

tau confers drug stability but not cold stability to microtubules in living cells

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SUMMARY

We previously defined two classes of microtubule polymer in the axons of cultured sympathetic neurons that differ in their sensitivity to nocodazole by roughly 35-fold (Baas and Black (1990) *J. Cell Biol.* 111, 495-509). Here we demonstrate that virtually all of the microtubule polymer in these axons, including the drug-labile polymer, is stable to cold. What factors account for the unique stability properties of axonal microtubules? In the present study, we have focused on the role of tau, a microtubule-associated protein that is highly enriched in the axon, in determining the stability of microtubules to nocodazole and/or cold in living cells. We used a baculovirus vector to express very high levels of tau in insect ovarian Sf9 cells. The cells respond by extending processes that contain dense bundles of microtubules

(Knops et al. (1991) *J. Cell Biol.* 114, 725-734). Cells induced to express tau were treated with either cold or 2 µg/ml nocodazole for times ranging from 5 minutes to 2 hours. The results with each treatment were very different from one another. Virtually all of the polymer was depolymerized within the first 30 minutes in cold, while little or no microtubule depolymerization was detected even after 6 hours in nocodazole. Based on these results, we conclude that tau is almost certainly a factor in conferring drug stability to axonal microtubules, but that factors other than or in addition to tau are required to confer cold stability.

Key words: tau, microtubule, Sf9 cell, neuron, axon, cold

INTRODUCTION

Microtubules (MTs) in the axons of nerve cells have unusual stability properties. A substantial portion of the MT mass within the axon turns over subunits more slowly and is more resistant to influences that depolymerize MTs than are MTs in typical mitotic or motile cells (for reviews see Meininger and Binet, 1989; Diaz-Nido et al., 1990; Joshi and Baas, 1993; see also Lim et al., 1990; Okabe and Hirokawa, 1990; Reinsch et al., 1991; Edson et al., 1993). The functional significance of this enhanced stability is manifold. Stable MTs are thought to be central to the generation and maintenance of many morphological features of neurons such as the long cylindrical appearance of axons (Mitchison and Kirschner, 1988). In addition, bidirectional organelle transport occurs along the surface of axonal MTs (for review see Brady, 1991), and the presence of a relatively stable MT array ensures that the transport of these organelles is not interrupted. Finally, stable MTs act as MT nucleating structures in the axon, and are essential for the regulation and expansion of the axonal MT array itself (Baas and Black, 1990; Baas and Ahmad, 1992). For all of these reasons, the stability properties of axonal MTs are a matter of great interest.

Perhaps the most common approach for studying MT stability has been the use of anti-MT drugs such as nocodazole. Nocodazole rapidly enters the cell and binds to unassembled

tubulin. MT assembly is blocked, while MT disassembly continues to occur (Hoebeke et al., 1976; Lee et al., 1980). As a result, the MTs disassemble at a rate that is presumably reflective of the normal rate of subunit turnover. In recent work, we quantified the effects of nocodazole on the MTs in the axons of cultured rat sympathetic neurons (Baas and Black, 1990; Baas et al., 1991a, 1993). We found that roughly half the MT mass in these axons is drug-labile, depolymerizing with a half-time of ≈3.5 minutes. The other half of the mass is relatively drug-stable, depolymerizing with a 35-fold slower half-time. The labile polymer assembles directly from the plus ends of the stable polymer and, as a result, the MTs consist of two distinct domains that differ in their stability to the drug (Baas and Black, 1990; Baas and Ahmad, 1992; Ahmad et al., 1993).

MTs also tend to depolymerize in response to cold, and hence the resistance of a MT to cold-depolymerization is also an indicator of its stability. However, cold stability is not well understood, and it is unknown whether cold stability and drug stability reflect the same or different properties of the MT. To date, most information on the cold stability of axonal MTs derives from studies on cellular extracts (Webb and Wilson, 1980; Brady et al., 1984; Binet et al., 1987; Watson et al., 1990), and these studies indicate that a substantial fraction of the polymer is cold-stable. However, the isolation of the cellular extracts could remove or alter factors that normally regulate MT stability. In fact, the common procedure for

isolating tubulin exploits cycles of cold and warm treatments, with the cold-stable polymer typically discarded during the purification procedure (for review see Dustin, 1984). Thus axons may contain far greater levels of cold-stable polymer than are indicated by previous biochemical work.

What are the mechanisms by which drug stability and/or cold stability are conferred upon axonal MTs? Available evidence suggests that MT stabilization in the axon is a complex process, and probably results from the interplay of many factors. However, MT stabilization in the axon appears not to be the direct result of end-caps on the MTs or post-translational modifications of tubulin such as detyrosination or acetylation. With regard to the former, it has been hypothesized that some types of MTs become stabilized by physical caps on their ends, the presence of which blocks MT disassembly (see Khawaja et al., 1980). However, the cap would also be expected to block MT assembly, and the results of nocodazole recovery experiments indicate that stable MTs in the axon are assembly-competent at their plus ends (Baas and Ahmad, 1992; see above). Also arguing against the presence of end-caps are findings that MT stability in the axon is not altered when new ends are created by severing the MTs along their lengths (White et al., 1987). With regard to tubulin modifications such as detyrosination or acetylation, data on a variety of cell types suggest that these modifications are the result, not the cause, of MT stabilization (Piperno et al., 1987; Khawaja et al., 1988; Webster et al., 1990). The more likely possibility is that MTs are stabilized by the binding along their length of accessory proteins termed MAPs (microtubule-associated proteins). In fact, biochemical evidence indicates that MTs assembled from purified brain tubulin are highly dynamic, and that the addition of MAPs to the MT reduces the frequency of dynamic events (see for example Bre and Karsenti, 1990; Drechsel et al., 1992; Pryer et al., 1992; see also, for review, Chapin and Bulinski, 1992).

With regard to MT stability in the axon, the most compelling MAP is tau. tau is abundant in the neuron and highly enriched in the axon (Binder et al., 1985; Peng et al., 1986; Kowall and Kosik, 1987; for review see Matus, 1988, 1991). A growing body of indirect evidence suggests that tau may play important roles in MT nucleation, assembly and bundling, as well as in stabilization (for reviews see Lee, 1990; Goedert et al., 1991). In addition, it appears from anti-sense studies that tau has a role in the differentiation of the axon (Caceres and Kosik, 1990). Unfortunately, the presence of an assortment of other neuronal MAPs, most probably including some as yet undiscovered, makes difficult the assignment of specific functions to tau or any individual MAP in the axon. For this reason, we recently developed a novel experimental system for analyzing the functional properties of individual neuronal MAPs within living cells. Normally round insect ovarian Sf9 cells are infected with a baculovirus vector containing a cDNA for the MAP, in this case tau. The cells express high levels of tau and, in response, they extend processes that contain dense bundles of MTs (Knops et al., 1991). This system offers a unique opportunity to study the effects of tau on the properties of MTs in living cells, separate from the myriad of other neuronal MAPs that coexist with tau in the axon. We have already used these cells to analyze the effects of tau on MT spacing and polarity orientation (Baas et al., 1991b). Here, we utilize the system to analyze the effects of tau on MT stability.

