γ-tubulin Distribution in the Neuron: Implications for the Origins of Neuritic Microtubules

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Abstract. Axons and dendrites contain dense microtubule (MT) arrays that are not attached to a traditional MT nucleating structure such as the centrosome. Nevertheless, the MTs within these neurites are highly organized with respect to their polarity, and consist of a regular 13-protofilament lattice, the two known characteristics of MTs nucleated at the centrosome. These observations suggest either that axonal and dendritic MTs arise at the centrosome, or that they are nucleated locally, following a redistribution of MT nucleating material from the centrosome during neuronal development. To begin distinguishing between these possibilities, we have determined the distribution of γ-tubulin within cultured sympathetic neurons. γ-tubulin, a newly discovered protein which is specifically localized to the pericentriolar region of nonneuronal cells (Zheng, Y., M. K. Jung, and B. R. Oakley. 1991. *Cell.* 65:817–823; Stearns, T., L. Evans, and M. Kirschner. 1991. *Cell.* 65:825–836), has been shown to play a critical role in MT nucleation in vivo (Joshi, H. C., M. J. Palacios, L. McNamara, and D. W. Cleveland. 1992. *Nature (Lond.).* 356:80–83). Because the γ-tubulin content of individual cells is extremely low, we relied principally on the high degree of resolution and sensitivity afforded by immunoelectron microscopy. Our studies reveal that, like the situation in nonneuronal cells, γ-tubulin is restricted to the pericentriolar region of the neuron. Furthermore, serial reconstruction analyses indicate that the minus ends of MTs in both axons and dendrites are free of γ-tubulin immunoreactivity. The absence of γ-tubulin from the axon was confirmed by immunoblot analyses of pure axonal fractions obtained from explant cultures. The observation that γ-tubulin is restricted to the pericentriolar region of the neuron provides compelling support for the notion that MTs destined for axons and dendrites are nucleated at the centrosome, and subsequently released for translocation into these neurites.

MICROTUBULES (MTs)[1] are highly organized throughout the neuron, but are organized differently in each of its compartments. In the axon, the MTs are all oriented with their plus ends distal to the cell body (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987, 1988, 1989, 1991), whereas in the dendrite, roughly equal proportions of the MTs are of each orientation (Baas et al., 1988, 1989, 1991; Burton, 1988). The minus-end distal MTs in the dendrite arise later in development than the plus-end distal MTs (Baas et al., 1989) suggesting that dendrites contain two populations of MTs of uniform but opposite orientation. In the cell body, MTs also appear to have a complex organization, funneling from the cell center toward the axons and dendrites (Bartlett and Banker, 1984; Stevens et al., 1988). Because MTs are key to the establishment of cellular architecture and directing organelle transport, these unique MT patterns provide a foundation for defining the shape and cytoplasmic composition of each neuronal compartment (for reviews, see Lasek, 1988; Black and Baas, 1989). Therefore, there is great interest in elucidating the mechanisms by which MTs are organized in each of these compartments.

Most current knowledge about the organization and assembly of MTs derives from in vitro studies, or from studies on nonneuronal cells. In the test tube, MT assembly can occur de novo by self-association of free subunits, but preferentially occurs by elongation from a nucleating structure (for review see Kirschner, 1978). Studies on nonneuronal cells indicate that self-association is strongly suppressed in favor of nucleated assembly, and that MT nucleation is spatially regulated by discrete templates or nucleating structures such as the centrosome (for review see Brinkley, 1985). Nucleation from these structures results in MT arrays of uniform polarity orientation (Heidemann and McIntosh, 1980; Euteneuer and McIntosh, 1981), and also results in a regular lattice structure of each MT within the array. With regard to the latter issue, in vitro studies on MT nucleation in the presence or absence of centrosomes indicate that the 13 protofilament lattice of the MT results from centrosomal

1. *Abbreviations used in this paper*: CBM, cell body mass; MT, microtubule.

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nucleation, and that in the absence of the centrosome, protofilament number varies (Evans et al., 1985; see also Scheele et al., 1982). The extent to which these observations on protofilament number can be extended to living cells is unclear. However, the observations that axonal and dendritic MTs typically have a regular 13-protofilament lattice (Tilney et al., 1973; Burton et al., 1975), and are highly organized with respect to their polarity are nevertheless provocative, suggesting the likelihood that axonal and dendritic MTs are nucleated by a structure or substance comparable in function to the centrosome.

