The Plus Ends of Stable Microtubules Are the Exclusive Nucleating Structures for Microtubules in the Axon

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Abstract. Microtubules (MTs) in the axon have a uniform polarity orientation that is recapitulated during recovery from episodes of MT depolymerization (Heidemann, S. R., M. A. Hamborg, S. J. Thomas, B. Song, S. Lindley, and D. Chu. 1984. *J. Cell Biol.* 99:1289–1295). This tight regulation of their organization indicates that axonal MTs are spatially regulated by discrete nucleating structures comparable in function to the centrosome. Several authors have proposed that an especially stable class of MTs in the axon may serve as these nucleating structures. In a previous report (Baas, P. W., and M. M. Black. 1990. *J. Cell Biol.* 111:495–509), we determined that the axons of cultured sympathetic neurons contain two classes of MT polymer, stable and labile, that differ in their sensitivity to nocodazole by roughly 35-fold. The stable and labile polymer represent long-lived and recently assembled polymer, respectively. We also determined that these two classes of polymer can be visually distinguished at the immunoelectron microscopic level based on their content of tyrosinated α-tubulin: the labile polymer stains densely, while the stable polymer does not stain. In the present study, we have taken advantage of these observations to directly identify MT nucleating structures in the axon. Neuron cultures were treated with nocodazole for 6 h to completely depolymerize the labile polymer in the axon, and substantially shorten the stable polymer. The cultures were then rinsed free of the drug, permitted to reassemble polymer for various periods of time, and prepared for immunoelectron microscopic localization of tyrosinated α-tubulin. Serial reconstruction of consecutive thin sections was undertaken to determine the spatial relationship between the stable MTs and the newly assembled polymer. All of the new polymer assembled in direct continuity with the plus ends of stable MTs, indicating that these ends are assembly competent, and hence capable of acting as nucleating structures. Our results further indicate that no self-assembly of MTs occurs in the axon, nor do any MT nucleating structures exist in the axon other than the plus ends of stable MTs. Thus the plus ends of stable MTs are the exclusive nucleating structures for MTs in the axon.

THE organization of microtubules (MTs)[1] in the axon is tightly regulated. The plus ends of the MTs are uniformly oriented away from the cell body, toward the axon tip (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987, 1988, 1989, 1991*b*). Moreover, this organization is recapitulated during recovery from episodes of MT depolymerization (Heidemann et al., 1984; Baas et al., 1987). These observations indicate that highly efficient mechanisms exist within the axon to ensure that all MTs are assembled with a plus-end-distal orientation. Because the plus end of the MT is, by definition, the end preferred for assembly (Binder et al., 1975; Bergen and Borisy, 1980), such an organization is likely to have important implications for the manner by which the MT array of the axon is elaborated. In addition, because different organelles appear to be preferentially translocated toward either the plus or the minus end of the MT, the polarity orientation of MTs in the axon will, in part, determine its cytoplasmic composition (Baas et al., 1988; Black and Baas, 1989). Although these observations clearly indicate an essential role for MT organization in defining the cytoarchitecture of the axon, surprisingly little is known about the mechanisms by which this organization is established and maintained.

Most information on MT assembly and organization has derived from in vitro studies, or from studies on nonneuronal cells. In the test tube, MT assembly can occur by self-association of tubulin subunits, but preferentially occurs by elongation from a MT template or nucleating structure (for review see Kirschner, 1978). Studies on MT assembly in intact cells indicate that self-association is strongly suppressed in favor of nucleated assembly, and that MT nucleation is spatially regulated by discrete nucleating structures such as the centrosome (for review see Brinkley, 1985). The minus ends of the MTs are embedded within the nucleating structure, while the plus ends radiate away from it, resulting in MT arrays of uniform polarity orientation (Haimo et al.,

1. *Abbreviation used in this paper*: MT, microtubule.

1979; Heidemann and McIntosh, 1980; Euteneuer and McIntosh, 1981). The uniform polarity orientation of axonal MTs suggests that they too may be spatially regulated by nucleating structures. However, the identity of these putative nucleating structures has been elusive. In fact, it is now well established that MTs in the axon do not emanate from a common centralized structure such as the centrosome (Lyser, 1968; Sharp et al., 1982). Instead, they appear to stop and start at multiple sites along the length of the axon, with no apparent specializations at their minus ends which could serve as nucleating structures (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981).

An intriguing hypothesis, suggested by several authors, is that a unique class of especially stable MTs may act as the MT nucleating structures in the axon (Morris and Lasek, 1984; Brady et al., 1984; Black et al., 1984; Heidemann et al., 1984; Baas and Heidemann, 1986; Sahenk and Brady, 1987). A key prediction of this hypothesis is that individual MTs in the axon will consist of a stable domain in direct continuity with a newly assembled, more labile domain. In a recent study, we were able to test this prediction using immunoelectron microscopy to visually distinguish stable and labile MT polymer in the axons of cultured sympathetic neurons (Baas and Black, 1990). We reported that roughly half the MT mass in these axons is labile, depolymerizing with a halftime of ~3.5 min in the presence of 2 μg/ml nocodazole, while the other half is relatively stable, depolymerizing with a halftime of ~130 min (see also Baas et al., 1991*b*). Staining for tyrosinated α-tubulin distinguishes the labile polymer from the stable polymer: the former is rich in tyrosinated α-tubulin, while the latter is deficient in this α-tubulin variant. In our immunoelectron micrographs, a significant proportion of the MT profiles showed stable polymer in direct continuity with labile polymer. In all such cases, the labile domain of the MT was situated at the plus end of the stable domain (see Baas and Black, 1990 for more details). These observations provide strong support for the hypothesis that stable MTs serve as MT nucleating structures in the axon, and further suggest that it is specifically the plus ends of the stable MTs from which new assembly occurs.

In the present study, we have sought to directly test these hypotheses by determining the distribution and identity of the MT nucleating structures in the axon. To accomplish these goals, we used a strategy similar to that which has been used previously to identify MT nucleating structures in nonneuronal cells (De Brabander et al., 1977, 1980; Brinkley, 1985; Gunderson et al., 1987; Bulinski et al., 1988). Neuron cultures were treated with nocodazole for a time sufficient to completely depolymerize the labile MT polymer in the axon. The drug was then rinsed from the cultures, and they were permitted brief periods of MT reassembly. To determine the distribution of MT nucleating structures in the axon, newly assembled polymer was visualized by immunofluorescence staining for tyrosinated α-tubulin along the length of the axon. To determine the identity of the nucleating structures, newly assembled polymer was visualized by immunoelectron microscopy, and its relationship with the stable polymer, or other potential nucleating structures, was determined by serial reconstruction of consecutive thin sections. The results of these studies were conclusive: all new assembly occurred specifically from the plus ends of stable MTs, which were distributed throughout the length of the axon. No assembly occurred from the minus ends, nor did any assembly occur independently of the stable MTs. These observations indicate that the plus ends of stable MTs are assembly competent, and are the exclusive MT nucleating structures in the axon.