The present study consists of two parts. First, we analyzed the cold stability of the MTs in the axons of cultured sympathetic neurons, and compared our findings with our previous knowledge of the drug stability of these MTs. Then, in order to evaluate the capacity of tau to confer onto the MTs of living cells drug stability, cold stability, both, or neither, we analyzed the stability to cold and nocodazole of the MTs in the processes extended by Sf9 cells induced to express tau.

MATERIALS AND METHODS

Cell culture

Explant cultures of rat sympathetic neurons were prepared as previously described (Baas and Black, 1990; Baas and Ahmad, 1992; see also Peng et al., 1986). Briefly, superior cervical ganglia from newborn rat pups were cut into 2-3 pieces and placed in collagen-coated plastic culture dishes with N2 medium (Moya et al., 1980) supplemented with 1% human placental serum, 50 ng/ml nerve growth factor, and 0.6% methyl cellulose. The following morning, the cultures were fed with N2 medium supplemented with 50 ng/ml nerve growth factor and 10 μ m cytosine arabinoside. Axons were permitted to grow for 5-10 days. For studies on axonal MTs, explant cultures are ideal because they generate a dense outgrowth of aligned axons in all directions from the centralized cell body mass.

Insect ovarian Sf9 cells were infected with a virus containing the transcript for either the three- or four-repeat isoform of tau, and cultured in 35 mm tissue culture dishes as previously described (Knops et al., 1991; see also Smith and Summers, 1987). These normally rounded cells, when infected in this manner, extend cellular processes. Ultrastructural analyses were performed after approximately 2 days in culture, by which time most of the cells had extended long processes, but had not yet begun to suffer from the toxic effects of the viral infection.

Nocodazole and cold treatments

Cultures were treated with nocodazole as described in our previous work (Baas and Black, 1990; Baas et al., 1991a, 1993; Baas and Ahmad, 1992). Briefly, a stock solution of 2 mg/ml nocodazole (Aldrich, Milwaukee, WI) was prepared in DMSO. This stock solution was used at 1:1000 in tissue culture medium for a final concentration of 2 μ g/ml nocodazole. Cultures were incubated with the drugged medium for the designated periods of time prior to fixation for electron microscopy. For cold treatment, it was necessary to chill the cultures as rapidly as possible and to get them as cold as possible without freezing. In addition, it was necessary that the pH remain relatively steady during the cold treatments. To accomplish these goals, the medium was removed from the cultures and replaced with medium that had been carefully adjusted to the proper pH and incubated in an ice/water bath for at least 1 hour. The culture dishes were filled to brimming with the ice-cold medium, and the cover was returned to each dish. Dishes were then sealed with parafilm, and special care was taken to ensure that little or no air entered during the sealing. Then, the cultures were submerged in an ice/water bath for the designated period of time prior to fixation with ice-cold fixative.

Electron microscopy

For transmission electron microscopy, cultures were prepared as previously described (Baas and Heidemann, 1986; Baas et al., 1987). Briefly, cultures were fixed in 2% glutaraldehyde for 10 minutes, rinsed in 0.1 M cacodylate, stained with 2 mg/ml tannic acid for 5 minutes, rinsed again, postfixed for 5 minutes with 1% osmium tetroxide, dehydrated with increasing concentrations of ethanol, and embedded in LX-112 (Ladd Res. Inds. Inc., Burlington, VA). After curing of the resin, specific regions of the culture were re-located by

phase-contrast microscopy, photographed with a video-printer (Sony Corporation, Japan), circled with a diamond marker objective, and sectioned parallel to the substratum using either a Reichert-Jung Ultracut E or Ultracut S Ultramicrotome (Reichert-Jung, Vienna). Thin sections were then stained with uranyl acetate and lead citrate, and observed with a JEOL CX-100 electron microscope (JEOL USA, Inc., Peabody, MA).

Quantification of microtubule mass

To quantify the effects of the experimental treatments on the levels of MT mass in axons and tau-induced processes, we used our previously described method (Baas and Black, 1990; Baas et al., 1991a). Briefly, total lengths of MT profiles were measured in the electron micrographs for each time point, and standardized to unit area of axoplasm. The latter was determined by trimming all empty space from the electron micrographs and then weighing them. For the explant cultures, a minimum of 200 square microns of axoplasm for each time point was quantified. In the case of the tau-induced processes, total MT length and total area were scored for each of 10 processes examined for each time point. Then for each time point, a mean \pm standard deviation was calculated for the group. Calculation of a standard deviation was particularly important in the case of the tau-induced processes because it is likely that there is variability in the levels of tau expressed among different infected Sf9 cells (see Knops et al., 1991). In the case of the densely bundled explant axons, it was impossible to determine which axonal profiles on each negative derived from the same axon, and hence no standard deviation could be calculated.

RESULTS

Microtubule stability in axons

In previous work, we analyzed the drug stability of MTs in the axons of cultured rat sympathetic neurons (Baas and Black, 1990; Baas et al., 1991a, 1993). For these studies, we treated explant or dissociated cultures for times up to 6 hours with 2 μ g/ml nocodazole, and then used electron microscopy to quantify the levels of MT mass remaining relative to control axons. We found that roughly half the MT mass in the mainshaft region of these axons is drug-labile, depolymerizing with a halftime of \approx 3.5 minutes, while the other half is relatively drug-stable, depolymerizing with a halftime of \approx 130 minutes. (We have used the term 'stable' to indicate relatively slow depolymerization.) In the present study, we used the same approach to analyze the cold stability of MTs in these axons. Explant cultures were treated with cold and the levels of MT mass remaining were calculated as described in Materials and Methods. Because the axons of explant cultures are densely bundled (see Fig. 1A), it was impossible to visualize morphological changes such as beading or retraction that often accompany MT depolymerization in the axon (Horie et al., 1983; Joshi et al., 1986). In the mainshaft region of control axons (Fig. 1B) and axons treated for 7.5 minutes, 15 minutes, 30 minutes, 2 hours and 6 hours (Fig. 1C), the total length of MT polymer in microns per square micron of axoplasm was 3.82, 3.19, 3.55, 3.29, 3.97 and 3.66, respectively. Unfortunately, the use of explant cultures, while permitting the acquisition of a large sample number, does not permit the acquisition of meaningful standard deviations (see Materials and Methods). However, results obtained with this system in previous studies (Baas and Black, 1990; Baas and Ahmad, 1992) have produced consistent results, indicating that indi-

vidual variation among the samples is probably low. Based on the assumption of low sample variation, and the present results, we conclude that very little or no MT depolymerization occurs in the mainshaft region of these axons as a result of cold treatment, even at 6 hours.

