

Tubulin Transport in Neurons

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Abstract. A question of broad importance in cellular neurobiology has been, how is microtubule cytoskeleton of the axon organized? It is of particular interest because of the history of conflicting results concerning the form in which tubulin is transported in the axon. While many studies indicate a stationary nature of axonal microtubules, a recent series of experiments reports that microtubules are recruited into axons of neurons grown in the presence of a microtubule-inhibitor, vinblastine (Baas, P.W., and F.J. Ahmad. 1993. *J. Cell Biol.* 120:1427–1437; Ahmad F.J., and P.W. Baas. 1995. *J. Cell Sci.* 108:2761–2769; Sharp, D.J., W. Yu, and P.W. Baas. 1995. *J. Cell Biol.* 130:93–103; Yu, W., and P.W. Baas. 1995. *J. Neurosci.* 15:6827–6833.). Since vinblastine stabilizes bulk microtubule-dynamics in vitro, it

was concluded that preformed microtubules moved into newly grown axons. By visualizing the polymerization of injected fluorescent tubulin, we show that substantial microtubule polymerization occurs in neurons grown at reported vinblastine concentrations. Vinblastine inhibits, in a concentration-dependent manner, both neurite outgrowth and microtubule assembly. More importantly, the neuron growth conditions of low vinblastine concentration allowed us to visualize the footprints of the tubulin wave as it polymerized and depolymerized during its slow axonal transport. In contrast, depolymerization resistant fluorescent microtubules did not move when injected in neurons. We show that tubulin subunits, not microtubules, are the primary form of tubulin transport in neurons.

BECAUSE microtubules serve as tracks for fast transport (400 mm/d) of many cellular components into and down the axon, an axonal microtubule array must be elaborated and maintained. To determine the mechanisms by which cytoskeletal components move from their place of synthesis in the cell body to their place of use in the axon, which lacks protein synthetic machinery, Hoffman and Lasek (1975) injected radiolabeled amino acids into neuronal cell bodies in the spinal cord and then followed the progression of the newly synthesized radiolabeled tubulin down the cat sciatic nerve. They observed tubulin movement down the axon as a discrete, but slow ($\sim 1\text{--}4$ mm/d), wave and concluded that polymers may assemble in the cell body and move slowly into and down the axon.

Hoffman and Lasek's conclusion was first challenged by Bamburg et al. (1986). They locally applied microtubule-disrupting drugs to the growing tip of the axon or to the cell body and then monitored neurite outgrowth. They found axonal elongation stopped when drug (<0.1 $\mu\text{g/ml}$) was applied to the growing tip, but axonal elongation con-

tinued when the same concentration of the drug was applied to the cell body. They concluded that microtubule polymerization at the growth cone was required for neurite outgrowth. These results have since been confirmed and greatly enlarged upon in several studies (Brown et al., 1992; Li and Black, 1996; Okabe and Hirokawa, 1988).

Nonetheless, the hypothesis of polymer transport gained support from subsequent experiments where neuronal microtubules were rendered fluorescent by growing them with injected fluorescent tubulin, and then creating a discrete marked zone in the axon by photobleaching. Although the initial study of this type reported the photobleached zone moved down the neurite at the rate of slow axonal transport (Keith, 1987), later experiments using an identical technique showed that the photobleached zone remained stationary (Lim et al., 1990; Okabe and Hirokawa, 1990). The reasons for these differing results are not clear. It is possible that fluorescent microtubules and their associated movement mechanisms may be sensitive to radiation energy used for photobleaching (Vigers et al., 1988). To address this issue, Reinch et al. (1991) used the technique of photoactivation. Photoactivation involves shining a brief pulse of laser light onto tubulins conjugated with a caged fluorophore (Mitchison, 1989). After illumination, the caged fluorophore becomes fluorescent. This technique is less damaging than photobleaching because it requires much lower radiation energy to photoactivate caged tubulin than it takes to photobleach fluorescent tu-

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1. *Abbreviation used in this paper:* EGS, ethylene glycol-bis-succinimidylsuccinate.

bulin. To monitor the movement of a photoactivated zone in *Xenopus* axons, embryos were injected with caged fluorescent tubulin and the embryos were allowed to develop a neural tube. The caged tubulin in the neurites of neurons from the neural tube was then photoactivated, and the position of the photoactivated region was monitored over time. Based upon these experiments, Reinch et al. (1991) reported that the photoactivated mark moved down the axons of *Xenopus* neurons. Subsequently, Okabe and Hirokawa (1992, 1993) reported that while both photobleaching and photoactivation procedures can show movement in *Xenopus* axons, these techniques reveal an absence of tubulin transport in mammalian axons. However, the movement of photobleached/photoactivated zone in the *Xenopus* axons were believed not to be due to slow axonal transport (Okabe and Hirokawa, 1992; Sabre et al., 1995), but rather as a consequence of passive pulling of the axon by the growth cone (Okabe and Hirokawa, 1992; Takeda et al., 1995; Popov et al., 1993). The stationary nature of the axonal microtubules has also been recently reported in vivo in grasshopper and zebra fish neurons (Takeda et al., 1995; Sabry et al., 1995).

An alternative approach to the study of microtubule transport has recently been taken by Baas and Ahmad (1993), Ahmad and Baas (1995), Sharp et al. (1995), and Yu and Baas (1995). They report that the relative mass of microtubules in the cell body is less than in the neurites when neurons are grown in nanomolar concentrations of vinblastine. Because low concentrations of vinblastine have been shown to inhibit both polymerization and depolymerization of microtubules in vitro (Toso et al., 1993), Baas and Ahmad (1993) concluded that microtubules that were pre-formed within the cell body must be transported into the newly grown axon.

One critical limitation of these studies is that microtubule polymerization rates were not measured under the low drug concentrations used. Thus, the conclusions of Baas and Ahmad (1993), Ahmad and Baas (1995), Sharp et al. (1995), and Yu and Baas (1995) were based upon the assumption that vinblastine stopped microtubule polymerization and depolymerization in cultured neurons. To draw firm conclusions from those observations, it is important to determine quantitatively the effects of vinblastine on microtubule dynamics in neurons. In this paper, we show substantial polymerization of microtubules throughout neurons in the presence of vinblastine concentrations used in previously published reports (Baas and Ahmad, 1993; Ahmad and Baas, 1995; Sharp et al., 1995; Yu and Baas, 1995). A quantitative analysis of microtubule polymerization reveals that injected tubulin moves from the cell body into the axon as a discrete wave of tubulin subunits, and fluorescent microtubules stabilized against depolymerization remain stationary within the neuronal cell bodies.

Materials and Methods

Reagents

Unless otherwise specified, all reagents were purchased from Sigma Chem. Co. (St. Louis, MO). All solutions for cell culture were sterilized by passage through a 0.22- μ m filter (Millipore Corp., Bedford, MA).

