Mutations in tau reduce its microtubule binding properties in intact cells and affect its phosphorylation

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Abstract In vitro evidence has suggested a change in the ability of tau bearing mutations associated with fronto-temporal dementia to promote microtubule assembly. We have used a cellular assay to quantitate the effect of both isoform differences and mutations on the physiological function of tau. Whilst all variants of tau bind to microtubules, microtubule extension is reduced in cells transfected with 3-relative to 4-repeat tau. Mutations reduce microtubule extension with the P301L mutation having a greater effect than the V337M mutation. The R406W mutation had a small effect on microtubule extension but, surprisingly, tau with this mutation was less phosphorylated in intact cells than the other variants.
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Key words: Tau; Fronto-temporal degeneration; Mutation; Microtubule; GSK-3

1. Introduction

Abnormal accumulations of the microtubule associated protein tau in paired helical or straight filaments characterise a number of neurodegenerative conditions including Alzheimer's disease, progressive supranuclear palsy and fronto-temporal degeneration [1]. The process whereby tau becomes deposited in these structures is not understood although evidence points to an early increase in phosphorylation in AD (reviewed in [2]). Linkage of a form of fronto-temporal degeneration to chromosome 17 (FTDP-17) was followed by the identification of mutations in the tau gene in a number of families [3–8].

The tau gene is alternatively spliced to produce six isoforms in adult human brain differing by possession of three or four microtubule binding domains and one or two amino-terminal inserts of unknown function. Some missense mutations associated with FTDP-17 are localised in the microtubule binding domain, whilst another (R406W) is in the adjacent C-terminal region. The intronic mutations have been shown to result in an increase in generation of isoforms with four microtubule binding domains probably because of an alteration in secondary structure of the splice donor site following exon 10 [5,6]. It has been suggested that an imbalance in the relative proportion of tau with either three or four microtubule binding domain repeats could lead to an excess of 4-repeat tau which may then aggregate [9]. As the missense mutations localise either within or adjacent to the critical microtubule binding domain, a prime hypothesis for the pathogenic effect of these mutations was a reduction in the ability of tau to bind microtubules resulting in the abnormal accumulation of tau and microtubule instability. Evidence in favour of this hypothesis was provided by in vitro studies demonstrating a reduction in the ability of tau to promote microtubule assembly [10,11]. However, this effect was most marked in the first few minutes of tubulin polymerisation and all tau variants increase microtubule assembly substantially after 10 min. Given that the observed loss of function of tau demonstrated in these studies was partial only, it remained possible that the mutations would not substantially affect this property of tau in situ. In order to establish whether the in vitro observations translate to differences in situ, we have characterised the ability of different tau variants to promote microtubule extension in intact cells.

2. Materials and methods

2.1. Cell culture and transfection

CHO cells were cultured in α-MEM supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all Gibco BRL). Human SHSY5Y neuroblastoma were cultured in DMEM/F12 supplemented with 10% (v/v) foetal calf serum and differentiated with staurosporine (20 nM). Human BE(2)-M17 neuroblastoma were grown in OPTI-MEM (Life Technologies) supplemented with 2.4 g/l sodium bicarbonate (Mallinckrodt Baker), 2 mM L-glutamine (Sigma) and 10% foetal bovine serum (Life Technologies). CHO and SHSY5Y cells were transiently transfected using Lipofectamine reagent (Gibco BRL) and BE(2)-M17 cells with Tfx-20 reagent (Promega), both according to the manufacturer's recommendations.

2.2. Expression of tau variants

Site-directed mutagenesis (Promega Gene Editor) was used to introduce a series of missense mutations (V337M, R406W and P301L) in human tau (exon 1, 4, 5, 7, 9, 11–13) containing 3 or 4 microtubule binding domain repeats (but without an NH₂-terminal insert) in pUC19. Sequences of mutagenic oligonucleotides were as follows: V337M 5′-CAGGAGGTGGCCAGATGGAAGTAAAATCT-G-3′, R406W 5′-GGGACACGTCTCCATGGCATCTCAGCAAT-3′, P301L 5′-CCGCGAGACCCACCCTTGGAGGCTCCAGATT-TATC-3′. The different tau constructs were then amplified with the Expand High Fidelity Kit (Boehringer Mannheim) using primers with *Bgl*II and *Sal*I linkers (ACTCAGATCTAGGATGGCTGAGCC and CCCAGTCGACTGATCACAAACCCT) for directional restriction site cloning into pEGFP (Clontech). All constructs were verified by sequencing. Untagged constructs were recloned into the *Sal*I site of pcDNA3.1(+) (Invitrogen).