Materials and Methods

Cell Culture

Sympathetic neurons from the superior cervical ganglia of newborn rat pups were cultured in two ways. Dissociated cultures were used for immunofluorescence analyses because they permit visualization along substantial lengths (hundreds of micrometers) of individual axons. Explant cultures were used for electron microscopic analyses because they maximize the number of aligned axons, and hence MTs of known polarity orientation, that can be sectioned simultaneously. Methods for the preparation of these cultures were as previously described (Baas and Black, 1990), except that dissociated cultures were grown at particularly low density to limit dendritic outgrowth (see Higgins et al., 1991). Fig. 1, *a* and *b*, shows phase-contrast micrographs of a dissociated and explant culture, respectively.

Nocodazole Treatments

Nocodazole, a potent anti-MT drug, was purchased from Aldrich Chemical Co. (Milwaukee, WI). A stock solution of 5 mg/ml was prepared in DMSO, and diluted in tissue culture medium at 1:2,500 or 1:500 for final concentrations of 2 and 10 μg/ml, respectively. 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 the cells for either 1 or 6 h, depending on the experiment. For recovery, cultures were rinsed twice briefly with drug-free tissue culture 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 1 min to 24 h. Previous studies have determined that nocodazole is immediately and completely reversible when rinsed from neuron cultures in this manner, even when the drug is used at higher concentrations than those used here (Baas and Heidemann, 1986).

Immunological Procedures

Immunofluorescence and immunoelectron microscopic analyses were performed as previously described (Baas and Black, 1990). Briefly, cultures were extracted in the presence of a MT stabilizing buffer to remove free tubulin, and then fixed with cold (−20°C) methanol for immunofluorescence, or glutaraldehyde for immunoelectron microscopy. For immunofluorescence, dissociated cultures were double labeled with YL 1/2 (Accurate Chemical and Scientific Corp., Westbury, NY), a rat mAb which recognizes the tyrosinated but not the detyrosinated form of α-tubulin (Kilmartin et al., 1982; Wehland et al., 1983), and a mouse general β-tubulin antibody purchased from Amersham Corp. (Arlington Heights, IL). These antibodies were used at concentrations of 1:100 and 1:300, respectively. The YL 1/2 was visualized with a fluorescein-conjugated goat anti-rat second antibody, and the β-tubulin antibody was visualized with a Texas red–conjugated goat anti-mouse second antibody. Both of these second antibodies were purchased from Accurate Chemical and Scientific Corp., and used at 1:100. For immunoelectron microscopy, explant cultures were labeled with YL 1/2, used at a concentration of 1:200. The second antibody was the Janssen 5-nm colloidal gold–conjugated goat anti-rat second antibody, purchased from Amersham Corp., and used at 1:2.

Serial Reconstruction of Axonal MTs

Immunoelectron microscopic localization of tyrosinated α-tubulin distinguishes the stable MT polymer in the axon from the more recently assembled polymer (Baas and Black, 1990). To determine the spatial relationship between these two types of polymer, we undertook serial reconstruction of consecutive thin sections using a modification of our previously described method (Baas and Heidemann, 1986; Joshi et al., 1986). In the original method, the MT profiles from consecutive sections are traced onto transparent plastic sheets, together with the cell membrane and internal membra-

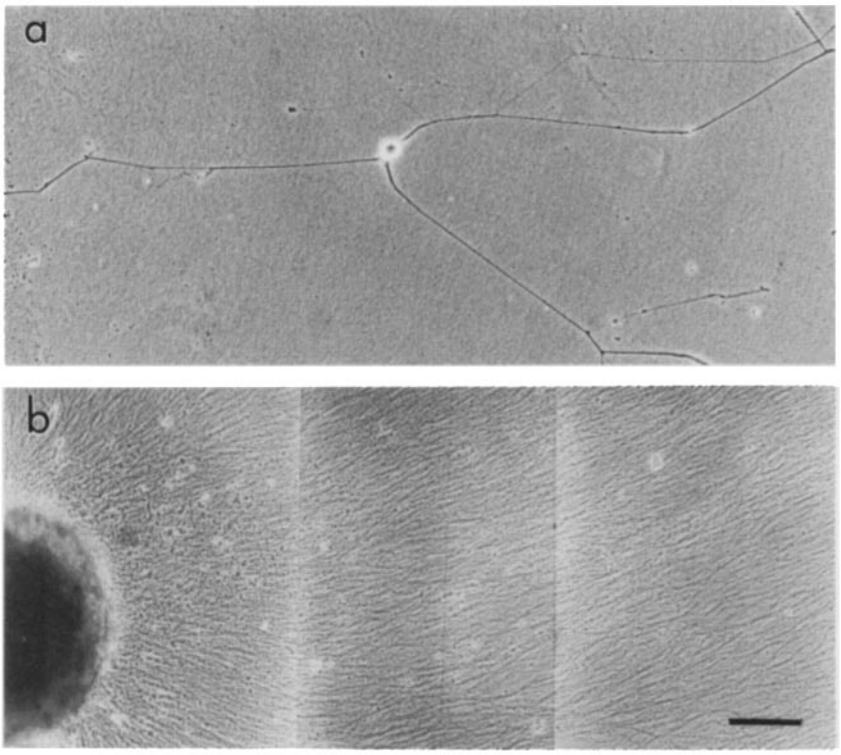

Figure 1. Phase-contrast micrographs of rat sympathetic neurons grown in two different ways for the present study. *a* shows a dissociated neuron culture, the type used for immunofluorescence analyses, while *b* shows an explant culture, the type used for electron microscopic analyses. See Materials and Methods for more details. Bar: (*a*) 100 μm; (*b*) 200 μm.

nous elements. Consecutive sections are aligned using the membranous elements as registration markers. This results in an accurate alignment of the MT profiles, revealing which profiles are actually portions of the same MT. Unfortunately, in the present study, it was necessary to extract the neurons under very harsh conditions for the immunoelectron microscopic procedure, resulting in the removal of virtually all membranous borders that would otherwise have served as registration markers. Therefore, it was necessary to develop an alternative technique for the alignment of consecutive sections.