These results suggest that all or nearly all of the MT polymer in the axon is cold-stable, including that portion which is drug-labile. To test this conclusion further, we compared the MT mass in control axons and axons treated in the cold for either 15 minutes or 6 hours specifically in their most distal regions, which contain exclusively drug-labile polymer (Baas and Black, 1990; Ahmad et al., 1993; Baas et al., 1993). In contrast to our previous studies in which the distal region was defined as \approx 10 μ m, here we focused on the few microns just proximal to the MT-deficient growth cone. This further maximized our chances of detecting the MT polymer that was most newly assembled, and hence most likely to be labile. In this region, control axon tips had relatively smooth borders (Fig. 1D), while their cold-treated counterparts showed varying degrees of beading that appeared to increase with increasing incubation time (Fig. 1F shows a 6 hour sample). The total length of polymer in microns per square micron of axoplasm was 1.93 and 1.48 for control and 6 hour cold-treated axons, respectively (Fig. 1E and G, respectively), indicating a reduction in MT mass of about 23%. The MT mass after 15 minutes in cold was 1.77 μ m MT/ μ m² cytoplasm, a reduction of \approx 8% relative to control levels, indicating that the majority of the polymer that depolymerized during the 6 hour treatment did so exceedingly slowly. These results indicate a slightly greater cold lability of newly assembled MT polymer but nevertheless, like the results on the mainshaft region of the axon described above, indicate that all or nearly all of the MT polymer in the axons of cultured rat sympathetic neurons depolymerizes either very slowly or not at all in response to cold.

Microtubule stability in tau-induced processes

We next examined the effects of cold and drug treatment on the tau-induced processes of Sf9 cells. Principal efforts focused on the 3-repeat form of tau, and all of the data and Figures presented here are from these studies. Consistent with our previous work (Knops et al., 1991; Baas et al., 1991b), results entirely similar to those obtained on the 3-repeat form were obtained in a limited number of studies on the 4-repeat form of tau. Unlike the axons of explant cultures, the tau-induced processes do not fasciculate, and hence it was possible to observe gross morphological changes during the experimental treatment. Many of the control processes showed beading along their length, but the number of beads exceeding twice the diameter of the process was generally fewer than 2-3 per process (Fig. 2A). Entirely similar results were obtained with the nocodazole-treated processes, even at 6 hours of drug treatment (Fig. 2B and C). In addition, no retraction of the processes was apparent at any time during treatment. In contrast to drug-treated processes, over half of the cold-treated processes retracted into the cell body, and virtually all of the processes that did not retract showed dramatic beading along their length within the first 5 to 7.5 minutes of cold treatment (Fig. 2D and E show two examples at 7.5 minutes). This beading was typically far more severe than any beading observed in the control axons, with broader diameter beads and higher numbers of beads along individual processes. In some

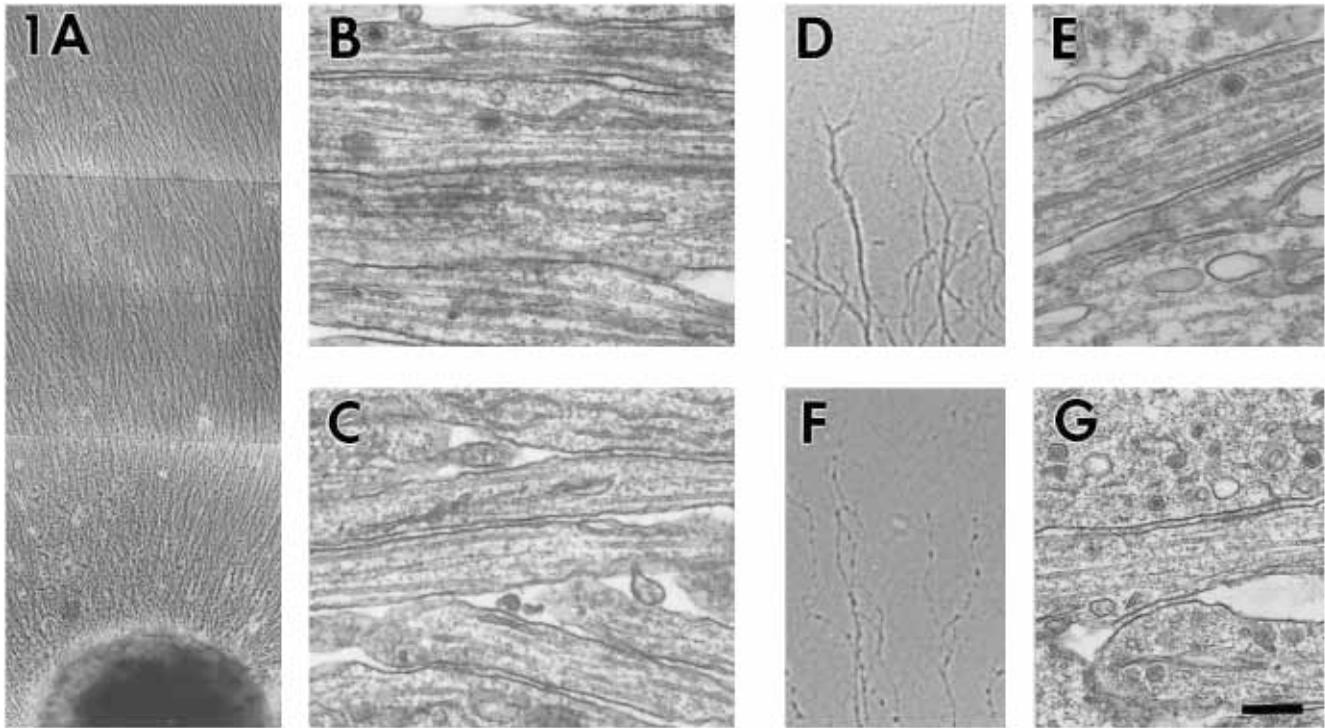


Fig. 1. Effects of cold treatment on MTs in the mainshaft and distal regions of axons. (A) A phase-contrast micrograph of a typical explant culture of rat sympathetic neurons. (B) An electron micrograph of a region of control axons, showing dense arrays of MTs. (C) An electron micrograph of a region of axons from a culture treated for 6 hours with cold. Quantitative analyses indicate that the levels of MT polymer in cold-treated axons are indistinguishable from control levels (see Results). (D) A phase-contrast micrograph from an explant culture of rat sympathetic neurons showing the most distal few microns of the axons (embedded for EM). (E) An electron micrograph of axons in this region, showing some variability in MT density, presumably relating to the precise distance from the MT-deficient growth cone. (F) A phase-contrast micrograph of the most distal few microns of the axons from a culture treated for 6 hours with cold (embedded for EM); substantial beading of the axons is apparent. (G) An electron micrograph of the distal regions of axons from a culture treated for 6 hours with cold. The MT mass has been reduced by about 23% (see Results). Bar: (A), 200 μm ; (D and F), 30 μm ; (B, C, E and G), 0.2 μm .

cases, the individual beads of cytoplasm became completely pinched off from one another. These morphological responses indicate that the MTs within the tau-induced processes may react very differently to cold versus nocodazole.

