Tau interaction with microtubules in vivo

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Summary

Tau is a major microtubule-associated protein which induces bundling and stabilization of axonal microtubules (MTs). To investigate the interaction of tau with MTs in living cells, we expressed GFP-tau fusion protein in cultured Xenopus embryo neurons and performed time-lapse imaging of tau-labeled MTs. Tau uniformly labeled individual MTs regardless of their assembly/disassembly status and location along the axon. Photobleaching experiments indicated that interaction of tau with MTs is very dynamic, with a half-time of fluorescence recovery of the order of 3 seconds. Treatment of cells with taxol, a drug that suppresses MT dynamics, rapidly induced detachment of tau from MTs. Although binding of tau to straight MTs was uniform, there was a heightened concentration of tau at the sites of high MT curvature. Our results suggest that dynamic interaction of tau with MTs may modify local mechanical properties of individual MTs and play a crucial role in the remodeling of the MT cytoskeleton during neuronal plasticity.

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Introduction

Neuronal microtubules (MTs) are essential for neurite extension, axonal guidance, and communication between the cell body and neuronal processes. Dynamic properties of individual MTs and the architecture of the MT array are regulated by MT-associated proteins (MAPs). One member of the MAP family, tau, has received particular attention since hyperphosphorylation of tau has been linked to the formation of abnormal filaments characteristic of Alzheimer’s and other neurodegenerative diseases (‘tauopathies’) (Garcia and Cleveland, 2001; Lee et al., 2001).

A large body of experimental evidence indicates that tau promotes MT stability and induces MT bundling in vitro (Drechsel et al., 1992; Kanai et al., 1992). Expression of tau in non-neuronal cells is sufficient to induce formation of neurite-like processes that contain arrays of parallel MTs (Baas et al., 1991; Knops et al., 1991; Lee and Rook, 1992), while lowering the level of tau expression in neuronal cells inhibits axonal growth (Caceres and Kosik, 1990). Although mice deficient in tau are essentially normal (Harada et al., 1994), knockout of both tau and MAP1B leads to severe neuronal defects (Takei et al., 2000), suggesting functional redundancy between these (and, perhaps other) MAPs. Interestingly, in humans, mutations in tau linked to some neurodegenerative diseases reduce the ability of tau to promote MT assembly (D’Souza et al., 1999; Hasegawa et al., 1999). Collectively, these data predict that tau-induced stabilization of the MT cytoskeleton is essential for proper development of nerve processes.

Tau decoration of MTs appears to be uniform (Murphy and Borisy, 1975; Sloboda and Rosenbaum, 1979), and its binding to MTs is non-cooperative (Biernat et al., 1992). Electron microscopy studies suggest that tau binds to the outer surface of MTs and stabilizes tubulin-tubulin interactions along protofilaments (Al-Bassam et al., 2002). Binding of tau to MTs is phosphorylation-dependent and is regulated by several protein kinases (Drewes et al., 1998). Different isoforms of tau include three or four MT-binding repeats which are highly conserved between different species and different members of the MAP family (Goedert et al., 1996; Heidary and Fortini, 2001). 3R-tau and 4R-tau are developmentally regulated, and imbalances in their expression have been linked to neurodegenerative diseases (Hutton et al., 1998; Spillantini et al., 1998), suggesting a distinct function for each isoform. Further, even though both 3R- and 4R-tau induce neurite outgrowth in transfected cells, the onset of this outgrowth in 4R-tau-expressing cells is significantly more rapid (Yu et al., 2002). Immunocytochemical evidence indicates that tau density progressively increases in a proximal-to-distal direction along the length of growing axons (Black et al., 1996). This somewhat paradoxical co-localization of tau with a dynamic subset of axonal MTs at the distal end of the axon has led to the suggestion that, in addition to its well-established function as MT stabilizer, tau may play a role in signaling during growth cone navigation (Shahani and Brandt, 2002). It has also been suggested that tau may determine the spacing between MTs (Chen et al., 1992), provide a link between MTs and other membrane or cytoskeletal structures (Brandt et al., 1995; Cunningham et al., 1997), alter mechanical properties of MTs (Felgner et al., 1996), and affect transport of membrane organelles along MTs (Ebneth et al., 1998; Stamer et al., 2002).

Progress towards understanding the functional properties of
tau-MT interaction in neuronal cells has been limited by the technical difficulties of detecting individual MTs in living neurons. In this study, we took advantage of a unique morphological feature of Xenopus embryo neurons in culture. When the cultures are prepared on strongly adhesive substrate, individual tau-decorated MTs can be resolved with fluorescence microscopy throughout the entire axonal length. Using a combination of time-lapse microscopy and a photobleaching method, we examined the dynamics of tau association with MTs in living cells. We report that association of tau with individual MTs in neurons is very rapid, does not depend on MT location along the axon, and is strongly affected by MT curvature. Unexpectedly, we found that the MT-stabilizing drug taxol displaced tau from MTs. Our results reveal unexpectedly rapid and complex interaction of tau with MTs in live neurons.

Materials and Methods

DNA constructs
pEGFP-Tub encoding a fusion protein consisting of EGFP and human α-tubulin was from Clontech (Palo Alto, CA). Preparation of GFP-tau constructs has been described previously (Yu et al., 2002).

