Microtubule Nucleation and Release from the Neuronal Centrosome

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Abstract. We have proposed that microtubules (MTs) destined for axons and dendrites are nucleated at the centrosome within the cell body of the neuron, and are then released for translocation into these neurites (Baas, P. W., and H. C. Joshi. 1992. *J. Cell Biol.* 119:171–178). In the present study, we have tested the capacity of the neuronal centrosome to act as a generator of MTs for relocation into other regions of the neuron. In cultured sympathetic neurons undergoing active axonal outgrowth, MTs are present throughout the cell body including the region around the centrosome, but very few (<10) are directly attached to the centrosome. These results indicate either that the neuronal centrosome is relatively inactive with regard to MT nucleation, or that most of the MTs nucleated at the centrosome are rapidly released. Treatment for 6 h with 10 μg/ml nocodazole results in the depolymerization of greater than 97% of the MT polymer in the cell body. Within 5 min after removal of the drug, hundreds of MTs have assembled in the region of the centrosome, and most of these MTs are clearly attached to the centrosome. A portion of the MTs are not attached to the centrosome, but are aligned side-by-side with the attached MTs, suggesting that the unattached MTs were released from the centrosome after nucleation. In addition, unattached MTs are present in the cell body at decreasing levels with increasing distance from the centrosome. By 30 min, the MT array of the cell body is indistinguishable from that of controls. The number of MTs attached to the centrosome is once again diminished to fewer than 10, suggesting that the hundreds of MTs nucleated from the centrosome after 5 min were subsequently released and translocated away from the centrosome. These results indicate that the neuronal centrosome is a highly potent MT-nucleating structure, and provide strong indirect evidence that MTs nucleated from the centrosome are released for translocation into other regions of the neuron.

MICROTUBULES (MTs)[1] are present throughout the neuron, but are organized differently within each of its compartments. In the axon, the MTs are uniformly oriented with their plus-ends-distal to the cell body (Heidemann et al., 1981; Burton and Paige, 1981), whereas in the dendrite, roughly equal proportions of the MTs are of each orientation (Baas et al., 1988; Burton, 1988). The minus-end-distal MTs arise later in development and generally do not extend as far into the dendrite as do the plus-end-distal MTs (Baas et al., 1989), suggesting that dendrites contain two populations of MTs of uniform but opposite polarity orientation. In the cell body, MTs are splayed in many directions (Baas et al., 1988; Burton, 1988), but some coalesce into organized bundles that funnel into the hillock regions of the neurites (Bartlett and Banker, 1984; Stevens et al., 1988). Because the shape and cytoplasmic composition of each neuronal compartment is principally defined by these unique MT arrays (for reviews see Lasek, 1988; Black and Baas, 1989), there is great interest in elucidating the mechanisms by which they are elaborated and organized.

In nonneuronal cells, MT arrays are typically organized by discrete MT nucleating structures known as centrosomes (for reviews see Kimble and Kuriyama, 1992; Tucker, 1992). The minus ends of the MTs are embedded within the centrosome, and the plus ends emanate away from it, resulting in MT arrays of uniform polarity orientation (Euteneuer and McIntosh, 1981). In addition, centrosomes help regulate the number and length of the MTs within a cell (for review see Brinkley, 1985), and may also play a role in determining the lattice structure of each MT within the array (Scheele et al., 1980; Evans et al., 1985). In light of the fact that axonal and dendritic MTs are highly organized, it is surprising that these MTs are not attached to any centrosome-like nucleating structure (Sharp et al., 1982; Lyser, 1964). Rather, axonal and dendritic MTs are discontinuous along the lengths of these neurites (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981; Sasaki et al., 1983), with neither minus nor plus ends attached to any visible structure that could regulate MT assembly or organization.

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1. *Abbreviation used in this paper*: MTs, microtubules.

Despite the lack of attachment of neuritic MTs to the centrosome, two sets of observations suggest that the centrosome may nevertheless be critical for their formation. First, we have reported evidence indicating that all MT assembly in the axon is limited to the elongation of existing MTs (Baas and Ahmad, 1992). This conclusion is attractive in that newly assembled polymer will inherit the polarity orientation and lattice structure of the polymer from which it elongates, thus preserving and perpetuating these characteristics as the axonal MT array expands. More relevant to the issue here, the inability of the axon to generate entirely new MTs implicates the cell body as the only potential site where new MTs for the axon could arise. Second, studies on the distribution of gamma-tubulin in the neuron also support a cell body origin for neuritic MTs, and in particular, suggest a centrosomal origin. Gamma-tubulin is a newly discovered protein (Oakley and Oakley, 1989) which has been shown to be essential for MT nucleation in nonneuronal cells (Joshi et al., 1992). We reasoned that if gamma-tubulin is also required for MT nucleation in neurons, then the distribution of gamma-tubulin could be used to reveal the sites where axonal and dendritic MTs originate. Our studies showed that gamma-tubulin is localized to the centrosome, and is not present in the axon, dendrite, or regions of the cell body other than the centrosome (Baas and Joshi, 1992). Based on these results, we concluded that the centrosome is, in fact, the site of origin for all MTs in the neuron, even those ultimately appearing within axons and dendrites at great distances from the cell body.

If this reasoning is correct, the neuronal centrosome is an extremely potent MT-nucleating structure, capable of generating hundreds upon hundreds of MTs for the growth and maintenance of elaborate axonal and dendritic arbors. In addition, it is implicit in our proposal that the neuronal centrosome has the capacity to rapidly release the MTs it nucleates, so that they can be exported into and down the lengths of the neurites. Electron microscopic analyses of different kinds of neurons at different developmental stages vary with regard to the appearance of the centrosome, but most studies reveal relatively few MTs directly attached to the centrosome (Lyser, 1964, 1968; Tennyson, 1965). In fact, our studies on cultured sympathetic neurons undergoing active axonal and dendritic outgrowth reveal fewer than 10 and often no MTs directly attached to the centrosome (Baas and Joshi, 1992; present study). One possibility is that neuritic MTs do not arise at the centrosome, and the neuronal centrosome is actually relatively inactive. Alternatively, however, the nucleation and release of MTs from the neuronal centrosome may be so rapid that there is insufficient time for substantial numbers of attached MTs to accumulate at the centrosome before they are released. These two possibilities have very different implications for the manner by which the MT arrays of the neuron are elaborated. Is the neuronal centrosome a relatively inactive structure, or is it a highly potent generator of MTs destined for release and transport into axons and dendrites?

In the present study, we have addressed this issue using the classic assay for identifying MT-nucleating structures in living cells (De Brabander et al., 1977, 1980; Brinkley, 1985; Gunderson et al., 1987; Bulinski et al., 1988). In this assay, existing MTs are depolymerized using nocodazole, a highly specific and reversible anti-MT drug. The drug is then rinsed from the cells, and MTs are permitted to reassemble. The sites where MTs reassemble correspond to MT-nucleating structures. In previous studies, we used this assay to study MT assembly in the axon (Baas and Ahmad, 1992). In this article, using the same approach to study the cell body of the neuron, we have been able to evaluate the capacity of the neuronal centrosome to nucleate MTs in the kinds of numbers that would be required to sustain the growth of axons and dendrites. In addition, by performing a series of studies at progressively longer recovery times, we have explored whether MTs nucleated at the neuronal centrosome are subsequently released and relocated into other regions of the neuron.