In control Sf9 processes, the MT mass was far greater and far denser than in the axon (Fig. 3A; see also Knops et al., 1991; Baas et al., 1991b). The average total length of MT polymer in microns per square micron of cytoplasm was 14.12 ± 6.16 , over 4 times that of the axon (although the potential for error was somewhat higher due to the tighter packing of these MTs). Because the lack of beading and process retraction in response to nocodazole suggested that MT depolymerization may be minimal, we first scored the MT mass in processes treated for the full 6 hours with nocodazole. We found it to be 13.48 ± 6.75 $\mu\text{m MT}/\mu\text{m}^2$ cytoplasm, statistically indistinguishable from control levels (Fig. 3D). As in the axon, the most distal region of the tau-induced processes is rich in the plus ends of MTs (Baas et al., 1991b), and these ends presumably correspond to the part of the MT that is most recently assembled and hence most likely to be labile. Nevertheless, examination of the distal tips of processes treated for 6 hours in drug (Fig. 3E and F) revealed no discernable diminution in MT mass relative to controls (Fig. 3B and C), although the MT mass often decreased in the distal regions relative to the proximal and middle portions of even the control processes (see Baas et al., 1991b). Results entirely similar to those

obtained at 6 hours of drug treatment were obtained for a small number of processes treated with drug for the shorter periods of time (data not shown). Collectively, these studies indicate that all or nearly all of the MT polymer in the tau-induced processes is drug-stable.

The rapid retraction and beading of the tau-induced processes in response to cold provided initial indication that a significant portion of the MT mass in the processes could be cold-labile. In addition, with increasing cold incubation, there was a progressive increase in the proportion of cytoplasm within the processes occupied by the viral gene product p10, an electron-opaque structure associated with the viral infection (Knops et al., 1991). In control processes, viral p10 inclusions occupied 0.15% of the cytoplasm. In processes treated with cold for 5, 7.5, 15 and 30 minutes, the proportion of cytoplasm occupied by the viral inclusions increased to 0.69%, 1.6%, 3.6% and 4.0%, respectively. At 6 hours, the proportion increased to 15.04% (see especially Fig. 4F). The reasons for these results on the viral inclusions are unknown, but may relate to a diminution in the steric hindrance of their movement from the cell body into the processes by a decrease in MT mass associated with the cold treatment.

Specifically with regard to the MTs, their levels decreased rapidly and dramatically in the presence of cold. At 5 (Fig. 4B), 7.5 (Fig. 4C), 15 (Fig. 4D) and 30 (not shown) minutes, there were 3.88 ± 3.80 , 0.50 ± 0.84 , 1.83 ± 2.17 , and 0.095 ± 0.17 μm

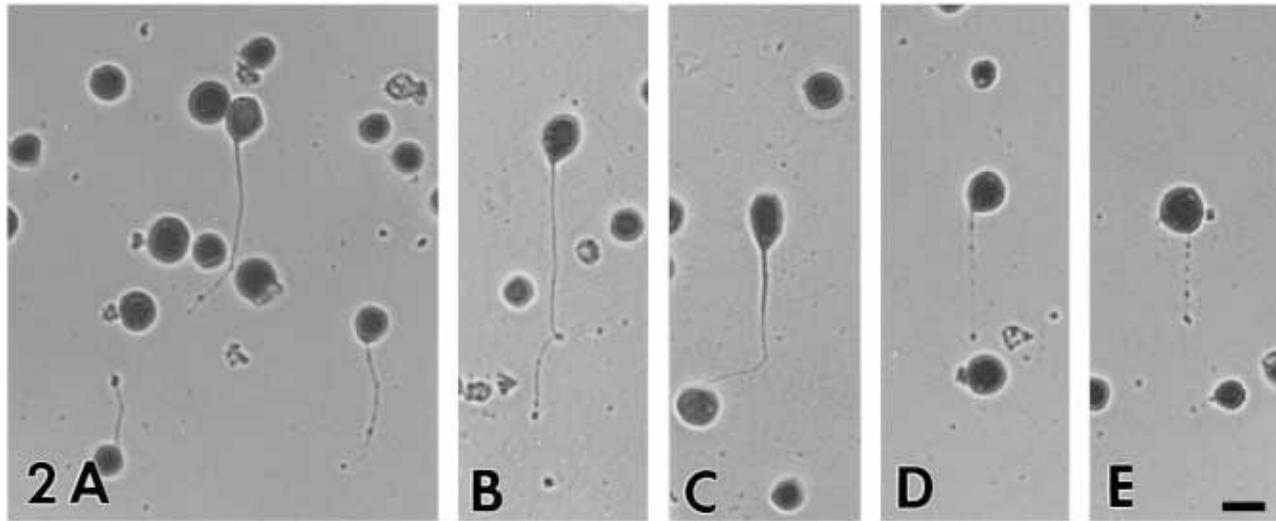


Fig. 2. Phase-contrast micrographs of Sf9 cells induced to express the three-repeat form of tau for two days (embedded for EM). As previously reported (Knops et al., 1991), the cells extend processes. (A) Control cells (not treated with cold or nocodazole). (B and C) Examples of cells treated with 2 $\mu\text{g}/\text{ml}$ nocodazole for 6 hours. (D and E) Examples of cells treated with cold for 7.5 minutes. The light microscopic morphology of the nocodazole-treated cells is indistinguishable from that of controls. In contrast, the processes of the cold-treated cells show substantial retraction and beading along their lengths. See Results for more details. Bar, 20 μm .

MT/ μm^2 cytoplasm, respectively, indicating approximate decreases of 73%, 96%, 87% and 99%, respectively, relative to control levels (another example of which is shown in Fig. 4A). By 6 hours of cold treatment, no MT polymer was detected (Fig. 4E and F). These observations indicate the absence of any detectable cold-stable polymer in these processes. Together with the drug studies, these results indicate that all or nearly all of the MT polymer in the tau-induced processes is simultaneously drug-stable and cold-labile.

DISCUSSION

Several lines of evidence suggest that tau can affect the stability properties of MTs both in the test tube as well as in living cells. The addition of tau to MTs assembled from purified tubulin results in a decrease in the frequency of catastrophic disassembly events and an increase in the frequency of rescue from such events (Bre and Karsenti, 1990; Pryer et al., 1992; Drechsel et al., 1992). In addition, the microinjection of tau into fibroblasts results in an increase in the levels of MTs that are stable to drug-induced depolymerization (Drubin and Kirschner, 1986). Consistent with this finding, Lee and Rook (1992) demonstrated that the MTs in CHO and 3T3 cells depolymerize more slowly in the presence of nocodazole when these cells are induced to express tau. In addition, they showed that the MTs accumulate higher levels of acetylated tubulin subunits, another indication of reduced rates of subunit turnover. Similar results were obtained in studies by Takemura et al. (1992) on COS cells induced to express tau. Our quantitative studies on the effects of tau expression in Sf9 cells are consistent with these previous results, but are even more dramatic. After 6 hours in nocodazole, we observed no change in the levels of MTs, indicating that all, or nearly all, of the MT polymer had become very drug-stable. The reasons for these more dramatic results are unknown, but may relate to different levels of tau expressed in each study, and/or the inter-

actions of other factors such as competing endogenous MAPs already present in each cell type that might alter the effects of tau.

