The natural osmolyte trimethylamine *N*-oxide (TMAO) restores the ability of mutant tau to promote microtubule assembly

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Abstract Coding region and intronic mutations in the gene for microtubule-associated protein tau cause frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). Most coding region mutations effect a reduced ability of tau protein to interact with microtubules and lead to the formation of a filamentous pathology made of hyperphosphorylated tau. Here we show that trimethylamine *N*-oxide (TMAO) restores the ability of tau with FTDP-17 mutations to promote microtubule assembly. To mimic phosphorylation, serine and threonine residues in tau were singly or multiply mutated to glutamic acid, resulting in a reduced ability of tau to promote microtubule assembly. With the exception of the most heavily substituted protein (27 glutamic acid residues), TMAO increased the ability of mutant tau to promote microtubule assembly. However, it had no significant effect on heparin-induced assembly of tau into filaments.

Key words: Tau protein mutation; Frontotemporal dementia; Phosphorylation; Microtubule assembly; Trimethylamine *N*-oxide

1. Introduction

The discovery of mutations in the gene for microtubule-associated protein tau in frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) has shown that dysfunction of tau protein is sufficient to cause neurodegeneration and dementia [1–4]. Known tau mutations are intronic mutations located close to the splice-donor site of the intron following exon 10, and missense, deletion or silent mutations in the coding region. A filamentous pathology made of hyperphosphorylated tau protein is an invariant neuropathological characteristic of FTDP-17 [5].

Six tau isoforms are produced in the adult human brain by alternative mRNA splicing from a single gene [6]. They differ from each other by the presence or absence of 29 or 58 amino acid inserts located in the amino-terminal half and an additional 31 amino acid repeat located in the carboxy-terminal half. Inclusion of the latter, which is encoded by exon 10 of the tau gene, gives rise to the three isoforms with four repeats each; the other three isoforms have three repeats each. Similar levels of three-repeat and four-repeat tau isoforms are present in normal adult human brain [7]. The repeats and some adjoining sequences constitute the microtubule-binding domains of tau [8].

The primary effect of the intronic and some coding region mutations in exon 10 is at the mRNA level [2–4,9–11]. This results in a change in the ratio of three- to four-repeat tau isoforms and the net overproduction of four-repeat tau. By contrast, most coding region mutations reduce the ability of mutant tau protein to interact with microtubules [12–18] and some of these mutations also stimulate the in vitro assembly of tau into filaments [19–21]. The reduced ability of tau to interact with microtubules probably leads to its hyperphosphorylation. This exacerbates the functional deficit, since hyperphosphorylated tau is known to be unable to bind to microtubules or to promote microtubule assembly [4].

Recent studies have shown that the organic osmolytes trimethylamine *N*-oxide (TMAO) and betaine increase tau-promoted assembly of microtubules [22] and restore the ability of tau phosphorylated by cAMP-dependent protein kinase or glycogen synthase kinase-3β to promote microtubule assembly [23]. Here, we have investigated the effects of TMAO on the ability of tau protein with FTDP-17 mutations to promote microtubule assembly. In addition, we have introduced glutamic acid in place of serine and threonine residues, most of which are phosphorylated in pathological tau, to mimic phosphorylation. The ability of these glutamate mutant tau proteins to promote microtubule assembly in the presence and absence of TMAO was also investigated.

2. Materials and methods

2.1. Expression and purification of wild-type and mutated tau proteins

Site-directed mutagenesis was used to change K257 to threonine or glutamic acid, G272 to valine, to delete K280, to change P301 to leucine, serine or glutamic acid and to change V337 to methionine in the four-repeat 412 amino acid isoform of human tau (expressed from cDNA clone tau46) (in the numbering of the 441 amino acid isoform of human tau). In addition, S235, S262, S356 and S396 were separately changed to glutamic acid in the four-repeat 441 amino acid isoform of human tau (expressed from cDNA clone tau40), to mimic phosphorylation at single sites. Three additional constructs (called Etau8, Etau18 and Etau27) were produced using polymerase chain reaction methods, in which multiple serine and threonine residues were changed to glutamic acid in the 441 amino acid isoform of human tau. In Etau8, residues downstream of the repeat region were replaced by glutamic acid. In Etau18, residues upstream of the repeats were replaced by glutamic acid. Etau27 represents a composite of Etau8 and Etau18, with the addition of S262E. The mutated residues were: S396, S400, T403, S404, S409, S412, S413 and S422 (for Etau8); S46, T50, T69, T111, T153, T175, T181, S198, S199, S202, T205, S208, S210, T212, S214, T217, T231 and S235 (for Etau18);