Materials and Methods

Cell Culture

Dissociated cultures of sympathetic neurons from the superior cervical ganglia of newborn rat pups were prepared as follows. After dissection, the ganglia were treated with 0.25% collagenase for 1 h followed by 0.25% trypsin for 45 min, and then triturated with a pasteur pipette into a single cell dispersion. The neurons were then plated onto "special dishes" that were prepared by adhering a glass coverslip to the bottom of a 35-mm plastic petri dish into which had been drilled a 1-cm diam hole (Whitlon and Baas, 1992; Ahmad et al., 1993). Before plating the cells, the glass-bottomed well of the special dish was treated for 3 h with 1 μg/ml polylysine, rinsed extensively, and then treated with 10 μg/ml laminin for 4 h (Higgins et al., 1991). Cells were plated in medium consisting of Leibovitz' L-15 (Sigma Chem. Co., St. Louis, MO) supplemented with 0.6% glucose, 2 mM *L*-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS (Hyclone, Logan, UT), and 100 μg/ml NGF. The cells were plated at a particularly high density (one ganglion per glass-bottomed well) to maximize the number of cell bodies that could be sectioned simultaneously for electron microscopy. Cells were cultured for roughly 20 h, during which time extensive axon outgrowth occurred (see Fig. 1).

Nocodazole Treatment

Nocodazole was purchased from Aldrich Chem. Co. (Milwaukee, WI). A stock solution of 10 mg/ml was prepared in DMSO, and diluted in tissue culture medium at 1:1,000 to achieve a final concentration of 10 μg/ml. Both the stock solution and the drugged medium were prepared fresh for each experiment, and fresh nocodazole was purchased each month. Nocodazole-containing medium was warmed to 37°C and applied to cells for 6 h. For recovery, cultures were rinsed twice briefly with drug-free medium, and then incubated for the designated recovery time in a third rinse of drug-free medium at 37°C. Timing for recovery began at the point at which the third rinse of drug-free medium was added, and recovery times ranged from 30 s to 30 min. Previous studies have determined that nocodazole is immediately and completely reversible when rinsed from neuron cultures in this manner (Baas and Heidemann, 1986; Baas and Ahmad, 1992).

Confocal Immunofluorescence Microscopy

For immunofluorescence microscopy, cultures were rinsed briefly in a MT stabilizing buffer termed PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9; Schliwa and van Blerkom, 1981), and then extracted for 3 min with 0.5% Triton X-100 in PHEM containing 10 μM taxol. PHEM supplemented with 10 μM taxol has been shown to stabilize existing MTs against disassembly during extraction of cultured sympathetic neurons without promoting artifactual MT assembly (Black et al., 1986; see also Baas and Black, 1990; Baas and Ahmad, 1992; Brown et al., 1992). After extraction, the cultures were fixed for 10 min in PBS containing 4% paraformaldehyde and 0.5% glutaraldehyde. After two rinses in PBS, cultures were treated four times for 5 min each in PBS containing 10 mg/ml sodium borohydride, and rinsed again in PBS. Cultures were then treated for 30 min in a blocking solution containing 2% normal goat serum and 1% BSA in PBS, and exposed to primary antibodies diluted in blocking solution overnight at 4°C. The following morning, the cultures were rinsed three times in PBS, treated again for 30 min in blocking solution, exposed to second antibodies diluted in blocking solution for 1 h at 37°C, and rinsed

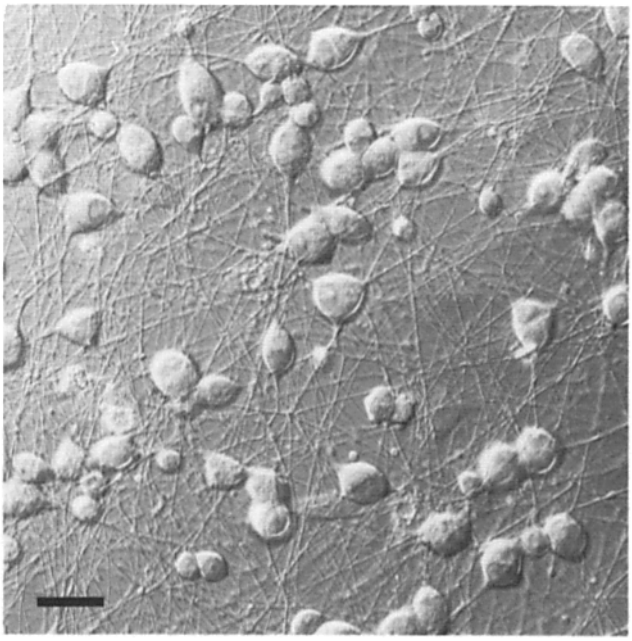

Figure 1. Differential-interference contrast (DIC) micrograph of a region of a culture of rat sympathetic neurons prepared as described in Materials and Methods. Neurons were plated at a particularly high density to maximize the number of cell bodies that could be sectioned and analyzed simultaneously for EM. For consistency, the same type of culture was used for immunofluorescence analyses. By 20 h after plating, dense axonal outgrowth is apparent. Bar, 30 μm.

four times for 5 min each in PBS. The glass coverslips were removed from the special dishes and mounted onto glass slides using nail polish to seal the edges. The mounting medium, which reduces photobleaching, consisted of 100 mg/ml DABCO and 1 mg/ml *p*-phenylenediamine dissolved in a solution containing 90% glycerol and 10% PBS. The primary antibodies were YL 1/2, a mouse monoclonal antibody against tyrosinated α-tubulin (Kilmartin et al., 1982; Wheland et al., 1983) which stains all MTs in the cell body of cultured sympathetic neurons (Baas et al., 1991), and a rabbit polyclonal antibody against centrin, a protein found in the pericentriolar material (Sanders and Salisbury, 1989). The former was purchased from Accurate Chem. & Sci. Corp. (Westbury, NY) and used at 1:100, while the latter was provided by Dr. Jeff Salisbury and used at 1:3,000. The second antibodies were a fluorescein-conjugated goat anti-rat and a Texas red–conjugated goat anti-rabbit. Both were purchased from Accurate Chem. & Sci. Corp. and used at 1:100. Cells were observed using the MRC-600 laser confocal microscope (Biorad, Cambridge, MA). This instrument is equipped with a KrAr laser, and is located at the Integrated Microscopy Resource (University of Wisconsin). Images of MT and centrin staining were collected from the same focal plane through the cell body, and particular attention was given to the optical sections containing the spot which stained brightest for centrin.

