

The Initiation of Neurite Outgrowth by Sympathetic Neurons Grown In Vitro Does Not Depend on Assembly of Microtubules

Carolyn L. Smith

Laboratory of Neural Control, and Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Abstract. Neurite formation by dissociated chick sympathetic neurons in vitro begins when one of the many filopodia that emanate from the cell body of a neuron is invaded by cytoplasm containing microtubules and other components of axoplasm (Smith, 1994). This study was undertaken to determine whether this process depends on assembly of microtubules. To inhibit microtubule assembly, neurons were grown in medium containing nocodazole or colchicine. In one series of experiments, neurons first were exposed to the microtubule-stabilizing drug, taxol, so that existing microtubules would remain intact while assembly of new microtubules was inhibited. The ability of neurons to form neurites was assessed by time-lapse video microscopy. Neurons subsequently were stained with antibodies against the tyrosinated and acetylated forms of α -tubulin and examined by laser confocal microscopy to visualize microtubules. Neurons were able to

form short processes despite inhibition of microtubule assembly and they did so in a way that closely resembled process formation in control medium. Processes formed by neurons that had not been pretreated with taxol were devoid of microtubules. However, microtubules were present in processes of taxol-pretreated neurons. These microtubules contained acetylated α -tubulin, as is typical of stable microtubules, but not tyrosinated α -tubulin, the form present in recently assembled microtubules. These findings show that the initial steps in neurite formation do not depend on microtubule assembly and suggest that microtubules assembled in the cell body can be translocated into developing neurites as they emerge. The results are compatible with models of neurite formation which postulate that cytoplasm from the cell body is transported into filopodia by actomyosin-based motility mechanisms.

THE outgrowth of an axon from the cell body of a neuron represents the first step in a developmental program that culminates in the formation of synaptic connections with appropriate target cells. Although the cytoskeletal mechanisms responsible for axonal elongation have been studied extensively (Bamburg et al., 1986; Forscher and Smith, 1988; Lim et al., 1989; Okabe and Hirokawa, 1990, 1992; Sabry et al., 1991; Reinsch et al., 1991; Keith and Farmer, 1993; Lin and Forscher, 1993) the mechanisms involved in the initial outgrowth of an axon remain largely unknown. Clues as to the types of mechanisms that are likely to be involved were recently obtained in a study of dissociated chick sympathetic neurons grown in defined medium on substrates coated with polyornithine (Smith, 1994). The changes that occur in neurons as they initiate neurite formation can be readily examined in this system because neurite formation is triggered by cell-cell contact. Observations made by time lapse video microscopy showed that freshly dissociated sympathetic neurons have multiple filo-

podia. They begin to form a neurite when one of their filopodia contacts an adjacent cell. This causes the filopodium to straighten and detach from the substrate. Then a wedge-shaped bulge of cytoplasm begins to move from the perinuclear region of the cell body outward, along a trajectory aligned with the filopodium. The bulge of cytoplasm invades the filopodium, converting it into a process several micrometers in diameter which then continues to elongate, becoming a definitive neurite with a growth cone at its distal end.

Neurite formation is accompanied by coordinated changes in the distributions of microtubules and actin microfilaments (Smith, 1994). Before a neuron begins to form a neurite, its microtubules are confined to the perinuclear region. Actin microfilaments are present in its filopodia and in the lamella that develops around its circumference after it attaches to the substrate. When cytoplasm from the perinuclear region invades a filopodium, the bundle of actin microfilaments forming the core of that filopodium extends proximally only to the leading edge of the cytoplasm, so the bundle must shorten as the cytoplasm advances. The bulge of cytoplasm is surrounded by a cortical actin network and contains a parallel array of microtubules. The array of microtubules extends distally to the leading edge of the cytoplasm indicating

Address all correspondence to C. L. Smith, LMB, NINDS, NIH Building 36, Room 2A21, Bethesda, MD 20892. Ph.: (301) 496-8893. Fax: (301) 480-1485.

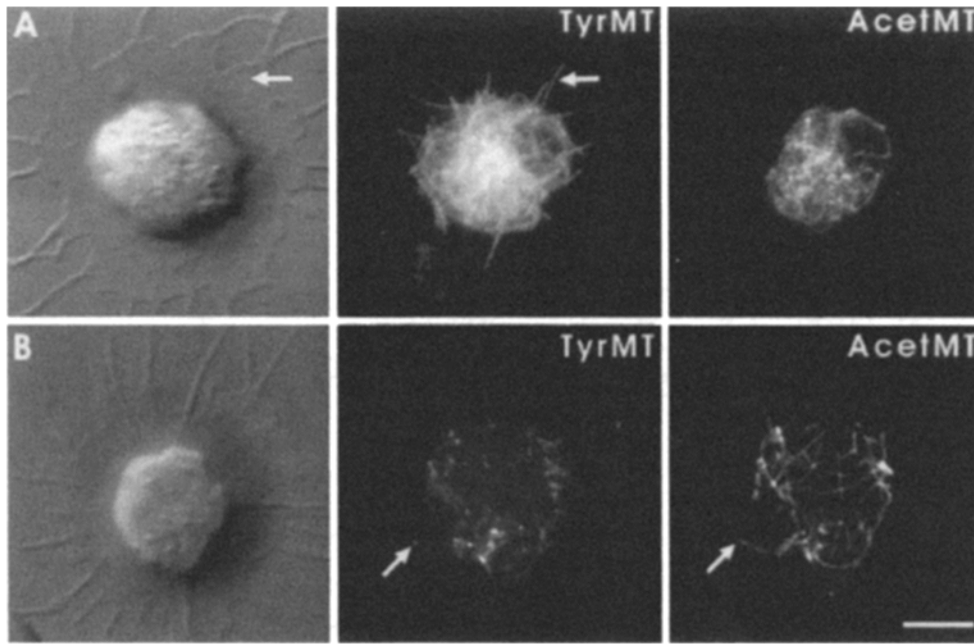


Figure 1. DIC and fluorescence images of neurons grown in control medium (*A*) or medium containing 2 $\mu\text{g/ml}$ nocodazole (*B*). The fluorescence images show microtubules immunolabeled with the antibodies against the tyrosinated and acetylated forms of α -tubulin (TyrMT and AcetMT, respectively). The neuron in *A* contained both tyrMT and acetMT, as is typical of neurons in control medium. AcetMT were confined to the perinuclear region but tyrMT extended into the lamella (arrows). The neuron in *B* was exposed to nocodazole for 2 h and many of its microtubules had depolymerized. The microtubules that remained had high concentrations of acetylated α -tubulin. Some microtubules

had an aggregation of tyrosinated α -tubulin at one end (arrows) or low levels of tyrosinated α -tubulin along their entire length. The fluorescent images are projections of optical sections collected with a laser scanning confocal microscope at 0.36- μm steps in the z-axis. The cell body of the neuron in *B* appears smaller in the DIC image than in the fluorescence images because the widest part of the cell body was not in the plane of focus in the DIC image. Bar, 5 μm .

outgrowth by sympathetic ganglion neurons grown in vitro: (a) does neurite formation depend on assembly of microtubules; and (b) can assembled microtubules be translocated into developing neurites. The first question was investigated by examining neurons grown in medium containing nocodazole, which binds tubulin and prevents its polymerization. In the second series of experiments, neurons first were exposed to taxol to stabilize existing microtubules (Schiff and Horwitz, 1980) and then grown in medium containing either nocodazole or colchicine to inhibit polymerization.

Polyornithine was used as the growth substrate so that neurite formation would require contact of a filopodium with another cell (Smith, 1994). The cultures were kept in control medium for one to two hours after plating. During this time, neurons developed a lamella around their circumference and formed several filopodia (Fig. 1 *A*). A few neurons contacted other neurons and began to form neurites. The cultures then were incubated in medium containing nocodazole (first series of experiments) or taxol (1 h) followed by nocodazole or colchicine (second series of experiments). The ability of neurons to form neurites was assessed by using video microscopy to monitor the behavior of neurons as they contacted adjacent neurons. Neurons subsequently were fixed and stained with antibodies against the tyrosinated and acetylated forms of α -tubulin (Wehland et al., 1983; Piperno et al., 1987) so as to visualize microtubules and verify that tubulin polymerization had been inhibited.

Time Course of Tubulin Acetylation in Neurons Recovering from Exposure to Nocodazole

Previous studies have shown that newly polymerized portions of microtubules contain the tyrosinated form of α -tubulin and that α -tubulin becomes detyrosinated and acetylated

after it polymerizes (Gunderson et al., 1987; Piperno et al., 1987; Bulinski et al., 1988). Consequently, the ages of microtubules can be inferred from their contents. The interpretation of the experiments reported here required knowing how rapidly after polymerization α -tubulin becomes converted from the tyrosinated form to the acetylated form in sympathetic neurons. To determine the time course, neurons were exposed to 2 or 10 $\mu\text{g/ml}$ nocodazole for 1 to 2 h to deplete them of microtubules and then returned to control medium so that microtubules would begin to polymerize. Neurons were doubly labeled with antibodies against the tyrosinated and acetylated forms of α -tubulin after 0, 4, 8, 16, or 32 min in control medium, and then examined by laser scanning confocal microscopy. Neurons grown in control medium were examined for comparison.