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S46, T50, T69, T111, T153, T175, T181, S198, S199, S202, T205, S208, S210, T212, S214, T217, T231, S235, S262, S396, S400, T403, S404, S409, S412, S413 and S422 (for Etau27). All constructs were verified by DNA sequencing and by mass spectrometric determination of the molecular masses of expressed proteins. Wild-type and mutated tau proteins were expressed in *Escherichia coli* BL21(DE3), as described [7]. Bacterial pellets were resuspended in 50 mM PIPES, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.5 μg/ml leupeptin, pH 6.8, followed by a 2×1-min sonication on ice using a Kontes Micro Ultrasonic Disrupter. The homogenates were centrifuged at 27 000×*g* for 15 min. Supernatants were loaded onto phosphocellulose columns (bed volume, 2 ml) equilibrated in extraction buffer. The columns were washed in extraction buffer, followed by extraction buffer+0.1 M NaCl. The protein was eluted batchwise with 6 ml extraction buffer containing 0.3 M NaCl and the eluates passed through 0.2 μm Acrodisc. This was followed by overnight dialysis against a saturated ammonium sulphate solution and precipitation by a 10-min centrifugation at 50 000 rpm (Beckman TL100). The pellets were resuspended in extraction buffer and reprecipitated by addition of an equal volume of saturated ammonium sulphate. Following centrifugation, the pellets were resuspended in 1 ml extraction buffer containing 0.5 M NaCl and 2% 2-mercaptoethanol, and held at 100°C for 5 min. Following a 10-min centrifugation at 50 000 rpm, the supernatants were loaded onto NAP10 columns equilibrated in 80 mM PIPES, 1 mM EGTA, 0.2 mM $MgCl_2$, 1 mM DTT (microtubule assembly buffer minus GTP) and eluted with 1.5 ml of the same buffer. GTP was added to 1 mM. Tau protein concentrations were determined using densitometry (Molecular Dynamics) of Coomassie blue-stained polyacrylamide gels and calibration against tau protein of known concentration. Bovine serum albumin was used as the standard for densitometry. In all experiments, wild-type and mutant tau proteins were expressed and purified in parallel.

2.2. Microtubule assembly

Purified recombinant wild-type and mutant htau40 and htau46 were used at 0.1 mg/ml. They were incubated with bovine brain tubulin (1 mg/ml, 20 μM, Cytoskeleton) in assembly buffer at 37°C, as described [12]. TMAO (Sigma Fine Chemicals) or betaine (Sigma Fine Chemicals) was used at 200 mM. The assembly of tubulin into microtubules was monitored over time by a change in turbidity at 350 nm. Maximum assembly rate was measured for each turbidity/time plot by taking the slope of the tangent at the steepest point of the curve and expressed as A_{350} nm/min. Relative assembly rates were calculated from the averages of the maximum rates in the presence or absence of TMAO.

2.3. Tau filament assembly

Tau proteins were expressed and purified as described [21]. Purified wild-type htau46 and htau46P301S (3 mg/ml) were incubated in the absence or the presence of various concentrations of heparin (100, 200 and 400 μg/ml; British Drug House) in 25 μl of 30 mM MOPS, 1 mM 4-(2-aminoethyl)benzenesulphonylfluoride (Calbiochem), pH 7.4, at

