

Progressive and Spatially Differentiated Stability of Microtubules in Developing Neuronal Cells

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Abstract. The establishment of neural circuits requires both stable and plastic properties in the neuronal cytoskeleton. In this study we show that properties of stability and lability reside in microtubules and these are governed by cellular differentiation and intracellular location. After culture for 3, 7, and 14 d in nerve growth factor-containing medium, PC-12 cells were microinjected with X-rhodamine-labeled tubulin. 8–24 h later, cells were photobleached with a laser microbeam at the cell body, neurite shaft, and growth cone. Replacement of fluorescence in bleached zones was monitored by digital video microscopy. In 3-d cultures, fluorescence recovery in all regions occurred by 26 ± 17 min. Similarly, in older cultures, complete

fluorescence recovery at the cell body and growth cone occurred by 10–30 min. However, in neurite shafts, fluorescence recovery was markedly slower (71 ± 48 min for 7-d and 201 ± 94 min for 14-d cultures). This progressive increase in the stability of microtubules in the neurite shafts correlated with an increase of acetylated microtubules. Acetylated microtubules were present specifically in the neurite shaft and not in the regions of fast microtubule turnover, the cell body and growth cone. During the recovery of fluorescence, bleached zones did not move with respect to the cell body. We conclude that the microtubule component of the neuronal cytoskeleton is differentially dynamic but stationary.

DEVELOPMENT of the nervous system requires that the neuronal cytoskeleton be endowed with the seemingly incompatible properties, morphological stability and plasticity. Morphological stability is critical for maintenance and proper function of neuronal circuits, while plasticity is required for cell growth and remodeling of cell processes during response to environmental inputs or to injury. How does the cytoskeleton adapt to these different requirements? Are there changes in the cytoskeleton that reflect the state of cellular differentiation?

Microtubule formation is required for the growth and structural integrity of neuronal processes (17, 18, 62, 63) and numerous studies have demonstrated that neuronal microtubules exhibit unusual stability properties. For example, a significant fraction of tubulin in brain is not solubilized by conventional methods of temperature-reversible, assembly-disassembly procedures (10, 13, 27, 50, 59). Further, the susceptibility of microtubules to depolymerization by colchicine diminishes with increased culture age (6, 17).

Several factors have been postulated to enhance microtubule stability. By binding microtubule-associated proteins microtubule stability increases (4, 40), and neurons contain a variety of microtubule-associated proteins of which MAP-1, MAP-2, tau, and chartins are quite prominent (5, 8, 9, 42, 44, 45). Neuronal tubulin also undergoes posttranslational modifications including detyrosination/tyrosination (2, 49), acetylation (7), and phosphorylation (21, 22). Although the physiological relevance of the differential binding reactions

with microtubule-associated proteins and the posttranslational modifications of tubulin is not known, they both are possible mechanisms for regulating the dynamic behavior of microtubules.

To evaluate the relationship between stability of microtubules and cell function, we need to identify regions of different stability and determine how they are affected by controlled conditions. Relative stabilities of microtubules have previously been assayed indirectly by measuring the amount of polymer that remains after cold- or drug-induced depolymerization (6, 10). Stable microtubules have been found along the length of axons treated with cold or depolymerizing drugs (27, 41) and tubulin can be incorporated into microtubules along the length of the axon (43). However, the rate of microtubule turnover and its possible modulation has not been directly assayed.

In this study, we have measured the rate of microtubule turnover in functionally distinct domains within the cell and at different developmental stages. We combined techniques of microinjection of X-rhodamine-labeled tubulin, fluorescence redistribution after photobleaching (31), and direct video microscopy (51) to determine directly the turnover time and hence the stability of microtubules.

We report a progressive increase in stability of microtubules in the neurite shaft of PC-12 cells as they matured in nerve growth factor (NGF)¹-containing medium. Within a

1. Abbreviation used in this paper: NGF, nerve growth factor.

cell, microtubules in the morphologically stable neurite shaft were less dynamic than those in growth cone and cell body. The intracellular distribution of stable microtubules and its enhancement during differentiation correlated with the distribution of acetylated alpha tubulin.

Materials and Methods

Cell Culture

PC-12 cells (gift from Dr. Erik Schweitzer, Department of Anatomy, University of Wisconsin, Madison, WI) were cultured in DME supplemented with 20 mM Hepes, 5% horse serum, and 5% calf serum at 37°C, 9% CO₂. In preparation for an experiment, trypsinized cells were plated onto 22-mm coverslips that had been previously carbon coated through a 400-mesh locator grid (Ted Pella, Inc., Tustin, CA) which was used as a mask to form a locator pattern in the carbon film. The newly trypsinized cells were allowed to settle overnight in NGF-free media. Thereafter, NGF (Sigma Chemical Co., St. Louis, MO) was added to 100 ng/ml and cells were continued in culture for 3, 7, or 14 d.

Protein Preparation

Microtubule protein was obtained from porcine brain by cycles of assembly and disassembly (11). Pure tubulin was prepared by DEAE-cellulose column chromatography (57). X-rhodamine labeling of tubulin was performed as previously described (51).

Microinjection

Before microinjection, the coverslips of cells were transferred to a 35-mm tissue culture dish with an 18-mm hole in the bottom, and adhered with a lining of vacuum grease (Dow Corning Corp., Midland, MI). Dishes were then returned to the incubator until microinjection.

Just before use, an aliquot of X-rhodamine tubulin was thawed and spun at 20,000 g for 30 min to remove particulates. Micropipettes with 1–2- μ m-diam tips were loaded with 0.25–0.5 μ l of X-rhodamine tubulin. Air pressure was provided by a gas-tight syringe (Hamilton Co., Reno, NV) that allowed a regulatable flow rate. A Leitz micromanipulator (Laborlux) was used for micromanipulation. Injected volumes were estimated by eye to be between 5 and 10% of the volume at the neuronal cell body.

Photobleaching

The photobleaching apparatus was assembled according to the methods of Petersen et al. (46). An argon ion laser (model 2020; Spectra-Physics Inc., Mountain View, CA) and a Zeiss IM35 microscope were anchored to a vibration table (Newport Research Corp., Mountain View, CA). A cylindrical lens was positioned to produce a focused, 4 \times 57- μ m beam cross section in the specimen plane when a neofluar 100 \times , 1.3 NA objective was used (51). The laser was operated at 300 MW for 300 ms and the beam attenuated by a 0.6 OD neutral density filter. Given the dimensions of the zone of irradiation, and correcting for the reflectivity of mirrors, lenses, and filters, the light intensity at the specimen was estimated to be 60 MW/m². This exposure (60 MW/m² \times 300 ms = 18 MJ/m²) was eightfold less than the exposure (71 MW/m² \times 2 s = 142 MJ/m²) reported to cause dissolution of microtubules *in vitro* (58).

Microinjected cells were allowed to equilibrate with the labeled tubulin for 8–24 h. The culture dishes were then transferred to the microscope stage and maintained at 34–35°C with an air-curtain incubator (Nicholson Precision Instruments, Bethesda, MD). An injected cell was selected and then photobleached one to three times at different locations. Phase-contrast images and fluorescence images were taken before and after photobleaching to record the progress of the cell during recovery of the bleaching zones.

To prevent excessive irradiation, cells were observed with fluorescence microscopy at intervals of 10–60 min. Recovery from photobleaching was assayed by comparing the radiance in the bleached zone with that in adjacent regions. The time of complete recovery of a bleached zone was the last time-point at which the radiance of the bleached zone was indistinguishable from that of adjacent regions. The relative radiance was estimated by eye and confirmed in test cases by measurements with an image processor (model QX-9200; Quantex Corp., Sunnyvale, CA).

Indirect Immunofluorescence Microscopy

PC-12 cells were rapidly rinsed with two changes of buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM MgCl₂, pH 6.95) at room temperature and the cells were lysed for 90 s in 0.15% Triton X-100 in the same buffer (53). Cells were then fixed for 20 min in 5 mM ethylene glycol bis (succinic acid *N*-hydroxy succinimide ester) (Sigma Chemical Co.) in the same buffer (23). After fixation, cells were rinsed several times with PBS (140 mM NaCl, 3 mM KCl, 10 mM PO₄, pH 7.3) containing 3 mM Na₂S₂O₃. The fixative was then quenched with 0.1 M glycine, pH 7.0, for 10 min. Cells were rinsed with PBS and processed for immunolabeling immediately or stored overnight at 4°C.

