Human Molecular Genetics, 2011, Vol. 20, No. 4 **763–778**
doi:10.1093/hmg/ddq521
Advance Access published on November 30, 2010

Strategies for diminishing katanin-based loss of microtubules in tauopathic neurodegenerative diseases

Haruka Sudo and Peter W. Baas*

Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 W. Queen Lane, Philadelphia, PA 19129, USA

Received October 4, 2010; Revised and Accepted November 25, 2010

It is commonly stated that microtubules gradually disintegrate as tau becomes dissociated from them in tauopathies such as Alzheimer's disease. However, there has been no compelling evidence to date that such disintegration is due to depolymerization of microtubules from their ends. In recent studies, we have shown that neurons contain sufficient levels of the microtubule-severing protein termed katanin to completely break down the axonal microtubule array if not somehow attenuated. The presence of tau on axonal microtubules renders them notably less sensitive to katanin, prompting us to posit that microtubule disintegration in tauopathies may result from elevated severing of the microtubules as they lose tau. In support of this hypothesis, we demonstrate here that pathogenic tau mutants that bind less strongly to microtubules than wild-type tau provide correspondingly less protection against katanin-based severing. Using cultured rat hippocampal neurons, we pursued two potential therapies for fortifying axonal microtubules against excess severing by katanin, under conditions of tau depletion. We found that either deacetylating the microtubules via overexpression of HDAC6 or treating the neurons with NAP, a microtubule-interacting neuroprotective peptide, resulted in notable protection of the microtubules against katanin-based loss. In both cases, we found that these treatments also diminished the characteristic increase in axonal branching that normally accompanies tau depletion, an effect that is also known to be directly related to the severing of microtubules. These observations may be useful in developing therapeutic regimes for preserving microtubules against loss in the axons of patients suffering from tauopathies.

INTRODUCTION

The severing of microtubules is critically important for the development of the nervous system and also for its maintenance throughout adult life (1,2). The severing of long microtubules into short ones is important, for example, for the ongoing transport of microtubules, given that only short microtubules are able to move in a rapid and concerted fashion within the axon (3). In addition, severing transforms single microtubules into many, thus creating greater numbers of free ends of microtubules (4,5). Greater mobility of microtubules and higher numbers of microtubules are especially crucial for axonal branch formation (2,6). Experimental diminution of microtubule-severing activity can have profoundly detrimental effects on neuronal development (7–10). Overexpression of microtubule-severing proteins can also produce detrimental effects, in that too much severing can degrade the microtubule array and render it unable to perform its normal functions such as maintenance of neuronal architecture and efficient transport of organelles (1,8).

P60-katanin (hereafter referred to as katanin), the most abundant microtubule-severing protein in neurons, is present at surprisingly high levels. Katanin is especially high during development and is present throughout the growing axon as well as throughout the dendritic arbor, where it appears to play major roles in sculpting the microtubule array (1). Although the levels in the axon are lower in adult, they remain robust compared with the amount of katanin needed to effectively sever purified microtubules. In fact, the levels are so high that the available katanin would theoretically sever the microtubules completely down to subunits, if the severing process were not somehow regulated (11). Any failure in this regulation could seriously jeopardize the

*To whom correspondence should be addressed. Tel: +1 2159918298; Fax: +1 2158439082; Email: pbaas@drexelmed.edu

health of the nervous system, as excess severing would gradually degrade the axonal microtubule array. Interestingly, we have found that the most significant factor in attenuating the sensitivity of axonal microtubules to katanin appears to be the presence on the microtubules of tau, a fibrous microtubule-associated protein (12,13). Because tau dissociates from microtubules when it is phosphorylated, localized phosphorylation of tau is a potent means for stimulating focal bursts of microtubule severing underlying axonal branch formation (2,6). A number of neurodegenerative disorders (termed tauopathies) involve hyperphosphorylation of tau, which causes it to permanently dissociate from the microtubules (14). When this happens, the microtubule array gradually disintegrates, although it is unclear exactly why.

We have posited that an increased sensitivity to katanin might be the basis for the loss of microtubules in tauopathic diseases (12,15). Here, we tested this idea by ascertaining whether pathogenic mutant forms of tau are diminished relative to wild-type tau in their capacity to protect microtubules against katanin. Then, using cultured neurons depleted of tau, we tested two potential strategies for protecting the microtubules from excess severing by katanin. The first strategy was to experimentally lower the acetylation state of the microtubules (13). The second was to employ a neuroprotective peptide called NAP, which is known to interact with neuronal microtubules (16).

RESULTS

Pathogenic tau species protect microtubules less well than wild-type tau from katanin-based severing

Our underlying premise is that tau gradually loses association from microtubules in the axon during various pathogenic situations and that this renders the microtubules abnormally sensitive to severing by the endogenous katanin in the neuron (15,17). Presumably, the ensuing microtubule degradation would be gradual in pathogenic conditions afflicting human patients. For experimental purposes, we hastened the effect by increasing katanin levels in cultured rat neurons via overexpression. We previously established that when ectopically expressed in RFL-6 rat fibroblasts, tau provides strong protection against overexpressed katanin (12). Correspondingly, when endogenous tau is depleted from cultured rat hippocampal neurons, the microtubules in the axon become notably more sensitive to a 12 h bout of katanin overexpression. To test the underlying premise of the present study, we compared the capacity of two different human tau mutants with that of wild-type human tau to protect microtubules from being severed by high levels of katanin. One of the mutants we tested has 10 Alzheimer's disease (AD)-related phosphorylation sites (S198, S199, S202, T231, S235, S396, S404, S409, S413 and S422) replaced by glutamates (18). This creates a mutant that behaves in cells as a pseudo-hyperphosphorylated tau (PHP). The other tau mutant (R406W) has a single point mutation that has been shown to be causative for hereditary frontotemporal dementia with parkinsonism linked to chromosome 17 (19). These tau mutants have a diminished capacity to interact with microtubules and/or promote their polymerization (19,20) and hence would presumably afford less protection against katanin, if our hypothesis is correct.

As in our previous studies (12,13), we started by imaging interphase microtubule arrays in rat fibroblasts ectopically expressing human wild-type tau together with either green fluorescent protein (GFP) alone or GFP-tagged rat p60-katanin. Then, we compared the effects of the tau mutants, relative to wild-type tau. For all of these studies, we chose medium expressers rather than cells expressing notably higher or lower levels of either katanin or tau within the population. In these cells, katanin levels were elevated 2.6 times the endogenous levels, whereas the ectopically expressed tau was roughly two to four times the amount of endogenous tau in cultured rat hippocampal neurons (Table 1). Microtubule levels and distribution were assessed in cells that had been fixed and prepared for immunofluorescence visualization of microtubules. When compared with controls (control; Fig. 1A), the wild-type tau expressers (wild-type; Fig. 1B) showed higher total levels of microtubules as well as a strong enhancement of microtubule bundling (as observed previously, see 12,13). Cells expressing PHP (PHP; Fig. 1C) showed less prominent microtubule bundle formation than cells expressing wild-type tau, but still showed elevated microtubule levels relative to control cells not expressing tau. Cells expressing R406W (R406W; Fig. 1D) displayed microtubule bundling and elevation above controls generally similar to the situation with wild-type tau. In cells overexpressing katanin but not expressing tau (katanin; Fig. 1E), there was dramatic loss of microtubule mass. When katanin was co-expressed with wild-type tau (wild-type + katanin; Fig. 1F), there was no apparent microtubule loss, and this was true of both bundled and unbundled microtubules. These results are essentially the same as we have previously reported (12,13). When katanin was overexpressed in cells expressing either of the mutant forms of tau, there were reductions in microtubule levels not observed with the wild-type tau. Microtubule loss in the case of the PHP (PHP; Fig. 1G) was greater than that in the case of the R406W (R406W; Fig. 1H), and this was true of both bundled and unbundled microtubules. In the case of the R406W mutant, microtubule loss was mainly restricted to the unbundled microtubules.

Quantification of the results was conducted as in our previous studies (12,13). Appropriate rigor was taken in image acquisition to avoid saturation, identical parameters were used for all cells and analyses were conducted using imaging software as described in our earlier studies (see also Materials and Methods). In terms of total microtubule levels, cells expressing the tau constructs alone showed about 40% increase above control levels (Fig. 1I). There was no significant difference among the various tau constructs, although the PHP-expressing cells show a trend toward lower microtubule levels than cells expressing the other tau constructs. Katanin overexpression induced a dramatic reduction in microtubule levels (59%) of control cells not expressing tau constructs, but no reduction in cells co-expressing the wild-type tau construct. Compared with the wild-type + katanin, there was a dramatic reduction in PHP + katanin and a moderate but still significant reduction in R406W + katanin (48 and 21%, respectively; both significant: $P < 0.01$). When PHP + katanin or R406W + katanin co-expressers were compared

Table 1. Levels of ectopically expressed proteins relative to endogenous levels in the present experiments

Cell name	Assessed protein	AFU (SD)	Ratios
RFL-6	Exogenously expressed tau	331 (116)	2.3 (ratio to endogenous tau in neuronal cell body) 3.6 (ratio to endogenous tau in neuronal axon)
Hippocampal neuron	Endogenously expressed tau (cell body)	143 (51)	—
Hippocampal neuron	Endogenously expressed tau (axon)	91 (15)	—
RFL-6	Endogenously expressed katanin	93 (21)	—
RFL-6	Exogenously expressed katanin	238 (82)	2.6 (ratio to endogenous katanin in RFL6 cell)
Hippocampal neuron	Endogenously expressed katanin (cell body)	186 (43)	—
Hippocampal neuron	Exogenously expressed katanin (cell body)	527 (124)	2.8 (ratio to endogenous katanin in neuronal cell body)

Levels were determined using quantitative immunofluorescence as described (see Materials and Methods). Upper three rows: the levels of ectopically expressed tau in RFL-6 cells were 2.3 and 3.6 times the endogenous levels in the cell body and axon of cultured hippocampal neurons, respectively. Middle two rows: ectopically expressed katanin in RFL-6 cells elevated the levels to 2.6 times the endogenous levels. Lower two rows: ectopically expressed katanin in hippocampal neurons elevated the levels to 2.8 times the endogenous levels.