Microinjection of DNA into Xenopus embryos

Xenopus eggs were fertilized and dejellied in vitro as described previously (Chang et al., 1998). At the two-cell stage, the eggs were injected with 10-15 nl of 20 μg/ml DNA plasmids using the air pressure injector Picospritzer II (General Valve, Fairfield, NJ). The diameters of the pipettes used for injection ranged from 9 to 18 μm. Injections were performed in Ringer’s solution (115 mM NaCl, 2 mM CaCl2, 2.5 mM KCl, and 10 mM HEPES, pH 7.6) supplemented with 5% Ficoll (Sigma). One hour after injection the embryos were transferred to 20% Ringer’s solution containing 5% Ficoll, and 2-3 hours later they were transferred to 20% Ringer’s solution. Development of embryos injected with the plasmid encoding GFP-tau23 or GFP-tau24 was slowed down by approximately 20% in comparison with control (uninjected or injected with vector encoding GFP alone) embryos. However, up to 25% of injected embryos developed normally (the actual number in a particular experiment depended on the quality of eggs). The eggs were allowed to develop to stages 19-24 and were then used for the preparation of neuronal cultures.

Microinjection of Cy3-tubulin into Xenopus embryos

Cy3-tubulin was a generous gift of Gary Borisy (Northwestern University, Chicago, IL). Xenopus embryos were injected with 10-25 nl of 10 mg/ml Cy3-tubulin as described elsewhere (Chang et al., 1998).

Cell cultures

Xenopus embryo neuronal cultures were prepared according to previously reported methods (Popov et al., 1993; Spitzer and Lamborghini, 1976). Briefly, the neural tubes of embryos at stages 19-24 were dissociated in Ca2+- and Mg2+-free solution (115 mM NaCl, 2.6 mM KCl, 10 mM HEPES, and 0.4 mM EDTA, pH 7.6) for 20-30 minutes. Dissociated cells were plated on glass coverslips precoated with concanavalin A (0.1-1.0 μg/cm²; Sigma). The cultures were kept at 20°C in a culture medium consisting of 50% (v/v) Ringer’s solution, 49% L-15 Leibovitz medium (GIBCO Life Technologies), and 1% fetal bovine serum (GIBCO). The neurons were used for experiments 14-20 hours after plating. Human 356 foreskin fibroblasts were kindly provided by T. Svitkina (Northwestern University) and were cultured in HAM’s F-10 medium supplemented with 10% fetal bovine serum (GIBCO). Okadaic acid and calyculin A were from Sigma.

Image acquisition and data analysis

We obtained digital images of cells at room temperature using an inverted microscope (TE2000, Nikon) equipped with a 60x PlanApo objective (NA 1.4) and a 100-W mercury lamp. The light passed through an infrared-blocking filter, neutral density filters, and a GFP-wide-band filter. Images were acquired with a charge-coupled device (CCD) camera (CoolSnapHQ, Roper Scientific) driven by IPLab imaging software (Scanalytics). The image was directly projected onto the CCD chip and the final magnification was =0.11 μm/pixel. Exposure time was 0.2-1.0 seconds, and images were collected at 1- to 3-second intervals. Typically, 100-200 frames were captured. The culture medium was supplemented with the oxygen-depleting preparation, Oxyrase (Oxyrase, Mansfield, OH), during observation, in order to reduce photodamage. Neither axonal morphology (as inspected with differential interference contrast microscopy) nor MT integrity was affected by exposure to the fluorescent light during image acquisition. Images were processed with IPLab and Photoshop (Adobe Systems). Quantification of data was performed with IPLab software.

Photobleaching

The beam of the 150-mW multimode argon laser (Spectra-Physics, Fremont, CA) was channeled into the epi-illumination port of a Nikon 200 microscope. A cylindrical lens with a 700 mm focal distance and a 60x, 1.4 NA objective lens was used to produce a 4 μm x 15 μm or 2 μm x 15 μm focused beam in the specimen plane. Exposure was 50-1000 milliseconds. Laser irradiation of neurons under these conditions had no detectable effect on axonal growth or on MT assembly/disassembly. Cells were imaged before and immediately after photobleaching. In general, a 2- to 4-μm-wide region on a single MT or MT bundle was bleached by a single laser pulse and allowed to recover. Images were typically collected at 1-second intervals. To evaluate the rate of fluorescence recovery of cytoplasmic GFP-tau, ten images per second were collected without shutter opening/closure. To measure fluorescence intensity of a single MT, a small linear region of interest, 1×10 pixels (≈0.1×1 μm) in size, was selected along the MT length, and the average fluorescence intensity was calculated. The background fluorescence was measured in a similar 1×10 pixel MT-free region of the cytoplasm. Typically, background fluorescence was averaged over 3-4 regions. Background-subtracted MT intensity was plotted as a function of time and fitted with a single exponential function to calculate the half-time t½ (Bulinski et al., 2001).

Total internal reflection fluorescence microscopy

A beam from a 150-mW argon laser (Spectra-Physics) was steered through the single mode fiber into an Olympus total internal reflection fluorescence (TIRF) illuminator and focused at the back focal plane of the objective lens (Planapo 60x, 1.45 NA, Olympus). The focal point was moved off-axis to the most peripheral position that still allowed the beam to pass through the objective. Light underwent total internal reflection at the glass interface. Images were recorded with a CoolSnapHQ cooled CCD camera (Roper Scientific) driven by IPLab software.

