FTDP-17 Mutations Compromise the Ability of Tau to Regulate Microtubule Dynamics in Cells*

Janis M. Bunker, Kathy Kamath, Leslie Wilson, Mary Ann Jordan, and Stuart C. Feinstein

From the Neuroscience Research Institute and Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California 93106

The neural microtubule-associated protein Tau binds directly to microtubules and regulates their dynamic behavior. In addition to being required for normal development, maintenance, and function of the nervous system, Tau is associated with several neurodegenerative diseases, including Alzheimer disease. One group of neurodegenerative dementias known as FTDP-17 (fronto-temporal dementia with Parkinsonism linked to chromosome 17) is directly linked genetically to mutations in the tau gene, demonstrating that Tau dysfunction can cause neuronal cell death and dementia. These mutations result either in amino acid substitutions in Tau or in altered Tau mRNA splicing that skews the expression ratio of wild-type 3-repeat and 4-repeat Tau isoforms. Because wild-type Tau regulates microtubule dynamics, one possible mechanism underlying Tau-mediated neurodegeneration is aberrant regulation of microtubule behavior. In this study, we microinjected normal and mutated Tau protein into cultured cells expressing fluorescent tubulin and measured the effects on the dynamic instability of individual microtubules. We found that the FTDP-17 amino acid substitutions G272V (in both 3-repeat and 4-repeat Tau contexts), ΔK280, and P301L all exhibited markedly reduced abilities to regulate dynamic instability relative to wild-type Tau. In contrast, the FTDP-17 R406W mutation (which maps in a regulatory region outside the microtubule binding domain of Tau) did not significantly alter the ability of 3-repeat or 4-repeat Tau to regulate microtubule dynamics. Overall, these data are consistent with a loss-of-function model in which both amino acid substitutions and altered mRNA splicing in Tau lead to neurodegeneration by diminishing the ability of Tau to properly regulate microtubule dynamics.

Microtubules are dynamic polymers with growing and shortening behaviors that are exquisitely regulated. The dynamic behaviors of microtubules are both temporally and spatially regulated, even within individual cells (1). For example, microtubule dynamics in migrating individual cells (1). For example, microtubule dynamics in migrating

*This work was supported by National Institutes of Health Grants NS53010 (to S. C. F.), NS13560 (to L. W.), and CA57191 (to M. A. J.).

1 To whom correspondence should be addressed: Neuroscience Research Institute, Bldg. 571, Rm. 6129, University of California, Santa Barbara, CA 93106. Tel: 805-893-2659; Fax: 805-893-2659; E-mail: feinstein@files.ucsb.edu.

The on-line version of this article (available at http://www.jbc.org) contains Movies 1–3.
reverse-phase liquid chromatography (DeltaPak-C18; Millipore, Billerica, MA). High pressure liquid chromatography fractions contained abilities to regulate microtubule dynamics relative to wild-type Tau. To test this prediction, we injected identical amounts of either wild-type or FTDP-17 mutant Tau into living MCF-7 cells, and we analyzed the effects upon the dynamic instability behavior of individual microtubules in the thin lamellar peripheral region of the cells.

**MATERIALS AND METHODS**

**Tau Protein Purification**—pRK expression vectors containing the human cDNA sequences for the shortest 4-repeat and 3-repeat Tau isoforms (encoding 383 and 352 amino acids, respectively) were the kind gifts from Dr. Kenneth Kosik (University of California, Santa Barbara) and Dr. Gloria Lee (University of Iowa). FTDP-17 mutations were introduced into both 4-repeat and 3-repeat Tau constructs using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (see Fig. 1A). The sequence of all constructs was verified by direct sequence analysis prior to use. Tau protein was expressed and purified as described previously (33, 34). Briefly, Tau expression was induced in BL21 (DE3) cells (Novagen, Madison, WI). Bacteria were lysed by sonication, and the lysate was clarified by centrifugation (12,000 × g, 15 min, 4 °C). Supernatants were boiled to precipitate heat-labile proteins and re-centrifuged. The heat-stable proteins were adsorbed to a phosphocellulose column and eluted with a 0.2 to 1.0 M NaCl gradient. Fractions containing Tau protein were pooled and further purified using reverse-phase liquid chromatography (DeltaPak-C18; Millipore, Billerica, MA). High pressure liquid chromatography fractions containing Tau were pooled, lyophilized, and resuspended in PBS2 (Fig. 1A).

The concentration of each Tau sample was determined by SDS-PAGE comparison with a "Tau mass standard," the concentration of which was established by amino acid analysis (35).

2 The abbreviations used are: PBS, phosphate-buffered saline; GFP, green fluorescent protein.

**FTDP-17 Mutations Compromise Tau Function**

**Cell Culture**—MCF7 human breast cancer cells (ATCC, Manassas, VA) stably expressing the GFP-tubulin plasmid pEGFP-Tub (Clontech) (33) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with nonessential amino acids, 10% bovine serum, antibiotic-antimycotic (Invitrogen), and geneticin (400 μg/ml; Invitrogen) at 37 °C and 5.5% CO2. Cells were seeded 36–48 h before injection on 12-mm CellLocate coverslips (Eppendorf, Hamburg, Germany) coated with poly-d-lysine (100 μg/ml; Sigma) followed by human fibronectin (20 μg/ml; Invitrogen) and laminin (10 μg/ml; Sigma). To induce a more flattened morphology, cells were serum-starved in media containing 2% bovine serum 12 h before injection.