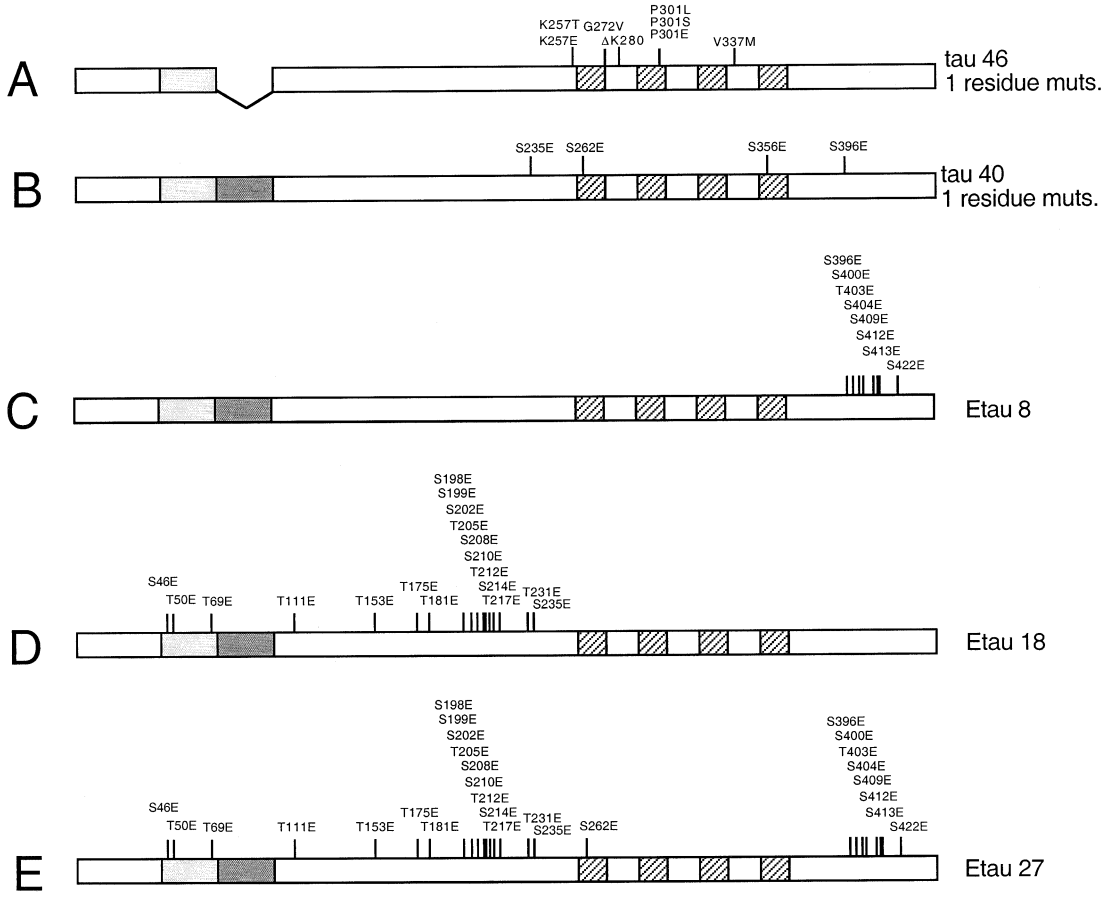

Fig. 1. Amino acid changes in tau protein whose effects on tau-promoted microtubule assembly were investigated. A: Schematic drawing of the 412 amino acid isoform of human tau (tau46). The four tandem repeats are shown as shaded bars, with the amino-terminal 29 amino acid insert shown in light grey. The positions of FTDP-17 mutations K257T, G272V, ΔK280, P301L, P301S and V337M are indicated. Amino acid substitutions K257E and P301E are also shown. All residues were mutated individually. B: Schematic diagram of the 441 amino acid isoform of human tau (tau40). The repeats are shown as shaded bars, with the two 29 amino acid amino-terminal inserts shown in light and dark grey, respectively. Amino acid substitutions S235E, S262E, S356E and S396E are indicated. The four residues were mutated individually. C–E: Schematic diagram indicating the positions of eight (C), 18 (D) or 27 (E) S or T to E mutations that were introduced into the 441 amino acid isoform of human tau to generate Etau8, Etau18 and Etau27.

37°C for up to 48 h, as described [24]. Experiments were carried out in the presence or absence of 100–800 mM TMAO. Aliquots were taken at 20 and 48 h, placed onto carbon-coated 400-mesh grids and stained with 1% lithium phosphotungstate. Micrographs were recorded at a nominal magnification of ×40 000 on a Philips model EM208S electron microscope. Each filament assembly experiment made use of freshly prepared batches of recombinant wild-type and mutant tau proteins that had been purified in parallel. Three separate assembly experiments were carried out.

