Changes in the Colchicine Susceptibility of Microtubules Associated with Neurite Outgrowth: Studies with Nerve Growth Factor-responsive PC12 Pheochromocytoma Cells

MARK M. BLACK and LLOYD A. GREENE

Department of Pharmacology, New York University Medical Center, New York, New York 10016. Dr. Black's present address is Department of Anatomy, Temple University School of Medicine, Philadelphia, Pennsylvania 19140.

ABSTRACT The PC12 line of nerve growth factor (NGF)-responsive rat pheochromocytoma cells was used as a model system to determine whether properties of microtubules change during neurite growth and maturation. In the absence of NGF, PC12 cells lack processes. After several days with NGF, PC12 cells begin extending neurites and, by 2–3 wk with NGF, PC12 cells have long (~1 mm), highly branched neurites. We examined the effect of colchicine on microtubules of PC12 cells grown without NGF or with NGF for 1 or 21 d. PC12 cells grown under the various conditions were exposed to 50 μM colchicine for 1 or 6 h, and were then assayed for their content of polymerized tubulin using a biochemical assay. Microtubule levels in drug-treated cultures were compared to those in non-drug-treated control sister cultures.

PC12 cells grown without NGF or with NGF for 1 d were depleted of MT by 1 h with colchicine. In contrast, microtubule levels in long-term NGF-treated cells exposed to colchicine for 6 h were reduced to only ~57% of those in control cells. Control experiments indicated that the observed differential susceptibility to colchicine was not due to differences in colchicine uptake or to the effects of colchicine on cell viability. These observations suggest that microtubules of PC12 cells grown without NGF or with NGF for 21 d differ in their properties. Such differences may be related to one or more of the changes in structure and/or motility that result from treatment with NGF.

The determinants of neuronal morphology are present within the neuron as well as within its environment (35, 39). Several lines of evidence indicate that the cytoskeleton is the structural basis for cell morphology (6, 10–12, 14, 15, 29, 44, 46, 50, 51). Thus, it is likely that the various determinants of neuronal morphology act either directly or indirectly on the neuronal cytoskeleton, specifying its structural properties and pattern of organization. The experiments presented here examine specific properties of the cytoskeleton during neurite growth and maturation to obtain a better understanding of how the cytoskeleton is involved in the elaboration and maintenance of neuron morphology.

Microtubules are major components of the neuronal cytoskeleton (33). Their involvement in neurite growth was first indicated by ultrastructural studies of the organization of microtubules in developing neurons (34, 43, 50) and, subsequently, by the demonstration that colchicine and other agents that promote microtubule depolymerization inhibit the initiation of neurite outgrowth *in vitro* and also cause neurites of cultured embryonic neurons to retract (10, 50). The ability of colchicine to promote neurite retraction *in vitro* appears to diminish with increasing culture age (12). One interpretation for this observation is that properties of microtubules change during neuronal growth and maturation. Such changes may be related to the elaboration and stabilization of neurite structure.

In the experiments reported here, the PC12 clonal line of rat pheochromocytoma cells (19) was used as a model system to determine whether properties of microtubules change during neurite growth and maturation. PC12 cells can be induced to extend neurites by treatment with nerve growth factor (NGF) (19), a protein involved in the growth and maturation of sympathetic and dorsal root ganglia neurons (26). PC12 cells grown without NGF proliferate and lack processes (19, 47). After exposure to NGF for several days, PC12 cells cease mitosis and express many features that are characteristic of neurons (19), including neurite outgrowth. Neurites of PC12 cells elongate at a rate of 30–50 μm/d, and neurite growth continues as long as NGF is present in the growth medium

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 NOVEMBER 1982 379-386

(18). In view of the evidence indicating that microtubules are involved in the elaboration and maintenance of cellular asymmetries (7, 15, 34, 44, 46), it is likely that microtubules are involved in NGF-induced neurite outgrowth by PC12 cells. This possibility is supported by the observations that PC12 neurites contain arrays of microtubules that are oriented parallel to their long axes (27, 47), and that colchicine and nocodazole, drugs that promote microtubule depolymerization (13, 28), inhibit the initiation of neurite outgrowth by PC12 cells (18).

We have used colchicine as a probe to compare microtubules of PC12 cells grown in the absence and in the presence of NGF. The results of this comparison demonstrate that microtubules of long-term NGF-treated cells (neurite-bearing) are much less susceptible to disruption by colchicine than are microtubules of cells grown in the absence of NGF (nonneurite-bearing). A preliminary account of this work has been published (2).

MATERIALS AND METHODS

Cell Culture

The experiments reported here used PC12 cells grown without NGF or with NGF for 1 or 21 d. In all experiments, cells were grown on Falcon culture dishes coated with rat-tail collagen. When cells were treated with colchicine, an appropriate volume of a 15 mM stock solution (freshly prepared) was added to the culture medium to a final concentration of 50 μM. Cells were grown in RPMI 1640 supplemented with 10% horse serum + 5% fetal calf serum (15% serum) or just 1% horse serum.

The assay of polymerized tubulin in PC12 cells (see below) requires that cultures consist of single cells or small cell clumps. To achieve this condition with cultures grown in the absence of NGF or in its presence for 1 d, cells from stock cultures were vigorously triturated and then plated onto collagen-coated culture dishes in medium containing 15% serum. Such cultures were maintained for a minimum of 2 d before use.