Electron Microscopy

To enhance contrast and optimize our ability to visualize MTs, we extracted cultures for EM precisely as described above for immunofluorescence microscopy. After extraction, cultures were fixed for 20 min by the addition of an equal quantity of PHEM containing 2% glutaraldehyde, rinsed in 0.1 M cacodylate, treated for 5 min with 2 mg/ml tannic acid, rinsed again in 0.1 M cacodylate, postfixed for 10 min in 1% OsO_4, dehydrated with increasing concentrations of ethanol, and embedded in LX-112 (Ladd Res. Inds. Inc., Burlington, VT). After curing, the glass coverslip was either removed by mechanical means (Whitlon and Baas, 1992) or dissolved using a 10-min treatment with hydrofluoric acid. Serial thin sections (90–120 nm) were obtained with a Reichert Jung Ultracut E or Ultracut S Ultramicrotome (Reichert Jung, Vienna), picked up on Butvar-coated slot grids, and stained with uranyl acetate and lead citrate. For each experimental condition, a minimum of 10 neurons were examined. Regions of the cell body containing the centrosome as well as regions not containing the centrosome were analyzed and photographed.

After identification of the centrosome in a thin section, all other sections through the same centrosome (5–8 sections) were located and photographed as well. In those cases in which there was a substantial accumulation of MTs in the region of the centrosome (namely the 5-min recovery), we wished to obtain estimates of the numbers of MTs that were attached and unattached to the centrosome. To obtain an estimate of attached MTs on each thin section, we scored those MTs that touched the pericentriolar material, which extends less than 0.1 μm in all directions around the centrosome (see Results). To obtain an estimate of the total number of attached and unattached MTs on each thin section, we scored the number of MTs intersecting a circle whose center was the centrosome and whose radius was half the average length of a MT emanating from the centermost section through the centrosome. The numbers obtained for each section were then summed for the entire 5–8 sections comprising each centrosome. Because each section was roughly four times as thick as a MT, and because we always scored in precisely the same two locations in each section, there was little chance of scoring the same MT twice. After obtaining estimates for the total number of MTs and the number of attached MTs, an estimate for the number of unattached MTs was obtained by subtraction. To confirm the effectiveness of this technique, in a few cases consecutive thin sections were aligned and reconstructed using the centrioles and other non-MT structures and debris as registration markers as previously described (Joshi et al., 1986; Baas and Heidemann, 1986; Baas and Ahmad, 1992; Baas and Joshi, 1992). Results from both techniques proved consistent with one another, although the latter was more troublesome due to the overlap of large numbers of MTs on the two-dimensional reconstructions.

Levels of MT polymer were quantified at increasing distances from the centrosome. Specifically, levels of polymer were measured in three distinct regions of the cell body, each region consisting of 2.6 square microns of cytoplasm per thin section. Region I was centered around the centrosome, Region III was located as far from Region I as possible without crossing the nucleus in an individual section, and Region II was located equidistant between Regions I and III. Center-to-center, the three regions were 2–4 μm apart from one another, depending on the size of the particular cell body. Levels of polymer were expressed as mean $\pm$ SD, and plotted on a bar graph.

Results

The goal of the present study was to evaluate the capacity of the neuronal centrosome to nucleate and release MTs for relocation into other regions of the cell. To accomplish this, we performed nocodazole-recovery experiments on dissociated cultures of rat sympathetic neurons (see Introduction and Materials and Methods). Cultures were treated for 6 h with 10 μg/ml nocodazole, a treatment previously shown to reduce the MT mass of the axon to ~5% of control levels (Baas and Ahmad, 1992). After this treatment, the cultures were rinsed free of the drug and permitted to reassemble MTs for times ranging from 30 s to 30 min. Confocal immunofluorescence and quantitative EM were then used to determine the levels and spatial organization of MT polymer in the cell body of control, drugged, and recovered neurons. MT polymer was analyzed in the region of the centrosome as well as in regions of the cell body not containing the centrosome.

Confocal Immunofluorescence Microscopy

Confocal immunofluorescence microscopy was used to obtain a global view of the sequence of events that occur during nocodazole treatment and recovery. As described in Materials and Methods, cultures were extracted to remove free tubulin, staining for tyrosinated α-tubulin was used to visualize MTs, and staining for centrin was used to visualize

the centrosome. Cell bodies were optically sectioned at a thickness of 0.5 μm, and particular attention was given to those sections which contained the spot staining brightest for centrin, presumably the centrosome. Confocal images of control neurons confirm that the cell body contains a dense and complex array of MTs that appear to originate from many sites and splay in many directions (Fig. 2 *A*). Centrin staining was somewhat patchy, perhaps due to nonspecific binding of the primary or secondary antibody, but uniformly showed a single particularly bright spot, often slightly hairy in appearance, that was presumably the centrosome (Fig. 2 *A'*). After nocodazole treatment, the MT staining in the cell body was almost completely obliterated (Fig. 2 *B*), leaving a spot that stains for tubulin. This spot stained the brightest for centrin, suggesting that it was the centrosome (Fig. 2 *B'*). Little MT reassembly was apparent after 30 or 90 s (data not shown). However, after 5 min of recovery, an extremely dramatic reassembly of MTs was apparent, with MTs emanating in all directions from a single focused region (Fig. 2 *C*). Once again, this focused region stained brightest for centrin (Fig. 2 *C'*). By 30 min of recovery, the appearance of the MT array (including the centrosome) was indistinguishable from that of controls (Fig. 2, *D* and *D'*).

Assuming that the spot staining brightest for centrin corresponds to the centrosome, the results on control neurons provide some support for the view that the centrosome is a "point source" from which MTs in the cell body emanate. The recovery experiments provide far stronger support for this conclusion, calling into question the notion that the neuronal centrosome is a relatively inactive structure. Instead, it appears that the neuronal centrosome is a highly potent MT-nucleating structure, similar to a mitotic centrosome in its capacity to nucleate tremendous numbers of MTs. Similar results, using standard fluorescence microscopy, have been reported for cultured neurons from chick dorsal root ganglion (Jacobs and Thomas, 1982) and rat hippocampus (Dotti and Banker, 1991). Unfortunately, the degree of resolution provided even by the confocal microscopic approach is not sufficient to provide the kind of quantitative and spatial information needed to evaluate these conclusions with great rigor. In addition, because the resolution of this technique is insufficient to document whether individual MTs are attached or unattached to the centrosome, the confocal images do not assist in evaluating whether MTs nucleated at the centrosome are subsequently released. Finally, there remains some question as to whether the immunofluorescence staining for centrin truly corresponds to the centrosome. For these reasons, we used EM as our principal method of analysis.

Electron Microscopic Analyses

EM was used to obtain high resolution information on the spatial relationship between the centrosome and the MTs in the cell body. In addition, this technique provided quantitative information on the levels of MT polymer in the region of the centrosome (Region I) and at increasing distances from it (Regions II and III; see Materials and Methods). In control neurons (Fig. 3), the centrosome consisted of two centrioles, although some sections contained only a single centriole of the pair. As expected of a postmitotic cell, there was variability regarding the orientation of the centrioles relative to one another. In some cases, the centrioles were precisely perpendicular to one another, at other times they were slightly off perpendicular, and in rare instances, they were actually parallel to one another (Fig. 3 *e*). Also as expected of a cell not undergoing mitosis, the pericentriolar material did not extend as a diffuse cloud away from the centrioles, but appeared in close proximity to them, extending no greater than 0.1 μm in all directions, and was typically difficult to distinguish from the centrioles themselves. MTs appeared in the region of the centrosome, but the density of MT polymer was not significantly greater than in the two other regions of the cell body examined (mean ± SD = 1.15 ± 0.155, 1.13 ± 0.110, 1.14 ± 0.105 μm polymer/μm² cytoplasm in Regions I, II, and III, respectively). Analyses of every section through each of the centrosomes revealed very few MTs directly attached to the centrosome (attached MTs were defined as those touching the pericentriolar material; see Materials and Methods). In all cases, the total number of attached MTs in all of the sections through an individual centrosome was fewer than 10 (mean ± SD = 5 ± 3 MTs/centrosome). Entirely similar observations were made in the 20 other neurons examined in our recent study on gamma-tubulin distribution in the neuron (Baas and Joshi, 1992).

