

# Microtubules released from the neuronal centrosome are transported into the axon

Fridoon J. Ahmad and Peter W. Baas\*

Department of Anatomy and Program in Cellular and Molecular Biology, The University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706, USA

\*Author for correspondence

## SUMMARY

There is controversy concerning the source of new microtubules required for the development of neuronal axons. We have proposed that microtubules are released from the centrosome within the cell body of the neuron and are then translocated into the axon to support its growth. To investigate this possibility, we have developed an experimental regime that permits us to determine the fate of a small population of microtubules nucleated at the neuronal centrosome. Microtubules within cultured sympathetic neurons were depolymerized with the anti-microtubule drug nocodazole, after which the drug was removed. Microtubules rapidly and specifically reassembled from the centrosome within three minutes of nocodazole removal. At this point, low levels of vinblastine, another anti-microtubule drug, were added to the culture to inhibit further microtubule assembly while not substantially depolymerizing the small population of microtubules that had already assembled at

the centrosome. Within minutes, released microtubules were apparent in the cytoplasm, and many of these had already translocated to the cell periphery by ten minutes. By one hour, virtually all of the microtubules had been released from the centrosome and were concentrated at the cell periphery. With increasing time, these microtubules appeared within and progressively farther down developing axons. Nonneuronal cells within the culture also reassembled microtubules at the centrosome, but only a small portion of these microtubules were released. These observations indicate that microtubules are released from the neuronal centrosome and transported into growing axons, and that microtubule release and relocation from the centrosome are especially active in neurons compared to nonneuronal cells.

Key words: microtubule, neuron, axon, centrosome

## INTRODUCTION

Neurons are terminally postmitotic cells that utilize most of their resources for the development of long and complex axonal arbors. The development of an axon is dependent upon the elaboration of a dense and highly organized array of microtubules. Microtubules are essential cytoskeletal elements that provide architectural support for the growth and maintenance of the axon, and also provide a substrate along which organelles are transported in both directions within the axon. Because of the fundamental importance of the axonal microtubule array, there is great interest in elucidating the mechanisms by which it is elaborated. A central issue regarding these mechanisms is the source of new microtubules required for axon growth.

In typical nonneuronal cells, new microtubules are generated via nucleation from a discrete centralized structure termed the centrosome (for reviews see Brinkley, 1985; Kellog et al., 1994). Nucleation from the centrosome regulates key features of the microtubules including their polarity orientation (Euteneuer and McIntosh, 1981), number (Brinkley et al., 1981), and lattice structure (Evans et al., 1985). Axonal microtubules are also tightly regulated with regard to these features

(Heidemann et al., 1981; Baas et al., 1988, 1991; Tilney et al., 1973; Yu and Baas, 1994), but are not attached to the centrosome (Lyser, 1964, 1968; Sharp et al., 1982). This apparent paradox has led to a great deal of confusion concerning the origins of axonal microtubules. Over the past several years, many workers in the field have focused on non-centrosomal mechanisms that might account for certain features of the axonal microtubule array. For example, significant efforts have focused on the dynamics of microtubules within the axon itself, and in particular at its distal tip (Bamburg et al., 1986; Robson and Burgoyne, 1988; Tanaka et al., 1995). However, the local dynamics of microtubules is an issue separate from their origins, and it is difficult to imagine how the same mechanisms that regulate the elongation and shortening of microtubules could also regulate their polarity orientation, number, or lattice structure.

Recent studies from our laboratory have led us back to the centrosome as a potential generator of microtubules for the axon. Specifically, we have proposed that microtubules are released from the centrosome within the cell body of the neuron and are then transported into the axon (Baas and Joshi, 1992; Joshi and Baas, 1993). To evaluate this proposal, it will be necessary for us to follow the fate of centrosomal micro-

tubules over space and time. In a first set of studies toward this end, cultured sympathetic neurons were treated with high doses of nocodazole to depolymerize existing microtubules, after which the drug was removed to permit microtubules to reassemble from their sites of origin (Yu et al., 1993). Within a few minutes of drug removal, hundreds of microtubules reassembled in the region of the centrosome, and most of these microtubules were clearly attached to the centrosome. With increasing time, fewer microtubules were attached and increasing numbers of unattached microtubules were apparent in the cytoplasm. These results suggested that microtubules had been released from the centrosome and were then relocated to other sites within the neuron. In a subsequent study, we demonstrated that the formation and growth of the axon are compromised if centrosome function is inhibited (Ahmad et al., 1994). The simplest interpretation of this latter result is that the microtubules nucleated at the centrosome are in fact the same microtubules used to elaborate the microtubule array of the axon.

In the present study, we sought to follow the fate of microtubules after their release from the centrosome to determine whether they are actually transported into the axon. This has proven to be a technical challenge because the cell body contains a great many microtubules oriented in many different directions. Even with the nocodazole-recovery regime, it is virtually impossible to follow the fate of the microtubules beyond the first few minutes of drug removal because of ongoing elongation of these microtubules as well as nucleation and elongation of additional microtubules. To simplify matters, we have now developed a kind of pulse-chase regime that permits the study of a small population of microtubules nucleated at the centrosome. After nocodazole recovery, we permit a three-minute pulse of microtubule assembly at the centrosome, after which nanomolar levels of vinblastine are added to the culture. At these low levels, vinblastine suppresses further microtubule assembly while not substantially depolymerizing existing microtubules (Jordan et al., 1991, 1992; Baas and Ahmad, 1993; Wilson and Jordan, 1994; Zheng et al., 1993; Tanaka et al., 1995). Thus any alterations in the microtubule array that occur after the addition of vinblastine result from the movement of existing microtubules from one location in the cell to another. Changes in the distribution of the microtubules were then determined at various time periods using immunofluorescence confocal microscopy.

## MATERIALS AND METHODS

### Cell culture

Cultures of sympathetic neurons from the superior cervical ganglia of newborn rat pups were prepared as follows. After dissection, the ganglia were treated with 0.25% collagenase and 0.25% trypsin for 15 minutes, and then triturated with a Pasteur pipet into a single cell dispersion. The cells were then plated onto 'special dishes' that were prepared by adhering a glass coverslip to the bottom of a 35 mm plastic Petri dish into which had been drilled a 1 cm diameter hole. Prior to plating the cells, the glass-bottomed well of the special dish was treated for 3 hours with 1 µg/ml polylysine, rinsed extensively, and then treated with 10 µg/ml laminin (Sigma Chemical Company, St Louis, IL) for 4 hours. Cells were plated in medium consisting of Leibovitz' L-15 (Sigma Chemical Company) supplemented with 0.6% glucose, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum (Hyclone, Logan, UT), and

100 ng/ml nerve growth factor (Upstate Biotechnology, Lake Placid, NY).