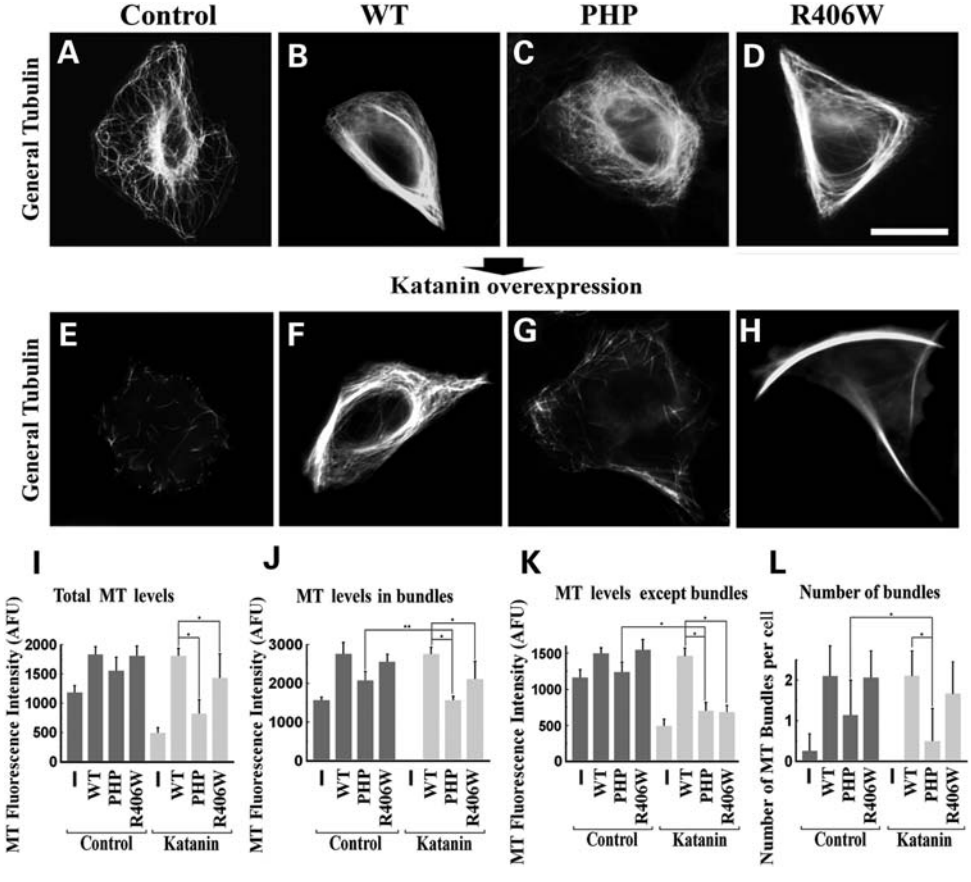

Figure 1. Effects of tau mutants on katanin-based microtubule severing in RFL-6 cells. Upper row images: cells were transfected with various tau constructs, fixed and then immunostained with a general tubulin antibody (**A**–**D**). Non-expressing control (A); cell expressing wild-type tau (B); cell expressing PHP (C) and cell expressing R406W (D). Both wild-type tau and R406W induced the formation of microtubule bundles. PHP was less effective at causing microtubule bundle formation, but, like wild-type and R406W, caused a notable increase in microtubule levels compared with control. Lower row images: cells were co-transfected with one of the three tau constructs together with katanin overexpression. Katanin alone (**E**); katanin + wild-type (**F**); katanin + PHP (**G**) and katanin + R406W (**H**). Cells overexpressing katanin alone showed efficient microtubule severing, whereas the katanin + wild-type cells showed no indication of microtubule severing or any obvious loss of microtubule mass. The katanin + PHP cells showed less resistance to loss of both bundled and unbundled microtubules, whereas the resistance was more specific to the microtubule bundles in the case of katanin + R406W. Bar: 20 μm. (**I**–**K**) Quantification of microtubule levels by general tubulin staining intensity. Total microtubule levels (I); bundled microtubule levels (J) and non-bundled microtubule levels (K). AFU, arbitrary fluorescence unit. (**L**) Number of microtubule bundles per cell. Asterisks and a double asterisk indicate that there are significant differences (Student's *t*-test, $P < 0.01$ and $P < 0.05$, respectively).

with cells only overexpressing katanin, there were significant differences in both cases (both $P < 0.01$).

Because bundled microtubules sometimes show higher resistance to severing than unbundled microtubules, we also performed separate quantification of bundled and unbundled

microtubules under the various experimental conditions (Fig. 1J and K). In the case of bundled microtubules (Fig. 1J), the levels in cells expressing wild-type tau or R406W were similar, whereas there was a 25% difference between wild-type tau and PHP. When katanin was overexpressed as well, there was no significant reduction in microtubule bundles in the case of cells expressing wild-type tau. Compared with the wild-type + katanin, PHP + katanin showed significant reduction (44%; $P < 0.01$), whereas R406W + katanin showed moderate but significant reduction (24%; $P < 0.01$). In PHP + katanin, there was a significant reduction of microtubules in bundles compared with PHP alone (25%: shown by double asterisks; $P < 0.05$). With regard to unbundled microtubules, the results were qualitatively similar to those observed for bundled microtubules. However, when we compared R406W + katanin with wild-type + katanin, we detected a larger reduction (54%) than seen in the total or bundled microtubule analyses. The results from analyses on microtubule bundle number (Fig. 1L) showed that both wild-type and R406W had similar numbers of bundles (both about 2 per cell), whereas PHP had fewer (about 1 per cell). Katanin overexpression did not change this tendency in wild-type or R406W (wild-type + katanin and R406W + katanin), whereas there was a significant reduction in the case of PHP (PHP + katanin: fewer than 0.5 per a cell; $P < 0.01$).

Collectively, these observations are consistent with a scenario by which the tau mutants retain some ability to protect the microtubules from severing by the overexpressed katanin, but notably less so than wild-type tau. However, another formal possibility is that there is little or no difference in the severing of the microtubules, but rather the difference lies in the ensuing depolymerization of microtubules that can follow the severing event. In other words, if a microtubule is very labile, then a severing event may cause it to depolymerize, but if it is very stable, then a severing event may not result in any change in total microtubule levels because no depolymerization would ensue. To investigate this, we tested the sensitivity of the microtubules after expressing various tau constructs to nocodazole, a drug that causes depolymerization of microtubules from their ends (21,22). For this, we expressed the tau constructs in RFL-6 cells and treated them with 7 μM nocodazole for 5–30 min (Fig. 2). At the starting point (0 min), relative to controls, we observed notable bundles in cells expressing the wild-type or R406W constructs, and somewhat less prominent bundles in cells expressing the PHP construct compared (Fig. 2A–D), as we observed above (Fig. 1A–D). At 5 min drug treatment, control cells showed dramatic reduction in microtubule levels, whereas cells expressing any of the tau constructs showed no reduction in microtubule levels, regardless of the differences in their initial bundle status (Fig. 2E–H). By 30 min, microtubule levels continued to drop in control cells, but still showed comparatively little change in any of the tau-expressing cells (Fig. 2 I–L).

In the control cell response curve, we found a biphasic pattern reminiscent of what we observed in earlier studies on the axon (23,24). In the case of the fibroblasts and the nocodazole concentration used for the present studies, the slope for the first 5 min of drug treatment was 96.0 arbitrary fluorescence unit (AFU)/min, whereas that for the next 25 min was 15.4 AFU/min. The magnitudes of the slope for the first 5 min for wild-type tau, PHP and R406W were 41, 31 and 34 AFU/min, respectively. When tau was expressed, the biphasic pattern was not as pronounced, and therefore we also compared the slopes of the curves for the entire 30 min period of time. The slope for the entire control response curve was 28.8 AFU/min, whereas those for wild-type, PHP and R406W were 16.3, 16.1 and 16.9 AFU/min, respectively. These results, summarized graphically in Figure 2M, show no notable differences in microtubule stability in cells expressing the various tau constructs. These results are consistent with previous reports (25–29). The overall conclusion of these drug studies was that the notable differences observed between wild-type tau and the mutant forms of tau in the katanin overexpression studies are not explicable on the basis of any detectable differences in microtubule stability, thus confirming our conclusion that the tau mutants are less effective than wild-type tau at attenuating the severing of the microtubules by katanin.

Studies on tau-depleted rat hippocampal neurons

Tauopathies are complicated diseases because the loss of tau from the microtubules is only one potential avenue leading to axonal degeneration. Therefore, rather than using complicated animal models to specifically test strategies for alleviating the sensitivity of the tau-deficient microtubules to excess severing by katanin, we chose to use cultured rat hippocampal neurons depleted of tau by siRNA (2,12). We felt that this was a better model for testing these strategies than using neurons from a tau knockout mouse because depleting tau from neurons that once had tau more closely parallels the disease situation. Using the regime from our previous studies, we introduced the tau siRNA (or control siRNA) by electroporation, after which we plated the cells for 2 days and then re-plated the cells to allow axons to grow anew. The tau protein levels under these conditions are 95–99% depleted by the fifth total day (3 days after re-plating) (12). In this experimental procedure, the timetable for axonal differentiation is similar to that of control neurons. In each case, about 80% of the neurons are in developmental stage III, which is the stage when axons differentiate according to the criteria of Dotti *et al.* (30), on the fifth total day. Two days after re-plating, some of the cultures were transfected to overexpress katanin for 12 h, prior to fixation. As in our previous studies, the phenotype obtained with this procedure is profound: the axon shows virtually no microtubule diminution, whereas the cell body and minor processes show dramatic microtubule loss (compare Fig. 3A with A′). Quantitative assessment of katanin staining in control and overexpressing cells indicates that the overexpression increased katanin levels by 2.8 times (Table 1). As in our earlier studies, compared with control cells (control; Fig. 3A), we did not observe any prominent morphological differences in the tau-depleted neurons (tau siRNA; Fig. 3B). In tau-depleted neurons overexpressing katanin, the minor processes showed a similar diminution in microtubule levels as observed in the minor processes of control neurons, but so too did the axon (Fig. 3B′). These results are consistent with those reported

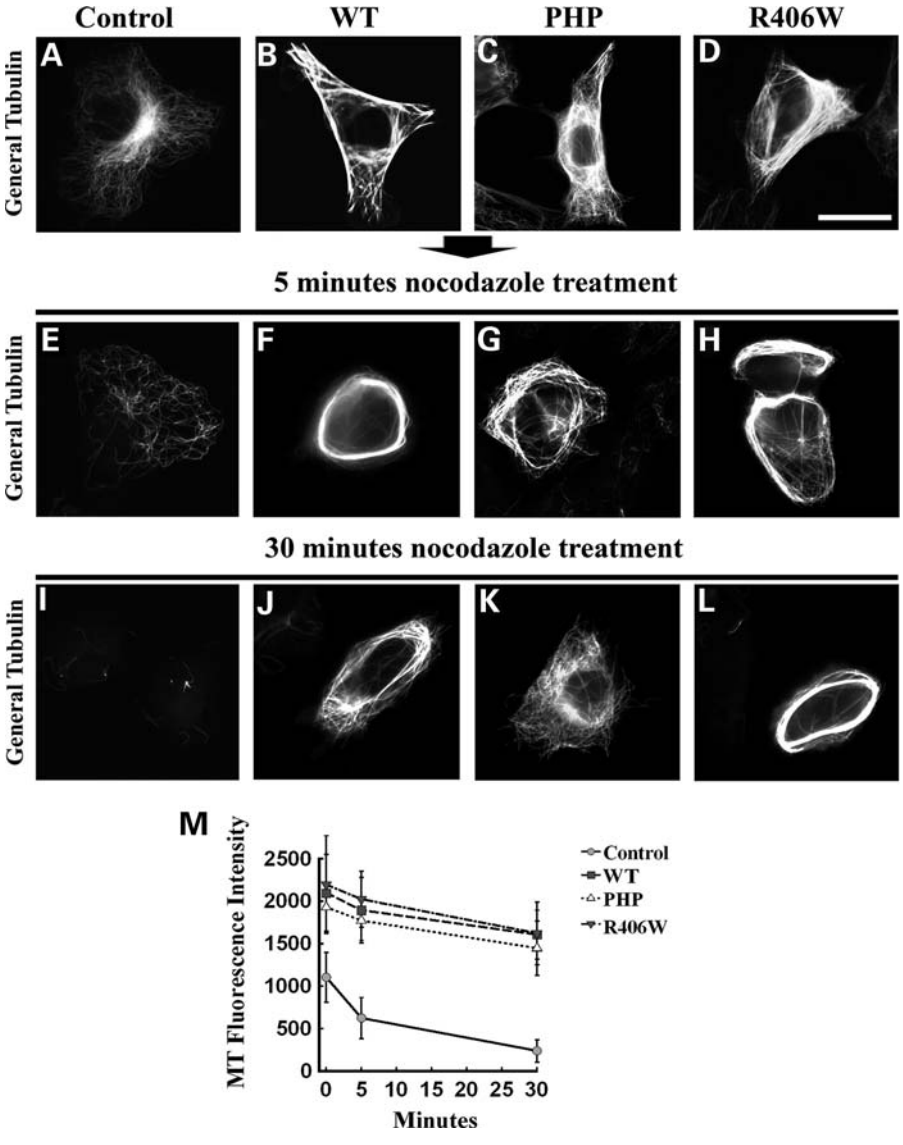

Figure 2. Effects of tau mutants on nocodazole-induced microtubule depolymerization in RFL-6 cells. Upper row images show microtubule arrays in cells not treated with nocodazole: cells were transfected with one of the three tau constructs, fixed and then immunostained for general tubulin (**A**–**D**). Control cell (A); cell expressing wild-type tau (B); cell expressing PHP (C) and cell expressing R406W (D). Middle row images show microtubule arrays after 5 min treatment with nocodazole: control (**E**), cell expressing wild-type tau (**F**), cell expressing PHP (**G**) and cell expressing R406W (**H**). Lower row images show microtubule arrays after 30 min treatment with nocodazole: control (**I**), cell expressing wild-type tau (**J**), cell expressing PHP (**K**) and cell expressing R406W (**L**). Bar: 20 μm. (**M**) Quantification of total microtubule levels by general tubulin staining. The microtubule mass (unit: AFU) response curves to nocodazole treatment for each condition are shown as a function of time (unit: minute). Although there was a tendency without statistical significance that PHP showed lower microtubule levels at all time points compared with wild-type and R406W, the slope for PHP was not notably different from that of the other two constructs (see Results for details).

previously (12) and provide a useful setting to test potential protective effects of our two proposed therapeutic strategies.