Detergent extraction

Neurons expressing GFP-tau were extracted in a MT-stabilizing buffer (60 mM PIPES, 1 mM MgCl2, 5 mM EGTA, 0.1% Triton X-100, 10 μM taxol, pH 6.8) and examined under the fluorescent microscope during extraction.
Data analysis
We made all position, intensity and length measurements on 12-bit digital images using the analysis function of the IPLab software. To determine significant differences between averages, unpaired t-tests assuming equal variance were performed.

Association of tau with microtubules in vitro
CHO cells in DMEM/F12 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum were cultured at 37°C with 5% CO₂. Cells were transfected with pcNA3 vector, wild-type tau, or GFP-tau plasmids. Twenty-four hours after transfection, cells were harvested and resuspended in G-PEM buffer (80 mM PIPES, 0.5 mM MgCl₂, 1 mM EGTA) containing Complete Protease Inhibitor Tablets (Roche Molecular Biochemicals, Indianapolis, IN). Cell suspensions were frozen and thawed twice and then homogenized with a teflon-glass homogenizer (Kontes Glass Company, Vineland, NJ). Unbroken cells and cellular debris were removed by centrifuging at 20,000 g at 4°C for 20 minutes. Each supernatant was divided into two 1.5 ml tubes and after incubation at 32°C for 3 hours, one tube of each group was treated with 5 µM taxol for 10 minutes. A high-speed centrifugation was applied to spin down microtubules at 146,000 g at 32°C for 30 minutes. Pellets were separated by 12% SDS-PAGE and transferred to Immobilon transfer membrane (Millipore, Bedford, MA). Blotting was done as described previously (Yu et al., 2002). Polyclonal antibody against the C-terminal of human tau and monoclonal antibody against α-tubulin were purchased from DakoCytomation (Carpinteria, CA) and Sigma (St Louis, MO), respectively.

Supplementary movie
Time-lapse sequences were linearly adjusted for brightness and contrast and converted to QuickTime movies (animation compression, medium quality, 12-bit depth) with IPLab software (see supplementary material).

Results
GFP-tau labels individual MTs in neurons and in fibroblasts
The plasmid encoding GFP-tau23 fusion protein (GFP-tau) was injected into one of the blastomeres of two-cell-stage

![Fig. 1. GFP-tau binds to individual MTs and induces MT bundling and the formation of neurite-like processes in Xenopus embryo cultures.](image)

(A-D) Fluorescence micrographs of fibroblast-like cells expressing GFP-tau. GFP-labeled polymeric structures can be observed in the cytoplasm. Individual polymers at the edge of the cell (marked by arrows in A) are similar in intensity and therefore are likely to represent single MTs. MT bundles can be found both in the soma (B) and inside neurite-like cell processes (C). Panels A and D show representative examples of cells demonstrating non-centrosomal and centrosomal patterns of MT organization. Bar, 10 µm. (E) Series of fluorescence images of the lamella region of a fibroblast transfected with GFP-tau. Both growing (arrows) and shortening (arrowheads) MTs are visible. Time (in seconds) is shown at the upper-right corner. Bar, 10 µm. (F) Quantitative analysis of GFP-tau binding to individual MTs. For each MT, the intensity of fluorescence was measured at distances of ~1 µm (open bar) and ~5-10 µm (hatched bar) from the tip. The background fluorescence at adjacent MT-free regions was subtracted from the measurements. Data from 50 MTs in seven different neurons are presented as means ± s.e.m. (G) Series of fluorescence images of GFP-tau-expressing fibroblasts (elapsed time is stated in seconds). Both individual MTs and MT bundles are detected at the edge of the cell. MT bundles arise through lateral interaction between individual MTs (marked by arrows). Bar, 10 µm.
Xenopus embryos. Injected embryos were allowed to develop to stage 19-24 and then were used for the preparation of cell cultures. Approximately 20-25% of cells expressed GFP-tau as visualized with fluorescence microscopy. Experiments were performed on neurons and on fibroblast-like cells present in cell cultures.

Both in fibroblasts (Fig. 1) and in neurons (Fig. 2), GFP-tau decorated individual MTs and MT bundles. The background fluorescence in MT-free regions of the cytoplasm of transfected cells reflected the presence of free cytoplasmic GFP-tau; this background fluorescence, however, was sufficiently low to allow for the detection of individual MTs. In fibroblasts, the architecture of the MT array strongly depended on the level of tau expression. At the lower levels of expression, individual tau-labeled MTs could be resolved throughout the cytoplasm. We observed both non-centrosomal (Fig. 1A) and centrosomal (Fig. 1D) organization. At the higher expression levels, the majority of MTs were packed into tight bundles (Fig. 1B). In agreement with previous reports (Knops et al., 1991; Lee and Rook, 1992), expression of tau in fibroblasts often led to formation of neurite-like processes filled with MT bundles along the axon. (A) Fluorescence image of a neuron expressing GFP-tau. (B,C) Higher-magnification views of the growth cone region (B) and the proximal axon (C) taken from areas marked by rectangles in A. Individual MTs can be resolved in both areas, allowing for direct comparison of tau binding to MTs in different axonal regions. Bar, 10 µm. (D) Quantitative analysis of tau binding to individual MTs at the growth cone and at the proximal axonal segment. Axons chosen for analysis were 150-250 µm in length. The fluorescence intensity of individual MTs was measured at the growth cone region and at the middle axonal segment and, after background subtraction, scaled to adjust for the different levels of tau expression in different neurons. Data are presented as means±s.e.m. Data from 40 MTs in five different neurons.