### Experimental regime

Stock solutions of nocodazole (Aldrich Chemical Company, Milwaukee, WI) and vinblastine sulfate (Sigma Chemical Company) were prepared and diluted into culture medium as previously described (Yu et al., 1993; Baas and Ahmad, 1993). Thirty minutes after plating, nocodazole was introduced into the cultures at a final concentration of 10 µg/ml, and the cultures were returned to the incubator. After six hours, the cultures were rinsed twice with warm drug-free medium, placed in a third rinse of warm drug-free medium, and then returned to the incubator. After three minutes of recovery, vinblastine was added to a final concentration of 50 nM, and cultures were once again returned to the incubator. Cultures were removed from the incubator at times ranging from 3 minutes to 6 hours and prepared for immunofluorescence microscopy.

### Preparation for immunofluorescence microscopy

Cultures were rinsed briefly in a microtubule-stabilizing buffer termed PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, pH 6.9), and then extracted for 3 minutes with 0.5% Triton X-100 in PHEM containing 10 µM taxol (provided as a gift from the National Cancer Institute). This treatment removes unassembled tubulin while preserving microtubules (see for example Black et al., 1986; Baas and Ahmad, 1992). The cultures were then fixed by the addition of an equal volume of PHEM containing 8% paraformaldehyde and 0.3% glutaraldehyde. After 10 minutes of fixation, the cultures were rinsed twice in PBS, treated 3 times for 5 minutes each in PBS containing 10 mg/ml sodium borohydride to reduce autofluorescence, and rinsed again in PBS. Cultures were then treated for 30 minutes in a blocking solution containing 2% normal goat serum and 1% BSA in PBS, and exposed overnight at 4°C to primary antibodies diluted in blocking solution. The following morning, the cultures were rinsed 3 times in PBS, treated again for 30 minutes in blocking solution, exposed for 1 hour at 37°C to second antibodies diluted in blocking solution, and rinsed 4 times for 5 minutes each in PBS. The cultures were then mounted in a medium consisting of 100 mg/ml DABCO and 1 mg/ml *p*-phenylenediamine (to reduce photobleaching) dissolved in a solution containing 90% glycerol and 10% PBS. The primary antibodies were a mouse monoclonal antibody against β-tubulin used at 1:500 and purchased from Amersham (Arlington, VA), a rabbit polyclonal antibody against neurofilament protein used at 1:500 and purchased from Chemicon (Temecula, CA), and a rabbit polyclonal antibody against centrin used at 1:3,000 and obtained as a gift from Dr Jeff Salisbury of the Mayo Clinic. The beta-tubulin antibody was used in all experiments. In some experiments, cultures were double-labeled for neurofilament protein to confirm that we could distinguish neurons from nonneuronal cells on the basis of morphology. In other experiments, cultures were double-labeled for centrin, a centrosomal protein (Sanders and Salisbury, 1989), to confirm that the single discrete spot of nonfilamentous tubulin staining was the centrosome. The second antibodies were a Cy-3-conjugated goat anti-mouse and a Cy-5-conjugated goat anti-rabbit. Both were purchased from Jackson Immunoresearch (West Grove, PA) and used at 1:100.

### Confocal microscopy and image analysis

Because the cell bodies of cultured sympathetic neurons are slightly flattened spheres, optical sections are required to visualize microtubules at the centrosome with necessary resolution and clarity (see Yu et al., 1993; Ahmad et al., 1994). For this reason, all images were obtained with the LSM 410 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY). Optical sections with a thickness of 0.5 µm were obtained, and all sections (not just those containing the centrosome) were examined and included in our quantitative analyses. Contrast and brightness were optimized for each image individually, but were also standardized so that quantitative comparison of micro-

tubule levels among different images would be meaningful. For quantification of microtubule levels in different regions of the neuronal cell body, each cell body was divided into three equal regions (center, intermediate, and peripheral), and an average pixel intensity was determined using NIH-Image software (provided free of charge from the NIH). Data were expressed in arbitrary fluorescence units (AFUs).

## RESULTS

The goal of this study was to follow the fate of microtubules nucleated at the centrosome to determine whether these microtubules are released and translocated into developing axons. To accomplish this, we developed an experimental regime in which a small population of microtubules nucleated at the centrosome could be observed over time. After extensive nocodazole treatment to depolymerize existing microtubules, a small burst of microtubule reassembly at the centrosome was permitted, after which subsequent microtubule assembly was inhibited by the addition of nanomolar levels of vinblastine. Cultures were extracted and fixed at various times thereafter, and changes in the distribution of the centrosomal microtubules were documented using confocal immunofluorescence microscopy.

### Efficacy of the drug treatment

Before considering changes in microtubule distribution, it was first necessary to validate the efficacy of the nocodazole and vinblastine treatments. Almost immediately after adhering to their substratum, the cells were exposed to 10  $\mu\text{g}/\text{ml}$  nocodazole for 6 hours. Over this period of time, the neurons remained adhered and were generally rounded in shape with no axonal outgrowth occurring in the presence of the drug. Cultures were then rinsed free of the drug, returned to the incubator for 3 minutes, and exposed to 50 nM vinblastine for various periods of time. This concentration of vinblastine was higher than the 4–16 nM concentrations previously reported to curtail microtubule assembly in cultured neurons (Baas and Ahmad, 1993; Zheng et al., 1993; Tanaka et al., 1995). This higher concentration has been shown to induce low levels of microtubule disassembly in sympathetic neurons (Baas and Ahmad, 1993), and therefore provides extra confidence against any consequential microtubule assembly occurring in the presence of the drug. At least 100 neurons were examined at each time point in each of 5 separate experiments. All optical sections (0.5  $\mu\text{m}$  thick) were examined to ensure that microtubules were properly identified as either attached or unattached to the centrosome and to ensure that quantitative analyses accurately reflected total microtubule levels within the cell. The centrosome was generally discernable as a single nonfilamentous spot of tubulin staining, and this was confirmed in cultures that were double-labeled with the antibody to centrin (data not shown; see Yu et al., 1993).

