Asparagine residue 368 is involved in Alzheimer’s disease tau strain–specific aggregation

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In tauopathies, tau forms pathogenic fibrils with distinct conformations (termed “tau strains”) and acts as an aggregation “seed” templating the conversion of normal tau into isomorphic fibrils. Previous research showed that the aggregation core of tau fibril covers the C-terminal region (243–406 amino acids (aa)) and differs among the diseases. However, the mechanisms by which distinct fibrous structures are formed and inherited via templated aggregation are still unknown. Here, we sought to identify the key sequences of seed-dependent aggregation. To identify sequences for which deletion reduces tau aggregation, SH-SY5Y cells expressing a series of 10 partial deletion (Del 1–10, covering 244–400 aa) mutants of tau-CTF24 (243–441 aa) were treated with tau seeds prepared from a different tauopathy patient’s brain (Alzheimer's disease, progressive supranuclear palsy, and corticobasal degeneration) or recombinant tau, and then seed-dependent tau aggregation was assessed biochemically. We found that the Del 8 mutant lacking 353–368 aa showed significantly decreased aggregation in both cellular and in vitro models. Furthermore, to identify the minimum sequence responsible for tau aggregation, we systematically repeated cellular tau aggregation assays for the delineation of shorter deletion sites and revealed that Asn-368 mutation suppressed tau aggregation triggered by an AD tau seed, but not using other tauopathy seeds. Our study suggested that 353–368 aa is a novel aggregation-responsible sequence other than PHF6 and PHF6*, and within this sequence, the Asn-368 residue plays a role in strain-specific tau aggregation in different tauopathies.

Tau is a natively highly soluble, unfolded protein (1, 2), and physiologically it participates in the assembly and stabilization of microtubules (MTs) (3). In the adult human brain, tau has six isoforms from alternatively spliced products (0N4R, 0N3R, 1N4R, 1N3R, 2N4R, and 2N3R) which differ from one another in containing zero, one, or two N-terminal projection domains (N1, N2) and the presence of four (4R) or three (3R) C-terminal microtubule-binding repeat domains (MBD) (4, 5). In Alzheimer’s disease (AD) and other tauopathies, aggregated forms of tau are observed in various brain regions as pathological structures (6).

Accumulated tau in AD patients’ brains forms distinct filamentous structures called paired-helical filament (PHF) or straight filament (7–9) by assembling with each other and generating a cross-β-sheet structure (10). In progressive supranuclear palsy (PSP), most filaments were found to be straight, and in corticobasal degeneration (CBD), twisted filaments were mainly detected (11). Biochemically, AD filaments were composed of both 3R and 4R tau, whereas PSP and CBD showed only 4R tau. Experimental evidence showed that tau aggregates, prepared from AD, PSP, or CBD brains or recombinant tau, can convert native tau into abnormal aggregated tau when internalized into cells, like prion protein (12–14). Therefore, the hypothesis that aggregated tau is propagated through a prion-like mechanism possibly explained the spread of neuropathologies and progression of neuronal death in not only AD but also in PSP and CBD (6, 15). Furthermore, a recent study showed that tau aggregates may form conformationally distinct structures in different tauopathy, which were termed as “tau strains,” and different strains determine the seeding potency in cellular and animal tauopathy models (16, 17).

Understanding of the mechanisms of tau aggregation is required to establish effective treatments for tauopathy. However, much about these mechanisms remains unclear. It has been revealed that MBD was mainly responsible for the core region of PHFs (18), and tau C-terminal fragments are prone to aggregation (19–21). Our previous report showed that C-terminal tau fragments (tau-CTF24: amino acid (aa) residues 243–441) were deposited in human brains with tauopathies as well as in the brains of aged transgenic mice (Tg601) overexpressing WT human tau (22). In addition, genetic analysis of patients with tauopathies revealed that nearly 80% of missense mutations in tau were localized in the C-terminal region (244–441 aa) (23). These findings suggested that the C-terminal region including the MBD plays an essential role in the aggregation of tau. This understanding was supported by the discovery of the PHF6 (306–311 aa: VQIVYK) and PHF6* (275–280 aa: VQIINK) sequences, which were found in the protease-resistant C-terminal fragment of tau and were shown to be involved in the initiation of tau aggregation (24, 25).
Here, we attempted to identify aggregation-responsible sequences in the C-terminal region of tau using a cellular seed-dependent aggregation model of tau-CTF24. To determine the key sequences of the tau C-terminal region systematically, we prepared 10 deletion mutants of tau-CTF24 that were expressed in SH-SY5Y cells and treated with tau aggregates prepared from a patient’s brain or recombinant tau. Then we identified Del 8 (353–368 aa) as a novel aggregation-responsible sequence, and this region overlapped with the core sequence of AD tau, which was recently determined by means of cryo-EM (26). The role of this sequence in tau aggregation was tested in an in vitro aggregation assay using recombinant tau mutants or peptides. We found that this sequence also affected the morphology of recombinant tau fibrils, but the sequence itself had no aggregation properties. In addition, we further investigated the smallest sequences involved in tau aggregation within 353–368 aa. We found that the aggregation property can be attributed to a single amino acid, Asn-368, whose mutations affected tau aggregation differently, depending on the type of tau strains.

Results

Seed-dependent tau aggregation assay of SH-SY5Y cells expressing tau-CTF24 deletion mutants

To explore the sequences of the tau C-terminal region that is responsible for aggregation, we constructed a series of plasmids expressing deletion mutants of tau-CTF24 (243–441 aa) lacking 16-amino acid residues: Del 1 (Δ244–259 aa), Del 2 (Δ259–274 aa), Del 3 (Δ275–290 aa), Del 4 (Δ290–305 aa), Del 5 (Δ306–321 aa), Del 6 (Δ322–337 aa), Del 7 (Δ338–353 aa), Del 8 (Δ353–368 aa), Del 9 (Δ369–384 aa), and Del 10 (Δ385–400 aa) (Fig. 1A). Deletion sites of Del 5 (306–321 aa) and Del 10 (385–400 aa) were designed to include the C-terminal neighboring region of this domain. PHF6 (306–311 aa) and PHF6* (275–280 aa) sites were located on Del 5 and Del 3 deletion sites, respectively.

Figure 1. Construction of a series of partial deletion mutants of tau C-terminal fragments. A, schematic diagrams of full-length 4R2N tau (1–441 aa) and the epitopes of tau antibodies used in this study. Deletion sites (1–10) were designed in the C-terminal region (244–400 aa) containing the microtubule-binding domain (M1–M4; 244–368 aa). B, schematic images of tau-CTF24 deletion mutants (Del 1–10). Each mutant possesses one of 10 deletions. The Del 1 to Del 8 mutants have deletions in the microtubule-binding domain (Del 1 (244–259 aa) and Del 2 (259–274 aa) on M1, Del 3 (275–290 aa) and Del 4 (290–305 aa) on M2, Del 5 (306–321 aa) and Del 6 (322–337 aa) on M3, and Del 7 (338–353 aa) and Del 8 (353–368 aa) on M4), and the deletion sites of Del 9 (369–384 aa) and Del 10 (385–400 aa) were designed to include the C-terminal neighboring region of this domain. PHF6 (306–311 aa) and PHF6* (275–280 aa) sites were located on Del 5 and Del 3 deletion sites, respectively.