Curiously, MTs in the axon and dendrite are not attached to any observable nucleating structure (Lyser, 1968; Sharp et al., 1982), but rather appear to be free in the cytoplasm, stopping and starting all along the lengths of these neurites (Bray and Bunge, 1981; Sasaki et al., 1983). Moreover, no evidence has emerged for any kind of structural cap at the minus end of the MT. These observations have resulted in much interest over the past several years with regard to the mechanisms by which the nucleation, assembly, organization, and lattice structure of axonal and dendritic MTs are regulated. In recent studies (Baas et al., 1989; Baas and Ahmad, 1992), we have discussed two possibilities with regard to the sites where new MTs for axons and dendrites may arise. One possibility is that axonal and dendritic MTs are, in fact, nucleated at the centrosome, after which they detach from this structure and move into axons and dendrites by an active transport mechanism. Alternatively, the MT nucleating material of the centrosome may redistribute during neuronal development, permitting new MTs to be nucleated locally within axons and/or dendrites. These two possibilities have clearly different implications with regard to the cascade of events by which the axonal and dendritic MT arrays are elaborated.

To begin to distinguish between these possibilities, we have focused our attention on γ-tubulin. γ-tubulin is a recently discovered member of the tubulin family (Oakley and Oakley, 1989), which has been shown to be associated with MT nucleating structures such as spindle-pole bodies in fungal cells (Oakley et al., 1990) and centrosomes in vertebrate cells (Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991). Genetic and immunocytochemical evidence suggests that γ-tubulin directly interacts with β-tubulin, and thereby nucleates the assembly of plus-end distal MT arrays (Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991). In support of this view, the microinjection of a γ-tubulin antibody into cultured mammalian cells blocks the reassembly of MTs from the centrosome following MT depolymerization (Joshi et al., 1992), confirming the requirement of functional gamma-tubulin for the nucleation of MTs from this structure.

In the present study, we have determined the distribution of γ-tubulin within cultured sympathetic neurons. We have identified γ-tubulin as a component of the pericentriolar material of the neuron, and have analyzed axons, dendrites, and other regions of the cell body in an effort to determine whether γ-tubulin is also present at any other locations in the neuron. Our results provide provocative new information regarding the origins of axonal and dendritic MTs.

Materials and Methods

Cell Culture

Cultures of sympathetic neurons from the superior cervical ganglia of newborn rat pups were grown in two different ways. Dissociated cultures were used for immunoelectron microscopy because they permit the identification of individual axons, dendrites, and cell bodies. Explants cultures were used for biochemical analyses because they permit the separation of pure axonal and highly enriched somatodendritic fractions. For dissociated cultures, ganglia were treated with 0.25 mg/ml collagenase for 1 h followed by 0.25 mg/ml trypsin for 45 min, and then triturated with a Pasteur pipette into a single cell dispersion. The neurons were plated onto collagen-coated plastic dishes in N2 medium (Moya et al., 1980) supplemented with 2.5% FBS, 5% human placental serum, and 50 ng/ml nerve growth factor. Neurons were plated at a particularly high density (2–3 ganglia per 35-mm dish) to promote rapid dendritic outgrowth. On the morning after plating, the cultures were fed with the same medium further supplemented with 5 μm cytosine arabinoside to reduce nonneuronal contamination. For biochemical analyses, each ganglion was cut into three pieces, and plated as explants onto collagen-coated dishes (six explants per dish). The explants were plated into N2 medium supplemented with 1% human placental serum, 50 ng/ml nerve growth factor, and 0.6% methyl cellulose. The following morning, the cultures were fed with N2 medium supplemented with 50 ng/ml nerve growth factor and 10 μm cytosine arabinoside. Axons were permitted to grow for 10 d, a time over which it has been determined that the protein content of the axonal halo becomes roughly equivalent to that of the cell body mass (CBM) (Peng et al., 1986).

Sample Preparation for EM

For immunoelectron 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), and then extracted for 5 min in PHEM containing 10 μM taxol and 1% Triton X-100. Cultures were fixed by the addition of an equal quantity of PHEM containing 4% paraformaldehyde and 0.4% glutaraldehyde. After 10 min, the cultures were rinsed briefly in PHEM, treated for 15 min with 2 mg/ml sodium borohydride, rinsed briefly in TBS-1 (10 mM Tris, 140 mM NaCl, pH 7.6), treated for three times, 10 min each with blocking solution containing 2% normal goat serum, 3% BSA, and 0.1% fish gelatin in TBS-1, and then incubated for 90 min at 37°C with our polyclonal γ-tubulin antibody (Joshi et al., 1992) diluted 1:50 in blocking solution. In a limited number of experiments, the blocking solution contained 5% normal goat serum in TBS-1, the γ-tubulin antibody was used at dilution of 1:25, and the incubation was at 4°C overnight. After antibody incubation, cultures were rinsed three times for 10 min each in TBS-2 (20 mM Tris, 140 mM NaCl, pH 8.2) containing 0.1% Triton X-100, treated with blocking solution (same protein composition, but dissolved in TBS-2), and then incubated for 3 h at 37°C with 5 nm gold-conjugated goat anti-rabbit second antibody (Amersham Corp., Arlington Heights, IL) diluted 1:2 in blocking solution. The cultures were then rinsed three times for 10 min each with TBS-2, fixed in 0.1 M cacodylate containing 1% glutaraldehyde and 2 mg/ml tannic acid, postfixed in 2% OsO_4 for 10 min, dehydrated in ethanols, and embedded in LX-112 resin (Ladd Research Inc., Burlington, VT).