PC12 cell cultures exposed to NGF for several weeks in medium containing 15% serum contain large cell clumps, regardless of the initial plating density or the absence of cell clumps at the time of plating (4). To obtain long-term NGF-treated cultures that were free of large cell clumps, PC12 cells were maintained with NGF in medium supplemented with 1% horse serum. Cultures prepared in this manner lack large cell clumps. In other respects, PC12 cells grown in RPMI supplemented with 1% horse serum or 15% serum respond similarly to NGF (16, 18). The following procedure was used to prepare long-term NGF-treated cultures maintained in 15% serum that were free of large cell clumps. A single cell suspension was obtained from PC12 cell cultures treated with NGF for 14 d (in medium containing 15% serum) by trypsinization (0.2% trypsin [Worthington Biochemicals, in phosphate buffered saline (PBS) for 45 min at 37°C) followed by vigorous trituration. These cells were then plated onto fresh collagen-coated dishes in media containing 15% serum and NGF, and the resulting cultures were maintained for an additional 7 d. The cells in these cultures occurred singly or in small cell clumps. Although the neurite network in these cultures was extensive, it appeared somewhat less dense than that in cultures maintained in 1% horse serum for 21 d without passaging. Furthermore, we had the impression that the percentage of cells in these cultures without neurites was less than that in cultures fed 1% horse serum plus NGF. For these reasons, most of the experiments involving long-term NGF-treated cells used cultures prepared with media supplemented with 1% horse serum.

Assay of Polymerized Tubulin

Cells were extracted while attached to the culture dish with a buffer containing 0.1% Triton X-100 to separate polymerized from unpolymerized tubulin. The extraction protocol is modified from Solomon et al. (40). Cells were rinsed twice with PBS, Ca^{++}-Mg^{++}-free, and then once with buffer 1 (0.1 M PIPES, pH 6.9, 2 M glycerol, 1 mM $MgSO_4$, 1 mM EGTA). Washed cells were then exposed to buffer 2 (buffer 1 + 0.1% Triton X-100) for two successive 10-min incubations at room temperature. Unpolymerized tubulin is soluble in buffer 2 and diffuses out of permeabilized cells, while polymerized tubulin is insoluble and remains associated with the detergent-extracted cell residues (see results and references 21 and 40). The detergent-extracted cell residues were rinsed once with buffer 3 (buffer 1 without EGTA) and then suspended in a Ca^{++}-containing buffer (0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 5 mM $CaCl_2$) to depolymerize and solubilize polymerized tubulin in the detergent-extracted cells. The suspension of detergent-extracted cells was incubated at 4°C for 10 min and then clarified by centrifugation (12,000 *g* for 10 min at 4°C) to obtain Ca^{++}-soluble and insoluble fractions (Ca^{++}-soluble fractions will be referred to as Ca^{++} extracts). Tubulin in the Ca^{++} extracts was analyzed by SDS PAGE (1-D PAGE, 24); gels were stained with Coomassie Blue. Analyses of Ca^{++}-insoluble fractions by 1-D and isoelectric focusing-SDS PAGE (2-D PAGE, 31) indicated that virtually all of the tubulin in detergent-extracted cells was solubilized by the Ca^{++}-containing buffer. Thus, Ca^{++} extracts contain most or all of the tubulin that was polymerized at the time of lysis of the cells.

To quantify the effects of colchicine on polymerized tubulin of PC12 cells grown in the absence or presence of NGF, proteins in Ca^{++} extracts prepared from control (non-drug-treated) and drug-treated cells were iodinated using the iodine chloride procedure (48). Samples from each experimental series were iodinated at the same time and with the same preparation of ^{125}I. Protein-bound ^{125}I was separated from free ^{125}I by chromatography on Sephadex G-25 (fine) equilibrated with 0.0625 M tris, pH 6.8, 1% SDS and 10% (wt/vol) glycerol. Greater than 95% of the protein-bound radioactivity applied to the columns was recovered in the void volume. No detectable protein degradation occurred during the iodination procedure. Iodinated samples were mixed with unlabeled brain microtubule protein (freshly prepared from pig or rat brain by two cycles of thermally induced assembly-disassembly [3]) and then analyzed by 2-D PAGE. Samples from each experimental series were loaded equally with respect to cpm. Gels were stained with Coomassie Blue to visualize brain tubulin. Autoradiographs were prepared from the stained gels and then the stained tubulin spots were excised from the gels and their ^{125}I content was determined. Control experiments demonstrated that, under the conditions of 2-D PAGE used, PC12 cell tubulin comigrated with brain tubulin. For each experimental series, the radioactivity associated with tubulin in Ca^{++} extracts prepared from drug-treated cells is expressed as a percentage of the radioactivity associated with tubulin in Ca^{++} extracts prepared from control cells (see Fig. 5).

Electron Microscopy

Cultures of intact cells or detergent-extracted cell residues were fixed for 40 min at room temperature with 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.5, containing 3% sucrose, and then rinsed three times with the phosphate buffer without glutaraldehyde. Fixed cultures were then postfixed with 1% O_xO_4 (in the phosphate buffer) for 1 h at 4°C, rinsed with 0.9% saline, stained *en bloc* for 1 h at 4°C with 0.5% (wt/vol) magnesium uranyl acetate (in saline), rinsed in saline, and then dehydrated and embedded in EPOK 812 (Fullam, Inc., Schenectady, NY). Representative areas from the cultures were cut out and re-embedded before sectioning.

Colchicine Uptake

Cells grown in the absence of NGF or in its presence for 21 d (in 1% horse serum) were incubated with 1.3 μM colchicine containing tracer amounts of [^{3}H]colchicine (New England Nuclear, Boston, MA; final sp act, 1 Ci/mmol). At several times between 0 and 180 min after addition of the drug, cultures were rapidly rinsed with PBS, and the rinsed cells were scraped into 500 μl of 0.4 (wt/vol) Nonidet P-40. Parallel operations were performed with collagen-coated culture dishes without cells to obtain values for nonspecific binding. The tritium content of the extracts was determined by scintillation counting. Cpm obtained from cultures with cells were corrected for nonspecific binding by subtracting cpm obtained from cultures without cells. Corrected data are expressed as cpm [^{3}H]colchicine per milligram protein (protein was measured with the Bio-Rad assay) or cpm [^{3}H]colchicine per 10^6 cells (see Fig. 6).

RESULTS

In the experiments reported here, colchicine was used as a probe to compare properties of microtubules of PC12 cultures grown in the absence of NGF or in its presence for 1 or 21 d. Each type of culture was treated with colchicine for 1 or 6 h and then was assayed for its content of polymerized tubulin. The levels of polymerized tubulin in drug-treated cultures were compared to those in sister cultures that were not treated with colchicine (control cultures).