2.3. Microscopy

To examine the co-localisation of tau and microtubules, SHSY5Y cells were rinsed with PBS and then incubated with 0.3% (v/v) glutaraldehyde in PBS for 10 min at room temperature before permeabilisation with 0.5% (v/v) Triton in PBS. Coverslips were treated with

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sodium borohydride (10 mg/ml, Sigma) in PBS for 10 min, incubated with 0.1 M glycine in PBS for 20 min and then blocked with 5% (v/v) horse serum in PBS for 30 min. A primary monoclonal antibody against alpha-tubulin (DM1A, 1:100, Sigma) was followed by a secondary Texas red-conjugated secondary antibody (Molecular Probes). In other experiments, M17 cells were rinsed with stabilisation buffer (SB) containing 80 mM PIPES, 30% glycerol, 1 mM $MgCl_2$, 1 mM EGTA and 1.1 mM GTP, and fixed for 15 min at room temperature in 2% (v/v) paraformaldehyde/0.1% (v/v) glutaraldehyde/SB. The fixed cells were exposed in sequence to methanol, sodium borohydride (1 mg/ml in PBS) and glycine (100 mM in PBS) prior to permeabilisation with 0.3% (v/v) Triton X-100/PBS. A blocking step in 3% (v/v) goat serum/0.1 M Tris saline (pH 7.6) preceded the incubation with a primary monoclonal antibody to beta-tubulin (clone TUB2.1, 1:100 dilution, Sigma) and a secondary rhodamine-conjugated goat anti-mouse IgG antibody (Zymed Laboratories). Fixed cells were visualised either using a Zeiss light microscope and Pentamax (Princeton instruments) camera or an Olympus Fluoview Confocal Laser Scanning Microscope.

2.4. Microtubule extension assay

CHO cells were transfected with 1 μg of cDNA encoding tau-EGFP variants and cultured at low density on coverslips overnight before treatment with 20 μM cytochalasin B (Sigma) for a further 24 h. Cells were carefully fixed with 4% (w/v) paraformaldehyde for 30 min so as to minimise disruption to processes. For analysis of microtubule extension formation, transfected cells were observed by fluorescence microscopy (Zeiss) and images acquired using a Pentamax camera (Princeton Instruments). For each tau variant, 50 images were taken entirely at random. The first 100 consecutive cells were analysed using MetaMorph software (Universal Imaging) both by counting the number of tau-filled cell processes and by measuring the length of each process. All experimentation and analysis was performed entirely blind. For statistical analysis the data from two separate experiments were combined and tau variant data were compared using ANOVA and the t-test for independent samples.

2.5. SDS-PAGE and immunoblotting

For analysis of tau phosphorylation, tau was isolated from CHO and COS cells transiently transfected with untagged tau constructs. Heat stable tau was harvested as previously described [12] and subjected to 10% (v/v) SDS-PAGE. Blots were incubated with primary antibodies at 4°C overnight and subsequently developed by enhanced chemiluminescence (ECL, Amersham). Antibodies used included the phosphorylation-independent rabbit polyclonal TP70 (1/10 000, [13]), and the phosphorylation-dependent mouse monoclonals recognising tau phosphorylated at Ser-202 and Thr-205 (AT8, 1/200, [14]), tau not phosphorylated at an overlapping epitope, Ser-199 and Ser-202 (Tau 1, 1/200 [15]) and tau phosphorylated at an epitope close to the R406W mutation Ser-396 and Ser-404 (PHF1, 1/200, [16])