Nocodazole binds free tubulin subunits, thereby subtracting them from the dynamics that regulate the monomer/polymer equilibrium. As a result, MTs disassemble at rates presumably reflective of their normal rates of subunit turnover. The fact that we were unable to detect MT disassembly in the tau-induced processes even after 6 hours in nocodazole suggests that the MTs in these processes either do not exchange subunits with a soluble tubulin pool or that they do so exceedingly slowly. In contrast to this 'hyperstability', the MTs in the axons of cultured sympathetic neurons consist of two domains one of which is labile, depolymerizing with a half-time of ≈ 3.5 minutes in the presence of nocodazole, and the other of which is relatively stable, depolymerizing with a half-time of ≈ 130 minutes (see Introduction). It is important to note that while these two types of axonal MT polymer differ in their sensitivity to nocodazole by ≈ 35 -fold, the stable polymer clearly depolymerizes and is reduced by greater than 90% during a 6 hour drug treatment. Thus the MT polymer in the tau-induced processes is far more stable to nocodazole than is the stable MT polymer in the axon.

How might tau assist in the generation of two types of MT polymer in the axon that differ so significantly in their stability? In considering this issue, it is first important to note that tau is present throughout the length of the axon, even in its most distal region (Robson and Burgoyne, 1988; our unpublished observations). Because this latter region contains exclusively labile polymer (Baas and Black, 1990; Ahmad et al., 1993), and because tau has a strong affinity to bind MTs, it seems fair to conclude that tau is present along the labile domain of the MT as well as the stable domain. In support of this conclusion, immunoelectron microscopic studies have thus far failed to reveal distinct classes of MT polymer in the axon that differ with regard to tau staining (Papasozomenos and Binder, 1987; our unpublished observations). However, the

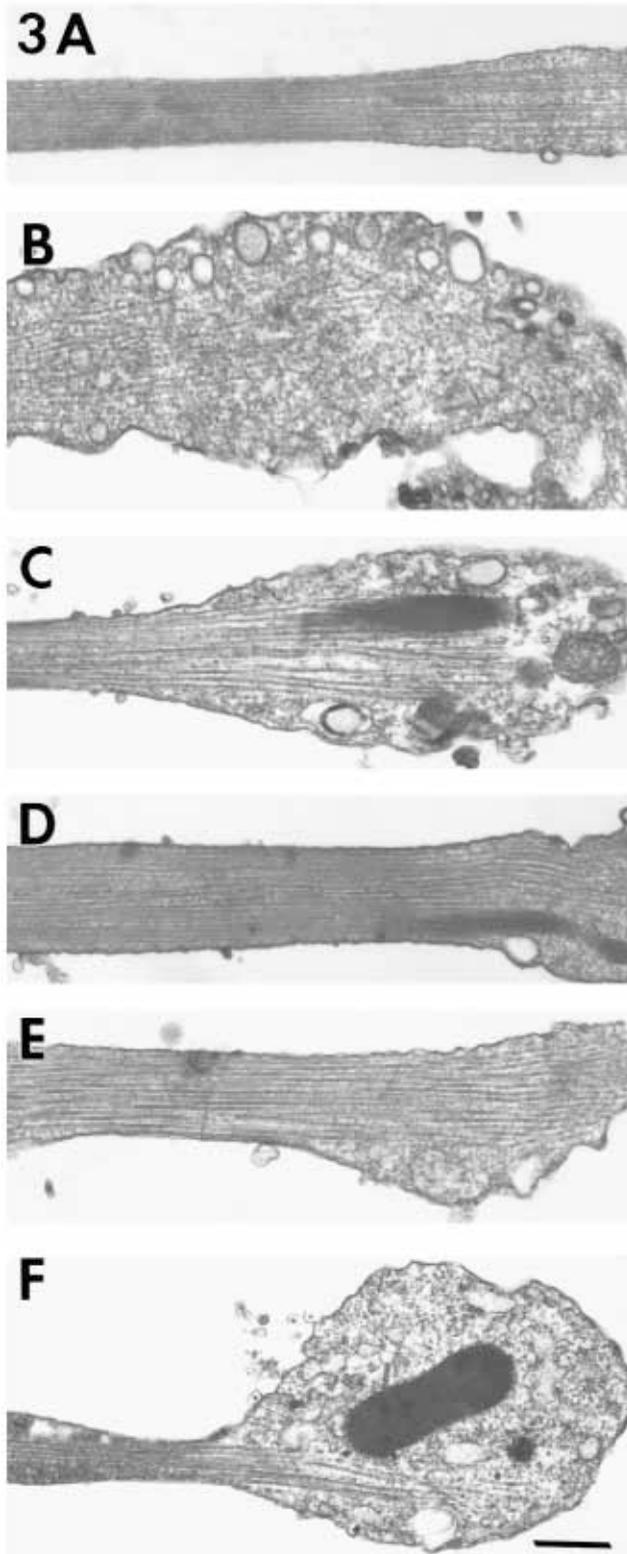


Fig. 3. Electron micrographs showing the effects of nocodazole on the MTs of tau-induced processes from Sf9 cells. (A-C) Regions of processes from undrugged cells, (D-F) regions of processes from cells treated for 6 hours with nocodazole. Panels (A) and (D) are from the middle regions of the processes, while the remaining panels are from the distal tips of the processes. MT levels are indistinguishable in similar regions of control and drugged processes. Bar, 0.5 μ m.

possibility remains that these studies were not sufficiently quantitative to reveal differences in the levels of tau along the two domains, or that existing tau becomes redistributed during preparation for immunoelectron microscopy. The simplest possibility suggested by the data is that the absolute levels of tau may not be very different between the two domains. If this is true, tau-stabilization of MTs may be dependent upon very subtle alterations in the levels of tau, or upon modifications of tau such as phosphorylation. Finally, as noted in the Introduction, axons contain a myriad of other MAPs, which may also affect MT stability (see, for example, work on MAP-1b by Takemura et al., 1992). Collectively, these considerations and the present data argue that tau plays a principal role in determining the drug stability and normal rates of subunit turnover for axonal MTs, but that the mechanisms by which tau accomplishes this and the factors with which it interacts are complex.