Effects of NAP on sensitivity of tau-depleted axonal microtubules to katanin

NAP (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln) is a membrane-permeable octapeptide whose sequence is contained within the neurotrophic factor activity-dependent neuroprotective protein. NAP has been shown to protect neurons against a wide variety of insults, when used in concentrations ranging from femtomolar to nanomolar (16,31–35). NAP has been shown to interact with neuronal microtubules and has been shown to promote neurite extension in primary cultured neurons (36–38). NAP is already showing promising results in animal models of neurodegenerative diseases (39–41) and clinical trials (42).

We chose to test the effects of two concentrations of NAP, namely 1 pM and 1 nM. In a first set of experiments with NAP, we treated cultured rat hippocampal neurons with 1 pM NAP for 2.5 days (from the time of re-plating to fixation). With this concentration, compared with controls, we detected no alterations in morphology or any changes in katanin's effects on microtubule levels after tau depletion. Consistent

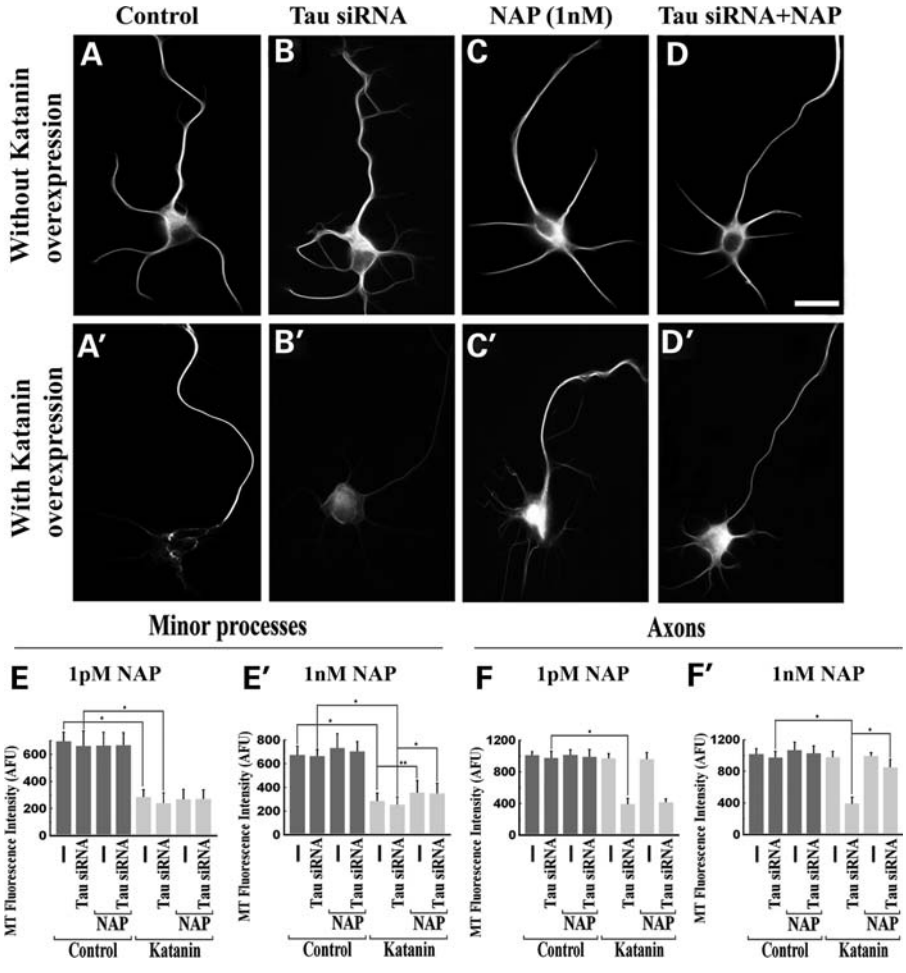

Figure 3. Effects of NAP on katanin-based microtubule loss in tau-depleted axons. Tau-depleted hippocampal neurons treated with NAP were tested for their microtubule sensitivity to overexpressed katanin. Upper row images show control [control; (**A**)], tau depletion alone [tau siRNA; (**B**)], 1 nM NAP treatment alone [NAP; (**C**)] and tau depletion together with NAP [tau siRNA + NAP; (**D**)]. Lower row images show katanin-overexpressing cells under the conditions corresponding to the upper panels. All images show general tubulin staining. In katanin overexpression alone [katanin; (**A**')], significant reduction in microtubule levels was observed in minor processes but not in axons. Tau depletion rendered the axonal microtubules sensitive to katanin [tau siRNA + katanin; (**B**')]. NAP treatment conferred some resistance to microtubule severing in minor processes [NAP + katanin; (**C**')]. NAP treatment under tau depletion restored axonal microtubules to the level of resistance observed under tau-normal conditions [tau siRNA + katanin + NAP; (**D**')]. Bar: 20 μm. (**E**, **E**', **F** and **F**') Quantification of microtubule levels in axons and minor processes assayed by intensity of general tubulin staining. AFU, arbitrary fluorescence unit. Single asterisks and a double asterisk indicate that there are significant differences (Student's *t*-test, $P < 0.01$ and $P < 0.05$, respectively). (E and F) Data on the effects of 1 pM NAP. In either axons or minor processes, there were no significant protective effects against katanin of NAP at this lower concentration. (E' and F') Data from experiments testing the effects of 1 nM NAP. Treatment by NAP at this higher concentration significantly inhibited 77% of microtubule severing in tau-depleted axons and significantly inhibited about 20% of microtubule severing in minor processes both with and without tau depletion.

with previously reported observations (38), neurons treated with 1 nM NAP showed more elongated morphologies compared with controls and somewhat straighter minor processes and axons (Fig. 3C). We then overexpressed katanin during the final 12 h of the experimental procedure in the presence of NAP (keeping the total exposure to NAP at 2.5 days). NAP-treated katanin-overexpressing neurons (NAP + katanin; Fig. 3C′) displayed somewhat increased microtubule levels in the minor processes compared with katanin-overexpressing neurons not treated with NAP (katanin; Fig. 3A′). Axons did not show any obvious difference in microtubule levels compared with the situation when katanin was overexpressed without NAP treatment. In tau-depleted neurons, NAP treatment (tau siRNA + NAP; Fig. 3D) resulted in morphologies that were only slightly different compared with NAP treatment without tau depletion (NAP; Fig. 3C). However, when katanin was overexpressed in the presence of NAP, the dramatic reduction in microtubule levels observed in axons treated in this manner without NAP (tau siRNA + katanin; Fig. 3B′) was not observed (tau siRNA + katanin + NAP; Fig. 3D′).

Quantification of microtubule levels in minor processes of neurons treated with 1 pM NAP resulted in data indicating no significant differences in the various experimental conditions without the drug being present (Fig. 3E). Quantification of microtubule levels in minor processes of neurons treated with 1 nM NAP resulted in data as follows: control (667 ± 80), tau siRNA (658 ± 61), NAP (726 ± 126), tau siRNA + NAP (695 ± 90), katanin (278 ± 74), tau siRNA + katanin (246 ± 74), katanin + NAP (350 ± 109) and tau siRNA + katanin + NAP (344 ± 90), as indicated by (mean microtubule

levels: AFU ± SD) formula ($n = 3$, 25 cells were analyzed for each condition). These data, summarized in Figure 3E′, indicate that NAP treatment increased microtubule levels by 8.7 and 5.7% without and with tau depletion, respectively, but these apparent differences were not statistically significant (in either case; $P > 0.05$). Katanin overexpression induced about 60% (control: 59% and tau depletion: 63%) significant microtubule reduction in minor processes regardless of whether tau was depleted or not (in either case; $P < 0.01$: as shown by asterisks in Fig. 3E′). This reduction was partially inhibited by 1 nM NAP significantly (in both cases reduction: 48%; $P < 0.05$ in control and $P < 0.01$ in tau depletion; as shown by asterisks in Fig. 3E′), suggesting small but significant resistance to katanin provided by NAP in minor processes.

Quantification of microtubule levels in axons of neurons treated with 1 pM NAP resulted in data, indicating no significant differences in the various experimental conditions without the drug being present (Fig. 3F). Quantification of microtubule levels in axons of neurons treated with 1 nM NAP resulted in data as follows: control (1010 ± 77), tau siRNA (966 ± 84), NAP (1056 ± 111), tau siRNA + NAP (1020 ± 101), katanin (967 ± 85), tau siRNA + katanin (380 ± 92), katanin + NAP (981 ± 53) and tau siRNA + katanin + NAP (839 ± 111), as indicated by (mean microtubule levels: AFU ± SD) formula ($n = 3$, 25 cells were analyzed for each condition). These data, summarized in Figure 3F', indicate an apparent microtubule reduction accompanying tau depletion of 5%, although this did not prove to be statistically significant ($P > 0.05$). Treatment with NAP alone increased the levels of microtubules by 4.5 and 5.5% without and with tau depletion, respectively, but again, these apparent differences did not prove to be statistically significant (in either cases; $P > 0.05$). Without tau depletion, katanin overexpression induced only 5% microtubule reduction (but not statistically significant), but with tau depletion, the reduction was 61% (statistically significant; $P < 0.01$) (as shown by an asterisk in Fig. 3F′). This reduction was notably inhibited by 1 nM NAP (reduction: 14%; $P < 0.01$; statistically significant) (as shown by an asterisk in Fig. 3F′). These data demonstrate that NAP, if used at nanomolar levels, has strong protective effects against katanin-based loss of tau-depleted microtubules.