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CBD patients’ brains were used as seeds (case descriptions and immunoblotting images are shown in Table S2 and Fig. S1). Del 1 and Del 2 were not included in the cellular experiments because these mutants were not expressed sufficiently in SH-SYSY cells. Tau aggregation was evaluated by biochemical analysis of sarkosyl-insoluble (insol.) fractions of cell lysates. After treatment with all the types of tau seeds, the amounts of ppt were significantly decreased in cells expressing the Del 5, Del 6, and Del 8 mutants (Fig. 2, A–D), regardless of the types of tau strains used as seed. There were no significant differences in the levels of soluble in the levels of soluble tau (sol.) detected irrespective of either deletion sites of mutants or the types of seeds used.

The effects of deletion on seed-induced cellular aggregation of tau were also analyzed by fluorescence immunocytochemistry. SH-SYSY cells expressing WT or deletion mutants were treated with recombinant 2N4Rtau seeds and the phosphorylated tau-specific antibody PHF-1 (27, 28) to visualize intracellularly formed tau aggregates but not recombinant 4R2N tau seed itself (not phosphorylated) remaining on the cell surface even after washing. Aggregated tau was detected as cytoplasmic inclusion-like structures (Fig. 3A, white arrowhead), which were morphologically distinct from diffusely expressed non-aggregated tau (Fig. 3A, white arrow). The results with Del 10 were not included because the PHF-1 antibody did not stain either expressed Del 10 monomer or aggregates. This was
probably because the PHF-1 epitope (pSer-396/404) overlapped with the Del 10 deletion sequence (Δ385–400 aa). The ratio of aggregate-positive cells was significantly decreased in cells expressing Del 3, Del 5, Del 8, and Del 9 compared with WT (Fig. 3B). The Del 6 mutant, which exhibited a significant decrease in tau aggregation in the biochemical analysis (Fig. 2, A–D), showed only a moderate reduction in tau aggregation in this setting. It is possible that the Del 6 mutation may

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**Figure 3. Immunocytochemical analysis of intracellular tau aggregation.** A, the representative images of confocal microscopic analysis of cellular aggregation of tau-CTF24 mutants after 2N4R seed treatment. Cells were stained with PHF-1 (green), anti-tubulin polyclonal antibody (red), and DAPI (blue). Tau aggregates are identified by white arrowheads, and nonaggregated tau are indicated by white arrows on the magnified images (magnified areas are indicated by the red square). Scale bar, 20 μm. B, total cells and cells with PHF-1-positive tau aggregates were counted, and the ratios of tau aggregate-positive cells were compared between WT + seed and D1–D9 + seed. The result for D10 was not included, because the PHF1 antibody did not stain either expressed D10 monomer or aggregates. Values are means ± S.D. (error bars) (n = 3). **, p < 0.01; ***, p < 0.001 by Student’s t test versus WT + seed.
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affect the tau aggregation, ability more mildly than Del 8 and Del 9, and thereby the detectable amount of Del 6 aggregates was preserved through the immunocytochemistry staining process but easily solubilized and monomerized by sarkosyl extraction. We also tried to analyze cells treated with other tau strains, but the seeds extracted from tauopathy patients’ brains were highly phosphorylated so that PHF-1 antibody strongly stained the seeds themselves, which remained on the coverslips or cell surface, and intracellular tau aggregates were indistinguishable from them.

Collectively, Del 5 (habering the aggregation-prone PHF6 sequence), Del 8, and Del 9 showed significant decreases in tau-CTF24 aggregation using both biochemical and histochemical analyses.

In vitro aggregation of recombinant tau-CTF24 deletion mutants

To further analyze the effect of deletion in vitro, we conducted ThS fluorescence assays using a series of recombinant tau-CTF24 deletion mutants. We successfully purified recombinant tau-CTF24 mutants except for Del 4, because most of the bacterially expressed Del 4 mutant was captured in inclusion bodies, and therefore, we were unable to prepare sufficient amounts of recombinant Del 4. Tau-CTF24 WT and deletion mutants (Del 1–10) were incubated at 37°C on a shaker in the presence or absence of heparin. Consistent with the results of cultured cells, Del 5 and Del 8 showed a significant decrease in ThS fluorescence compared with the WT after incubation for 96 h, suggesting that the deleted regions in Del 5 and Del 8 are critical for tau aggregation (Fig. 4A). Del 3 also exhibited a slight reduction in fluorescence, and Del 6 showed significantly decreased aggregation at 24 and 48 h, but ThS fluorescence elevated rapidly and showed almost identical levels to WT at 72 and 96 h. Other mutants, including Del 9, showed almost the same or higher ThS fluorescence compared with WT. In particular, Del 2 exhibited significantly higher ThS fluorescence levels than WT. We were not able to clarify the mechanisms for the increased aggregation of Del 2 and Del 4 (inclusion bodies in bacteria), but considering the fact that the deletion sites of these mutants (Del 2, 259–274 aa; Del 4, 290–305 aa) were N-terminally adjacent to PHF6a (275–280 aa) or PHF6 (305–311 aa), we speculated that these sequences might be an obstacle to the aggregation-prone motif. All of the mutants and WT showed no increased ThS fluorescence in the absence of heparin.

Recombinant tau-CTF24 aggregates were negatively stained with phosphotungstate and observed by transmission EM (TEM). Other than Del 5 and Del 8, TEM observations of tau-CTF24 aggregates showed straight filamentous structures of 10–15-nm width. Del 5 showed shorter fibrils gathering each other, and Del 8 indicated a characteristic tangled string-like shape that clearly differed from WT or other mutants (Fig. 4B). However, there were inconsistent results between cellular biochemical analyses and the in vitro aggregation assay of Del 6 and Del 9. Previous studies have demonstrated that structural differences exist between heparin-induced tau filaments and those from brain tissues and that different seeding properties were observed between tau filaments seeded by AD tau and those assembled by heparin (29–31). Hence, we speculated that tauopathy seed-induced cellular aggregation and heparin-induced in vitro aggregation occurred in a different manner, so that inconsistent results were obtained, even using the same tau deletion mutants.