For conventional EM, cultures were fixed for 15–20 min in 0.1 M cacodylate buffer containing 2% glutaraldehyde and 2 mg/ml tannic acid, postfixed for 10 min in 1% OsO_4, dehydrated in ethanols, and embedded in LX-112 resin.

EM and Serial Reconstruction

After curing, the resin was removed from the plastic dish, and regions of interest were relocated by phase-contrast microscopy, circled with a diamond-marker objective, and sectioned using a Reichert Jung Ultracut E Ultramicrotome (Reichert Jung, Vienna). Serial sections roughly 140-nm thick (about twice as thick as a typical thin section) were picked up on butvar-coated slot grids, and stained with uranyl acetate and lead citrate. Initial observations were made on the 7 1 MeV high voltage electron microscope at the University of Wisconsin (Madison, WI) HVEM facility. Most observations and all micrographs were taken using a conventional JEOL CX-100 electron microscope (JEOL USA Inc., Peabody, MA), which was found to provide sufficient resolution for our purposes.

In one set of studies, we wished to identify the minus ends of MTs in axons and dendrites. Because portions of individual MTs may be present on more than one section, serial reconstruction was necessary for this identification. The use of thicker sections substantially eased the task of reconstruction, with the MTs from an individual axon typically occupying <6 sections and a typical dendrite typically occupying <20 sections. To identify minus ends of MTs, we used an abbreviated version of our previously described method for reconstructing axonal MTs (Joshi et al., 1986; Baas and Heidemann, 1986). Sets of three consecutive sections were aligned using non-MT structures (any membranous elements or other cellular debris remaining after extraction) as registration markers (see Baas and

Ahmad, 1992). Any MT terminating in the middle section (not continuous onto either of the two flanking sections) was known to be a true MT end. Lengths of reconstructed axons or dendrites were 20–60 μm, and generally 0–3 MT ends were identified in each trio of sections. In the axon, all of the MTs have a plus-end distal orientation (Baas et al., 1991), and thus the minus ends were known to be those directed toward the cell body. In the dendrite, roughly half the ends are expected to be of each type (Baas et al., 1991), so the ends we identified were assumed to be a mixture of both plus and minus ends. The direction of axon growth, needed to determine MT polarity orientation, was not always apparent at the phase level, and therefore was assessed at the electron microscopic level based on continuity of the proximal region of the axon with the cell body.

Biochemical Analyses

To further analyze the γ-tubulin content of the axonal and somatodendritic compartments of the neuron, we performed immunoblot analyses on pure axonal and highly enriched somatodendritic fractions obtained from explant cultures. For these analyses, the cell body masses (CBMs) were dissected with forceps from the explant cultures, and collected in an epindorph tube. The CBMs and axon halos were then separately dissolved/homogenized in 1% SDS containing a cocktail of protease inhibitors (0.2 TIU aprotinin and 10 μg/ml each of leupeptin, antipain, and chymostatin). The samples were precipitated with nine volumes of cold methanol, dissolved in 1× Laemmli gel sample buffer, and boiled for 3 min. SDS-PAGE was then performed as described by Laemmli (1970). Proteins were transferred (3 h at 1 Amp) to nitrocellulose (BA83; Schleicher and Schuell, Inc., Keene, NH) in half-strength Laemmli gel running buffer plus 20% methanol. Nitrocellulose filters containing transferred samples were stained with Ponceau S (0.2% Ponceau S in 3% TCA) to identify the positions of molecular weight standards. Nonspecific protein binding sites were blocked by incubation for 15 min with PTX-BSA (0.2% Triton X-100, 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.5, 1 mM EGTA, 4% BSA). After blocking, the filters were incubated overnight with primary γ-tubulin antibody diluted in PTX-BSA. The following morning, unbound primary antibody was removed, and the filters were washed four times, 15 min each in wash buffer (0.5% Triton X-100, 50 mM triethanolamine, pH 7.4, 0.1 M NaCl, 0.1 mM EDTA, 0.1% SDS). The filters were then incubated for 1 h at room temperature with ^{125}I-labeled protein A in PTX-BSA, and then washed four times 15 min each in wash buffer to remove unbound protein A. Binding was detected by autoradiography using Dupont Lightening Plus intensifying screens (Dupont Instruments, Wilmington, DE) and Kodak XAR film (Eastman Kodak Co., Rochester, NY).