Neurons grown in control medium (Fig. 1 *A*) had numerous microtubules containing tyrosinated α -tubulin (tyrMT) and most also had numerous microtubules containing acetylated α -tubulin (acetMT). The few cells that appeared to be neuronal but did not have acetMT probably were neuroblasts or newly generated neurons (Smith, 1994). Comparison of fluorescence and DIC images of neurons showed that microtubules were largely confined to the perinuclear region but some tyrMT extended into the lamella that surrounded neuronal cell bodies (Fig. 1 *A*, arrows). The perinuclear microtubules were interwoven in a dense plexus, making it impossible to see the entire lengths of individual microtubules. However, segments of microtubules could be seen. Segments that contained tyrosinated α -tubulin usually did not appear to contain acetylated α -tubulin, and vice versa, but some segments appeared to contain both forms of α -tubulin.

After 1 h or more in medium containing 2 or 10 $\mu\text{g/ml}$

that it advances at the same rate as the cytoplasm (up to 10 $\mu\text{m}/\text{min}$).

Two familiar cytoskeletal mechanisms have been invoked as possible explanations of these observations (Smith, 1994). Microtubules could invade filopodia by polymerizing at their distal ends (Baas et al., 1987; Okabe and Hirokawa, 1988). Microtubules serve as substrates for organelle transport (Allen et al., 1985; Schnapp et al., 1985) so their entry into a filopodium would provide a mechanism for bringing in other characteristic components of axoplasm. According to this scenario, invasion of a filopodium by microtubules would be the pivotal step in its transformation into a neurite. Alternatively, the driving force for neurite formation could be provided by actomyosin-based motility mechanisms similar to those that are thought to be involved in cell migration (Abercrombie, 1980; Albrecht-Buehler, 1987; Lee et al., 1993). If microtubules can be translocated by such a mechanism, then neurite formation might not require assembly of microtubules. Similar mechanisms previously have been invoked to explain neurite elongation (Letourneau, 1973; Kirchner and Mitchison, 1986; Bray and White, 1988; Forscher and Smith, 1988; Mitchison and Kirschner, 1988; Goldberg, et al., 1991; Heidemann et al., 1991; Lin and Forscher, 1993; O'Connor and Bentley, 1993).

The present study uses drugs that inhibit the dynamic turnover of microtubules to test certain aspects of these models. The results demonstrate that microtubules are not required for the initiation of neurite outgrowth and provide evidence that the microtubules that invade a filopodium during its transformation into a neurite may be pulled outward from the cell body as assembled microtubules. These observations support the second type of model, in which the outgrowth of neurites is driven by actomyosin-based movements.

Materials and Methods

Cell Culture

Dissociated cell cultures were prepared from sympathetic ganglia of 8- or 9-d old chick embryos using methods that have been described previously (Smith, 1994). The cells were plated on polyornithine-coated glass coverslips in a defined medium consisting of Ham's F14 (GIBCO BRL, Gaithersburg, MD) with 20 ng/ml 7s NGF, 10 $\mu\text{g}/\text{ml}$ avian transferrin, 5 $\mu\text{g}/\text{ml}$ insulin, 5 ng/ml selenium, 1 mg/ml bovine serum albumin, 100 U/ml penicillin, 100 U/ml streptomycin and 250 ng/ml Fungizone (Marusich et al., 1986). Coverslips photoetched with a grid pattern (Bellco, Vineland, NJ) were used to facilitate relocation of individual cells in experiments in which neurons were examined first by video microscopy and then by laser scanning confocal microscopy.

Experimental Treatments

Stock solutions of nocodazole (Sigma Chemical Co., St. Louis, MO) were prepared in DMSO (2 or 5 $\mu\text{g}/\text{ml}$) and stored at 4°C. Freshly thawed nocodazole stock solutions were used for each experiment because stock solutions that were repeatedly warmed and cooled were less effective in inhibiting tubulin polymerization. Taxol (Molecular Probes, Eugene, OR) was dissolved in DMSO (2 mM) and stored at 4°C. Colchicine (Sigma Chemical Co.) was dissolved in sterile water (1 mg/ml) and stored at -20°C. Stock solutions were diluted in warm cell culture medium for use.

To determine the timecourse by which neurons recover from exposure to nocodazole, cultures were incubated for 1 to 2 h in medium containing 2 or 10 $\mu\text{g}/\text{ml}$ nocodazole, rinsed three times in control medium, and then incubated in control medium for varying periods of time. Recovery times given in the text do not include time during rinsing (~ 2 min).

Video Microscopy

The methods and equipment used for video microscopy were the same as described previously (Smith, 1994). Briefly, cultures were examined on a Zeiss Axiovert set up for differential interference contrast (DIC)¹ optics with a 100 \times (1.3 NA) objective and 1.4 NA condenser. Images were collected with a Newvicon camera (Dage MTI, Michigan City, IN), processed to enhance contrast using a computer based system (Image 1; Universal Imaging, West Chester, PA), and stored on an optical memory disk recorder (Panasonic, Secaucus, NJ). Images were transferred to a Macintosh computer for editing (see below).

Fixation and Staining

Cultures were briefly rinsed with PBS and then simultaneously fixed and permeabilized for 15 min in PHEM buffer (60 mM Pipes and 25 mM Hepes) containing 3.7% paraformaldehyde, 0.25% glutaraldehyde, 5 mM EGTA, 3% sucrose, and 1% Triton X-100 (modified from Falconer et al. 1989; Schliwa and van Blerkom, 1981). Video microscopic observations showed that the shapes of neurons did not significantly change during fixation.

To visualize microtubules, cultures were doubly labeled with rat monoclonal antibodies specific for the tyrosinated form of α -tubulin (YL1/2; Accurate Chemical Corp., Westbury, NY) and mouse monoclonal antibodies specific for acetylated α -tubulin (6-11B-1; a gift of G. Piperno, The Rockefeller University, New York). Staining was done sequentially to minimize cross-reaction (for protocol see Smith, 1994). Secondary antibodies were biotinylated anti-rat IgG, followed by fluorescein-avidin (Vector Labs, Burlingame, CA), and anti-mouse IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, Westgrove, PA). Coverslips were mounted in Vectashield (Vector Labs).

Laser Scanning Confocal Microscopy

Fluorescent images were collected with MRC-600 laser scanning confocal imaging system (Bio-Rad Laboratories, Richmond, CA) on a Zeiss microscope. For each cell, a series of optical sections was collected at a spacing of 0.36 μm in the z-axis and then superimposed to form a "z-series" projection. These z-series projections were transferred to a Macintosh computer for editing and processing. Image contrast and gain were adjusted so as to make individual microtubules appear bright. In some instances, the brightness in areas of cells containing large numbers of microtubules was reduced to avoid signal saturation. Fluorescence images, together with corresponding video images, were printed with a Tektronix Phaser II printer.

Analysis of Fluorescence Intensity

To compare the numbers of microtubules in neurons exposed to different treatments, images of individual cells were collected with a silicon-intensified tube (SIT) camera on a video microscope set up for epifluorescence microscopy. The gain and contrast of the camera were set such that the background had a gray level of 30 and the brightest images (those produced by YL1/2 staining in control cells) had a gray level of 250 (in a 256 level gray scale). The intensity of the fluorescein and rhodamine signals in individual neurons was measured by using the "area brightness" function of Image 1 (Universal Imaging) to draw an outline around the cell body and determine the average intensity within the outline. Data was collected from at least 49 neurons subjected to each treatment in three separate experiments. Data from one experiment were used to make the histograms in Fig. 6. The intensity scales in these histograms were rescaled to extend from 0 to 250 for both fluorescence channels.

Results

This study uses drugs that inhibit the dynamic turnover of microtubules to investigate two questions concerning the cytoskeletal mechanisms involved in the initiation of neurite

1. *Abbreviations used in this paper:* acetMT, microtubules containing acetylated α -tubulin; DIC, differential interference optics; SIT, silicon-intensified tube; tyrMT, microtubules containing tyrosinated α -tubulin.

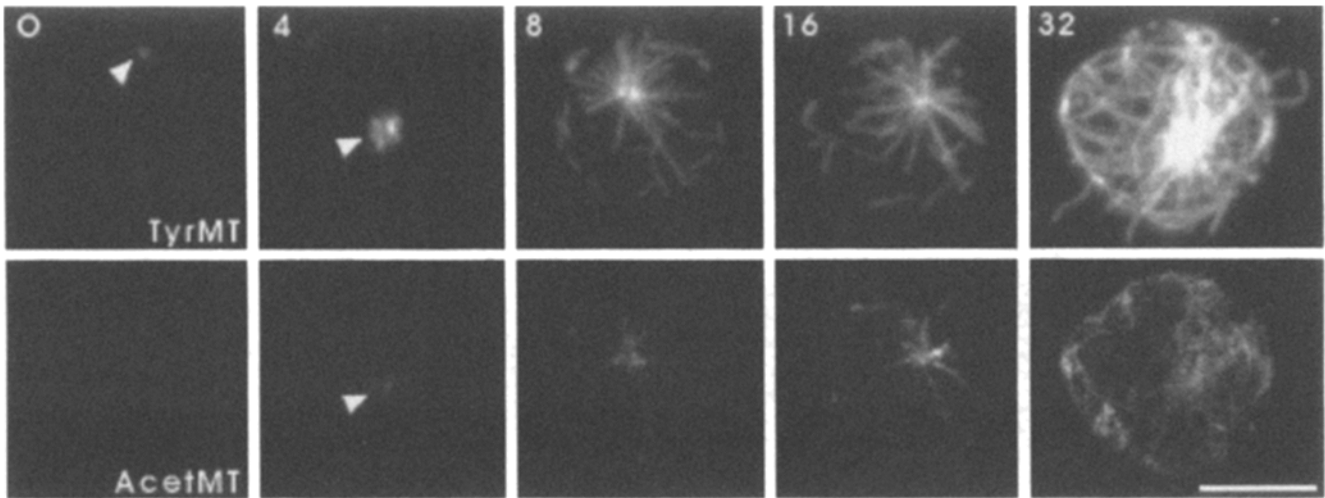


Figure 2. Fluorescence images illustrating the reappearance of tyrMT and acetMT in neurons recovering from exposure to nocodazole. Neurons are shown after 0-, 4-, 8-, 16-, or 32-min recovery in control medium. A neuron examined immediately after exposure to nocodazole (0-min recovery) had a small aggregation of tyrosinated α -tubulin (*arrowhead*) which probably represents the centrosome. A neuron that recovered for 4 min had a small aster (*arrowheads*) composed predominantly of tyrosinated α -tubulin but with acetylated α -tubulin at its center. Neurons that recovered for 8 or 16 min had longer astral microtubules and more acetylated α -tubulin. A neuron that recovered for 32 min had a dense plexus of tyrMT, but fewer acetMT than control neurons (compare to neuron in Fig. 1 *A*). Bar, 5 μ m.