For double-label indirect immunofluorescence, cells were incubated with one of the two sets of primary antibodies: (a) rabbit anti-tyr-tubulin (61) (a gift from Dr. Chloe Bulinski, Columbia University, New York) and mouse anti-acetylated tubulin (47, 48) (a gift from Dr. Gianni Piperno, Rockefeller University, New York); (b) rat anti-tyr-tubulin (33) (Accurate Chemical and Scientific Corp., Westbury, NY) and rabbit anti-glu-tubulin (58) (gift from Dr. Chloe Bulinski). After three rinses of 15 min each in PBS, the coverslips were incubated with the corresponding secondary antibodies, fluorescein-conjugated anti-rabbit IgG, Texas red-conjugated anti-mouse IgG, and Texas red-conjugated anti-rat IgG. After rinsing in PBS the coverslips were mounted in a polyvinyl alcohol antibleaching solution (51).

To confirm the pattern of distribution of the acetylated tubulin and the glu-tubulin, antibodies to each were processed individually for indirect immunofluorescence microscopy. Their distribution was consistent with those observed for double-label experiments.

Results

Experimental Rationale

A principal advantage of the PC-12 cell line for examining neurite extension and maintenance is that the cells form processes in response to NGF, but do not require the factor for viability. Hence cells can be compared before and after various times of NGF treatment. In the absence of NGF, PC-12 cells proliferate and express the phenotypic properties of adrenal chromaffin cells, their nonneoplastic counterparts. Exposure of cells to NGF causes an alteration in phenotype such that they acquire numerous characteristics of sympathetic neurons including outgrowth of long branching neurites containing parallel arrays of microtubules (24).

Labeled tubulin microinjected into PC-12 cells became incorporated into polymeric structures within 1 h. This was evidenced by the appearance of nonextractable, labeled material that remained after detergent lysis (43; Sammak, P. J., S. S. Lim, and G. G. Borisy, manuscript submitted for publication). However, to ensure that experiments were done at steady state, the microinjected cells were incubated for an additional 8–24 h before photobleaching. For the photobleaching experiments to report faithfully on microtubule dynamics, it was essential that the intensity of the laser beam be selected such that it bleached the fluorophore without disrupting the integrity of the microtubules. Immediately afterwards, a video image was captured and the cell processed for indirect immunofluorescence with antitubulin to assay for laser-induced microtubule damage (Fig. 1). Under the conditions of our experiments, microtubules were continuous through the bleached zones. Other criteria for lack of perturbation by either microinjection or photobleaching included absence of beading or retraction of neurites, normal growth of neurites, and activity of growth cones during the periods of observation.

The extent and spatial pattern of fluorescence recovery was analyzed by image intensification and digital-imaging techniques. Complete recovery was estimated as the time when

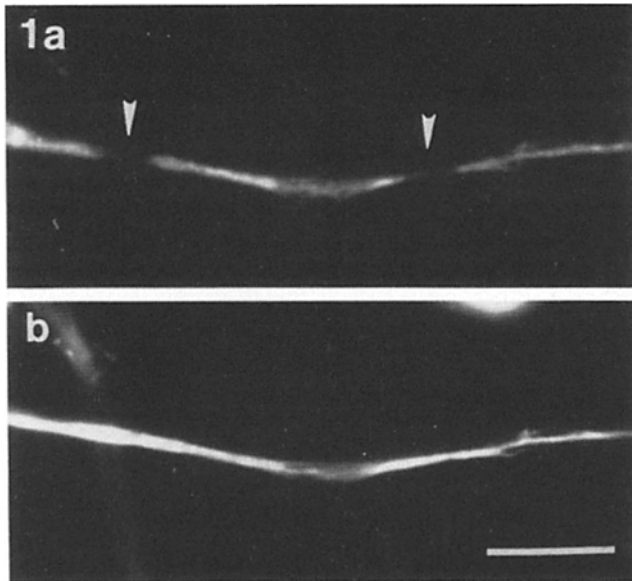


Figure 1. Microtubules are continuous through bleached zones. About 8–16 h after microinjection with X-rhodamine-tubulin, small regions of PC-12 neurites were photobleached (*a*, arrowheads). 7 min after photobleaching, this cell was lysed and fixed and subsequently processed for indirect immunofluorescence microscopy using an antitubulin antibody (YL1/2) and a FITC-conjugated anti-rat secondary antibody (*b*). Comparison of the two images show that microtubules are continuous through the bleached zones and were not broken by the photobleaching. Bar, 5 μ m.

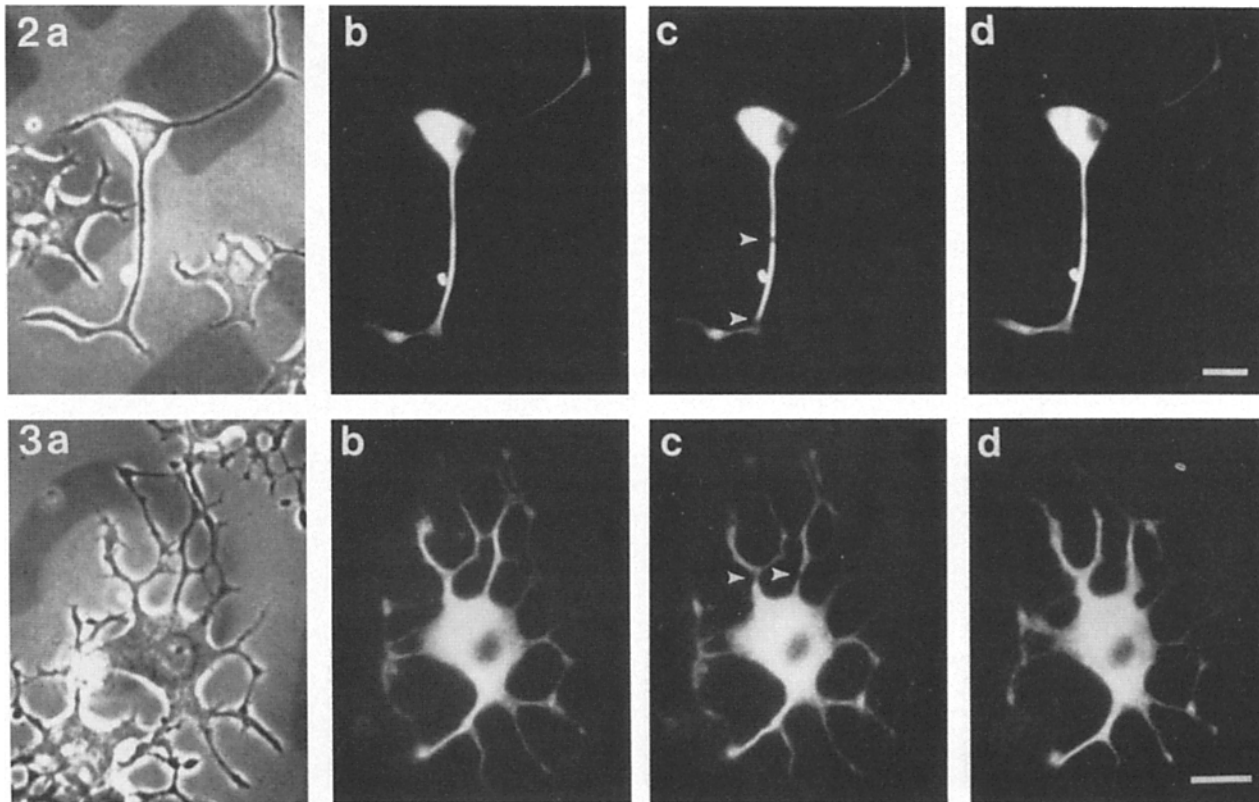
the bleached zones were no longer discernable from adjacent regions. The position of bleached zones was monitored with respect to the grid patterned coverslip on which the cells were grown. The cell body, neurite branch points, and pieces of debris also provided useful markers.