Deletion effects of 306–321 (Del 5) and 353–368 (Del 8) aa on the aggregation of full-length tau

We further tested the effects of 306–321 (Del 5) and 353–368 (Del 8) aa deletions on the aggregation of full-length tau (2N4R and 2N3R isoforms). Cellular and in vitro aggregation experiments revealed that the 353–368 aa deletion in 2N4R and 2N3R tau caused decreased in tau aggregation and formation of abnormal fibrous structures (Fig. 5). These data showed that deletion of 353–368 aa affects the aggregation of tau, regardless of the length (C-terminal region or full length) and the presence or absence of microtubule-binding repeat 2 (2N4R or 2N3R). The results in Figs. 2–4 are summarized in Fig. 6A. This schematic diagram indicates that the deletions of 306–321 aa (Del 5) and 353–368 aa (Del 8) in tau showed decreased aggregation in all experimental conditions. Moreover, TEM observation of Del 8 fibrils revealed abnormal structures, which were clearly distinct from WT or other mutant fibrils. Focusing on the core regions of each tau strain (Fig. 6A, solid and broken lines), the Del 5 sequence overlapped with the all of the core regions, but the Del 8 sequence was not included in the recombinant 4R2N core. The cause of the different filament morphology is not clear, but it is possible that sequences outside of the core region also can participate in the molecular process of tau aggregation or that the remarkable ultrastructural versatility of 4R2N fibrils (Zhang et al. distinguished at least four types of them: snake, twister, jagged, and hose (29)) may contribute to this result.

Because 306–321 aa (Del 5) includes a previously reported and well-established aggregation-prone sequence (PHF6), we concluded that 353–368 aa was a candidate for a novel tau aggregation–responsible sequence, which was also likely to affect the morphology of fibrous structures of tau aggregates.

Further examinations of aggregation-related properties of the 353–368 aa sequence (Del 8)

We next investigated whether the 353–368 aa (Del 8) sequence itself has aggregation properties like PHF6 or PHF6a (24, 25). We prepared a synthetic peptide corresponding to the deletion sequence of Del 8 (Pep D8: 353–368 aa) as well as control peptides, Del 5 (Pep D5: 306–321 aa), Del 7 (Pep D7: 338–353 aa), and Del 9 (Pep D9: 369–384 aa) (Fig. 6A). Pep D5 (including the PHF6 (306–311 aa)) showed a marked increase in ThS fluorescence, and other peptides, including Pep D8, exhibited no fluorescence elevation (Fig. 6B). TEM observations of the peptide solution after the incubation revealed that only Pep D5 showed a filamentous structure, which was consistent with the results obtained in the ThS assay (Fig. 6C). These data indicated that the 353–368 aa sequence itself has no aggregation properties like PHF6, although the 353–368 aa deletion from tau-CTF24 and full-length 2N4R and 2N3R tau
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A

B

WT

Del 1

Del 2

Del 3

Del 5

Del 6

Del 7

Del 8

Del 9

Del 10

WT

Del 1

Del 2

Del 3

Del 5

Del 6

Del 7

Del 8

Del 9

Del 10
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caused significant decreases in tau aggregation. Thus, 353–368 aa is likely to be involved in tau aggregation in a different way from that of the PHF6 sequence.

The aggregation-related characteristics of 353–368 aa were further tested on cells using a tau-CTF24 Ala 8 construct, in which all of 353–368 aa was replaced with alanine, to check the involvement of side chains in 353–368 aa on tau aggregation (Fig. 6, D and E). Of note, Del 8 showed decreased aggregation with all the types of tau seeds, but Ala 8 exhibited no decreased aggregation with Rec, PSP, and CBD seeds. In contrast, with AD tau seed, both Del 8 and Ala 8 showed decreased aggregation of tau, and this consistent result suggested that the 353–368 aa sequence reacts with tau seeds in different manners, depending on the type of seed (AD or not AD). Considering the result that alanine simplification of 353–368 aa in tau-CTF24 exhibited decreased aggregation induced by AD brain lysate, AD tau seed might require molecular interaction with the 353–368 aa sequence more specifically than the other types of tau seeds.

Identification of detailed aggregation-responsive regions within 353–368 aa of tau-CTF24

Next, to identify the most important sites within the 353–368 aa sequence, we took a narrowing down approach using an AD tau seed–induced cellular tau-CTF24 aggregation model. As shown in Fig. 7A, we systematically repeated cellular tau aggregation assays and delineation of shorter deletion sites to identify the minimum sequence for which deletion significantly reduces tau aggregation. First, we identified 358–363 aa (Del 8-2) and 363–368 aa (Del 8-3), and then 361–363 aa (Del 8-2-2) and 366–368 aa (Del 8-3-2) were identified as aggregation-responsive sequences (Fig. S2).

Based on these findings, we constructed the six deletion mutants that lacked single amino acids in 361–363 aa and 366–368 aa as follows: tau-CTF24 Δ361 (Thr), Δ362 (His), Δ363 (Val), Δ366 (Gly), Del 367 (Gly), and Δ368 (Asn). As a result, Δ366, Δ367, and Δ368 showed significant decreases in tau aggregation compared with WT (Fig. 7B). In vitro aggregation assays and TEM analysis were also conducted using the recombinant deletion mutants in the presence of heparin, but most of the data were not consistent with the results of the cellular models (Fig. 7C and D) and Fig. S3, as observed in Fig. 2 (cellular) and Fig. 4 (in vitro). We speculated that this discrepancy also reflected the distinct conformational differences between disease-derived tau and in vitro fibrils, as considered above. Taken together, we found that single-amino acid deletion of Gly-366, Gly-367, and Asn-368 was sufficient to decrease tau-CTF24 aggregation in the cellular experiment using AD tau seed.

To exclude the contribution of deletion itself and assess the involvement of the side chains of Gly-366, Gly-367, and Asn-368 on tau aggregation formation, we constructed single-amino acid replacement mutants of tau-CTF24. First, tau-CTF24 G366I and G367I were constructed to test the small side-chain effect of Gly by replacing it with an amino acid containing a bulky side chain, such as Ile. Fig. 7E shows that the replacement of Gly-366 and Gly-367 by Ile did not affect tau aggregation, unlike Δ366 and Δ367. This suggested that in the Gly-366 and Gly-367 residues, the side chain of Gly itself does not play an essential role in cellular tau aggregation induced by AD tau seed.

Next, to simplify the side chain of Asn-368, we constructed tau-CTF24 N368A, and to assess the specific roles of the Asn-368 side chain in tau aggregation, we also prepared tau-CTF24 N368D, N368Q, and N368L, in which the chemical structures of the side chains resemble that of Asn (Fig. 7F): Asp replacement included a negative charge (Δ368D), Glu replacement elongated the Asn side chain by one methylene group (Δ368Q), and Leu replacement converted the amido group of the Asn side chain into saturated hydrocarbons (Δ368L). Of note, in cells expressing all mutants, tau-CTF24 aggregation levels were dramatically decreased.