Observations made on the drug-treated and recovered neurons were consistent with the results obtained with confocal microscopy. In neurons treated with 10 μg/ml nocodazole for 6 h, the levels of MT polymer in the cell body were drastically reduced in all three regions of the neuron examined (0.010 ± 0.003, 0.034 ± 0.006, 0.033 ± 0.007 μm MT polymer/μm² cytoplasm in Regions I, II, and III, respectively), with these levels reflecting reductions relative to control levels of over 99% in the region of the centrosome and ~97% in the other two regions (Fig. 4). After 30 s of recovery as well as after 90 s of recovery, a few MTs (<5) could be observed attached to the centrosome and total polymer levels were only slightly higher than in the drugged neurons (data not shown). At 5 min of recovery, the levels of MT assembly from the centrosome were dramatic. Numerous MTs could be visualized in the region of the centrosome, emanating away from it in numbers reminiscent of a mitotic centrosome (Fig. 5). Unlike a mitotic centrosome, and as noted above for control neurons, there was not a diffuse pericentriolar cloud. As a result, there was little ambiguity as to whether a MT was attached or unattached to the centrosome. Using the procedure described in Materials and Methods, we determined that, for the 10 cells examined, 301 ± 32 MTs were localized in the region of the centrosome, and 200 ± 24 of these were clearly attached to the centrosome. Thus by subtraction, an average of ~101 MTs within each centrosomal array were not attached to the centrosome. Similar numbers were obtained when serial sections were aligned so that individual MTs could be traced from end-to-end. Formal serial reconstructions (Fig. 6) also provided consistent information, but proved less useful (more ambiguous) due to the overlap of large numbers of MTs in the two-dimensional reconstruction. It is of special importance to note that, of the unattached MTs, the vast majority were aligned alongside the attached MTs, suggesting that they may have been attached at one time, and then released from the centrosome.

The total levels of polymer at 5 min of recovery were 7.15 ± 0.730 μm polymer/μm² cytoplasm in Region I, and 0.445 ± 0.146 and 0.115 ± 0.063 μm polymer/μm² cyto-

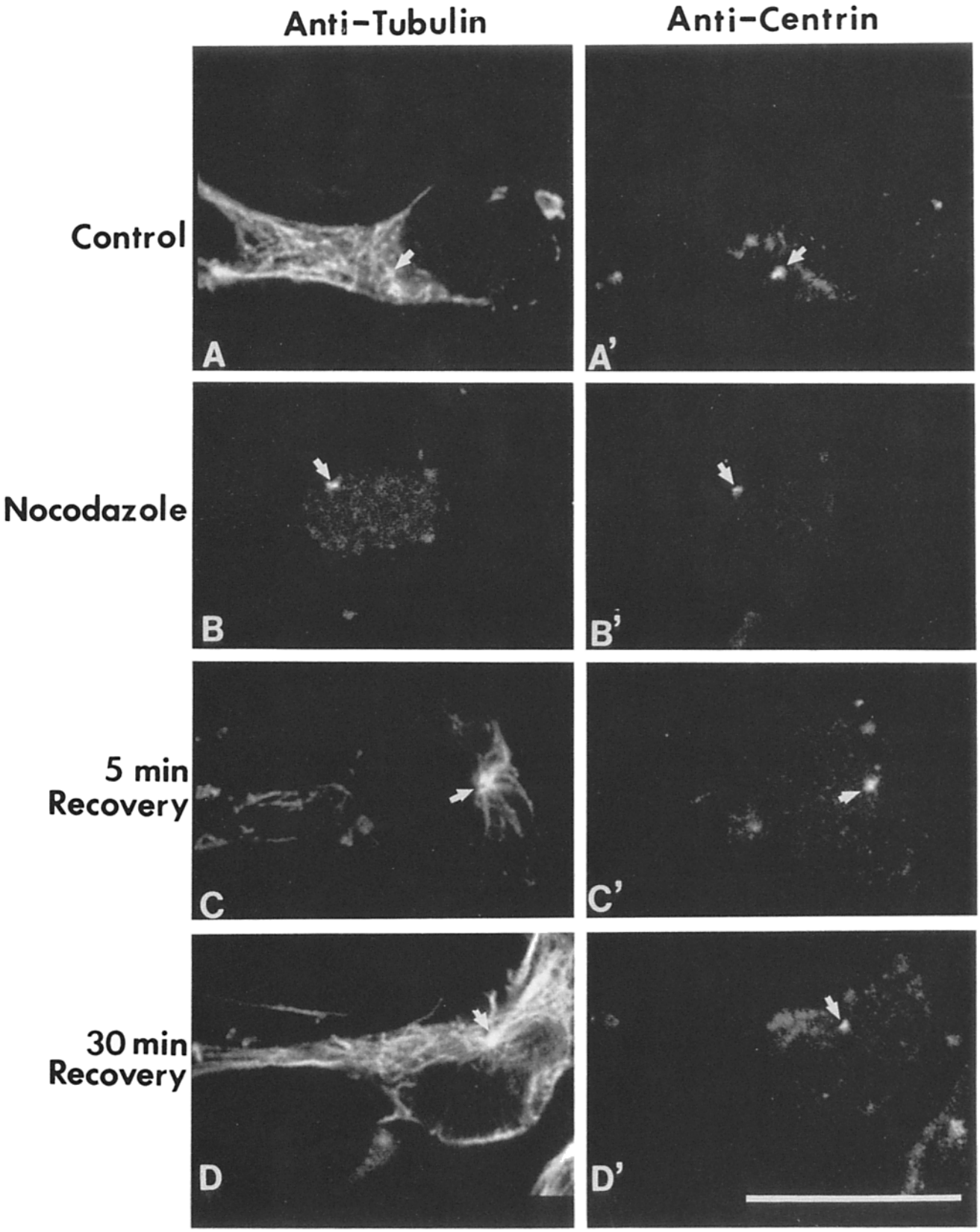

Figure 2. Immunofluorescence micrographs of cultured rat sympathetic neurons obtained with a confocal microscope. Shown are a control neuron (*A* and *A'*), a neuron treated for 6 h with 10 μg/ml nocodazole (*B* and *B'*), and neurons recovered from nocodazole treatment for 5 min (*C* and *C'*) and 30 min (*D* and *D'*). Before fixation, neurons were extracted in a MT stabilization buffer to remove free tubulin and double-stained for tyrosinated α-tubulin (*A*, *B*, *C*, and *D*) and centrin (*A'*, *B'*, *C'*, and *D'*). In control neurons, a dense array of splayed MTs are apparent throughout the cell body. After drug treatment, the staining for MTs is almost completely obliterated, with a bright spot apparent (*arrow*). By 5 min of recovery, a dramatic array of MTs has assembled in all directions from a single focused region (*arrow*). By 30 min, the MT array is indistinguishable from controls. In double-label analyses, centrin staining appeared somewhat patchy (perhaps due to some nonspecific binding), but uniformly consisted of a single brightest spot that was somewhat hairy in appearance, presumably the centrosome (*arrows* mark position of this spot in both micrographs of each pair). Bar, 30 μm.