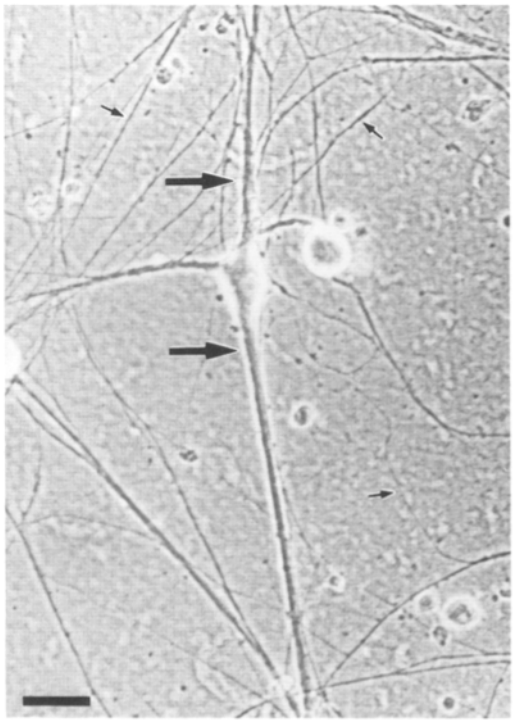


Figure 1. Phase-contrast micrograph of a dissociated culture of rat sympathetic neurons grown on a collagen-coated plastic culture dish as described in Materials and Methods. Cell bodies, axons, and dendrites are readily identifiable by morphological criteria. Axons are longer, thinner processes that are uniform in diameter, while dendrites are thicker, shorter processes that taper with distance from the cell body. Smaller arrows mark examples of axons, while larger arrows mark examples of dendrites. The reliability of these morphologic criteria to distinguish the axons and dendrites of these cultures from one another has been confirmed by immunological and ultrastructural criteria (Baas et al., 1991). Bar, 20 μm.

Results

γ-tubulin Is Present in the Pericentriolar Material of the Neuron

Our goal in the present studies was to determine the distribution of γ-tubulin in the neuron. For these studies, immunoelectron microscopic analyses were performed on dissociated cultures of rat sympathetic neurons. These neurons extend distinct axons and dendrites that are readily distinguished from one another both at the light and electron microscopic levels (Fig. 1). Axons are longer, thinner processes of uniform diameter, whereas dendrites are shorter, broader processes that taper with distance from the cell body. In previous studies using immunoelectron microscopy to localize γ-tubulin in nonneuronal cells, it was found that γ-tubulin is specifically localized to the pericentriolar region of these cells (Stearns et al., 1991). Therefore, our first efforts herein were focused at determining whether the pericentriolar region of the neuron also contains γ-tubulin. These studies were more problematic on neurons compared with nonneuronal cells because of the comparatively larger size and rounder geometry of the neuronal cell body. The cell body of the neuron is a slightly flattened sphere, ~20–40 μm in diameter, and as such, occupies hundreds of typical thin sections (70-nm thick). The centrosome lies on roughly five of these sections. To better our chances of observing the centrosome, we used somewhat thicker sections (140 nm), and analyzed serial sections picked up on butvar-coated slot grids (see Materials and Methods).

We initially wished to explore whether our extraction protocol altered the appearance or integrity of the centrosome and/or the pericentriolar material. For this reason, we compared the appearance of centrosomes in extracted cultures with their appearance in unextracted cultures. Fig. 2 *a* shows a centrosome from an unextracted culture. The pericentriolar cloud is not dramatic in appearance, but is apparent as a hazy substance asymmetrically surrounding the centrosome. In some sections, the centrosome was manifest as only one centriole of the pair, while in other cases, both centrioles appeared in a single section. In all cases, and as expected in the case of a postmitotic cell, the centrioles were not perfectly perpendicular to one another. MTs were generally observed in the region of the centrosome, and throughout the cell body, but it was unclear whether or not individual MTs were emanating directly from the centrosome. In extracted neurons, the appearance of the centrosome was essentially similar to that in unextracted neurons with regard to all of the features discussed here (Fig. 2, *b* and *c*).

Fig. 2 *b* shows an extracted culture immunolabeled for γ-tubulin using the antibody at 1:25 and a blocking solution consisting of 5% normal goat serum (see Materials and Methods). The pericentriolar regions of this and all other neurons examined were labeled with gold particles. In addition, we occasionally found small clusters of gold particles situated on MTs, neurofilaments, or cellular debris. The lack