To investigate whether AD brain lysate from another case of AD can reproduce our results, we first prepared new lysates from two other AD patients’ brains (AD 2 and AD 3, Table S2 and Fig. S1). The results with AD 2– and AD 3–treated cells were almost identical to the experiments in which AD 1 lysate was used as the tau seed (top three panels in Fig. 8, A and B), except for the case of N368A (seeding effects of AD 2 and AD 3 were not as negative as AD 1 on this mutant). Previous findings revealed that tau aggregates from multiple AD cases have very similar structures (32, 33); thus, this similarity probably contributed to this result. Collectively, these data suggest that Gly-366 and Gly-367 play no essential roles in AD tau-induced cellular tau aggregation, but in the case of Asn-368, the side chain of this amino acid is essential for the process.

Effects of single-amino acid deletion or replacement on tau aggregation seeded by PSP, CBD, or FTDP-17 tau

To examine the effect of mutation of Asn-368 on tau aggregation induced by non-AD seeds, we used 2N4R recombinant tau fibrils and 4R tauopathy brains, such as PSP, CBD, and FTDP-17 (N279K) (Table S2 and Fig. S1). We expressed single-amino acid deletion mutants (Δ361, Δ362, Δ363, Δ366, Δ367, and Δ368) and Asn-368 replacement mutants of tau-CTF24 (N368A, N368D, N368Q, and N368L) in SH-SY5Y cells, and subsequently the cells were treated with non-AD seeds.

After 2–3 days’ incubation, intracellular tau aggregation was analyzed by immunoblotting. Surprisingly, a decrease in tau aggregation in the single-amino acid deletion mutants (Δ366, Δ367, and Δ368), which was observed in cells treated with AD seeds, was abolished in cells treated with three cases of PSP, three cases of CBD, and one case of FTDP-17 (N279K) patients’ brain lysates and recombinant tau seed (Fig. 8A). A decrease in

Figure 4. In vitro aggregation of recombinant tau-CTF24 deletion mutants. A recombinant tau-CTF24 (Del 1 to Del 10) solutions were incubated at 37°C on a shaker in the presence or absence of heparin (Hep). Tau aggregation was detected using ThS assays at the indicated times. Data are means ± S.D. (error bars) (n = 3). Data values at 96 h were analyzed statistically using Student’s t test versus the value of WT at 96 h (***, p < 0.001). a.u., arbitrary units; n.s., not significant. B, after >7 days of incubation with heparin, aggregated recombinant tau-CTF24 deletion mutants were analyzed using TEM. Magnification level in the micrographs is ×30,000. Scale bar, 200 nm.
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tau aggregation in cells expressing N368A, N368D, N368Q, and N368L was not observed in non-AD tauopathies (Fig. 8B). Furthermore, we confirmed that the deletion of Asn-368 also affected the aggregation of full-length 2N4R tau induced by AD tau seeds but not by other tauopathies or recombinant seeds (the results of FTDP-17 seeds were excepted because these seeds showed no aggregation property on 4R2N tau) (Fig. S4).

To confirm these results, we tested AD tau seed–specific effects on tau-CTF24 Δ368 using fluorescent immunocytochemical analysis. We constructed hemagglutinin (HA)-tagged tau-CTF24 and HA-tau-CTF24 Δ368, because the PHF-1 antibody strongly detected not only intracellular tau aggregates but also AD tau seeds, which should remain on the cell surface. Without seeds, SH-SY5Y cells expressing these constructs were homogenously stained with anti-HA antibody; however, AD brain lysate or recombinant 2N4R tau fibril induction demonstrated densely packed intracellular staining (Fig. 8C). Higher-magnification images revealed that some densely stained structures looked like aggregates (Fig. 8D, white arrowheads). When the cells were treated with recombinant tau seed or no seed, the ratio of intracellular aggregate–positive/–negative cells was almost same between cells expressing HA-tau-CTF24 and HA-tau-CTF24 Δ368, whereas in AD tau seed–induced cells expressing HA-tau-CTF24 Δ368, the positive/negative cell ratio was significantly decreased compared with HA-tau-CTF24–expressing cells (Fig. 8E), coincident with the results of immunoblotting analyses. Taken together, we concluded that the deletion or replacement effects of Asn-368 on tau aggregation can occur in a disease-specific manner; only AD tau strain–specific aggregation was affected.

The mutation effect of Δ368 on microtubule assembly of tau

We also investigated the effect of Δ368 mutation on the physiological function of tau: promoting MT assembly. Previous experiments have shown that the cellular function of tau on MT assembly is detectable using MT-bundling assays of tau-overexpressing cultured cell models (34–36).

Because tau-CTF24 has been reported to lose its ability to bind to MTs (22), we overexpressed full-length 4R2N tau (WT and Δ368) to Neuro2a cells, and MT bundles were detected as round-shaped fluorescence structures on tau-overexpressing cells (Fig. S5A, white arrowhead on magnified images), using anti-acetylated tubulin and tauC antibodies. We found that the Δ368 mutant showed significantly decreased MT bundling (Fig. S6B). To confirm this result, we prepared recombinant 4R2N tau (WT and Δ368) and conducted in vitro MT assembly assays as described previously (22, 37). As a result, the levels of MT assembly were significantly reduced with the Δ368 mutant compared with WT.

Collectively, we found that deletion of the Asn-368 residue also affected the MT-binding ability of tau, as seen for single-residue deletion mutants ΔK280 and ΔN296 (38–40).

Effects of Ala replacement of Val-318, Thr-319, and Ser-320 on tau aggregation

Recently, high-resolution core structures of AD tau filaments were determined by means of cryo-EM. These structures showed that the cores consist of two identical C-shaped units (306–378 aa), which have eight β-sheets (β1–β8) and a C-shaped architecture (Fig. 9A) (26, 33). Asn-368 is located in “β8 (368–378 aa)” opposing “β1 (306–311 aa, PHF6)” and “β2 (313–322 aa)” at a packing interface. In this structure, Val-318, Thr-319, and Ser-320 (on “β2”) are closely aligned with Asn-368 (on “β8”).

Therefore, we speculated that Val-318, Thr-319, or Ser-320 was also involved in Asn-368–associated tau aggregation. To examine this hypothesis, we constructed three types of Ala replacement mutants of tau-CTF24 (V318A, T319A, and S320A). As a result, only S320A aggregation was significantly decreased in cells treated with AD seed (Fig. 9B), but this effect was not observed in cells treated with non-AD tauopathy lysates or recombinant tau fibrils (Fig. 9C). On the other hand, the V318A mutation affected tau aggregation seeded by Rec, CBD, and FTDP-17 (N279K) tau.

These results indicated that Ser-320, the amino acid residue that is closely associated with Asn-368 in the core structures of AD tau filaments, is also essential for tau aggregation induced by AD tau seed but not by other tauopathies.