In the present technique, individual MTs were reconstructed one at a time. Particularly small blockfaces (~0.75 × 0.1 mm) were carved from the axonal regions of explant cultures such that a chain of 15 consecutive serial sections could be picked up on a single butvar-coated slot grid. Next, the observation screen on an electron microscope (model CX100; JEOL U.S.A. Inc., Peabody, MA) was removed from the instrument, and a meshwork consisting of 2-mm squares was drawn on the screen with a sharp lead pencil, after which the screen was returned to the electron microscope. For ease of relocation, regions of the sections destined for serial reconstruction were first selected by their proximity to one of the four corners of the section. Then, a single MT profile was selected from the middle section of the series (the 8th section of 15), and its precise location, length, and shape within the meshwork were drawn onto a corresponding map. The map contained a meshwork identical to that on the screen, and at the magnification used on the electron microscope, distances as little as the diameter of a single MT (24 nm) could be accurately discerned and translated onto the map. Also recorded on the map were any residual bits of cellular material or debris that could serve as registration markers to align the next section. Using this system, the MT of interest was followed out in both directions, and MT profiles continuous with the original profile were drawn onto the map. Once all 15 sections were examined, the MT was considered as a data point only if both ends of the MT terminated within the 15 sections. Otherwise, the MT was discarded as uninterpretable for our purposes. Regions of the MT that were labeled or unlabeled with gold particles were also recorded on the map.

MT Polarity Determination

To determine the polarity orientation of MTs in control, drug-treated, and drug-recovered neuron cultures, we used the standard "hooking" protocol (Heidemann and McIntosh, 1980; Baas et al., 1989; for review see Heidemann, 1991). In this procedure, cultures are lysed in the presence of a special MT assembly buffer containing exogenous brain tubulin, and then prepared for EM by conventional means. The exogenous tubulin adds onto existing MTs in the form of lateral sheets that appear as "hooks" on the MTs when viewed in cross section. The curvature of the hook reveals the polarity orientation of the MT; a clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indicates the opposite. In the present study, control and experimentally manipulated explant cultures were rinsed in PBS and then treated for 30 min at 37°C with 0.06% Brij 58 in a MT assembly buffer (0.5 M Pipes, 0.1 mM EGTA, 0.01 mM EDTA, 0.1 mM $MgCl_2$, 2.5% DMSO, 0.5 mM GTP) containing 1.2 mg/ml MT protein. Cultures were fixed by the addition of an equal quantity of 4% glutaraldehyde. After preparation and embedment for EM, cross sections of the axons were taken, and hooks were interpreted and scored as previously described (Heidemann and McIntosh, 1980; Heidemann, 1991). As in our previous work, hooks were judged as clockwise or counterclockwise from the vantage point of the growth cone.

Results

Distribution of MT Nucleating Structures in the Axon

The strategy of the present studies was to visualize MT nucleating structures in the axon by their capacity to initiate the reassembly of MTs during recovery from nocodazole treatment. To determine the distribution of these nucleating structures, immunofluorescence microscopy was used to visualize newly assembled polymer along the length of the axon. Because the stable MT polymer within the axon is a candidate for the MT nucleating structures, and because shortening the stable polymer would alter the distribution of its ends, it was necessary for these studies to choose a drug treatment that would completely depolymerize the labile polymer without substantially shortening the stable polymer. Our previous determinations for the halftimes of depolymerization of the stable and labile polymer in the presence of 2 μg/ml nocodazole indicate that 1 h of drug treatment at this concentration depolymerizes virtually 100% of the labile polymer, but <20% of the stable polymer (Baas and Black,