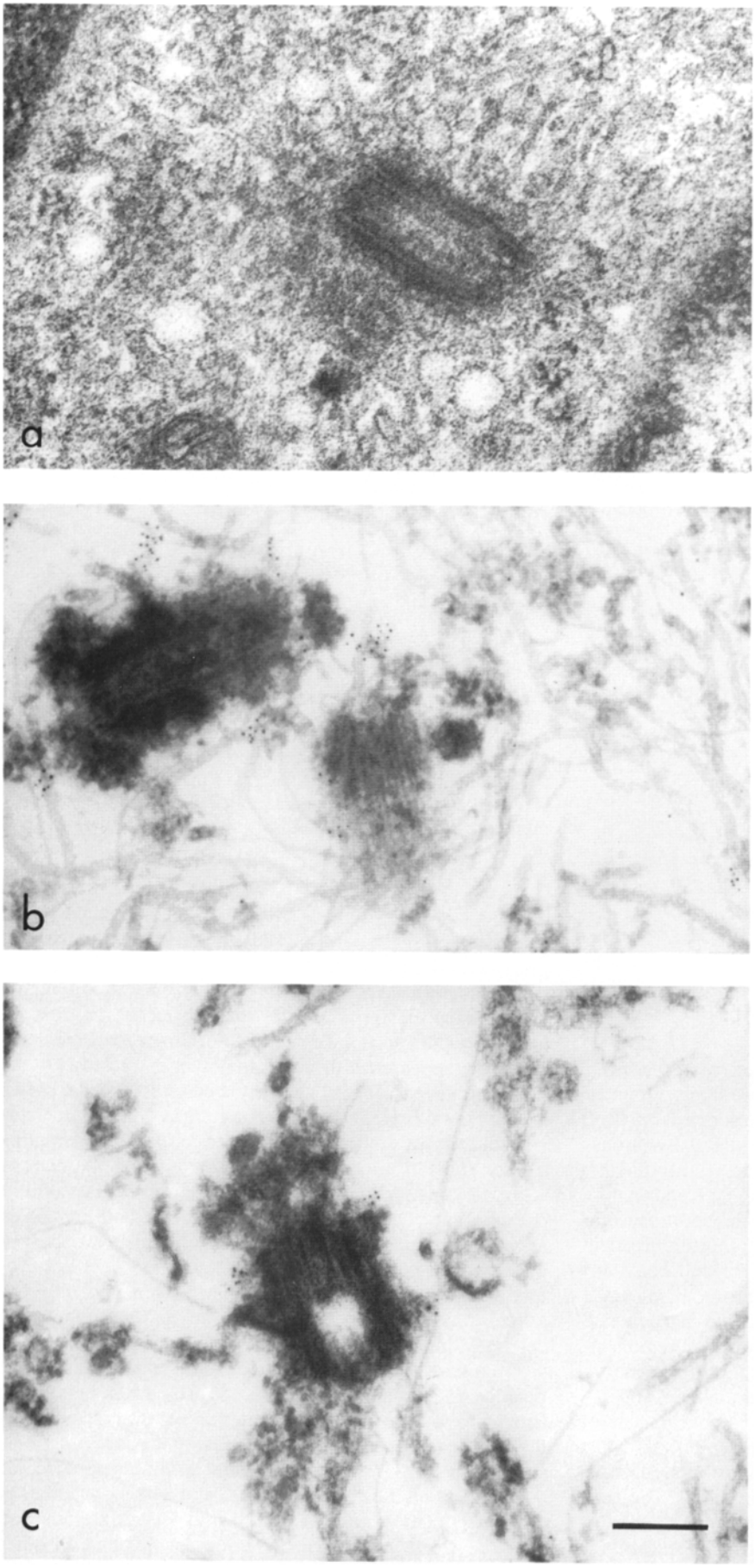


Figure 2. Electron micrographs of centrosomes and surrounding regions from the cell bodies of cultured rat sympathetic neurons. *a* shows a conventional electron micrograph of a centrosome from an unextracted culture, while *b* and *c* show immunoelectron micrographs of centrosomes from extracted cultures immunostained with a polyclonal γ-tubulin antibody (Joshi et al., 1992), and an appropriate 5-nm gold-conjugated second antibody. In both unextracted and extracted cells, the centrosome is clearly identifiable, and is asymmetrically surrounded by a cloud of pericentriolar material (see Results for more details). In all immunostain experiments, the pericentriolar material labeled for γ-tubulin. The protocol used in *b* resulted in a higher density of staining of this material, but also resulted in higher levels of background staining (manifest as small clusters of gold particles of apparently random distribution). The protocol used in *c* resulted in a somewhat lower density of staining at the centrosome, but also resulted in the reduction of background staining to negligible levels. (It is worth noting that higher levels of gold particles are present within the pericentriolar material than are apparent upon casual examination of our micrographs. For example, in *c*, there are ~15 gold particles that are readily noticeable. In addition, however, several more gold particles are buried within darkly osmicated pericentriolar material, and are difficult to accentuate in micrographs of our thick sections.) Bar, 0.25 μm.