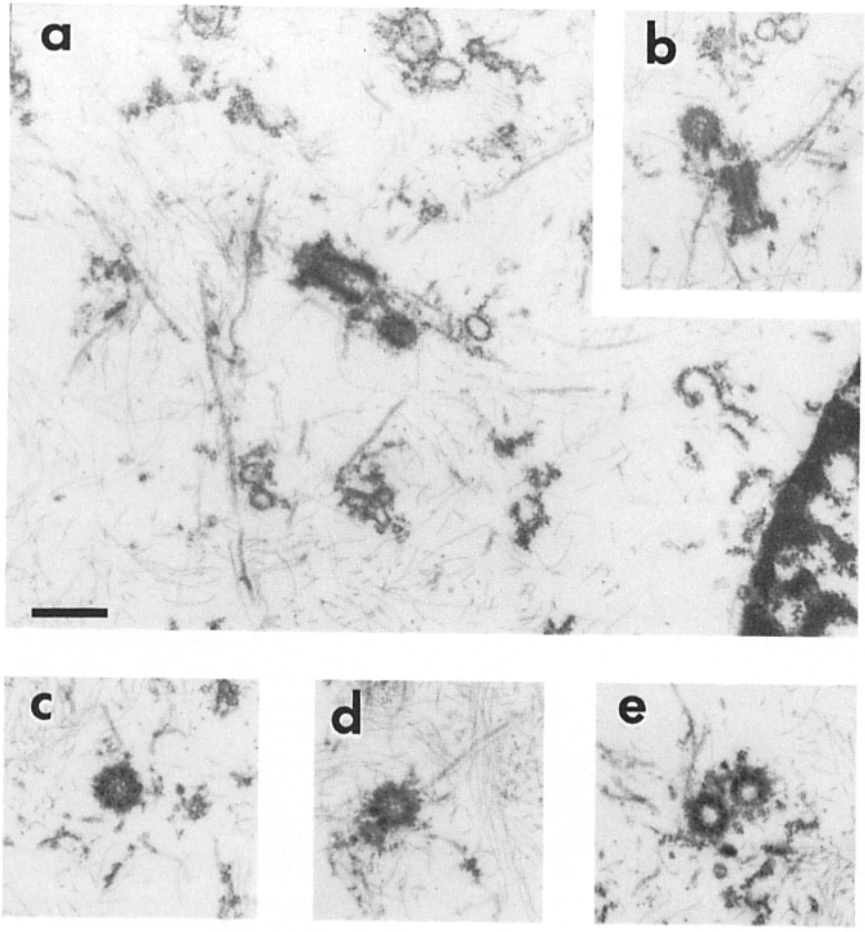

Figure 3. Electron micrographs of control neurons in the region of the centrosome. *a* shows the centrosome (*middle of panel*), a portion of the nucleus (*lower right*), and many unattached MTs. *b* shows a different section through the same centrosome shown in *a*. A small number of MTs can be seen attached to the centrosome in each section. *c–e* show centrosomes from three other control neurons, each with MTs in the vicinity. *c* shows no attached MTs, while *d* and *e* each show one attached MT. Analyses of all sections through each centrosome reveal a total of fewer than 10 attached MTs per centrosome. In all cases, centrosomes consisted of two centrioles, which were usually perpendicular to one another (*a* and *b*). In some cases, only one centriole of the pair appeared on an individual thin section (*c* and *d*). In other cases, as expected of a postmitotic cell, the two centrioles were off-perpendicular, and even parallel to one another in a few rare instances (*e*). Bar, 0.4 μm.

plasm in Regions II and III, respectively. These results indicate a sixfold increase in MT polymer levels above control levels in the region of the centrosome. (Interestingly, a comparable increase in MT levels in the region of the centrosome has been reported to occur when nonneuronal cells shift from interphase to mitosis; see Kuriyama and Borisy, 1981). In contrast, the levels of polymer in Regions II and III were still under 40 and 10% of control levels, respectively. Compared to the drugged cultures, these data indicate increases in polymer levels of 13-fold and 3.5-fold in Regions II and III, respectively, and over 700-fold in the region of the centrosome.

These results indicate that the centrosome is a highly specialized site within the cell body that is particularly active with regard to MT nucleation and assembly. The decreasing levels of MT polymer at increasing distances from the cen-

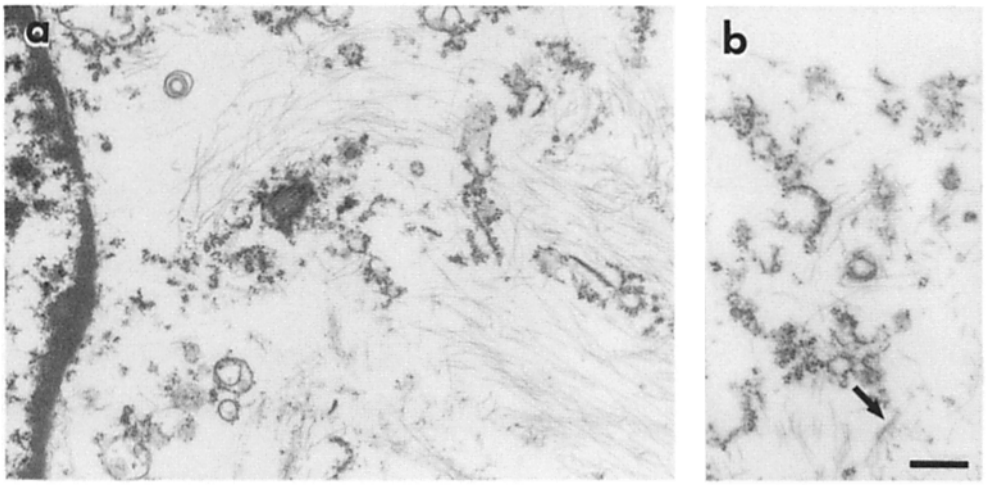

Figure 4. Electron micrographs of neurons treated for 6 h with 10 μg/ml nocodazole. *a* shows the region of the centrosome (*Region I*), while *b* shows a region some distance from the centrosome (*Region II*). No MTs are apparent in *a*, and one short MT fragment is apparent in *b* (*arrow*). See Materials and Methods and Results for more details. Bar, 0.4 μm.