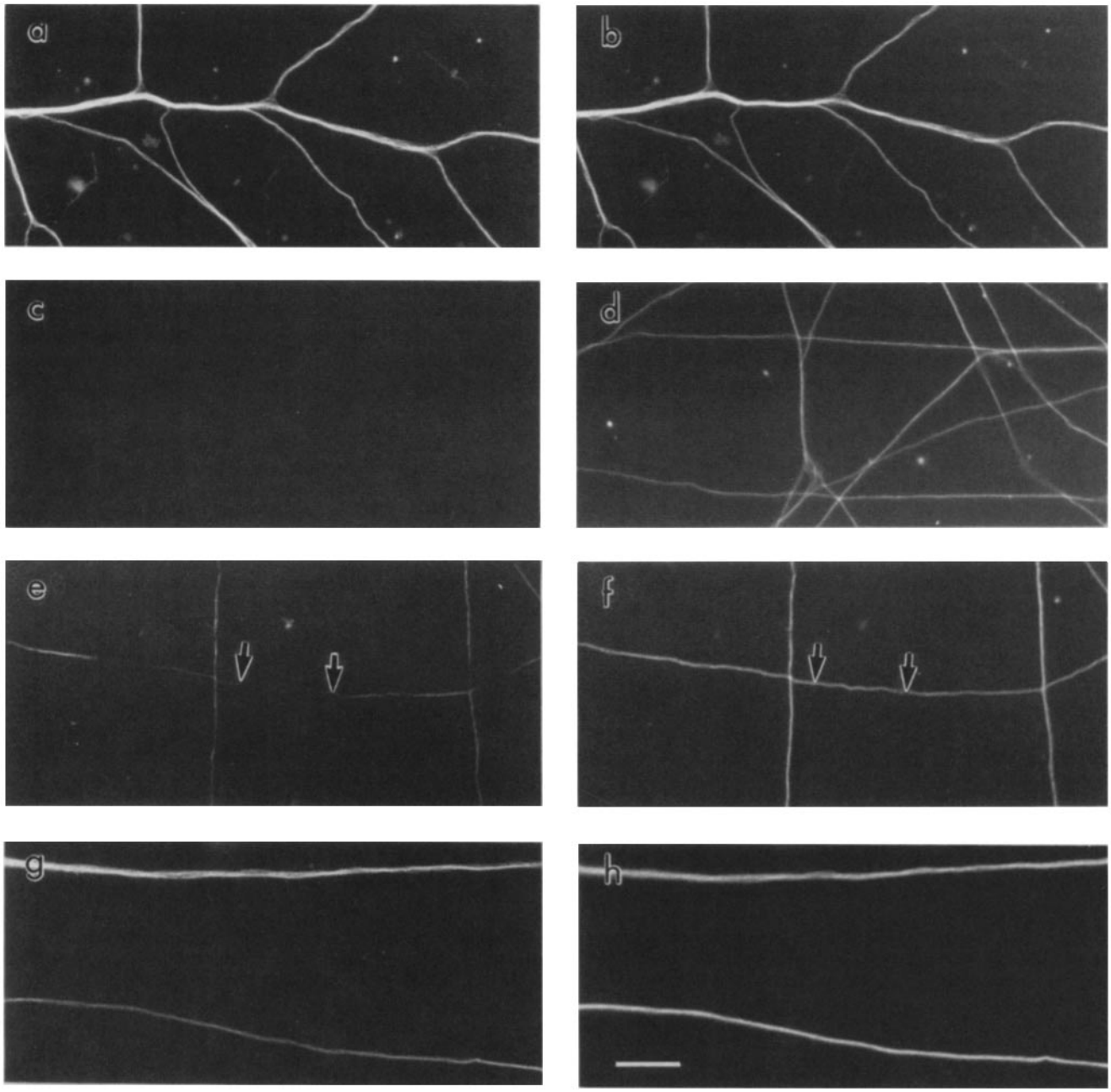

Figure 2. Double-label immunofluorescence analyses on the axons of cultured sympathetic neurons. The YL 1/2 antibody against tyrosinated α-tubulin was used to localize newly assembled MT polymer in the axon (*a*, *c*, *e*, and *g*), and a general β-tubulin antibody was used to visualize total polymer (*b*, *d*, *f*, and *h*). *a* and *b* are control axons; *c* and *d* are axons from cultures treated with 2 μg/ml nocodazole for 1 h; *e* and *f* are axons recovered from such nocodazole treatment for 5 min; and *g* and *h* are axons recovered for 30 min. Staining for both antibodies is present throughout the length of control axons. Staining for tyrosinated polymer, but not total polymer, is virtually eliminated after nocodazole treatment. During recovery, new polymer assembles in discrete segments that are apparent along the axon's length at 5 min of recovery (area between arrows is unstained for tyrosinated tubulin but stains for total tubulin indicating the presence of stable but not new polymer in this region). By 30 min of recovery, axons once again stain along their lengths for new as well as total polymer. Bar, 20 μm.

1990; Baas et al., 1991*b*; see introduction). After such treatment, nocodazole was rinsed from the cultures, and periods of recovery ranging from 1 min to 24 h were permitted. If MT assembly in the axon is regulated by nucleating structures, as indirect evidence suggests (see introduction), we would predict that new assembly should appear in discrete regions of the axon. On the other hand, if de novo initiation of MT assembly occurs in the axon, we would expect relatively uniform levels of recovery to occur along the axon's length.

The results of these experiments are shown in Fig. 2. As described in Materials and Methods, the cultures were double stained with an antibody for tyrosinated α-tubulin to visualize the newly assembled polymer, and a general β-tubulin antibody to visualize total polymer. In control axons, both antibodies stain relatively evenly along the length

of the axon, indicating that labile polymer and probably stable polymer are present throughout the axon (Fig. 2, *a* and *b*; see also Baas and Black, 1990; Baas et al., 1991*b*). After nocodazole treatment, the staining for tyrosinated α-tubulin is diminished almost completely (Fig. 2 *c*), while the staining for the general tubulin antibody is somewhat dimmer, but still relatively continuous along the length of the axon (Fig. 2 *d*). The latter observation confirms the widespread distribution of stable polymer along the axon's length. After 1 min of recovery from nocodazole, discrete segments of staining for tyrosinated α-tubulin begin to become apparent, but are difficult to capture in a photograph (not shown). After 5 min of recovery, the segments are more dramatic, and are manifest as 20–100-μm stretches of relatively bright staining flanked by unstained or more dimly stained regions of somewhat shorter lengths (5–50 μm; Fig. 2 *e*). After 10 min of recovery, the stained regions are significantly longer, such that only infrequent unstained regions can be seen (not shown). By 30 min, staining for tyrosinated α-tubulin is continuous along the axon's length (Fig. 2 *g*). Correspondingly, staining for the general tubulin antibody becomes more intense with increasing recovery times (Fig. 2, *f* and *h*). These observations are consistent with the hypothesis that MT assembly in the axon occurs from discrete nucleating structures, and indicate that these nucleating structures have a widespread (but uneven) distribution throughout the length of the axon.

A slight variation on these results was observed in the distal region of the axon contiguous with the growth cone. After nocodazole treatment, this region (spanning ~10–50 μm from the axon tip) showed no staining for either the antibody against tyrosinated α-tubulin or the general tubulin antibody. This result is consistent with our previous observation that all of the MT polymer in this region is labile (Baas and Black, 1990). During recovery, this region always showed a bright segment of staining for tyrosinated α-tubulin, which appeared to extend from the mainshaft of the axon where stable MTs are present. These data, not shown in figures, are entirely similar to previous observations of this type on the growth cone region of shorter axons (Arregui et al., 1991). These observations indicate that no MT nucleating structures are present in the distal region of the axon. Instead, the nucleating structures for the distal axon are located more proximally, in the axon's mainshaft.

Identification of MT Nucleating Structures in the Axon

To determine the identity of the MT nucleating structures in the axon, nocodazole recovery experiments were performed at the immunoelectron microscopic level. Our strategy was to visualize newly assembled MT polymer by its staining for tyrosinated α-tubulin, and then identify the structures from which the new polymer elongates. Because the thin sections required for EM contain only portions of individual MTs, it was necessary to perform serial reconstructions of consecutive thin sections to accurately determine the spatial relationship between the new polymer and potential nucleating structures. In previous work, we serially reconstructed segments of the axon, using membranous borders as registration markers to align the MT profiles in consecutive sections (Baas and Heidemann, 1986; Joshi et al., 1986). For the present work, this method was impossible because of the loss of membranous borders as a result of the extraction necessary for immunogold labeling. Therefore, we developed an alternative technique that results in the reliable reconstruction of individual MTs within serial sections of extracted axons (see Materials and Methods for details). Because the stable polymer is a likely candidate for the nucleating structures, it was necessary for these studies to capture both the plus and minus ends of the stable polymer, as well as both ends of the new polymer. To ensure that a majority of the MTs in our preparations were encompassed within the 15 sections that were practical for use in this technique (see Materials and Methods), we wished to minimize the length of both the stable and new polymer. To substantially shorten the stable polymer, nocodazole treatment was extended to 6 h and the concentration of the drug was increased to 10 μg/ml. This treatment shortened the majority of MTs to within a few micrometers in length (see later in text). To determine the recovery time that would be most practical for our serial reconstructions, we assessed the levels of MT reassembly at 1, 5, and 30 min, and at 24 h of recovery.