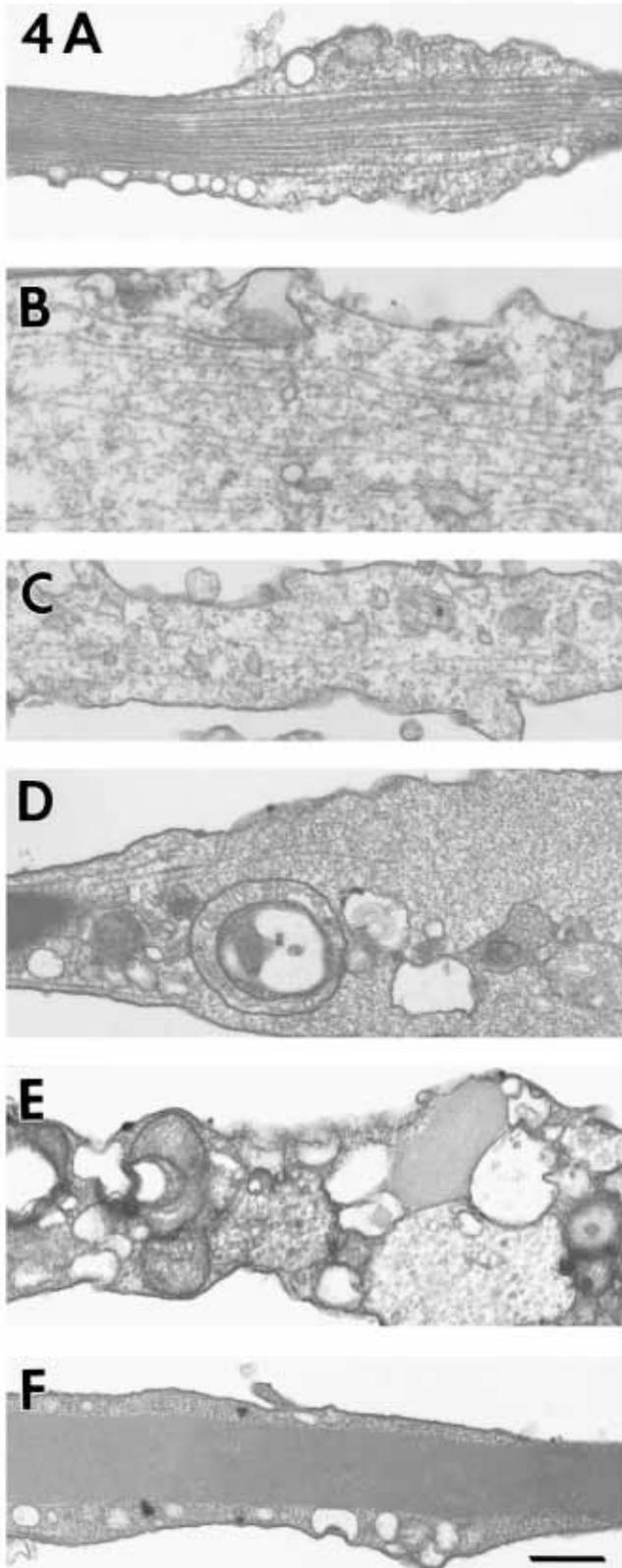
tau and cold stability of microtubules

We were initially surprised by our inability to detect cold-labile MT polymer in the axon, in that numerous biochemical studies indicate the existence of cold-labile as well as cold-stable polymer in axonal extracts (Webb and Wilson, 1980; Brady et al., 1984; Watson et al., 1990; see also, for review, Dustin, 1984). In addition, morphological studies on a variety of animals indicate substantial MT depolymerization in pieces of dissected nerve incubated in the cold (Rodriguez-Echandia and Piezzi, 1968; Banks et al., 1975; Brimijoin et al., 1979; Donoso, 1986; Heidemann et al., 1984; Sahenk and Brady, 1987). However, just as the isolation of cellular extracts may result in the loss or alteration of factors that confer cold stability upon the MTs, the same may be true in the case of nerve dissection. In support of this interpretation, Pannese et al. (1982) observed no difference in MT levels in nerves from whole lizards first incubated at 3°C or 30°C for 90-95 days, and then dissected from the animals. In addition, our results showing little or no cold-labile MT polymer in the axon are consistent with those of a study on the cold stability of MTs in rat brain (Jones et al., 1980). In this study, thick slices of brain were incubated for 1 hour at 0°C, and no MT depolymerization was detected. In contrast, when the brain slices were homogenized in buffer either at 0°C or 37°C, substantially less polymer was detected in the cold homogenate compared to the warm. The authors concluded that virtually all of the MT polymer in the brain is cold-stable, but that the factors that confer cold stability to these MTs are easily lost during preparative procedures.

Evidence for the co-existence of cold-labile and cold-stable MT polymer in the axon has been obtained in a small number of studies on intact neurons, but most of these studies were performed on neurons that were immature and still developing. We detected cold-labile as well as cold-stable MT polymer in the axons of chick sensory neurons grown in culture for less than 24 hours (Baas and Heidemann, 1986; Baas et al., 1987), and in the neurites of developing PC12 cells (Joshi et al., 1986). In addition, Cohen et al. (1987) detected a cold-labile fraction among the MTs in the developing axons of mouse tectal plate. These data support a scenario holding that the MT cold-stabilizing factors, easily lost during invasive preparative procedures, steadily increase during neuronal development and axon growth, until most or all of the MT polymer in the axon becomes cold-stable. However, in one study on intact adult

nerves, Alvarez and Fadic (1992) incubated eviscerated toads at 0°C and observed substantial MT depolymerization in their lumbosacral nerves. Thus, the levels of cold-stable and cold-labile polymer may vary among animal species and among different types of neurons, as well as developmentally.

The fact that the populations of cold-stable and drug-stable



polymer in the axon are only partially overlapping suggests that cold stability and drug stability result from different mechanisms. Our data on the tau-induced processes from Sf9 cells support this conclusion. After only 15 minutes in cold, no detectable MT polymer remains in these processes. Thus, although all or nearly all of the MT polymer in the tau-induced processes is hyperstable to nocodazole (see above), little or none is stable to cold. These results are compelling in that considerably higher levels of tau are present in the tau-induced processes compared to the axon, and yet the MTs in the axon are far more cold-stable than those in the tau-induced processes. The simplest interpretation of these results is that tau is not the factor that confers cold stability to axonal MTs. However, at present we cannot dismiss the possibility that tau contributes to cold stability in a way that we do not yet understand. For example, in order to cold-stabilize MTs, tau may have to undergo certain post-translational modifications or splicing events. Of note, however, are the findings that the tau expressed in Sf9 cells does become highly phosphorylated (Cheley et al., 1992), and that neither the three- nor the four-repeat form of tau appears to be able to confer cold stability to MTs.