Morphology of Control and Colchicine-treated Cultures

PC12 cells grown without NGF are round to polygonal in shape and lack neurites (defined as cell processes >2 cell body

diameters in length) (Fig. 1). PC12 cells in cultures grown with NGF for 1 d are also round to polygonal in shape, although they are more flattened than cells grown without NGF. In such cultures, <10% of the cells have neurites (8, 19); in most of the experiments reported here, the proportion of cells having neurites after 1 d of NGF treatment ranged from 1 to 3%. Exposure of PC12 cells grown without NGF or with NGF for 1 d to colchicine for 1 or 6 h caused them to round up and appear more phase bright than control cells (Fig. 1). Cell margins, which were normally smooth, frequently become crenated in the presence of drug.

In cultures of PC12 cells exposed to NGF for 3 wk, >90% of the cells have long, highly branched neurites (Fig. 1, and references 8 and 19) (long-term NGF-treated cells will be referred to as neurite-bearing). Exposure of neurite-bearing cells to colchicine for up to 6 h had no discernible effect on the appearance of the cell bodies (Fig. 1). Individual neurites and neurite bundles, however, became progressively more irregular in outline with increasing time with colchicine; this effect was slight after 1 h, but after 6 h, it was often quite pronounced (Fig. 1). We had the impression that after 6 h with colchicine, but not after 1 h, the lushness of the neurite network in cultures treated with colchicine was slightly diminished compared to control cultures.

Effect of Colchicine on the Content of Polymerized Tubulin

The susceptibility of microtubules of PC12 cells grown in the absence or presence of NGF to colchicine-induced depolymerization was determined by comparing the amount of polymerized tubulin in drug-treated cultures with that in control cultures. Cultures were extracted with a buffer containing 0.1% Triton X-100 (see Materials and Methods). The extraction conditions used, effectively removed unpolymerized tubulin from the cells (see below). Polymerized tubulin in detergent-extracted residues was then quantitatively solubilized by a Ca^{++}-containing buffer (see Materials and Methods), and the resulting Ca^{++}-extracts were analyzed by 1-D PAGE. The fraction of polymerized tubulin that may be solubilized during detergent extraction is unknown. However, others have shown that microtubule networks in detergent-extracted cells closely resemble those of intact cells when stained with antibody to tubulin (21, 40), indicating that loss of polymerized tubulin during detergent extraction is minimal. Also, electron microscopic analyses of intact and detergent-extracted PC12 cells (see Fig. 4) further indicate that their microtubules are stable to detergent-extraction. It is also unlikely that significant tubulin polymerization occurs during detergent extraction because (*a*) the detergent-containing buffer lacks GTP, which is required for the assembly of tubulin into microtubules (37), (*b*) unpolymerized tubulin in the cells will be rapidly diluted as a result of detergent extraction, and (*c*) in colchicine-treated cells, it is likely that most or all unpolymerized tubulin is complexed with colchicine and therefore is able to assemble into microtubules very poorly, if at all (28, 41).

As seen in Figs. 2 and 3, material comigrating with tubulin represents heavily stained bands in 1-D gels of Ca^{++} extracts prepared from control cells (most of the material that comigrates with brain tubulin in 1-D PAGE also comigrates with brain tubulin in 2-D PAGE). Visual inspection of the gels indicates that the levels of tubulin-like material relative to the other proteins in Ca^{++} extracts changes as a function of exposure to NGF. In particular, tubulin-like material is a much less prominent component of the total protein in Ca^{++} extracts prepared from cells grown without NGF (Fig. 2) than it is in the corresponding material prepared from cells grown with NGF for 21 d (Figs. 2 and 3).

Cultures were exposed to 50 μM colchicine for 0, 1, or 6 h and then assayed for their content of polymerized tubulin. Polymerized tubulin of cells grown without NGF or with NGF for 1 d was very labile to treatment with colchicine (Fig. 2). After a 1-h exposure to 50 μM colchicine, very little polymerized tubulin remained in these cells (similar results were obtained with 5 μM colchicine). In contrast to this finding, polymerized tubulin of neurite-bearing cells was much less labile to colchicine treatment (Fig. 2). Fig. 3 shows that relatively high levels of polymerized tubulin persist in neurite-bearing cells even after 6 h of treatment with 50 μM colchicine.

Many polypeptides in addition to tubulin are present in Ca^{++} extracts prepared from PC12 cells grown in the absence or presence of NGF (Figs. 2 and 3). The relative abundance of most of these nontubulin polypeptides in Ca^{++} extracts was not detectably altered by treatment with colchicine (see also Solomon et al., 40).

Cultures of neurite-bearing cells, either treated or untreated with 50 μM colchicine for 6 h, were examined by electron microscopy (Fig. 4). Microtubules were abundant in control and colchicine-treated neurite-bearing cells (Fig. 4 *a* and *b*), and these microtubules were stable to detergent extraction (Fig.

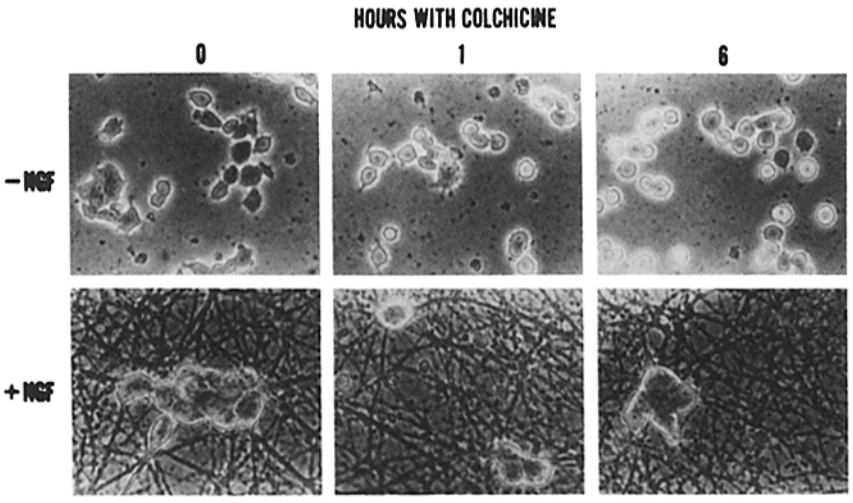

FIGURE 1 Phase micrographs of PC12 cells grown in the absence of NGF or in the presence of NGF for 25 d and exposed to 50 μM colchicine for 0, 1, or 6 h.