For these initial studies, the total length of MT polymer and the total length of polymer which did or did not label for tyrosinated α-tubulin were measured in five randomly selected spaces on our 200-mesh EM grids. The results of these experiments are shown in Fig. 3, and the data are presented graphically in Fig. 4. In presenting our data, the levels of MT mass are all calculated as percentages relative to total control levels. In control axons, roughly half the polymer is labeled for tyrosinated α-tubulin, and roughly half is unlabeled (Fig. 3*a*; see also Baas and Black, 1990; Baas et al., 1991*b*). As in our previous studies, a small but significant number of profiles show a labeled domain in direct continuity with the plus end of an unlabeled domain. After nocodazole treatment, 5% of the MT mass is remaining, and all of this is unlabeled (Fig. 3 *b*). The lengths of individual MT profiles captured in the nocodazole-treated axons were also clearly shorter than those in control axons. After 1 min of recovery (Fig. 3 *c*), the levels of unlabeled MT mass were unchanged, but segments of labeled polymer were now present. The levels of labeled polymer were equivalent to 5% of the total MT mass in control axons. After 5 and 30 min (Fig. 3, *d* and *e*, respectively), unlabeled polymer still showed no significant change, but labeled polymer had increased to 17 and 90%, respectively. After 24 h of recovery (Fig. 3 *f*), the levels of MT mass and proportions of labeled and unlabeled polymer are indistinguishable from those in controls. At each point in recovery, a small number of profiles displayed labeled and unlabeled domains. As in the controls, the labeled (i.e., newly assembled) domain was always situated at the plus end of the unlabeled (i.e., stable) domain (Fig. 3, *c*, *e*, and *f*). These results indicate a rapid reassembly of polymer that approaches control levels after only ~30 min, and a relatively slower rate of α-tubulin detyrosination after assembly. In addition, these results provide initial data favoring the hypothesis that new assembly occurs specifically from the plus ends of stable polymer.

Assuming that MTs in the axons of cultured neurons average ~100 μm in length (Bray and Bunge, 1981), these results further indicate that 1 min of recovery is well suited for our serial reconstruction studies. For these studies, two approaches were used. In one set of experiments, for each reconstruction, we selected a labeled (i.e., newly assembled)

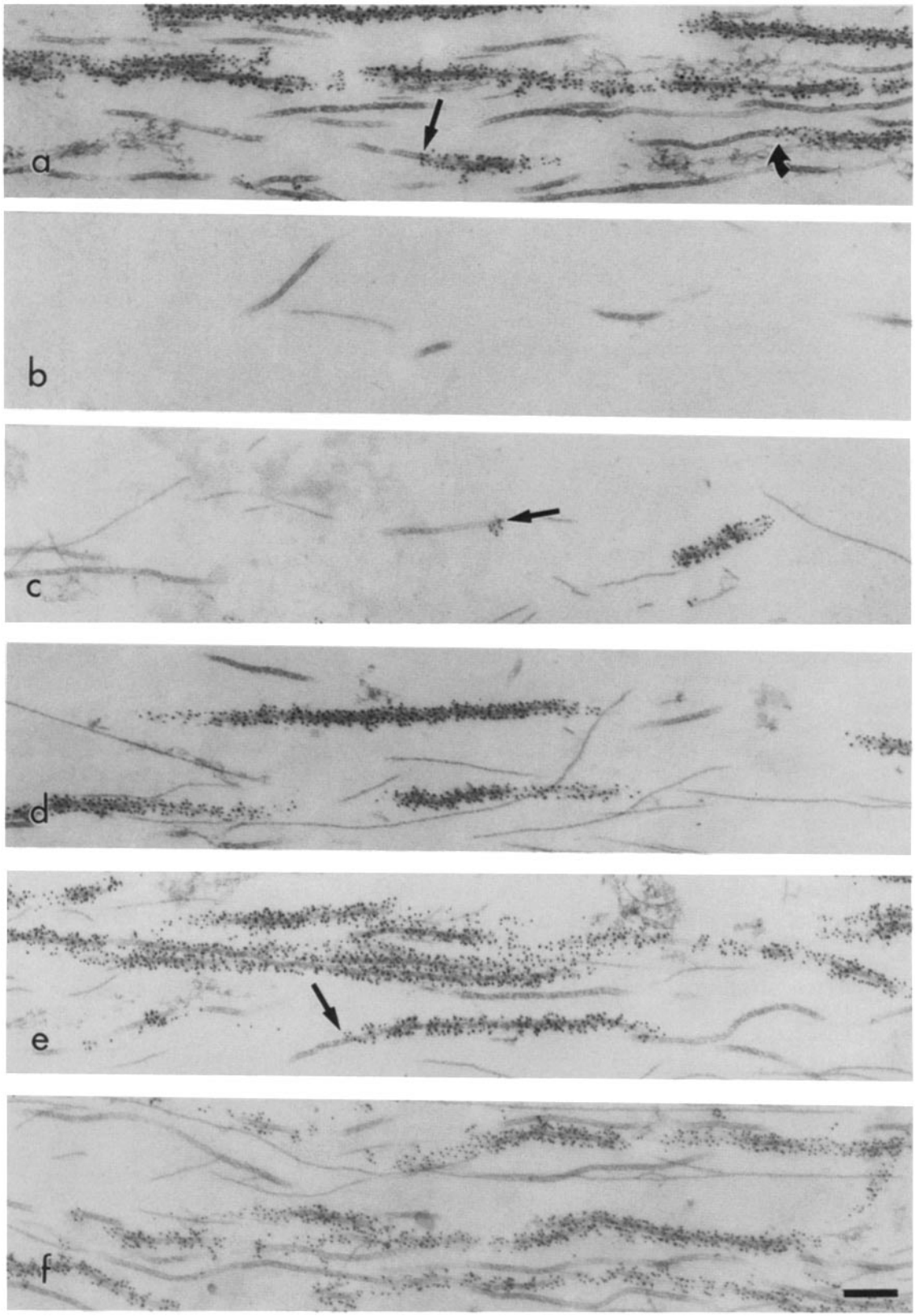

Figure 3. Immunoelectron microscopic analyses on the axons of cultured sympathetic neurons. The YL 1/2 antibody against tyrosinated α-tubulin was used to differentiate newly assembly MT polymer from stable polymer (see text). In control axons (*a*), roughly equal levels of polymer are labeled and unlabeled. After 6 h of treatment with 10 μg/ml nocodazole (*b*), all of the MT profiles are unlabeled, and appear to be reduced in length relative to the controls. After 1 min of recovery (*c*), profiles of labeled polymer, roughly similar in length to the unlabeled profiles, are present. After 5 and 30 min of recovery (*d* and *e*, respectively), the unlabeled profiles show no apparent change, but the levels of labeled polymer have increased substantially. After 24 h of recovery (*f*), the levels of labeled and unlabeled polymer