Table 1. Dynamics of tau-MT interaction: photobleaching analysis

|                      | GFP-tau<sub>23</sub> |                      | GFP-tau<sub>24</sub> |
|----------------------|----------------------|----------------------|----------------------|
|                      | Axonal shaft, single MT | Growth cone, single MT | Axonal shaft, single MT | Fibroblast, cytoplasmic MT bundle | Fibroblast, MT bundles in cell processes | Axonal shaft, single MT | Fibroblast, MT bundles in cell processes |
| T<sub>0</sub> (seconds) | 2.8±1.1  | 2.5±0.5  | 2.8±0.3  | 3.2±0.4  | 9.5±0.5  | 16.7±2.6  | 2.5±0.5  | 12.2±2.3  |
| % Recovery           | 69.3±15.2 | 85±10.5  | 54.6±3.5 | 88±6.5   | 69.5±6.3 | 56.2±5.6  | 53±11.3  | 24.5±5.3  |

Background-subtracted fluorescence intensity of MTs after photobleaching as a function of time was fitted with a single exponential to obtain the slope<sub>0</sub> of fluorescence recovery and the percent of recovery (% recovery); the latter was calculated as a difference between fluorescence intensity immediately before photobleaching and that more than 5 minutes after photobleaching normalized to initial fluorescence. Values represent means±s.e.m. The number of experiments for each datum point varied from 15 to 20. No statistically significant difference was found between 3R-tau and 4R-tau in the dynamics of MT binding (P>0.05 for corresponding series of experiments).
MT bundles often splay into individual polymers at the lateral edges of the axonal shaft and/or in the vicinity of the branch points (Fig. 2A). This allows for the direct comparison of tau binding to MTs at different axonal segments (Fig. 2B-D). In contrast to previous immunochemical studies that documented a higher concentration of tau at the distal MTs in comparison with the proximal axon in rat sympathetic neurons (Black et al., 1996), we found that, in *Xenopus* neurons, the density of MT-bound tau did not depend on MT location along the axon (Fig. 2D).

To compare the concentrations of free cytoplasmic GFP-tau at the different axonal regions, we measured the intensity of fluorescence in flat MT-free regions of cytoplasm with TIRF microscopy. The fluorescence signal in this case is proportional to the concentration of cytoplasmic GFP-tau in the thin (=100 nm) submembrane layer of the cytoplasm. The fluorescence intensities at the growth cone region and at the proximal axon (300-400 µm from the growth cone) were 43±5.5 and 54±7.3, respectively (arbitrary units; means±s.e.m., data from six different neurons). These data are consistent with the notion that the concentration of tau is uniform throughout the axoplasm.

Taken together, the results of these experiments indicate that, within the resolution limit of light microscopy, binding of tau to neuronal MTs is uniform along individual MTs, does not depend on MT location in the axonal shaft, and is independent of the dynamic state of the MT.

**Binding of tau to individual MTs depends on their curvature**

Local mechanical properties of a MT (such as flexural rigidity) on a submicrometer scale are likely to be affected by the local density of MT-bound MAPs. It is usually assumed that the concentration of MT-bound MAPs does not vary along the length of the MT. To test this assumption, we performed time-lapse imaging of fluorescent tau-labeled MTs in living fibroblasts and in neurons. We focused on the flat regions of the cells (leading edge of fibroblasts and growth cone-like protrusions at the lateral edge of the axonal shaft), where...
individual tau-decorated MTs could be resolved. Results of a typical experiment are presented in Fig. 3. At the lamella region of the cell and at the growth cone, MTs often buckled, presumably because of the activity of retrograde actin flow (Waterman-Storer and Salmon, 1997) or because of compressive forces generated by tubulin polymerization itself. As described above (Figs 1, 2), relatively straight MTs displayed a uniform pattern of GFP-tau labeling. However, for buckled MTs the concentration of GFP-tau was significantly higher at the regions of higher MT curvature (Fig. 3; see also Movie 2 in supplementary material). The density of MT-bound tau rapidly adjusted to MT shape (Fig. 3B-D). For quantitative analysis of the data we measured fluorescence intensity of MTs as a function of the radius of MT curvature (Fig. 3F). The dependence of tau density on the radius of MT curvature was similar for fibroblasts and neurons. Concentration of tau at the region with a radius of curvature of 0.5 µm was approximately twice as high as that at the straight segment of a MT. Similar quantitative analysis of individual MTs in GFP-tubulin-expressing neurons (Fig. 3F) indicated that fluorescence intensity of MTs remains uniform regardless of their curvature. This suggests that enhanced binding of tau to highly curved MT regions is not an optical artifact because of our CCD detection system.

