGE. After transfer, the membranes were blocked in 5% milk dissolved in TBS for 1 h. Membranes probed with phosphorylation-specific antibodies were blocked in 5% BSA dissolved in TBS for 1 h. The membranes were incubated in primary antibody overnight at 4 °C at dilutions of 1:1000 for 3026 tau antibody, β-tubulin antibody, and tau phosphorylation-specific antibodies AT8 and PHF-1. The membranes were washed in TBS and incubated in goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch) at 1:4000 dilution for 1 h. After TBS washes, the membranes were exposed and imaged after adding Western Lightning Plus ECL reagents (PerkinElmer Life Sciences).

**Statistical analysis**

The specific signals in each lane were quantified based on densitometric analysis with ImageJ. Statistical comparison tests were performed on GraphPad Prism for one-way or two-way analysis of variance (ANOVA), with post hoc analysis using Dunnett’s test to compare each group to the control.

**Immunofluorescence of fixed cells**

Double immunofluorescence was performed on cells similar to previous studies except for the fixation step (102, 105). Cell fixation was performed at −20 °C in 95% acetic acid and 5% methanol for 30 min. After PBS washes, the cells were blocked in PBS with 2% FBS and 0.1% Triton X-100 for 30 min. Primary antibody such as 3026 at 1:4000 and β-tubulin 1:1000 were incubated for 1 h. After the PBS wash, the slides were incubated in the dark for 1 h in Alexa-fluor 488- or 594-conjugated secondary antibodies (Invitrogen). Afterward, the slides were incubated in 4’,6-diamidino-2-phenylindole (Invitrogen) for 5 min to stain nuclei and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). Immunofluorescent images were captured with an Olympus BX51 fluorescence microscope (Olympus, Center Valley, PA).

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