If tau is not among the factors that cold-stabilize axonal MTs, then what are the cold-stabilizing factors? Consideration of available data suggests that other conventional neuronal MAPs are also unlikely candidates. MAP-2 is somatodendritic-specific, and thus could not stabilize axonal MTs. MAP1b levels decrease during development, and this contrasts with an apparent increase in MT cold stability during development (see above). MAP1a increases during development (Matus, 1991), and may be a possibility. Interestingly though, none of the conventional MAPs including those similar to MAP1a appear to be overexpressed in the neurons of antarctic fishes, in which the MTs are extremely cold-stable, even in the test tube (Detrich et al., 1990). Collectively, these observations strongly suggest that some other unknown or less studied factors confer cold stability to axonal MTs. One such possibility is STOP, a protein isolated from neuronal tissue, which avidly stabilizes *in vitro* MT preparations to cold (Margolis et al., 1986; Pirolet et al., 1992). In addition, there is some evidence for a dynein-associated factor that can confer cold stability to MTs in the test tube (Eyer et al., 1990). Finally, in some axons, as yet unidentified post-translational modifications appear to alter the mobility of the α -tubulin in cold-stable MTs on SDS gels, and these modifications may be a direct cause of MT stability (Brady et al., 1984). We do not favor the idea that different tubulin isotypes comprise cold-stable and cold-labile MTs because available evidence indicates that stable MTs are derived from the *stabilization* of labile MTs both in the test tube and in living cells (see Introduction). Far more work will

Fig. 4. Electron micrographs showing the effects of cold on the MTs of tau-induced processes from Sf9 cells. (A) A control process (not treated with cold), (B and D) processes from cells treated with cold for 5, 7.5 and 15 minutes, respectively. A rapid and dramatic loss of MT mass with time in cold is apparent. At times greater than 30 minutes, no remaining MT polymer could be detected. (E and F) Processes treated for 6 hours with cold; no MTs remain. With increasing cold treatment, an increasing proportion of the cytoplasm was occupied by viral p10 inclusions, a dramatic illustration of which can be seen in (F). Bar, 0.5 μ m.

be required to elucidate the mechanisms by which MTs are cold-stabilized in the axon.

In conclusion, we have sought to better characterize the stability properties of axonal MTs, and to explore whether tau could contribute to the drug stability and/or cold stability of these MTs. Our results indicate that tau has a potent capacity to stabilize MTs against drug-induced depolymerization in living cells, and thus most probably contributes to the drug stability (and normal rates of subunit turnover) of axonal MTs. However, we were unable to find evidence consistent with the idea that tau can stabilize MTs against cold-induced depolymerization. We conclude that tau is an important stabilizer of MTs in the axon, but that other factors are also required to fully define the stability properties of axonal MTs and, in particular, their stability to cold.

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REFERENCES

- Ahmad, F. J., Pienkowski, T. P. and Baas, P. W. (1993). Regional differences in microtubule dynamics in the axon. *J. Neurosci.* **13**, 856-866.
- Alvarez, J. and Fadic, R. (1992). Assembly and disassembly of axonal microtubules of the toad *Xenopus laevis* under the effect of temperature. *J. Exp. Zool.* **264**, 261-266.
- Baas, P. W. and Heidemann, S. R. (1986). Microtubule reassembly from nucleating fragments during the regrowth of amputated neurites. *J. Cell Biol.* **103**, 917-927.
- Baas, P. W., Sinclair, G. I. and Heidemann, S. R. (1987). Role of microtubules in the cytoplasmic compartmentation of neurons. *Brain Res.* **420**, 73-81.
- 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.
- Baas, P. W., Slaughter, T., Brown, A. and Black, M. M. (1991a). Microtubule dynamics in axons and dendrites. *J. Neurosci. Res.* **30**, 134-153.
- Baas, P. W., Pienkowski, T. P. and Kosik, K. S. (1991b). Processes induced by tau expression in Sf9 cells have an axon-like microtubule organization. *J. Cell Biol.* **115**, 1333-1344.
- Baas, P. W. and Ahmad, F. J. (1992). The plus ends of stable microtubules are the exclusive nucleating structures for microtubules in the axon. *J. Cell Biol.* **116**, 1231-1241.
- Baas, P. W., Ahmad, F. J., Pienkowski, T. P., Brown, A. and Black, M. M. (1993). Sites of microtubule stabilization for the axon. *J. Neurosci.* **13**, 2177-2185.
- Banks, P., Mayor, D. and Owen, T. (1975). Effects of low temperatures on microtubules in the non-myelinated axons of post-ganglionic sympathetic nerves. *Brain Res.* **83**, 277-292.
- Binder, L. I., Frankfurter, A. and Rebhun, L. I. (1985). The distribution of tau in the mammalian nervous system. *J. Cell Biol.* **101**, 1371-1378.
- Binet, S., Cohen, E. and Meininger, V. (1987). Heterogeneity of cold-stable and cold-labile tubulin in axon- and soma-enriched portions of the adult mouse brain. *Neurosci. Lett.* **77**, 166-170.
- Brady, S. T., Tytell, M. and Lasek, R. J. (1984). Axonal transport and axonal tubulin: biochemical evidence for cold stability. *J. Cell Biol.* **99**, 1716-1724.
- Brady, S. T. (1991). Molecular motors in the nervous system. *Neuron* **7**, 521-533.
- Bre, M. H. and Karsenti, E. (1990). Effects of brain microtubule-associated proteins on microtubule dynamics and the nucleating activity of centrosomes. *Cell Motil. Cytoskel.* **15**, 88-98.
- Brimijoin, S., Olsen, J. and Rosenson, R. (1979). Comparison of the temperature-dependence of rapid axonal transport and microtubules in nerves of the rabbit and bullfrog. *J. Physiol.* **287**, 303-314.
- Caceres, A. and Kosik, K. S. (1990). Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* **343**, 461-463.
- Chapin, S. J. and Bulinski, J. C. (1992). Microtubule stabilization by assembly-promoting microtubule-associated proteins: a repeat performance. *Cell Motil. Cytoskel.* **23**, 236-243.
- Cheley, S., Kosik, K. S., Paskevich, P., Bakalis, S. and Bayley, H. (1992). Phosphorylated baculovirus p10 is a heat-stable microtubule-associated protein associated with process formation in Sf9 cells. *J. Cell Sci.* **102**, 739-752.
- Cohen, E., Binet, S. and Meininger, V. (1987). In situ appearance of the cold-stable microtubules in the growing axons of tectal plate of mouse investigated immunocytochemically after polyethyleneglycol (PEG) embedding. *Dev. Brain Res.* **36**, 171-180.
- Detrich, H. W. III, Neighbors, B. W., Slaboda, R. D. and Williams, R. C. Jr (1990). Microtubule-associated proteins from antarctic fishes. *Cell Motil. Cytoskel.* **17**, 174-186.
- Diaz-Nido, J., Hernandez, M. A. and Avila, J. (1990). Microtubule proteins in neuronal cells. In *Microtubule Proteins* (ed. J. Avila), pp. 193-257. CRC Press, Inc., Boca Raton.
- Donoso, J. A. (1986). Microtubule stability along mammalian peripheral nerves. *J. Neurobiol.* **17**, 383-403.
- Drechsel, D. N., Hyman, A. A., Cobb, M. H. and Kirschner, M. W. (1992). Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *Mol. Biol. Cell* **3**, 1141-1154.
- Drubin, D. G. and Kirschner, M. W. (1986). Tau protein function in living cells. *J. Cell Biol.* **103**, 2739-2746.
- Dustin, P. (1984). *Microtubules*, second edition. Springer-Verlag, New York.
- Edson, K. J., Lim, S.-S., Borisy, G. G. and Letourneau, P. C. (1993). A FRAP analysis of the stability of the microtubule population along the neurites of chick sensory neurons. *Cell Motil. Cytoskel.* **25**, 59-72.
- Eyer, J., White, G. and Gagnon, C. (1990). Presence of a new microtubule cold-stabilizing factor in bull sperm dynein preparations. *Biochem. J.* **270**, 821-824.
- Goedert, M., Crowther, R. A. and Garner, C. C. (1991). Molecular characterization of microtubule-associated proteins tau and MAP-2. *Trends Neurosci.* **14**, 193-199.
- Heidemann, S. R., Hamborg, M. A., Thomas, S. J., Song, B., Lindley, S. and Chu, D. (1984). Spatial organization of axonal microtubules. *J. Cell Biol.* **99**, 1289-1295.
- Hoebeke, J., VanNijen, G., and DeBrabander, M. (1976). Interaction of nocodazole (R17934), a new anti-tumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.* **69**, 319-324.
- Horie, H., Takenaka, T. and Kaiho, M. (1983). Effects of disruption of microtubules on translocation of particles and morphology in tissue culture neurites. *Brain Res.* **288**, 85-93.
- Jones, D. H., Gray, E. G. and Barron, J. (1980). Cold stable microtubules in brain studied in fractions and slices. *J. Neurocytol.* **9**, 493-504.
- Joshi, H. C., Baas, P., Chu, D. T. and Heidemann, S. R. (1986). The cytoskeleton of neurites after microtubule depolymerization. *Exp. Cell Res.* **163**, 233-244.
- Joshi, H. C. and Baas, P. W. (1993). A new perspective on microtubules and axon growth. *J. Cell Biol.* **121**, 1191-1196.
- Khawaja, S., Gunderson, G. G. and Bulinski, J. C. (1988). Enhanced stability of microtubules enriched in detyrosinated tubulin is not a direct function of detyrosination level. *J. Cell Biol.* **106**, 141-150.
- Knops, J., Kosik, K. S., Lee, G., Pardee, J. D., Cohen-Gould, L. and McConlogue, L. (1991). Overexpression of tau in non-neuronal cells induces long cellular processes. *J. Cell Biol.* **114**, 725-734.
- Kowall, N. W. and Kosik, K. S. (1987). Axonal disruption and aberrant localization of tau protein characterize the neuropil pathology of Alzheimer's disease. *Ann. Neurol.* **22**, 639-643.
- Lee, G. (1990). Tau protein: an update on structure and function. *Cell Motil. Cytoskel.* **15**, 199-203.
- 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.
- Lee, J. C., Field, D. J. and Lee, L. L. Y. (1980). Effects of nocodazole on structures of calf brain tubulin. *Biochemistry* **19**, 6209-6215.
- Lim, S.-S., Edson, K. J., Letourneau, P. C. and Borisy, G. G. (1990). A test of microtubule translocation during neurite elongation. *J. Cell Biol.* **111**, 123-130.