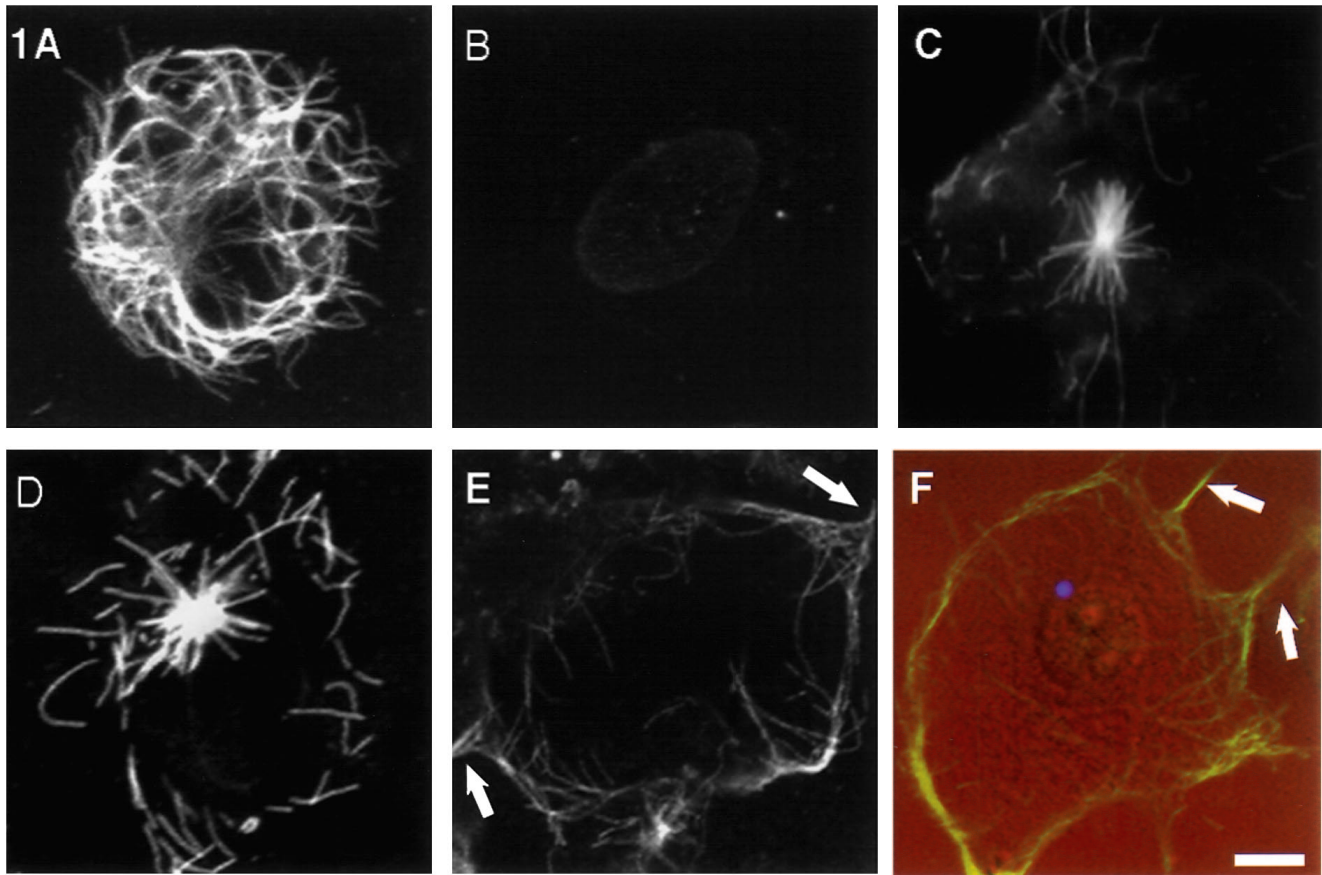
Microtubule staining patterns in control and nocodazole-treated neurons were similar to those reported in our previous studies (Yu et al., 1993; Ahmad et al., 1994). Control neurons showed complex staining with no apparent focus from which microtubules emanated even within individual thin sections taken at the level of the centrosome (Fig. 1A). Most (>90%) of the nocodazole-treated neurons showed no detectable microtubules (Fig. 1B), but a small portion of the cells showed

varying levels of microtubules remaining. After 3 minutes of recovery, >90% of the neurons showed an array of short microtubules at the centrosome. The microtubules were 1–5  $\mu\text{m}$  in length, slightly shorter than in our previous studies using a 5 minute recovery time. The remaining  $\approx$ 10% of the cells showed higher levels of microtubules throughout, and we suspect that these cells correspond to those in which the nocodazole was less effective in depolymerizing existing microtubules that might act as nucleating structures (see Ahmad et al., 1994). A similar proportion of cells contained these higher levels of polymer at subsequent stages of the experiment, and these cells were not included in our analyses. In the more typical cells, individual microtubules remained 1–5  $\mu\text{m}$  in length, and total microtubule levels did not increase over time (Fig. 1C–F). As expected with the 50 nM concentration of vinblastine, there was actually a slow but significant decrease in total microtubule levels with increasing time in drug. After zero minutes, three minutes, ten minutes, and one hour in vinblastine, there were  $1830 \pm 220$ ,  $1565 \pm 385$ ,  $1492 \pm 154$ , and  $1308 \pm 493$  AFUs, respectively, within the neurons. In statistical analyses (Student's *t*-test), these levels differed from one another with *P* values less than 0.05. Analyses on cells with more dispersed microtubules indicate that these fluorescence intensities corresponded to  $\approx$ 500–700  $\mu\text{m}$  of microtubule polymer per cell.

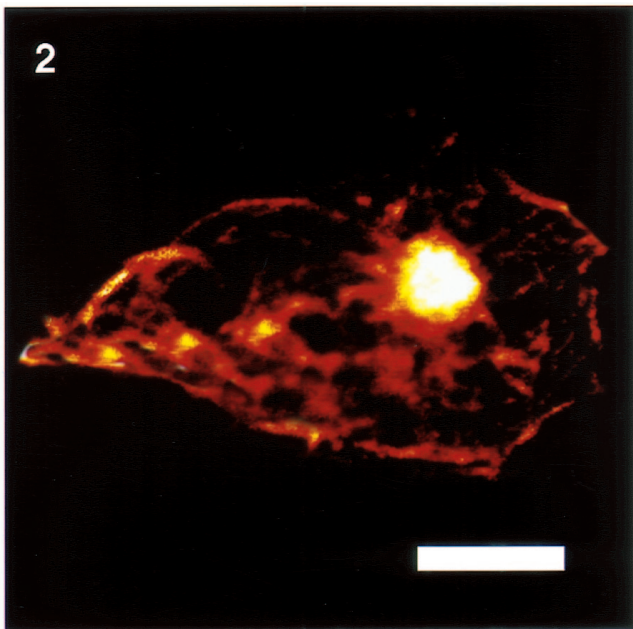
### Changes in microtubule distribution over time