4*c* and *d*). All neurites examined in colchicine-treated cultures contained microtubules (Fig. 4*b*). These data demonstrate that the polymerized tubulin detected biochemically in colchicine-treated neurite-bearing cells corresponds at least in part to microtubules.

To quantify the colchicine sensitivity of microtubules of PC12 cells in the various types of cultures, proteins in Ca^{++} extracts were labeled with ^{125}I and analyzed by 2-D PAGE (see Materials and Methods). Representative autoradiographs of

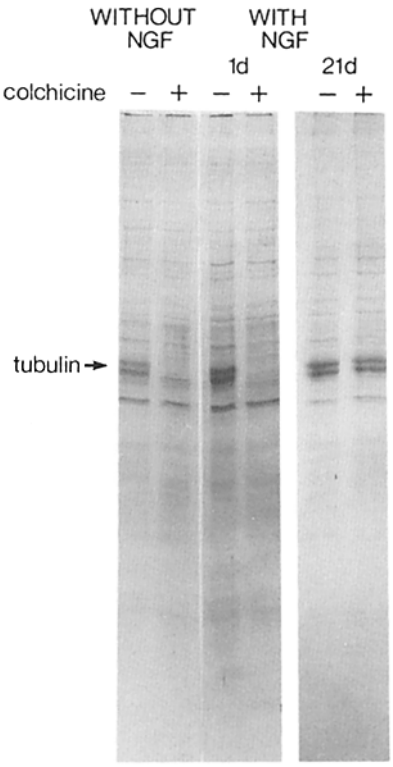

FIGURE 2 Coomassie Blue-stained gels of polypeptide in Ca^{++}-extracts prepared from control cells (−) and cells exposed to 50 μM colchicine for 1 h (+). Cells were grown in the absence of NGF or in its presence for 1 or 21 d. Ca^{++} extracts were prepared and electrophoresed as described in Materials and Methods. The samples from each set of cultures were loaded equally with respect to protein. The gel contained a 8.5–17% gradient of acrylamide.

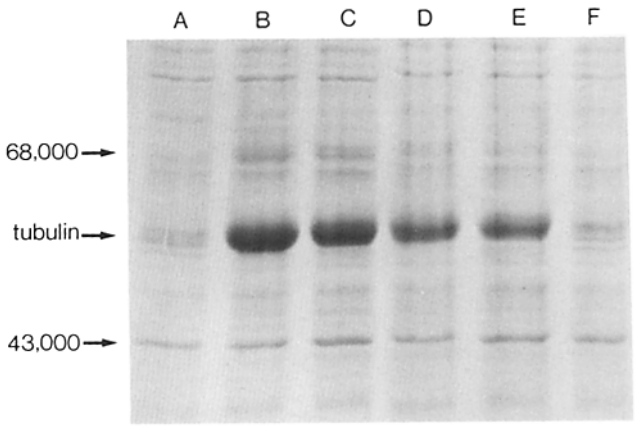

FIGURE 3 Coomassie Blue-stained polyacrylamide gels (10% acrylamide) of Ca^{++}-soluble fractions prepared from neurite-bearing cells exposed to 50 μM colchicine for 0, 1, 2, and 6 h (*B*, *C*, *D*, and *E*, respectively) before extraction. *A* and *F* depict the pattern of polypeptides in Ca^{++}-extracts prepared from neurite-bearing cells that were detergent-extracted in the presence of 5 mM $CaCl_2$ (see text for details); in *A*, cells were not treated with colchicine, while in *F*, the cells were treated with colchicine for 2 h before detergent extraction.