Microtubule Turnover Is Slower in Neurites of More Mature Cells

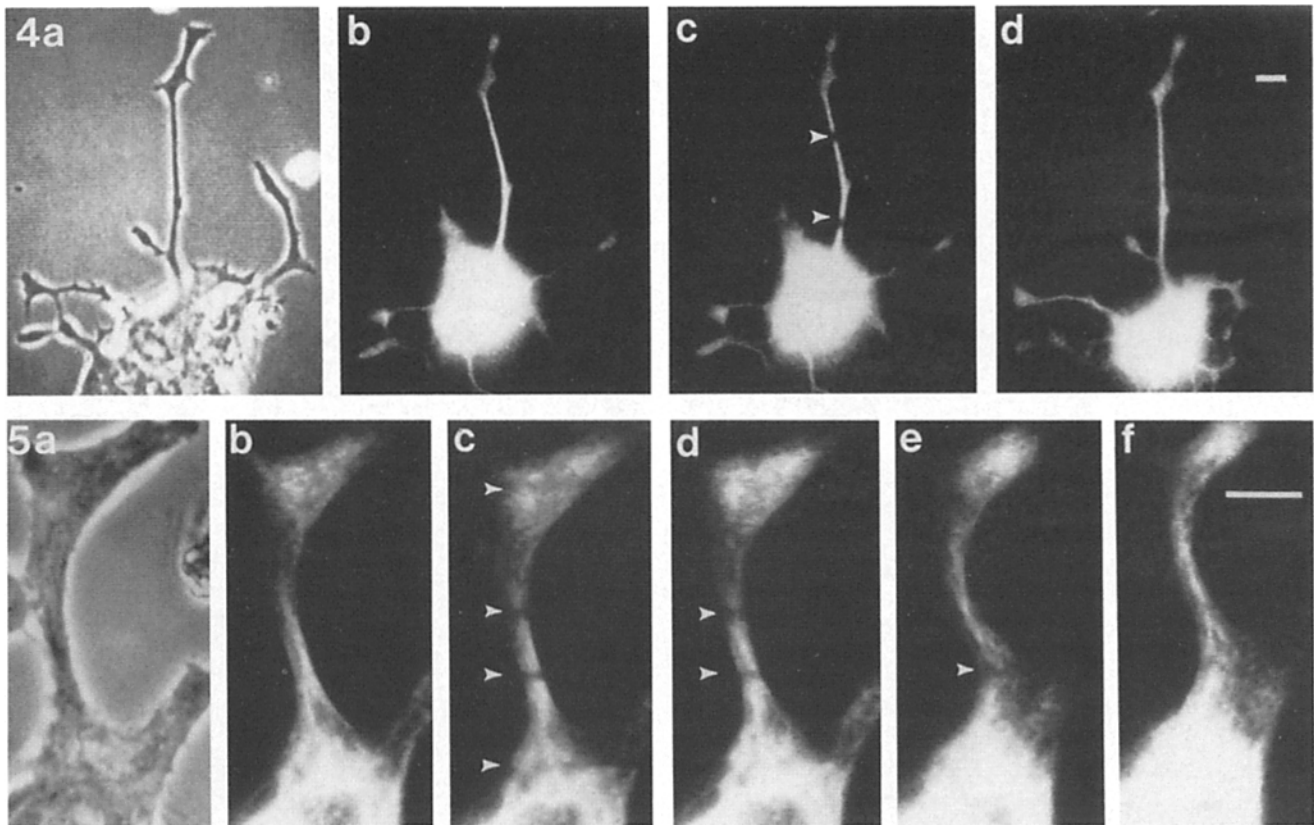
PC-12 cells were grown in NGF-containing medium for 3, 7, or 14 d, after which the cells were microinjected with X-rhodamine tubulin, allowed to equilibrate for 8–24 h, and photobleached. The response of PC-12 cells to NGF was not homogeneous as evidenced by the variation of the morphology of neurites within a population exposed to NGF-containing medium for the same amount of time.

Figs. 2 and 3 are examples at low magnification of 3-d cells with simple and highly branched neurites. Although the cells differed in neurite length and pattern, the recovery times were similar and reflective of the general behavior of cells grown for 3 d in NGF medium. Analysis of a total of 15 bleached zones in 12 cells indicated a mean recovery time 26 ± 17 min.

Analysis of a total of 52 bleached zones in 35 cells in the older cultures (7 and 14 d) indicated progressively slower microtubule turnover. In 7-d cultures, recovery in the neurite was 71 ± 48 min. As for younger cultures, there was no systematic dependence of the rate of recovery on neurite length or general morphology. Fig. 4 shows at low magnification a



Figures 2 and 3. Microtubule turnover is fast in neurites of young cultures (3 d in NGF medium). Cells with simple and complex neurite morphologies were analyzed. Comparison of the phase contrast image (*a*) and the subsequent direct fluorescence images (*b–d*) identifies the cell microinjected with the X-rhodamine-tubulin. The neurites were uniformly fluorescent before photobleaching (*b*). After irradiation with a bar-shaped beam of laser light, a discrete bleached zone was produced (arrows in Figs. 2 *c* and 3 *c*), time designation being 0 min. Recovery of the bleached zones (*d*) was almost complete within 17 (Fig. 1 *d*) and 18 min (Fig. 2 *d*). Bars, 16 μ m.



Figures 4 and 5. Microtubule turnover in neurites slows with increased time in culture (7 d in NGF medium). The rate of microtubule turnover in the neurite shaft differs from that in the cell body and growth cone. (a) Phase images; (b) fluorescent images of injected cells just before photobleaching; (c) fluorescent images captured immediately after photobleaching, time designation being 0 min. Complete turnover of microtubules in the neurite shaft required 1 (Fig. 4 d) to 2 h (Fig. 5 f). Four bleached zones (arrowheads) are apparent at the growth cone, neurite, and cell body in the cell at (Fig. 5 c, $t = 0$). 4 min later (Fig. 5 d), recovery was almost complete in the cell body and growth cone while the zones in the neurite were still detectable at 1 h 17 min (Fig. 5 e) recovering almost completely by at 1 h 56 min (Fig. 5 f). A bundle of microtubules is seen across the bleached zone during recovery (Fig. 5 d). Bars, 8 μm .

cell with two bleached zones placed on the neurite shaft. Both recovered at the same rate.

However, in both the 7- and 14-d cultures, microtubule dynamics in the cell body and growth cone were different from that in the neurite, as illustrated by the cell shown in Fig. 5. Here four bleached zones were produced, one in the cell body, two on the neurite shaft, and one in the growth cone. A portion of the cell is shown at higher magnification to facilitate comparison. The bleached zones placed in the cell body and growth cone clearly recovered before those in the neurite. Indeed, in this cell, substantial recovery occurred at these sites before the first video image after photobleaching was recorded and recovery was almost complete by 4 min (Fig. 5 d). In contrast, recovery in the neurite shaft required nearly 2 h and at this magnification, filamentous structures could be observed in the recovering bleached zone (Fig. 5 d). In some instances, bleached zones in the most proximal and distal segments of the neurites recovered at rates comparable to that in cell bodies and growth cones. However, all bleached zones placed within the central 3/5 of a neurite length consistently recovered more slowly.

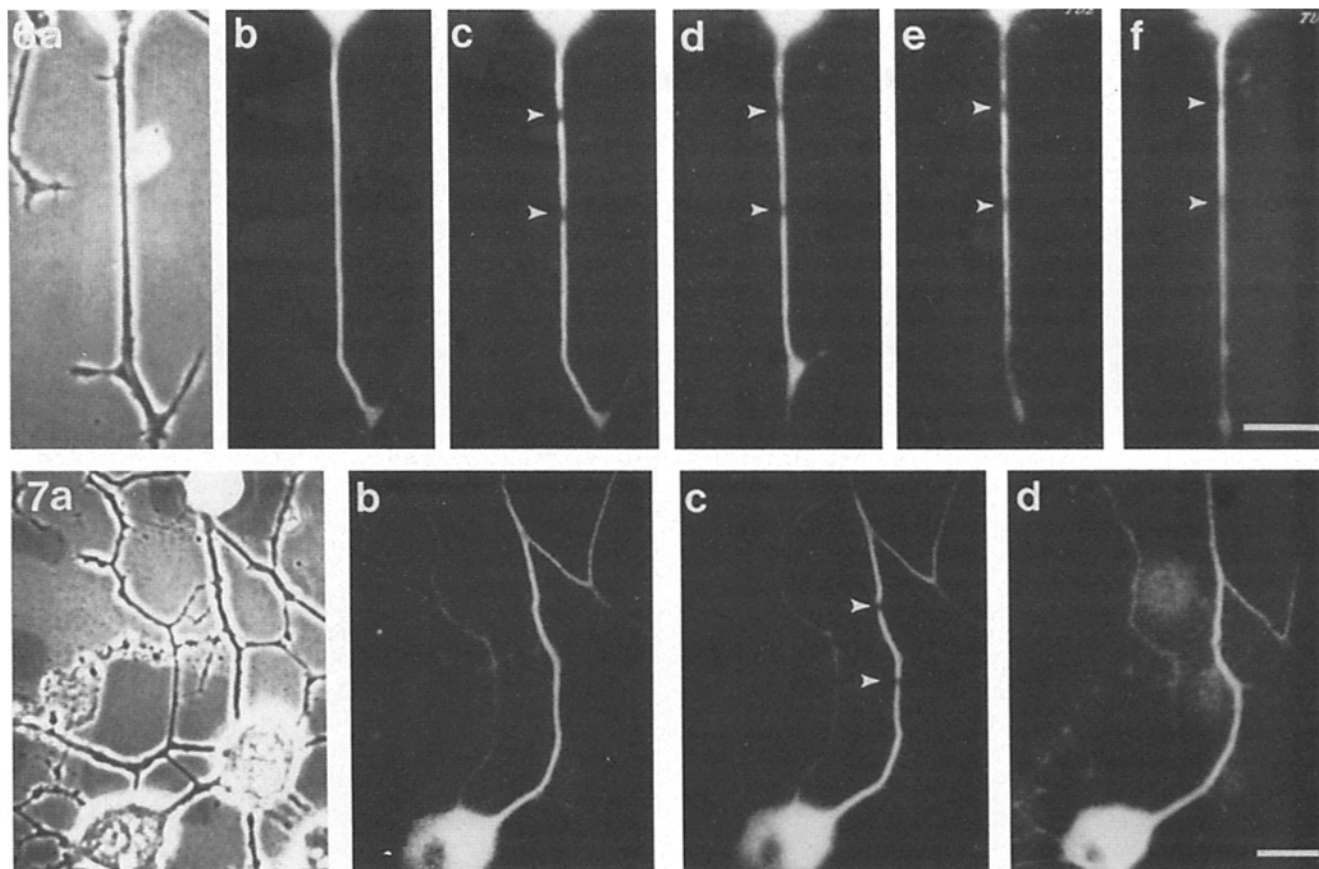
Microtubule dynamics were even slower in neurites of cells cultured for 14 d in NGF medium (recovery time = 201 ± 94 min). Cells by this time have quite elaborate networks of neurites and an even greater variation in neurite lengths.