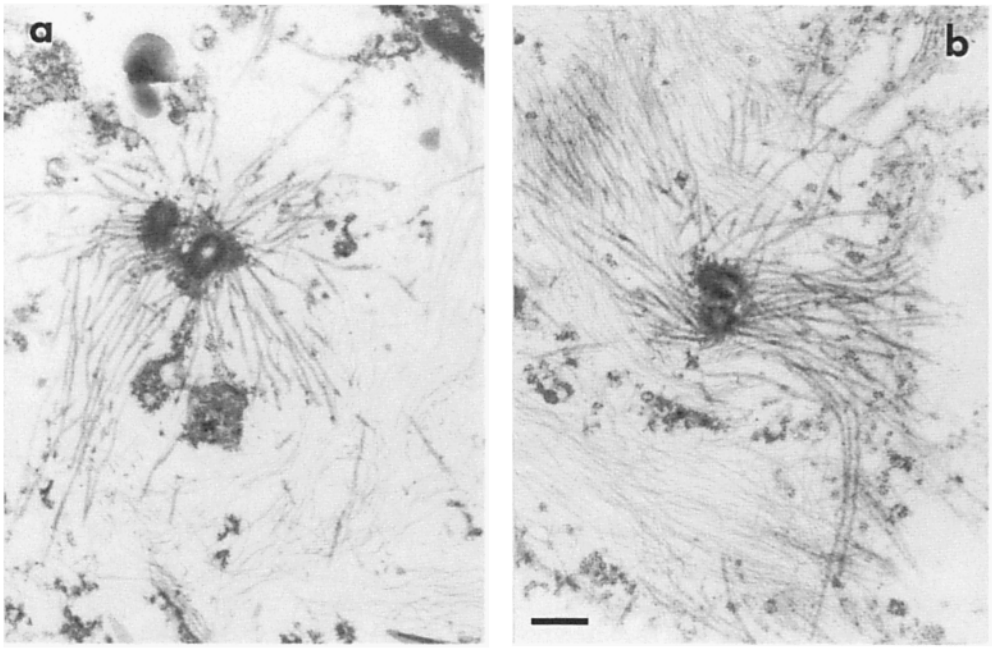

Figure 5. Electron micrographs of two different neurons treated for 6 h with 10 μg/ml nocodazole, rinsed free of the drug, and permitted to recover for 5 min. MT reassembly from the centrosome is dramatic, with high levels of attached MTs. Also apparent are MTs not directly attached to the centrosome. These MTs are aligned with the attached MTs as if they were once attached, and then released from the centrosome. Analyses of every section through each centrosome were required to define and score attached and unattached MTs (see Materials and Methods and Results). Bar, 0.4 μm.

trosome support the view that the centrosome acts as a "point source" from which MTs are nucleated and released. It is likely that MT reassembly can also occur by elongation from the short fragments of polymer resisting the drug treatment, as is the case within the axon (Baas and Ahmad, 1992). However, the modest levels of assembly that occur in Region III, the region farthest from the centrosome, argue that this assembly is relatively low compared to that occurring at the centrosome, at least over the first 5 min of recovery. In addition, the elongation of these fragments, which are fairly evenly distributed throughout the cell body, could not account for the decreasing gradient of MT polymer that occurs with increasing distance from the centrosome. Taken together, these observations support the view that the increased levels of unattached MTs appearing at sites distant from the centrosome are due principally to the continuous nucleation, release, and movement of MTs from the centrosome.

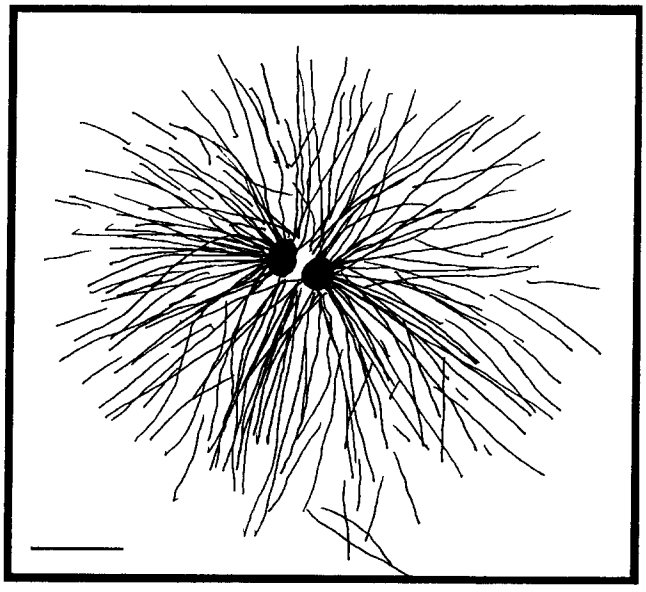

Figure 6. Serial reconstruction of the centrosomal MT array of a neuron that recovered from a 6-h nocodazole treatment for 5 min. Both attached and unattached MTs are apparent. Bar, 0.8 μm.

At 30 min of recovery, shown in Fig. 7, the polymer levels in all three regions of the neuron have returned to levels indistinguishable from controls (1.24 ± 0.145, 1.34 ± 0.243, 1.19 ± 0.120 μm polymer/μm² cytoplasm in Regions I, II, and III, respectively) as has the number of MTs directly attached to the centrosome (6 ± 3 MTs/centrosome). These observations provide additional support for the conclusion that the hundreds of MTs attached to the centrosome at 5 min of recovery were subsequently released from the centrosome and relocated to other regions throughout the neuron.

Curiously, in one unusual case at 30 min of recovery, one of the two centrioles can be seen giving rise to a third new centriole (Fig. 7 *b*). While it is possible that this is simply an anomaly unrelated to the experimental treatment, another possibility is that the extended nocodazole treatment triggered mechanisms in this particular cell normally associated with cell division, causing the centriole to replicate.

Quantitative data on the levels of MT polymer in the three neuron regions under each of the experimental conditions are summarized graphically in Fig. 8.

Discussion

In our previous study on gamma-tubulin distribution in the neuron, we proposed that MTs destined for axons and dendrites originate from the centrosome within the cell body of the neuron, and are then released and translocated into and down these neurites (Baas and Joshi, 1992). For this to be correct, the neuronal centrosome would have to be an ex-