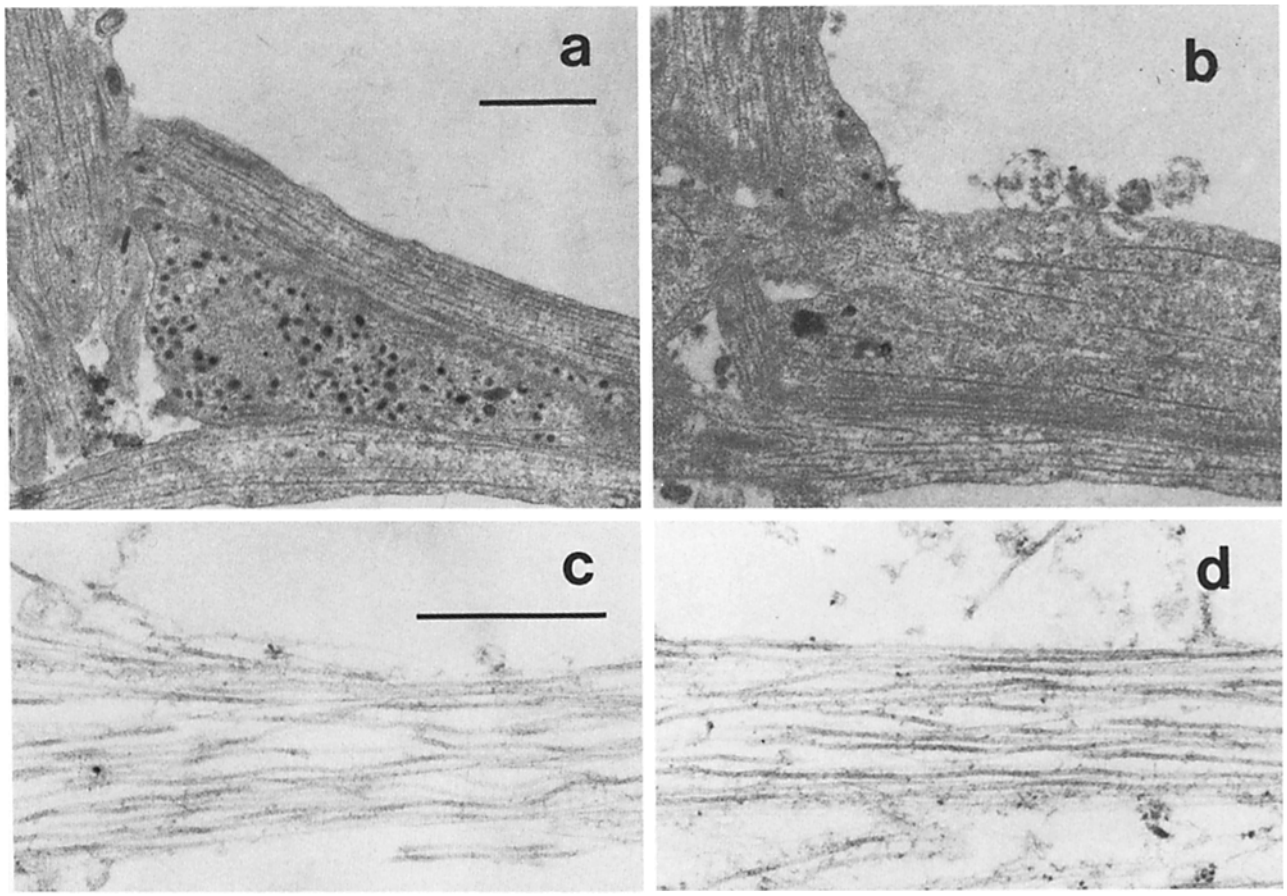

FIGURE 4 Electron microscopic demonstration of microtubules in colchicine-treated neurite-bearing PC12 cells. Cells treated with NGF for 21 d were incubated for 6 h without (*a*, *c*) or with (*b*, *d*) 50 μM colchicine. The cells depicted in *c* and *d* were extracted with the Triton-containing stabilization buffer before fixation. Bar, 1 μm. (*a*, *b*) × 15,000. (*c*, *d*) × 25,000.

gel patterns of iodinated samples prepared from cells grown with NGF for 1 or 21 d are shown in Fig. 5. Ca^{++} extracts prepared from control cultures grown with NGF for 1 d contain major labeled spots that comigrate with brain tubulin (panel *a*), while such extracts prepared from colchicine-treated cultures contain very little material that comigrates with brain tubulin (panel *b*). Similar results were obtained with cells grown without NGF. Panels *d–g* show that Ca^{++} extracts prepared from control and colchicine-treated cultures of neurite-bearing cells contain relatively high levels of labeled material that comigrates with tubulin. Fig. 6 shows quantitative data for this type of experiment. For cultures grown without NGF or with NGF for 1 d, the amount of polymerized tubulin remaining after exposure to colchicine for 1 or 6 h was ~10 and ~3%, respectively, of that in control cultures. The amount of polymerized tubulin in long-term NGF-treated cells exposed to colchicine for 1, 2, or 6 h was ~76, ~65, and ~57%, respectively, of that in control cells.

The above-described differential colchicine susceptibility of polymerized tubulin of PC12 cultures grown without or with NGF could reflect differences in the polymerized tubulin in each type of culture, or could merely reflect differences in (*a*) the ability of colchicine to enter these cells, (*b*) the effects of colchicine on cell viability, or (*c*) the ability of the detergent-containing buffer to remove unpolymerized tubulin. Several control experiments were performed to distinguish among these possibilities.

Removal of Unpolymerized Tubulin

The assay for polymerized tubulin requires that unpolymerized tubulin be quantitatively removed from the cells during