Despite the inhibition of microtubule assembly by the vinblastine treatment, there were notable changes in the distribution of microtubules over time. Three minutes after the addition of vinblastine, most microtubules remained attached to the centrosome, but a small number were unattached (Fig. 1C). By 10 minutes, fewer microtubules were attached, more microtubules were unattached, and the unattached microtubules appeared at greater distances from the centrosome (Fig. 1D). Many of the unattached microtubules had already reached the cell periphery (just under the cell membrane) by 10 minutes, indicating that these microtubules were transported at a rate of  $\approx$ 1  $\mu\text{m}/\text{minute}$ . By 30 minutes, many of the neurons showed a clear diminution of microtubules from cell center. By 1 hour, the vast majority of the neurons showed few or no microtubules at cell center, with virtually all microtubules concentrated at the cell periphery (Fig. 1E and F). The centrosome (bereft or nearly so of microtubules at this point) was typically located above the nucleus, whereas the peripheral concentration of microtubules appeared at the level closest to the substratum, the same level at which axons emerge (Fig. 1F). These observations suggested that the microtubules were cascading radially from the centrosome while simultaneously moving downward toward the substratum. Rotation of the three-dimensional images of reconstructed cells gave this same impression. Many of the cells began to extend short processes (<10  $\mu\text{m}$  in length) during this time frame, and microtubules could be visualized funneling from the cell body into these nascent processes (Fig. 1E and F). Fig. 2 is a pseudocolor image of a three-dimensional reconstruction of a cell (similar to the cell shown in Fig. 1D) after 10 minutes in vinblastine. Microtubules can be visualized cascading from the centrosome toward the cell periphery.

Not surprisingly, there was variability among the cells with regard to the distribution of microtubules at these various time points. To provide a more quantitative sense of the

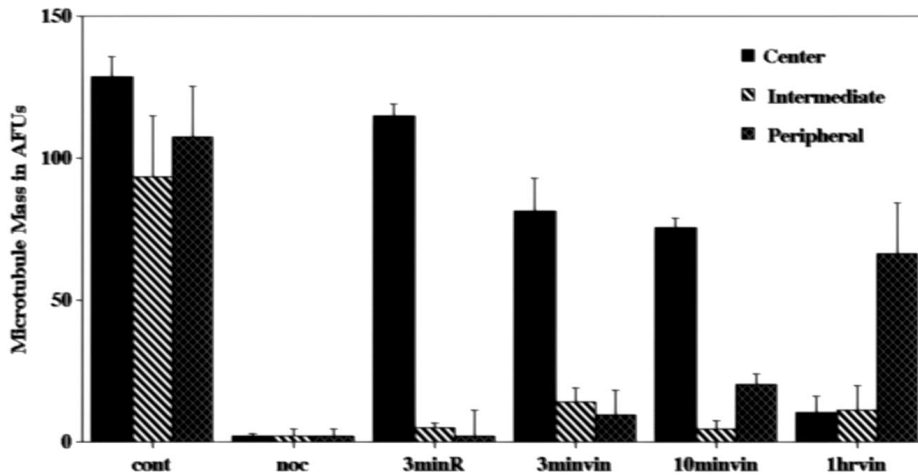


**Fig. 1.** Release and translocation of microtubules from the neuronal centrosome to the cell periphery. Control neurons showed a dense array of microtubules (A), while nocodazole-treated neurons showed few or none (B). Neurons recovered for 3 minutes showed a small population of microtubules emanating from the centrosome (not shown). After 3 minutes in vinblastine, most microtubules remained attached but a small number of unattached microtubules were apparent (C). After 10 minutes, both attached and unattached microtubules were apparent, with some of the unattached microtubules appearing at the cell periphery (D). By 1 hour, microtubules were concentrated at the periphery of the cell body, and some could be seen funnelling into developing axons (E and F; arrows mark developing axons). In F, superimposed on the immunofluorescence image of microtubules (in green) are the differential-interference-contrast image showing the cell's morphology (in orange) and the immunofluorescence image of the centrosome (in purple), which appears in a different optical section (see text). No microtubules were attached to the centrosome at 1 hour. Bar, 5  $\mu\text{m}$ .



change in microtubule distribution over time and the variability among the different cells examined, we measured fluorescence intensity in three regions of the neuronal cell bodies. For this, cells were selected in which the centrosome was roughly centralized within the cell body. The radius of the cell was trisected and the cytoplasm was correspondingly divided into three regions, center (not including the centrosome itself), peripheral (just under the plasma membrane), and the intermediate region between them. To correct for differences in the volume of cytoplasm in each of these regions,

**Fig. 2.** Serial reconstruction of a neuron releasing microtubules from the centrosome. Shown in the figure is a neuron similar to the one shown in Fig. 1D. The neuron was recovered from nocodazole for 3 minutes, treated with vinblastine for 10 minutes, prepared for immunofluorescence visualization of microtubules, and optically sectioned. The optical sections were then reconstructed. The single optical section shown in Fig. 1D shows gaps between the peripheral microtubules and the centrosomal microtubules. The serial reconstruction shown here demonstrates that there is actually a continuous stream of microtubules from the centrosome to the cell periphery. Bar, 5  $\mu\text{m}$ .