Cell Culture

Superior cervical ganglia neurons from postnatal day one rats were harvested as described by Higgins et al. (1991), incubated in 2.5 μ g/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in PBS for 1 h, and then 2.5 μ g/ml trypsin (Worthington Biochemical Corp.) in PBS for 45 min. After enzymatic digestion, the ganglia were triturated once and plated at 10 cells/mm² onto photoetched glass coverslips treated as described below, and grown in a humidified incubator set at 37°C in L-15 (Leibovitz) supplemented with glucose 0.6%, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (L-15 GGPS) plus 4% FCS (KC Biological Inc., Lenexa, KS), 2% horse serum (Gibco, Grand Island, NY), and nerve growth factor at 100 ng/ml (L-15 GGPSHFN). Photoetched, α -numerically labeled coverslips from Bellco (Vineland, NJ) were first cleaned as described by Goslin and Banker (1991), and then coated with 120 μ l of fresh solution of 1 mg/ml poly-L-lysine in borate buffer (0.1 M pH 8.5) for 5 min at room temperature, washed once with distilled H₂O, and coated with 120 μ l of 100 ng/ml laminin in L-15 GGPS for 3 h at 37°C in a humidified atmosphere. The L-15 GGPS containing laminin was then replaced with L-15 GGPSHFN for 15 min, and this was removed immediately before plating.

Microinjection

Microinjecting pipettes (borosilicate glass 1.0 mm outside diameter, D., 0.75 mm internal diameter with filling fiber from Medical Systems Corp., Greenvale, NY) were pulled on a Brown and Flaming horizontal pipette puller (model P-87), and then back-loaded with the injection solutions. Microinjections were conducted using a Kinetic Systems Inc. Vibraplane table (Roslindale, MA), visualized with a Zeiss 35M Axiovert microscope (West Germany) using phase optics with a 32 \times objective (Achromat 44 01 49), and pressure regulation was provided by Narishige USA Inc. (Greenvale, NY) nitrogen pressure system. To begin injections, the pressure going to the pipette was set at 1 pound/inch², and the pipette was lowered into the injection bath, which contained the normal growth medium for superior cervical ganglion neurons, L-15 GGPSHFN. For the microinjection of ethylene glycol-*bis*-succinimidylsuccinate (EGS) stabilized microtubules (see below), the microtubules were first sheared to an average length of 1–10 μ m by up and down pipetting of the microtubule solution with an Eppendorf p200 pipette tip. Average microtubule length was determined by direct visualization of the microtubules using fluorescence microscopy. After the microtubules had been sheared, they were taken through serial dilutions (1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:500) until a concentration of microtubules was found that was low enough to avoid clogging of injection pipettes, but high enough to allow microtubules to flow out of the end of the injection pipette. Neurons were then injected with the microtubule solution without the aid of fluorescence, and the location of injected cells was recorded with the aid of photoetched α -numeric grids on the coverslips.

Preparation and Characterization of Labeled Tubulin

Phosphocellulose tubulin was purified (Vallee, 1986; Sloboda and Rosenbaum, 1982) and labeled with tetramethylrhodamine succinimidyl ester (Molecular Probes Inc.), and stored at -80°C as described by Hyman and Mitchison, (1991). Before injection, tubulin was thawed, stored at 4°C, and back-loaded into pipettes prechilled to 4°C. For the preparation of the stable microtubules, aliquots of rhodamine-tubulin in PEM (Pipes 80 mM, EGTA 1 mM, MgCl₂ 1 mM) were thawed, GTP was added to bring the final concentration to 1 mM, and the microtubules were polymerized at 37°C for 30 min. Next, warm (37°C) 15 mM EGS in dry DMSO was gently added to bring the final concentration of EGS to 1.5 mM. The solution was incubated at 37°C for 15 min and then 10 vol of PEM with 10 mM potassium glutamate, 10 mM β -mercaptoethanol, 50% (vol/vol) sucrose, pH 6.8, were added for 1 h at room temperature to stop the reaction. The microtubules were then isolated by centrifugation at 100,000 g for 10 min at room temperature, and the pellet was then washed three times with PEM and resuspended in a minimal volume of PEM.

Determination of Microtubule Segment Length and Distribution

Cells grown in 1 nM vinblastine (VB) for 20 h were microinjected with rhodamine-tubulin (10 μ g/ml). Neurons were lysed to remove free tubulin and fixed simultaneously as described previously (Joshi, 1993) at different

times after injection. Briefly, cells were incubated for 1 min at room temperature with freshly prepared 1% Triton X-100 and 0.5% glutaraldehyde in Hank's buffered salt solution containing 5 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (Pipes), 2 mM MgCl₂, and 2 mM ethylene glycol-bis(β-aminoethyl ether-N,N,N', N'-tetraacetic acid (EGTA)), pH 6, Geuens et al. (1986). The injected neurons were then relocated, and the newly polymerized microtubules were photographed using fluorescence microscopy. Images of the newly polymerized fluorescent microtubule segments were projected onto paper at a final magnification of 1 mm = 0.4 μm, and then each fluorescent segment and the outline of each cell was traced and the length of each labeled microtubule and its distance from the axon origin was measured with a flexible ruler. The results were expressed as the average plus or minus the standard mean error.

DNA Plasmid Injections

Plasmid phyt-myc (Shu and Joshi, 1995) encoding human γ-tubulin cDNA, driven by the long terminal repeats of the Rous sarcoma virus, was microinjected in a solution containing 0.01 μg/μl of plasmid DNA. The solution had an osmolarity of 300 mosmol, a pH of 7.3, and was filtered with a 0.22-μm filter (Millipore). To visualize the expression of the myc tagged, γ-tubulin plasmid, coverslips were rinsed with 0.1 M TBS, pH 7.6, fixed with -20°C methanol for 5 min, and then rehydrated at room temperature in TBS for 1 min. Next, the cells were incubated with 1% BSA in TBS (TBS/BSA) at room temperature for 15 min, and then incubated in a humidified atmosphere with a 1:200 dilution of mouse α-myc in TBS/BSA for 1 h at 37°C. After incubation with the primary antibody, the cells were washed twice for 10 min with TBS/BSA, and incubated with 1:200 tetramethyl-rhodamine-labeled goat anti-mouse in TBS/BSA for 1 h at 37°C. To remove excess secondary antibody, the cells were washed twice for 10 min with TBS/BSA, and this was followed by mounting the coverslips on slides with Aquamount (Lerner Laboratories).

Results

Low Concentrations of Vinblastine Inhibit Neurite Outgrowth

It has been reported that low concentrations of vinblastine stop microtubule polymerization but not neurite outgrowth (Baas and Ahmad, 1993; Ahmad and Baas, 1995; Sharp et al., 1995; Yu and Baas, 1995). Because comprehensive data on the effect of vinblastine on axonal elongation were not available, we grew neurons in different concentrations of vinblastine (0, 1, 2, and 8 nM), and measured rates of neurite outgrowth over 6 h. The neurons were randomly chosen and the neurite lengths were determined from phase micrographs at different time points after plating. Our results show that the rate of neurite outgrowth at 0 nM vinblastine was 42 ± 9 μm/h (SEM), at 1 nM vinblastine it was 36 ± 7 μm/h, at 2 nM vinblastine it was 27 ± 7 μm/h, and at 8 nM vinblastine it was 7 ± 1 μm/h (*n* = 10 neurons for each concentration). These data reveal that neurite outgrowth decreases with increases in vinblastine concentration (Fig. 1 a).

Low Concentrations of Vinblastine Do Not Eliminate Microtubule Polymerization

Although under several in vitro situations it is possible to achieve neurite outgrowth in the absence of microtubules (see for example, Lamoureux et al., 1990; Morris and Hollenbeck, 1995), microtubule polymer is thought to be important physiologically for neurite outgrowth (see for example, Yamada et al., 1970). Therefore, we wanted to determine if microtubule polymerization also occurs at low vinblastine concentrations. To do this, we microinjected fluorescent tubulin into the cell bodies of neurons

grown in the presence of vinblastine. Neurons were then allowed to grow for 1 h followed by permeabilization to remove free labeled tubulin before fixation. We found that fluorescently labeled tubulin became incorporated into both cell body and axonal microtubules in the range of concentrations of vinblastine that have been previously reported to stop microtubule polymerization in vivo (Baas and Ahmad, 1993; Ahmad and Baas, 1995; Sharp et al., 1995; Yu and Baas, 1995).