- Margolis, R. L., Rauch, C. T. and Job, D.** (1986). Purification and assay of a 145-kDa protein (STOP₁₄₅) with microtubule-stabilizing and motility behavior. *Proc. Nat. Acad. Sci. USA* **466**, 639-643.
- Matus, A.** (1988). Microtubule-associated proteins: their potential role in determining neuronal morphology. *Annu. Rev. Neurosci.* **11**, 29-44.
- Matus, A.** (1991). Microtubule-associated proteins and neuronal morphogenesis. *J. Cell Sci. Suppl.* **15**, 61-67.
- Meininger, V. and Binet, S.** (1989). Characteristics of microtubules at the different stages of neuronal differentiation and maturation. *Int. Rev. Cytol.* **114**, 21-79.
- Mitchison, T. and Kirschner, M.** (1988). Cytoskeletal dynamics and nerve growth. *Neuron* **1**, 761-772.
- Moya, F., Bunge, M. B. and Bunge, R. P.** (1980). Schwann cells proliferate but fail to differentiate in defined medium. *Proc. Nat. Acad. Sci. USA* **77**, 6902-6906.
- Okabe, S. and Hirokawa, N.** (1990). Turnover of fluorescently labelled tubulin and actin in the axon. *Nature* **343**, 479-482.
- Pannese, E., Arcidiacono, G., Rigamonti, L., Procacci, P. and Ledda, M.** (1982). Stability at low temperatures of neuronal microtubules in spinal ganglia and dorsal roots of the Lizard (*Lacerta muralis*). *J. Ultrastruct. Res.* **79**, 18-30.
- Papasozomenos, S. C. and Binder, L. I.** (1987). Phosphorylation determines two distinct species of tau in the central nervous system. *Cell Motil. Cytoskel.* **8**, 210-226.
- Peng, I., Binder, L. I. and Black, M. M.** (1986). Biochemical and immunological analyses of cytoskeletal domains of neurons. *J. Cell Biol.* **102**, 252-262.
- Piperno, G., LeDizat, M. and Chang, X.** (1987). Microtubules containing acetylated α -tubulin in mammalian cells in culture. *J. Cell Biol.* **104**, 289-302.
- Pirollet, F., Margolis, R. L. and Job, D.** (1992). Ca²⁺-calmodulin regulated effectors of microtubule stability in neuronal tissues. *Biochem. Biophys. Acta* **1160**, 113-119.
- Pryer, N. K., Walker, R. A., Skeen, V. P., Bourns, B. D., Soboeiro, M. F. and Salmon, E. D.** (1992). Brain microtubule-associated proteins modulate microtubule dynamic instability in vitro. Real-time observations using video microscopy. *J. Cell Sci.* **103**, 965-975.
- Robson, S. J. and Burgoyne, R. D.** (1988). Differential levels of tyrosinated, detyrosinated and acetylated alpha-tubulins in neurites and growth cones of dorsal-root ganglion neurons. *Cell Motil. Cytoskel.* **12**, 273-282.
- Rodriguez-Echandia, E. L. and Piezzi, R. S.** (1968). Microtubules in the nerve fibers of the toad *Bufo Arenarum* Hensel. *J. Cell Biol.* **39**, 491-497.
- Reinsch, S. S., Mitchison, T. J. and Kirschner, M.** (1991). Microtubule polymer assembly and transport during axonal elongation. *J. Cell Biol.* **115**, 365-379.
- Sahenk, Z. and Brady, S. T.** (1987). Axonal tubulin and microtubules: morphologic evidence for stable regions on axonal microtubules. *Cell Motil. Cytoskel.* **8**, 155-164.
- Smith, G. E. and Summers, M. D.** (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin no. 1555*.
- 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.
- Watson, D. F., Hoffman, P. N. and Griffin, J. W.** (1990). The cold stability of microtubules increases during axonal maturation. *J. Neurosci.* **10**, 3344-3352.
- Webb, B. C. and Wilson, L.** (1980). Cold-stable microtubules from brain. *Biochemistry* **19**, 1993-2001.
- Webster, D. R., Wheland, J., Weber, K. and Borisy G. G.** (1990). Detyrosination of alpha tubulin does not stabilize microtubules in vivo. *J. Cell Biol.* **111**, 113-122.
- White, L. A., Baas, P. W. and Heidemann, S. R.** (1987). Microtubule stability in severed axons. *J. Neurocytol.* **16**, 775-784.

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