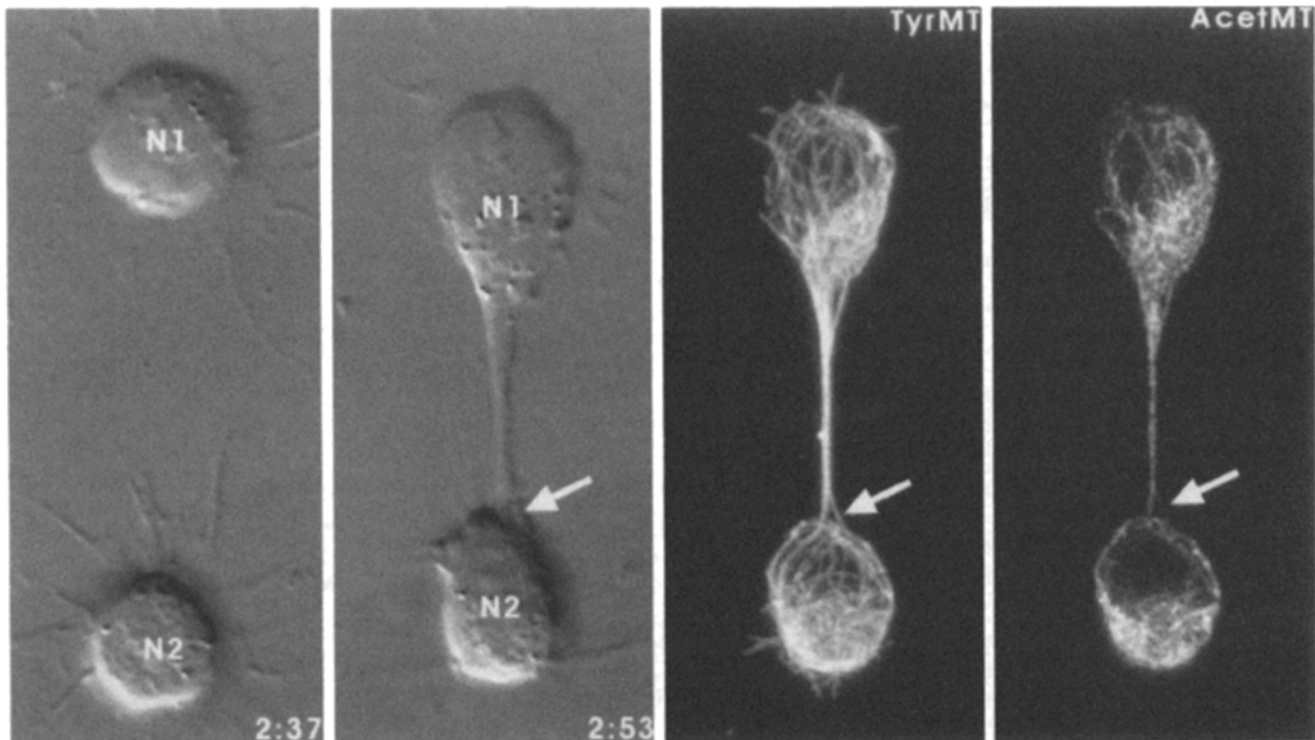


Figure 3. DIC and fluorescence images showing contact-induced neurite formation by a neuron in control medium. Time after plating is given in hours and minutes on the DIC images. A filopodium of a neuron (*N1*) was invaded by cytoplasm, forming a process (*arrow*) that terminated on the cell body of an adjacent neuron (*N2*). The cell body of *N1* moved toward *N2* while the process formed. The process contained a bundle of microtubules labeled with antibodies against tyrosinated and acetylated α -tubulin (*TyrMT* and *AcetMT*, respectively). Notice that staining for tyrosinated α -tubulin extends farther distally than staining for acetylated α -tubulin (*arrows*), suggesting that microtubules were oriented such that their “plus” ends were located distally. Bar, 5 μ m.

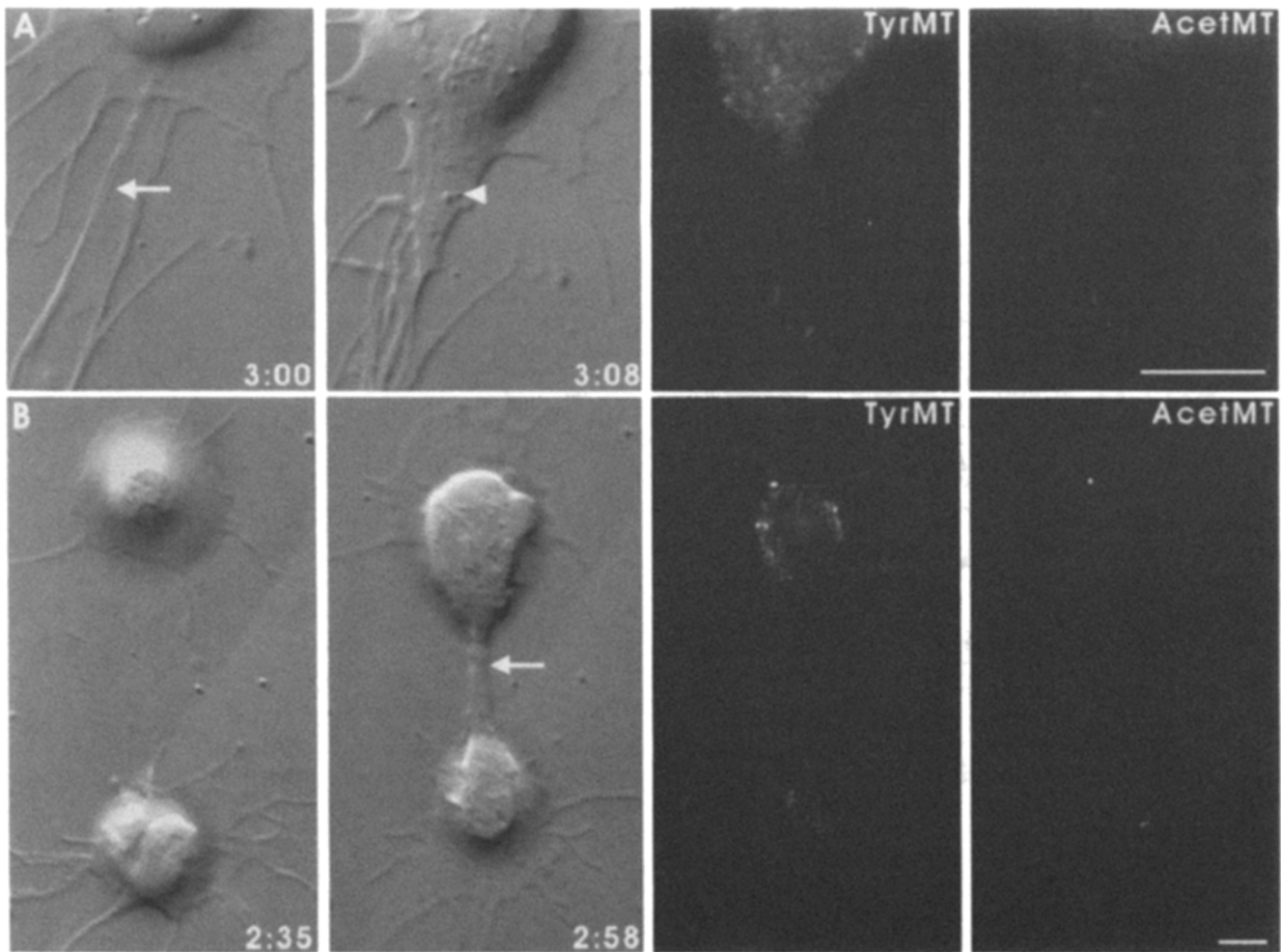


Figure 4. DIC and fluorescence images showing contact-induced process formation by neurons depleted of microtubules by treatment with 2 $\mu\text{g}/\text{ml}$ nocodazole. Time in nocodazole is given in hours and minutes on the DIC images. DIC images in *A* show highly magnified views of a neuron just after one of its filopodia (*arrow*) contacted an adjacent neuron (not visible), and 8 min later, after cytoplasm containing organelles (*arrowhead*) had begun to invade this filopodium. DIC images in *B* show two neurons before contact and after process formation. The processes of the two neurons (*arrow*) were closely apposed and cannot be individually resolved. The cell bodies of the neurons migrated toward each other while the processes formed. Neurons in both *A* and *B* were fixed within 20 s after the second DIC image was collected and then stained with antibodies against the tyrosinated and acetylated forms of α -tubulin. The neurons contained small aggregations of α -tubulin but no intact microtubules. The two short microtubules in the lower part of *A* were in processes of an adjacent neuron. Bars, 5 μm .

nocodazole, most neurons had few or no microtubules (Fig. 1 *B*; see also Figs. 5 and 6). Most of the remaining microtubules appeared to contain only acetylated α -tubulin. However, some microtubules had low concentrations of tyrosinated α -tubulin along their entire length and a few microtubules had small aggregations of tyrosinated α -tubulin at one end (Fig. 1 *B*). Experiments in which microtubules were stained with antibodies that recognize β -tubulin or all forms of α -tubulin likewise showed that few or no microtubules remained intact in most neurons after treatment with nocodazole (not illustrated).