We sought to determine if there was a relationship between microtubule polymerization and neurite outgrowth. The first step was to establish that after the injection of fluorescent tubulin, the rate of microtubule polymerization was linear. To do this, we grew neurons in the presence of different concentrations of vinblastine for 20 h. We then injected the fluorescent tubulin into neurons, waited 5–15 min, extracted the soluble tubulin, and fixed the neurons (Fig. 2). As expected from our earlier observations, the injected fluorescent tubulin incorporated into cellular microtubules (Fig. 2). Fortunately, in the presence of vinblastine the number of microtubules that extended at early time points (up to 15 min) was low enough that individual segments of newly polymerized fluorescent microtubule were clearly discernible throughout the axon for a period of as long as 15 min (Fig. 2). This allowed us to accurately measure the position and the extent of newly polymerized fluorescent microtubule segments in the axon as a function of time after the injection of fluorescent tubulin. In 1 nM vinblastine, the average length of labeled segments was 0.81 ± 0.05 (SEM) μm at 5 min, 1.5 ± 0.08 μm at 8 min, and 2.4 ± 0.1 μm at 11 min. In the higher concentration (4 nM) of vinblastine, the average lengths of the labeled segments were 0.5 ± 0.02 μm at 4 min, 1.0 ± 0.08 μm at 8 min, 1.9 ± 0.14 μm at 12 min, and 2.7 ± 0.14 μm at 16 min (*n* = 3 at each time point). Analysis of these data reveals polymerization rates of 0.26 ± 0.016 (SEM) μm/min at 1 nM vinblastine (*n* = 9) and 0.18 ± 0.009 μm/min at 4 nM vinblastine (*n* = 12) (Fig. 1 b). Like the effect of vinblastine on the neurite outgrowth, microtubule polymerization decreased with increase in vinblastine concentration. These data are thus consistent with the hypothesis that vinblastine inhibits neurite outgrowth by inhibiting microtubule polymerization.

Fluorescent Tubulin Injection Results in Brighter MTs Nearer the Injection Site and Dimmer MTs Farther Away

During our analysis of the microtubule polymerization within the axon at low concentrations of vinblastine, we noticed that the fluorescent axonal microtubules which are near the cell body are brighter than those far from the cell body (Fig. 2). Since we had introduced a pulse of fluorescent tubulin at time 0 min, which begins to be diluted by unlabeled cellular tubulin as more and more microtubules turn over with time, microtubules that assemble at early time points after injection are brighter than those assembled later. The presence of lighter microtubules farther from the cell body near the growth cone, and brighter microtubules near the cell body, indicated that we may be observing new microtubule assembly near the growth cone as suggested by Bamberg et al. (1986); Lim et al. (1990);

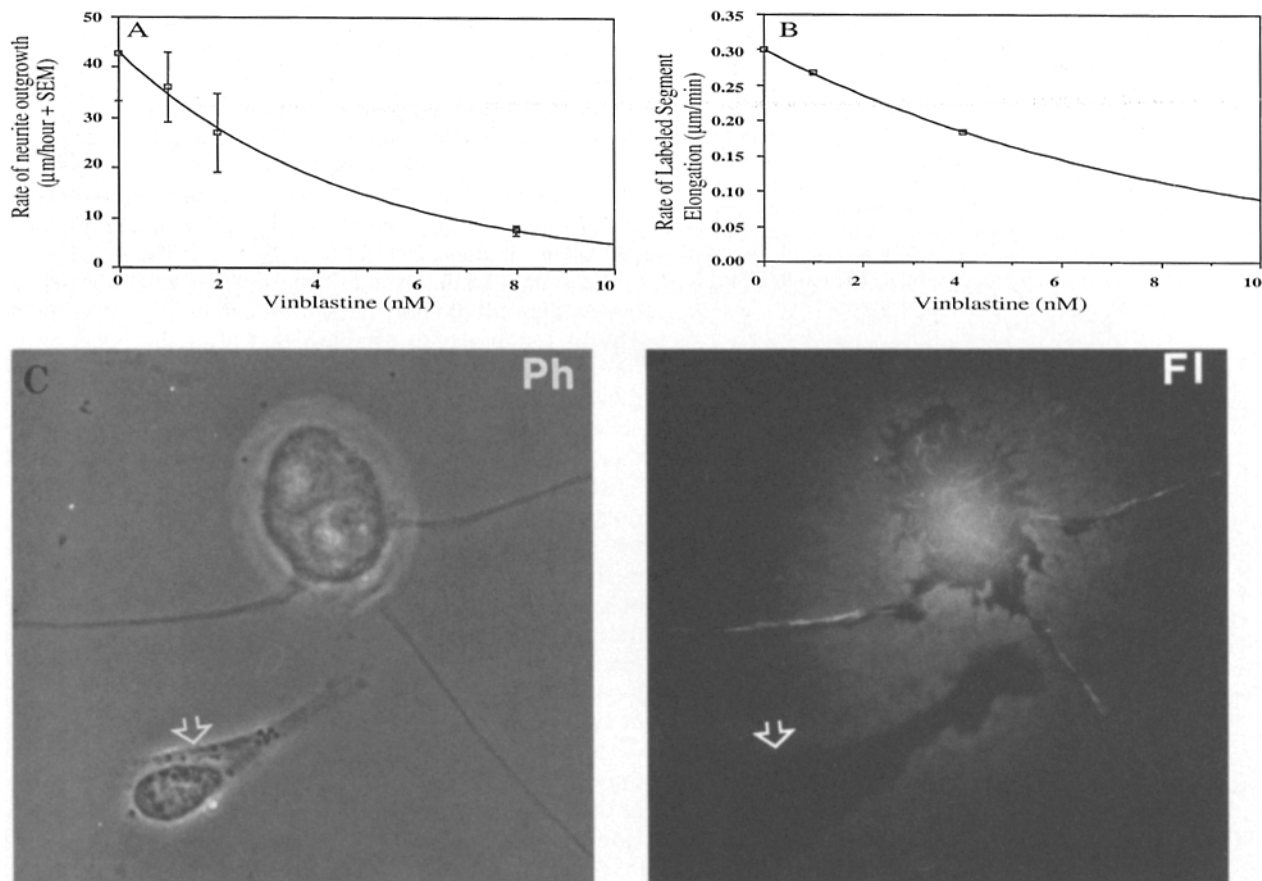


Figure 1. Neurite outgrowth and microtubule polymerization in low concentrations of vinblastine. (A) Rate of neurite elongation at different concentrations of the microtubule drug, vinblastine. Neurons were plated in L-15 GGPSHFN on coverslips coated with poly-L-lysine and laminin. 1 h after plating, the concentration of vinblastine was adjusted to 0, 1, 2, or 8 nM. The neurons were then grown for five additional hours and the average neurite lengths were measured. (B) The rate of microtubule polymerization, as determined from the labeled lengths of fluorescent microtubules at different time points and concentrations of vinblastine ($n = 10$). (C) Phase contrast (Ph) and a fluorescence (Fl) micrograph from an example of a neuron 10 min after injection with fluorescent tubulin grown under the 4 nM vinblastine concentration. Note that the free tubulin escapes out of the cell during lysis and fixation producing a halo of fluorescent tubulin around the cell body. The uninjected fibroblast (arrow) shows absolutely no fluorescent background.