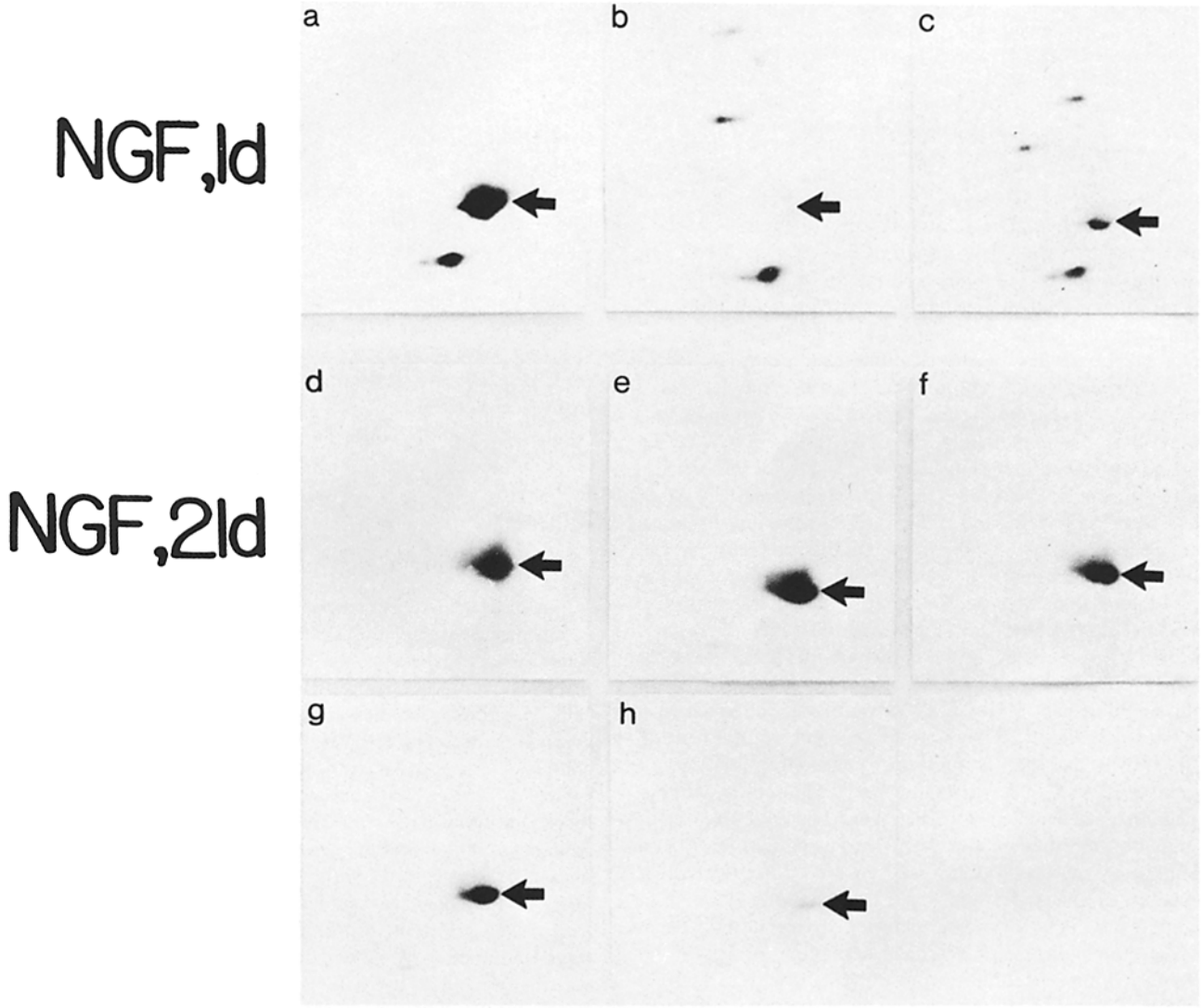

FIGURE 5 Autoradiographs of 2-D polyacrylamide gels depicting ^{125}I-labeled proteins in Ca^{++}-extracts prepared from control and colchicine-treated PC12 cells. The cells were grown in medium supplemented with 1% horse serum in the presence of NGF for 1 d (panels *a*-*c*) or 21 d (panels *d–h*). Proteins in Ca^{++} extracts prepared from control and colchicine-treated cells were iodinated as described in Materials and Methods. Iodinated samples were mixed with unlabeled brain microtubule protein and then analyzed by 2-D PAGE (the second dimension consisted of 10% acrylamide). The region of the gels containing tubulin is shown; the arrow indicates the position of carrier brain tubulin as revealed by staining with Coomassie Blue. Panels *a* and *b*: Ca^{++} extracts from cells grown with NGF for 1 d and then exposed to 50 μM colchicine for 0 or 1 h, respectively. Panel *c*: Ca^{++} extract from control cells (NGF for 1 d) that were detergent extracted in the presence of 1 mM $CaCl_2$ (see text for details). Panels *d–g*: Ca^{++} extracts from cells grown in the presence of NGF for 21 d and then exposed to 50 μM colchicine for 0, 1, 2, or 6 h, respectively. Panel *h*: Ca^{++} extract from neurite-bearing control cells that were detergent extracted in the presence of 5 mM $CaCl_2$.

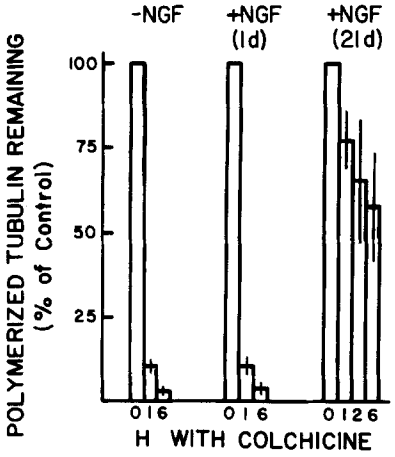

FIGURE 6 Quantitation of colchicine susceptibility of polymerized tubulin of PC12 cells grown in the absence or presence of NGF. Proteins in Ca^{++} extracts prepared from control and colchicine-treated cells were iodinated, mixed with unlabeled brain tubulin, and then subjected to 2-D PAGE (see Materials and Methods). Iodinated samples of control and colchicine-treated cells from each experiment were loaded equally with respect to cpm. Gels were stained with Coomassie Blue to reveal the carrier brain tubulin, and, after obtaining autoradiographs of the gels, the spots corresponding to brain tubulin were excised and their ^{125}I content was determined. The amount of radioactivity associated with tubulin in Ca^{++} extracts prepared from control and colchicine-treated cells is expressed as a percentage of that from control cells. The mean ± SE is shown for each experimental series (*n*, 3 trials for each experimental series).

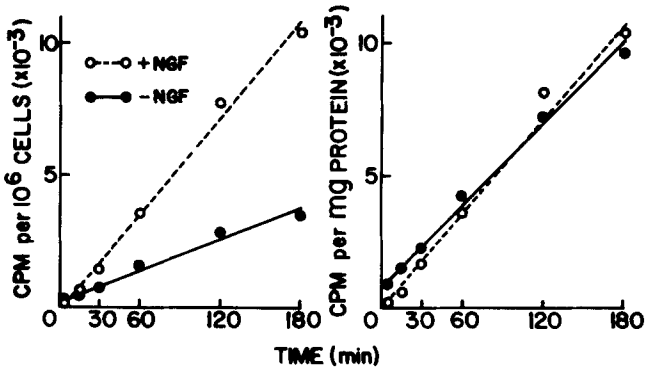

FIGURE 7 Colchicine uptake by cells grown in the absence of NGF or in its presence for 21 d. Each experiment was done with a set of sister cultures. Colchicine, along with tracer [^3H]colchicine was added to each culture to a final concentration of 1.3 μM (final sp act, 1 Ci/mmol). At several times after the addition of drug, the cells were rapidly rinsed with Ca^{++}-Mg^{++}-free PBS. The amount of radioactivity remaining with the cells was determined by scintillation counting. Each data point is the average of at least two cultures.