**Microinjection**—Seeded cells were transferred to serum-free Dulbecco’s modified Eagle’s medium lacking bicarbonate and containing 25 mM HEPES and 4.5 g/liter glucose (recording media) (Invitrogen). All wild-type and mutant Tau proteins were diluted to a concentration of 13.3 μM in PBS plus 1.4 mM β-mercaptoethanol. Immediately prior to microinjection, the solution was centrifuged (50,000 × g, 15 min, 4 °C) to remove any aggregates or debris. Pressure microinjection was performed using an Eppendorf Transjector 5246 and Injectman. The injection volume was ~10% of the cell volume (36), resulting in an ~1.3 μM final Tau concentration in the cells. Injected cells were returned to normal media and incubated 2–3 h at 37 °C to allow equilibration of Tau within the cells.

The quantity of Tau injected into cells was based on the following rationale. Based upon the work of Dhamodharan and Wadsworth (37), we estimated that the total tubulin concentration in the cell was ~20 μM (38) with 65% in polymer during interphase (39), resulting in 13 μM polymerized tubulin. With respect to Tau, Drubin et al. (40) determined that the Tau:polymeric tubulin molar ratio in neuronally differentiated rat PC12 cells is ~1:5, whereas it is ~1:34 in undifferentiated PC12 cells. Thus, we concluded that a 1:10 Tau:polymeric tubulin molar ratio represents a reasonable approximation of in vivo neuronal conditions.

**Immunocytochemistry**—Cells were rinsed once with PBS and fixed by the rapid addition of 100% methanol (4 °C). Fixed cells were incubated overnight in blocking buffer (3% bovine serum albumin, 0.1% Triton-X-100, and 1% horse serum in PBS). Cells were incubated first with mouse monoclonal Tau 5 antibody (1:100; BioSource International, Camarillo, CA), then Cy3-conjugated donkey anti-mouse secondary antibody (1:100; Jackson ImmunoResearch, West Grove, PA), followed by fluorescent iso-thiocyanate-conjugated mouse monoclonal tubulin antibody DM1α (1:50; Sigma). All incubations were for 1 h at room temperature, followed by four 15-min washes in blocking buffer. Coverslips were mounted on glass slides using Prolong (Molecular Probes, Eugene, OR). Images were obtained using a laser-scanning confocal microscope (MRC 1024; Bio-Rad). Images were captured with an inverted fluorescence microscope (100 objective lens, maintained at 36.5 ± 1 °C. Thirty images per cell were taken at 4-s intervals using a Hamamatsu Orca II (Middlesex, NJ) digital camera driven by MetaMorph software (Universal Imaging, Media, PA).

**Analysis of Microtubule Dynamic Instability**—To determine the percentage of microtubules that displayed visually detectable growing and shortening dynamics, we analyzed the total change in length (growing plus shortening) of at least 50 randomly selected microtubules from four independent cells microinjected with a given Tau isoform. A region of
FTDP-17 Mutations Compromise Tau Function

the cell periphery was randomly boxed, and 10–20 microtubules within 1–2 boxes per cell were analyzed. A microtubule was considered to be not detectably dynamic if the total change in length during the 2-min time-lapse sequence was less than or equal to 1.0 μm.

Analysis of microtubule dynamic instability behavior was performed as described previously (33, 42). The positions of the plus ends of individual microtubules over time were recorded using MetaMorph software, exported to Microsoft Excel, and analyzed using RTM software (43). The lengths of individual microtubules were plotted as a function of time. Changes in length greater than 0.5 μm were designated as growth or shortening events. Periods in which length changes were less than 0.5 μm were designated as phases of attenuated microtubule dynamics (or pause). A catastrophe was defined as a transition from either growth or attenuation to shortening. The catastrophe frequency was calculated either as the total number of catastrophes divided by the total time spent growing and attenuated or as the total number of catastrophes divided by the total length grown. A rescue was defined as a transition from shortening to either growth or attenuation. Frequencies of rescue were calculated either as the total number of rescues divided by the total amount of time spent shortening or as the total number of rescues divided by the total length shortened. Dynamicity was calculated as the total length grown and shortened divided by the total time measured.

On-line Supplemental Material—Time-lapse movies of enhanced GFP-microtubules undergoing dynamic instability in cells are available as Supplemental Material. Each movie consists of 30 frames taken at 4-s intervals and is played 30 times faster than real time. Movie 1 shows microtubule dynamics in a control cell injected with buffer; Movie 2 shows microtubule dynamics in a cell injected with wild-type 4-repeat Tau; and Movie 3 shows microtubule dynamics in a cell injected with 4-repeat Tau containing the ΔK280 mutation.