Okabe and Hirokawa (1990); Takeda et al. (1995); Sabry et al. (1995).

The Number and Length Distributions of New Polymer Segments Vary Systematically

We also noticed that the injected labeled tubulin polymerization within the axon follows a precise distribution pattern that changes systematically with time after injection. To determine quantitatively this precise spatial relationship of number, and length distributions of labeled microtubules in the axon at different times (5, 10, and 15 min) after the fluorescent tubulin injection, we divided axons into eight 20- μm bins beginning 5 μm from their origin, and then calculated the average length and number of labeled microtubules in each of the bins (Fig. 3). At 5 min after injection, a large number of short-labeled microtubules formed near the cell body (15 and 35 μm), but not at intermediate or far distances from the cell body (55–155 μm). At 10 min, labeled microtubule number and length increased both near the cell body (15 and 35 μm) and at intermediate distances from the cell body (55–95 μm). At

15 min, a few short-labeled microtubules appeared far from the cell body (115–155 μm), a large number of medium length-labeled microtubules were at an intermediate distance from the cell body (55–95 μm), and a small number of long-labeled microtubules near the cell body (15 and 35 μm) followed by a gap in the proximal axon where labeled microtubules were only occasionally found. Thus, distribution of labeled microtubule lengths and numbers in the axon changes with time in a meaningful way (see below for discussion).

A Gap Observed between New Polymer in the Cell Body and in the Distal Axon at Longer Times after Injection

Shown in Fig. 5 is a sequence of tubulin transport events at 5 min, 15 min, and 30 min after the injection of rodaminated tubulin subunits in the cell body of these neurons. Strikingly, a gap of no labeled microtubules develops in the proximal axon between the labeled microtubules within the cell body and the labeled microtubules in the distal axon near the growth cone. The analysis of our data

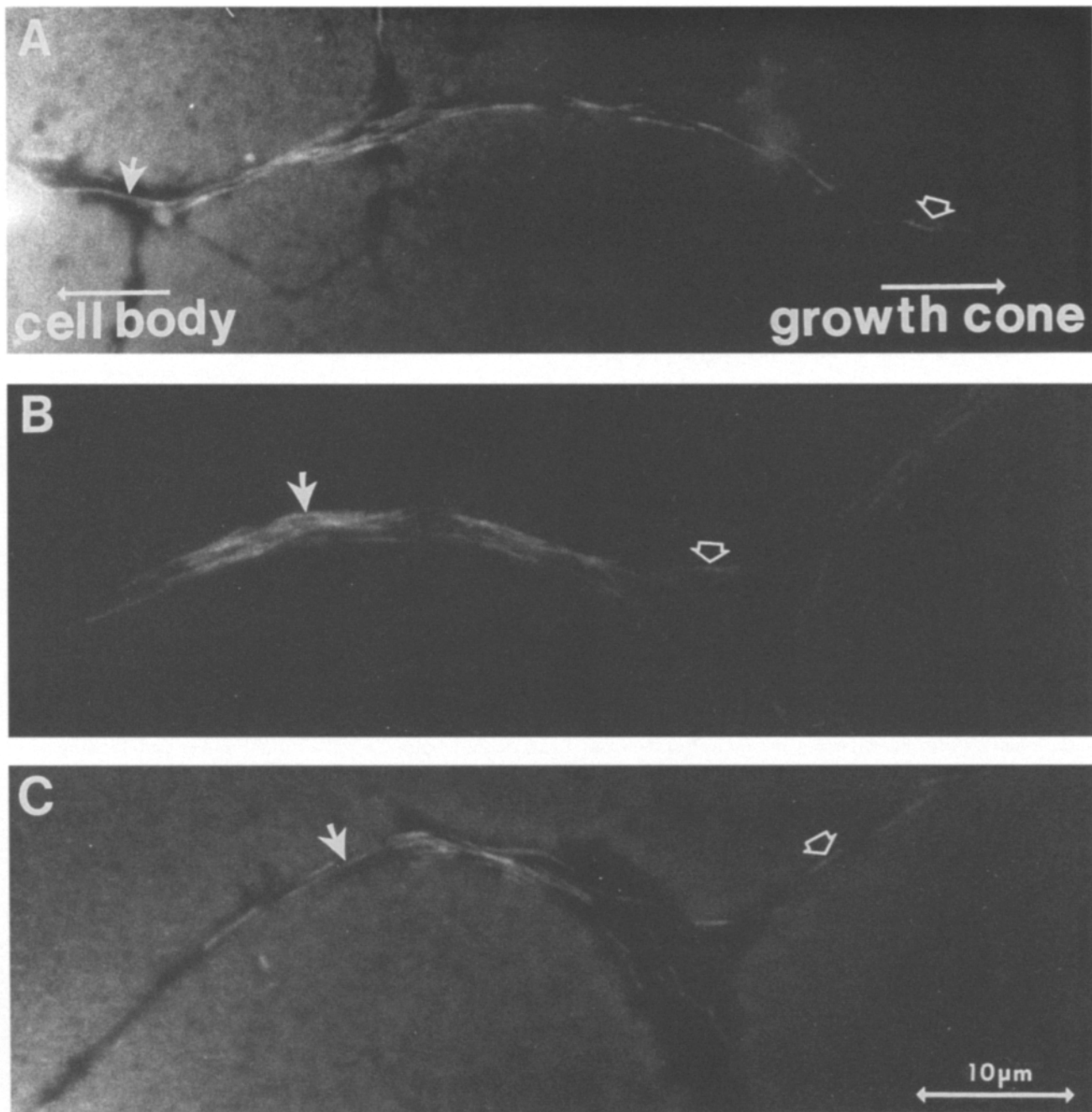


Figure 2. Visualization of microtubule number, length, and fluorescence distribution within the axons of neurons 15 min after the injection of fluorescent tubulin. Three representative examples (*A*, *B*, and *C*) are shown from three independent experiments. Images like these were used for the quantitation of the number and length distribution of microtubules shown in Fig. 3. Note that fewer long bright microtubules occur toward the cell body (*solid arrows*) and lightly labeled shorter microtubules occur toward the growth cone (*open arrows*).

revealed that the average position of labeled microtubule mass in the neurite shifts in position away from the cell body as a coherent wave at an average rate of 4.8 ± 1.1 (SEM) $\mu\text{m}/\text{min}$ (Fig. 4). While this rate of transport is higher than the rate of transport reported by Lasek (1988), there is indication that the rate of slow axonal transport is higher in growing axons (for review see McQuarrie et al., 1989). In any event, this methodology makes it possible to study slow axonal transport over very short time periods, and allows manipulations that are not possible in vivo.