detergent extraction. The virtual absence of tubulin in Ca^{++} extracts prepared from cells grown in the absence of NGF or in its presence for 1 d and exposed to colchicine (Fig. 2) indicates that unpolymerized tubulin is completely removed from these cells during detergent extractions. For further evaluation of the ability of the detergent-containing buffer to remove unpolymerized tubulin from the cells, the cells were extracted with a detergent-containing buffer (buffer 2 without EGTA) that also contained 1 or 5 mM $CaCl_2$. By including Ca^{++} in this buffer, polymerized tubulin as well as unpolymerized tubulin should be solubilized in permeabilized cells. If all cells are permeabilized, and if diffusion of solubilized tubulin out of permeabilized cells is unhindered, then the resulting cell residues should be depleted of tubulin and subsequent extraction of the residues with the Ca^{++} buffer should not solubilize additional tubulin. As seen in Fig. 5*a* and *c*, relatively little tubulin is present in Ca^{++} extracts prepared from cells exposed to NGF for 1 d when they are first extracted with the Ca^{++}-containing detergent buffer. Similar results were obtained with cells grown without NGF (not shown) and neurite-bearing cells (Figs. 3 and 5). In addition, quantitative analyses of tubulin levels in Ca^{++} extracts prepared from cultures of neurite-bearing cells treated with detergent buffer (buffer 2) or detergent buffer that contained Ca^{++} indicated that 80–90% (n = 3) of unpolymerized tubulin was removed during detergent extraction. Both 1 and 5 mM $CaCl_2$ were equally effective in removing tubulin from the cells during detergent extraction. Because the conditions of detergent extraction effectively remove unpolymerized tubulin from PC12 cells with or without neurites, tubulin in Ca^{++} extracts prepared from neurite-bearing cells treated with colchicine reflects polymerized tubulin that was not depolymerized by colchicine.

Permeability to Colchicine

Colchicine uptake by PC12 cultures grown without NGF or with NGF for 3 wk (in 1% horse serum) is linear for at least 3 h (Fig. 7) (correlation coefficients from linear regression analyses of the data were 0.96 ± 0.02 [mean ± SE, n = 3] or 0.96 ± 0.02 [n = 4] for cells grown without or with NGF, respectively). The linear regression analyses also provided an estimate of the rate of colchicine uptake. When the data are expressed on a per cell basis, neurite-bearing cells take up colchicine ~2.5 times more rapidly than do cells grown without NGF (52 ± 12 cpm/10^6 cells min [n = 4] vs 22 ± 3 cpm/10^6 cells min [n = 3]). On a per-milligram-protein basis, the rates of colchicine uptake by cultures grown with or without NGF are similar to one another (59 ± 17 cpm/mg protein min [n = 4] vs 50 ± 10 cpm/mg protein min [n = 3] for NGF-treated and untreated cells, respectively), reflecting the fact that long-term NGF-treated cells contain ~2.5 times more protein than cells grown without NGF (19). Thus, the difference in colchicine susceptibility of polymerized tubulin of cells grown in the absence or presence of NGF is apparently not due to differences in drug permeability.

Effects of Colchicine on Cell Viability

The following observations indicate that the regimen of colchicine treatment used in these studies is not toxic to the cells. First, visual inspection of control and drug-treated cultures revealed no obvious differences in the number of dead cells, regardless of whether the cultures were grown in the absence or presence of NGF. This subjective impression was confirmed quantitatively for cells grown in the absence of NGF by experiments which compared the values for cell number, incorporation of [^3H]amino acids into trichloroacetic acid-precipitable material, and the percentage of cells excluding trypan blue in control cultures with those values obtained from cultures exposed to 50 μM colchicine for 6 h. Cell number in drug-treated cells was 104% of that in control cultures (cell number was determined in 10 sister cultures, 5 treated with drug and 5 not treated with drug). The values for incorporation of [^3H]-amino acids in drug-treated cultures were 95% of those obtained with control cultures (9 sister cultures were analyzed, 5 treated with drug and 4 not treated with drug). Finally, there were no detectable differences in the percentage of cells excluding trypan blue when 200–300 cells were scored in each of three control and drug-treated cultures (92–96% of the cells in each type of culture excluded trypan blue). Thus, the difference in colchicine susceptibility of polymerized tubulin of cells grown in the absence or presence of NGF is not due to differences in the effects of colchicine on cell viability.

Effect of Colchicine on Polymerized Tubulin of NGF-treated Cells Grown in 15 or 1% Serum

Because cells were grown in medium supplemented with different levels of serum (see Materials and Methods), we examined whether the serum content of the medium influenced the susceptibility of polymerized tubulin to disruption by colchicine. The colchicine-lability of polymerized tubulin in PC12 cultures grown with NGF for 1 d in medium containing 15% serum was indistinguishable from that of cells exposed to NGF for 1 d in medium containing 1% horse serum (compare Fig. 2 with Fig. 4, panels *a* and *b*). In another experiment, neurite-bearing cultures free of large cell clumps were prepared in medium containing 15% serum plus NGF for 21 d (see Materials and Methods). These cultures were treated with 50 μM colchicine for 1 h and then assayed for polymerized tubulin as described previously. Ca^{++} extracts prepared from such cells contained a substantial amount of tubulin relative to the tubulin levels in Ca^{++} extracts prepared from control cells. Because all of the tubulin in these cultures could be solubilized with buffers containing detergent plus Ca^{++} (1 mM), tubulin in Ca^{++} extracts prepared from colchicine-treated cultures represents polymerized tubulin that was not depolymerized by exposure to drug. Thus, polymerized tubulin that is colchicine-resistant appears in PC12 cells treated with NGF regardless of whether the medium contains 10% horse serum plus 5% fetal calf serum or just 1% horse serum.