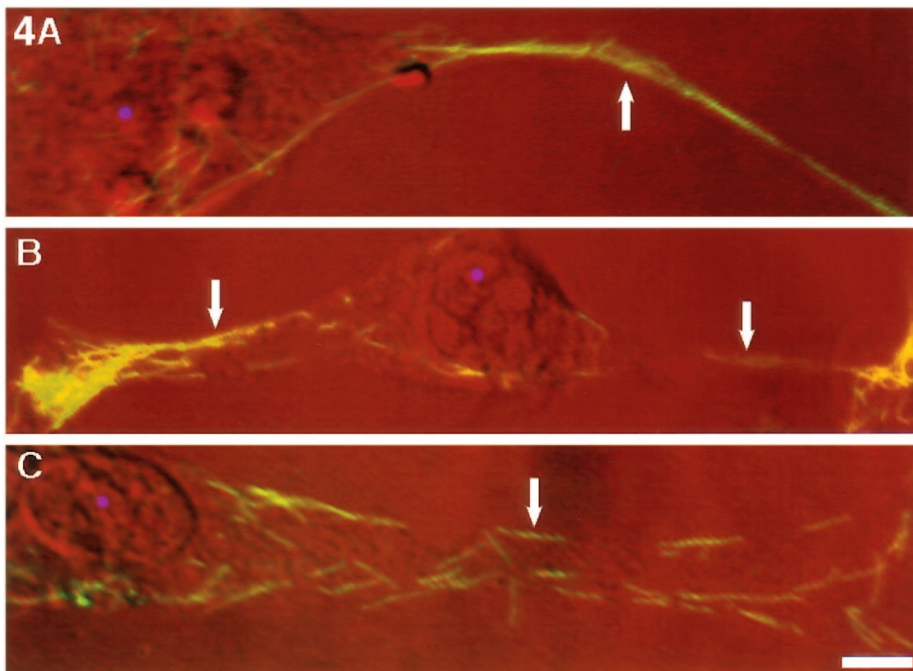
**Fig. 3.** Quantitative data on the levels of microtubules in different regions of the neuron. Cells were selected in which the centrosome was roughly centralized over the nucleus. The radius of the cell was trisected and the cytoplasm was correspondingly divided into three regions, center (not including the centrosome itself), peripheral (just under the plasma membrane), and the intermediate region between them. Data were expressed as an average pixel intensity in arbitrary fluorescence units. Control neurons showed high levels of microtubule polymer in all three regions, with somewhat higher levels in the central region. Nocodazole-

recovered cells showed most polymer in the central region and this was also true after three and ten minutes in vinblastine. By one hour in vinblastine, most of the polymer had become concentrated in the peripheral region.

the data were expressed as an average pixel intensity. The data were then displayed in the form of a bar graph (Fig. 3). Control neurons showed high levels of microtubule polymer in all three regions, with somewhat higher levels in the central region. Cells recovered from nocodazole for 3 minutes showed most polymer in the central region and this was also true after three and ten minutes in vinblastine. By one hour in vinblastine, most of the polymer was concentrated in the peripheral region.

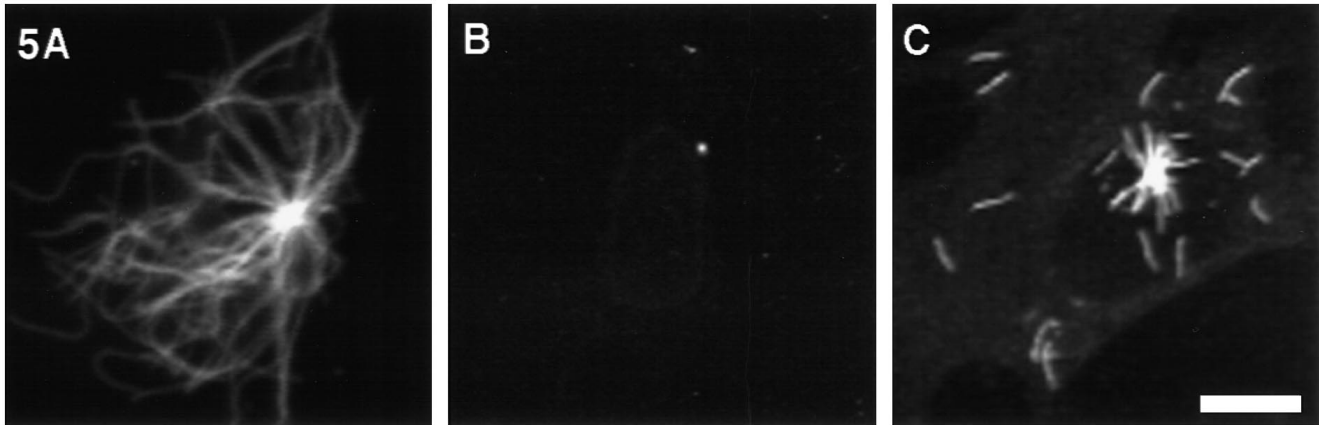
Also not surprisingly, the efficiency of axon growth was compromised by the lower levels of microtubules inherent in our paradigm. Most of the neurons failed to initiate and grow axons over the next several hours, and microtubules remained

concentrated in the peripheral region of the cell body and in the short nascent processes. Nonetheless  $\approx 20\%$  of the neurons grew bona fide axons over this time frame, and in all cases microtubules were present in these axons. It is worth noting that these axons were 20–50  $\mu\text{m}$  in length, longer than any lamellopodial veils that extended from the cell bodies. Thus it is clear that these axons represent actual outgrowths from the cell body and not the collapse of a lamellopodium around stationary microtubules. In addition, there was a concomitant diminution of microtubules from the cell body as the axons formed. Some of these axons showed a relatively even distribution of microtubules (Fig. 4A), while other axons showed a clustering of microtubules in their more distal



**Fig. 4.** Translocation of centrosomal microtubules into and down the axon. This figure is essentially a continuation of Fig. 1, showing neurons that had been treated with nocodazole, recovered for 3 minutes, and exposed to vinblastine for 6 hours. As in Fig. 1F, immunofluorescence images of microtubule staining (in green) are superimposed on differential-interference-contrast images (in orange) to better demonstrate the morphology of the neurons. Also superimposed on the composite image is the immunofluorescence image of the centrosome (in purple) from another optical section. Shown in this figure are neurons that had extended axons during vinblastine treatment. In each case, a small number of microtubules remained in the cell body (none were attached to the centrosome), but most had translocated into and down the axon. (A) A cell with an axon showing a relatively even distribution of

microtubules. (B) A cell with two axons, each showing a clustering of microtubules in the distal region of the axon. (C) An advantageous axon in which the ends of individual microtubules can be discerned. Arrows mark axons. Bar, 5  $\mu\text{m}$ .



**Fig. 5.** Release of microtubules from the centrosome of nonneuronal cells. Shown in this figure are nonneuronal fibroblastic cells from the same cultures (treated under the same experimental paradigm) as those containing the neurons shown in Fig. 1. (A) A control cell with a characteristic microtubule array emanating from the centrosome. (B) A cell treated with nocodazole for 6 hours, with no detectable microtubules remaining. (C) A cell treated with nocodazole for 6 hours, recovered for 3 minutes, and then exposed to vinblastine for 30 minutes. Most microtubules remained attached to the centrosome. Unattached microtubules were apparent, but numbered fewer than 20 and appeared dispersed in the cytoplasm, not concentrated at the cell periphery. Bar, 5  $\mu$ m.

regions (Fig. 4B). In most cases, the microtubules were clustered together obscuring their ends, but in favorable cases it was clear that the microtubules were still 1-5  $\mu$ m in length (Fig. 4C). These results demonstrate that after the microtubules released from the centrosome are transported to cell periphery, they are then transported into and down the length of developing axons.