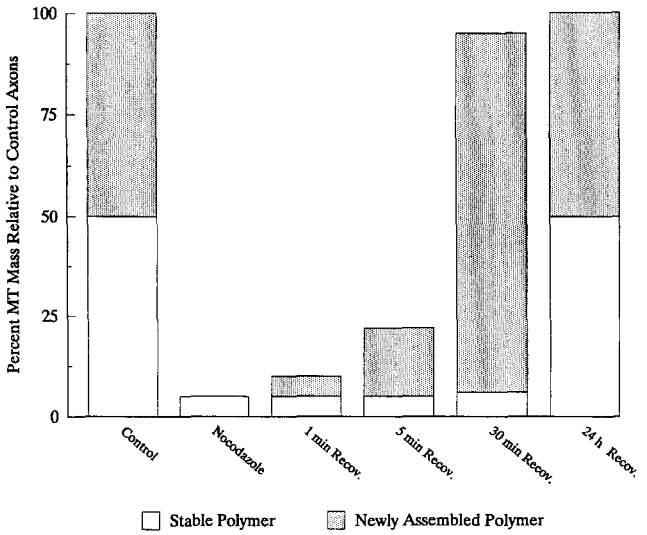

Figure 4. Levels of MT mass in experimentally manipulated axons relative to controls. The lengths of newly assembled and stable polymer were summed separately over an area spanning five open spaces on a 200-mesh transmission EM grid. The white areas of the graph indicate stable polymer, while the hatched areas indicate newly assembled polymer (distinguished electron microscopically by the specific staining of the latter for tyrosinated α-tubulin). In control axons, half of the total MT mass was of each type. After 6 h of nocodazole treatment at 10 μg/ml, only 5% of the total MT mass remained, and all of this was unlabeled (i.e., stable). After 1, 5, and 30 min of recovery, the levels of stable polymer were unchanged, but the levels of new polymer increased progressively. At 24 h of recovery, levels of stable and new polymer were indistinguishable from controls.

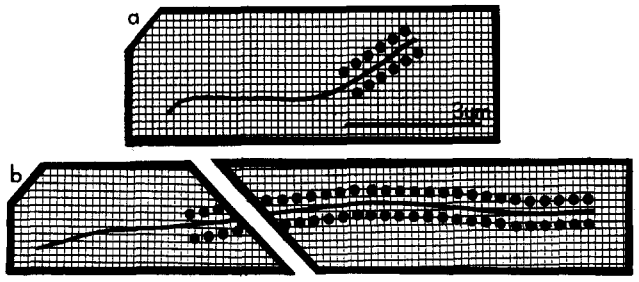

Figure 5. Serial reconstructions of axonal MTs in neuron cultures treated for 6 h with 10 μg/ml nocodazole followed by recovery for 1 min (*a*) or 5 min (*b*). New polymer, distinguished electron microscopically by its staining for tyrosinated α-tubulin, is indicated with surrounding black dots. In all cases, new polymer assembled directly from the plus ends of stable polymer. Open space in *b* represents 8.5 μm of labeled polymer. See text for more details, and Table I for quantitative data. Width of MTs on reconstructions is not to scale. Bar, 3 μm.

MT profile in the center-most section of our series of 15, and followed it out in both directions through the serial sections to determine its association with stable polymer or other potential nucleating structures (see Materials and Methods). In 25 such profiles examined, 24 were found to be continuous with the plus ends of stable polymer (Fig. 5 *a*; see also 3 *c*), none were continuous with the minus end of stable polymer, none were found unassociated with stable polymer, and one was ambiguous because of folds in the epon/butvar (Table I). In a second set of experiments, for each reconstruction, the MT profile chosen as a starting point was unlabeled (i.e., a stable MT). In 25 such profiles, none were continuous with the plus end of new polymer, 19 were continuous with the minus end of new polymer, 5 were not continuous with new polymer, and 1 was ambiguous (Table I). The average length of new polymer after 1 min was 3.5 $\pm$ 1 μm. (Although this figure is probably an underestimate in that our method biases against the selection of the longer MT profiles [see Materials and Methods], it is interesting to note that remarkably similar figures have been reported for the rate of MT assembly in nonneuronal cells [Schulze and Kirschner, 1986], and for the rate of MT-dependent regrowth of amputated axon segments [Baas and Heidemann, 1986]).

Collectively, these results suggest that all new polymer assembles directly from the plus ends of stable MTs in the axon. The stable MTs from which no assembly occurred presumably represent either a subpopulation of stable MTs in the axon which are not assembly competent, or perhaps more likely, stable MTs that are no different from the rest, but from which no assembly had yet occurred at this short recovery time.

A possibility worth considering is that the plus ends of stable MTs are the preferred, but not exclusive, sites of new MT assembly in the axon. That is, if we had extended the recovery time somewhat longer, new polymer may have arisen independently of these nucleating structures. To test this hypothesis, we performed serial reconstruction studies at 5 min of recovery, after which substantial MT reassembly had occurred (with polymer levels reaching 20–25% of control levels; Figs. 3 *d* and 4). These experiments were more difficult in that, because of the greater lengths of new polymer, the vast majority of MTs we pursued did not terminate within our 15 sections, and hence had to be discarded from our data (see Materials and Methods). Despite this difficulty, we were able to locate three labeled profiles and three unlabeled profiles that were usable for our analyses. The average length of new polymer in these six MTs was 17.2 $\pm$ 2 μm, almost exactly five times the length at 1 min of recovery. Consistent with our results at 1 min of recovery, all three of the labeled profiles (i.e., new polymer) were continuous with the plus ends of stable polymer, and all three of the unlabeled profiles (i.e., stable polymer) were continuous with the minus ends of new polymer (Fig. 5 *b* and Table I).