Fig. 6 displays a cell with a well-defined neuritic process. A growth cone to the left of the cell body (Fig. 6 a) belonged to an adjacent uninjected cell and therefore was not fluorescent. Bleached zones placed on the neurite shaft were not quite fully recovered by 3.5 h. The cell in Fig. 7 had several neuritic branches and from the phase image alone, its neurites could not be distinguished from those of nearby cells, the culture having formed an interconnected network. The recovery of the bleached zones in this cell also fell within the range observed for 2-wk cultures. Therefore, recovery in a neurite shaft did not seem to depend on whether it was isolated or in contact with other cells.

Fig. 8 shows the recovery times of individual bleached zones in PC-12 cells grown in NGF-medium for 3, 7, and 14 d. While a range of recovery times was apparent within a population, it clearly demonstrates that increased time in NGF-containing medium can be correlated with a slowing of microtubule turnover as reflected by the increase in recovery times of the bleached zones.

Microtubule Turnover in Growth Cones Does Not Change with Increased Time in NGF-sustained Culture

While recovery times of bleached zones in neurite shafts increased with maturation of PC-12 cells in NGF-containing



Figures 6 and 7. Microtubule turnover in neurites of 14-d cultures is very slow. (a) Phase images; (b) fluorescent images of injected cells just before photobleaching. Immediately after photobleaching, two bleached zones are apparent on the neurite of each cell whose morphologies are quite different (compare Figs. 6 c and 7 c, $t = 0$). Subsequent images show the persistence of these bleached zones at (Fig. 6 d) $t = 30$ min, (Fig. 6 e) $t = 2$ h 22 min, with recovery almost complete by (Fig. 6 f) $t = 3$ h 29 min. The cell in Fig. 7 d required 4 h 30 min for complete recovery. During these periods of observation, the bleached zones remained stationary. Bars, 21 μ m.

medium, a correlative increase in recovery times for growth cones was not observed (Fig. 9). Fig. 9 a shows a distal end of a neurite terminating in two growth cones. The growth cone to the left consists of two large flattened processes. A bleached zone was first placed in the upper process (Fig. 9

c, arrow). Immediately afterwards, a bleached zone was placed on the second process (Fig. 9 c, arrowhead). The edge of this irradiating beam also bleached an adjacent portion of the neurite shaft, providing an internal comparison of recovery times for neurite and growth cone.

Bleached zones in the growth cone are generally not as distinct as those placed on neurites. Two factors contribute to this. First, the fluorescence intensity at growth cones is inherently lower because of a lesser density of microtubules as revealed by indirect antitubulin immunofluorescence. Second, due to the quick turnover of microtubules in this area, some recovery has already occurred between the time the cell was photobleached and the time the first possible video image can be captured. The lack of contrast between the bleached zone and its adjacent regions gives rise to less distinct bleached zones. Nevertheless, it was evident that bleached zones on the growth cone were fully recovered by 5.5 min (Fig. 9 d) while the bleached zone on the neurite persisted for an additional 2 h (Fig. 9, d-f), indicating that the growth cone was significantly more dynamic. During this time it can be seen that the tip of the neurite was actively changing its morphology.

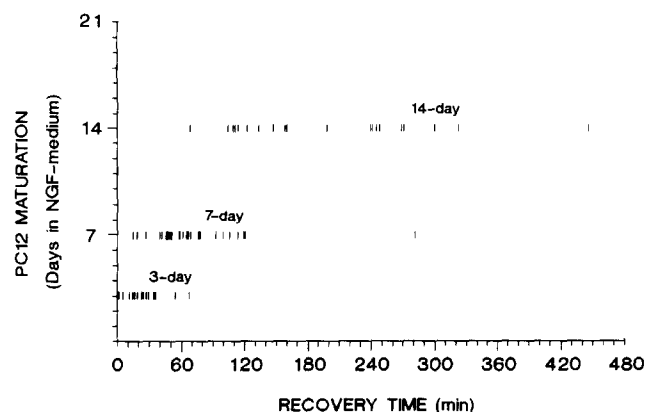


Figure 8. Longer times are required for recovery of bleached zones in more mature cultures of PC-12 cells. Recovery times for individual bleached zones are displayed for the three different cultures (3, 7, and 14 d). Mean recovery times were 26 ± 17 min ($n = 15$) for 3-d cells, 71 ± 48 min ($n = 30$) for 7-d cells, and 201 ± 94 min ($n = 20$) for 14-d cells.

Regions of Slow Microtubule Turnover Correlate with the Distribution of Acetylated Alpha Tubulin

The development of differential stability of microtubules

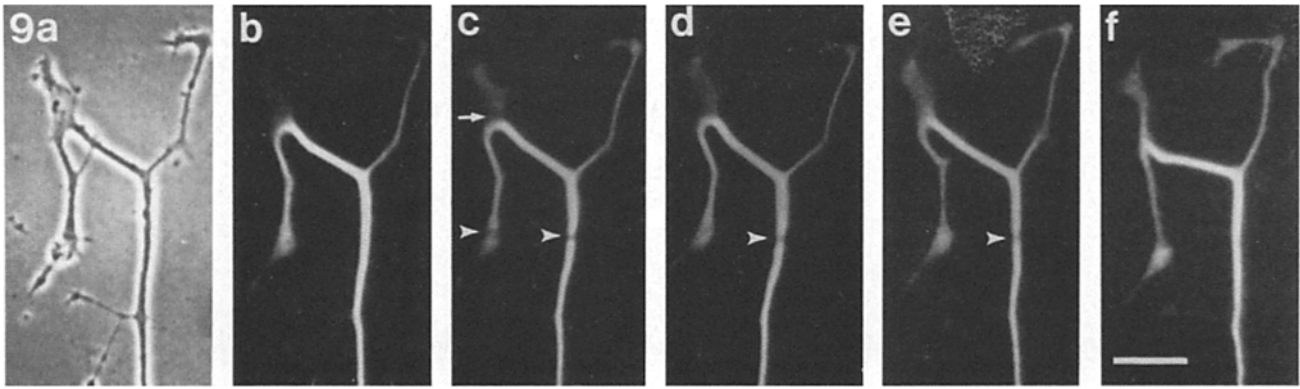


Figure 9. The slowing of microtubule turnover in neurites of older cultures is not observed in microtubules at the growth cone. This cell was incubated for 14 d with NGF-containing medium before microinjection. Two pulses of irradiation were made. The first (arrow) bleached one growth cone while the second (arrowheads) resulted in bleached zones in the growth cone and an adjacent neurite shaft. 6 min after photobleaching (d), bleached zones at the growth cones are no longer visible while the bleached zone on the neurite shaft was still apparent at (e) $t = 43$ min until recovery at (f) $t = 2$ h 29 min. Bar, 10 μm .

with maturation of PC-12 cells in NGF medium points toward some heterogeneity in the microtubule population within the cell and to progressive changes of the microtubules during maturation. One possible chemical modification of tubulin is an acetylation that can be tested with an antibody specifically reactive with acetylated alpha tubulin (47, 48, 60). In cells cultured for only 3 d in NGF medium, no reaction was observed except for labeling at the midbodies of occasional cells that were still undergoing mitosis (Fig. 10, *a* and *a'*). Cell bodies and neurites of these cells were not reactive indicating the absence of acetylated tubulin in young cultures.