Injected Nondynamic Microtubules Do Not Enter the Axon

If tubulin subunits move from the cell body down the axon, polymerizing onto and depolymerizing from the stationary dynamic axonal microtubules, free heterodimers of tubulin must be required for tubulin transport in our assay. To directly test this, we injected fluorescent but nondynamic microtubules, and then looked for transport of fluorescent label. To inhibit microtubule dynamics, we

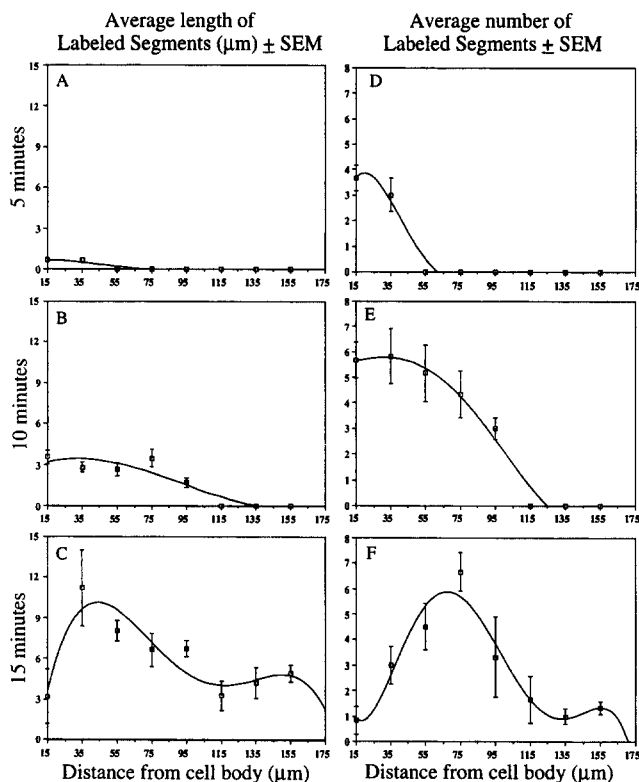


Figure 3. Quantitative analysis of the labeled microtubule number and lengths in the neurite. The average number of the labeled microtubule segments and the average length of the labeled microtubule segments were measured from images such as shown in Fig. 2 at short times after the injection of rhodamine-labeled tubulin into neuronal cell body. To analyze the distribution of the labeled segments, the neurites were divided into eight bins of equal length (20 μm). A, B, and C show the average length of labeled microtubule segments vs distance from the cell body at 5, 10, and 15 min. D, E, and F show the average number of labeled microtubule segments vs distance from the cell body at 5, 10, and 15 min.

chose the cross-linking agent ethylene glycol-*bis*-succinimidylsuccinate (EGS) because such microtubules do not depolymerize, but they can polymerize and can support anterograde as well as retrograde microtubule transport in cell extracts (Hyman and Mitchison, 1991). 30 min–30 h after the injection, the injected fluorescent microtubules were always retained within the cell body, and among 130 injected neurons there was not a single instance when microtubules were found in the axon (Fig. 5).

Minus End-binding Microtubule Protein, γ -Tubulin, When Overexpressed in Neurons Is Retained within the Cell Body and Does Not Enter the Axon

There are some cellular events when microtubules are thought to translocate. For example, in Diatoms during the spindle elongation in anaphase-B, the antiparallel microtubules of the central spindle slide against each other (for a review see Hogan and Cande, 1990). In plants, during the reorganization of microtubule arrays following mitosis, microtubules are thought to translocate (Cyr and Pa-

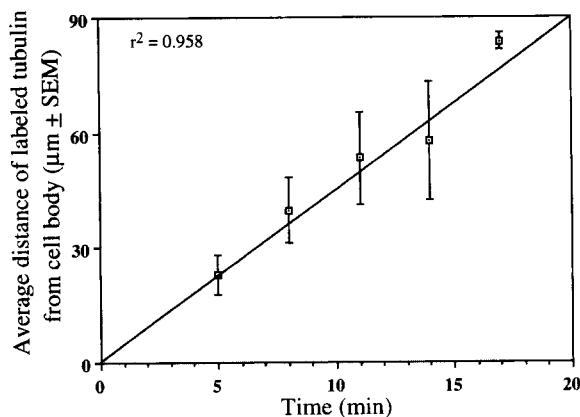


Figure 4. The average rate of fluorescence transport down the axon. Superior cervical ganglion neurons grown in the presence of 1 nM vinblastine for 20 h were microinjected with RITC-tubulin and allowed to incubate for 5–17 min before lysis and fixation. Photographs of newly polymerized fluorescently labeled segments of microtubules were used to determine the average distance of labeled tubulin from the cell body at different time points. The average rate of transport was 4.8 ± 1.1 (SEM) μm/min.

levitz, 1995; Yuan et al., 1994; Panteris et al., 1995). In actively dividing animal cells, during the formation of the mid-body in telophase, microtubules are thought to be recruited from the central spindle (Mastroratte et al., 1993; McIntosh and Landis, 1971; Mullins and Biesele, 1977). In all of these cases, a microtubule minus end-binding protein (Li and Joshi, 1995; Moritz et al., 1995; Zheng et al., 1995), γ -tubulin, is thought to be carried away along with the translocating microtubules (Julian et al., 1993; Shu et al., 1995). We asked if the microtubule minus end-binding protein, γ -tubulin, when overexpressed, is carried into neurites by microtubules translocating from the cell body. To do this, we injected a plasmid construct designed to overexpress mammalian γ -tubulin in the cell bodies of neurons and then observed the distribution of this protein 40 h after transfection (Shu and Joshi, 1995). In 20 out of 20 cells, like the endogenous γ -tubulin (Baas and Joshi, 1992), the expressed γ -tubulin was retained within the cell bodies of neurons (Fig. 6).

Discussion

In this manuscript, we present seven pieces of evidence which collectively show that neurons support axon growth by transporting tubulin subunits, but not microtubules. What follows is a discussion of our evidence in relation to previous work.

Although some experiments involving photobleaching or photoactivation of injected tubulin in neurons suggest microtubules are primarily stationary within the axons (Lim et al., 1989, 1990; Okabe and Hirokawa, 1990; Takeda et al., 1995; Sabry et al., 1995), a recent series of studies challenges this notion by reporting that low concentrations of vinblastine stop microtubule polymerization but not neurite outgrowth (Baas and Ahmad, 1993; Ahmad and Baas, 1995; Sharp et al., 1995; Yu and Baas, 1995). Our first line of evidence shows that the premise

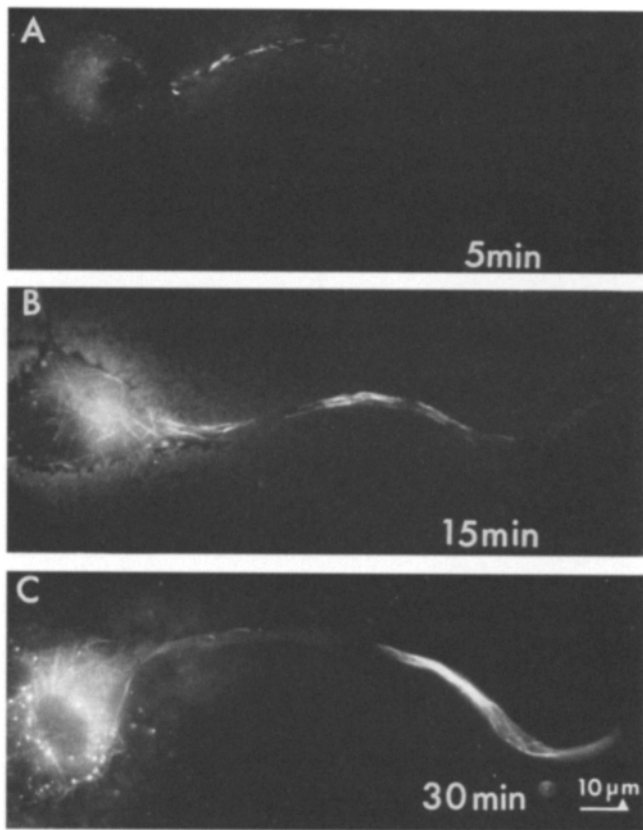


Figure 5. A gap of no labeled microtubules observed between the labeled polymer in the cell body and in the distal axon at longer times after injection. Neurons growing in 1 nM vinblastine containing medium were injected with rhodamine tubulin. Neurons were then lysed and fixed at 5 min (A), 15 min (B), or 30 min after the injection and photographed under fluorescent optics.

that low concentrations of vinblastine stop microtubule polymerization is faulty. We clearly show that neurite outgrowth and microtubule polymerization can occur in the presence of low concentrations of vinblastine, but at reduced average rates. Thus, the challenge posed by studies using vinblastine is not relevant to the issue of tubulin transport. Nevertheless, we must point out that the data reported in the studies of Baas and Ahmad (1993), Ahmad and Baas (1995), Sharp et al. (1995), and Yu and Baas (1995) are correct. In the absence of the availability of direct data of tubulin polymerization in neurons grown under these vinblastine concentrations, these data were misinterpreted.