**Fig. 4.** Tau-MT interaction is highly dynamic. (A-E) Series of fluorescence micrographs of *Xenopus* fibroblasts (A,C), *Xenopus* neurons (B,D), and human 356 fibroblasts (E) expressing GFP-tau. The first images were acquired immediately before photobleaching. Individual MTs are marked with arrows in A and B. Elapsed time after photobleaching is shown in seconds at the right corner of each image. The boxed regions mark the approximate location of the bleached zone. The size of the bleached regions is ≈4 µm. For each time-lapse sequence, the background-subtracted fluorescence intensity of MTs (arbitrary units) was plotted against time in seconds. Fluorescence recovery after photobleaching along individual MTs (A,B,E) is rapid and uniform along MT length. Fluorescence recovery for neurite-like processes produced by fibroblasts (C) and for axons (D) is much slower.

Tau interaction with MTs is highly dynamic: fluorescence recovery after photobleaching analysis

The ability of tau to follow rapidly changing MT shape suggests that interaction of tau with individual MTs is very
rate constant for the photobleaching recovery is identical to the measure t
argon laser and time-lapse imaging of cells was performed to
Small regions of cells were illuminated with a brief flash of
bundles both in fibroblasts and in neurons expressing GFP-tau.
performed photobleaching experiments on single MTs and MT
interaction, we performed photobleaching experiments on single MTs and MT
photobleaching experiments on single MTs and MT bundles both in fibroblasts and in neurons expressing GFP-tau.
Small regions of cells were illuminated with a brief flash of
argon laser and time-lapse imaging of cells was performed to
rate constant for the photobleaching recovery is identical to the
dissociation rate constant, k_{dis} (Bulinski et al., 2001). Fig. 4
illustrates results of a typical photobleaching experiment
performed on fibroblasts (Fig. 4A,C) and on neurons (Fig.
4B,D). In both cell types, some recovery of fluorescence along
single MTs (Fig. 4A,B) was visible within the first few seconds
and recovery appeared to be complete within 5-10 seconds
after the bleaching. Fluorescence recovery along neurite-like
processes produced by fibroblasts (Fig. 4C) was significantly
slower. To address a potential problem of our approach with
regard to the full functionality of human tau in a heterologous
Xenopus system, we repeated photobleaching experiments in
human 356 fibroblasts. As with Xenopus cells, in 356
fibroblasts we observed rapid fluorescence recovery of GFP-
tau-decorated single MTs after photobleaching (Fig. 4E) with
a characteristic t_{rec}=5.8±0.9 s (n=7). This rate of recovery was
comparable with that observed in Xenopus cells (see Table 1
below).
Previous studies have demonstrated that photobleaching
does not significantly affect cytoskeletal dynamics; in
agreement with these data, brief exposure to the laser beam in
our experiments had no detectable effect on growth cone
motility or the integrity of axonal MTs. Moreover, t_{rec} did not
depend on the duration of the pulse or the intensity of
illumination, and multiple cycles of tau binding could be
observed after repeated photobleaching of the same axonal
regions (data not shown). Therefore, it seems unlikely that
photobleaching significantly affects tau-MT interaction.
Two processes contribute to the rate of fluorescence recovery
of individual MTs after photobleaching. The first is the
diffusion of free cytoplasmic GFP-tau into the bleached zone;
the second is the dissociation of bleached GFP-tau from MTs.
Only the second (but not the first) process reflects the true
dynamics of tau-MT interaction. To estimate the contribution
of diffusion of cytoplasmic tau to the fluorescence recovery of
individual MTs, we repeated photobleaching experiments on
GFP-tau-expressing neurons incubated overnight at 4°C and
pretreated with nocodazole (10 µg/ml, 1 hour). Under these
experimental conditions, some of the well-spread regions of the
axonal shaft were devoid of MTs (Fig. 5). Photobleaching of a
small (≈4 µm) region in these neurons was followed by rapid
recovery, with a characteristic t_{rec}=400±52 ms (n=7). Therefore,
for the typical bleached area (≈4 µm), fluorescence recovery
of cytoplasmic GFP-tau is 10-50 times faster than that of
individual MTs and MT bundles (see below). In this situation,
the experimentally measured t_{rec} of the fluorescence recovery of
individual MTs reflects dynamics of tau-MT interaction with
an accuracy of 2-10%.
In each photobleaching experiment performed on GFP-tau-
decorated MTs, we measured t_{rec} for single MTs or MT bundles
and the extent of fluorescence recovery (the intensity of
fluorescence in the bleached segment ≈1-5 minutes after
photobleaching normalized to the initial fluorescence signal).
The results of these measurements are summarized in Table 1.
As suggested by the visual analysis of time-lapse sequences
after photobleaching, association/dissociation of tau along
single MT was dynamic both in neurons and in fibroblasts, with
t_{rec}=2-3 seconds. The slowest recovery of tau fluorescence was
observed in neurite-like processes produced by fibroblasts
(t_{rec}=16 seconds). In this case, as well as for MT bundles in the
axonal shaft, fluorescence recovery was incomplete (=55%
recovery). This suggests that a fraction of MT-bound tau is
tightly associated with MTs. Somewhat unexpectedly, no
significant difference was found between 3R-tau and 4R-tau in
their association with MTs (Table 1).