By 7 d in culture, acetylated tubulin was apparent in neurites. Double-label indirect immunofluorescence using antibodies to both acetylated alpha tubulin and tyrosinated alpha tubulin (33) revealed spatial differences in the distribution of the two microtubule populations. In general, most neurites at this stage contained acetylated tubulin. However, the cell bodies as well as the distalmost segments of the neurites (Fig. 10, *b* and *b'*) lacked acetylated tubulin even though they contained microtubules. This pattern of labeling persisted in the 2-wk cultures. Neurite branching was more elaborate in these cultures, yet the growth cones, distalmost segments of neurites, and the cell body still lacked the acetylated tubulin (Fig. 10, *c* and *c'*). In some neurites, acetylated tubulin was distributed along the length of the neurite, but stopped just short of the growth cone (Fig. 10, *d* and *d'*). The increase in acetylated tubulin in neurites from 3–14 d in NGF culture correlated with the cellular domains that contained increasingly stabilized microtubules.

Regional Differences in Microtubule Turnover Do Not Correlate with the Distribution of Glu-Tubulin

Another posttranslational modification of alpha tubulin involves the removal of carboxy-terminal tyrosine leaving glu-

tamic acid as the carboxy-terminal residue. Although the function of this unique posttranslational modification is unknown, many studies have associated it with alterations of the cytoskeleton, especially during differentiation (26). Double-label indirect immunofluorescence using antibodies specific for glu- (61) or tyr-tubulin (33) did not show any apparent difference in the localization of the two populations of microtubules at 3, 7, and 14 d in culture. Glu-tubulin was present in the cell bodies and growth cones of the neurons (Fig. 11, *a* and *a'* and *b* and *b'*) as well as being distributed along the neurite length. The distribution of glu-tubulin did not correlate specifically with cytoplasmic domains containing stabilized microtubules.

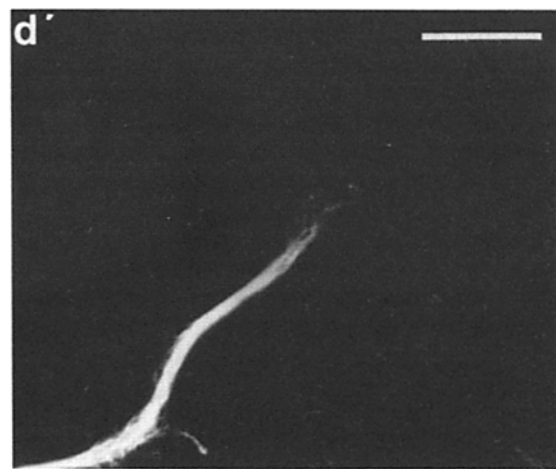
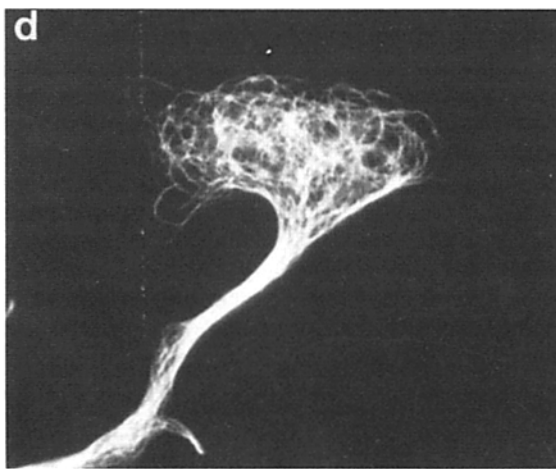
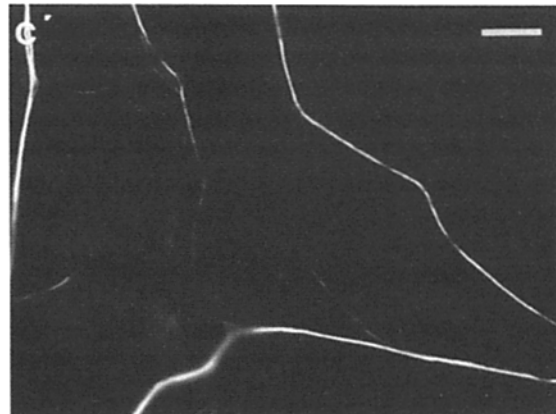
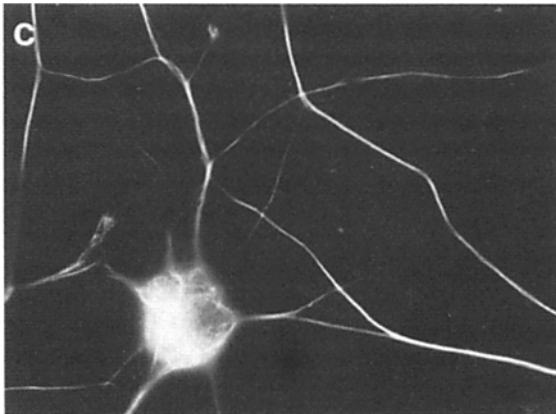
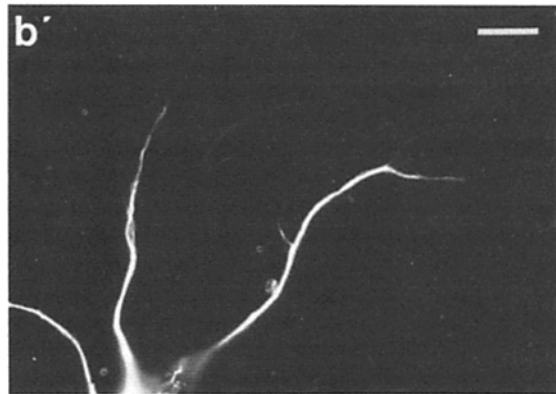
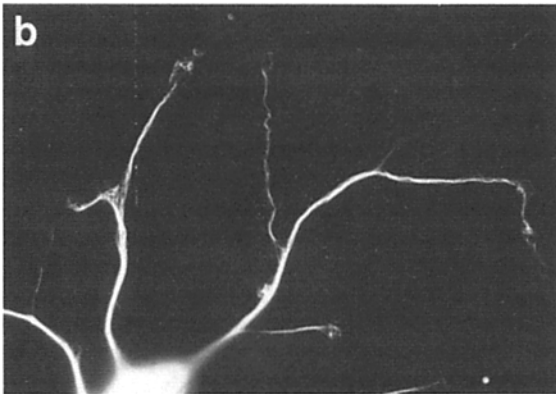
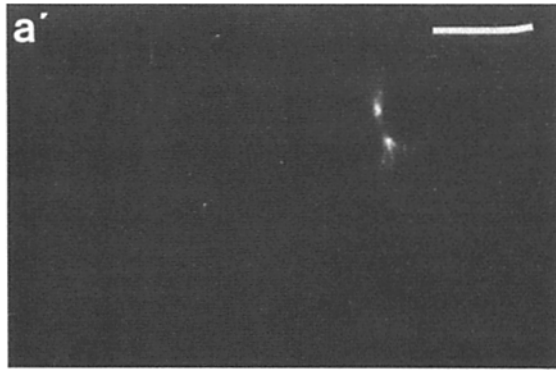
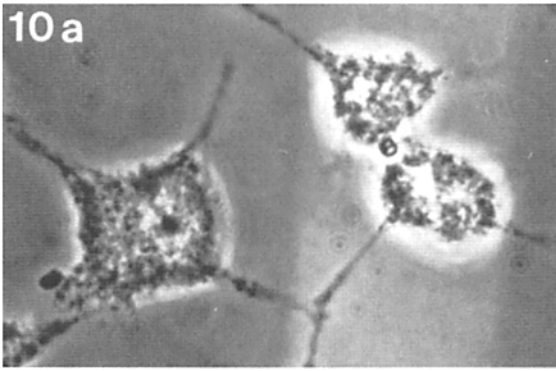
Microtubules Are Dynamic but Stationary with Respect to Their Position from the Cell Body

Bleached zones provide a marker for the microtubules in the irradiated zone. In all cells used in this study, no movement of the bleached zones occurred during the course of recovery. Since microtubule turnover was relatively fast in 3-d cultures, the bleached zones were lost in a shorter period of observation (10–40 min) than in older cultures. The more persistent bleached zones in the older cultures permitted analysis of possible motion in the neurites over a 1–4-h interval. During this period, the bleached zones showed no statistically significant movement with respect to the cell body ($0.0 \pm 0.7 \mu\text{m}/\text{h}$; Sammak, P. J., S. S. Lim, and G. G. Borisy, manuscript submitted for publication). In addition, in multiply bleached cells, the distance between bleached zones remained constant throughout the recovery process (Fig. 6).