NAP protection of microtubules from excess severing by katanin is dependent upon beta-III tubulin

The question arises as to how NAP affords protection against excess microtubule severing by katanin. In studies on purified tubulin, it has been shown that NAP does not impact the dynamic properties of microtubules (43), and hence it is unlikely that the observed effects of NAP in our studies are explicable by an attenuation of microtubule depolymerization that might ensue as a result of the severing of the microtubules. In addition, the levels of NAP that are efficacious are too low to function by simply occupying sites on the microtubule that would otherwise be occupied by tau. Interestingly, it has been shown that it is specifically the beta-III tubulin isotype that interacts with NAP (38), which leads us to believe that the microtubule lattice may undergo a conformational shift due to the interaction between NAP and beta-III tubulin that impacts the sensitivity of the microtubule to katanin. To begin to explore this idea, we wished to confirm that the presence of beta-III tubulin in the microtubules is indeed crucial for the effects we have observed. To investigate this, we took advantage of the high degree of resolution afforded by the very flat RFL-6 fibroblasts, which do not endogenously express beta-III tubulin. We compared the effects of katanin overexpression on control fibroblasts with fibroblasts in which we ectopically expressed beta-III tubulin. This was done with or without the addition of 1 nM NAP for 24 h. Neurons were then immunostained for beta-III tubulin and general tubulin, as well as for the GFP-tagged katanin. As seen in Figure 4, in the absence of NAP, katanin overexpression resulted in efficient severing of microtubules (Fig. 4C) compared with controls (Fig. 4A). Beta-III-tubulin expression alone slightly increased the total microtubule levels (Fig. 4C). When both proteins were expressed simultaneously (Fig. 4D), we observed no change in severing compared with katanin alone. In the presence of NAP alone, there was no observable change in total microtubule levels (Fig. 4E). Cells expressing beta-III tubulin in the presence of NAP displayed the same apparent slight increase in microtubule levels observed with beta-III expression alone (Fig. 4F). As predicted, NAP did not show any protective effects in cells expressing katanin alone (Fig. 4G). However, in cells expressing katanin and beta-III tubulin, there was a clear reduction in the degree of microtubule severing compared with cells overexpressing katanin without beta-III tubulin. Some beta-III-tubulin-expressing cells showed more microtubule loss than others, but even the cells displaying the most microtubule loss were distinguishable from those lacking beta-III tubulin under otherwise identical conditions (Fig. 4H).

Quantification showed that in the absence of NAP, katanin and katanin + beta-III tubulin provided 56 and 66% reduction in total microtubule mass, respectively. The difference between them was not significant in statistical analysis ($P > 0.01$). In the presence of NAP, the same sets of constructs expression provided 52 and 36% reduction, respectively. Statistically, there were significances between katanin + NAP and katanin + beta-III tubulin + NAP or between katanin + beta-III tubulin and katanin + beta-III tubulin + NAP (both $P < 0.01$). Thus, we observed significant inhibition by NAP of katanin-based microtubule severing only in the presence of beta-III tubulin.

Experimental deacetylation of axonal microtubules diminishes their sensitivity to overexpressed katanin under conditions of tau depletion

In our previous study, we reported that highly acetylated microtubules are the preferred substrates for severing by katanin (13). We also showed that less acetylated microtubules are more resistant to katanin and that the presence of tau affords protection against katanin regardless of the acetylation state of the microtubule. Given that axonal microtubules are highly acetylated (24,44), they would be prime targets for katanin after the loss of tau. Therefore, we reasoned that tau-depleted microtubules could be experimentally protected against excess severing by katanin by deacetylating them. This can be accomplished by overexpression of HDAC6, a

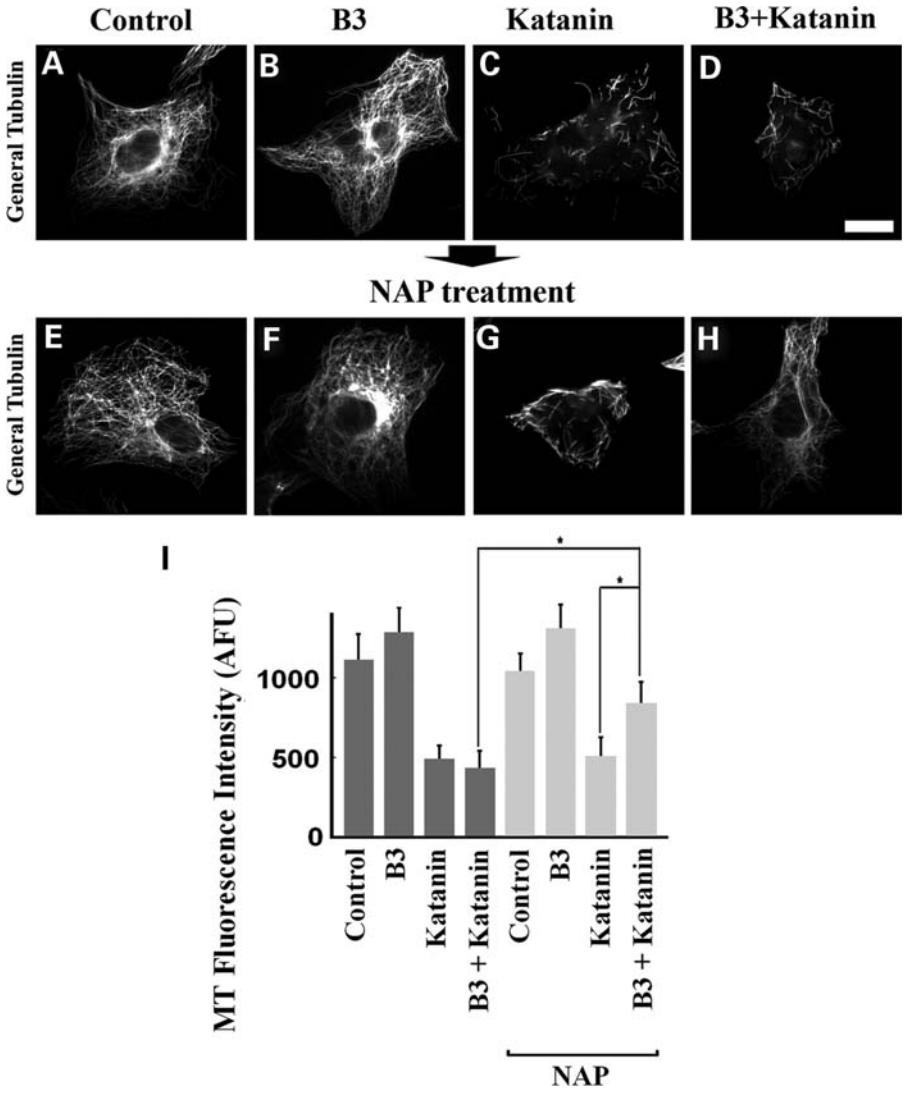

Figure 4. Effects of NAP on katanin-based microtubule severing in RFL-6 fibroblasts induced to express beta-III tubulin. Upper row images: cells were transfected with katanin and/or beta-III tubulin expression constructs, fixed and then immunostained with a general tubulin antibody (**A**–**D**). Non-expressing control (control) (A); cell expressing beta-III tubulin (B3) (B); cell expressing katanin (katanin) (C) and cell expressing both beta-III tubulin and katanin (B3 + katanin) (D). Robust microtubule severing and notable reduction in microtubule mass were observed in both katanin and beta III-tubulin + katanin. Lower row images: cells were transfected with the same sets of constructs as in upper row and simultaneously treated with 1 nM NAP for 24 h. Cells treated with NAP alone (**E**). Beta-III tubulin expressing cells treated with NAP (**F**). Cells overexpressing katanin alone (**G**) showed as efficient microtubule loss as seen in (C) and (D). In contrast, beta III-tubulin + katanin (**H**) showed dramatically less microtubule loss compared with comparable cells not expressing beta-III tubulin. Bar: 20 μm. (**I**) Quantification of microtubule levels by general tubulin staining. Total microtubule levels were assessed. AFU, arbitrary fluorescence unit. Asterisks indicate that there are significant differences (Student's *t*-test, $P < 0.01$).

deacetylase that is highly specific for tubulin [for details, see the previous study (13)]. The experimental design was essentially the same as that for the NAP experiments described earlier, except that the cells were transfected to overexpress HDAC6 rather than treating them with NAP.

We first analyzed the efficacy of the HDAC6 approach (Fig. 5A–D) by immunostaining with an antibody specific for acetylated tubulin as well as the general tubulin antibody (Fig. 5E–H). These studies revealed that, compared with control neurons (Fig. 5A), tau depletion only mildly reduced the acetylation of microtubules in axons (Fig. 5B). HDAC6 overexpression induced efficient reduction in microtubule acetylation in the axons of both control and tau-depleted neurons (Fig. 5C and D). The effects of HDAC6 on minor processes were less clear than in axons because the microtubules in minor processes of stage III neurons originally had lower levels of acetylation. The staining for general tubulin revealed that there were no obvious differences in morphology or microtubule organization compared with control neurons (Fig. 5E) and tau-depleted neurons with no HDAC6 overexpression (Fig. 5F) of neurons overexpressing HDAC6 but with no tau depletion (Fig. 5G), whereas HDAC6-overexpressing tau-depleted neurons showed a tendency to have slightly shortened minor processes (Fig. 5H).

We then analyzed control and tau-depleted neurons in which we overexpressed katanin. Katanin overexpression reduced microtubule levels in minor processes but not axons, over the 12 h time-frame of the experiment. No such resistance to