3. Results

3.1. Mutated tau binds microtubules in non-neuronal and in neuronal cells

When expressed in transfected cells, tau binds microtubules and promotes the formation of microtubule bundles [17]. In order to establish first whether this property of tau is abolished by mutations associated with neurodegeneration, we transiently transfected a non-neuronal cell line (CHO) and human neuroblastoma cells (M17 and SHSY5Y) with wild-type 4-repeat and 3-repeat EGFP-tau and EGFP-tau with R406W, V337M and, in the case of 4-repeat, P301L mutations. Microtubules were labelled by immunostaining using primary antibodies to tubulin. In all transiently transfected cell lines there was no obvious qualitative difference between wild-type and mutated tau or between 4-repeat and 3-repeat isoforms. In each case, tau bound microtubules and induced the formation of tau-decorated microtubule bundles which, in neuroblastoma but not in non-neuronal cells, tended to fill neurite-like extensions (Fig. 1).
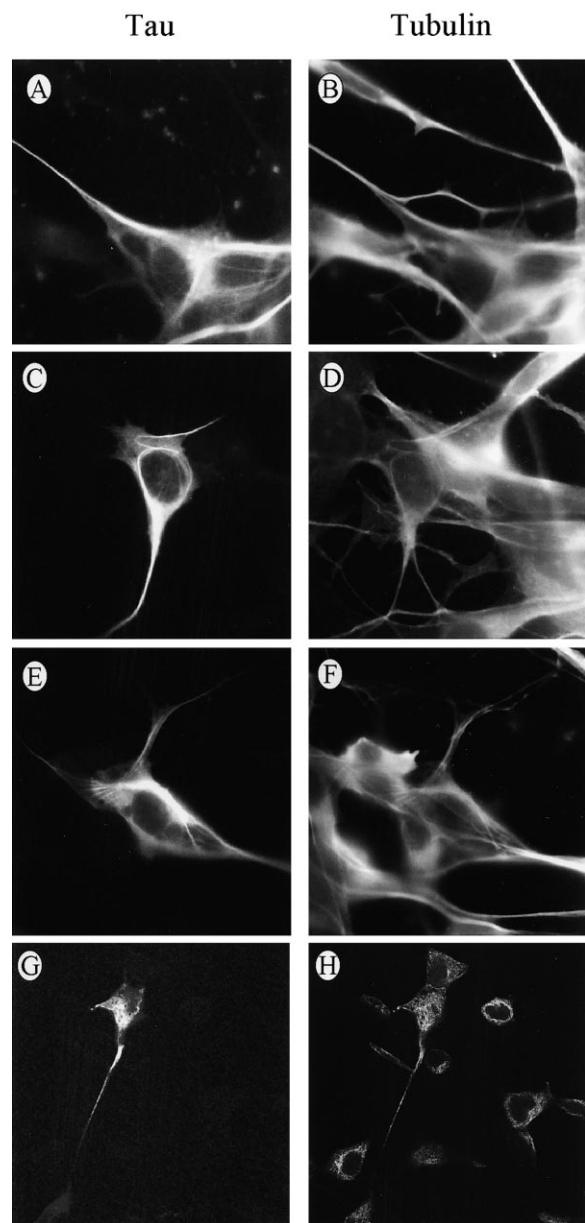
Tau　　　　　　　　Tubulin

Fig. 1. Tau with and without mutations binds to microtubules. SHSY5Y neuroblastoma cells (A–F) and M17 neuroblastoma cells (G, H) transfected with wild-type 4-repeat tau (A, B), R406W mutant tau (C, D) and P301L mutant tau (E–H). In all cases, tau was visualised with the EGFP tag (A–G) and microtubules with the antibody DM1A recognising tubulin. SHSY5Y cells were examined with light microscopy and M17 cells with confocal microscopy.

3.2. Effects of tau variants on the ability of tau to promote microtubule polymerisation in cells

In non-neuronal cells, expression of tau induces a rearrangement of the normal cytoskeleton with long, straight microtubules dissociated from the normal microtubule organising centre and microtubule bundles arranged around the periphery of the cell [18]. Other MAPs have similar effects and it has been previously demonstrated that if the actin cytoskeleton is disrupted by cytochalasin B, these stiff microtubule bundles push extensions out of the cell and thereby assume some of the properties of neuritic extensions [19]. We used this approach to quantitate the effects of tau variants on microtubules (Fig. 2). Measurements of microtubule exten-
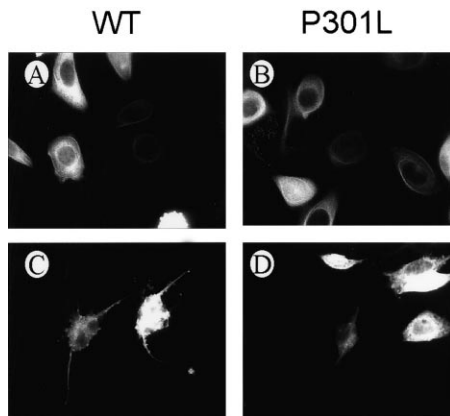
Fig. 2. Cytochalasin B induces microtubule extensions in tau-transfected cells. The microtubule arrangements in CHO cells expressing tau are altered with the appearance of bundles that tend to circumnavigate the perimeter of the cell (A, B). No difference between wild-type tau and mutated tau was seen (only P301L data shown). The addition of cytochalsin B to disrupt the actin cytoskeleton allowed the extension of microtubule bundles into cellular extensions (C, D).