Discussion

Microtubule dynamics in mammalian cells in culture have previously been studied in fibroblasts and epithelial cells (51,

Figure 10. Slow microtubule turnover correlates with the distribution of acetylated tubulin. PC-12 cells grown in NGF-containing medium for 3 (*a*), 7 (*b*), and 13 d (*c* and *d*) were processed for indirect immunofluorescence and double stained with antitubulin (*b–d*) and anti-acetylated tubulin (*a', b', c', and d'*). In 3-d cultures, acetylated tubulin was not detected in neurites, but was present in the midbody of a dividing cell (*a'*, compare with phase image in *a*). By 7 d in culture (*b'*), acetylated tubulin was present in most neurites except at the distalmost ends. This absence of acetylated tubulin at the cell body and growth cones persisted in cells cultured for 14 d (*c'* and *d'*). Bar, 10 μm .



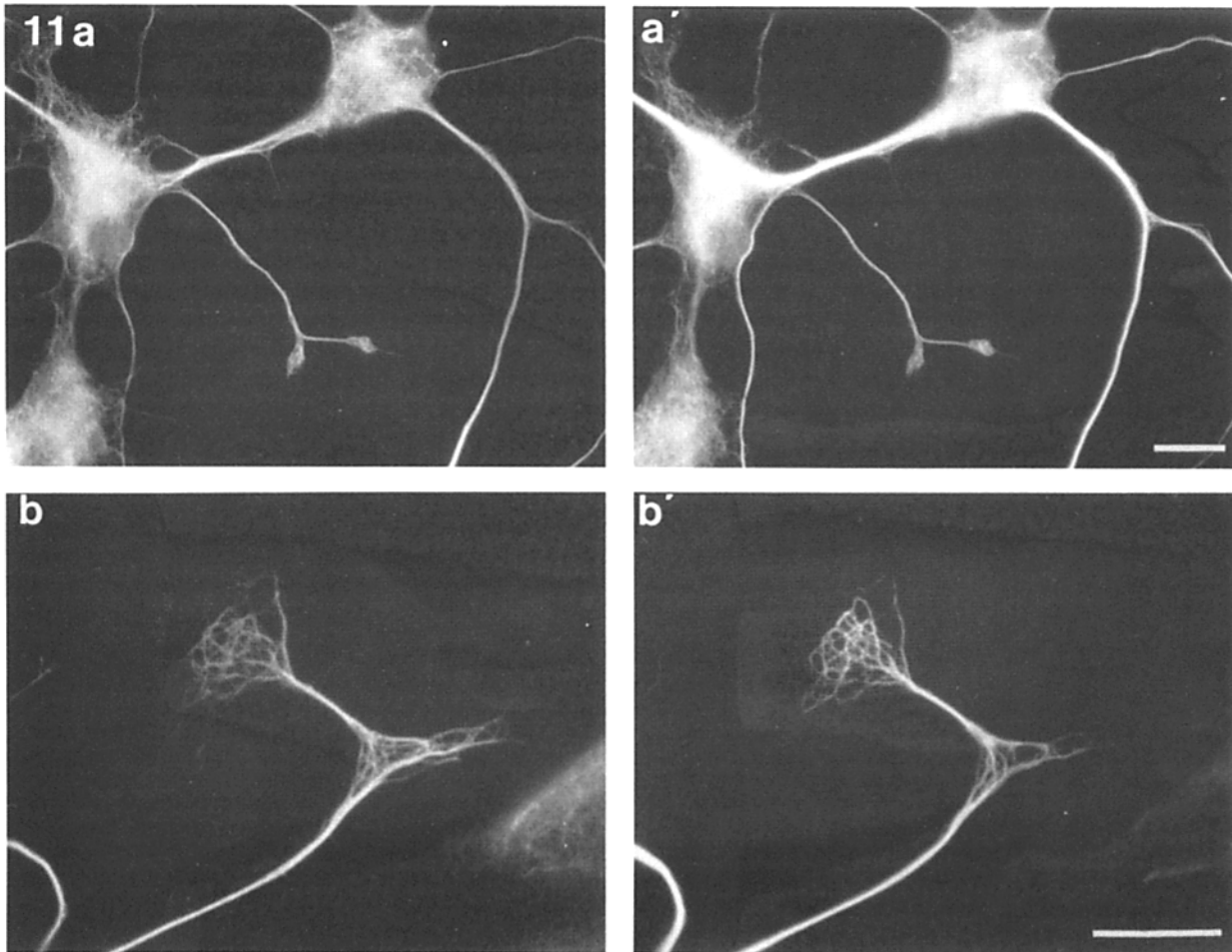


Figure 11. Regional differences in microtubule turnover do not correlate with the distribution of glu-tubulin. PC-12 cells grown in NGF-containing medium were processed for indirect immunofluorescence and double stained with anti-tyr-tubulin (*a* and *b*) and anti-glu-tubulin (*a'* and *b'*). The distribution of glu-tubulin was coincident with the tyr-microtubule population, and not restricted to regions of slow microtubule turnover. Bars, 10 μm .

52, 54–56). The major findings have been that the microtubule network turns over rapidly (half-times of 20–40 min) although subsets of stable microtubules do exist (26, 54, 60, 61). This study presents evidence that neuronal microtubules are dynamic. However, the progressive stabilization of microtubules with cellular differentiation and the localization of stable microtubules to a specific region are aspects of microtubule dynamics not described previously. Unlike fibroblasts which divide and undergo shape changes throughout their cell cycle, differentiated PC-12 cells cease division and have the capacity to form stable processes. The differences in microtubule dynamics that we describe could be a reflection of functional distinctions between the two cell types.

For a marker protein to report accurately on the behavior of the native tubulin, it should be able to compete effectively with the endogenous tubulin for assembly into polymer. Previous studies have established that derivatized brain tubulin assembles normally *in vitro* (51), incorporates into the bulk array of microtubules in a variety of cell types including PC-12 cells (43, 55, 56), and incorporates (although more slowly) into subsets of microtubules defined by the posttranslational modifications of deetyrosination and acetylation (60,

61). Therefore, we assume that the X-rhodamine-tubulin used in this study may be taken to report faithfully on the behavior of microtubules in neuronal processes.

Progressive Stabilization of Microtubules during Differentiation

Differences in the rate of recovery of bleached zones were observed in cultures that were in NGF for different durations. With increased time in culture, recovery became slower. What could account for the slowing in microtubule turnover? One explanation could lie in an increase of the average microtubule length. If turnover occurs at microtubule ends, longer microtubules will require more time to disassemble and reassemble, even if the rate constants remain unchanged. However, microtubules do not become dramatically longer in differentiating PC-12 neurites. In 3-d cultures, the average microtubule length has been reported to be 18 μm while that in 8-d cultures is $\sim 22 \mu\text{m}$ (30). The 20% increase in average length of microtubules cannot account for the measured fourfold increase in turnover time.

Alternatively, the difference in turnover time could be a reflection of the balance between the labile and progressively

stabilized microtubules. If labile microtubules predominate in younger cultures, this would result in the more rapid recovery of bleached zones. If stable microtubules predominate in older cultures, recovery of the bleached zones would be slower.