3. Results

The amino acid changes in tau protein whose effects on tau-promoted microtubule assembly were studied are shown schematically in Fig. 1. Tau proteins with the FTDP-17 mutations K257T, G272V, ΔK280, P301L, P301S and V337M [1,2,14, 16,18] showed a reduced ability to promote microtubule assembly, in confirmation of our previous results [12,14,16,18] (Fig. 2). The same was true of tau proteins with artificial

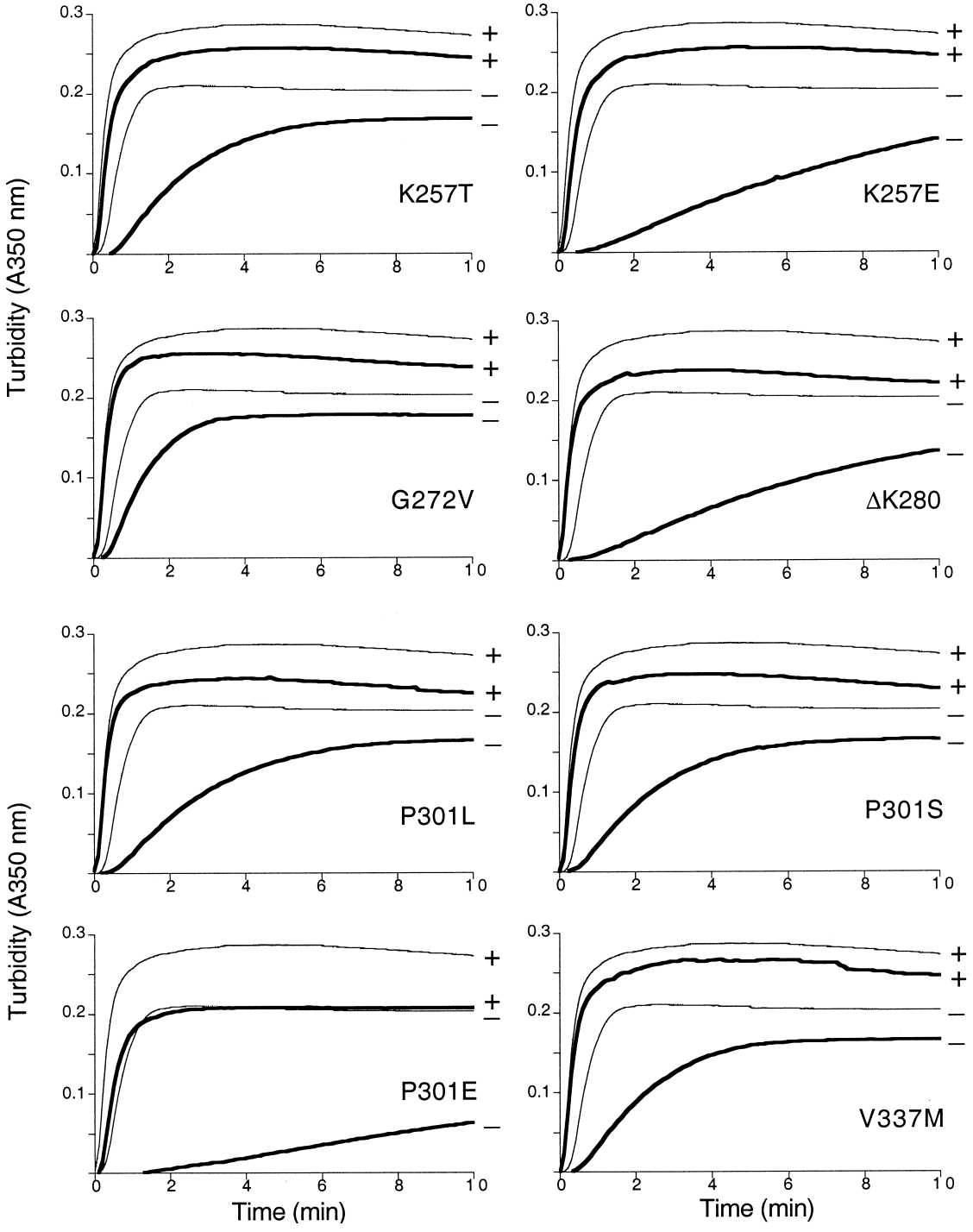

Fig. 2. Effects of tau mutations K257T, K257E, G272V, ΔK280, P301L, P301S, P301E and V337M on the ability of tau to promote microtubule assembly (in bold), as compared with wild-type 412 tau46 (regular). Experiments were carried out in the absence (−) or the presence (+) of 200 mM TMAO. Polymerisation of tubulin was monitored over time by turbidimetry. A typical experiment is shown; similar results were obtained using at least three separate preparations of each mutant protein.