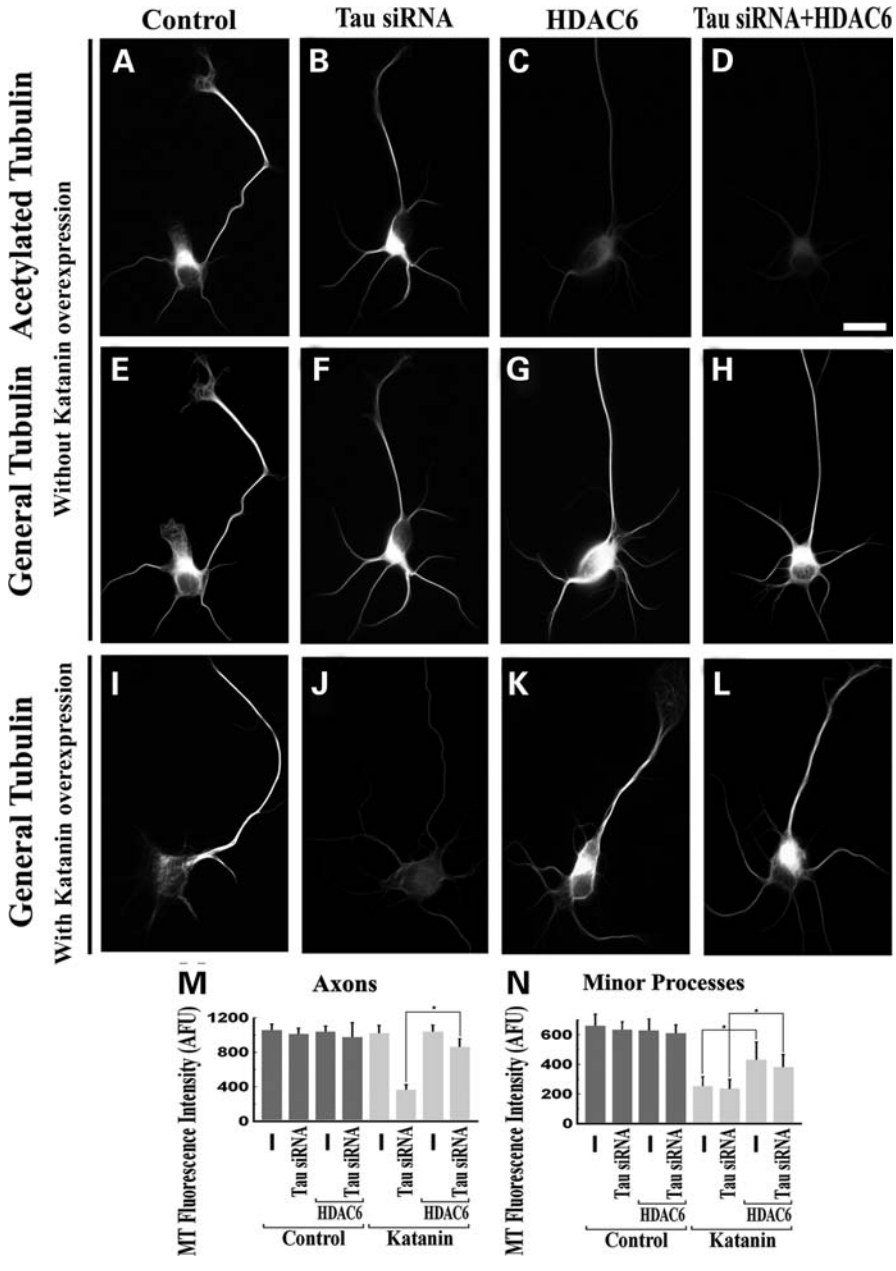

Figure 5. Effects of microtubule deacetylation by HDAC6 overexpression on axonal microtubule loss induced by katanin in tau-depleted neurons. Tau-depleted hippocampal neurons overexpressing HDAC6 were tested for their microtubule sensitivity to overexpressed katanin. Upper row images show acetylated tubulin staining of cells in control [control; (**A**)], tau depletion alone [tau siRNA; (**B**)], HDAC6 overexpression alone [HDAC6; (**C**)] and tau depletion and HDAC6 [tau siRNA + HDAC6; (**D**)]. Middle row images show general tubulin staining of the same neurons as corresponding upper images. Lower row images show katanin overexpressers stained for general tubulin under the conditions corresponding to the upper panels. Tau depletion induced only mild reduction in microtubule acetylation in axons (B). HDAC6 overexpression induced notable reduction in microtubule acetylation in axons and relatively mild reduction in minor processes (C and D). In general tubulin staining, there was no prominent change among the conditions without katanin overexpression (**E**–**H**), but a tendency of a modest reduction in the length of minor processes was observed in the case of tau siRNA + HDAC6 (H). In katanin overexpression alone [katanin; (**I**)], significant reduction in microtubule levels was observed in minor processes but not in axons. Tau depletion rendered the axonal microtubules sensitive to katanin [tau siRNA + katanin; (**J**)]. HDAC6 overexpression conferred mild resistance of microtubules in minor processes against katanin [HDAC6 + katanin; (**K**)]. HDAC6 overexpression under tau depletion restored the level of resistance observed in tau-normal axons [tau siRNA + katanin + HDAC6; (**L**)]. Bar: 20 μm. (**M** and **N**) Quantification of microtubule levels in axons and minor processes by assessing the intensity of general tubulin staining. (M and N) Data from axons and minor processes, respectively. AFU, arbitrary fluorescence unit. Asterisks indicate that there are significant differences (Student's *t*-test, $P < 0.01$). Overexpression of HDAC6 significantly inhibited 76% of microtubule severing in tau-depleted axons and significantly inhibited about 40% of microtubule severing in minor processes both with and without tau depletion.

katanin was observed in the neurons depleted of tau (katanin; Fig. 5I and tau siRNA + katanin; Fig. 5J). Without tau depletion, HDAC6 overexpression induced no change in axonal microtubules but induced a modest increase in minor process microtubule levels (katanin + HDAC6; Fig. 5K) compared with neurons only overexpressing katanin (katanin).

However, under conditions of tau depletion, there was a more notable elevation in microtubule levels in axons (tau siRNA + katanin + HDAC6; Fig. 5L) compared with tau siRNA + katanin. The effects on minor processes were similar to those observed in neurons without tau depletion.

Quantification of microtubule levels in axons of neurons overexpressing HDAC6 resulted in data as follows (Fig. 5M): control (1046 ± 79), tau siRNA (1004 ± 75), HDAC6 (1028 ± 74), tau siRNA + HDAC6 (963 ± 179), katanin (1011 ± 100), tau siRNA + katanin (354 ± 70), katanin + HDAC6 (1030 ± 82) and tau siRNA + katanin + HDAC6 (853 ± 01), as indicated by (mean microtubule levels: AFU ± SD) formula ($n = 3$, 25 cells were analyzed for each condition). These data indicate that neurons overexpressing HDAC6 also undergoing tau depletion (tau siRNA + HDAC6) display an apparent 7% microtubule reduction compared with controls, but this proved not to be statistically significant ($P > 0.05$). Tau depletion alone (tau siRNA) or HDAC6 overexpression alone (HDAC6) showed less than 5% apparent decrease ($P > 0.05$), but again this was not statistically significant. Without tau depletion, katanin overexpression induced only 4% apparent but not statistically significant microtubule reduction (katanin), but with tau depletion, katanin overexpression resulted in a 65% reduction ($P < 0.01$; statistically significant), similar to what we observed in the NAP experiments. This reduction was largely inhibited by HDAC6 overexpression (statistically significant reduction of 15%; $P < 0.01$) (as shown by an asterisk in Fig. 5M). These data demonstrate that deacetylation has protective effects against excess severing of tau-depleted axonal microtubules by katanin.

Quantification of microtubule levels in minor processes of neurons overexpressing HDAC6 showed data as follows (Fig. 5N): control (656 ± 83), tau siRNA (628 ± 59), HDAC6 (623 ± 83), tau siRNA + HDAC6 (606 ± 61), katanin (247 ± 70), tau siRNA + katanin (230 ± 69), katanin + HDAC6 (427 ± 125) and tau siRNA + katanin + HDAC6 (376 ± 91), as indicated by (mean microtubule levels: AFU ± SD) formula ($n = 3$, 25 cells were analyzed for each condition). These data indicate that HDAC6 overexpression decreased microtubule levels by 5.0 and 4.6% in HDAC6 alone and tau siRNA + HDAC6 conditions, respectively, but these apparent differences were statistically not significant (in either case; $P > 0.05$). Katanin overexpression induced about 60% (control; 62% and tau depletion; 63%) significant microtubule reduction in minor processes regardless of tau depletion (in either case; $P < 0.01$). This reduction was inhibited by HDAC6 overexpression (katanin + HDAC6: 35% and tau siRNA + katanin + HDAC6: 40%; both cases, $P < 0.01$, as shown by asterisks in Fig. 5N) at a level indicating somewhat more modest but still significant protection of microtubules in minor processes.

Changes in axonal branching as an indicator of alternations in microtubule severing

In a final set of studies (shown in Fig. 6), we investigated the effects on axonal branching of the two experimental strategies designed to attenuate katanin-based severing of microtubules. Axonal branch formation is known to be increased when microtubule severing is enhanced in the axon (2,6). In the case of katanin, the effect is mediated by tau, as evidenced by the fact that tau depletion results in a notable increase in axonal branching that is dependent upon katanin (2). Spastin-induced branching appears to be independent of tau. Consistent with our previous studies, here we found that, compared with controls (Fig. 6A), there was a clear enhancement of axonal branching in neurons depleted of tau (tau siRNA; Fig. 6B). Compared with controls, 1 nM NAP treatment reduced axonal branching, which is consistent with less microtubule severing, even in the presence of tau (NAP; Fig. 6C). In tau-depleted neurons, the augmentation of branching relative to controls was completely obliterated by NAP treatment, with the branching levels reduced even lower than controls without NAP treatment (tau siRNA + NAP; Fig. 6D). Similar results were obtained with HDAC6 overexpression on control neurons (HDAC6; Fig. 6E) and tau-depleted neurons (tau siRNA + HDAC6; Fig. 6F).

In order to meaningfully quantify the number of branches, we first assayed the length of axons (Fig. 6G). Relative to controls, the axons of tau-depleted neurons showed a tendency of slight shortening, which is consistent with our previous report (12). NAP-treated cells showed a slight tendency of increased axonal lengths in both control and tau-depleted neurons, which is consistent with previously reported findings on NAP (38). However, these differences were not statistically significant, with the vast majority of axons in all experimental conditions existing within a range of 130–150 μm in length. We limited our branch analyses to axons within this length range.

Axonal branches were quantified by the same approach as used in our previous studies, and the results are shown in Figure 6H. Data are expressed as mean branch number per axon ± SD ($n = 3$, 25 cells were analyzed for each condition). Compared with control neurons (2.9 ± 0.7 branches per axon), tau-depleted neurons showed significantly greater branch numbers (tau siRNA: 4.4 ± 1.1; $P < 0.01$). This increase was obliterated by NAP treatment or HDAC6 overexpression (tau siRNA + NAP: 1.9 ± 0.7 and tau siRNA + HDAC6: 2.2 ± 0.7; both $P < 0.01$ compared with tau siRNA). These results provide independent confirmation that these two strategies (NAP treatment or HDAC6 overexpression) are effective at protecting axonal microtubules from being severed by katanin when tau is depleted. The fact that control axons were also diminished in their branching levels (NAP: 1.4 ± 0.6 and HDAC6: 1.6 ± 0.6; both $P < 0.01$ compared with control) is consistent with the fact that normal branching is mediated in part by an endogenous katanin/tau pathway.

DISCUSSION

Tauopathies consist of many potential avenues for producing neurodegeneration, some of which are directly related to the loss of microtubules and some of which are not. For example, there are studies suggesting that Abeta, the main constituent of amyloid plaques, when applied to tau-expressing cells, can result in rapid loss of microtubules (45). This mode of microtubule loss does not occur in cells that do not express tau and does not appear to be related to whether or not the tau is bound to microtubules. ApoE4, another protein that is linked to AD, also results in microtubule loss when

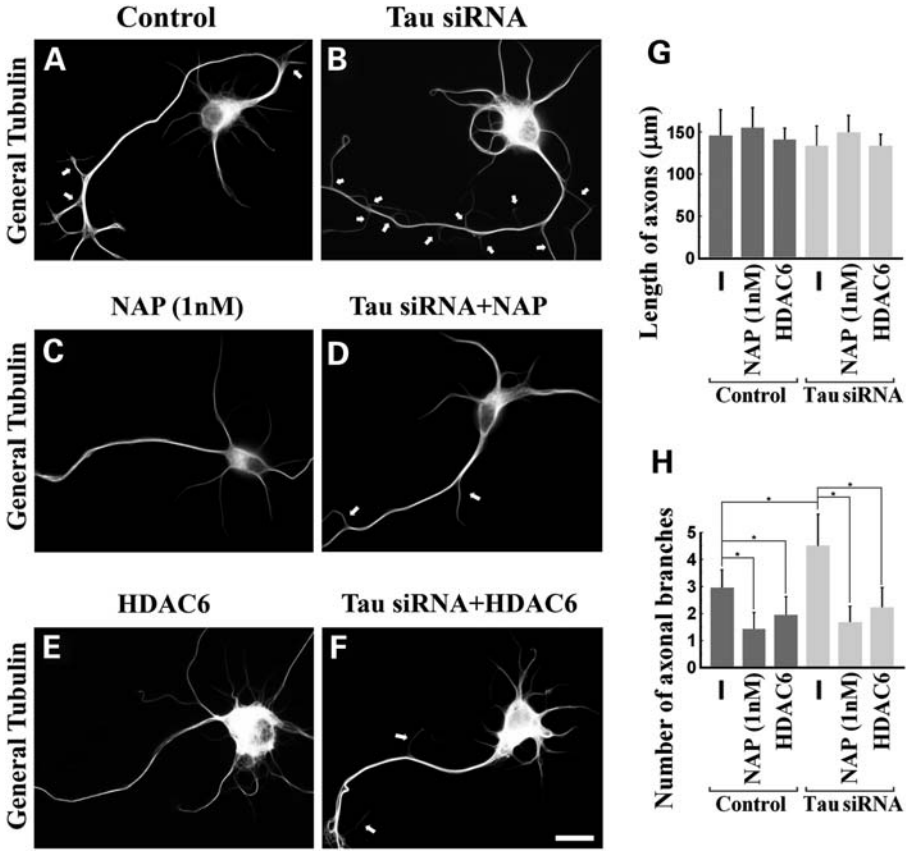

Figure 6. Microtubule deacetylation or NAP treatment diminishes katanin-based axonal branching. Axonal branching was analyzed in control or tau-depleted neurons treated with 1 nM NAP or transfected to overexpress HDAC6 overexpression. General tubulin staining images (**A**), (**C**) and (**E**) show control neurons without treatment (A); with NAP treatment (C) or with HDAC6 overexpression (E); whereas images (**B**), (**D**) and (**F**) show tau-depleted neurons without treatment (B), with NAP treatment (D) or with HDAC6 overexpression (F), respectively. Compared with controls (A), tau-depleted neurons showed increased numbers of axonal branches (B). The increase was not observed under conditions of NAP treatment (D) or HDAC6 overexpression (F). Arrows show axonal branches. Bar: 20 μm. (**G** and **H**) Quantification of axonal length (G) and axonal branch number per axon (H). No significant differences in axonal length were observed. (H) Average branching number per axon from neurons with axonal length of 100–150 μm. Compared with controls, tau-depleted neurons display 1.5 times more axonal branches (asterisk, Student's *t*-test, $P < 0.01$). This increase was significantly repressed by 1 nM NAP treatment or HDAC6 overexpression (asterisks, Student's *t*-test, $P < 0.01$). Interestingly, the basal level of axonal branching was also decreased by NAP and HDAC6, and the differences were significant (asterisks, Student's *t*-test, $P < 0.01$).