Neurons that were recovering from exposure to nocodazole contained a radially oriented array of microtubules. Such arrays, referred to as "asters", represent microtubules assembling at the centrosome (Osborn and Webster, 1976; Brinkley, 1985). Some neurons also had microtubules that were not associated with asters but, for simplicity, the time course of tubulin polymerization and acetylation was analyzed in neurons that only had astral microtubules (Fig. 2).

After 4 min recovery, astral microtubules were very short (<2 μm) and contained predominantly the tyrosinated form of α -tubulin. At 8 min, they were 2–4- μm long and portions nearest the astral center (the oldest portions) had low concentrations of acetylated α -tubulin. At 16 min, some astral microtubules were over 5- μm long and larger portions contained acetylated α -tubulin. By 32 min, neurons had a dense plexus of microtubules and some microtubules had concentrations of acetylated α -tubulin almost as high as in microtubules in neurons kept in control medium. These observations indicate that microtubules begin to develop significant concentrations of acetylated tubulin between 16 and 32 min after polymerization.

The Initial Steps in Neurite Formation Can Occur during Inhibition of Tubulin Polymerization

Comparison of cultures that were grown in medium containing nocodazole with control cultures showed that nocodazole

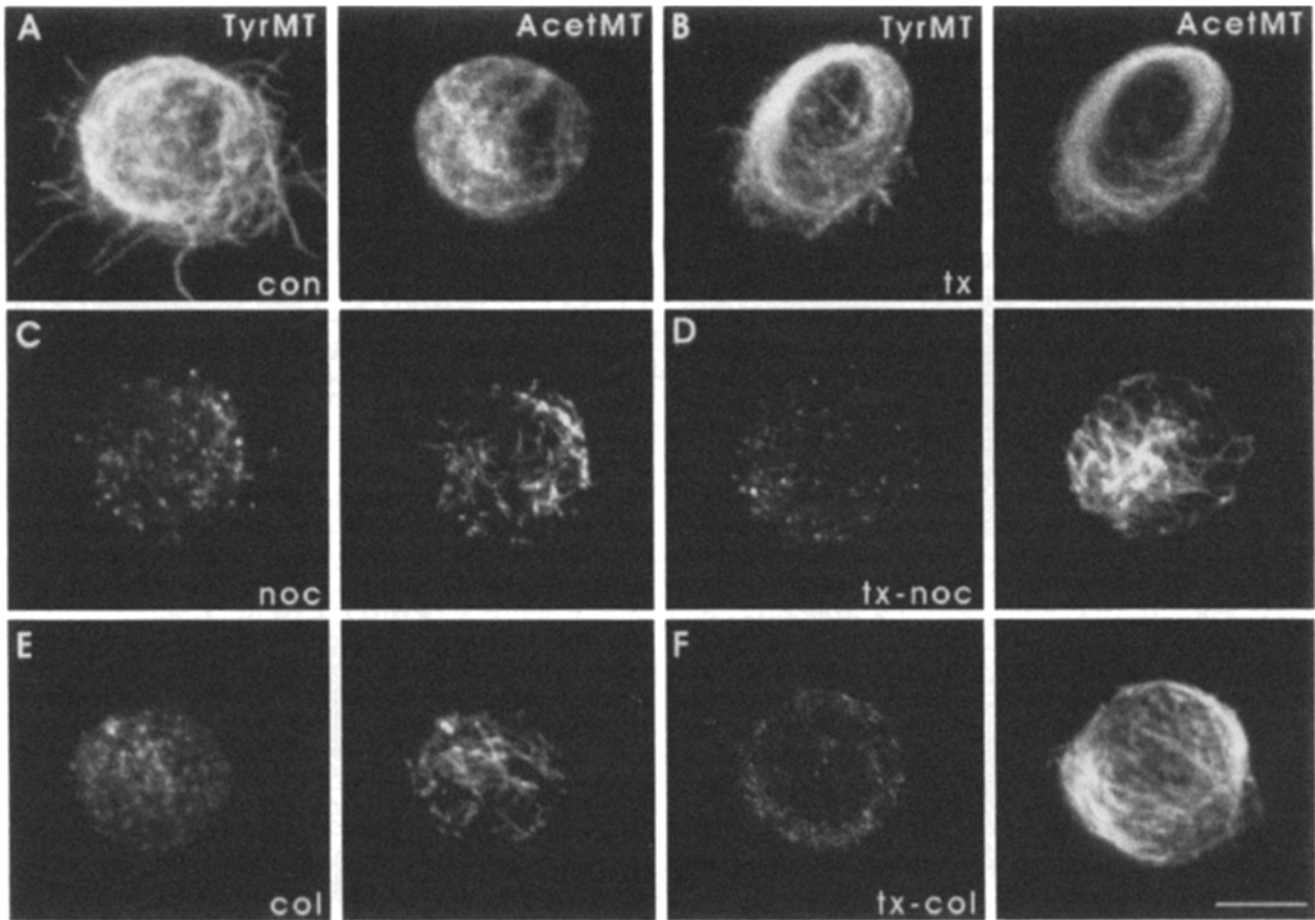


Figure 5. Fluorescence images showing the effects of nocodazole and colchicine on microtubules in control and taxol-pretreated sympathetic neurons. Neurons were labeled with antibodies against tyrosinated and acetylated tubulin following: (A) 1 h in control medium; (B) 1 h in 1 μ M taxol; (C) 3 h in 2 μ g/ml nocodazole; (D) 1 h in 1 μ M taxol followed by 3 h in 2 μ g/ml nocodazole; (E) 3 h in 2 μ g/ml colchicine; (F) 1 h in 1 μ M taxol followed by 3 h in 2 μ g/ml colchicine. Notice that taxol pretreated neurons retain more acetMT than neurons that were not pretreated with taxol. Bar, 5 μ m.

interferes with neurite formation. In control cultures examined 3 to 4 h after plating, between 6 and 35% of neurons had contacted an adjacent cell and begun to form a process (Fig. 3) and some neurons had long neurites. By contrast, in cultures that were exposed to 2 or 4 μ g/ml nocodazole for 2 to 3 h (after 1 h in control medium), fewer than 1% of the neurons had processes and these processes were very short (<25 μ m). Drugs that inhibit microtubule assembly long have been known to prevent neurite elongation (Yamada et al., 1970; Daniels, 1972) and it was widely believed that they prevent neurite formation as well.

However, when neurons growing in medium containing nocodazole were examined by time-lapse video microscopy, they sometimes were observed to form short processes (Fig. 4) and they did so in a way that closely resembled process formation by neurons in control medium (Smith, 1994; Fig. 3). Process formation was initiated by contact of a filopodium with an adjacent neuron. The filopodium straightened suggesting that it was under tension. Then a wedge-shaped bulge of cytoplasm from the perinuclear region began to move outward through the lamella along a path corresponding to the long axis of the filopodium (Fig. 4 A). The bulge of cytoplasm subsequently invaded the filopodium, convert-

ing it into a process several microns in diameter (Fig. 4 B). The interval between the initial movement of cytoplasm from the cell body into the lamella and its arrival at the distal end of the filopodium was 15 to 30 min, within the range found for neurons in control medium (Smith, 1994). The fastest rate at which cytoplasm was observed to advance was \sim 4 μ m/min, half as fast as the fastest cytoplasmic movements seen in neurons in control medium.

Processes formed by neurons during exposure to nocodazole contained organelles (Fig. 4 A). When these organelles were examined at high magnification in real time, they were observed to undergo short, randomly oriented movements similar to those of particles in Brownian motion. By contrast, organelles in processes of neurons in control medium often move rapidly over distances of several microns because they are translocated along a microtubule (Allen et al., 1985; Schnapp et al., 1985). The random movements of organelles in nocodazole-treated neurons suggests that they were not attached to microtubules.

Process formation was accompanied by movement of the nucleus and perinuclear cytoplasm (Fig. 4 B). Before neurons began to form a process, their nucleus and perinuclear cytoplasm were located centrally within the surrounding

lamella. When one of their filopodia contacted another cell and was invaded by cytoplasm, the nucleus and cytoplasm surrounding it moved toward the base of that filopodium. In some neurons, the nucleus and perinuclear cytoplasm migrated into the thickened portion of the filopodium (not illustrated). Similar movements of the nucleus and perinuclear cytoplasm were observed in neurons forming neurites in control medium (Smith, 1994; Fig. 3).