RESULTS

Localization of Microinjected Wild-type and FTDP-17 Mutated Tau in Cells—Prior to assessing the effects of FTDP-17 mutations on dynamic instability, we first sought to assess the subcellular distribution of injected wild-type and FTDP-17-mutated Tau. We injected MCF7 cells, which do not express any endogenous Tau, with either wild-type or FTDP-17 mutant Tau and then returned the cells to the 37 °C incubator for 2 h to allow sufficient time for the Tau to equilibrate throughout the cells. We then fixed the cells and double-stained for Tau and tubulin. We found that wild-type and the various FTDP-17-mutated Tau proteins associated with microtubules in injected cells, whereas no Tau was detected in un.injected cells, as expected (Fig. 2). Tau staining was evenly distributed along the microtubules, with little or no Tau staining elsewhere in the cells. Additionally, there was no detectable difference in the intensity of Tau staining on microtubules between wild-type Tau and Tau containing the various FTDP-17 mutations. Thus, differences in the abilities of various Tau molecules to regulate microtubule dynamics most likely reflect intrinsic mechanistic differences in the Tau molecules being tested rather than differential binding of particular Tau molecules to the microtubules.

Microinjection of Exogenous Wild-type or FTDP-17 Mutant Tau Does Not Markedly Affect the Fraction of Microtubules That Display Dynamic Instability—To visualize dynamic instability behavior, we captured time-lapse images of microtubules in the flat peripheral region of living cells stably transfected with GFP-tubulin. As shown in Fig. 3, and the online supplemental movies, the microtubules were well resolved, and their ends were clearly visible. Initial analyses revealed that all cells possess two populations of microtubules, a stable population and a pop-ulation that exhibits visually detectable dynamic behavior. To determine whether the different Tau isoforms used in this study differentially affected the percentage of dynamic versus nondynamic microtubules in cells, we operationally defined dynamic microtubules as those exhibiting total length changes (growth plus shortening lengths) of 1.0 μm or more during 2 min of observation (dynamicity ≥0.5 μm/min); stable microtubules were defined as those that grew and/or shortened less than 1.0 μm during the same period. Using these criteria, there were no significant differences in the percentage of dynamic microtubules in cells injected with control buffer versus any of the Tau-injected cells (Table 1). Most cells had ~80% dynamic microtubules. Cells injected with either 4-repeat wild-type Tau or ΔK280 had slightly fewer dynamic microtubules than the control cells, but these differences were not statistically significant (p = 0.20 and p = 0.39, respectively, using χ² test). These data are in contrast to our earlier assertion, based on nonquantitative assessments, suggesting that Tau might increase the percentage of nondynamic microtubules in the cells (33). In retrospect, it is likely that our earlier subjective observation was based upon the fact that Tau markedly increases the percentage of time microtubules spend in the attenuated state (33).
**TABLE 1**

| No. microtubules | Dynamic                |
|------------------|------------------------|
|                  | % of total             |
| Buffer control   | 60                     |
| 4R WT            | 63                     |
| 4R G272V         | 70                     |
| 4R ΔK280         | 60                     |
| 4R P301L         | 78                     |
| 4R R406W         | 50                     |
| 3R WT            | 60                     |
| 3R G272V         | 65                     |
| 3R R406W         | 78                     |

---

**TABLE 2**

| Fraction of time spent | Buffer control | 4R WT** | 4R G272V† | 4R ΔK280# | 4R P301L‡ | 4R R406W | 3R WT** | 3R G272V† | 3R R406W |
|------------------------|---------------|---------|-----------|-----------|-----------|---------|---------|-----------|---------|
| Growing                | 0.39          | 0.24    | 0.34      | 0.38      | 0.36      | 0.26    | 0.31    | 0.35      | 0.34    |
| Shortening             | 0.24          | 0.18    | 0.18      | 0.21      | 0.18      | 0.15    | 0.16    | 0.21      | 0.14    |
| Attenuated             | 0.37          | 0.58    | 0.48      | 0.41      | 0.46      | 0.59    | 0.52    | 0.44      | 0.53    |

---

**FIGURE 4.** Life history plots of the growth and shortening dynamics of individual microtubules. The positions of the ends of individual microtubule were tracked in cells injected with buffer, 4-repeat wild-type Tau, or FTDP-17 mutated Tau and then measured the changes in length of individual microtubules over time by tracking the positions of microtubule ends (see "Materials and Methods"). Typical microtubule life history plots for buffer-injected, wild-type 4-repeat Tau-injected and ΔK280-injected cells are presented in Fig. 4. From these plots, we determined the dynamic instability parameters (as described under "Materials and Methods").