Figure 3. Immunoelectron microscopic analyses for γ-tubulin in a region of an axon. *a* shows the middle section of three consecutive sections, while *b* shows a serial reconstruction of MTs on all three sections. Ends of MTs were identified on the middle section by their lack of continuity onto either of the two surrounding sections. Minus ends were distinguished from plus ends by the orientation of the former toward the cell body. Two minus ends are indicated by arrows. Neither of these ends, or any of the other 10 minus ends observed, labeled for γ-tubulin. Bar, 0.15 μm.

of consistency with regard to these latter gold particles argues that they are the result of nonspecific background staining, which is not an uncommon problem with the use of peptide-derived polyclonal antibodies such as ours. To minimize background, most studies were performed with a lower antibody concentration (1:50), and the concentration and variety of proteins in the blocking solution were increased (see Materials and Methods; see also Stearns et al., 1991). This resulted in a somewhat lower density of labeling at the centrosome, but also reduced the background labeling to negligible levels (see Fig. 2 *c*). Careful inspection of multiple sections through each cell body examined ($n = 20$) revealed no labeling for γ-tubulin anywhere in the cell body other than within the pericentriolar material.

γ-tubulin Is Undetectable in Axons and Dendrites

We next focused our efforts on axons and dendrites. In searching for γ-tubulin in these processes, we initially used the same method as was used for the cell body, which entailed simply scanning multiple sections of several different axons and dendrites ($n = 10$ each). These searches revealed virtually no gold particles. Nevertheless, to control for the possibility that sites of γ-tubulin staining were missed due to their paucity, we wished to specifically identify the most likely sites where γ-tubulin might be localized in axons and dendrites, and then carefully examine these sites for the presence or absence of gold particles. Because MT nucleation sites are generally attached to the minus ends of MTs, we chose to examine these ends as the most likely sites where γ-tubulin might be localized in axons and/or dendrites. To identify and study the minus ends of MTs, we examined sets of three consecutive serial sections, searching for ends of MTs on the middle section that were not continuous onto either of the flanking sections (see Materials and Methods). In the dendrite, we assumed, because roughly equal levels of dendritic MTs are of each orientation (Baas et al., 1991), that roughly half the ends that we randomly identified were minus ends. In these studies, we identified 12 MT ends (all minus) in axons (Fig. 3), and 14 MT ends (~half minus) in dendrites (Fig. 4). In no case did we find labeling for γ-tubulin (i.e., there were no gold particles) at any of these ends.

Biochemical Analyses

In a final set of experiments, we confirmed the absence of γ-tubulin from the axon by SDS-PAGE/immunoblot analyses of pure axonal fractions obtained from explant cultures. Half of a ganglion, containing roughly 10^4 neurons (see Higgins et al., 1991) was used for each explant (Fig. 5 *a*). Given that there is one centrosome per neuron and 10^4–10^5 γ-tubulin molecules per centrosome (Stearns et al., 1991), and assuming that all of the γ-tubulin in the CBM is localized to the centrosome, we calculate that each CBM should contain 10^8–10^9 molecules of γ-tubulin. If γ-tubulin were present at the minus ends of axonal MTs, how much γ-tubulin would we expect to find in the axonal halo? We obtained a rough estimate for this value based on the approximate cross-sectional areas of the axonal halo (170 mm^2) and of an individual axon (.006 mm^2), the number of axons comprising the width of the halo (10–12 axons), the average length of the axons (6 mm), the average cross-sectional density of MTs in an axon (10; Baas et al., 1991), the average length of each axonal MT (100 μm; Bray and Bunge, 1981), and the minimum number of γ-tubulin molecules expected to be present at the minus end of a MT (13, assuming that at least one γ-tubulin molecule is bound to each of the 13 protofilaments of axonal MTs). Taking these factors into account, we calculate that, if γ-tubulin is present at the minus ends of axonal MTs, then each halo should contain about 3×10^9 molecules of γ-tubulin, or at least three times the number of γ-tubulin molecules present in the CBM.

Fig. 5 *b* shows the immunoblot comparing the γ-tubulin content of axons and CBMs. Lanes *1* and *3* contain equal amounts of total protein obtained from axons and CBMs respectively, while lane *2* contains twice this amount of axonal protein. The immunoblots reveal the presence of γ-tubulin in the CBMs, as expected, but show no detectable γ-tubulin present in the axon halos. This point is reinforced by the lack of γ-tubulin signal even when the amount of axonal material loaded is increased to an amount containing a number of minus ends of MTs corresponding to over six times the amount of γ-tubulin estimated to be present in the CBM. These results indicate that γ-tubulin is not present at the minus ends of axonal MTs, and thus are entirely consistent with the immunoelectron microscopic results indicating