DISCUSSION

The experiments presented here have examined the *in situ* colchicine susceptibility of polymerized tubulin of PC12 cells with or without neurites. The data in Figs. 2–6 show that conditions of colchicine treatment which cause complete disruption of polymerized tubulin of PC12 cells grown without or with NGF for 1 d have relatively little effect on polymerized tubulin of neurite-bearing cells. We were unable to detect differences in the effects of colchicine on viability and in the rate and extent of colchicine uptake by PC12 cells grown without or with NGF, and preliminary experiments indicate that soluble extracts prepared from cells grown with or without NGF have similar colchicine binding properties (M. Black, unpublished observations). These observations indicate that the difference in the colchicine susceptibility of polymerized tubulin of nonneurite- and neurite-bearing PC12 cells reflects differences in the polymerized tubulin itself. This interpretation is supported by recent experiments which indicate that polymerized tubulin of cells grown in the absence of NGF is much more susceptible to cold (4°C)-induced depolyermization than polymerized tubulin of long-term NGF-treated cells (M. Black, unpublished observations).

The effects of NGF on PC12 cells have been divided into two general classes, short-latency and long-latency (17). Short-latency effects become manifest within minutes to hours of exposure to NGF, while long-latency effects first appear on the order of days of exposure to NGF. Polymerized tubulin that shows colchicine resistance appears in PC12 cultures only after several days of treatment with NGF. Thus, the change in colchicine susceptibility of polymerized tubulin induced by NGF appears to be associated with the class of relatively long-latency effects of NGF.

Several considerations indicate that the polymerized tubulin identified biochemically is primarily in the form of microtubules. First, microtubules are the principal tubulin-containing structures in cells (49). Second, the polymerized tubulin studied here behaves like microtubules in that it is insoluble in the detergent-containing buffer (Buffer 2) and soluble in the Ca^{++}-containing buffers. Third, essentially all of the polymerized tubulin of PC12 cells grown in the absence of NGF or in its presence for 1 d shows lability to colchicine that is typical of cytoplasmic microtubules. Similarly, ~40% of the polymerized tubulin of neurite-bearing cells is labile to treatment with colchicine (Fig. 6). Although the other 60% of polymerized tubulin of neurite-bearing cells is colchicine-stable under the experimental conditions used, microtubules are abundant in the neurites of long-term NGF-treated cells exposed to 50 μM colchicine for 6 h (Fig. 4). On the basis of these considerations, we conclude that the colchicine susceptibility of microtubules of long-term NGF-treated cells differs from that of cells grown without or with NGF for 1 d.

Microtubules of PC12 cells grown in the absence or presence of NGF may differ in their sensitivity to the action(s) of colchicine. Colchicine binds to monomeric tubulin and thereby interferes with the tubulin monomer-polymer equilibrium (28, 41). Thus, the differential colchicine susceptibility of microtubules of PC12 cells grown in the absence or presence of NGF may reflect differences in the cellular equilibrium conditions between tubulin monomer and polymer. It is also possible that differences in the length of microtubules of nonneurite- and neurite-bearing cells contribute to their differential susceptibility to colchicine.

A possible clue to a mechanism that contributes to the NGF-induced change in microtubule susceptibility to colchicine stems from the observation that the pattern of microtubule organization differs in PC12 cells grown with or without NGF. In long-term NGF-treated cells, many microtubules are organized into tightly packed, linear arrays or bundles that extend from the cell body into the neurites (27, 47). Such microtubule arrays have not been observed in cells grown without NGF. Microtubules in tightly packed bundles often show resistance to colchicine (1, 5, 45). Thus, the cellular condition(s) that leads to the formation of microtubule bundles in NGF-treated cells may also be responsible for the appearance of colchicine-resistant microtubules. In this regard, it may be relevant to note that microtubule-associated proteins (MAPs) influence the center-to-center spacing of microtubules in cells (23) and, under in vitro conditions, MAPs decrease the susceptibility of microtubules to several disassembly-promoting conditions (30, 38) including treatment with colchicine (20, 42). In addition, there is evidence suggesting that the MAPs of process-bearing neuroblastoma cells differ in some way from the MAPs of process-free neuroblastoma cells (32, 36). These observations raise the possibility that NGF-induced changes in MAPs of PC12 cells could be involved in the changes that occur in the spatial organization of microtubules as well as the changes that occur in their sensitivity to colchicine.

What is the functional specialization of colchicine-resistant microtubules in long-term NGF-treated PC12 cells? Several observations indicate that microtubules are involved in elaborating and maintaining cellular morphology, conferring spatial organization on cytoplasm, and participating in various aspects of motility (7, 10, 15, 34, 44, 46, 51). PC12 cells undergo a number of changes in structure and motility after treatment with NGF (Fig. 1 and references 27 and 47), and, thus, it seems reasonable that the NGF-induced change in microtubules of PC12 cells is related to one or more of these changes in cell structure and/or motility. The fact that NGF, a protein involved in neuronal development, initiates the sequence of

events that lead to modifications of microtubules in the PC12 model system supports the possibility that properties of neuronal microtubules also change during growth and maturation.

As discussed in the introduction, the elaboration and maintenance of a particular morphology reflect processes that result in the production and stabilization of a particular pattern of cytoskeletal organization. The properties of the individual components of the cytoskeleton will influence its pattern of organization as well as the tendency of a particular pattern to persist over time. In this regard, it is intriguing to consider the possibility that the change in microtubules described here is one of a class of cytoskeletal modifications used during neuronal development to modulate the structural properties of neurons as well as the relative plasticity or stability of neuronal morphology (9, 22, 25, 29).

We thank Dr. Adriana Rukenstein for assistance with tissue culture and Ms. Margaret DiPiazza and Jeffrey Kurdyla for technical assistance. We would like to express our appreciation to Drs. Scott T. Brady, P. John Seeley, Mike Shelanski, and Frank Solomon for valuable discussions during the course of the investigations presented here. We also thank Ms. Lois Bricken for carrying out the electron microscopy and Dr. Paulette Bernd for advice concerning electron microscopy.

The work reported here was supported by grants from the March of Dimes Defects Foundation and (U. S. Public Health Service (USPHS) (NS 16036) to L. A. Greene and USPHS (Biomedical Research Support grant S 07 RR054170 from the Division of Research Resources and NS 17681) to M. M. Black. Dr. Black was the recipient of a USPHS postdoctoral fellowship.

Received for publication 16 February 1982, and in revised form 27 July 1982.

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