Because vinblastine decreases the number of microtubules that polymerize within the axon, the use of this drug allowed us to observe the detailed polymerization behavior of the injected fluorescent tubulin into microtubules. Thus, the third line of evidence we provide is that the fluorescent tubulin injection results in brighter axonal microtubules near the cell body (the injection site) and dimmer axonal microtubules farther away. In this pulse-chase study, a pulse of fluorescent tubulin at time 0 min begins to be diluted by unlabeled cellular tubulin as more and more microtubules turn over with time, the presence of lighter distal microtubules and brighter proximal microtubules indicates that new microtubule assembly occurs near the

growth cone as suggested by Bamberg et al. (1986), Lim et al. (1990), Okabe and Hirokawa (1990), Takeda et al. (1995), and Sabry et al. (1995). Although it is less likely, an alternative explanation could be that the dimmer shorter microtubules at the distal axon and brighter long microtubules in the proximal axon may reflect the concentrations of the fluorescent tubulin in those axonal locations. If microtubules were transported, it would be expected that at least a few brightly labeled microtubules would be present in the distal axon far from the cell body.

The fourth line of evidence that we offer is the pattern of the number and length distributions of new polymer segments varies systematically and in a meaningful way. Like microtubules in other cell types, axonal microtubules have been shown to be dynamic (Reinch et al., 1991; Tanaka and Kirschner, 1991; Okabe and Hirokawa, 1990; Takeda et al., 1995; and Sabry et al., 1995). In the cell types where it is possible to observe the behavior of individual microtubules, it is shown that microtubules *in vivo* follow the generalized property of the dynamic instability (Cassimeris et al., 1988; Sammak and Borisy, 1987). This property of microtubule dynamics predicts a precise pattern of changes with time in the number and lengths of microtubules during their polymerization. Initially, many short microtubules begin polymerizing slowly, but occasionally some individual microtubules undergo a transition from their growing phase to a rapid shrinking phase in which they lose most or all of their length. As a result, a large number of short growing microtubules changes to a small number of long microtubules (Mitchison and Kirschner, 1984).

In our experiments, a pulse of labeled tubulin was introduced into the cell body, and the initial events of labeled tubulin polymerization in the axon were observed. If microtubules rather than the soluble tubulin move from the cell body into the axon, the leading labeled segments of microtubules would be the oldest and hence would be represented by a few long-labeled segments of microtubules that have not undergone the phase transition and lost the labeled segment during their passage. If however, the pulse of the labeled tubulin subunits moves, the leading edge of the pulse will be represented by growing labeled microtubule segments toward the growth cone and the lagging end of the pulse will be represented by fewer long-labeled microtubule segments. The long remaining microtubule segments may even disappear with time due to the complete loss of the labeled microtubule segments in the proximal axon after the free tubulin wave has passed away from this region. Thus, whether free tubulin subunits or microtubules move down the axon, they dictate different length distributions of microtubules in the axon. Indeed, as shown in the results section, a careful analysis of the length distribution places the young growing-labeled microtubule segments in the distal tip and longer old-labeled microtubule segments, eventually disassembling completely, toward the cell body. Thus, in light of the dynamic instability model of microtubule assembly, the observed distribution of microtubule lengths and numbers in the axon fits the prediction that the assembly of new polymers occurs toward the growth cone.

An additional piece of information provided by the distribution of labeled segments of microtubules along the

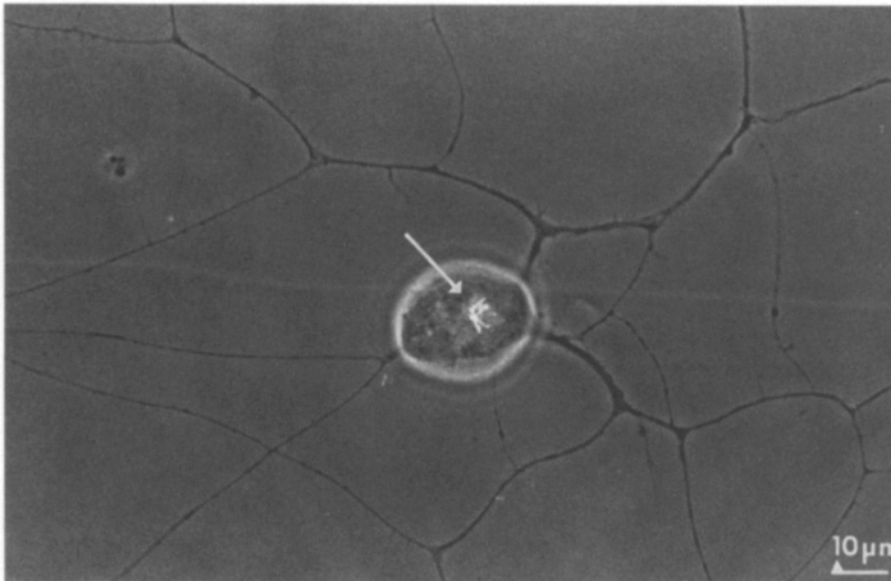


Figure 6. Stable injected fluorescent microtubules remain stationary within the cell body. This figure shows a combination of phase contrast and fluorescence micrograph of a cell showing microinjected stable fluorescent microtubules within the cell body. Phosphocellulose purified and fluorescently labeled tubulin was polymerized, stabilized with EGS, and microinjected before observation at times between 30 min to 30 h. Bar, 10 μ m.

axon is that the fluorescent microtubule segments increase in their lengths within the axon overtime. Thus, the labeled segments undergo significant elongation by incorporating fluorescent tubulin within the axon. This cannot be explained if microtubules that became fluorescent in the cell body were moving in the axon, which would lack the fluorescent tubulin subunits. However, the elongation with time of the fluorescent microtubules in the axon would be the predicted outcome if fluorescent tubulin was transported actively into the axon.