Dynamic microtubules transition among three phases: growth, shortening, and attenuation (Fig. 4). To begin our analyses, we first assessed the ability of each wild-type and FTDP-17 mutant Tau isoform to influence the fraction of time that dynamic microtubules spent in each phase relative to the total time tracked. Wild-type 3-repeat and 4-repeat Tau increased the fraction of time microtubules spent attenuated while reducing the fraction of time spent growing, relative to microtubules in buffer-injected control cells (33) (see also Table 2). In contrast, four of the FTDP-17 Tau mutations (3-repeat G272V, 4-repeat G272V, ΔK280, and P301L) were significantly compromised in their ability to influence the phase distribution relative to their wild-type counterparts (Table 2). For example, although wild-type 4-repeat Tau reduced the fraction of time microtubules spent growing by 37% (from 0.39 to 0.24; see Table 2), 4-repeat G272V, ΔK280, and P301L only caused 2–12% reductions. Similarly, whereas 4-repeat wild-type Tau strongly increased the fraction of time microtubules spent attenuated (from 0.37 to 0.58, an increase of 58%), 4-repeat G272V, ΔK280, and P301L Tau exhibited markedly reduced effects (10–29%). Thus, these FTDP-17 Tau isoforms have a decreased ability to regulate the dynamic behavior of cellular microtubules. In contrast to these large effects, the C-terminal tail mutation R406W, in both 4-repeat and 3-repeat contexts, exhibited no loss of effect relative to the respective wild-type Tau isoforms. In fact, the R406W mutation slightly enhanced the ability of Tau to decrease the time microtubules spent shortening in both 4-repeat and 3-repeat Tau.

---

*Most FTDP-17 Missense Mutations Reduce the Ability of Tau to Regulate the Percentage of Time Dynamic Microtubules Spend Growing, Shortening, or Attenuated—The FTDP-17 mutations examined here all map to regions of Tau known to be important for normal Tau function (see Fig. 1). G272V is present in both 3-repeat and 4-repeat Tau, residing in the first repeat. ΔK280 and P301L are both encoded by the alternatively spliced exon 10 and therefore are present only in 4-repeat Tau; these mutations reside in the R1-R2 inter-repeat and at the end of repeat 2, respectively. Each of these mutations map to the region of Tau believed to interact directly with microtubules (44–47). An additional mutation, R406W, is present in both 3-repeat and 4-repeat Tau and resides in the flanking sequence on the carboxyl side of the repeat-inter-repeat region; these flanking sequences are believed to influence Tau action indirectly via protein folding and/or phosphorylation effects (47, 48). To assess the effects of the various Tau molecules on the regulation of microtubule dynamic instability, we injected cells either with buffer, wild-type Tau, or FTDP-17 mutated Tau and then measured the changes in length of individual microtubules over time by tracking the positions of microtubule ends (see "Materials and Methods"). Typical microtubule life history plots for buffer-injected, wild-type 4-repeat Tau-injected and ΔK280-injected cells are presented in Fig. 4. From these plots, we determined the dynamic instability parameters (as described under "Materials and Methods"). Dynamic microtubules transition among three phases: growth, shortening, and attenuation (Fig. 4). To begin our analyses, we first assessed the ability of each wild-type and FTDP-17 mutant Tau isoform to influence the fraction of time that dynamic microtubules spent in each phase relative to the total time tracked. Wild-type 3-repeat and 4-repeat Tau increased the fraction of time microtubules spent attenuated while reducing the fraction of time spent growing, relative to microtubules in buffer-injected control cells (33) (see also Table 2). In contrast, four of the FTDP-17 Tau mutations (3-repeat G272V, 4-repeat G272V, ΔK280, and P301L) were significantly compromised in their ability to influence the phase distribution relative to their wild-type counterparts (Table 2). For example, although wild-type 4-repeat Tau reduced the fraction of time microtubules spent growing by 37% (from 0.39 to 0.24; see Table 2), 4-repeat G272V, ΔK280, and P301L only caused 2–12% reductions. Similarly, whereas 4-repeat wild-type Tau strongly increased the fraction of time microtubules spent attenuated (from 0.37 to 0.58, an increase of 58%), 4-repeat G272V, ΔK280, and P301L Tau exhibited markedly reduced effects (10–29%). Thus, these FTDP-17 Tau isoforms have a decreased ability to regulate the dynamic behavior of cellular microtubules. In contrast to these large effects, the C-terminal tail mutation R406W, in both 4-repeat and 3-repeat contexts, exhibited no loss of effect relative to the respective wild-type Tau isoforms. In fact, the R406W mutation slightly enhanced the ability of Tau to decrease the time microtubules spent shortening in both 4-repeat and 3-repeat Tau.*
FTDP-17 Mutations Compromise Tau Function

When the data for each mutant was plotted as percentage of the wild-type Tau effect (for 4-repeat or 3-repeat wild-type Tau, as appropriate), it is clear that 4-repeat G272V, 3-repeat G272V, ΔK280, and P301L all exhibit marked loss-of-function effects upon the ability of Tau to regulate the time microtubules spend in each phase (Fig. 5). In contrast, both the 4-repeat and 3-repeat R406W mutants exhibit near wild-type effects.