To determine whether neurons that formed processes during exposure to nocodazole contained microtubules, cultures were fixed and stained with antibodies against the tyrosinated and acetylated forms of α -tubulin after filopodia of two neurons had come into contact and begun to thicken by invasion with cytoplasm (two experiments). Three neurons were devoid of microtubules (Fig. 4), while the fourth had a few, very short ($<3 \mu\text{m}$) microtubules. By contrast, processes formed by neurons grown in normal medium contained numerous microtubules (Fig. 3).

These observations show that characteristic steps underlying neurite formation by sympathetic neurons, including the invasion of a filopodium by cytoplasm and the translocation of the nucleus and perinuclear cytoplasm toward the base of this filopodium, can occur in neurons treated with concentrations of nocodazole that inhibit microtubule assembly and lead to depolymerization of most if not all microtubules. This indicates that these steps do not depend on assembly of microtubules or on motors that require assembled microtubules.

Taxol-treated Neurons Retain Microtubules during Exposure to Nocodazole or Colchicine

To determine whether microtubules can be translocated into developing neurites, a paradigm was needed for producing neurons that contain microtubules but are incapable of further microtubule assembly. Taxol is known to stabilize microtubules and it has been reported that cells that have been exposed to taxol retain microtubules during subsequent exposure to nocodazole (De Brabander et al., 1982). These findings suggested that a culture preparation with the desired properties might be obtained by using taxol in conjunction with nocodazole.

Initial experiments showed that neurons that had been exposed to $1 \mu\text{M}$ taxol for one hour and then grown for several hours in medium with both taxol ($1 \mu\text{M}$) and nocodazole (2 or $4 \mu\text{g/ml}$) had microtubules with regions several microns long at their ends that contained high concentrations of tyrosinated α -tubulin (not illustrated). The presence of tyrosinated α -tubulin at the ends of microtubules suggested that microtubules may continue to assemble when simultaneously exposed to taxol and nocodazole.

In a second series of experiments, neurons were exposed to $1 \mu\text{M}$ taxol for one hour and then grown in medium containing 2 or $4 \mu\text{g/ml}$ nocodazole (without taxol). Neurons that were pretreated with taxol had more acetMT following subsequent exposure to nocodazole than neurons in parallel cultures that were exposed to nocodazole without such pretreatment (Figs. 5 and 6), indicating that taxol pretreatment stabilizes microtubules. After 3 h in nocodazole, approximately 75% of the neurons in taxol-pretreated cultures had a fairly dense plexus of acetMT (Figs. 5 D and 6 H), as compared to approximately 25% of the neurons in cul-

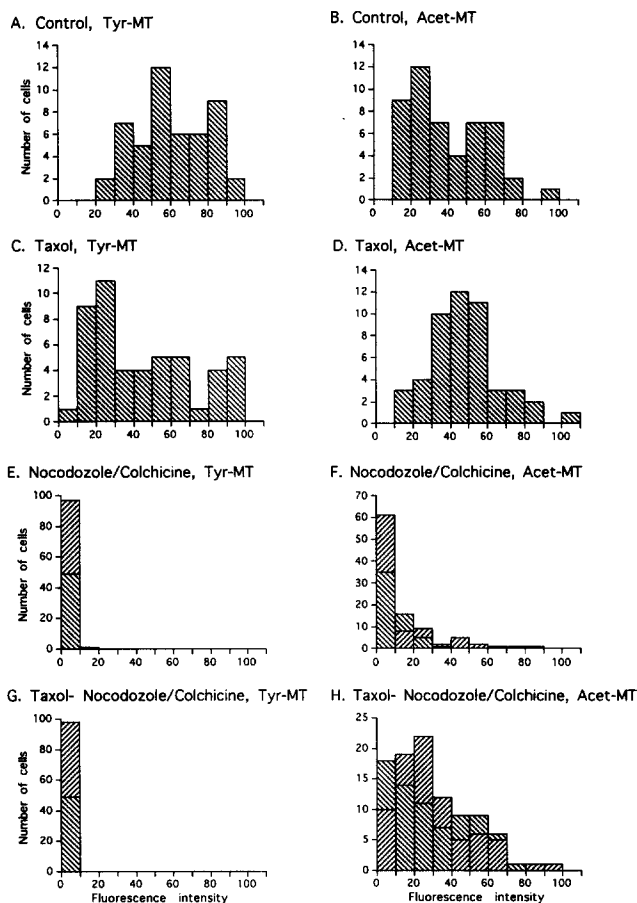


Figure 6. Histograms showing the fluorescence intensity of tyrMT and acetMT in neurons after 1 h in control medium (A and B); 1 h in taxol (C and D); 3 h in nocodazole or colchicine (E and F); 1 h in taxol followed by 3 h in nocodazole or colchicine (G and H). Data from nocodazole and colchicine-treated cultures were combined (right- and left-hand cross hatching) because these drugs had similar effects. Taxol was used at $1 \mu\text{M}$; nocodazole and colchicine at $2 \mu\text{g/ml}$. Fluorescence intensity was measured in the cell bodies of 49 neurons subjected to each treatment (see Materials and Methods for additional details).

tures that had not been exposed to taxol (Figs. 5 C and 6 F). Most microtubules appeared to contain only the acetylated form of α -tubulin, but a few also were weakly stained with anti-tyrosinated α -tubulin (Fig. 5 D). In addition, neurons had small aggregations of tyrosinated tubulin, some of which were aligned with or at the ends of microtubules (Fig. 5 D).

Because the presence tyrosinated α -tubulin at the ends of microtubules might mean that microtubules continued to polymerize during exposure to nocodazole, similar experiments were done using higher concentrations of nocodazole ($10 \mu\text{g/ml}$) or up to $10 \mu\text{g/ml}$ colchicine, another commonly used inhibitor of tubulin polymerization. The results were virtually identical to those obtained with 2 or $4 \mu\text{g/ml}$ nocodazole (Figs. 5, E and F and 6, E-H). Neurons had small aggregations of tyrosinated α -tubulin, some of which were aligned with acetMT. Saturating concentrations of colchicine can completely block tubulin polymerization in vitro (Wilson and Jordan, 1994). Since the concentrations of

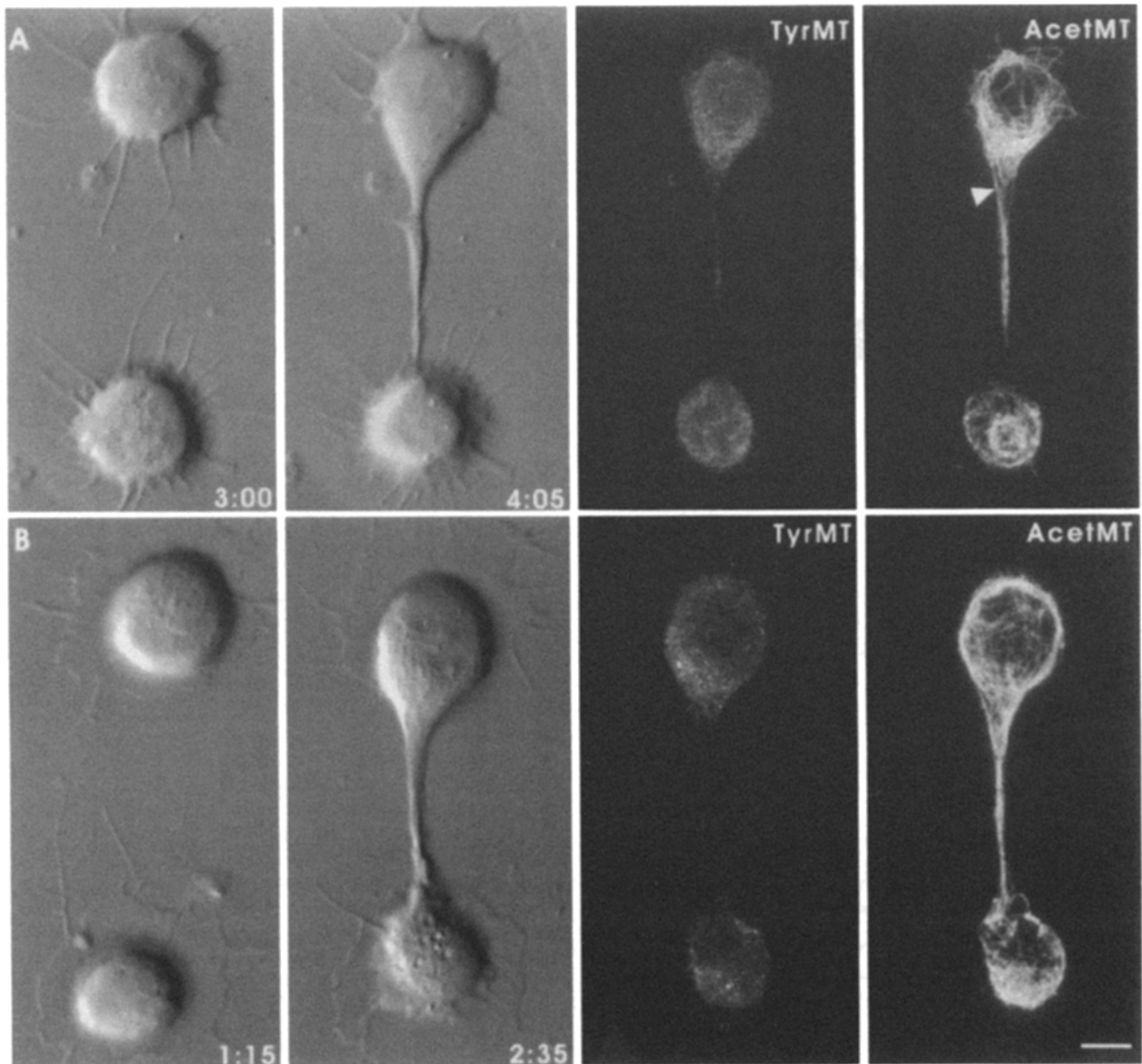


Figure 7. Contact-induced process formation by neurons that were pretreated with taxol to stabilize microtubules and then grown in medium containing 2 $\mu\text{g/ml}$ nocodazole (*A*) or colchicine (*B*) to inhibit assembly of microtubules. The DIC images show neurons before and after process formation. Time in nocodazole or colchicine is given in hours and minutes. The fluorescence images show microtubules labeled with antibodies against the tyrosinated and acetylated forms of α -tubulin (TyrMT and AcetMT, respectively). Microtubules in the cell bodies and processes of neurons had high concentrations of acetylated α -tubulin along their entire lengths. The neurons lacked tyrMT, but contained small aggregates of tyrosinated α -tubulin. Bar, 5 μm .