The fifth line of evidence that supports tubulin subunit transport is the observation of a gap of unlabeled microtubules between labeled polymer in the cell body and in the distal axon. Despite a large reservoir of labeled microtubules in the cell body, a gap in the adjacent axon would not be expected to form if microtubules move from the cell body into the axon. One explanation for the gap is that when the labeled tubulin is injected, it first incorporates in the microtubules in the cell body. Subsequently, free labeled tubulin moves into the axon as a fluorescent pulse. This pulse is then followed by a "chase" of unlabeled recently synthesized tubulin. A high rate of tubulin synthesis in neurons is not unreasonable. In our experiments, the average rate of neurite elongation was $0.6 \pm 0.2 \mu\text{m}/\text{min}$. Thus, enough protein would need to be synthesized to form 1 μm of axon every 2 min. To supply an adequate amount of tubulin for the growing axon, tubulin subunit synthesis and transport must be robust. As the unlabeled newly synthesized tubulin chases the fluorescent tubulin pulse farther away from the proximal axon, the fluorescent microtubule-segments in the cell body and within the proximal axon face a certain probability of losing the fluorescent segments due to the dynamic nature of microtubules. Because the cell body harbors many more fluorescent microtubule-segments compared to the proximal axon, the fluorescent segments of microtubules in the proximal axon would be expected to disappear more often than in the cell body. In any event, the formation of a distinct gap of unlabeled microtubule segments in the proximal axon, coupled with the evidence of longer brighter mi-

cro-tubules toward the cell body and shorter dimmer microtubules away from the cell body indicates that tubulin subunits move down the axonal microtubule cytoskeleton in a manner analogous to a protein band moving down a column chromatography matrix.

The two hypotheses of whether neurons supply tubulin to the growing axon in the form of subunits or the polymer predict different fates for the injected fluorescent tubulin. If polymer is the transported form of tubulin, the pre-existing axonal polymer adjacent to the growth cone will supply the new microtubules for the newly grown tip of the axon. If, however, tubulin is supplied in the form of subunits which can then be transported down to the site of new growth, the newly synthesized tubulin will be found assembled at the growing tip. In our experiments, we find that 30 min after injection of the fluorescent tubulin within the cell body the fluorescent tubulin is incorporated in the growing tip of the axon.

There are two lines of evidence in the literature that need to be addressed in light of the transport of tubulin subunits. First, Yu et al.(1993) observed repolymerization of microtubules after nocodazole induced depolymerization in cultured neurons. They showed an enormous burst of microtubule polymerization at the neuronal centrosome immediately after the removal of the drug. At later time points after drug removal, microtubules appeared in the cell periphery and the number of microtubules at the centrosome returned to a normal lower level. They interpreted this to mean that neuronal microtubules form at the centrosome and then are chased into the axon. However, in these correlative studies there was no means of confirming the source of microtubules within the axon or at the cell periphery. An alternative explanation for these data is that microtubule drugs such as colchicine are reported to inhibit slow axonal transport (Komiya and Kurokawa, 1980). If transport of tubulin subunits away from the cell body is arrested, it would be expected that there would be an accumulation of newly synthesized tubulin within the cell body. In the cell body, there resides a potent microtubule nucleation site, the centrosome.

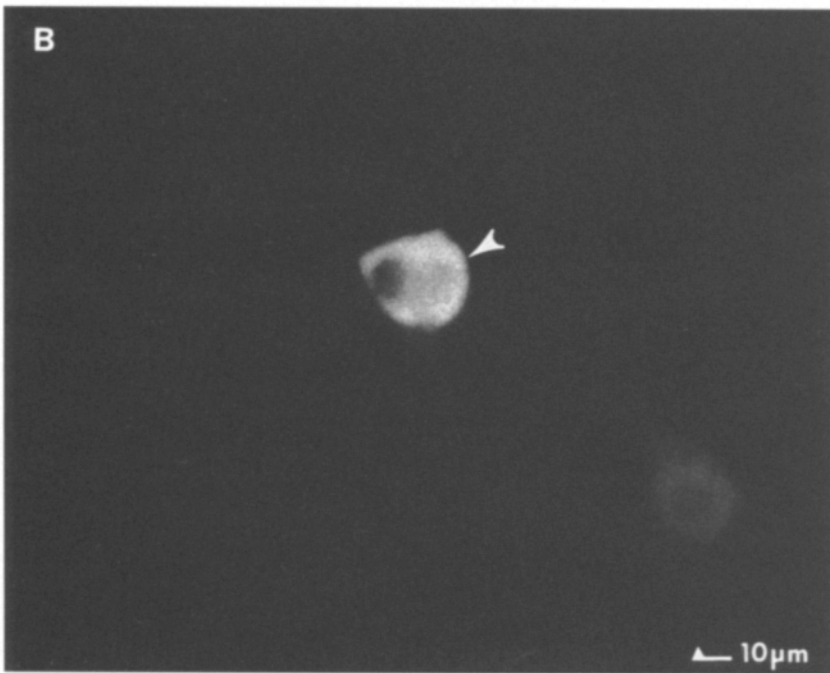
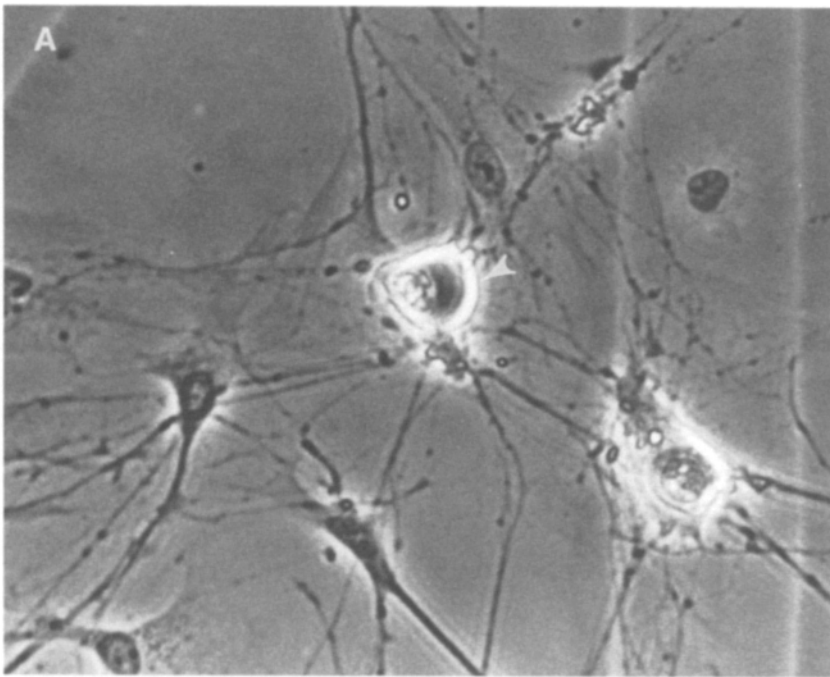


Figure 7. The minus end tag of microtubules, γ -tubulin, when overexpressed, is restricted to the cell bodies of neurons. Cultured rat superior cervical ganglion neurons plated on photoetched coverslips were microinjected with plasmid encoding human γ -tubulin cDNA. Immunocytochemistry was performed 40 h later to visualize the expressed γ -tubulin. (A) A phase contrast photograph of a field of neurons, one of which is injected with γ -tubulin expression plasmid (arrow). (B) γ -Tubulin staining reveals that it is restricted to the cell body ($n = 20$).

Hence, upon washing out the drug, it is natural to see extensive microtubule nucleation from the centrosome. As the tubulin transport resumes in the absence of the drug, the concentration of tubulin subunits within the cell body is expected to fall. Under these conditions, centrosomes would not be able to maintain the nucleated assembly of as many microtubules. Furthermore, as discussed earlier, the very fundamental dynamic instability property of microtubule polymerization predicts that microtubules begin polymerization as a population of many short microtubules which changes, over time, to a population of a few long microtubules.