Collectively, the results of our serial reconstructions indicate that all new MT assembly in the axon occurs from the plus ends of stable polymer. No new polymer arises independently of the stable polymer, indicating that no self-assembly of MTs occurs in the axon, nor do any MT nucleating structures exist in the axon other than the plus ends of stable MTs. Based on these observations, we conclude that the plus ends

are indistinguishable from controls. Arrows mark MT profiles in which a newly assembled domain can be visualized in direct continuity with the plus end of a stable domain (*curved arrow* marks a somewhat ambiguous example of the same). Plus ends of MTs are oriented to the right of the panels. See text for more details and Fig. 4 for quantitative data. Bar, 0.2 μm.

Table I. Summary of Data from Serial Reconstruction of Axonal MTs

	Total MT examined	Number at [+] end of opposite type	Number at [−] end of opposite type	Number not continuous with other type	Ambiguous
1 min recovery*	25	24	0	0	1
1 min recovery‡	25	0	19	5	1
5 min recovery*	3	3	0	0	0
5 min recovery‡	3	0	3	0	0

MTs in cultures recovered for 1 or 5 min from treatment with 10 μg/ml nocodazole for 6 h were serially reconstructed as described in the text (see Materials and Methods and Results). In one set of experiments, a profile labeled for tyrosinated tubulin (i.e., newly assembled polymer) was selected as a starting point, whereas in a second set of experiments, an unlabeled profile (i.e., stable polymer) was selected as a starting point. In all cases, newly assembled polymer arose in direct continuity with the plus ends of stable polymer (see text for more details; see also Fig. 5).

* Serial reconstructions were based on the initial selection of a labeled MT profile. Therefore, the "opposite type" refers to unlabeled polymer.

‡ Serial reconstructions were based on the initial selection of an unlabeled MT profile. Therefore, the "opposite type" refers to labeled polymer.

Table II. MT Polarity Orientation in Control and Experimental Axons

	CW*	CCW*	AMB*	UHK*	% HK‡	% CW‡
Control	500	21	35	115	83 ± 3	96 ± 3
6 h nocodazole	54	3	7	11	85 ± 5	95 ± 5
1 min recovery	75	4	9	13	88 ± 5	95 ± 4
5 min recovery	152	10	14	32	84 ± 6	94 ± 2
30 min recovery	488	21	22	94	85 ± 7	96 ± 3
24 h recovery	522	25	44	136	81 ± 6	95 ± 6

MT polarity orientation was determined using the standard "hook" procedure (see Baas et al., 1989 and Materials and Methods). In this procedure, neurons are lysed in a special MT assembly buffer in the presence of exogenous brain tubulin. The exogenous tubulin adds onto existing MTs in the form of lateral protofilament sheets that appear on the MTs as hooked appendages when viewed in cross section electron microscopically. A clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indicates that the minus end of the MT is directed toward the observer. MT polarity orientation was analyzed in control axons, as well as axons treated for 6 h with 10 μg/ml nocodazole, and axons recovered from such treatment for 1, 5, and 30 min, and 24 h. In all of these situations, hooks were predominantly clockwise as viewed from the growth cone, indicating uniform MT polarity orientation, plus ends distal to the cell body. *CW*, microtubules with clockwise hooks as viewed from the tip of the axon looking toward the cell body; *CCW*, microtubules with counterclockwise hooks as viewed from same; *AMB*, microtubules with ambiguous hooks; *UHK*, microtubules with no hooks; *HK*, microtubules with hooks.

* Indicates sum of all MTs for all 50 axons of each type analyzed.

‡ Mean ± SD for all 50 axons scored.

of stable MTs are the exclusive MT nucleating structures in the axon.

MT Polarity Analyses

Before closing, it is important to consider the possibility that the short MTs remaining after nocodazole treatment may have shifted orientation in the axon, such that the minus ends of some MTs in our experimental axons may have been directed away from the cell body. If such an inversion has occurred, then the conclusion drawn from our serial reconstruction studies with respect to MT polarity is incorrect. To test this possibility, we analyzed MT polarity orientation in control and experimentally manipulated cultures. For these analyses, we used the standard "hook" protocol, in which neurons are lysed in a special MT assembly buffer containing exogenous tubulin. The exogenous tubulin adds onto existing MTs as lateral sheets that appear as "hooks" on the MTs when viewed in cross section. A clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indicates the opposite. For the present study, we analyzed MT polarity orientation in the axons of control cultures, cultures treated with 10 μg/ml nocodazole for 6 h, and cultures permitted to recover from such drug treatment for 1, 5, and 30 min, and 24 h. In all of these situations, 94–96% of the hooks were clockwise as viewed from the growth cone, indicating uniform MT polarity orientation, plus ends distal to the cell body (see Fig. 6 and Table II). These results indicate that no inversion of MTs occurred during nocodazole treatment or recovery, are consistent with the conclusion that no haphazard MT assembly occurs within the axon, and validate the strong conclusions derived from our serial reconstruction studies.

It should be mentioned that our MT polarity results differ

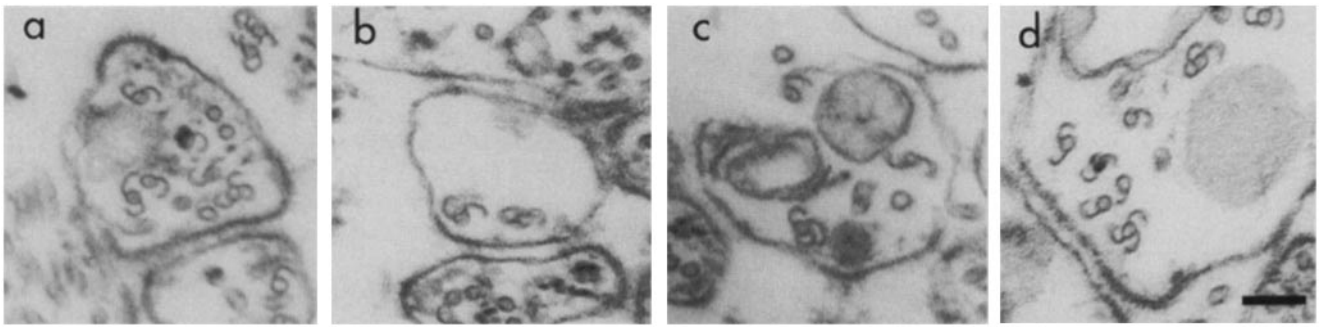

Figure 6. Analyses on MT polarity orientation in the axons of cultured sympathetic neurons using the standard "hook" technique (see Materials and Methods). As viewed from the vantage point of the growth cone, clockwise hooks on the MTs indicate that the plus ends of the MTs are distal to the cell body, and counterclockwise hooks indicate the opposite. In the axons of control cultures (*a*), cultures treated with 10 μg/ml nocodazole for 6 h (*b*), and cultures recovered from such treatment for 1 (not shown), 5 (*c*), and 30 (*d*) min, and 24 h (not shown), hooks were predominantly clockwise, indicating uniform MT polarity orientation, plus ends distal to the cell body. Bar, 0.1 μm.