Discussion

In this study, we identified 353–368 aa as a novel aggregation–responsible sequence of tau-CTF24 (243–441 aa) and full-length tau (2N4R, 2N3R) using a series of deletion mutants of tau on cellular and in vitro tau aggregation models. In addition, we further explored the region that is more actively involved in tau aggregation by designing minor-deletion sequences in 353–368 aa, and Asn-368 was found as an essential single amino acid for aggregation of tau-CTF24 induced by AD tau seed. Of note, the Asn-368 deletion or replacement mutants of tau-CTF24 showed decreased aggregation only when the cells were treated with AD tau seed, not with recombinant or other tauopathies, including PSP, CBD, and FTDP-17 (N279K) tau seeds.

The 353–368 aa sequence is located in the fourth repeat of the MBD and is included in the Pronase-resistant core region of PHF (18). This region also harbors one hyperphosphorylation site (Ser–356) (41, 42), and several missense mutations (S356T, V363A/I, P364S/R, G366R) have been found in patients with frontotemporal dementia (FTD) (42–45), but the involvement of this sequence in tau aggregation has not yet been demonstrated.
been confirmed. Furthermore, we confirmed that PHF6 harboring 306–321 aa (Del 5) sequence itself can form ThS-positive fibrils in \textit{in vitro} like the 6-amino acid length PHF6 peptide.

On the contrary, 353–368 aa (Del 8) sequence showed no aggregation. In addition, TEM analysis revealed that recombinant tau fibrils with 353–368 aa (Del 8) deletion showed

\begin{itemize}
  \item \textbf{Peptide D5 (306–321):} VQIVYKPVDSLKVTSK
  \item \textbf{Peptide D7 (338–353):} EVKSEKLDKDRVQSK
  \item \textbf{Peptide D8 (353–368):} KIGSLDNITHVPGGGN
  \item \textbf{Peptide D9 (369–384):} KKIETHKLTFRENAKA
\end{itemize}
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abnormal morphology, compared with that of WT or other mutants. Taken together, we speculated that 353–368 aa (Del 8) sequence, which has no self-aggregation propensity, does not participate in tau fibrillation as a “driver” of aggregation like PHF6 but acts as a “regulator” of distinct fibrous structure formation.

Of note, regarding the 353–368 aa mutant tau, some inconsistent results seemed to depend on the experimental system (cellular or in vitro); the tau-CTF24 mutants of Del 8, Δ366, Δ367, and Δ368 showed decreased AD-seeded aggregation (Figs. 2A and 7B), but in a heparin-induced in vitro aggregation assay, only Del 8 exhibited reduced aggregation and abnormal filamentous EM structure (Figs. 4 and 7 (C and D)). As summarized in Fig. 6A, the Del 8 sequence (353–368 aa) is included in the AD tau core (274–379 aa) but does not exist in the core region of heparin-induced tau filaments (272–330 aa), and cryo-EM described their distinctly different structures. Therefore, the single-amino acid mutations of Δ366, Δ367, and Δ368 are outside of the heparin-induced tau core and caused no decrease in in vitro aggregation in the presence of heparin. On the other hand, deletion of the 353–368 aa sequence (15-aa length) reduced tau aggregation not only in a cellular AD model but also in a heparin-induced in vitro model, despite the fact that 353–368 aa is not included in the core region. We speculated that 1) 353–368 aa might include a specific region that plays a role in an early intermediate process of conformation change toward the fibrils but was finally excluded from the core of the completed heparin-induced fibrils, and 2) residues 366, 367, and 368 may not be included in that specific region so that the deletion of these amino acids did not affect heparin-induced tau aggregation. We were not able to clearly explain the exact cause of these discrepancies at the molecular level, but our data seem to reflect the structural and biochemical differences between human-derived AD tau and heparin-induced in vitro filament that have been discussed to date (30, 31, 46). In the cryo-EM structures of AD tau filament cores shown in Ref. 26 and Fig. 9A, 353–368 aa (Del 8) sequence ranges from the end of “β6” to the N-terminal tip of “β8”, and this sequence is opposite to the region ranging from the middle of “β2” to “β4” strand (318–339 aa, which almost corresponds with Del 6: 322–337 aa) in the core structure. Focusing on the amino acids Gly-366, Gly-367, and Asn-368, of which deletion from tau-CTF24 caused decreased aggregation in cellular models, the two glycine residues are located in the spacer region between “β7” and “β8”, and this region was at the sharp corner of the polypeptide chain. We first speculated that glycine residues play an essential role in this narrow space by taking advantage of its minimal side chain (one hydrogen atom), but contrary to our expectation, replacement of glycine residues with isoleucine, a hydrophobic amino acid that has a bulky side chain, did not cause any disturbance of cellular tau aggregation induced by AD tau seed (Fig. 7E). In the case of Asn-368, this residue is located at the N-terminal tip of “β8” opposing “β2” sheet in the atomic model of the AD tau filament. Tau-CTF24 mutants of N368A, N368D, N368Q, and N368L were prepared to investigate the involvement of the Asn residue in tau aggregation by altering the chemical structure of the side chain. The amido group of the Asn residue is known to undergo deamidation (47, 48) and N-glycosylation (49) and interact with the side chain of other amino acid residues or the peptide backbone via hydrogen bonds. Therefore, we predicted that N368A, N368D, and N368L, whose side chains no longer harbored an amido group, can affect tau aggregation. Despite our expectation, not only N368A, N368D, and N368L, but also N368Q, exhibited dramatic decreased tau aggregation in the cellular model, and this probably meant that the one-methylene group length displacement of the amido group was enough to cause the inhibition of AD tau-seeded tau aggregation. On the other hand, N368A mutant seeded with AD 2 and AD 3 seed showed a smaller decrease in the aggregation effect than that with AD 1 (Fig. 8B), despite the fact that these AD tau seeds showed almost the same typical AD banding pattern (Fig. S1). These data might indicate that AD seeds from different patients share similar structural and biochemical properties (32, 33), but they may not be completely identical, and in specific experimental conditions, these subtle differences will manifest as different results. It is difficult to clearly determine the reason why this event occurred only in N368A mutants, but considering the fact that only N368A mutation shortened its side chain among Asn-368 substitution mutants, we speculated that substitution of Asn-368 to Ala caused slightly increased local peptide flexibility around “Ala-368” and this might affect the receptivity against different AD seeds. Taken together, these data indicated that the proper configuration of the amido group of Asn-368 on tau aggregation processes was likely to be important for the formation of tau fibrils.