applied to neuronal cells in culture (46). Other studies suggest that it is actually the abnormal filaments formed by the pathological tau that are most dangerous to the axon, as the filaments result in de-activation of molecular motor proteins that move along the microtubules (47). It is unclear whether there is a close causative relationship between microtubule loss and axonal degeneration, given that the most dramatic observations of microtubule loss have been reported in axons that had already suffered a great deal of degeneration. Despite this complexity, it is clear from decades of work that microtubule loss is a hallmark feature of tauopathic axons (48–52), and this is the case in pure tauopathies, more complicated diseases such as AD and animal models. In fact, drugs that preserve microtubules have produced some encouraging results as potential treatments for tauopathies (53–55). Therefore, despite the myriad mechanisms that undoubtedly contribute to axonal degeneration in these diseases, there remains a compelling argument that the microtubules, as they gradually lose tau, become more sensitive to whatever in the axon causes them to shift from polymer to subunits and that this contributes in a significant way to axonal degeneration.

In schematic illustrations, terms such as 'disintegrate' or 'fall apart' are often used to describe this effect on the microtubules as they lose tau, with the general impression being that the microtubules depolymerize from their ends as they lose a critical stabilizing protein. Experimental studies, however, have never revealed any evidence that depleting axonal microtubules of tau render them more sensitive to depolymerization (56). This makes sense because axons contain a variety of other proteins that can potentially stabilize microtubules even when tau is lost, such as doublecortin (57), STOP (58), plakins (59) and other fibrous microtubule-associated proteins (60). Although it is not inconceivable that the loss of tau could tip the balance just enough so that the microtubules would depolymerize over time, we have proposed an alternative hypothesis to explain the gradual degradation of the tau-compromised microtubules. It is our hypothesis that the microtubules become gradually more sensitive to the endogenous severing proteins as they lose tau and in particular to katanin (17). Support for this idea first came from our studies

showing that tau-depleted microtubules are extremely sensitive to severing by katanin (12) and our observation that the levels of the severing proteins in neurons are unexpectedly high, sufficiently high to completely sever the microtubules to subunits, if not somehow attenuated (11). This is not to say that abnormal microtubule severing is the principal explanation for axonal degeneration in these diseases, but it could be one of the more clinically treatable factors. If so, preventing or reversing the loss of microtubules by this pathway could help stave off degeneration and hence provide patients with additional years of better quality of life.

Our first set of studies presented here was designed to test the hypothesis that pathogenic tau species are not as good as wild-type tau at protecting microtubules from excess severing by katanin. In our earlier studies, we established that tau must bind to microtubules to elicit its protective effects against katanin-based severing (12), and thus it seems reasonable that tau species that bind less avidly to the microtubules would protect them less. Our present results indicate that two different tau mutants, one that is expressed in humans with tauopathy and the other designed to mimic pathogenic hyperphosphorylated tau, both suppress microtubule depolymerization about as well as wild-type tau when highly expressed in fibroblasts. However, the degree of protection they afford against overexpressed katanin is markedly diminished compared with wild-type tau and correlates well with the impact that each mutant has on the microtubule array. PHP enhances microtubule levels but does not produce marked alterations in microtubule organization or bundling, whereas R406W has a markedly more dramatic effect to induce bundling as well as to increase microtubule levels. Correspondingly, the PHP mutant affords notably less protection than does the R406W mutant against katanin-based severing of microtubules, while both afford less than wild-type tau. Thus, we would conclude that pathogenic situations in which tau binds less avidly to the microtubules can render microtubules more sensitive to severing by katanin relative to the case with normal tau. This is not an all-or-nothing scenario and hence is consistent with a gradual enhancement of sensitivity to abnormal microtubule severing in human patients as the tauopathic conditions worsen.

Our hypothesis that experimental deacetylation of the microtubules in tau-compromised axons might mitigate such sensitivity derives from our recent studies indicating that highly acetylated microtubules are the favored substrates for katanin (13). Axonal microtubules are both highly acetylated (24,44) and rich in tau (61), which means that if they lose their tau, the axonal microtubules would be a prime target for abnormal severing. This is supported by observations from other studies, which were somewhat confusing at the time, that the more stable microtubules in the axon seem to be preferred for destruction in pathogenic situations over the more labile microtubules (62). At present, there are no simple clinical therapies for altering the acetylation status of axonal microtubules, but a growing body of evidence suggests that such a strategy might be useful for treating a number of neurodegenerative disorders (63,64). This is because acetylation and other post-translational modifications of tubulin render them better or worse substrates for various microtubule-related proteins such as molecular motors (65) and perhaps even tau itself (66). Also for this reason, such potential therapies must be considered with caution because deacetylating the microtubules might help with their sensitivity to katanin but might also create problems with their capacity to interact with other important proteins. Even so, the results of our 'proof or principle' experiments are compelling evidence that experimentally deacetylating axonal microtubules can reverse their greater sensitivity to katanin in conditions under which tau is lost. Interestingly, there are observations that during tauopathy, axonal microtubules can become somewhat less acetylated without clinical intervention (67), suggesting that the neuron may use this mechanism in an attempt to fight back against excess severing of microtubules as they become tau-compromised.

Our results using the same experimental paradigm as we used for the deactylation studies demonstrate that NAP has a potent capacity to protect microtubules in the axon from excess severing when tau is lost. Although the mechanism for this remains unclear, NAP has been shown not to influence microtubule dynamics (43), which suggests to us that a more likely mode of action is for NAP to alter the microtubule lattice in such a way as to make it less susceptible to katanin. The levels at which NAP is efficacious are too low for NAP to simply occupy sites on the microtubule that would otherwise be occupied by tau, which also suggests a more subtle and yet broader-based impact on the microtubule lattice itself. Moreover, we would argue that this is a very specific effect relevant to microtubule severing rather than microtubule dynamics because NAP also attenuates axonal branch formation in the case of tau-normal as well as tau-depleted axons.

The dependence of NAP efficacy on the presence of beta-III tubulin suggests a potential role for tubulin isotypes in regulating the sensitivity of microtubules to severing in certain situations. However, there is no compelling evidence for different classes of microtubules in the axon that differ in their composition of tubulin isotypes, which suggests that the relative sensitivity of individual microtubules in the axon to katanin is not determined by this aspect of their composition. At a practical level, this is a potential advantage for the use of NAP to prevent microtubule loss during neurodegenerative diseases, in that its protective effects may extend to microtubules in other neuronal compartments as well, such as cell bodies and dendrites, in which microtubules are also rich in beta-III tubulin (68).

Interestingly, it has been known for many years that there can be enhanced sprouting of axons in patients with AD (69–71), and this is entirely consistent with an increase in katanin-based severing as tau is lost from the microtubules. More difficult to explain on the basis of our observations is the fact that dendrites sometimes also display microtubule loss (49,72–74) as well as aberrant sprouting (75) during AD and other tauopathies. Dendrites are normally not so rich in tau nor are their microtubules highly acetylated. However, it is known that pathogenic tau often forms aggregates that absorb and thereby deplete other microtubule-associated proteins from neurons (76). Some of these proteins, such as MAP2, might play a protective role against excess severing of microtubules in dendrites. Additional work will be required to better evaluate the importance of katanin in the loss of

microtubules from axons and dendrites of neurons afflicted with compromised tau function. The present results may be useful in developing therapeutic regimes for preserving microtubules against loss in the neurons of patients suffering from tauopathies.

MATERIALS AND METHODS

DNA constructs and siRNA

Constructs used for these studies were the following: pEGFP-C1 (control construct; BD Biosciences, Boston, MA, USA) (GFP, green fluorescent protein); pEGFP-C1-p60 (C-terminally EGFP-tagged rat p60-katanin) (12); pcDNA-human HDAC6-flag (ID:13823) (addgene, Cambridge, MA, USA); pRC/CMV-Flag-htau441wt (flag-tagged human four-repeat tau); pRC/CMV-Flag-hPHPtau441 (PHP); pRC/CMV-Flag-htau441R406W (tau harboring a causative mutation for hereditary frontotemporal dementia with parkinsonism linked to chromosome 17) and human beta-III tubulin expression construct (backbone vector: pCMV6-XL5) (SC116313-20) (Origene, MD, USA). All tau constructs were provided by Dr R. Brandt, University of Osnabrück, Barbarastrasse, Germany. For tau depletion, we used a mixture of four different siRNA duplexes designed against different regions of the molecule using Dharmacon (Lafayette, CO, USA) custom SMARTpool siRNA service. The Dharmacon accession number for rat tau is NM_017212. The non-specific duplex III (Dharmacon) was used as control.

Pharmacological tools

Nocodazole was purchased from Sigma (St Louis, MO, USA) and used as described previously (23,24). NAP was purchased from Abbiotec (San Diego, CA, USA), aliquoted and stocked as a 1 mM solution in 5% dimethyl sulfoxide at −20°C.

Cell culture, transfection and drug treatments

RFL-6 cells. Rat RFL-6 fibroblasts were cultured as described previously (12) and transfected with pEGFP-C1-P60, pHDAC6-flag or pRC/CMV-Flaghtau441wt and its two mutants using a Nucleofector (Amaxa, Gaithersburg, MD, USA) with the manufacturer's program G-13. Fifteen micrograms of each plasmid and 10^6 cells were used for each transfection. The cells were plated at a density of 4000/well on glass cover slips mounted in the bottom of 35 mm diameter Petri dishes with 1 cm holes drilled in the bottom. In some experiments, nocodazole (used at a final concentration of 7 μM) was added to the culture 24 h after transfection, and the cells were fixed after 5 or 30 min incubation in the drug.

Hippocampal neurons. Rat hippocampal neurons were prepared as described previously (77). Neurons were cultured using hippocampal neuron plating medium (neurobasal medium supplemented with 2% B27, 0.3% glucose, 1 mM glutamine and 5% fetal bovine serum). Neurons were prepared, transfected with siRNA against rat tau by Nucleofection (protocol: G-13) as described previously (12) and then plated on 35 mm diameter Petri dishes coated with poly-L-lysine. Two days after plating, neurons were re-plated at a density of 3000 cells/well on poly-L-lysine-coated glass cover slips mounted onto the bottom of 35 mm diameter Petri dishes with holes drilled in the bottom. In some cases, NAP was then added to the culture medium. The transfections of pEGFP-C1-P60, pHDAC6-flag or pRC/CMV-Flaghtau441wt and its mutants were performed 2 days after the re-plating. For transfections of those constructs, we used Lipofectamine 2000 (Invitrogen, San Diego, CA, USA). Two micrograms of DNA constructs and 5 μl of Lipofectamine 2000 were used per dish. The cells were incubated in the DNA/lipofectamine-containing medium for 5 h. Then neurons were transferred to fresh 37°C hippocampal neuron plating medium. In experiments using NAP, the peptide was added to the medium at this time. Twelve hours later, cells were fixed for immunostaining. Transfection efficiency was generally 5–10% for neuronal cultures and 15–30% for RFL-6 cell cultures.