nocodazole and colchicine used in the present experiments were 10 \times to 100 \times the concentration that depolymerizes most microtubules in sympathetic neurons (unpublished observations) it seems likely that they would be sufficient to block polymerization.

Microtubules Invade Processes Formed during Inhibition of Tubulin Polymerization

Video microscopic observations showed that neurons that had been exposed to taxol and then grown in medium containing nocodazole or colchicine resembled control neurons

in overall shape and often formed processes when one of their filopodia contacted an adjacent cell (Figs. 7 and 8). Process formation entailed invasion of filopodia by cytoplasm and was accompanied by translocation of the cell body, as was described above for neurons that were exposed to nocodazole alone, and was previously found for neurons grown in control medium (Smith, 1994). The cytoplasm that invaded filopodia contained organelles that underwent rapid, saltatory movements, as is typical of organelles being translocated along a microtubule. The processes were just as thick as processes formed by neurons in control medium. However, they differed in that they never became any longer than

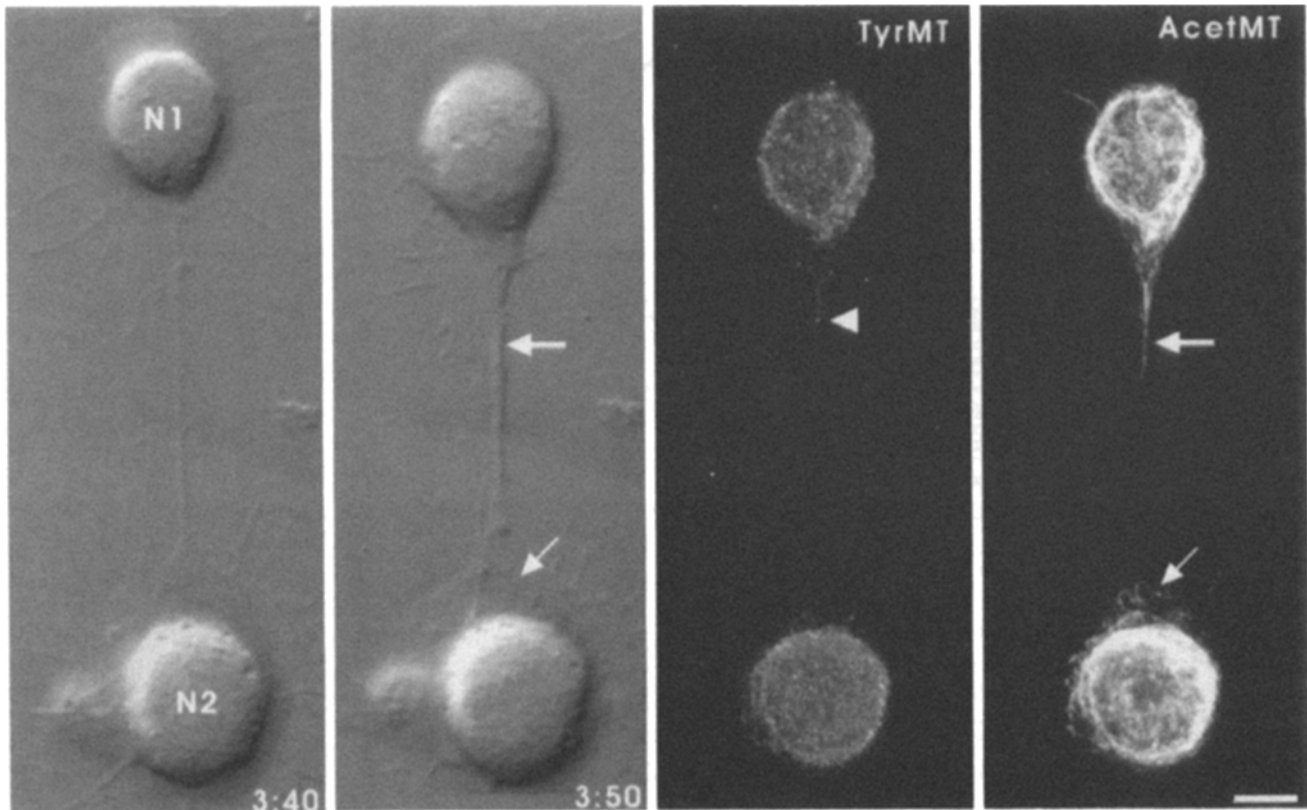


Figure 8. DIC and fluorescence images showing early stages in process formation by neurons (*N1* and *N2*) that were pretreated with taxol and then grown in medium with 2 $\mu\text{g/ml}$ colchicine. Time in colchicine is indicated on the DIC images. In the first DIC image, a filopodium of *N1* and *N2* were in contact. The filopodia cannot be individually resolved because they were closely apposed. 10 min later, cytoplasm from the perinuclear region *N1* had invaded proximal portions of the filopodium contacting *N2* (*thick arrow*). The portions of the filopodium that had been invaded contained a bundle of acetMT (*thick arrow* in AcetMT image). There was a small aggregation of tyrosinated α -tubulin along the bundle (*arrowhead* in TyrMT image), but the microtubules that extended farthest from the cell body did not appear to contain tyrosinated α -tubulin. In *N2*, a sector of the lamella (*thin arrow* in DIC image) had been invaded by cytoplasm and contained curved acetMT (*thin arrow* in AcetMT image). Bar, 5 μm .

the filopodium they replaced. By contrast, neurons grown for equivalent times in control medium often have processes that are 50 μm or more in length (Smith, 1994; and unpublished observations).

The time course by which neurons formed processes was investigated in time-lapse recordings collected at 30- or 60-s intervals (six experiments). The fastest rate at which cytoplasm was observed to advance was approximately 3 $\mu\text{m}/\text{min}$. The average rate of advance, as determined from the time cytoplasm began to invade the lamella surrounding the cell body until it reached half the distance to the tip of the filopodium was $1.43 \pm 0.49 \mu\text{m}/\text{min}$. Both the maximum and average rates of advance were approximately one third as fast as found in neurons grown in control medium (Smith, 1994).

Portions of a filopodium that had been invaded by cytoplasm typically contained a bundle of acetMT which extended from the cell body distally to the leading edge of the cytoplasm (Figs. 7 and 8). AcetMT were present in processes formed by 23 neurons during exposure to 2 or 4 $\mu\text{g/ml}$ nocodazole and by 15 neurons during exposure to up to 10 $\mu\text{g/ml}$ colchicine. Many of the microtubules were straight, as is typical of microtubules in processes formed in

control medium, but some were “U”-shaped (Fig. 7 *A*, *arrow*). Processes formed by two neurons did not contain a bundle of microtubules but, instead, had a few short (<5 μm) microtubules which were not contiguous with microtubules in the cell body (not illustrated). All microtubules in both the cell bodies and processes of neurons had high concentrations of acetylated α -tubulin along their entire lengths and most appeared to contain no tyrosinated α -tubulin (Figs. 7 and 8). Even microtubules in portions of a process that had formed only a few minutes prior to fixation had high concentrations of acetylated α -tubulin along their entire lengths (Fig. 8). The composition of the microtubules suggests that they were not polymerizing at the time of fixation, and is consistent with the idea that they assembled before polymerization was inhibited.

Discussion

The present study used two experimental paradigms to investigate the roles of microtubules in the formation of neurites by sympathetic neurons grown in vitro. In the first paradigm, neurons were grown in medium containing nocodazole to inhibit microtubule assembly, thereby eventually

depleting them of microtubules. In the second, neurons were exposed to taxol to stabilize existing microtubules and then incubated in medium containing nocodazole or colchicine to inhibit tubulin polymerization. Neurite initiation was monitored by time lapse video microscopy in both types of experiments.