By focusing on the structures around Asn-368, we observed that the side chains of Val-318 and Ser-320 were arranged facing inward toward Asn-368 at the cross-β interface between “β1-2” and “β8” (only the Thr-319 side chain faced outward) (Fig. 9A). As a result of Ala replacement, we showed that the S320A mutant significantly decreased AD seed–induced tau aggregation. In contrast, T319A showed no decrease, and V318A, which seems to be the closest to Asn-368, showed only a mild decrease in tau aggregation. These findings showed that Ser-320 also participated in the tau aggregation and may be present at the interface between Asn-368 and Ser-320, such as hydrogen bonding between the amido group of Asn-368 and

Figure 6. The characteristics of candidate sequences involved in tau aggregation. A, the results of Figs. 2–4 are summarized as a schematic diagram in the left panel. The deletion mutants, which showed significantly decreased tau aggregation, are marked with check symbols in the table. The core regions of AD (red), 4RN recombinant (blue), PSP (green), and CBD (purple) tau fibrils are indicated by solid (determined by the cryo-EM method (26, 29)) and broken (tryptic-resistant fragments (32)) lines. The right panel shows synthetic peptides derived from the deletion sequences. B, peptides corresponding to 306–321 aa (Del 5) (which harbors the PHF6 sequence), newly identified 353–368 aa (Del 8), and its adjacent sequences 338–353 aa (Del 7) and 369–384 aa (Del 9) were prepared and designated as Pep D5, Pep D7, Pep D8, and Pep D9 and subjected to Ths assays. Data are means ± S.D. (error bars) (n = 3). C, incubated peptides were observed by TEM. Magnification level was ×30,000. Scale bar, 200 μm. D, schematic image of tau-CTF24 Del 8 and Ala 8, a mutant in which the 16 amino acids 353–368 aa were all substituted with alanine. E, aggregation assay of the cellular tau aggregation model expressing tau-CTF24 Del 8 and Ala 8 mutants. Cells expressing these mutants were treated with AD, PSP, or CBD brain lysates and recombinant 2N4R tau fibrils (Rec). After 2–3 days’ incubation, A68 buffer-soluble (sol) and sarkosyl-insoluble (insol) fractions were immunoblotted and detected using the T46 antibody.
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A

B

C

D

E

F
the hydroxyl group of Ser-320. However, we are unable to exclude the possibility that the S320A mutation decreased tau aggregation independently of Asn-368.

To date, a number of pathogenic missense mutations on MAPT have been reported, and their effects on tau function were also assessed experimentally (38, 42). Among them, single-amino acid deletion mutants ΔK280 and ΔN296 have been reported to lose their ability for MT assembly, and ΔK280 also showed increased filament formation and the local structural changes observed in the second repeat domain of this mutant (39, 40, 50). Here, we also showed that Asn-368 deletion not only decreased AD-seeded tau aggregation but also attenuated the MT-binding ability of tau. By analogy with these findings, we proposed that deletion of Asn-368 possibly induced a minor structural disturbance in soluble tau, which might be the cause of decreased MT-binding ability.

Previous reports have indicated that pathological tau aggregates of each tauopathy are highly diverse in their histological and biochemical patterns (32, 51) and showed different seeding potency in cellular and animal tauopathy models (16, 17). These diversities are termed as “tau strains.” In this study, tau-CTF24 mutants of Δ366, Δ367, Δ368, N368A/D/Q/L (Fig. 8, A and B), and S320A (Fig. 9C) showed significantly decreased aggregation only when cells expressing these mutants were treated with AD tau strain, but not with recombinant 2N4R or other non-AD tauopathy strain. As for the difference between tau strains, recent cryo-EM studies clearly indicated that different tau strains have distinct core structures as “C-shaped” (AD) (26, 33), “kinked hairpin” (recombinant 4R2N tau) (29), “elongated structure” (Pick’s disease) (52), and “four-layered fold” (CBD) (53, 54). Considering these findings, we speculated that the side chain of Asn-368 (and possibly Ser-320) is specifically involved in the formation of “C-shaped” core structure, and thereby tau Asn-368 mutant was not templated by AD tau seed. More recently, the cryo-EM method revealed that chronic traumatic encephalopathy tau also adopts a “C-shaped” core structure, which is similar to that of AD (55), and future studies testing the chronic traumatic encephalopathy tau seed effects on the aggregation of tau Asn-368 mutant can strengthen our hypothesis. Cryo-EM structures of other 4R tauopathies (PSP, FTDP-17) have not been reported, but considering our results, the structures of these 4R tauopathies are suspected to be distinctly different from those of AD. Recently, a combination of cryo-EM and MS revealed that AD tau has a pattern of post-translational modifications (acetylation, ubiquitination, trimethylation, and phosphorylation) distinct from that of CBD, and these modifications may mediate the formation of distinct tau strains (54). According to previous reports, the Lys-369 residue, which is adjacent to Asn-368, differed in its modifications in tauopathy cases as acetylated in AD and ubiquitinated in CBD. Although the detailed mechanisms of how these posttranslational modifications contribute to the formation of strain-specific fibril structures are not fully understood, we speculated on another possibility, that the Asn-368 residue might play a role in distinguishing the differences in tau strains (in this case, AD or CBD) by the molecular interaction between these modified residues.

Besides the Del 5 and Del 8 sequences, other deletion mutants also indicated the strain difference. Deletion of 275–290 aa (Del 3) significantly decreased PSP and CBD tau–seeded aggregation (recombinant 4R2N also showed mildly decreased aggregation) but not AD-seeded aggregation (Fig. 2A). Del 3 includes another aggregation-prone sequence, PHF6 (275–280 aa), and remarkably, only AD tau does not harbor this sequence in the core region (29, 32) (Fig. 6A). Based on these findings, we speculated that the presence or absence of an aggregation-prone sequence (PHF6 and PHF6*) in the regions expected to be included in an aggregation core is an important factor in the formation of tau aggregates. In addition, the deletion of 338–353 aa (Del 7) and 369–384 aa (Del 9) sequences, which do not include any aggregation-prone sequences and showed no self-aggregation ability in vitro (Fig. 6B and C), also indicated the strain difference: Del 7 mutation affected only PSP tau–seeded aggregation, and Del 9 mutation did not decrease CBD tau–seeded aggregation. It is difficult to explain the detailed mechanisms of these differences, but we speculated that these results may reflect the distinct structural differences between CBD and PSP tau for which cryo-EM structures have not yet been reported.

As a result of attempts to search for drugs that can inhibit the aggregation of tau, some chemical compounds have been tested in in vitro or in vivo tauopathy models and identified as tau aggregation inhibitors (56–61). In addition, a new drug-development strategy targeting specific regions of tau to efficiently inhibit tau aggregation has been tested, and antibodies (62) and PHF6-targeting peptide drugs (63–65) have been analyzed. Clinical trials of immunotherapy including an active vaccine, AAD-vac, targeting taupeptide sequence 294KDNKHKVPGG305 in the second repeat region, are in progress (66, 67). Most previous studies of tau-based therapy used mutated tau constructs or in vitro heparin assay systems; however, we used human AD seed–dependent cell models and referred to the result of cryo-EM analysis of human AD brain. In this respect, our data will be more accurate for searching for AD therapeutic targets.

