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)–containing medium. Within a
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, 5% 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., Tuspin, 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 (1). 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 20,000g for 30 min to remove particulates. Micropipettes with 1.2-μm diam tips were loaded with 0.25–0.5 μl 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 × 37-μm beam cross section in the specimen plane when a neofluar 100×/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 × 300 ms = 18 MJ/m2) was eightfold less than the exposure (71 MW/m2 × 2 s = 142 MJ/m2) reported to cause dissolution of microtubules in vivo (58).

**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 antibulin 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
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 (YLI/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 μm.

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
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 #m.

Fig. 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 #m.

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 medium, growth cones did not show this same increase in recovery times. The rate of microtubule turnover in growth cones was constant over the range of culture times studied, with similar bleached zones and recovery times observed in all instances. This suggests that the microtubule turnover rate in growth cones is not affected by increased time in NGF-containing medium.
Figures 6 and 7. Micromtubule 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. 6c and 7c, $t = 0$). Subsequent images show the persistence of these bleached zones at (Fig. 6d) $t = 30$ min, (Fig. 6e) $t = 2$ h 22 min, with recovery almost complete by (Fig. 6f) $t = 3$ h 29 min. The cell in Fig. 7d required 4 h 30 min for complete recovery. During these periods of observation, the bleached zones remained stationary. Bars, 21 μm.

medium, a correlative increase in recovery times for growth cones was not observed (Fig. 9). Fig. 9a 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. 9c, arrow). Immediately afterwards, a bleached zone was placed on the second process (Fig. 9c, 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. 9d) while the bleached zone on the neurite persisted for an additional 2 h (Fig. 9d–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.

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

The development of differential stability of microtubules...
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 cell bodies as well as the 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 glutamic 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 ± 0.7 μm/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,
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.

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 ~22 μm (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 neuron'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 locomotory 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 (dichlorotriazinylarnino 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.

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