sions were made on a total of 1400 cells (2728 separate cell extensions, Fig. 3). The wild-type 4-repeat tau induced more extensions than the wild-type with only 3 repeats (mean 2.04 compared to 1.50; S.D. 2.0 and 1.4; Independent t-test value $= 3.11$; $P < 0.005$). We then compared the sum of the length of extensions per cell finding a longer mean extension length in 4-compared to 3-repeat tau (ANOVA $P < 0.05$). Of the tau mutants, the greatest effect was found for the P301L mutation, which reduced the extension length of 4-repeat tau by 33.8% (from 39 μm to 26 μm; ANOVA, $P < 0.005$). The R406W mutation did not appear to significantly affect the mean extension length for either 3- or 4-repeat tau whilst the V337M reduced the extension length in both cases by 17%. This consistent reduction in extension length, caused by the mutation, just failed to reach significance when considering the different isoforms separately (ANOVA; $P = 0.06$ for 3-repeat and $P = 0.08$ for 4-repeat). However combining isoforms (V337M or R406W 3- and 4- repeat compared to wild-type 3- and 4-repeat), a significant reduction in extension length was seen for both mutations, albeit larger and more significant for the V337M variant (37 μm versus 30 μm for V337M; ANOVA $P < 0.01$; 37 μm versus 34 μm for R406W ANOVA $P < 0.05$).

3.3. Effects of mutations on the phosphorylation of tau by endogenous kinases and by GSK-3β

In order to examine the effects of the mutations on tau phosphorylation, tau with both three and four microtubule binding domains was transiently expressed in CHO cells. As in previous studies [20] three bands recognised by the phosphate-independent antibody TP70 were apparent following SDS-PAGE (Fig. 4, only 4-repeat data shown). All three bands were recognised by an antibody TAU1, recognising tau not phosphorylated at Ser-199/202, which also labelled a very faint faster migrating band, possibly a degradation product. None of the species identified by TP70 or TAU1 were recognised by AT8 but another phosphorylation-dependent antibody, PHF-1, did faintly label tau in these cells. As previously reported [12], when co-transfected with cDNA encod-

ing glycogen synthase kinase-3β (GSK-3β) one or two bands of wild-type tau are labelled with TP70 and the slowest migrating band of this pair is recognised by phosphorylation-dependent antibodies such as AT8 raised to epitopes of tau that are phosphorylated in AD. TAU-1 immunoreactivity was decreased in the co-transfected cells and PHF-1 immunoreactivity was increased.

We then expressed 3-repeat tau with the V337M and R406W mutations and 4-repeat tau with V337M, R406W and P301L mutations alone and in co-transfection experiments with GSK-3β. In co-transfection experiments, all variants of tau were indistinguishable from wild-type tau in both the migration pattern and immunoreactivity indicating that all were similarly phosphorylated by GSK-3β overexpression. In the absence of GSK-3β, the variants of tau possessing V337M and P301L mutations were also indistinguishable from wild-type tau but both 3- and 4-repeat tau with the R406W mutation resolved as a single band co-migrating with the fastest migrating band of wild-type tau (Fig. 4, only 4-repeat data shown). Repeated transfection experiments gave identical results. This single band of 3-repeat R406W and 4-repeat R406W tau was recognised by TP70 and TAU1 but not by AT8 and PHF-1 indicating that it was not phosphorylated at the epitopes identified by these antibodies. Notably, a band of wild-type tau and P301L and V337M tau was recognised by PHF-1 in the absence of GSK-3β. As previous studies have demonstrated by alkaline phosphatase treatment that the different species of tau seen when expressed in non-neuronal cells are due to differential phosphorylation [20], we treated CHO cells, transfected with wild-type tau, with lithium chloride (25 mM) for 4 h. Lithium inhibits GSK-3β and in doing so it alters tau phosphorylation in neurons and in CHO cells [21–23]. In CHO cells, the slowest migrating band was not visible after lithium treatment but two bands remained, including that co-migrating with tau R406W.