Figure 7. Narrowing down of the aggregation-responsive sequences in Del 8 (Δ353–368 aa). A, schematic diagram of the experiment. First, the Del 8-1, Del 8-2, and Del 8-3 mutants were tested using an AD tau–induced cellular aggregation model. Del 8-2 and Del 8-3 showed significantly decreased tau aggregation (Fig. S2A). Next, Del 8-2, Del 8-2-1, Del 8-2-2, Del 8-3-1, and Del 8-3-2 were tested, and Del 8-2-2 and Del 8-3-2 showed significantly decreased aggregation (emphasized in red) (Fig. S2B). B, the mutants of tau-CTF24 Δ351, Δ352, Δ353, Δ356, Δ367, and Δ368–expressing cells were treated with AD brain lysate and cultured for 2–3 days. A68 buffer–soluble (sol) and Sarkosyl-insoluble (insol) fractions were immunoblotted and detected using the T46 antibody. Values are means ± S.D. (error bars) (n = 3), **p < 0.01; ***p < 0.001 by Student’s t test versus WT + seed. a.u., arbitrary units; n.s., not significant. C, in vitro aggregation of a series of recombinant tau-CTF24 mutants harboring single aa deletions (Δ366, Δ367, and Δ368). Aggregation was assessed using a ThS assay. Data are means ± S.D. (n = 3). Data values at 96 h were analyzed statistically using Student’s t test versus AD at 96 h (*, p < 0.05; a.u., arbitrary units; n.s., not significant. D, photographs of aggregated tau-CTF24 mutants obtained by TEM. Magnification level in the micrographs is ×30,000. Scale bar, 200 nm. E and F, tau-CTF24 mutants that harbor replaced single amino acids (G366I or G367I) (Δ) and (N368A, N368D, N368Q, or N368L) (F) were generated by site-directed mutagenesis. The mutants were overexpressed in SH-SYSY cells and treated with AD brain lysates. A68 buffer–soluble (sol) and Sarkosyl-insoluble (insol) fractions of the cells were prepared and analyzed by immunoblotting. Data are means ± S.D. (n = 3), **p < 0.01 by Student’s t test versus WT + seed. a.u., arbitrary units; n.s., not significant.

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(A) Soluble and insoluble fractions of AD and Rec seeds. (B) Soluble and insoluble fractions of PSP and CBD seeds. (C) Immunofluorescence images of HA-CTF WT and HA-CTF Δ368 in the absence and presence of seed. (D) Immunofluorescence images of WT, Δ368, WT + Rec, and Δ368 + AD seeds. (E) Bar graphs showing the percentage of aggregates positive cells in the absence and presence of Rec and AD seeds.
Collectively, we expected that antibodies, peptides, or other chemical compounds that specifically recognize and block 353–368 aa or Asn-368 might be promising candidates as tau aggregation inhibitors. In this study, we found that Asn-368 of tau was an essential single amino acid for the formation of tau aggregates in tau-CTF24 cell models. This effect was only observed in AD seed

![Diagram](image)

**Figure 8.** The effect of single-amino acid deletions or replacements on tau aggregation induced by different types of tauopathy seeds. A and B, series of single-amino acid deletion or replacement mutants of tau-CTF24 (Δ361, Δ362, Δ363, Δ366, Δ367, or Δ368 (A) or N368A, N368D, N368Q, N368L, or Δ368 as positive control (B)) were transfected into SH-SYSY cells. Subsequently, cells were treated with the different types of tauopathy brain lysates (including two other cases of AD, three cases of PSP, three cases of CBD, and one case of FTDP-17) or recombinant 4R2N tau fibrils. Soluble and insoluble fractions of cells were prepared and analyzed by immunoblotting. The AD1 blots in C were the reuse of B.

C, HA-tagged tau-CTF24 WT or Δ368 mutant was expressed in SH-SY5Y cells and treated with AD brain lysates or recombinant 4R2N tau fibrils. Cells were stained with an anti-HA mAb (green), an anti-tubulin polyclonal antibody (red), and DAPI (blue). D, the selected area (red square) on the confocal microscopic images was magnified, and distinct tau aggregates detected using an anti-HA antibody are marked with white arrowheads. E, total cells and cells with HA-positive tau aggregates were counted, and the ratios of tau aggregate–positive cells were compared between WT and Δ368-expressing cells treated with AD brain lysates or recombinant 4R2N tau fibrils. Values are means ± S.D. (error bars) (n = 3). *** p < 0.001 by Student’s t test. n.s., not significant.
induction, not with PSP, CBD, or FTDP-17 (N279K) seeds. These findings will be helpful not only for the development of tau aggregation inhibitors but also in the elucidation of the molecular mechanisms of tau-seeding events and detailed characteristics of tau strains.

**Experimental procedures**

**Construction of plasmids**

To construct a series of deletion mutants lacking sequences of 20 amino acid residues in tau-CTF24 and full-length tau (4R2N and 3R2N isoforms), we conducted site-directed mutagenesis of pcDNA3-tau-CTF24 (22), pcDNA3-4R2N tau, and pcDNA3-3R2N tau for cellular expression and pRK172-tau-CTF24, pRK172-4R2N tau, and pRK172-3R2N tau for bacterial expression. PCR was carried out using a site-directed mutagenesis kit (Stratagene) and a KOD-plus mutagenesis kit (TOYOBO) with a set of primers (Table S1).

**Antibodies**

The following tau antibodies were used. A monoclonal tau antibody (clone T46, Thermo Fisher Scientific) that recognizes the C-terminal region of tau and an mAb (PHF-1) that recognizes phosphorylated Ser-396 and Ser-404 of tau were generated in the C-terminal region of tau and an mAb (PHF-1) that recognizes phosphorylated Ser-396 and Ser-404 of tau were gener-

**Preparation of patient brain lysate**

All experimental procedures for brain autopsy and the use of the human brain sample were approved by the Juntendo University School of Medicine Ethics Committee (approval number 2012068). This research was conducted according to the principles expressed in the Declaration of Helsinki. Human brain samples were obtained from the Juntendo University Hospital brain bank. Samples were from pathologically proven patients with tauopathies, including the frontal cortices of AD (n = 3), pons of PSP (n = 3), frontal cortices of CBD (n = 3), and pons of frontotemporal dementia with MAPT (N279K mutation) (Table S2).

Frozen brain tissues were cut into 0.2-g blocks and immediately homogenized in 2 ml of A68 buffer (10 mM Tris-HCl, pH 7.5, 10% sucrose, 0.8 mM NaCl, 1 mM EGTA) using a Dounce homogenizer. Lysates were sonicated using an ultrasonic homogenizer (VP-050N, TAITEC) for 1 min (power 25.0%) and centrifuged at 3,000 × g for 10 min at 4 °C. The supernatants were separated into 15–20 samples in 1.5-l plastic tubes (each 30–50 μl) and stored at −80 °C. For confirmation of the presence of aggregated pathological tau, sarkosyl-insoluble fractions of brain lysates were prepared and analyzed biochemically by immunoblotting as follows. 30–50 μl of brain lysates were resuspended in 10 volumes (v/v) of A68 buffer containing 1% sarkosyl and sonicated using an ultrasonic homogenizer for 1 min (power 25.0%). Samples were centrifuged at 99,000 × g for 20 min, and resulting pellets were washed by centrifugation with 0.5 ml of sterile saline solution. The pellets were solubilized in SDS-sample buffer and subjected to 15% polyacrylamide gel SDS-PAGE. Proteins in gels were transferred onto a polyvinylidene difluoride membrane and visualized by an ABC method using a T46 antibody (Fig. S1).