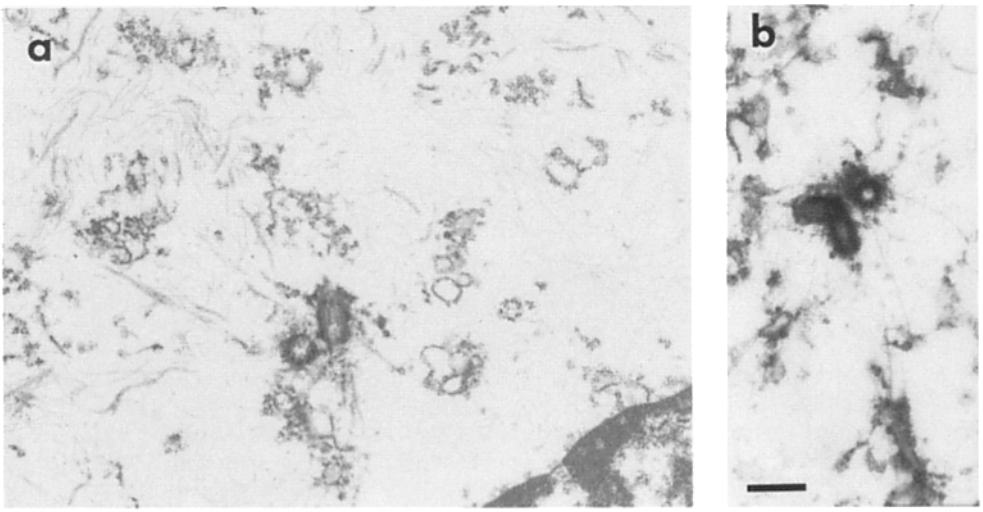

Figure 7. Electron micrographs of neurons treated for 6 h with 10 μg/ml nocodazole, rinsed free of the drug, and permitted to recover for 30 min. The MT array is indistinguishable from controls, with fewer than 10 MTs attached to the centrosome, and unattached MTs appearing throughout the cell body. *a* shows a typical cell body. *b* shows the centrosome of a rather unusual case, in which one of the two centrioles can be seen giving rise to a third new centriole (see Results). Bar, 0.4 μm.

tremely potent MT-nucleating structure, capable of nucleating the hundreds upon hundreds of MTs required for the growth and maintenance of these neurites. Paradoxically, the appearance of the neuronal centrosome is not dramatic, with only a very few MTs directly attached to it. One possibility is that the neuronal centrosome is actually a relatively inactive structure with regard to MT nucleation. The other possibility, consistent with our proposal, is that MT nucleation and release occur so rapidly that there is insufficient time for great numbers of attached MTs to ever accumulate at the centrosome. In the present studies, we sought to resolve this issue with nocodazole-recovery experiments, the classic approach for identifying MT-nucleating structures in living cells. Our results indicate that the neuronal centrosome is a highly active structure. During the first 5 min of recovery from nocodazole treatment, hundreds of MTs are nucleated and assembled from the centrosome, and this compares with exceedingly low levels of MT reassembly elsewhere in the cell body. Also a great number of MTs within the centrosomal array are clearly not attached to the centrosome, and unattached MTs appear elsewhere in the cell body at decreasing levels with increasing distance from the centrosome. Within 30 min, the hundreds of MTs that were nucleated from the centrosome within the first 5 min of recovery are no longer present at the centrosome. Instead, unattached MTs are present throughout the cell body at levels similar to controls. The simplest explanation for these results is that, by synchronizing the nucleation and assembly of a great number of MTs from the centrosome, we have revealed the active nucleation and release of MTs that presumably occur continuously within the neuron.

It should be noted that MT nucleation, release, and relocation is not the only scenario that could explain the present results. The alternate possibility is that MT nucleation is

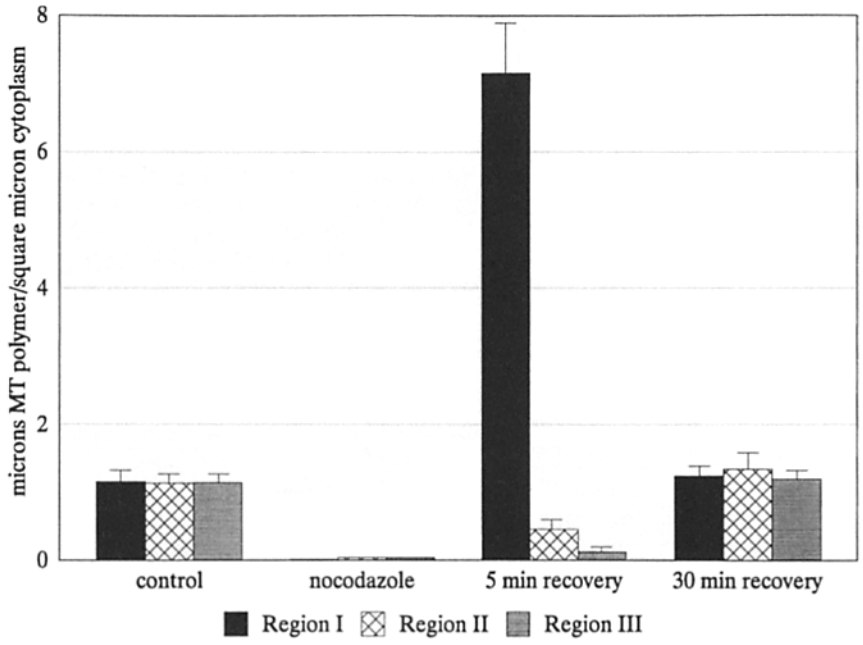

Figure 8. Bar graph showing the levels of MT polymer in three distinct regions of the neuronal cell body as described in Materials and Methods. Region *I* was centered around the centrosome. Region *III* was located as far from the centrosome as possible without crossing the nucleus. Region *II* was located equidistant between Regions *I* and *III*. Levels of MT polymer in each of the three regions were scored for control neurons, neurons treated with 10 μg/ml nocodazole for 6 h, and neurons recovered from nocodazole treatment for 5 and 30 min. Values are expressed as the average length of MT polymer per square μm of cytoplasm. Ten neurons were analyzed for each case, and SD are provided in the form of error bars. See Results for more details.

suppressed at sites in the cell body other than at the centrosome during the first 5 min of recovery, and that after 5 min, the hundreds of MTs nucleated at the centrosome are rapidly disassembled and replaced by other MTs nucleated elsewhere within the cell body. Given the potential for very rapid MT dynamics at the centrosome (for review see Kirschner and Mitchison, 1986), this possibility should not be dismissed out of hand. However, we know of no precedent for such a rapid and complete shift in the action of two different types of MT-nucleating structures (centrosomal and noncentrosomal), as one would have to hypothesize for this scenario to be true. In addition, there is increasing evidence that MT release from the centrosome is an important event that occurs in many different cell types (Kitanishi-Yumura and Fukui, 1987; McBeath and Fujiwara, 1990). In fact, centrin, the protein used to identify the centrosome in our studies is thought to have a potent MT severing function (Sanders and Salisbury, 1989). In nonneuronal cells, it is thought that the release of a MT is followed by its rapid depolymerization from both ends (Kitanishi-Yumura and Fukui, 1987). In neurons, MTs are generally far more stable (as evidenced by the extensive 6-h nocodazole treatment required to diminish MT levels in the present study), and hence would likely not depolymerize after release, and this could be a critical factor which distinguishes the generation of neuronal vs nonneuronal MT arrays. Taking all of this into account, the most compelling conclusion that can be drawn from the present data is that the neuronal centrosome is a highly potent MT-nucleating structure, capable of generating tremendous numbers of MTs for their active release and relocation into other regions of the neuron.