4. Discussion

The accumulation of tau-containing inclusions accompanying diverse neurodegenerative disorders suggests that disruption of the normal function of this microtubule-associated
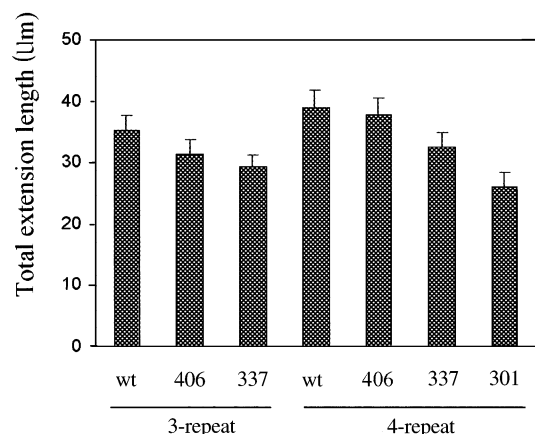

Fig. 3. Microtubule extension length induced by tau variants. The sum length of microtubule extensions induced by cytochalsin B in tau-transfected cells was greater in 4- than in 3-repeat tau. The mutations reduce the extension length with the P301L mutation showing the greatest and the R406W mutation the least effect.
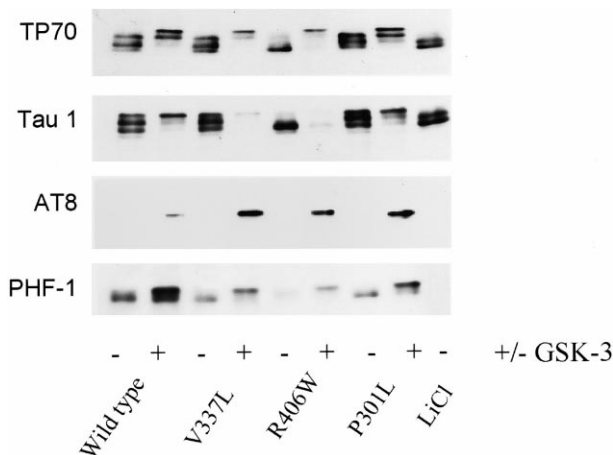
Fig. 4. Phosphorylation of tau variants in CHO cells. Wild-type tau expressed in CHO cells alone (−) and together (+) with GSK-3 was examined by Western blotting using the phosphorylation-independent antibody TP70 and the phosphorylation-dependant antibodies TAU1, AT8 and PHF-1. The migration pattern of tau was altered from three TP70 labelled bands by the R406W mutation and to two bands by lithium. PHF-1 labelling was reduced by the R406W mutation and abolished by lithium.

protein, and resulting aggregation, is a critical factor in pathogenesis [9]. However the mechanism whereby this occurs is not understood. Although progress has been made in demonstrating that tau in the paired helical filaments of AD is highly phosphorylated, in determining the phosphorylation sites and in identifying the kinases and phosphatases responsible, it has proven difficult to determine whether phosphorylation is a cause or a result of aggregation [2]. Finding mutations in tau in FTDP-17 offers a unique opportunity to explore further the sequence of events resulting in the aggregation of tau.

In vitro studies have demonstrated that mutations in tau reduce but do not abolish the ability of tau to promote microtubule assembly [10,11]. However, the results obtained on the relative effects of the different mutations have been contradictory. Hasewaga et al. [10] found only a relatively modest effect of the R406W mutation and a maximal effect of the P301L mutation. In contrast, Hong et al. [11] found a maximal effect of the R406W mutation and little, if any effect, of the V337M mutation. The reason for these discrepancies is currently unclear. We have developed an assay for analyzing the effect of the FTDP-17 mutants on microtubule formation in transfected cells. This quantitative assay has the further advantage that we are also able to observe differences between 4-repeat and 3-repeat tau isoforms in the same system (exon 10 splice site mutants increase the level of 4-repeat tau).