**Purification of recombinant tau and preparation of tau fibrils**

*Escherichia coli* BL21 (DE3) cells were transformed with the expression plasmid pRK172 harboring tau sequences (tau-CTF24, 4R2N, 3R2N, and their mutants). After mass culture of transformed BL21 in 500 ml of 2× YT medium, recombinant tau proteins were purified as described previously (56, 68, 69). After dialysis against 1× PBS, samples were centrifuged at 99,000 × g for 20 min, and the supernatant was used as a recombinant tau monomer. The concentration of tau protein was estimated using a BCA Protein Assay Kit (Pierce). To prepare tau fibrils, 350 μl of 1 mg/ml recombinant tau with 0.1 mg/ml heparin, 10 mM DTT, and 0.1% sodium azide (NaN3) were incubated on a shaker at 37 °C. After 3–7 days’ incubation, the samples were centrifuged at 99,000 × g for 20 min at room temperature, and the pellets were recovered in 100 μl of 1× PBS containing 0.1% NaN3.

**In vitro thioflavin S aggregation assay of recombinant tau**

100 μl of 15 μM recombinant tau in 30 mM Tris-HCl (pH 7.5) were mixed with 0.1 mg/ml heparin, 1 mM DTT, and 0.1% NaN3 as described above and incubated on a shaker at 37 °C. Then 10-μl aliquots of samples were collected at the indicated times and mixed with 200 μl of 20 mM MOPS buffer (pH 6.8) containing 5 μM thioflavin S (ThS). After a 30-min incubation in the dark at room temperature, ThS fluorescence (436-nm excitation/535-nm emission) was measured at the indicated times (0, 6, 24, 48, 72, and 96 h) using a Flexstation II (Molecular Devices).

**Cell culture**

SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (Sigma) supplemented with 10% fetal calf serum, MEM NonEssential Amino Acid Solution (Gibco), penicillin-streptomycin-glutamine (Gibco). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in a culture chamber.

**Transfection of plasmids and induction of tau seeds into cells**

SH-SY5Y cells were grown to 70% confluence in 6-well culture dishes, and transfection of expression plasmids into the cells was carried out using X-treme GENE9 (Roche Applied Science) in accordance with the manufacturer’s instructions. Tau seeds (recombinant tau fibrils or patients’ brain lysates) were suspended in 62.5 μl of Opti-MEM (Gibco) and mixed with 30 μl of Multifectam (Promega) for transfection into SH-SY5Y cells. After a 30-min incubation at room temperature, 30 μl of Opti-MEM was added and further incubated for 5 min at room temperature. The seed mixture was added to SH-SY5Y cells immediately after transfection of the plasmid, and cells were further incubated at 37 °C in a CO2 chamber for 2–3 days.

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**Extraction of sarkosyl-insoluble tau from cultured cells and immunoblotting analysis**

Transfected SH-SY5Y cells were incubated in a 6-well plate for 2–3 days and harvested in 1,000 μl of 1× PBS. Cells were collected by centrifugation at 14,000 × g for 10 min and suspended in 150 μl of A68 buffer. After sonication for 1 min (power 25.0%) in iced water, cell lysates were centrifuged at 99,000 × g for 20 min at 4°C. The supernatants were recovered as an A68 buffer–soluble fraction, and the pellets were suspended in A68 buffer containing 1% Triton X-100. The samples were centrifuged at 99,000 × g for 20 min at 4°C, and the resulting pellets were suspended in A68 buffer containing 1% sarkosyl and further centrifuged under the same conditions. The pellets were lysed in SDS-sample buffer and heated at 100°C for 5 min to prepare a sarkosyl-insoluble fraction (ppt). Protein concentration was estimated using a BCA Protein Assay Kit. Samples were loaded on 15% SDS-polyacrylamide gels and electrophoresed with a Tris-glycine buffer system. Proteins in the gels were transferred onto polyvinylidene difluoride membrane (Millipore) and blocked with 3% gelatin. The blots were incubated overnight with the indicated primary antibodies in 10% calf serum at an appropriate dilution (1:1,000–5,000) at room temperature. Membranes were washed and incubated for 2 h with a biotin-labeled secondary antibody (Vector) at room temperature. Signals were detected using an ABC staining kit (Vector), and resulting blots were analyzed using Image software (National Institutes of Health).

**Transmission EM**

For TEM, aliquots (2 μl) of recombinant tau fibril solution (after a 96-h incubation on a shaker at 37°C) were placed on collodion-coated 300-mesh copper grids and stained with 2% (v/v) sodium phosphotungstate. Micrographs were recorded at a nominal magnification of ×30,000 on a HT7700 electron microscope (HITACHI).

**Immunocytochemistry**

Transfection of expression plasmids and induction of tau seeds were conducted as described above, using SH-SY5Y cells grown on coverslips. After incubation for 2 days in a CO2 incubator, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Fixed cells were washed with 1× PBS and permeabilized with 0.2% Triton X-100 in 1× PBS for 10 min. After blocking for 30 min in 5% BSA (in 1× PBS), samples were stained with the indicated primary antibodies at 1:1,000 dilution. After incubation for 1 h at 37°C, the cells were washed three times with 1× PBST and further treated with secondary antibodies (anti-rabbit IgG-conjugated Alexa-555 or anti-mouse IgG-conjugated Alexa-488, Invitrogen) at 1:500 dilution. After 1 h, the cells were washed three times with 1× PBST and mounted on microslides using ProLong Diamond Antifade Mountant with DAPI (Molecular Probes) and analyzed using an LSM5 Pascal confocal microscope (Carl Zeiss).

**Statistical analysis**

All values in the figures are expressed as mean ± S.D. Biochemical data were statistically analyzed using the unpaired, two-tailed Student’s t test. p ≤ 0.05 was considered to be statistically significant.

**Data availability**

All data are provided in the article, and raw data are available upon request.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: MT, microtubule; AD, Alzheimer’s disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; MBD, microtubule-binding domain; CTF, C-terminal fragment; ThS, thioflavin S; TEM, transmission electron microscopy; HA, influenza hemagglutinin; PHF paired-helical filament; aa, amino acids; ABC, avidin-biotin complex; DAPI, 4′,6-diamino-2-phenylindole.

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