The amount of time a microtubule spends growing and shortening is in part determined by transition frequencies. In other words, Tau might affect how long a microtubule grows or remains attenuated before it begins to shorten (defined as the catastrophe frequency), or conversely, Tau might affect how long a microtubule shortens before it becomes attenuated or begins to grow (defined as the rescue frequency). Wild-type Tau has a small effect on the catastrophe and rescue frequencies, which is most likely because of its regulatory effects on the growth and shortening rates (33). We calculated the catastrophe and rescue frequencies of microtubules in cells injected with FTDP-17 mutant Tau proteins (calculated both per unit of time and per unit of length grown or shortened) and found no significant difference between wild-type Tau and any of the mutants Tau proteins tested in this study (data not shown).

**FTDP-17 Tau Missense Mutations Exhibit Loss-of-Function Effects on Microtubule Shortening Events**—As shown previously, 4-repeat wild-type Tau significantly reduced the average length a microtubule shortened during a shortening event in cells; in contrast, 3-repeat Tau had a minimal effect on shortening events (33) (also see Table 3). When we examined the effects of the various 4-repeat FTDP-17 mutated Tau isoforms on the average length that a microtubule shortened during a shortening event in cells; in contrast, 3-repeat Tau had a minimal effect on shortening events (33) (also see Table 3). When we examined the effects of the various 4-repeat FTDP-17 mutated Tau isoforms on the average length that a microtubule shortened during a shortening event in cells; in contrast, 3-repeat Tau had a minimal effect on shortening events (33) (also see Table 3). When we examined the effects of the various 4-repeat FTDP-17 mutated Tau isoforms on the average length that a microtubule shortened during a shortening event in cells; in contrast, 3-repeat Tau had a minimal effect on shortening events (33) (also see Table 3). When we examined the effects of the various 4-repeat FTDP-17 mutated Tau isoforms on the average length that a microtubule shortened during a shortening event in cells; in contrast, 3-repeat Tau had a minimal effect on shortening events (33) (also see Table 3). When we examined the effects of the various 4-repeat FTDP-17 mutated Tau isoforms on the average length that a microtubule shortened during a shortening event in cells; in contrast, 3-repeat Tau had a minimal effect on shortening events (33) (also see Table 3).
**TABLE 3**

Dynamic instability parameters of microtubules in cells injected with wild-type or FTDP-17 mutated Tau

| No. of microtubules/ no. of cells | Growth rate | Growth length | Shortening rate | Shortening length | Dynamicity |
|----------------------------------|-------------|---------------|-----------------|-------------------|------------|
| Buffer control                   | 56/18       | 13.3 ± 0.4    | 2.7 ± 0.2       | 27.1 ± 1.5        | 5.2 ± 0.4  | 11.7       |
| 4R WT                            | 27/7        | 9.40 ± 0.3**  | 1.2 ± 0.1**     | 16.5 ± 0.9**      | 2.9 ± 0.4**| 5.2        |
| 4R G272V                         | 27/9        | 10.1 ± 0.3    | 1.9 ± 0.11      | 20.0 ± 1.41       | 3.2 ± 0.4  | 7.1        |
| 4R ΔK280                         | 27/8        | 11.3 ± 0.5    | 2.4 ± 0.3       | 17.4 ± 1.00       | 2.8 ± 0.3  | 8.0        |
| 4R P301L                         | 27/11       | 9.6 ± 0.5     | 1.9 ± 0.21      | 21.0 ± 1.51       | 4.2 ± 0.6  | 7.2        |
| 4R R406W                         | 27/10       | 9.5 ± 0.4     | 1.8 ± 0.21      | 20.5 ± 1.9        | 3.6 ± 0.6  | 5.5        |
| 3R WT                            | 27/8        | 11.6 ± 0.5*   | 1.8 ± 0.2**     | 22.3 ± 1.5*       | 3.6 ± 0.4* | 7.3        |
| 3R G272V                         | 25/9        | 11.7 ± 0.5    | 2.2 ± 0.2       | 23.8 ± 1.5        | 4.3 ± 0.6  | 9.0        |
| 3R R406W                         | 25/8        | 12.2 ± 0.6    | 2.5 ± 0.31      | 25.6 ± 1.6        | 3.8 ± 0.5  | 7.3        |

The length a microtubule shortens is determined by both the duration of the shortening event and the rate of shortening. When we compared the effects of wild-type and FTDP-17 mutant Tau isoforms on the average duration of shortening, we found that the mutations had little or no effect, which might be expected because wild-type Tau has little effect on this parameter (data not shown).

On the other hand, one of the most potent effects of wild-type 4-repeat Tau on dynamic instability in vitro and in cells is to reduce the shortening rate (14, 33, 35). When we examined the effect of the FTDP-17 Tau mutations on the microtubule shortening rate, we found that most of them (4-repeat G272V, P301L, and R406W) weakened the ability of Tau to reduce the shortening rate (Table 3; Fig. 5). Interestingly, ΔK280 did not alter the effect of Tau on the shortening rate. In the 3-repeat Tau constructs, the FTDP-17 mutations had insignificant effects on the ability of Tau to modify the shortening rate. However, it would be difficult to detect a loss of effect on the shortening rate in these isoforms because 3-repeat wild-type Tau itself has little effect on this parameter either in vitro or in cells (33, 35).