**Taxol induces rapid detachment of tau from single MTs**

Biochemical and structural studies concerning interaction of
tau with MTs are typically performed in the presence of the
MT-stabilizing drug taxol (Gustke et al., 1994; Littauer et al.,
1986; Melki et al., 1991). Recent studies have demonstrated
that in the presence of taxol, the dynamics of association of
ensconsin (E-MAP-115) with MTs is slowed down by an order
of magnitude (Bulinski et al., 2001). To investigate whether
interaction of tau with MTs can be modified by exposure to
taxol, we attempted to conduct photobleaching experiments on
GFP-tau-expressing cells in the presence of taxol (1 µM).
Unexpectedly, within minutes of taxol incubation, both in
fibroblasts and in neurons (Fig. 6; see also Movie 3 in

![Fig. 5. Recovery of cytoplasmic GFP-tau after photobleaching. (A) Fluorescence images of a GFP-tau-transfected neuron after overnight incubation at 4°C followed by nocodazole treatment (10 µg/ml, 1 hour). The first image was acquired immediately before photobleaching. The time after photobleaching is shown at the upper-right corner of each panel. The size of the bleached zone was ≈4 µm. No single MTs can be detected with fluorescence microscopy, and therefore fluorescence intensity reflects the concentration of cytoplasmic GFP-tau. Dark elongated structures visible throughout the entire sequence correspond to membrane organelles (most likely mitochondria) which exclude GFP-tau. (B) Fluorescence intensity of the bleached zone (I) normalized to the initial fluorescence (I_o) as a function of time.](image-url)
supplementary material) most single MTs decorated with tau became undetectable with fluorescence microscopy. Simultaneously, we observed an increase in the level of background fluorescence in the cytoplasm. In contrast, bundles of cytoplasmic MTs were still visible after taxol incubation although the intensity of their fluorescence had decreased. Incubation of *Xenopus* cell cultures with vinblastine (1 µM) for 10 minutes had no visible effect on association of GFP-tau with MTs (Fig. 6C,F). These results suggest that taxol induces rapid dissociation of tau from single MTs.

Incubation of *Xenopus* neurons with low concentrations of taxol does not change the total amount of polymeric tubulin (Zakharenko and Popov, 1998); hence it seems unlikely that the observed effect of taxol is because of rapid depolymerization of MTs. To test the effect of taxol on the integrity of single MTs directly, we repeated experiments on cells loaded simultaneously with Cy3-tubulin (to observe the architecture of the MT array) and with GFP-tau. As expected, single MTs could easily be detected in the cytoplasm after exposure to taxol (Fig. 7D). In contrast, no association of GFP-tau with MTs was observed (Fig. 7C). As with *Xenopus* cells, incubation with taxol induced rapid dissociation of tau from MTs in human 356 fibroblasts (Fig. 8; see also Movie 4 in supplementary material). This suggests that the effect of taxol is not specific to the heterologous *Xenopus* expression system.

The taxol-induced dissociation of tau from MTs in living cells was totally unexpected. We wanted to investigate whether a similar phenomenon can be observed after detergent extraction of cells in a MT-stabilizing buffer containing taxol (Tanaka and Kirschner, 1991). Such extraction is known to preserve individual MTs while rapidly removing free tubulin dimers and other cytoplasmic proteins. Within seconds after the onset of extraction, single GFP-tau-labeled microtubules became significantly dimmer and acquired a characteristic ‘speckled’ pattern (Fig. 9A; see also Movie 5 in supplementary material), indicating rapid dissociation of tau from individual MTs (Waterman-Storer et al., 1998). Interestingly, both in GFP-tau-transfected fibroblasts (Fig. 9B) and in neurons (Fig. 9C) a significant fraction of GFP-tau remained associated with MT bundles for as long as 30 minutes after extraction. This effect was particularly robust for MT bundles in fibroblasts (Fig. 9B). These data are in general agreement with the results of photobleaching experiments, which indicated that a significant fraction of tau is tightly associated with MT bundles (Table 1).

Results from living cell studies showed taxol-induced dissociation of GFP-tau from MTs, which was found both in *Xenopus* cells and in human 356 fibroblasts. It is not feasible to observe the effect of taxol on wild-type tau association with MTs in living cells using fluorescence techniques. To estimate further the dissociation of endogenous tau from MTs promoted by taxol treatment, CHO cells were transfected with wild-type tau or with GFP-tau and homogenized. Standard cold-heating cycle was used to induce MT polymerization and MT pellet from the cellular supernatant was isolated on SDS-PAGE gels. Western blots indicated that both wild-type tau and GFP-tau expressed in CHO cells co-precipitated with microtubules (Fig. 10A). Ten-minutes taxol (5 µM) treatment dramatically decreased the amount of wild-type tau or GFP-tau co-precipitation with MTs (Fig. 10A). Taxol treatment increased microtubule formation in CHO cells that did not express tau protein. However, this did not occur in CHO cells expressing either wild-type tau or GFP-tau (Fig. 10B). It thus appears that wild-type tau and GFP-tau are displaced from microtubules by tau in vitro. This is consistent with the results of experiments in living cells (Figs 7, 8).