Brown et al. (1992) performed a quantitative study where they measured the location of newly assembled mi-

cro-tubules in cultured superior cervical ganglion neurons. These authors relied upon the knowledge that α -tubulin in stable microtubules accumulates an unusual posttranslational modification, the removal of the COOH-terminal tyrosine residue. Thus, the presence of tyr- α -tubulin in microtubules is indicative of their young age. These authors measured the distribution of the tyr- α -tubulin in microtubules, and found that it is enriched at the proximal and at the distal segments of the axon. They concluded that the cell body and distal axon are the principal sites for the formation of new polymer in the growing axon. Therefore, they suggested that the high levels of polymer assembly in the cell body contributed to the net addition of polymer to the axon. However, this reasoning falls short of explaining

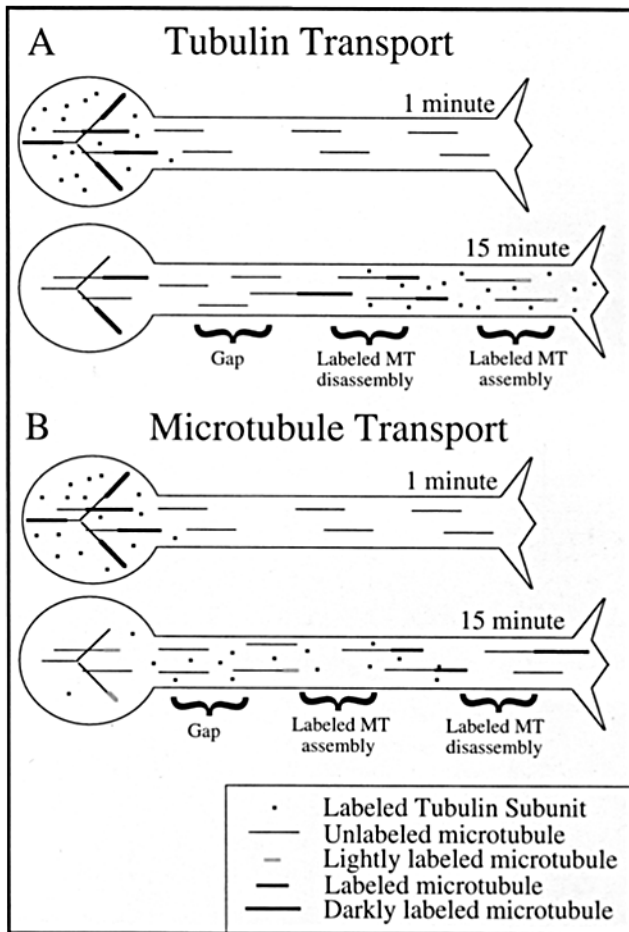


Figure 8. A schematic showing the predicted fate of injected fluorescent tubulin in the cell bodies of neurons. (A) If tubulin is actively transported into the axon as subunits, the fluorescent subunits will move down the axonal column as a discrete wave. As the subunit wave passes down the axon, subunits will add and fall off from the plus ends of the axonal microtubules, and the leading edge of the wave will denote young dimmer MTs and the lagging edge of the wave will denote old brightly labeled microtubules. Free fluorescent tubulin is in front of the labeled microtubules. This scenario is analogous to a protein band moving down a chromatography column. (B) If however, MT are actively transported, instead of the tubulin-subunits, the fluorescent MTs will move down the axonal column as a discrete wave. As the MT wave passes down the axon the leading edge of the wave will be made up of old brightly labeled MTs and the lagging edge of the wave will be made up of young dimly labeled microtubules. Free fluorescent tubulin in the axon in this model is postulated to be present behind the fluorescent MTs due to the dynamic nature of the transported MTs. The difference between the two models is where net assembly and disassembly of the labeled tubulin occurs. In the tubulin translocation model, old brightly labeled microtubules disassemble proximally, the labeled tubulin subunits move towards the growth cone and assemble into new, fainter microtubules. In the old microtubule translocation model, microtubules assemble proximally and the brightly labeled old microtubules should be found towards the growth cone.

how free tubulin arrived at the end of the axon where it also generated newly assembled microtubules. An alternative explanation for their observed pattern is that the tubulin polymerizes shortly after its synthesis in the cell body and in the final destination for tubulin transport—at the site of new axon growth. These data are thus not inconsistent with our hypothesis of tubulin subunit transport.

The sixth line of evidence that we present is that injected nondynamic microtubules do not enter the axon. Among 130 neurons injected with microtubules, there was not an instance when microtubules were found in the axon. Although this is compelling evidence for the stationary microtubules, one caveat is that it is a negative result. It is possible that the mechanism of microtubule translocation, if it does exist, may be inhibited by EGS stabilization of microtubules. Nevertheless, microtubule stabilization with EGS does not interfere with the molecular motors from cellular extracts (Koshland et al., 1988). The simplest interpretation of these data is that microtubules do not translocate from the cell body into the axon.

Recently, there has been a report that several objects including negatively charged plastic beads, dyes, phalloidin-actin, and fluorescently labeled microtubules, translocate when introduced into squid giant axon (Terasaki et al., 1995). The results of this report are not inconsistent with our data. Terasaki et al. (1995) showed that only objects (beads and dyes) 500 nm or less were transported in an anterograde direction. To see movement of fluorescently labeled microtubules, they had to triturate fluorescent microtubules 20 times, and in such preparation the fluorescence appeared as a point source of light (the wavelength at which rhodamine emits is ~ 550 nm). This suggests that their vigorous shearing may have broken microtubules into short oligomers. Furthermore, in these experiments, they observed the movement of only a small fraction of the fluorescence created by shearing fluorescent-microtubules.

In our experiments, the microtubules were clearly resolvable as lines of fluorescence. The lengths of microtubules that we injected in the neurons ranged from 1 to 10 μm . Microtubules in our studies were larger than the objects that were observed to be transported in the experiments of Terasaki et al. (1995). Based on those experiments, our long fluorescently labeled microtubules and the long endogenous microtubules in the axon would be predicted to be stationary. In addition, because negatively charged dye was observed to be actively transported, and because $\sim 40\%$ of the total tubulin is soluble and has a negative charge, it is predicted from the experiments of Terasaki et al. (1995) that the soluble tubulin could be transported. Together, this is consistent with the hypothesis that tubulin subunits or very small extractable tubulin oligomers are the primary form in which neurons supply neurites and axons with tubulin.

A final piece of evidence we provide is that the minus end-binding microtubule protein, γ -tubulin, when overexpressed in neurons is retained within the cell body and does not enter the axon. While this result is not compelling on its own, we believe that it is worth mentioning in the context of other lines of evidence. γ -Tubulin is thought to be essential for the *in vivo* nucleation of microtubules

(Oakley et al., 1990; Joshi et al., 1992; Zheng et al., 1995). This raises a question—how does the assembly of axonal microtubules occur? This is still an open issue, and further studies will be necessary to address this question. One possibility is that alternative mechanisms exist in neurons to nucleate axonal microtubule assembly. Consistent with this idea is a recent report that blocking the microtubule assembly at the centrosome by microinjection of a γ -tubulin antibody does not eliminate axon assembly, although it does compromise it (Ahmad et al., 1994). Another possibility is that microtubules might be fragmented physiologically within axons, and short oligomeric fragments of microtubules may be transported down the axon. These oligomeric fragments might then also be substrates for the transport and provide nuclei for the new assembly of axonal microtubules. This is consistent with the studies of Terasaki et al. (1995), who reported movement of a fraction of sheared bits of microtubules in squid giant axon.

We conclude that tubulin transport in neurons is largely in the form of tubulin subunits or oligomers.

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