Microtubules and the Neuronal Centrosome

A centrosomal origin for neuritic MTs is not a new hypothesis. As early as 1965, before "spindle tubules" and "neurotubules" were both identified as "microtubules," Gonatas and Robbins (1965) examined the lattice structure of neurotubules in chick embryo retina, found it to be indistinguishable from that of spindle tubules, and concluded that "neurotubules probably arise from the centrioles." Similarly, in ultrastructural studies on rabbit embryo dorsal root ganglion neuroblasts, Tennyson (1965) concluded that neurotubules "probably originate from the centriole . . ." and "migrate into the neurite." Unfortunately, since this early work, studies on the neuronal centrosome have been sparse, partially because of the technical difficulty of locating the centrosome within the almost spherical neuronal cell body, and partially because of a strong bias in recent years that local mechanisms within the neurites are sufficient to generate their MT arrays. The fact that neuritic MTs are not directly attached to the centrosome has contributed to this bias, with little attention given to the possibility that MTs are actively released from the centrosome after their nucleation. Our observations support the view expressed by the earlier authors, that MTs are actively released from the centrosome, and transported away from it into other regions of the cell body, and presumably into axons and dendrites as well.

As noted in the Introduction, the electron micrographs of neuronal centrosomes that are currently available in the literature vary somewhat with regard to the numbers of MTs that are attached to them, although these numbers are consistently low compared for example to those of a mitotic centrosome, or the centrosomes in our 5-min recovery experiments. It may be important that unlike earlier work, our studies were performed on cultured neurons which are undergoing extremely rapid axonal regeneration on a laminin substratum. One possibility is that the rates of MT nucleation and release from the centrosome are somehow regulated by the need for new MTs required to accommodate different rates of neuritic growth. The mechanism by which this regulation might occur is unknown, but is probably related to the manner by which cells monitor and autoregulate levels of assembled and unassembled tubulin (for review see Cleveland, 1988). When a MT leaves the cell body, we would expect the monomer/polymer equilibrium to shift toward assembly, in turn lowering the concentration of free tubulin in the cell body. As a result, the cell will synthesize more free tubulin, once again driving assembly of more polymer. If nucleation of MTs from the centrosome is strongly favored over elongation of existing polymer as our data suggest, this will result in an increased production of MTs at the centrosome. Studies are currently underway to test these ideas, and to determine how this cascade of events might also regulate the rate at which MTs are released from the centrosome.

In order for MTs released from the centrosome to move away from it, and ultimately move into axons and dendrites, there must be an active mechanism for MT translocation. Our data suggesting that the tremendous numbers of MTs nucleated by the centrosome are rapidly carried away from it strongly support the conclusion that such an active transport mechanism exists. In fact, such a mechanism would be consistent with a growing body of evidence concerning the capacity of molecular motors to create MT movements within living cells. In particular, and relevant to the centrosome, it has now been established that motors such as kinesin are essential for the formation of the mitotic spindle as well as for MT movements that occur during mitosis (for review see Fuller and Wilson, 1992). Perhaps in a postmitotic cell such as the neuron, similar mechanisms account for the movement of MTs away from the centrosome after they are released. Pertinent to this issue are studies on motile cells in which the position of the centrosome relocates to face the direction of cell movement (Kupfer et al., 1982). This appears to be a consequence rather than the cause of cell movement, and occurs in the culture dish to different degrees depending on the nature of the substratum on which the cells are grown (Schutze et al., 1991) as well as in situ (Rogers et al., 1992). Interestingly, the repositioning of the centrosome requires MTs that extend to the cell periphery (Euteneuer and Schliwa, 1992), and in at least one cell type, 3T3 fibroblasts, it has been documented that a subclass of stable MTs attached to the centrosome are "captured" by the leading edge of the cell, after which the centrosome moves in that direction (Gunderson and Bulinski, 1988). Based on these observations, it seems likely that there is some kind of pulling force on the MTs, and that this force results in the movement of the attached centrosome. We speculate that the release of MTs from the centrosome is far more active in neurons than in these motile cells, and that the pull on the MTs does not cause the centrosome to change location, but instead carries the MTs away from the centrosome toward the leading edge of the cell. As a result, the cell body remains relatively stationary, the centrosome does not change location, and the freed MTs are transported toward the leading

edge of the cell. MT-rich neurites are formed between the stationary cell body and the motile leading edge, which becomes a growth cone. If these speculations are correct, the capacity of the neuron to actively release MTs from the centrosome is fundamental to the differentiation and growth of axons and dendrites.

Mechanisms for Elaborating the Axonal and Dendritic Microtubule Arrays

What evidence do we have that MTs released from the centrosome are actually translocated into axons and dendrites? Presently, there is very little information available on the regulation of dendritic MTs. However, a strong argument can be made in the case of the axon. The results of earlier nocodazole-recovery experiments indicate that all MT assembly in the axon is limited to the elongation of preexisting polymer, and that no entirely new MTs arise within the axon itself (Baas and Heidemann, 1986; Baas and Ahmad, 1992; see Introduction). By the process of elimination, these results implicate the cell body as the only potential source of new MTs for the expansion of the axonal array. In support of this conclusion, we have recently demonstrated evidence for the active transfer of MTs from the cell body into the axon. In these studies, neurons were cultured under conditions that arrest MT assembly without depolymerizing the existing MTs in the cell body (Baas and Ahmad, 1993). Under these conditions, there is a progressive increase in MTs in the axon and a concomitant depletion of MTs from the cell body, indicating a net transfer of MTs from one compartment to the other. These observations, coupled with the present data indicating that the centrosome is the principal or perhaps exclusive source of MTs throughout the cell body, strongly argue that axonal MTs originate at the centrosome.

A centrosomal origin for axonal MTs has important implications for the cascade of events by which the axonal MT array is elaborated. It is clear that the MTs released from the centrosome are short, a fraction of the diameter of the cell body into which they must fit. The MTs emanating from the centrosome after 5 min of recovery from nocodazole are a few microns in length, and this probably represents the maximum length that MTs normally achieve before release (any attached MTs observed in control neurons were shorter). In contrast, the average length of an axonal MT is on the order of 100 μm (Bray and Bunge, 1981). To achieve these lengths, the MTs released from the centrosome must undergo substantial elongation. Because the lengths of MTs within the cell body are limited by the diameter of the cell body ($\sim$20 μm), this elongation must occur principally within the axon, and not within the cell body. We favor the view that many short MTs are transported into the axon. Many of these MTs give up their subunits for the elongation of others, resulting in a shift during transit from a large number of short MTs to a smaller number of longer MTs (Lasek, 1988; Ahmad et al., 1993; Baas and Ahmad, 1993). This scheme is attractive in that it provides a mechanism for the transport of tubulin subunits over long distances in the axon. In addition, MT transport from the cell body into the axon occurs exclusively in a direction with plus-ends-leading, thereby establishing the characteristic polarity orientation of axonal MTs (Baas and Ahmad, 1993). Collectively, these considerations indicate that MT transport and assembly work together to generate the MT array of the axon (for more detail, see Joshi and Baas, 1993).

In summary, the present results strongly suggest that the neuronal centrosome is capable of acting as a generator of MTs for relocation into other regions of the neuron. Real-time imaging of the neuronal centrosome will be required to confirm this conclusion, and such experiments are presently underway. In addition, direct studies are underway to determine whether MTs released from the centrosome are actually translocated into axons and dendrites, and whether centrosomally derived MTs are required for the growth of these neurites.

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