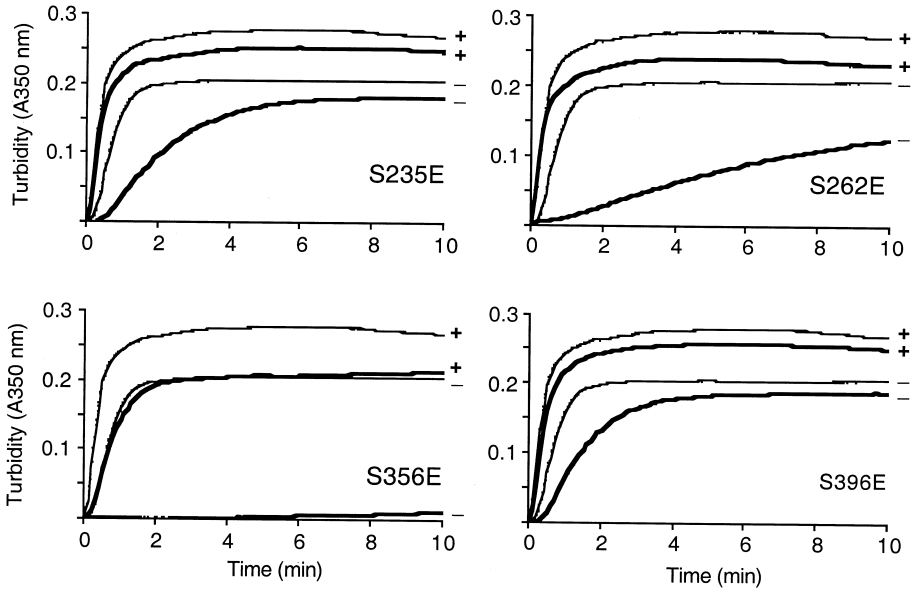

Fig. 3. Effects of tau mutations S235E, S262E, S356E and S396E on the ability of tau protein to promote microtubule assembly (in bold), as compared with wild-type tau40 (regular). Experiments were carried out in the absence (−) or the presence (+) of 200 mM TMAO. Polymerisation of tubulin was monitored over time by turbidimetry. A typical experiment is shown; similar results were obtained using at least three separate preparations of each mutant protein.

mutations K257E and P301E. When compared with the effects of the K257T, P301L and P301S mutations, the presence of glutamic acid at codons 257 and 301 resulted in a much greater reduction in the ability of tau to promote microtubule assembly (Fig. 2). When 200 mM TMAO was included in the assembly mixture containing tau and tubulin, the rate and extent of the increase in turbidity given by the tau mutants were equivalent or greater than those seen with wild-type tau in the absence of TMAO (Fig. 2). In the presence of TMAO, wild-type tau also assembled at a faster rate and to a greater extent, in confirmation of previous findings [22,23] (Fig. 2). The relative maximum rate for assembly in the presence and absence of TMAO was 2.7 for wild-type htau46 and ranged between 4.6 and 47 for the mutants examined. Similar results were obtained using betaine instead of TMAO (not shown).