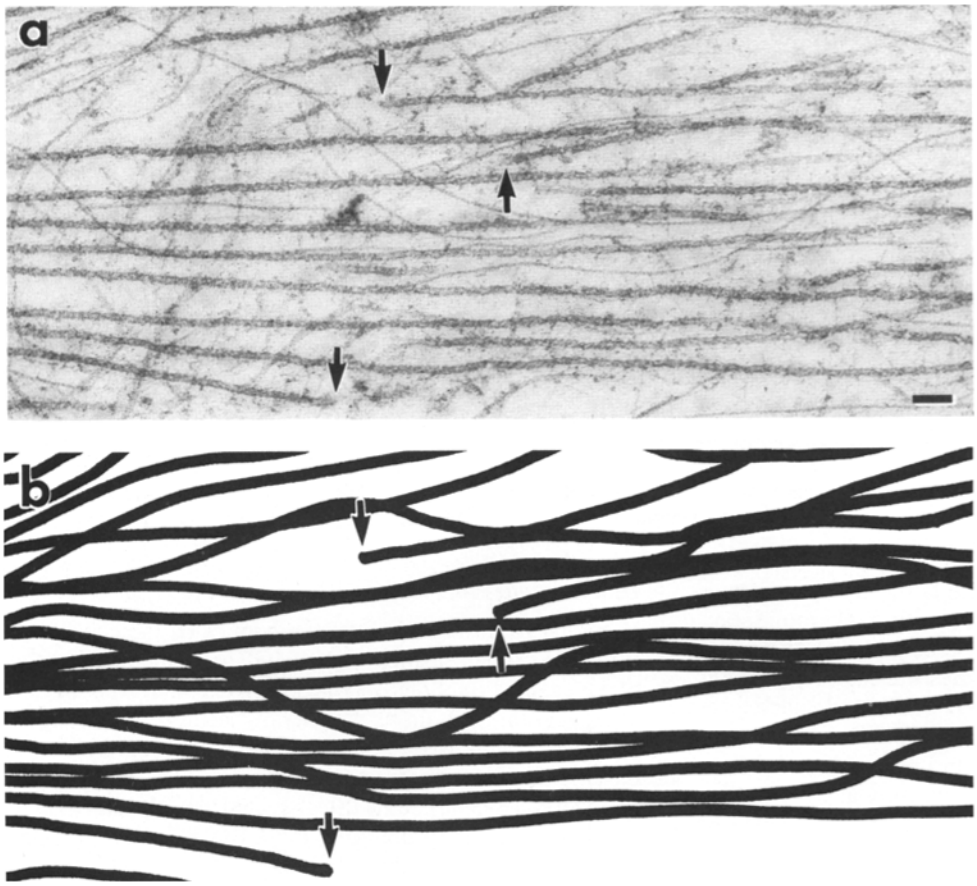


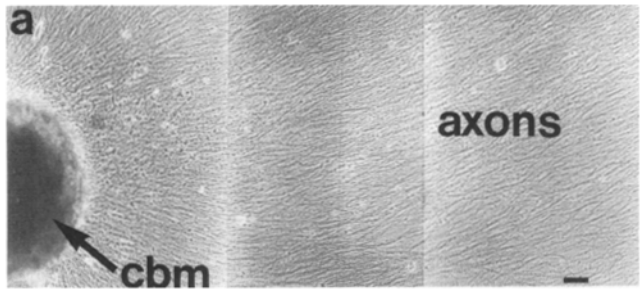
Figure 4. Immunoelectron microscopic analyses for γ-tubulin in a region of a dendrite. *a* shows the middle section of three consecutive sections, while *b* shows a serial reconstruction of MTs on all three sections. Ends of MTs were identified on the middle section by their lack of continuity onto either of the two surrounding sections. In the dendrite, roughly half the MTs are of each orientation (Baas et al., 1991), so it was impossible to distinguish a minus end from a plus end. Three MT ends are indicated by arrows. None of these ends, or any of the other twelve ends observed, labeled for γ-tubulin. These results suggest that neither minus nor plus ends of MTs in the dendrite contain γ-tubulin. Bar, 0.15 μm.

that γ-tubulin is present exclusively in the pericentriolar region of the neuron.

Discussion

The results of the present study indicate that the distribution of γ-tubulin in neurons, despite their highly specialized MT arrays, is similar to that in nonneuronal cells. γ-tubulin is present within the pericentriolar region, but is undetectable elsewhere in the cell. We found no γ-tubulin in the axon, the dendrite, or anywhere in the cell body other than at the centrosome. While we cannot exclude the possibility that γ-tubulin may be present at levels too low to detect, the following lines of reasoning argue against this possibility. First, we have used immunoelectron microscopy as our principal means of detection. This method affords very high sensitivity and resolution. Moreover, we used two different protocols, each with a different balance of signal-to-noise, and neither method resulted in the specific labeling for γ-tubulin at any site other than the pericentriolar material. Second, the absence of γ-tubulin from the axon is also indicated by immunoblot analyses; no γ-tubulin was detected in the axonal halo of explant cultures. Finally, the levels of γ-tubulin within the neuron are exceedingly low, and are on the order of the levels present in nonneuronal cells. If γ-tubulin did regulate the assembly of MTs within neuronal processes, which grow to be hundreds of microns in length, it seems likely that the total levels of γ-tubulin in these processes would exceed those present at the centrosome (see Results). Thus, all available data are consistent with the conclusion that γ-tubulin is localized exclusively to the pericentriolar region of neurons.