Immunofluorescence techniques

Cy3-conjugated monoclonal anti-β-tubulin (1:150; for general tubulin staining), monoclonal anti-acetylated tubulin (6-11B-1) (1:400), monoclonal anti-flag (M2) (1:500) and monoclonal anti-GFP (1:250; for the enhancement of GFP signals in experiments of neuron) antibodies were purchased from Sigma. Monoclonal anti-tau antibody (Tau5) (1:1000), which recognizes both human and rat tau regardless of phosphorylation status, was provided by Dr Lester Binder and was used for quantification of tau protein levels. Rabbit polyclonal anti-human HDAC6 (H-300) (1:200) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-katanin antibody (1:1000) was used to quantify katanin protein levels (6). Polyclonal anti-beta-III tubulin antibody (1:600) was purchased from Covance and used for the identification of beta-III tubulin in transfected RFL-6 cells.

For immunofluorescence studies, cultures were briefly washed with 37°C phosphate buffered-saline (PBS) and then simultaneously fixed and extracted with 4% paraformaldehyde, 0.2% glutaraldehyde and 0.1% Triton X-100 for 15 min. Cultures were washed with PBS three times for 5 min, quenched with 2 mg/ml sodium borohydride three times for 10 min and then blocked with 10% normal goat serum and 10 mg/ml bovine serum albumin in PBS for 1 h. For immunostaining of the expressed HDAC6, tau constructs and/or katanin, samples were prepared as described previously (8). In experiments using RFL-6, we detected the GFP signals of GFP-katanin without further enhancement. In experiments using neurons, we detected the GFP signals of GFP-katanin with enhancement by staining with the monoclonal anti-GFP antibody. We detected ectopically expressed HDAC6 with anti-human HDAC6 antibody [see our previous study (13)]. We detected tau-derived constructs with anti-flag antibody in RFL-6 cells.

To quantify microtubule levels, both fibroblasts and neurons were simultaneously fixed and extracted to remove free tubulin as described previously (12,13) and then immunostained for HDAC6, flag and/or GFP. Cultures were then treated with appropriate fluorescently conjugated secondary antibodies and subsequently with Cy3-conjugated anti-β-tubulin

antibody. So that samples could be compared against one another, and microscope settings were kept consistent. In experiments on cultured neurons, we selected only neurons that were sufficiently separated from neighboring cells that we could distinguish axons from minor processes on the basis of morphology. Images were acquired with an AxioVert 200M microscope (Carl Zeiss, Oberkochen, Germany) coupled with an Orca-ER Digital CCD (Hamamatsu, Shizouka, Japan) and a 100× Plan-Neorofluar/1.3 numerical aperture objective. Images to be compared were taken at identical settings of exposure time, brightness and contrast and analyzed with Axiovision 4.0 software. Measures of microtubule levels were taken as total fluorescence intensity per cell in the case of RFL-6 cells and total fluorescence intensity in 10 μm regions of axons or minor processes using the analytical command of densitometricmean in Axiovision 4.0. Regarding quantification in axons, because microtubule levels could sometimes vary along the length of the axon, we assessed the microtubule amount of a defined region with a constant length (10 μm) per axon which had the highest signal among all regions of that axon. Statistics were done using Student's *t*-test. In RFL-6 cells experiments, we performed subdivided microtubule quantification analyses. For this, we assessed densitometricsum, densitometricmean and densitometricarea values of the entire microtubule mass or bundled microtubule mass (i.e. only the microtubules that were closely spaced and aligned with other microtubules) in each cell. Non-bundled microtubule levels were then calculated for the cell by calculation with the following formula: [densitometricsum (entire) − densitometricsum (bundle)]/[densitometricarea (entire) − densitometricarea (bundle)]. Values were expressed as AFUs.

To quantify endogenous and ectopically expressed tau levels, wild-type tau was ectopically expressed in RFL-6 cells, after which the cultures were fixed and prepared for immunofluorescence side-by-side with control cultures of rat hippocampal neurons. The cultures were stained with the Tau5 antibody, which equally recognizes human and rat tau, followed by an appropriate fluorescent secondary antibody. The signals from the entire cell in the case of the tau-expressing RFL-6 cells were quantified using Axiovision software (unit: densitometricmean), whereas signals from cell bodies and axons in neurons were separately assessed in the case of the neurons. Twenty-five cells were analyzed in each case. To quantify endogenous and ectopically expressed katanin levels, cultures of RFL-6 cells and hippocampal neurons were induced to overexpress katanin and then fixed and stained for katanin using our polyclonal katanin antibody and an appropriate fluorescent secondary antibody. The signals from the entire cell were assessed in the case of the RFL-6 cells, whereas the signals from cell bodies only were assessed in the case of the neurons (as staining in the axons was too dim to provide confidence in the quantification with this method). Twenty-five cells were analyzed. The results are shown in Table 1.

ACKNOWLEDGEMENTS

We thank Dr Roland Brandt for generously providing the tau mutant constructs and for ongoing advice and support. We thank Dr Lester Binder for generously providing tau antibodies as well as ongoing advice and support. We thank Drs Wenqian Yu and Liang Qiang for conducting preliminary experiments related to this work.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants to P.W.B. from the Alzheimer's Association, the National Institutes of Health, the National Science Foundation, the Hereditary Spastic Paraplegia Foundation and the State of Pennsylvania Tobacco Settlement Funds.

REFERENCES

1. Yu, W., Solowska, J.M., Qiang, L., Karabay, A., Baird, D. and Baas, P.W. (2005) Regulation of microtubule severing by katanin subunits during neuronal development. *J. Neurosci.*, **25**, 5573–5583.
2. Yu, W., Qiang, L., Solowska, J.M., Karabay, A., Korulu, S. and Baas, P.W. (2008) The microtubule-severing proteins spastin and katanin participate differently in the formation of axonal branches. *Mol. Biol. Cell*, **19**, 1485–1498.
3. Wang, L. and Brown, A. (2002) Rapid movement of microtubules in axons. *Curr. Biol.*, **12**, 1496–1501.
4. Roll-Mecak, A. and Vale, R.D. (2006) Making more microtubules by severing: a common theme of noncentrosomal microtubule arrays? *J. Cell Biol.*, **175**, 849–851.
5. Baas, P.W., Vidya Nadar, C. and Myers, K.A. (2006) Axonal transport of microtubules: the long and short of it. *Traffic*, **7**, 490–498.
6. Qiang, L., Yu, W., Liu, M., Solowska, J.M. and Baas, P.W. (2010) Basic fibroblast growth factor elicits formation of interstitial axonal branches via enhanced severing of microtubules. *Mol. Biol. Cell*, **21**, 334–344.
7. Ahmad, F.J., Yu, W., McNally, F.J. and Baas, P.W. (1999) An essential role for katanin in severing microtubules in the neuron. *J. Cell Biol.*, **145**, 305–315.
8. Karabay, A., Yu, W., Solowska, J.M., Baird, D.H. and Baas, P.W. (2004) Axonal growth is sensitive to the levels of katanin, a protein that severs microtubules. *J. Neurosci.*, **24**, 5778–5788.
9. Wood, J.D., Landers, J.A., Bingley, M., McDermott, C.J., Thomas-McArthur, V., Gleadall, L.J., Shaw, P.J. and Cunliffe, V.T. (2006) The microtubule-severing protein Spastin is essential for axon outgrowth in the zebrafish embryo. *Hum. Mol. Genet.*, **15**, 2763–2771.
10. Riano, E., Martignoni, M., Mancuso, G., Cartelli, D., Crippa, F., Toldo, I., Siciliano, G., Di Bella, D., Taroni, F., Bassi, M.T., Cappelletti, G. and Rugarli, E.I. (2009) Pleiotropic effects of spastin on neurite growth depending on expression levels. *J. Neurochem.*, **108**, 1277–1288.
11. Solowska, J.M., Morfini, G., Falnikar, A., Himes, B.T., Brady, S.T., Huang, D. and Baas, P.W. (2008) Quantitative and functional analyses of spastin in the nervous system: implications for hereditary spastic paraplegia. *J. Neurosci.*, **28**, 2147–2157.
12. Qiang, L., Yu, W., Andreadis, A., Luo, M. and Baas, P.W. (2006) Tau protects microtubules in the axon from severing by katanin. *J. Neurosci.*, **26**, 3120–3129.
13. Sudo, H. and Baas, P.W. (2010) Acetylation of microtubules influences their sensitivity to severing by katanin in neurons and fibroblasts. *J. Neurosci.*, **30**, 7215–7226.
14. Wang, J.Z. and Liu, F. (2008) Microtubule-associated protein tau in development, degeneration and protection of neurons. *Prog. Neurobiol.*, **85**, 148–175.
15. Falnikar, A. and Baas, P.W. (2009) Critical roles for microtubules in axonal development and disease. *Results Probl. Cell Differ.*, **48**, 47–64.
16. Gozes, I., Stewart, A., Morimoto, B., Fox, A., Sutherland, K. and Schmeche, D. (2009) Addressing Alzheimer's disease tangles: from NAP to AL-108. *Curr. Alzheimer Res.*, **6**, 455–460.
17. Baas, P.W. and Qiang, L. (2005) Neuronal microtubules: when the MAP is the roadblock. *Trends Cell Biol.*, **15**, 183–187.