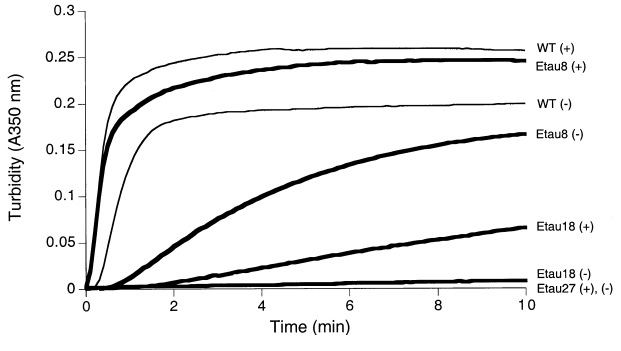

Fig. 4. Effects of constructs Etau8 (S396E, S400E, T403E, S404E, S409E, S412E, S413E, S422E), Etau18 (S46E, T50E, T69E, T111E, T153E, T175E, T181E, S198E, S199E, S202E, T205E, S208E, S210E, T212E, S214E, T217E, T231E, S235E) and Etau27 (mutations of Etau8+Etau18+S262E) on the promotion of microtubule assembly (in bold), as compared with wild-type htau46 (regular). Experiments were carried out in the absence (−) and the presence (+) of 200 mM TMAO. Polymerisation of tubulin was monitored over time by turbidimetry. A typical experiment is shown; similar results were obtained using at least three separate preparations of each mutant protein.

To mimic the effects of phosphorylation, we separately replaced serine residues 235, 262, 356 or 396 with glutamic acid. A reduced ability to promote microtubule assembly was observed for each of the mutant tau proteins (Fig. 3). It was particularly pronounced for tau with the S262E and S356E mutations, with smaller effects for tau with the S235E and S396E mutations. In the presence of TMAO, tau proteins with the S262E and S356E mutations promoted microtubule assembly at a similar or faster rate than wild-type four-repeat tau (Fig. 3). Tau proteins with the S235E and S396E mutations were better at promoting microtubule assembly in the presence of TMAO than wild-type tau in the absence of the osmolyte. The relative maximum rate for assembly in the presence and absence of TMAO was 1.7 for wild-type htau40. It was 6.3 for S235Etau40, 24 for S262Etau40, 144 for S356Etau40 and 3.5 for S396Etau40. No microtubule assembly was observed when tubulin and 200 mM TMAO were incubated in the absence of tau (data not shown).

Tau proteins were produced in which 8, 18 or 27 glutamic acid residues replaced serines and threonines (Etau8, Etau18, Etau27). Many of these residues are phosphorylated in pathological tau. Etau8, Etau18 and Etau27 showed a greatly reduced ability to promote microtubule assembly (Fig. 4). However, in the presence of TMAO, Etau8 promoted microtubule assembly to a greater extent than wild-type tau. TMAO only had a small effect on the ability of Etau18 to promote microtubule assembly, with no effect on Etau27 (Fig. 4).

The effect of TMAO on the heparin-induced assembly of tau into filaments was investigated by electron microscopy using wild-type htau46 and htau46 with the P301S mutation. No significant effect of TMAO (100, 200, 400 or 800 mM) was observed on either the rate of assembly or the number of filaments after 48 h.

4. Discussion

Most coding region mutations in *Tau* associated with

FTDP-17 reduce the ability of tau protein to promote microtubule assembly [12–18]. This is believed to be their primary effect. Here we show that the naturally occurring osmolyte TMAO is able to overcome the functional deficit caused by these mutations.

In the presence of 200 mM TMAO, recombinant tau proteins with six different coding region mutations (K257T, G272V, ΔK280, P301L, P301S or V337M) promoted microtubule assembly at a faster rate and to a more significant extent than wild-type tau. Of the known mutations in *Tau*, K257T and P301S create potential new phosphorylation sites. To mimic phosphorylation, we produced K257E and P301E recombinant four-repeat tau. Both proteins were much poorer at promoting microtubule assembly than tau proteins with the K257T or P301S mutations, indicating that phosphorylation of T257 and S301 in FTDP-17 brain could significantly exacerbate the primary effects of these missense mutations. In the presence of 200 mM TMAO, K257Etau46 and P301Etau46 promoted microtubule assembly to wild-type levels.

In all diseases with tau pathology, whether sporadic or familial, filamentous tau is abnormally hyperphosphorylated [4,5]. Many studies have helped to identify phosphorylation sites, as well as protein kinases and protein phosphatases that may be involved. In order to mimic the effects of phosphorylation, we mutated serine and threonine residues, either singly or multiply, to glutamic acid. Of the four singly mutated sites (S235E, S262E, S356E and S396E), tau protein with the S356E mutation showed a greatly reduced ability to promote microtubule assembly. A large effect was also observed for tau40 with the S262E mutation, with smaller effects resulting from mutations S235E and S396E. In the presence of 200 mM TMAO, the mutant tau proteins behaved like wild-type four-repeat tau in their ability to promote microtubule assembly. These results are in line with findings showing that phosphorylation of the KIGS motifs in the repeat region leads to a marked reduction in the ability of tau to interact with microtubules [25,26]. Phosphorylation of S262 and S356 in tau appears to play a major part in regulating its ability to interact with microtubules.