These results have important implications with regard to the mechanisms by which the axonal and dendritic MT arrays are generated. As explained in the Introduction, both the organization and regular 13-protofilament lattice of axonal and dendritic MTs suggest the likelihood that they are nucleated by a structure or substance comparable in function to the centrosome. We have recently utilized a functional assay to demonstrate an essential role for γ-tubulin in the nucleation of MTs in vivo (Joshi et al., 1992; see introduction). While we cannot completely dismiss the possibility that another protein such as a microtubule-associated protein (MAP) with the same functional properties may substitute for γ-tubulin in axons and dendrites, such a scenario seems unlikely at present. MAPs are abundant throughout cells, but do not appear to promote MT nucleation independent of defined MT nucleating structures (Brinkley, 1985). Assuming that neuronal MTs, like nonneuronal MTs, require γ-tubulin for their nucleation, one can use the distribution of γ-tubulin in the neuron to determine the sites where axonal and dendritic MTs originate. One possibility is that the MT nucleating material of the centrosome takes on a widespread distribution during neuronal development, permitting the local nucleation of MTs within axons and/or dendrites. The alternative possibility is that axonal and dendritic MTs are, in fact, nucleated by the centrosome, after which they detach from this structure and translocate into the neurites. Our results on γ-tubulin are inconsistent with the former possibil-



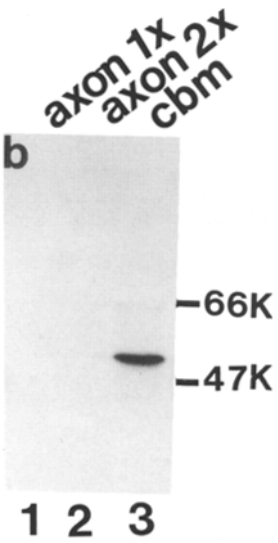


Figure 5. SDS-PAGE/immunoblot analyses on γ-tubulin content in pure axonal and highly enriched somatodendritic fractions obtained from explant cultures. *a* shows a phase contrast micrograph of a portion of an explant culture of rat sympathetic neurons. The CBM contains all of the cell bodies, dendrites, and initial segments of the axons, while the axonal halo contains exclusively axonal material (Peng et al., 1986). *b* shows the immunoblot, with CBMs in lane *3*, and axons in lanes *1* and *2*. Lane *1* has an equivalent amount of protein as in lane *3*, while lane *2* has twice as much protein as in lanes *1* or *3*. γ-tubulin is present in the CBMs, but is undetectable in the axonal halo, even with twice the amount of protein loaded. Bar, 100 μm.

ity, and therefore provide indirect support for the idea that axonal and dendritic MTs are nucleated at the centrosome.

Several additional lines of evidence support the notion of a centrosomal origin for neuritic MTs. In recent studies on the regulation of local MT dynamics in the axon, we demonstrated that all MT assembly occurs via elongation from the plus ends of preexisting stable MTs (Baas and Ahmad, 1992). The inability of the axon itself to generate entirely new MTs (see also Baas and Heidemann, 1986) points to the cell body as the only potential source of new MTs for the elongating axon. Consistent with this conclusion, the time interval between the synthesis and assembly of tubulin in the neuron is surprisingly short, suggesting that MT nucleation occurs at a site close to tubulin synthesis (Black et al., 1986), and tubulin synthesis is known to occur only in the cell body (Bruckenstein et al., 1991). Moreover, studies on the sites of MT stabilization in the neuron indicate that the net addition of new MT polymer to the growing axon occurs principally in the cell body and/or most proximal region of the axon (Baas, P. W., F. J. Ahmad, T. P. Pienkowski, A. Brown, and M. M. Black, 1991. *J. Cell Biol.* 115:174a). All of these observations support the long-standing polymer translocation model of axon growth (Lasek, 1982), which has recently garnered direct experimental support from studies using real-time imaging to observe the movement of fluorescently labeled MTs in the axon (Reinsch et al., 1991; Tanaka and Kirschner, 1991; Sabry et al., 1991). Finally, there is some precedent for MT detachment from the centrosome in other cell types (McBeath and Fujiwara, 1990), and at least two proteins with MT severing capacity have been identified (Sanders and Salisbury, 1987; Vale, 1991).

In conclusion, our results indicate that γ-tubulin is localized to the pericentriolar region of the neuron, and does not redistribute into axons or dendrites. When considered together with our previous results indicating that γ-tubulin is essential for MT nucleation in vivo, this observation provides strong indirect support for the notion that MTs destined for axons and dendrites are nucleated at the centrosome, and then released for translocation into these neurites. If this is correct, then new questions arise regarding the mechanisms by which differences in MT organization are established in axons and dendrites, in the absence of regional differences in MT nucleating capacity. Clearly, substantial efforts will be required to resolve this issue, and to test our proposal regarding a centrosomal origin for neuritic MTs.

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