The differentiation of neighboring microtubules could be the result of some stabilizing factor which acts on microtubules directly or indirectly. This factor could appear by induction at some specific threshold of NGF exposure or it could accumulate progressively as a result of NGF activity. It has been shown that after treatment with NGF, PC-12 microtubules become less sensitive to depolymerization induced by colchicine or low temperature (6). Concomitant with this increase in stability of microtubules was the parallel induction of several factors known to affect microtubule stability. These include MAP-1 (19, 25), MAP-2 (9), and tau (19, 20), which increase dramatically after long-term treatment with NGF. Posttranslational modifications such as phosphorylation of beta tubulin (22), the reversible tyrosination/detyrosination of alpha tubulin (2), and the acetylation of alpha tubulin (7) have also been documented. Our study suggests that the progressive acetylation of microtubules could be one factor that contributes to their increased stability during differentiation.

The modulation of microtubule dynamics in cultures at different stages of differentiation is consistent with changes in neuronal behavior. PC-12 cells are highly labile in morphology shortly after NGF activation (29). More dynamic microtubules may permit rapid and reversible changes which contribute to the neurons's ability to reorganize during active growth. In vivo, neuritic elongation diminishes as relatively permanent connectivity between nerve cells is required for maintenance of neuronal function. Similarly, in PC-12 cells the gross morphology becomes more stable after 1 wk in NGF (29). Stable microtubules may be an important intracellular factor that constrain the capacity of the neuron to change its morphology, facilitating the maintenance of a preferred organization.

Microtubule Dynamics and Intracellular Location

Another aspect of the present study is the localization of stable and labile microtubules in specific regions within a single cell. With maturation in NGF-containing medium, we observed the preferential stabilization of the neurite shaft while the lability of the growth cone was maintained. These results are consistent with the behavior of neurons as seen in culture. Activity at the growth cones is often apparent while the neurite shaft remains relatively quiescent (1, 15, 16, 29). Repeated movement is observed as growth cones advance, divide, retract, and start again until a particular contact becomes stable. These changes in cell shape and locomotive behavior would require rapid assembly and disassembly of microtubules at the growth cone while microtubules in the neurite shaft could remain stabilized. Such spatial control of microtubule dynamics might also play a role in the observed promotion of neuritic branching at the growing nerve tip, while minimizing collateral sprouting along the neurite shaft (14).

Microtubule Dynamics and Posttranslational Modification of Tubulin

We observed that regions of stable microtubules correlated

with the distribution of acetylated alpha tubulin. The more labile microtubules in the neurites of 3-d cultures were not acetylated. A progressive increase in the distribution of acetylated microtubules occurred in parallel with a slowing down of microtubule turnover in neurites of older cultures. The regions of differential stabilities within a cell also demonstrate this relationship. Even though increasingly prevalent in the older cultures, acetylated tubulin is restricted to the neurite shaft and not to the regions of fast microtubule turnover, the growth cones, and cell bodies. These observations are consistent with previous work that links acetylation of alpha tubulin to increased resistance of microtubules to conditions that promote disassembly (38, 39, 47, 48). Microtubule domains containing acetylated tubulin are observed to turnover much more slowly than the nonacetylated bulk array of microtubules (60).

In similar experiments conducted using the anti-glu-tubulin antibody, no correlation could be made between the progressive stabilization of microtubules and the distribution of detyrosinated tubulin. Microtubules containing detyrosinated (glu) tubulin were present in the 3-d cultures (not shown) as well as in the 14-d cultures. Within a cell, their distribution was not confined to the more stable regions of the neurite, even though other studies have correlated an elevated level of detyrosinated subunits with a stable subset of microtubules (35, 61).

Posttranslational modifications such as the acetylation of alpha tubulin, represent a means for dynamically altering the chemistry of tubulin and microtubules at sites that are spatially removed from the protein synthetic machinery. This local control of the cytoskeleton could be important in enabling the cell to respond quickly to changes in the internal and external milieu and may be of particular relevance in the neuronal cells as growth cones are very often some distance from the cell body.

Sites of Microtubule Assembly

The structural hypothesis (37) holds that microtubules are assembled at the cell body and as microtubules, are transported distally along the neurite at rates which constitute slow axonal transport (12, 28, 36). The direct prediction would be that a bleached zone placed on fluorescent microtubules in the neurite would not recover its fluorescence but would move distally. However, in this and a previous study (Sammak, P. J., S. S. Lim, and G. G. Borisy, manuscript submitted for publication), the ability of all bleached zones to recover their fluorescence and the lack of movement of the bleached zones during these periods of observation suggests that a substantial portion of the microtubules are stationary but dynamic. These results are consistent with tip growth (3) and with the recent electron microscopic study of microtubule dynamics in PC-12 cells that showed rapid incorporation of labeled tubulin into the distal ends of microtubules minutes after microinjection (43).

In contrast to our results, Keith (32) reported movement of bleached zones on fluorescently labeled microtubules in regenerating neurites of PC-12 cells previously primed with NGF. Using cells under these culture conditions and our experimental methods, we could not reproduce his observations. Instead, we found that bleached zones in regenerating neurites show complete fluorescent recovery between 5 and

20 min during which time the bleached zones did not move. Although we cannot account for the differences in results, it should be noted that in Keith's study (32), tubulin was labeled with a different fluorophore (dichlorotriazinylamino fluorescein) and photobleached locally with 5 min of a mercury arc lamp filtered through the 450–490-nm fluorescein excitation filter.

The neuronal system is an example of how two elemental properties, plasticity and stability, are carefully balanced such that neuronal form can be developed and molded while at the same time selective stabilization of preferred forms is allowed. In this study, we present evidence that properties of lability and stability reside within neuronal microtubules, and that this differential stability is a function of both maturity and spatial location within the PC-12 cells.

We thank Dr. Erik Schweitzer for his gift of PC-12 cells, Dr. Chloe Bulinski for the use of glu-tubulin and tyr-tubulin antibodies, and Dr. Gianni Piperno for the acetylated tubulin antibody. We also thank John Pelouquin for the preparation of the X-rhodamine-tubulin.

This work was supported by National Institutes of Health grant GM25062.

Received for publication 16 December 1988 and in revised form 27 March 1989.