18. Maas, T., Eidenmüller, J. and Brandt, R. (2000) Interaction of tau with the neural membrane cortex is regulated by phosphorylation at sites that are modified in paired helical filaments. *J. Biol. Chem.*, **275**, 15733–15740.
19. Brandt, R., Hundelt, M. and Shahani, N. (2005) Tau alteration and neuronal degeneration in tauopathies: mechanisms and models. *Biochim. Biophys. Acta*, **1739**, 331–354.
20. Eidenmüller, J., Fath, T., Hellwig, A., Reed, J., Sontag, E. and Brandt, R. (2000) Structural and functional implications of tau hyperphosphorylation: information from phosphorylation-mimicking mutated tau proteins. *Biochemistry*, **39**, 13166–13175.
21. Hoebeke, J., Van Nijen, G. and De Brabander, M. (1976) Interaction of nocodazole (R 17934), a new antitumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.*, **69**, 319–324.
22. Lee, J.C., Field, D.J. and Lee, L.L. (1980) Effects of nocodazole on structures of calf brain tubulin. *Biochemistry*, **19**, 6209–6215.
23. Baas, P.W. and Black, M.M. (1990) Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol., 111*, 495–509.
24. Baas, P.W., Slaughter, T., Brown, A. and Black, M.M. (1991) Microtubule dynamics in axons and dendrites. *J. Neurosci. Res.*, **30**, 134–153.
25. Drubin, D.G. and Kirschner, M.W. (1986) Tau protein function in living cells. *J. Cell Biol.*, **103**, 2739–2746.
26. Lee, G. and Rook, S.L. (1992) Expression of tau protein in non-neuronal cells: microtubule binding and stabilization. *J. Cell Sci.*, **102**, 227–237.
27. Takemura, R., Okabe, S., Umeyama, T., Kanai, Y., Cowan, N.J. and Hirokawa, N. (1992) Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. *J. Cell Sci.*, **103**, 953–964.
28. Ding, H., Matthews, T.A. and Johnson, G.V. (2006) Site-specific phosphorylation and caspase cleavage differentially impact tau–microtubule interactions and tau aggregation. *J. Biol. Chem.*, **281**, 19107–19114.
29. Pérez, M., Lim, F., Arrasate, M. and Avila, J. (2000) The FTDP-17-linked mutation R406W abolishes the interaction of phosphorylated tau with microtubules. *J. Neurochem.*, **74**, 2583–2589.
30. Dotti, C.G., Sullivan, C.A. and Banker, G.A. (1988) The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.*, **8**, 1454–1468.
31. Zemlyak, I., Manley, N., Sapolsky, R. and Gozes, I. (2007) NAP protects hippocampal neurons against multiple toxins. *Peptides*, **28**, 2004–2008.
32. Zemlyak, I., Sapolsky, R. and Gozes, I. (2009) NAP protects against cytochrome *c* release: inhibition of the initiation of apoptosis. *Eur. J. Pharmacol.*, **618**, 9–14.
33. Gozes, I., Divinski, I. and Piltzer, I. (2008) NAP and D-SAL: neuroprotection against the beta amyloid peptide (1–42). *BMC Neurosci.*, **9**, S3.
34. Greggio, S., Rosa, R.M., Dolganov, A., de Oliveira, I.M., Menegat, F.D., Henriques, J.A. and Dacosta, J.C. (2009) NAP prevents hippocampal oxidative damage in neonatal rats subjected to hypoxia-induced seizures. *Neurobiol. Dis.*, **36**, 435–444.
35. Incerti, M., Vink, J., Roberson, R., Benassou, I., Abebe, D. and Spong, C.Y. (2010) Prevention of the alcohol-induced changes in brain-derived neurotrophic factor expression using neuroprotective peptides in a model of fetal alcohol syndrome. *Am. J. Obstet. Gynecol., 202*, e1–e4.
36. Gozes, I. and Divinski, I. (2004) The femtomolar-acting NAP interacts with microtubules: novel aspects of astrocyte protection. *J. Alzheimers Dis.*, **6**, S37–S41.
37. Gozes, I. and Divinski, I. (2007) NAP, a neuroprotective drug candidate in clinical trials, stimulates microtubule assembly in the living cell. *Curr. Alzheimer Res.*, **4**, 507–509.
38. Divinski, I., Holtser-Cochav, M., Vulih-Schultzman, I., Steingart, R.A. and Gozes, I. (2006) Peptide neuroprotection through specific interaction with brain tubulin. *J. Neurochem.*, **98**, 973–984.
39. Matsuoka, Y., Gray, A.J., Hirata-Fukae, C., Minami, S.S., Waterhouse, E.G., Mattson, M.P., LaFerla, F.M., Gozes, I. and Aisen, P.S. (2007) Intranasal NAP administration reduces accumulation of amyloid peptide and tau hyperphosphorylation in a transgenic mouse model of Alzheimer's disease at early pathological stage. *J. Mol. Neurosci.*, **31**, 165–170.
40. Matsuoka, Y., Jouroukhin, Y., Gray, A.J., Ma, L., Hirata-Fukae, C., Li, H.F., Feng, L., Lecanu, L., Walker, B.R., Planel, E. *et al.* (2008) A neuronal microtubule-interacting agent, NAPVSIPQ, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer's disease. *J. Pharmacol. Exp. Ther.*, **325**, 146–153.
41. Shiryaev, N., Jouroukhin, Y., Giladi, E., Polyzoidou, E., Grigoriadis, N.C., Rosenmann, H. and Gozes, I. (2009) NAP protects memory, increases soluble tau and reduces tau hyperphosphorylation in a tauopathy model. *Neurobiol. Dis.*, **34**, 381–388.
42. Geerts, H. (2008) AL-108 and AL-208, formulations of the neuroprotective NAP fragment of activity-dependent neuroprotective protein, for cognitive disorders. *Curr. Opin. Investig. Drugs*, **9**, 800–811.
43. Yenjerla, M., LaPointe, N.E., Lopus, M., Cox, C., Feinstein, S.C. and Wilson, L. (2010) The neuroprotective peptide NAP does not directly affect polymerization or dynamics of reconstituted neural microtubules. *J. Alzheimers Dis.*, **19**, 1377–1386.
44. Cambray-Deakin, M.A. and Burgoyne, R.D. (1987) Posttranslational modifications of alpha-tubulin: acetylated and detyrosinated forms in axons of rat cerebellum. *J. Cell Biol.*, **104**, 1569–1574.
45. King, M.E., Kan, H.M., Baas, P.W., Erisir, A., Glabe, C.G. and Bloom, G.S. (2006) Tau-dependent microtubule disassembly initiated by prefibrillar beta-amyloid. *J. Cell Biol.*, **175**, 541–546.
46. Nathan, B.P., Chang, K.C., Bellosta, S., Brisch, E., Ge, N., Mahley, R.W. and Pitas, R.E. (1995) The inhibitory effect of apolipoprotein E4 on neurite outgrowth is associated with microtubule depolymerization. *J. Biol. Chem.*, **270**, 19791–19799.
47. LaPointe, N.E., Morfini, G., Pigino, G., Gaisina, I.N., Kozikowski, A.P., Binder, L.I. and Brady, S.T. (2009) The amino terminus of tau inhibits kinesin-dependent axonal transport: implications for filament toxicity. *J. Neurosci. Res.*, **87**, 440–451.
48. Flament-Durand, J. and Couck, A.M. (1979) Spongiform alterations in brain biopsies of presenile dementia. *Acta Neuropathol.*, **46**, 159–162.
49. Ellisman, M., Ranganathan, R., Deerinck, T., Young, S., Terry, R. and Mirra, S. (1987) Neuronal fibrillar cytoskeleton and endomembrane system organization in Alzheimer's disease. *Adv. Behav. Biol.*, **34**, 61–75.
50. Terry, R.D. (1998) The cytoskeleton in Alzheimer disease. *J. Neural Transm. Suppl.*, **53**, 141–145.
51. Tanemura, K., Murayama, M., Akagi, T., Hashikawa, T., Tominaga, T., Ichikawa, M., Yamaguchi, H. and Takashima, A. (2002) Neurodegeneration with tau accumulation in a transgenic mouse expressing V337M human tau. *J. Neurosci.*, **22**, 133–141.
52. Cash, A.D., Aliev, G., Siedlak, S.L., Nunomura, A., Fujioka, H., Zhu, X., Raina,, A.K., Vinters, H.V., Tabaton, M., Johnson, A.B. *et al.* (2003) Microtubule reduction in Alzheimer's disease and aging is independent of tau filament formation. *Am. J. Pathol.*, **162**, 1623–1627.
53. Lee, V.M., Daughenbaugh, R. and Trojanowski, J.Q. (1994) Microtubule stabilizing drugs for the treatment of Alzheimer's disease. *Neurobiol. Aging*, **15**, S87–S89.
54. Zhang, B., Maiti, A., Shively, S., Lakhani, F., McDonald-Jones, G., Bruce, J., Lee, E.B., Xie, S.X., Joyce, S., Li, C. *et al.* (2005) Microtubule-binding drugs offset tau sequestration by stabilizing microtubules and reversing fast axonal transport deficits in a tauopathy model. *Proc. Natl Acad. Sci. USA*, **102**, 227–231.
55. Michaelis, M.L., Ansar, S., Chen, Y., Reiff, E.R., Seyb, K.I., Himes, R.H., Audus, K.L. and Georg, G.I. (2005) {beta}-Amyloid-induced neurodegeneration and protection by structurally diverse microtubule-stabilizing agents. *J. Pharmacol. Exp. Ther.*, **312**, 659–668.
56. Tint, I., Slaughter, T., Fischer, I. and Black, M.M. (1998) Acute inactivation of tau has no effect on dynamics of microtubules in growing axons of cultured sympathetic neurons. *J. Neurosci.*, **18**, 8660–8673.
57. Tint, I., Jean, D., Baas, P.W. and Black, M.M. (2009) Doublecortin associates with microtubules preferentially in regions of the axon displaying actin-rich protrusive structures. *J. Neurosci.*, **29**, 10995–11010.
58. Slaughter, T. and Black, M.M. (2003) STOP (stable-tubule-only-polypeptide) is preferentially associated with the stable domain of axonal microtubules. *J. Neurocytol.*, **32**, 399–413.
59. Sonnenberg, A. and Liem, R.K. (2007) Plakins in development and disease. *Exp. Cell Res.*, **313**, 2189–2203.
60. Matus, A. (1988) Microtubule-associated proteins: their potential role in determining neuronal morphology. *Annu. Rev. Neurosci.*, **11**, 29–44.
61. Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985) The distribution of tau in the mammalian central nervous system. *J. Cell Biol.*, **101**, 1371–1378.
62. Merrick, S.E., Trojanowski, J.Q. and Lee, V.M. (1997) Selective destruction of stable microtubules and axons by inhibitors of protein serine/threonine phosphatases in cultured human neurons. *J. Neurosci.*, **17**, 5726–5737.

63. Dompierre, J.P., Godin, J.D., Charrin, B.C., Cordelières, F.P., King, S.J., Humbert, S. and Saudou, F. (2007) Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation. *J. Neurosci.*, **27**, 3571–3583.
64. Nguyen, L., Humbert, S., Saudou, F. and Chariot, A. (2010) Elongator$\frac{3}{4}$an emerging role in neurological disorders. *Trends Mol. Med.*, **16**, 1–6.
65. Reed, N.A., Cai, D., Blasius, T.L., Jih, G.T., Meyhofer, E., Gaertig, J. and Verhey, K.J. (2006) Microtubule acetylation promotes kinesin-1 binding and transport. *Curr. Biol.*, **16**, 2166–2172.
66. Saragoni, L., Hernández, P. and Maccioni, R.B. (2000) Differential association of tau with subsets of microtubules containing posttranslationally-modified tubulin variants in neuroblastoma cells. *Neurochem. Res.*, **25**, 59–70.
67. Hempen, B. and Brion, J.P. (1996) Reduction of acetylated alpha-tubulin immunoreactivity in neurofibrillary tangle-bearing neurons in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.*, **55**, 964–972.
68. Lieven, C.J., Millet, L.E., Hoegger, M.J. and Levin, L.A. (2007) Induction of axon and dendrite formation during early RGC-5 cell differentiation. *Exp. Eye Res., 85*, 678–683.
69. Geddes, J.W., Anderson, K.J. and Cotman, C.W. (1986) Senile plaques as aberrant sprout-stimulating structures. *Exp. Neurol.*, **94**, 767–776.
70. Cotman, C.W., Geddes, J.W. and Kahle, J.S. (1990) Axon sprouting in the rodent and Alzheimer's disease brain: a reactivation of developmental mechanisms? Prog. *Brain Res.*, **83**, 427–434.
71. Masliah, E., Mallory, M., Hansen, L., Alford, M., Albright, T., DeTeresa, R., Terry, R., Baudier, J. and Saitoh, T. (1991) Patterns of aberrant sprouting in Alzheimer's disease. *Neuron*, **6**, 729–739.
72. Gray, E.G. (1986) Spongiform encephalopathy: a neurocytologist's viewpoint with a note on Alzheimer's disease. *Neuropathol. Appl. Neurobiol.*, **12**, 149–172.
73. Gray, E.G., Paula-Barbosa, M. and Roher, A. (1987) Alzheimer's disease: paired helical filaments and cytomembranes. *Neuropathol. Appl. Neurobiol.*, **13**, 91–110.
74. Paula-Barbosa, M., Tavares, M.A. and Cadete-Leite, A. (1987) A quantitative study of frontal cortex dendritic microtubules in patients with Alzheimer's disease. *Brain Res.*, **417**, 139–142.
75. Ihara, Y. (1988) Massive somatodendritic sprouting of cortical neurons in Alzheimer's disease. *Brain Res.*, **459**, 138–144.
76. Alonso, A.D., Grundke-Iqbal, I., Barra, H.S. and Iqbal, K. (1997) Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proc. Natl Acad. Sci. USA*, **94**, 298–303.
77. Yu, W. and Baas, P.W. (1994) Changes in microtubule number and length during axon differentiation. *J. Neurosci.*, **14**, 2818–2829.