Our findings are in line with that of the in vitro data provided by Hasegawa et al. [10] in that the P301L mutation reduced the microtubule extension length to the greatest extent followed by the V337M mutation. Furthermore the ability of 4-repeat tau to induce extensions was considerably greater than that of 3-repeat tau. We conclude therefore that the pathogenic mechanism of the FTDP-17 V337M and P301L missense mutations is likely to involve a partial loss of the microtubule assembly/binding properties of tau. Neurodegeneration might result from this partial loss of function, leading to altered axonal transport or increased vulnerability of neurons. Alternatively, it is possible as suggested by Hong et al. [11] that a toxic gain of function would result from

increased unbound cytoplasmic tau and the subsequent aggregation. The mechanism of the FTDP-17 splice site mutants [5,6] which increase the proportion of 4-repeat tau remains unclear, however it is obvious from these experiments that the 4-repeat and 3-repeat isoforms do differ in the properties they confer to bound microtubules. It is therefore possible that the alteration in microtubule dynamics, caused by an increase in 4-repeat tau, is sufficient to cause neurodegeneration. Alternatively, the increase in 4-repeat isoforms might lead to increased levels of unbound cytoplasmic 4-repeat tau and thus to aggregation.

Our cell-based studies did not find a significant effect of the R406W FTDP-17 mutation with each isoform separately but a small and significant effect of combined 3-repeat R406W and 4-repeat R406W tau compared to combined wild-type isoforms. A slight decrease in microtubule extensions might represent a very small loss of function, enough to cause late onset disease, and this might account for the relatively mild phenotype in families with this mutation [24]. However, it is possible that some other factor accounts for the pathogenic effect of this mutation. To further examine the effect of the R406W mutation on the properties of tau, we have studied the effect of the FTDP-17 missense mutations on tau phosphorylation.

Phosphorylation regulates the properties of tau principally tubulin binding and microtubule polymerisation [2,25]. Many kinases can phosphorylate tau in vitro but increasing evidence suggests that GSK-3β is the predominant physiological tau kinase acting alone, or in conjunction with other kinases. All tau variants, including the FTPD-17 R406W mutation, were phosphorylated by overexpression of GSK-3β. However, it was apparent that R406W tau was not completely phosphorylated when compared to the other variants and the phosphorylation of R406W expressed in CHO cells in the absence of GSK-3β was different to that of wild-type tau and to tau bearing other FTDP-17 mutations. The multiple bands of tau, observed when expressed in non-neuronal cells, represent different phosphorylation states [20] and the faster migrating of the two bands of tau when expressed in CHO cells aligns with equivalent recombinant tau [12]. As both 3-repeat and 4-repeat R406W tau only appears as this single lower band, we conclude that this mutation reduces the phosphorylation of tau by endogenous kinases in CHO cells. Further evidence for a change in the phosphorylation in the R406W mutant tau comes from immunoblotting with an antibody raised to the closest phosphorylated epitope to the site of the mutation, PHF-1, recognising an epitope including phosphorylated Ser-404. [16]. A proportion of tau is phosphorylated at the PHF-1 epitope in CHO cells by endogenous kinases and this is reduced by the R406W mutation, and by lithium, but not the other mutations. Lithium, an inhibitor of GSK-3β, also reduces the number of bands recognised by TP70 from three to two. However GSK-3β is unlikely to be solely responsible for the phosphorylation changes in R406W tau as the migration pattern of tau induced by lithium treatment is not identical to the migration pattern of R406W tau. It is possible that a number of endogenous kinases, including GSK-3β, are responsible for the phosphorylation changes induced by the mutation and also that sites other than Ser-404 are affected.

To date, the relationship between phosphorylation and tau interaction with microtubules has been thought to be straight-

forward in that an increase in phosphorylation at one or more sites decreases the ability of tau to promote microtubules. We observe a simultaneous decrease in phosphorylation and a small decrease in microtubule extension. It might be that this change in phosphorylation renders tau more susceptible to phosphorylation by other kinases at sites that do not alter the electrophoretic mobility whilst reducing microtubule binding ability. Alternatively the relationship between phosphorylation and microtubule binding ability in tau might be more complex than hitherto suspected as it is for MAP2c where both an increase and a decrease in phosphorylation reduces the ability to promote microtubule elongation [26].

In summary, our results and those of others suggest that several different pathogenic mechanisms may result from the mutations in tau that are associated with FTDP-17. Intronic mutations lead to an excess of 4-repeat tau which may disrupt microtubule dynamics or might lead to increased amounts of cytosolic unbound 4-repeat tau which then aggregates to form paired helical or straight filaments. Mutations in the microtubule binding repeat domains reduce the affinity of tau for tubulin/microtubules. Finally the R406W mutation may exert its effect via a phosphorylation-dependent mechanism which could result in a reduction in microtubule binding and an excess of free tau or in a direct increase in aggregation. Clearly, future studies are needed to explore these possibilities further.

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