However, phosphorylation at these sites in filamentous tau is variable. Although S262 and S356 are phosphorylated to some extent in filamentous tau in Alzheimer's disease brain [27,28], they are not phosphorylated in Pick bodies in Pick's disease [29] or in the Pick body-like inclusions in cases of FTDP-17 with mutations K257T and G272V in tau [18,30]. This contrasts with S235 and S396 which are phosphorylated in all the known filamentous tau deposits [4]. Tau protein with the S235E or S396E mutations showed a somewhat reduced ability to promote microtubule assembly.

In the tauopathies, numerous serine and threonine residues are hyperphosphorylated [4]. We therefore produced Etau8, Etau18 and Etau27. In Etau8, mutations were located downstream of the repeats, in the carboxy-terminal region of tau. Etau8 was still able to promote microtubule assembly, albeit inefficiently, with TMAO restoring it to full activity. Etau18, with mutations in the amino-terminal half of tau, was greatly impaired in its ability to promote microtubule assembly. TMAO was able to increase that ability, but not to control levels. Etau27 contained S262E, together with the combined Etau8 and Etau18 mutations. It was totally unable to promote microtubule assembly, either in the presence or the absence of 200 mM TMAO. These findings are consistent with the fact that hyperphosphorylated tau protein from Alzheimer's disease brain is unable to promote microtubule assembly [4]. They suggest that TMAO would not be able to overcome the functional deficit caused by hyperphosphorylation of filamentous tau. In a recent study, a tau mutant with 10 glutamic acid residues instead of serines was produced [31]. It was still able to bind to taxol-stabilised microtubules, but was unable to interact with the membrane cortex. Based on the present findings, it appears probable that this Etau10 mutant would show a greatly reduced ability to promote microtubule assembly.

TMAO can force thermodynamically unfolded proteins to fold by causing a preferential hydration of exposed polypeptide backbone and side chains [32–34]. It has been found to raise the free energy of the unfolded state of proteins and to promote folding by a solvophobic effect. Previous studies have shown that organic osmolytes inhibit the conversion of the cellular prion protein to the protease-resistant form associated with transmissible spongiform encephalopathies [35,36]. This potential protective effect in prion diseases contrasts with the fibril-enhancing effect of TMAO on the β-amyloid protein (Aβ) associated with Alzheimer's disease [37].

Tau is a highly flexible, extended molecule with little secondary structure [21,38,39]. By circular dichroism spectroscopy, it appears as a random coil [38], even when carrying FTDP-17 mutations [21] or in the presence of TMAO [22]. Binding of tau to microtubules generates some ordered structures, indicating that microtubules can induce conformational changes in tau [40]. TMAO probably induces a tubulin and/or tau conformational change that favours tubulin assembly. The FTDP-17 missense and deletion mutations, as well as the glutamic acid mutations, appear to change the conformation of tau in such a way that it is less able to interact with microtubules. TMAO overcomes this effect, presumably by promoting the tubulin-induced folding of tau. The FTDP-17 mutations that reduce the ability of tau to interact with microtubules will favour assembly into filaments, because they increase the proportion of unfolded or partially folded conformations. This is reminiscent of the mechanism believed to underlie the assembly of other amyloidogenic proteins [41,42]. Hyperphosphorylation of tau is likely to exacerbate this process.

Unlike its effects on microtubule assembly, TMAO did not influence heparin-induced assembly of tau into filaments. It suggests that distinct conformations are involved in physiological tau folding and pathological tau aggregation. This is borne out by findings showing that known missense and deletion mutations reduce the ability of tau to promote microtubule assembly [12–18], whereas some of the same mutations increase heparin-induced assembly of tau into filaments [19–21]. The accelerated formation of tau assemblies from unfolded protein is probably a second mechanism underlying the formation of tau pathology in FTDP-17.

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