Interpretation of these experiments is critically dependent on verifying that the treatments had the expected effects on microtubules. This concern was addressed by staining neurons with two antibodies, one specific for tyrosinated α -tubulin (Wehland et al., 1983), the form of α -tubulin in the tubulin dimers that polymerize to make microtubules, and the other specific for acetylated α -tubulin (Piperno et al., 1987), the form of α -tubulin present in stable microtubules. Exposure of neurons to nocodazole or colchicine clearly inhibited tubulin polymerization because it led to depolymerization of most or, in some neurons, all microtubules. Neurons that were pretreated with taxol retained microtubules during subsequent exposure to either nocodazole or colchicine, consistent with reports that taxol stabilizes microtubules (Schiff and Horwitz, 1980; De Brabander et al., 1982). The microtubules that remained had high concentrations of acetylated α -tubulin along their entire lengths, as is typical of stable microtubules (Piperno et al., 1987; Bulinski et al., 1988; Baas et al., 1991). Most of these stable microtubules did not contain tyrosinated α -tubulin, although some had low concentrations, as also has been found in previous studies (Baas et al., 1991). In addition, some microtubules had small aggregations of tyrosinated α -tubulin at their ends. This was true of microtubules in neurons that had not been pretreated with taxol (Figs. 1 *B* and 5, *C* and *E*) as well as in neurons that had been pretreated (Figs. 5, *D* and *F*). While the presence of tyrosinated α -tubulin at the ends of microtubules could indicate that tubulin polymerized despite the presence of nocodazole or colchicine, an alternative explanation is that these aggregations consisted of tubulin-inhibitor complexes. Tubulin-colchicine complexes are known to bind to the ends of microtubules (Wilson and Jordan, 1994). Indeed, the binding of these complexes is thought to be the mechanism by which colchicine blocks polymerization. Nocodazole has structural similarities to colchicine and may inhibit polymerization by the same mechanism.

Neurons Can Form Short Processes Despite Depolymerization of Their Microtubules

Neurons grown in medium containing nocodazole were able to form short processes despite depolymerization of their microtubules. Process formation involved invasion of a filopodium by organelle-rich cytoplasm from the cell body and was accompanied by translocation of the nucleus and the perinuclear cytoplasm, thereby resembling process formation in control medium. The observation that these morphological changes occurred in neurons that contain few or no microtubules (Fig. 4) indicates they do not depend on assembly of microtubules or on microtubule based transport mechanisms.

The idea that assembly of microtubules might be necessary for neurite formation derives from several previous studies which showed that neurons grown in the presence of drugs that inhibit tubulin polymerization fail to form neurites (Yamada et al., 1970; Daniels, 1972; Solomon and Magen-

dant, 1981; Joshi et al., 1985; although see Lamoureaux et al., 1990). However, short processes such as those observed in the present experiments probably would not have been detected in the previous studies. The present findings demonstrate that microtubule assembly is not required for the initial steps in neurite formation by sympathetic neurons grown on polyornithine, but are consistent with the idea that assembly of microtubules is essential for neurite elongation. Indeed, processes formed by sympathetic neurons during exposure to nocodazole never developed into definitive neurites with growth cones and they failed to elongate. The observation that neurites fail to elongate when subjected to treatments that inhibit tubulin polymerization without depolymerizing existing microtubules also suggests that assembly of microtubules may be required for neurite elongation (see also Keith, 1990; Bamburg, et al., 1986).

Microtubules Invade Neurites Formed during Inhibition of Tubulin Polymerization

Neurons that had been treated with taxol formed short processes during exposure to nocodazole or colchicine and, in many instances, their processes contained microtubules. The most parsimonious explanation of these findings is that the microtubules in these processes assembled before polymerization was inhibited and were translocated into the processes as they emerged. The fact that the microtubules contained high concentrations of the acetylated form of α -tubulin and little or no tyrosinated α -tubulin supports the idea that they were taxol-stabilized microtubules that had assembled before polymerization was inhibited. The alternative explanation, that microtubules were not translocated but instead extended into processes by elongating, seems unlikely by the following reasoning. If microtubules invaded processes by polymerizing, then microtubules in recently formed portions of a process would be expected to contain the tyrosinated form of α -tubulin rather than the acetylated form (Piperno et al., 1987; Baas and Ahmad, 1992). Indeed, if the rate of acetylation in processes is similar to the rate observed in the cell bodies of neurons recovering from nocodazole (Fig. 2) or in 3T3 cells (Piperno et al., 1987), then microtubules would not contain high concentrations of acetylated tubulin until more than 16 min after assembly. However, even portions of processes that had formed only a few minutes before fixation had microtubules which, in most instances, contained only the acetylated form of α -tubulin. Although it is possible that acetylation occurs more rapidly in processes than cell bodies, the fact that no tyrosinated α -tubulin could be detected in most microtubules suggests that they were not polymerizing. Another reason to believe that taxol-treated neurons were unable to assemble new microtubules during exposure to nocodazole or colchicine is that the processes they formed remained short. If these neurons were able to assemble microtubules, then they might be expected to form longer processes. Also, sympathetic neuroblasts grown under these conditions were unable to complete mitosis (unpublished observations), another cellular process that is known to depend on assembly of microtubules.

The rate at which microtubules were translocated into filopodia can be inferred from the rate at which cytoplasm was observed to invade filopodia (up to 3 $\mu\text{m}/\text{min}$) because microtubules extended to the leading edge of the cytoplasm. The average rate of advance (1.46 $\mu\text{m}/\text{min}$; $n = 6$) was ap-

proximately one third as fast as found for neurons grown in control medium (4.68 $\mu\text{m}/\text{min}$; $n = 44$; Smith, 1994). Invasion of filopodia by microtubules during neurite formation in control medium probably involves elongation of microtubules by polymerization as well as translocation of microtubules and may occur more rapidly for this reason.

Two recent studies also used drug treatments to determine whether microtubules are translocated into developing neurites (Zheng et al., 1993; Baas and Ahmad, 1993). They examined neurons treated with concentrations of vinblastine which are thought to inhibit tubulin polymerization without causing depolymerization of extant microtubules (Jordan and Wilson, 1990; Wilson and Jordan, 1994). In one study, neurite formation was induced by attaching a pipette to the margin of a neuronal cell body and pulling outward (Zheng et al., 1993). Processes induced by this technique in control medium contained microtubules (Zheng et al., 1991), but those formed during exposure to vinblastine did not. One possible explanation of this discrepancy between the results of this previous study and the present findings is that processes induced by pulling on the margin of a cell with a pipette may not have an organized actin cytoskeleton capable of exerting a pulling force on microtubules (see below). A second study (Baas and Ahmad, 1993), showed that sympathetic neurons grown on extracellular matrix material (ECM) not only can initiate neurite formation during exposure to vinblastine, but can form long neurites (50 μm or more) that contain numerous microtubules. However, the microtubules in these neurites had high concentrations of tyrosinated α -tubulin which could indicate that vinblastine did not completely block tubulin polymerization.

The question of whether microtubules in neurites are translocated during neurite elongation has been investigated by visualizing fluorescence-labeled microtubules in living cells. Microtubules in neurites of avian and mammalian neurons appeared to be stationary in most studies (Lim et al., 1989, 1990; Okabe and Hirokawa, 1990; but see Keith, 1987; Keith and Farmer, 1993). However, microtubules in neurites of amphibian neurons moved anterogradely during neurite elongation at rates of up to approximately 1 $\mu\text{m}/\text{min}$ (Reinsch et al., 1991; Okabe and Hirokawa, 1992), similar to the rate at which the present experiments suggest that microtubules move during the initiation of neurite outgrowth.

The Movements of Cytoplasm That Lead to Neurite Formation Probably Are Produced by an Actomyosin Based Motility Mechanism

The observation that neurons can form processes following depletion of their microtubules rules out models of the initiation of neurite outgrowth, such as the first of the two discussed in the Introduction, which postulate an essential role for microtubules. However, the present findings are consistent with the idea that neurite formation is mediated by actomyosin-based motility mechanisms. An hypothesis about how myosin molecules might interact with actin filaments to produce the movements of cytoplasm that lead to the formation of a neurite was described previously (Smith, 1994). According to this hypothesis, neurite formation involves migration of an entire sector of perinuclear cytoplasm outward along a filopodium. The driving force for this migration is provided by myosin motor molecules associated

with actin filaments in the cortex surrounding perinuclear cytoplasm. These myosin motors drag cortical actin filaments outward along filopodial actin filaments while other components of perinuclear cytoplasm are transported passively. Evidence that cortical actin filaments are translocated during neurite formation has been reported previously (Smith, 1994). The observation that microtubules invaded processes formed by taxol-treated neurons during inhibition of tubulin polymerization (Figs. 7 and 8) suggests that microtubules also are translocated during neurite formation, thereby providing further support for this model.

The morphological changes that occur in the periphery of a neuron following contact of a filopodium with another cell resemble changes observed in growth cones following contact of a filopodium with an adhesive object such as another neurite (Heidemann et al., 1990; Lin and Forscher, 1993) or a guide post cell (O'Connor et al., 1990; Sabry et al., 1991). Contact is followed by extension of microtubules as well as other components of axoplasm into the periphery of the growth cone, along a trajectory aligned with the filopodium. Under normal conditions, extension of microtubules is due, at least in part, to growth of microtubules by polymerization at their distal ends (Bamburg et al., 1986; Robson and Burgoyne, 1987; Lim et al., 1989; Baas and Ahmad, 1992). However, microtubules in growth cones also may be translocated (Tanaka and Kirschner, 1991). These similarities between growth cone extension and neurite initiation suggests that the two processes probably are mediated by common mechanisms. Therefore, the present conclusions concerning the mechanisms that produce the cytoplasmic movements that lead to the initiation of neurite outgrowth are likely to be relevant to growth cones as well.

I thank Drs. Gary Banker, Thomas Reese, and Robert Burke for helpful discussions and critical reading of this manuscript.

Received for publication 25 July 1994.