**FTDP-17 Missense Mutations Decrease the Ability of Tau to Regulate Microtubule Growth Events**—Both 4-repeat and 3-repeat wild-type Tau significantly reduced the average length that microtubules grew during a growth event (33) (Table 3). To determine whether FTDP-17 mutations in Tau affect its ability to reduce the length grown during a growth event, we compared the average length grown per event in cells injected with buffer, wild-type Tau, or FTDP-17-mutated Tau. We found that all FTDP-17 mutations tested decreased the ability of Tau to regulate the length of a growth event (Table 3; Fig. 5). Although growth events in control cells averaged 2.7 ± 0.2 μm, growth events in wild-type 4-repeat Tau-injected cells averaged 1.2 ± 0.1 μm; the average growth event for the different 4-repeat FTDP-17 mutant Tau isoforms ranged between 1.8 ± 0.2 and 2.4 ± 0.3 μm. Similar magnitudes of changes occurred with FTDP-17 mutant 3-repeat Tau isoforms.

The length a microtubule grows during a growth event is a function of the duration of the growth event and the growth rate. We found that the FTDP-17 mutations affected both of these parameters. Whereas wild-type 4-repeat Tau decreased the average growth duration by 32%, 4-repeat Tau containing FTDP-17 mutations decreased the average growth duration by no more than 12% (data not shown). In addition, some FTDP-17 mutant Tau proteins tested were also compromised in their ability to inhibit the growth rate (Table 3; Fig. 5). The strongest loss-of-function occurred with ΔK280, which caused a 50% reduction in the ability of Tau to regulate the growth rate when compared with 4-repeat wild-type Tau. Two mutations, P301L and R406W (in both 4-repeat and 3-repeat isoforms), had almost no effect on this parameter, as they affected growth rates similarly to wild-type Tau.

Thus, the mechanistic effects of the FTDP-17 mutations on the regulation of dynamic instability could be analyzed in the absence of potential effects of the FTDP-17 mutations on microtubule shortening or growth. The consequence of this loss in the regulatory ability of Tau is that microtubule growth proceeds more rapidly and/or for longer durations in cells with FTDP-17 mutant Tau when compared with wild-type cells.
FTDP-17 Mutations Compromise Tau Function

Initially complicating effects on dynamics contributed by wild-type Tau isoforms. However, there are possible caveats to consider when extrapolating our data in MCF-7 cells to human neuronal cells. Important differences between MCF-7 cells and neuronal cells are that they express different tubulin isotypes and different kinds of microtubule-associated proteins, both of which could influence the ability of Tau or its mutated forms to regulate dynamic instability. Specifically, MCF-7 cells express primarily the β1 tubulin isoform (49), whereas brain cells express primarily the βIII isoform along with significant amounts of βIII (50). At the same time, our recent work shows that differences in the abilities of wild-type 3-repeat and 4-repeat Tau to regulate microtubule dynamics first observed in in vitro assays is fully mirrored when these isoforms are expressed and analyzed in MCF-7 cells (33, 35). Furthermore, analysis of the same mutant Tau isoforms tested here in MCF7 cells demonstrated almost identical effects in in vitro reactions. Thus, although differences may indeed be found between the mechanistic effects of FTDP-17 Tau mutations when present in MCF7 cells versus brain cells, it is likely that the data obtained in MCF7 cells provide a reasonable prediction of how the mutations will affect microtubule dynamics in neurons.

An additional point worthy of note is that the only Tau present in our experimental system is the wild-type or mutated Tau that we microinjected. In contrast, consistent with the dominance of the FTDP-17 mutations, cells in affected patients possess both wild-type and mutant Tau. However, the key point is that we are testing for the possible existence of mutation-induced alterations in the ability of Tau to regulate microtubule dynamics in a cellular environment. The simultaneous presence of both wild-type and mutant Tau could blur our ability to detect such alterations. Indeed, recent in vitro work in our laboratories indicates that the level of microtubule dynamics regulatory activity exerted by a mixture of two different Tau isoforms falls in between the levels of each isoform acting alone. Keeping in mind that subtle alterations in microtubule dynamics can have powerful cellular effects (51), we have sought to maximize the sensitivity of our assays by introducing only mutant Tau into the cells. Indeed, this strategy has been used by many investigators studying the cellular effects of various Tau isoforms (for example, see Refs. 52 and 53).

FTDP-17 Missense Mutations Exhibit Loss-of-Function Phenotypes with Respect to the Ability of Tau to Regulate Microtubule Dynamic Instability—We found that all of the FTDP-17 Tau mutations we examined caused loss-of-function effects (Tables 2 and 3; summarized graphically in Fig. 5). For example, all of the mutations except R406W greatly reduced the ability of Tau to increase the amount of time microtubules spent attenuated, and all of the mutations reduced the ability of Tau to regulate the length of microtubule growth during individual growth events. The most dramatic loss-of-function effects were exhibited by the mutations that map to regions of Tau believed to interact directly with microtubules (3-repeat G272V, 4-repeat G272V, P301L, and ΔK280), whereas less marked effects occurred with 4-repeat R406W and 3-repeat R406W, both of which map outside the microtubule binding domain.