#### Observations on nonneuronal cells

Given the dramatic results obtained with neurons, we wished to investigate whether the release and translocation of centrosomal microtubules are events specific to neuronal cells. For these studies, we took advantage of the fact that the same cultures which contained the sympathetic neurons were contaminated by small numbers of fibroblastic nonneuronal cells. These cells were easily discernable from the neurons in that they were flatter than the neurons, comprising significantly fewer optical sections (2-4 compared to 15-20 for the neurons). Our ability to distinguish nonneuronal cells on the basis of their morphology was confirmed by their lack of staining for neurofilament protein in some experiments (not shown). Fig. 5A shows a control nonneuronal cell, with a typical centrosomal microtubule array prior to drug treatment. Nocodazole-treated cells showed no detectable microtubules remaining (Fig. 5B), and cells recovered from nocodazole for 3 minutes showed an array of short microtubules at the centrosome similar to that observed in neurons (not shown). Following the addition of vinblastine, unattached microtubules were observed at all time points and these unattached microtubules were found throughout the cytoplasm. However, the vast majority of the microtubules remained attached to the centrosome. The unattached microtubules did not exceed 10-20 in number and did not concentrate at the cell periphery even after 1 hour (Fig. 5C shows 30 minutes in vinblastine). These results indicate that microtubules are also released and transported from the centrosome of nonneuronal cells, but less actively compared to neurons.

#### DISCUSSION

The present study is the third in a series of reports aimed at experimentally testing our proposal that axonal microtubules have a centrosomal origin. In the first of these reports, we used the classic nocodazole-recovery regime to document that the neuronal centrosome is an extremely potent microtubule nucleating structure, capable of generating microtubules in the kinds of numbers that would be needed to support the growth of the axon (Yu et al., 1993). In the second of these reports, we experimentally inhibited microtubule nucleation at the neuronal centrosome using a function-blocking antibody to gamma-tubulin (Ahmad et al., 1994). The result of centrosomal inhibition was an impaired ability of the neuron to reassemble microtubules during recovery from nocodazole treatment and a concomitant impairment in the ability of the neuron to grow axons. These studies provided strong support for the possibility that the microtubules nucleated at the neuronal centrosome may be the same microtubules utilized for the construction of the axonal microtubule array. In the present study, we sought to test this idea by documenting the fate of microtubules nucleated at the neuronal centrosome. Our studies, using a modification of the nocodazole-recovery regime, indicate that microtubules nucleated at the neuronal centrosome are released into the cytoplasm and then transported to the periphery of the cell body and ultimately into the axon.

#### Behavior of centrosomal microtubules in neurons and nonneuronal cells

Microtubule release from the centrosome is not an unprecedented idea. Prior to our work, a small number of studies had already documented evidence for this phenomenon in studies on nonneuronal cells (Kitanishi-Yumura and Fukui, 1987; McBeath and Fujiwara, 1990; Belmont et al., 1990). The most compelling of these studies utilized live-cell techniques to directly visualize the release and movement of microtubules from centrosomes in cytoplasmic extracts (Belmont et al., 1990). Our observations on the fibroblastic nonneuronal cells

that contaminate our sympathetic neuron cultures also demonstrate release of microtubules from the centrosome. However, direct comparison with the neurons in these cultures indicates that microtubule release and translocation from the centrosome are far less active in nonneuronal cells compared to neurons. In neurons, most or all of the microtubules were released within an hour of their nucleation, while in nonneuronal cells, most of the microtubules remained attached to the centrosome even hours after their nucleation. In addition, the translocation of microtubules after their release from the centrosome was sluggish in nonneuronal cells compared to neurons, with the released microtubules never concentrating at the cell periphery.

In interpreting our results, it is useful to consider in greater detail the effects of vinblastine on microtubule dynamics in the axon. Microtubules normally undergo rapid bouts of assembly and disassembly at their plus ends. Observations on the effects of vinblastine both in the test tube and in living cells indicate that nanomolar levels of the drug markedly reduce the frequency and extent of these dynamic events (Wilson and Jordan, 1994; Tanaka et al., 1995). However, the precise concentration of the drug needed to inhibit net assembly varies under different experimental conditions, and even at this concentration the ends of the microtubules still undergo oscillations, i.e. short bouts of assembly and disassembly. This is evidenced by the staining of the microtubules for tyrosinated tubulin even after prolonged vinblastine treatment (Baas and Ahmad, 1993) and by their incorporation of low levels of fluorescent tubulin microinjected into the cells (P. W. Baas and F. J. Ahmad, unpublished observations). In the present study, we found that a concentration of 50 nM, significantly higher than used in previous studies on neurons, effectively curtailed net microtubule assembly over the time period of the experiment and actually induced low levels of microtubule disassembly. While oscillations at the ends of the microtubules undoubtedly can still occur under these conditions, it is unlikely that these oscillations resulted in substantial microtubule elongation in that individual microtubules remained remarkably similar in length throughout the time course of the experiment (see also Baas and Ahmad, 1993, wherein we performed serial reconstructions from electron micrographs). Taken together, these results indicate that while not impossible, it is unlikely that microtubule assembly events contributed in any substantial way to the redistribution of microtubules once vinblastine was added to the cultures.

Even though our vinblastine treatment favors net disassembly of microtubules, it is notable that the drug modulates disassembly events as well as assembly events that might otherwise occur (Wilson and Jordan, 1994; Tanaka et al., 1995). This may be particularly relevant to our observations on nonneuronal cells, whose microtubules are normally more labile than those in neuronal cells. In nonneuronal cells, it is thought that the release of a microtubule is followed by its rapid depolymerization from both ends (Kitanishi-Yumura and Fukui, 1987). If this is correct, the use of vinblastine in our studies may have permitted us to visualize released microtubules in nonneuronal cells that would otherwise have depolymerized. In contrast to the nonneuronal cells, neurons normally contain high levels of microtubule-stabilizing factors such as tau, MAP-2, and STOP (for discussion see Baas et al., 1994). These factors may normally prevent centrosomal microtubules from depolymerizing after their release, permitting them to live

long enough to become further stabilized and elongate to the great lengths typical of axonal microtubules. In this manner, differences in the stability of neuronal and nonneuronal microtubules may also contribute to their fate after release from the centrosome.