**Discussion**

Tau is a major neuron-specific MAP implicated in MT stabilization. Tau binds to MTs with affinity in the low micromolar range and demonstrates neither enzymatic activity (e.g. nucleotide hydrolysis) nor high affinity binding for non-polymeric tubulin (Mandelkow and Mandelkow, 1995). As a
step towards understanding tau-MT interaction in living cells, we expressed a fetal isoform of tau (tau_23) fused to GFP in *Xenopus* embryo neurons in culture and performed imaging of fluorescent tau using a combination of time-lapse microscopy and photobleaching. We were able to detect individual MTs decorated with tau both in neurons and in non-neuronal cells. We interpret fluorescence intensity of single MTs in GFP-tau-transfected cells as a quantitative measure of tau binding to MTs. We also assume that photobleaching studies provide information on the dynamics of tau-MT interaction in living cells. The validity of our experimental approach is based on several assumptions. First, the fusion of GFP to the N-terminus of tau does not affect the functional properties of tau-MT interaction. Second, human tau used in this study is functional in *Xenopus* cell cultures. Third, photobleaching does not significantly alter the dynamics of tau-MT interaction. To a large extent, our rationale behind all these assumptions is based on several previous studies in which similar assumptions have been made and tested (e.g. Chang et al., 1998; Dehmelt et al., 2003; Kaech et al., 1996; Murphy and Borisy, 1975; Ozer and Halpain, 2000; Rodionov et al., 1994; Yu et al., 2002). In agreement with these findings, we further provide evidence that in our specific experimental conditions, GFP-tau binds to individual MTs, does not interfere dramatically with MT assembly/disassembly, induces MT bundling in non-neuronal cells, and binds to MTs in a phosphorylation-dependent manner (Figs 1, 2; see also Fig. S1 in supplementary material).

We provide evidence indicating that the major results of this study can be reproduced in human cell line (Figs 4, 8) and in vitro (Fig. 10). Finally, as discussed in Results, we performed several conventional controls for potential phototoxic effects of photobleaching. Therefore, it seems likely that our video microscopy approach provides reliable information concerning tau-MT interaction in living cells.

There are four unexpected findings in this report: (a) association of tau with MTs is very fast, with a $t_{1/2}$ of 3-5 seconds; (b) binding of tau to axonal MTs does not depend on MT location in the axonal shaft or on MT assembly/disassembly status; (c) binding of tau to MTs varies with MT curvature; and (d) treatment of cells with taxol induces rapid dissociation of tau from individual MTs in living cells.

There is now an abundance of evidence suggesting rapid interaction of MAPs with MTs. Thus, previous photobleaching studies reported MAP-MT interactions with $t_{1/2}$ ranging from 44 seconds to 5 minutes for different members of the MAP family (Bulinski et al., 2001; Olmsted et al., 1989; Scherson et al., 1984). Recent measurements of $t_{1/2}$ for ensconsin, a protein expressed predominantly in epithelial tissue, suggested a remarkably rapid interaction of this protein with MTs ($=4$ seconds) in non-neuronal cells (Bulinski et al., 2001). Despite the central role of tau in the regulation of the MT array, direct measurements of the dynamics of tau association with MTs in living neurons have not been reported. This is probably partly because of the difficulties in detecting individual MTs in living cells.
neurons. Indirect evidence based on the differential rates of slow axonal transport of tau and MAP2 indicates that the association of tau with MTs is dynamic (Mercken et al., 1995).

Both in neurons and in fibroblast-like cells present in Xenopus embryo cell cultures, we were able to detect individual MTs labeled with GFP-tau. In agreement with previous studies (Kaech et al., 1996), the GFP-tau fusion protein appeared to retain functional activity (Fig. 1), making it a faithful reporter of tau binding to MTs in living cells. In this study, we have not attempted to estimate the concentration

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**Fig. 8.** Taxol induces rapid dissociation of tau from MTs in a human cell line. Fluorescence images of a 356 fibroblast expressing GFP-tau. The first image was acquired immediately before application of taxol (1 µM). The time after taxol application is given in seconds. Individual MTs (arrows) and MT bundles (arrowhead) are becoming progressively dimmer, whereas the level of background cytoplasmic fluorescence increases during incubation. Bar, 10 µm.

**Fig. 9.** Detergent extraction of GFP-tau-expressing cells. Fluorescence images of fibroblasts (A,B) and a neuron (C) expressing GFP-tau before (top images) and after (bottom images) extraction in a buffer containing 10 µM taxol. The time after the onset of extraction is shown in the lower-right corner of each panel. Individual MTs (arrows in A) rapidly lose associated GFP-tau. In contrast, GFP-tau remains associated with MT bundles (arrowheads in B,C) for as long as 30 minutes after extraction. Bars, 10 µm.
of exogenous (GFP-labeled) tau in the cytoplasm. However, a strong fluorescence signal associated with individual GFP-tau-decorated MTs and low background fluorescence (because of unbound cytosolic GFP-tau) suggests that the concentration of exogenous tau was significantly lower than $K_d$ (which is in the low micromolar range for tau and other members of MAP family).