The differences we observed could possibly be explained by differences in the binding affinity of the mutated Tau isoforms for the microtubule surface. Indeed, it has been demonstrated that many of the FTDP-17 mutations used in this study decrease the binding affinity of Tau for microtubules (23, 54). Therefore, the FTDP-17 Tau isoforms could have a reduced ability to regulate microtubule dynamics simply by virtue of there being fewer molecules of mutated Tau bound to the microtubules. We do not believe this to be the case for several reasons. First, when we calculated the percentage of Tau that should be bound to microtubules using previously published microtubule binding constants (47) and our estimates of the Tau and tubulin concentrations in the cell (see “Materials and Methods”), we determined that >95% of both the wild-type and the FTDP-17 Tau molecules should be bound to the microtubules. Additionally, not all dynamic parameters were affected equally by the presence of mutations in Tau, as would be expected if the mutated Tau were not binding to microtubules as well as wild-type Tau. Furthermore, the R406W mutation has been shown to have a reduced affinity for microtubules (23), yet in our assays, it affects most dynamic instability parameters comparably to wild-type Tau. Thus, we conclude that the loss-of-function effects of FTDP-17 mutant Tau are manifested once the mutated Tau binds to microtubules.

FTDP-17 Missense Mutations May Act through Multiple Mechanisms—The different FTDP-17 mutations we examined had varying effects on dynamic instability. As noted above, the four mutations in the microtubule-binding region (3-repeat G272V, 4-repeat G272V, ΔK280, and P301L) can be grouped together relatively easily. These mutations all reduced the regulatory effects of wild-type Tau on growth length, on the fraction of time the microtubules spent growing and attenuated, and on dynamics. There are likely to be subtle mechanistic differences even within this group, given that each mutation exhibits its own unique features. For instance, recalling that 4-repeat Tau has a much stronger ability to repress the rate and extent of shortening than does 3-repeat Tau, it is notable that the 4-repeat Tau-specific mutant P301L is especially weak with respect to regulating the rate and extent of shortening. This raises the possibility that this proline may play an important role in the ability of 4-repeat Tau to suppress these parameters.

The R406W mutation behaved very differently than the other mutations, having only modest effects upon the ability of both 4-repeat and 3-repeat Tau to regulate dynamic instability. Notably, this mutation does not map to the microtubule-binding region of Tau; rather, it resides in the regulatory C-terminal tail. Rather than affecting the Tau-microtubule interaction directly, this mutation may act primarily by affecting the phosphorylation of Tau at nearby sites. Indeed, other studies have found that phosphorylation of Tau was significantly altered by the presence of the R406W substitutions (2, 48, 52, 55).

We propose that the 3-repeat G272V, 4-repeat G272V, ΔK280, and P301L mutations directly affect the Tau-microtubule interaction. In contrast, we suggest that the direct effect of the R406W mutation is on the regulation of Tau itself (for example, phosphorylation), which in turn impacts upon the Tau-microtubule interaction and the regulation of microtubule dynamics. This conclusion agrees with that of others using completely different bioassays (48).

Are These Losses of Function in the Ability of Tau to Regulate Microtubule Dynamic Instability Meaningful to a Cell?—It is well established that inappropriate alterations in microtubule dynamics can have major consequences during mitosis. Specifically, previous work has shown that low concentrations of taxol that suppress microtubule dynamics by only ~31% are sufficient to impair mitotic progression, which may in turn lead to apoptosis (51, 56). At the other extreme, increases in microtubule dynamicity of ~50–60% inhibit mitotic progression in taxol-dependent cells unless microtubule dynamics are suppressed by treatment with taxol (42). Thus, both overdynamic and underdynamic microtubules can have severe consequences for cells.

Proper regulation of microtubule dynamics is critical in neurons as well. Disruption of the regulation of microtubule dynamics has been shown to interfere with axonal stability and the formation of synaptic boutons (57, 58). Furthermore, increasing microtubule stability with 3 S. Levy and S. C. Feinstein, unpublished observations.
4 A. LeBoeuf and S. C. Feinstein, unpublished observations.
microtubule-binding drugs ameliorates the axonal transport defects observed in a mouse model of tauopathy (54).

Based on these considerations and our recent in vitro and cellular work with wild-type 3-repeat and 4-repeat Tau (33, 35), we have proposed that microtubule dynamics in neuronal cells must be maintained within an acceptable range of activity levels. The model further suggests that, outside of this range, microtubules cannot function normally, resulting in cumulative damage and eventual cell death. The work presented here demonstrates that the FTDP-17 missense mutations mapping to the microtubule binding region increase microtubule dynamicity by 37–54% when present at physiologically relevant levels, i.e. they significantly compromise the ability of Tau to regulate microtubule dynamicity. Neurons harboring these mutations, and thus containing a mixture of wild-type and mutant Tau, are to likely possess overly dynamic microtubules. This decrease in microtubule stability may not have an immediate effect on neuronal development and function, but over time, cumulative damage could lead to neurodegeneration.