The differences we have documented are consistent with the very different microtubule arrays generated and maintained by neurons versus nonneuronal cells. Typical nonneuronal cells are mitotic and motile, while neurons are terminally postmitotic and consist of a stationary cell body from which processes grow. In nonneuronal cells, the centrosome nucleates and organizes the microtubules of the mitotic spindle, as well as the microtubules that extend to the leading edge of the interphase cell to guide its motility. In neurons, microtubules are utilized for a very different purpose, the growth of the axon. It seems reasonable that the more active release of microtubules from the neuronal centrosome may be related to the unique functional and morphological demands inherent in these differences. It is interesting in this regard that neurons differentiate from pleuripotent precursor cells, such as neural crest cells, that are highly motile and mitotic. We suspect that major alterations in the behavior of centrosomal microtubules occur during neuronal commitment and differentiation, and that these alterations may be quintessential for the development of axons and the acquisition of a neuronal phenotype. If this is correct, it may follow that the centrosome continues to act as a kind of time-clock for neuronal differentiation, increasing and decreasing its activity during subsequent developmental milestones such as the formation of dendrites. Studies are underway in our laboratory to investigate these interesting possibilities.

### **Implications of a centrosomal origin for axonal microtubules**

A centrosomal origin for axonal microtubules has profound implications for the cascade of events by which the axonal microtubule array is elaborated. For example, there has been a great deal of controversy in recent years concerning whether or not microtubules are transported from the cell body down the axon. Much of this controversy has stemmed from the failure of many studies to visualize microtubule movements within neurons using fluorescence-based live-cell approaches (for discussion see Joshi and Baas, 1993). Direct visualization of microtubule transport is clearly an important goal, but at present live-cell work is fraught with technical pitfalls, and one cannot draw strong conclusions from negative results. Our approach, while not permitting us to directly observe microtubules in motion, is free of these technical pitfalls, and has permitted us to document the transport of microtubules from one site in the neuron to another. In addition, a need for microtubule transport is a logical necessity if microtubules destined for the axon originate at a structure located within the cell body. All of these considerations indicating a fundamental role for microtubule transport in the axon are consistent with the classic work of Lasek and collaborators. On the basis of the kinetics of tubulin transport down the axon, these authors concluded that tubulin is transported in the form of the assembled microtubule (Hoffman and Lasek, 1975; Black and Lasek, 1980; Brady and Lasek, 1982). Our experimental paradigm is not optimal for studying the rates of microtubule transport in the axon given the impaired ability of the neurons to grow axons under conditions of such low microtubule mass

(see also Tanaka et al., 1995, in which vinblastine was found to inhibit axon growth in short-term experiments). Interestingly, however, the rate of microtubule transport from cell center to cell periphery is virtually identical to the rates of tubulin transport reported in Lasek's studies.

In addition to the need for microtubule transport, a centrosomal origin for axonal microtubules points to other key events that must occur during the elaboration of the axonal microtubule array. For example, the nucleation and release of microtubules within the cell body are presumably coordinated with the growth requirements of the axon. Regarding microtubule nucleation, the activity of the centrosome has been shown in nonneuronal cells to depend on the phosphorylation state of certain proteins in the pericentriolar material (Centonze and Borisy, 1990), and the same might be true in neurons. Another possibility is that the nucleation activity is related to the manner by which cells monitor and autoregulate levels of assembled and unassembled tubulin (for review see Cleveland, 1988). When a microtubule leaves the cell body, we would expect the monomer/polymer equilibrium to shift toward assembly, in turn lowering the concentration of free tubulin in the cell body. As a result, the cell will synthesize more free tubulin, once again driving assembly of more polymer. If nucleation of microtubules from the centrosome is strongly favored over elongation (as our data suggest, see Yu et al., 1993), this will result in an increased production of microtubules at the centrosome. With regard to microtubule release, proteins with microtubule severing activity may be involved. For example, centrin, the centrosomal protein we have used to identify the centrosome (see also Yu et al., 1993), has been shown to have a calcium-modulated microtubule severing activity at the basal bodies of flagellates (Sanders and Salisbury, 1989). Other microtubule severing proteins and activities have also been reported (see for example McNally and Vale, 1994) and may play a role in microtubule release from the neuronal centrosome.

Finally, a centrosomal origin for axonal microtubules is consistent with the need for local elongation of these microtubules once they arrive in the axon. Centrosomal microtubules are short, a few microns in length, whereas microtubules in the axon can become well over a hundred microns in length (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). We have argued that during their transit down the axon, many microtubules shorten or completely depolymerize to provide the subunits needed for the elongation of others (see Baas and Ahmad, 1993; Yu and Baas, 1994; Joshi and Baas, 1993). This idea is attractive in that it accommodates a centrosomal origin for axonal microtubules, fits the predictions of the dynamic instability model for the behavior of microtubules in a population (Kirschner and Mitchison, 1986), and is entirely consistent with observations from several different laboratories on the need for local microtubule assembly in the axon. Thus the release and transport of centrosomal microtubules are critical components of an elegant cascade of events that work together to generate the microtubule array of the axon.

This work was supported by grants from the National Institutes of Health and the National Science Foundation to P. W. Baas, who is also the recipient of a Research Career Development Award from the National Institutes of Health. We thank Dr Jeff Salisbury of the Mayo Clinic for providing the antibody to centrin, Dr Anthony Brown of

the Ohio University for critically reading an earlier version of the manuscript, and Ms Roxanne Rios for technical assistance.