References

- Aletta, J. M., and L. A. Greene. 1988. Growth cone configuration and advance: a time-lapse video-enhanced differential interference contrast microscopy. *J. Neurosci.* 8:1425–1435.
- Arce, C. A., M. E. Hallak, J. A. Rodriguez, H. S. Barra, and R. Caputo. 1978. Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of the incorporation of these amino-acids on tubulin assembly. *J. Neurochem.* 31:205–210.
- Bamburg, J. R., D. Bray, and K. Chapman. 1986. Assembly of microtubules at tips of growing axons. *Nature (Lond.)* 321:788–790.
- Binder, L. L., A. Frankfurter, and L. I. Rebhun. 1986. Differential localization of MAP2 and tau in mammalian neurons in situ. *Ann. NY Acad. Sci.* 466:145–166.
- Binder, L. I., A. Frankfurter, H. Kim, A. Caceres, M. R. Payne, and L. I. Rebhun. 1984. Heterogeneity of microtubule-associated protein 2 during rat brain development. *Proc. Natl. Acad. Sci. USA.* 81:5613–5617.
- Black, M. M., and L. A. Greene. 1982. Changes in the colchicine susceptibility of microtubules associated with neurite outgrowth. Studies with nerve growth factor-responsive PC-12 pheochromocytoma cells. *J. Cell Biol.* 95:379–386.
- Black, M. M., and P. Keyser. 1987. Acetylation of alpha-tubulin in cultured neurons and the induction of alpha-tubulin acetylation in PC-12 cells by treatment with nerve growth factor. *J. Neurosci.* 7:1833–1842.
- Black, M. M., and J. T. Kurdyla. 1983. Microtubule-associated proteins of neurons. *J. Cell Biol.* 97:1020–1028.
- Black, M. M., J. M. Aletta, and L. A. Greene. 1986. Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. *J. Cell Biol.* 103:545–557.
- Black, M. M., J. M. Cochran, and J. T. Kurdyla. 1984. Solubility properties of neuronal tubulin: evidence for labile and stable microtubules. *Brain Res.* 295:255–263.
- Borisy, G. G., J. M. Marcum, J. B. Olmstead, D. B. Murphy, and K. A. Johnson. 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly in vitro. *Ann. NY Acad. Sci.* 253:107–132.
- Brady, S. T., and R. J. Lasek. 1982. Axonal transport: a cell biological method for studying proteins that associate with the cytoskeleton. *Methods Cell Biol.* 25:365–398.
- Brady, S. T., M. Tytell, and R. J. Lasek. 1984. Axonal tubulin and axonal microtubules: biochemical evidence for cold stability. *J. Cell Biol.* 99:1717–1724.
- Bray, D. 1982. Filopodial contraction and growth cone guidance. In *Cell Behaviour*. R. Bellairs, A. Curtis, and G. Dunn, editors. Cambridge University Press, Cambridge, England. 299–317.
- Bray, D. 1973. Branching patterns of individual sympathetic neurons in culture. *J. Cell Biol.* 56:702–712.
- Bray, D. 1970. Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci. USA.* 65:905–910.
- Daniels, M. P. 1975. The role of microtubules in the growth and stabilization of nerve fibers. *Ann. NY Acad. Sci.* 253:535–544.
- Daniels, M. P. 1972. Colchicine inhibition of nerve fiber formation in vitro. *J. Cell Biol.* 53:164–176.
- Drubin, D. G., S. C. Feinstein, E. M. Shooter, and M. W. Kirschner. 1985. Nerve growth factor-induced neurite outgrowth in PC-12 cells involves the co-ordinate induction of microtubule assembly and assembly-promoting factors. *J. Cell Biol.* 101:1799–1807.
- Drubin, D. G., D. Caput, and M. W. Kirschner. 1984. Studies on the expression of the microtubule-associated protein, tau, during mouse brain development, with newly isolated complementary DNA probes. *J. Cell Biol.* 98:1090–1097.
- Eipper, B. A. 1972. Rat brain microtubule protein: purification and determination of covalently bound phosphate and carbohydrate. *Proc. Natl. Acad. Sci. USA.* 69:2283–2287.
- Gard, D. L., and M. W. Kirschner. 1985. A polymer-dependent increase in phosphorylation of beta-tubulin accompanies differentiation of a mouse neuroblastoma cell line. *J. Cell Biol.* 100:764–774.
- Gorbsky, G. J., P. J. Sarnak, and G. G. Borisy. 1987. Microtubule dynamics and chromosome motion visualized in living anaphase cells. *J. Cell Biol.* 104:9–18.
- Green, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.
- Green, L. A., R. K. H. Liem, and M. L. Shelanski. 1983. Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. *J. Cell Biol.* 96:76–83.
- Gundersen, G. G., S. Khawaja, and J. C. Bulinski. 1987. Postpolymerisation dephosphorylation of alpha-tubulin: a mechanism for subcellular differentiation of microtubules. *J. Cell Biol.* 105:251–264.
- Heidemann, S. R., M. Hamborg, S. Thomas, B. Song, S. Lindley, and D. Chiu. 1985. Spatial organization of axonal microtubules. *J. Cell Biol.* 99:1289–1295.
- Heriot, K., P. Gambetti, and R. J. Lasek. 1985. Proteins transported in slow components a and b of axonal transport are distributed differently in the transverse plane of the axon. *J. Cell Biol.* 100:1167–1172.
- Jacobs, R. J., and J. K. Stevens. 1987. Dynamics of behaviour during neuronal morphogenesis in culture. *Cell Motil. Cytoskeleton.* 8:250–260.
- Jacobs, R. J., and J. K. Stevens. 1986. Changes in the organization of the neuritic cytoskeleton during nerve growth factor-activated differentiation of PC12 cells: a serial electron microscopic study of the development and control of neurite shape. *J. Cell Biol.* 103:895–906.
- Jacobson, K., E. Elson, D. Koppel, and W. Webb. 1983. International workshop on the application of fluorescence photobleaching techniques to problems in cell biology. *Fed. Proc.* 42:72–79.
- Keith, C. H. 1987. Slow transport of tubulin in the neurites of differentiated PC12 cells. *Science (Wash. DC)* 235:337–339.
- Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal anti-tubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576–582.
- Kirschner, M., and E. Schulze. 1986. Morphogenesis and the control of microtubule dynamics in cells. *J. Cell Sci.* 5(Suppl.):293–310.
- Kreis, T. E. 1987. Microtubules containing detyrosinated tubulin are less dynamic. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2597–2606.
- Lasek, R. J. 1986. Polymer sliding in axons. *J. Cell Sci.* 5(Suppl.):161–179.
- Lasek, R. J., and S. T. Brady. 1981. The structural hypothesis of axonal transport: two classes of moving elements. In *Axoplasmic Transport*. D. G. Weiss, editor. Springer-Verlag GmbH & Co., Berlin. 397–405.
- LeDizet, M., and G. Piperno. 1986. Cytoplasmic microtubules containing acetylated alpha-tubulin in *Chlamydomonas reinhardtii*: spatial arrangement and properties. *J. Cell Biol.* 103:13–22.
- L'Hernault, S. W., and J. L. Rosenbaum. 1983. *Chlamydomonas* alpha-tubulin is posttranslationally modified in the flagellate during flagellar assembly. *J. Cell Biol.* 97:258–263.
- Matus, A. 1988. Microtubule-associated proteins: their potential role in determining neuronal morphology. *Annu. Rev. Neurosci.* 11:29–44.
- Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192–198.
- Murphy, D. B., K. A. Johnson, and G. G. Borisy. 1977. Role of tubulin associated proteins in microtubule nucleation and elongation. *J. Mol. Biol.* 117:33–52.
- Okabe, S., and N. Hirokawa. 1988. Microtubule dynamics in nerve cells: analysis using microinjection of biotinylated tubulin into PC12 cells. *J. Cell Biol.* 107:651–664.
- Peng, I., L. I. Binder, and M. M. Black. 1986. Biochemical and immunological analyses of cytoskeletal domains of neurons. *J. Cell Biol.* 102:252–262.
- Peng, I., L. I. Binder, and M. M. Black. 1985. Cultured neurons contain a variety of microtubule-associated proteins. *Brain Res.* 361:200–261.
- Petersen, N. O., S. Felder, and E. L. Elson. 1986. Measurement of lateral diffusion by fluorescence photobleaching recovery. In *Handbook of Experimental Immunology*. 4th Ed. D. M. Weir, editor. Blackwell, Oxford. 1:24.1–24.22.
- Piperno, G., and M. T. Fuller. 1985. Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* 101:2085–2094.
- Piperno, G., M. LeDizet, and X. J. Chang. 1987. Microtubules containing

- acetylated alpha-tubulin in mammalian cells in culture. *J. Cell Biol.* 104:289-302.
49. Raybin, D., and M. Flavin. 1977. Modification of tubulin by tyrosylation in cells and extracts and its effects on assembly in vitro. *J. Cell Biol.* 73:492-504.
 50. Sahenk, Z., and S. T. Brady, 1987. Axonal tubulin and microtubules: Morphological evidence for stable regions on axonal microtubules. *Cell Motil. Cytoskeleton.* 8:155-164.
 51. Sammak, P. J., and G. G. Borisy. 1988. Detection of single fluorescent microtubules and methods for determining their dynamics in living cells. *Cell Motil. Cytoskeleton.* 10:237-245.
 52. Saxton, W. M., D. L. Stemple, R. J. Leslie, E. D. Salmon, M. Zavortink, and J. R. McIntosh. 1984. Tubulin dynamics in cultured mammalian cells. *J. Cell Biol.* 99:2175-2186.
 53. Schliwa, M., and J. van Blerkom. 1981. Structural interaction of cytoskeletal components. *J. Cell Biol.* 90:222-235.
 54. Schulze, E., and M. Kirschner. 1987. Dynamic and stable populations of microtubules in cells. *J. Cell Biol.* 104:277-288.
 55. Schulze, E., and M. Kirschner. 1986. Microtubule dynamics in interphase cells. *J. Cell Biol.* 102:1020-1031.
 56. Soltys, B., and G. G. Borisy. 1985. Polymerization of tubulin in vivo: direct evidence for assembly onto microtubule ends and from centrosomes. *J. Cell Biol.* 100:1682-1689.
 57. Vallee, R. B., and G. G. Borisy. 1978. The non-tubulin component of microtubule protein oligomers. *J. Biol. Chem.* 253:2834-2845.
 58. Vigers, G. P. A., M. Coue, and J. R. McIntosh. 1988. Fluorescent microtubules break up under illumination. *J. Cell Biol.* 107:1011-1024.
 59. Webb, B. C., and L. Wilson. 1980. Cold-stable microtubules from brain. *Biochemistry.* 19:1993-2001.
 60. Webster, D. R., and G. G. Borisy. 1988. Microtubules are acetylated in domains which turn over slowly. *J. Cell Sci.* 92:57-66.
 61. Webster, D. R., G. G. Gundersen, J. C. Bulinski, and G. G. Borisy. 1988. Differential turnover of tyrosinated and detyrosinated microtubules. *Proc. Natl. Acad. Sci. USA.* 84:9040-9044.
 62. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* 49:614-635.
 63. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1970. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. USA.* 66:1206-1212.