These data complement our recent cellular work relevant to the FTDP-17 Tau mRNA splicing mutations (33). In this work, we demonstrated that wild-type 4-repeat Tau exhibits quantitatively and qualitatively different mechanistic capabilities than does wild-type 3-repeat Tau, leading to the proposal that neurons harboring Tau mRNA splicing mutations should also exhibit markedly different patterns of microtubule dynamics relative to normal neurons. Taken together, our data suggest that both classes of FTDP-17 mutations, amino acid substitutions and altered Tau mRNA splicing, compromise the ability of Tau to properly regulate the dynamic behavior of microtubules. These data are consistent with the model in which defects in Tau or its regulation lead to disruption of microtubule function and consequent neuronal cell death.

Acknowledgments—We are extremely grateful to Herb Miller for technical assistance and data analysis, to Herb Waite for performing the mass spectrometry and to Michelle Massie for assistance with figures. We also thank Allen Stewart-Oaten and Carol Vandenbarg for valuable discussions, as well as Dmitri Leonoudakis for comments on the manuscript.

REFERENCES

1. Akhammer, A., and Hoogenraad, C. C. (2005) Curr. Opin. Cell Biol. 17, 47–54
2. Small, J. V., and Kaverina, I. (2003) Curr. Opin. Cell Biol. 15, 40–47
3. Imai, H., Sampao, P., and Sunkel, C. E. (2004) Int. Rev. Cytol. 241, 53–153
4. Zhou, J., and Giannakakou, P. (2005) Curr. Opin. Med. Chem. Anti-Cancer Agents 5, 65–71
5. Jordan, M. A., and Wilson, L. (2004) Microtubules in Health and Disease (Fojo, T., ed) Humana Press Inc., Totowa, NJ, in press
6. Machelsky, L. M., and Bornens, M. (2003) Curr. Opin. Cell Biol. 15, 2–5
7. Zhou, F. Q., and Snider, W. D. (2006) Science 308, 211–214
8. Riederer, B. M., Pellier, V., Antonsson, B., Di Paolo, G., Stimpson, S. A., Lutjens, R., Catsicas, S., and Grenningloh, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 741–745
9. Nakao, C., Itoh, T. J., Hotani, H., and Mori, N. (2004) Cell 117, 1799–1807
10. DeTure, M., Ko, L. W., Yen, S., Nacharaju, P., Easson, C., Lewis, J., van Slegtenhorst, M. H., Catsicas, S., and Grenningloh, G. (1997) ed) Humana Press Inc., Totowa, NJ, in press
11. Tirnauer, J. S., Grego, S., Salmon, E. D., and Mitchison, T. J. (2002) J. Cell Biol. 158, 1533–1539
12. Clark, L. N., Poorkaj, P., Wszolek, Z., Geschwind, D. H., Nasreddine, Z. S., Miller, B., Li, D., Payami, H., Awert, F., Markopoulou, K., Andreadis, A. D., D’Souza, I., Lee, V. M., Reed, L., Trojanowski, J. Q., and Zhai, Y., and Borisy, G. G. (1994) J. Cell Biol. 119, 6136–6144
13. Hasegawa, M., Smith, M. J., and Goate, M. (1996) FEBS Lett. 437, 207–210
14. Cassimeris, L. (2002) Mol. Cell. Biol. 15, 755–762
15. Butner, K. A., and Kirschner, M. W. (1991) J. Biol. Chem. 266, 15134–15143
16. Brandt, R., and Lee, G. J. (1999) J. Biol. Chem. 274, 11885–11890
17. Dhamodaran, R., and Wadsworth, P. (1995) J. Cell Sci. 108, 1679–1689
18. Miller, B., and Aisen, P. (1992) J. Biol. Chem. 267, 22090–22098
19. Budui, D., and Feuer, M. H., Wilson, L., and Feinstein, S. C. (1997) Mol. Cell Biol. 17, 533–535
20. Zhai, Y., and Borisy, G. G. (1994) J. Cell Biol. 127, 881–890
21. Drubin, D. G., Feinstein, S. C., Sholl, E. M., and Kirschner, M. W. (1985) J. Cell Biol. 101, 1799–1807
22. Rose, G. G., Porner, C. M., Shindler, T., and Trunnell, J. (1958) J. Biophys. Biochem. Cytol. 4, 761–764
23. Connelly, A., Brager, D., Kamath, K., Martello, L., Briand, C., Horwitz, S., Wilson, L., and Jordan, M. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11737–11742
24. DeTure, M., Ko, L. W., Yen, S., Nacharaju, P., Easson, C., Lewis, J., Prihar, G., Pickering-Brown, S., Duff, K., and Hutton, M. (1999) J. Biol. Chem. 274, 15134–15143
25. Overman, M. J., and Gajewski, T. (2000) Cell 101, 1799–1807