## REFERENCES

- Ahmad, F. J., Joshi, H. C., Centonze, V. E. and Baas, P. W. (1994). Inhibition of microtubule nucleation at the neuronal centrosome compromises axon growth. *Neuron* **12**, 271-280.
- Baas, P. W., Deitch, J. S., Black, M. M. and Banker, G. A. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc. Nat. Acad. Sci. USA* **85**, 8335-8339.
- Baas, P. W., Slaughter, T., Brown, A. and Black, M. M. (1991). Microtubule dynamics in axons and dendrites. *J. Neurosci. Res.* **30**, 134-153.
- 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. and Joshi, H. C. (1992). Gamma-tubulin distribution in the neuron: implications for the origins of neuritic microtubules. *J. Cell Biol.* **119**, 171-178.
- Baas, P. W. and Ahmad, F. J. (1993). The transport properties of axonal microtubules establish their polarity orientation. *J. Cell Biol.* **120**, 1427-1437.
- Baas, P. W., Pienkowski, T. P., Cimbalka, K. A., Toyama, K., Bakalis, S., Ahmad, F. J. and Kosik, K. S. (1994). Tau confers drug stability but not cold stability to microtubules in living cells. *J. Cell Sci.* **107**, 135-143.
- Bamburg, J. R., Bray, D. and Chapman, K. (1986). Assembly of microtubules at the tip of growing axons. *Nature* **321**, 788-790.
- Belmont, L. D., Hyman, A. A., Sawin, K. E. and Mitchison, T. J. (1990). Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell* **62**, 579-589.
- Black, M. M. and Lasek, R. J. (1980). Slow components of axonal transport: two cytoskeletal networks. *J. Cell Biol.* **86**, 616-623.
- Black, M. M., Keyser, P. and Sobel, E. (1986). Interval between the synthesis and assembly of cytoskeletal proteins in cultured neurons. *J. Neurosci.* **6**, 1004-1012.
- Brady, S. T. and Lasek, R. J. (1982). Axonal transport: a cell biological method for studying proteins which associate with the cytoskeleton. *Meth. Cell Biol.* **25**, 365-398.
- Bray, D. and Bunge, M. B. (1981). Serial analysis of microtubules of cultured rat sensory neurons. *J. Neurocytol.* **10**, 589-605.
- Brinkley, B. R. (1985). Microtubule organizing centers. *Annu. Rev. Cell Biol.* **1**, 145-172.
- Brinkley, B. R., Cox, S. M., Pepper, D. A., Wible, L., Brenner, S. L. and Pardue, R. L. (1981). Tubulin assembly sites and the organization of cytoplasmic microtubules in cultured mammalian cells. *J. Cell Biol.* **90**, 557-562.
- Centonze, V. E. and Borisy, G. G. (1990). Nucleation of microtubules from mitotic centrosomes is modulated by a phosphorylated epitope. *J. Cell Sci.* **95**, 405-412.
- Cleveland, D. W. (1988). Autoregulated instability of tubulin mRNAs: a novel eukaryotic regulatory mechanism. *Trends Biochem. Sci.* **13**, 339-343.
- Euteneuer, U. and McIntosh, J. R. (1981). Structural polarity of kinetochore microtubules in PTK2 cells. *J. Cell Biol.* **98**, 338-345.
- Evans, L., Mitchison, T. and Kirschner, M. (1985). Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* **100**, 1185-1191.
- Heidemann, S. R., Landers, J. M. and Hamborg, M. A. (1981). Polarity orientation of axonal microtubules. *J. Cell Biol.* **91**, 661-665.
- Hoffman, P. and Lasek, R. J. (1975). The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* **66**, 351-366.
- Jordan, M. A., Thrower, D. and Wilson, L. (1991). Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res.* **51**, 2212-2222.
- Jordan, M. A., Thrower, D. and Wilson, L. (1992). Effect of vinblastine, podophyllotoxin and nocodazole on mitotic spindles: implications for the role of microtubule dynamics in mitosis. *J. Cell Sci.* **102**, 402-416.
- Joshi, H. C. and Baas, P. W. (1993). A new perspective on microtubules and axon growth. *J. Cell Biol.* **121**, 1191-1196.
- Kellog, D. R., Moritz, M. and Alberts, B. M. (1994). The centrosome and cellular organization. *Annu. Rev. Biochem.* **63**, 639-674.
- Kirschner, M. and Mitchison, T. J. (1986). Beyond self-assembly: from microtubules to morphogenesis. *Cell* **45**, 329-342.



- Kitanishi-Yumura, T. and Fukui, Y.** (1987). Reorganization of microtubules during mitosis in Dictyostelium: dissociation from MTOC and selective assembly/disassembly in situ. *Cell Motil. Cytoskel.* **8**, 106-117.
- Lyser, K. M.** (1964). Early differentiation of motor neuroblasts in the chick embryo as studied by electron microscopy. I. General aspects. *Dev. Biol.* **10**, 433-466.
- Lyser, K. M.** (1968). An electron microscopic study of centrioles of differentiating neuroblasts. *J. Embryol. Exp. Morphol.* **20**, 343-354.
- McNally, F. J. and Vale, R. D.** (1993). Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell* **75**, 419-429.
- McBeath, E. and Fujiwara, K.** (1990). Microtubule detachment from the microtubule-organizing center as a key event in the complete turnover of microtubules in cells. *Eur. J. Cell Biol.* **52**, 1-16.
- 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.
- Sanders, M. A. and Salisbury, J. L.** (1989). Centrin mediated microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **108**, 1751-1760.
- Sharp, G. A., Weber, K. and Osborn, M.** (1982). Centriole number and process formation in established neuroblastoma cells and primary dorsal root ganglion neurons. *Eur. J. Cell Biol.* **29**, 97-103.
- Tanaka, E., Ho, T. and Kirschner, M. W.** (1995). The role of microtubule dynamics in growth cone motility and axonal growth. *J. Cell Biol.* **128**, 139-155.
- Tilney, L. G., Bryan, J., Bush, D. J., Fujiwara, K., Mooseker, M. S., Murphy, D. B. and Snyder, D. H.** (1973). Microtubules: evidence for 13 protofilaments. *J. Cell Biol.* **59**, 267-275.
- Tsukita, S. and Ishikawa, H.** (1981). The cytoskeleton of myelinated axons: serial section study. *Biomed. Res.* **2**, 424-437.
- Wilson, L. and Jordan, M. A.** (1994). Pharmacological probes of microtubule function. In *Microtubules* (ed. J. S. Hyams and C. W. Lloyd), pp. 59-84. Wiley-Liss, New York.
- Yu, W., Centonze, V. E., Ahmad, F. J. and Baas, P. W.** (1993). Microtubule nucleation and release from the neuronal centrosome. *J. Cell Biol.* **122**, 349-359.
- Yu, W. and Baas, P. W.** (1994). Changes in microtubule number and length during axon differentiation. *J. Neurosci.* **14**, 2818-2829.
- Zheng, J., Buxbaum, R. E. and Heidemann, S. R.** (1993). Investigation of microtubule assembly and organization accompanying tension-induced neurite initiation. *J. Cell Sci.* **104**, 1239-1250.

(Received 20 March 1995 - Accepted 2 May 1995)