Systematic analysis of tau-MT binding with the photobleaching method indicated that tau association with single MTs in *Xenopus* cells is very dynamic, with $t_{1/2}$ of the order of 3 seconds (see Table 1). Similar kinetics of association with single MTs has been reported for enscinsin (Bulinski et al., 2001) and for tau-MT binding in vitro (Makrides et al., 2004). Although enscinsin does not affect MT dynamics (Faire et al., 1999), tau is believed to be the major promoter of MT polymerization. It may seem paradoxical that the association of this MT stabilizer with MTs is so transient. Although the functional significance of such rapid tau-MT interaction remains unclear, it is tempting to speculate that it may be advantageous for proper activity of the kinesin-based axonal transport system. Indeed, in vitro tau reduces the probability of attachment of kinesin to MTs (Trinczek et al., 1999), and overexpression of tau in N2A neuroblastoma cells inhibits transport of vesicles and mitochondria (Ebneth et al., 1998). The transient nature of tau-MT interaction is likely to decrease the probability of irreversible steric interference between kinesin or its cargo and tau and, thus, to facilitate transport activity. It should be noted that photobleaching measurements (Table 1) indicate that fluorescence recovery of tau-decorated MT bundles is incomplete. This suggests that a significant fraction of tau is tightly associated with MT bundles (but not with individual MTs). This conclusion is further supported by the results of detergent extraction experiments (Fig. 9). Therefore, the dynamics of tau interaction with single MTs and with MT bundles appear to be significantly different.

Axonal MTs at the growth cone region are generally believed to be dynamic, whereas those along the axonal shaft are relatively stable (Baas et al., 1993). This difference in MT stability is reflected by different posttranslational modifications of tubulin at different axonal regions. In addition, the distal tips of growing MTs are distinct from the bulk of the MT lattice both structurally and biochemically (Desai and Mitchison, 1997). These regional differences in MT structure/molecular composition may lead to preferential association of tau with various axonal segments. It should be noted, however, that the studies concerning distribution of tau along nerve processes were primarily based on immunocytochemistry. Although this method generally reliably reports localization of MAPs such as MAP2, in the case of tau results strongly depend on a particular fixation/extraction protocol (Black et al., 1996). The ability to detect individual tau-decorated MTs in live *Xenopus* neurons allows for a direct comparison of the amount of MT-bound tau at different axonal regions. Our results indicate that the concentration of cytosolic tau (as measured with TIRF microscopy) and the binding of tau to MTs are similar in different axonal regions. Furthermore, results of our photobleaching experiments suggest that technical difficulties associated with immunocytochemical localization of tau may be related to the unusually fast dynamics of tau-MT interaction. It is possible, however, that the difference in the results obtained with photobleaching and immunocytochemical methods can be related to different experimental systems.

One intriguing possibility concerning the functional significance of rapid tau-MT interaction is suggested by the results of experiments in which we performed time-resolved measurements of tau association with buckling MTs. Previous experimental evidence (Felgner et al., 1996) suggests that tau and other MAPs are evenly distributed along the length of MTs. Moreover, biochemical evidence indicates that binding of tau to MTs is non-cooperative. Therefore, observations of strong preferential binding of tau to MT regions with higher curvature (Fig. 3) came as a surprise. Previous biophysical measurements of MT flexural rigidity (Felgner et al., 1996; Gittes et al., 1993; Kis et al., 2002) were done under the assumption that MTs can be modeled as isotropic and homogeneous beams; in this case their flexural rigidity (or bending stiffness) can be characterized by a single parameter, Young’s modulus. Our results indicate that association of tau with buckling MTs is highly non-uniform. Because the association of MAPs with MTs has a profound effect on the flexural rigidity of MTs, we hypothesize that transient association of tau with highly curved MT segments may increase local flexural rigidity of the MT to resist compressive forces acting on the MT lattice. This is likely to contribute to a relatively straight shape of neuronal MTs and to prevent formation of MT ‘tangles’. It is noteworthy that the rapid interaction of tau with MTs that we observe with photobleaching is crucial for tau’s ability to adjust to a changing MT shape. The molecular mechanisms of preferential association of tau with highly curved MT segments remain to be investigated.

A large number of previous biochemical and structural studies have investigated the interaction of MAPs with MTs; these studies have typically been performed on taxol-stabilized MTs. Under these experimental conditions, tau binds to the outer surface of a MT (Al-Bassam et al., 2002; Santarella et al., 2004). Because taxol binds to a specific site on $\beta$-tubulin.
located inside a MT (Nogales et al., 1999), rapid dissociation of tau from individual MTs after incubation with taxol (Figs 6, 8; see also Movies 3 and 4 in supplementary material) came as a complete surprise to us. Taxol may influence tau binding to MTs indirectly by affecting the phosphorylation state of several proteins (Shitl et al., 1999; Yuriji et al., 1999) which may be involved in the regulation of tau-MT interaction. Although at present we cannot completely exclude this hypothesis, the very rapid effect of taxol on the association of tau with MTs makes this possibility unlikely. Therefore, we hypothesize that taxol binding induces a conformational change of a MT lattice that is sufficient to affect binding of tau to the MT. Alternatively, tau and taxol may compete for the same binding site on a MT (Kar et al., 2003). Regardless of the molecular mechanism of interaction between tau and taxol, our results demonstrate that within minutes after the onset of treatment with 1 μM taxol, tau largely dissociates from single MTs in live cells. This is likely to change the mechanical properties of the MT array and contribute to neuropathy which is a common complication of taxol-induced toxicity (Peltier and Russell, 2002).

Taken together, the results of our experiments reveal unexpectedly rapid dynamics of tau-MT interaction in live cells and suggest that this phenomenon may contribute to local changes in the mechanical properties of MTs. The ability to visualize tau-MT interaction in live cells should provide further information on the involvement of this MAP in axonal growth and neuronal plasticity.

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