References

- Abercrombie, M. 1980. The crawling movement of metazoan cells. *Proc. Roy. Soc. Lond. B.* 207:129-147.
- Albrecht-Buehler, G. 1987. Role of cortical tension in fibroblast shape and movement. *Cell Motil. Cytoskeleton.* 7:54-67.
- Allen, R. D., D. G. Weiss, J. H. Hayden, D. T. Brown, H. Fujiwaki, and M. Simpson. 1985. Gliding movement of and bidirectional organelle transport along single native microtubules from squid axoplasm: evidence for an active role of microtubules in cytoplasmic transport. *J. Cell. Biol.* 100:1736-1752.
- Bamburg, J. R., D. Bray, and K. Chapman. 1986. Assembly of microtubules at the tip of growing axons. *Nature (Lond.)* 321:778-790.
- Baas, P. W., L. A. White, and S. R. Heidemann. 1987. Microtubule polarity reversal accompanies regrowth of amputated neurites. *Proc. Natl. Acad. Sci. USA.* 85:8335-8339.
- Baas, P. W., and M. M. Black. 1990. Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol.* 111:495-509.
- Baas, P. W., T. Slaughter, A. Brown, and M. M. Black. 1991. Microtubule dynamics in axons and dendrites. *J. Neurosci. Res.* 30:134-153.
- Bass, P. W., and F. J. Ahmad. 1992. The plus ends of stable microtubules are the exclusive nucleating structures for microtubules in the axon. *J. Cell Biol.* 116:1231-1241.
- Baas, P. W., and F. J. Ahmad. 1993. The transport properties of axonal microtubules establish their polarity orientation. *J. Cell Biol.* 120:1427-1437.
- Bray, D., and J. G. White. 1986. Cortical flow in animal cells. *Science (Wash. DC)* 239:883-888.
- Brinkley, B. R. 1985. Microtubule organizing centers. *Annu. Rev. Cell Biol.* 1:145-172.
- Bulinski, J. C., J. E. Richards, and B. Piperno. 1988. Post-translational modifications of α -tubulin: Detyrosination and acetylation differentiate populations of interphase microtubules in cultured cells. *J. Cell Biol.* 106:1213-1220.
- Daniels, M. P. 1972. Colchicine inhibition of nerve fiber formation in vitro.

- J. Cell Biol.* 53:164-176.
- De Brabander, M., G. Geuens, R. Nuydens, R. Willebrords, and J. De Mey. 1982. Microtubule stability and assembly in living cells: the influence of metabolic inhibitors, taxol and pH. *Cold Spring Harbor Symp. Quant. Biol.* 46:227-240.
- Forscher, P., and S. J. Smith. 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* 107:1505-1516.
- Forscher, P., and S. J. Smith. 1990. Cytoplasmic actin filaments move particles on surface of a neuronal growth cone. In *Optical Microscopy for Biology*. B. Herman and K. Jacobson, editors. Wiley-Liss, New York. 459-471.
- Forscher, P., C. H. Lin, and C. Thompson. 1992. Novel form of growth cone motility involving site-directed actin filament assembly. *Nature (Lond.)*. 357:515-518.
- Goldberg, D. J., D. W. Burmeister, and R. J. Rivas. 1991. Video microscopic analysis of events in the growth cone underlying axon growth and the regulation of these events by substrate-bound proteins. In *The Nerve Growth Cone*. P. C. Letourneau, S. B. Kater, E. R. Macagno, editors. Raven Press, New York. 79-96.
- Gundersen, G. G., S. Khawaja, and J. C. Bulinski. 1987. Postpolymerization detyrosination of α -tubulin: a mechanism for subcellular differentiation of microtubules. *J. Cell Biol.* 105:251-264.
- Heidemann, S. R., P. Lamoureux, and R. E. Buxbaum. 1991. On the cyto-mechanics and fluid dynamics of growth cone motility. *J. Cell Sci. Suppl.* 15:35-44.
- Joshi, H. C., P. Baas, D. T. Chu, and S. R. Heidemann. 1986. The cytoskeleton of neurites after microtubule depolymerization. *Exp. Cell. Res.* 163: 233-245.
- Jordan, M. A., and L. Wilson. 1990. Kinetic analysis of tubulin exchange at microtubule ends at low vinblastine concentrations. *Biochemistry*. 29: 2730-2739.
- Keith, C. H. 1990. Neurite elongation is blocked if microtubule polymerization is inhibited in PC12 cells. *Cell Motil. Cytoskeleton*. 17:95-105.
- Keith, C. H., and M. A. Farmer. 1993. Microtubule behavior in PC12 neurites: variable results obtained with photo bleach technology. *Cell Motil. Cytoskeleton*. 25:345-357.
- Kirchner, M., and T. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. *Cell*. 45:329-342.
- Lamoureux, P., V. L. Steel, C. Regal, L. Adgate, R. E. Buxbaum, and S. R. Heidemann. 1990. Extracellular matrix allows PC12 neurite elongation in the absence of microtubules. *J. Cell Biol.* 110:71-79.
- Lee, J., A. Ishihara, J. A. Theriot, and K. Jacobson. 1993. Principles of locomotion for simple-shaped cells. *Nature (Lond.)*. 362:167-171.
- Letourneau, P. C. 1982. Nerve fiber growth and its regulation by extrinsic factors. In *Neuronal Development*. N. C. Spitzer, editor. Plenum Press, New York. 213-254.
- Lim, S. S., P. J. Sammak, and G. G. Borisy. 1989. Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.* 109:253-263.
- Lim, S.-S., J. E. Edson, P. C. Letourneau, and G. G. Borisy. 1990. Progressive and spatially differentiated stability of microtubules in developing neuronal cells. *J. Cell Biol.* 111:123-130.
- Lin, C.-H., and P. Forscher. 1993. Cytoskeletal remodeling during growth cone-target interactions. *J. Cell Biol.* 121:1369-1383.
- Marusich, M. F., K. Pourmehr, and J. A. Weston. 1986. The development of an identified subpopulation of avian sensory neurons is regulated by interaction with the periphery. *Dev. Biol.* 118:505-510.
- Mitchison, T., and M. Kirschner. 1988. Cytoskeletal dynamics and nerve growth. *Neuron*. 1:761-772.
- O'Connor, T., J. Duerr, and D. Bentley. 1990. Pioneer growth cone steering decisions mediated by single filopodial contacts in situ. *J. Neurosci.* 10:3935-3946.
- O'Connor, T., and D. Bentley. 1993. Accumulation of actin in subsets of pioneer growth cone filopodia in response to neuronal and epithelial guidance cues in situ. *J. Cell Biol.* 123:935-948.
- Okabe, S., and N. Hirokawa. 1988. Microtubule dynamics in nerve cells: analysis using injection of biotinylated tubulin into PC12 cells. *J. Cell Biol.* 107:647-659.
- Okabe, S., and N. Hirokawa. 1990. Turnover of fluorescently labeled tubulin and actin in the axon. *Nature (Lond.)*. 343:479-482.
- Okabe, S., and N. Hirokawa. 1992. Differential behavior of photoactivated microtubules in growing axons of mouse and frog neurons. *J. Cell Biol.* 117:105-120.
- Osborn, M., and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structures toward the plasma membrane. *Proc. Natl. Acad. Sci. USA*. 73:867-871.
- Piperno, G., M. Li Dizet, and X. Chang. 1987. Microtubules containing acetylated α -tubulin in mammalian cells in culture. *J. Cell Biol.* 104: 289-302.
- Reinsch, S. S., T. J. Mitchison, and M. Kirschner. 1991. Microtubule polymer assembly and transport during axonal elongation. *J. Cell Biol.* 115:365-379.
- Sabry, J. H., T. P. O'Connor, L. Evans, A. Toroian-Raymond, M. Kirschner, and D. Bentley. 1991. Microtubule behavior during guidance of pioneer neuron growth cones in situ. *J. Cell Biol.* 115:381-395.
- Schiff, P. B., and S. B. Horwitz. 1980. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA*. 77:1561-1565.
- Schnapp, B. J., R. D. Vale, M. P. Sheetz, and T. S. Reese. 1985. The structure of cytoplasmic filaments involved in organelle transport in the squid giant axon. *Cell*. 40:455-462.
- Smith, C. 1994. Cytoskeletal movements and substrate interactions during initiation of neurite outgrowth by sympathetic neurons in vitro. *J. Neurosci.* 14:384-398.
- Tanaka, E., and M. Kirschner. 1991. Microtubule behavior in the growth cones of living neurons during axon elongation. *J. Cell Biol.* 115:345-363.
- Wehland, J., M. C. Willingham, and I. V. Sandoval. 1983. A rat monoclonal antibody reacting specifically with the tyrosylated form of α -tubulin. I. Biochemical characterization, effects on microtubule polymerization in vitro, and microtubule polymerization and organization in vivo. *J. Cell Biol.* 97:1467-1475.
- Wilson, L., and M. A. Jordan. 1994. Pharmacological probes of microtubule function. In *Microtubules*. J. S. Hyams, and C. W. Lloyd, editors. Wiley-Liss, New York. 59-84.
- Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1970. Axon growth: role of microfilaments and microtubules. *Proc. Natl. Acad. Sci. USA*. 66: 1206-1212.
- Zheng, J., R. E. Buxbaum, and S. R. Heidemann. 1993. Investigation of microtubule assembly and organization accompanying tension-induced neurite initiation. *J. Cell Sci.* 104:1239-1250.