somewhat from those of Heidemann et al. (1984), who performed the original MT polarity work of this kind on short segments of sympathetic axons isolated from the cat. These authors found that the proportion of plus-end-distal MTs was somewhat lower after recovery from bouts of MT disassembly (at most 83% clockwise hooks), suggesting that either some de novo assembly occurred, or that some of the stable MTs did, in fact, change orientation. Also in contrast with our results, Heidemann et al. observed an extremely rapid reassembly of MT polymer, the levels of which exceeded control levels after only 5 min of recovery. The reasons for these differing results are unclear. However, in light of the consistency of our polarity and immunoelectron microscopic results, and the noninvasive nature of our experimental system, we have confidence in the strong conclusions that can be drawn from our results. One possibility is that the experimental conditions of Heidemann et al., who performed all of their manipulations on dissected pieces of tissue, may have introduced some nonphysiological factors that had some effect on MT assembly and organization in their samples.

Discussion

In the present study, we have sought to determine the distribution and identity of the MT nucleating structures in the axon. Our strategy was to visualize the nucleating structures by their capacity to initiate MT reassembly during recovery from nocodazole treatment, a strategy previously used to identify MT nucleating structures in nonneuronal cells (De Brabander et al., 1977, 1980; Brinkley, 1985; Gunderson et al., 1987; Bulinski et al., 1988). New polymer was distinguished from stable polymer by the preferential staining of the former for tyrosinated α-tubulin (Baas and Black, 1990; Baas et al., 1991*b*). This permitted us to directly test the long-standing hypothesis that stable MTs serve as MT nucleating structures in the axon (see introduction). Our results at the immunofluorescence level indicate that MT nucleating structures have a widespread (but uneven) distribution along the length of the axon, with the exception of its most distal region contiguous with the growth cone, which contains no nucleating structures. Our results at the immunoelectron microscopic level reveal the identity of these nucleating structures. These data indicate that the plus ends of stable MTs in the axon are assembly competent, and moreover, that all MT assembly in the axon occurs via elongation from these plus ends. No assembly occurs from the minus ends of stable MTs, nor does any assembly occur independently of the stable MTs. Thus the plus ends of stable MTs are the exclusive MT nucleating structures in the axon.

These results complement the results of previous studies, and have important implications for the mechanisms by which the MT array of the axon is established and maintained. For example, the conclusion that all MT assembly in the axon occurs via elongation from stable MTs is supported by previous data indicating that no MT reassembly can occur if axon segments are experimentally depleted of stable as well as labile MT polymer (Baas and Heidemann, 1986). The present results are also consistent with our previous data suggesting that most or all MTs in the axon consist of a labile domain situated at the plus end of a stable domain (Baas and Black, 1990), and with studies demonstrating that when biotinylated tubulin is microinjected into PC12 cells, it incorporates specifically at the plus ends of MTs in their axon-like neurites (Okabe and Hirokawa, 1988). Finally, our results provide a simple explanation for the mechanism by which the uniform polarity orientation of MTs is maintained in the axon. Because all new assembly occurs from the plus ends of preexisting MTs, the new polymer will share the plus-end-distal polarity orientation already established for the stable polymer. In this manner, the specialized assembly properties of axonal MTs can account for the maintenance of their organization.

Interestingly, assembly competence is a property not generally shared by stable MTs in other cell types (Webster et al., 1987). In nonneuronal cells, MTs are thought to be stabilized by end caps, the presence of which blocks disassembly, but also blocks new assembly from the ends (Webster et al., 1987; Khawaja et al., 1988). In the axon, the assembly competence of stable MTs at their plus ends indicates that these MTs are stabilized by a mechanism other than end caps, presumably the binding of accessory proteins along the length of the MT. Although the identity of these factors is unknown, principal candidates include the microtubule-associated proteins tau and STOP. Tau is an axon-enriched protein which has been shown to promote MT assembly and nucleation in the test tube (Bre and Karsenti, 1990), and to increase the levels of stable MTs when microinjected into living cells (Drubin and Kirschner, 1986). In addition, expression of tau in certain nonneuronal cells causes them to develop processes with an axon-like MT organization (Baas et al., 1991*a*). STOP, a protein abundant in neuronal tissue, has been shown to markedly enhance the stability of MTs in the test tube (Margolis et al., 1986), and to preferentially associate with the stable MTs of the mitotic spindle in neuronal precursor cells (Margolis et al., 1990). The lack of assembly of the stable MTs at their minus ends suggests that the minus end may be capped by an as yet unidentified structure. Alternatively, there might be factors in the axoplasm that strongly support plus-end assembly and/or suppress minus-end assembly. Precedent for such factors exists in *Xenopus* eggs (Gard and Kirschner, 1987) and surf clam oocytes (Suprenant, 1991). Current efforts are aimed at elucidating the potential roles that factors such as those discussed here may play in determining the stability and assembly properties of MTs in the axon.

The results of our studies raise another interesting and quite fundamental question concerning the mechanisms by which the axonal MT array is elaborated. Because of the great length that axons can grow, the length of any MT within the axon will generally be short relative to that of the axon (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). Thus axon growth clearly requires the generation of new MTs, as well as the elongation of existing MTs. If no new MTs are generated within the axon itself, as our data indicate, where do these new MTs arise? Previous speculations pointed to the distal region of the axon behind the growth cone as a potential site for the generation of new MTs (Mitchison and Kirschner, 1988). Our data are inconsistent with this notion, and by the process of elimination, point to the cell body as the only potential source of new MTs for the axon. If this reasoning is correct, then MTs destined for the axon must first arise in the cell body, and then move into the axon by an active transport mechanism. It is intriguing to contem-

plate that such movement, if it exists, may be unidirectional with respect to the polarity of the MT, thereby establishing the uniform polarity orientation of the axon (Baas et al., 1991*a*).

The concept of MT nucleation in the cell body followed by transport into the axon is a long-standing hypothesis (Black and Lasek, 1980; Lasek, 1982) that has fallen into disfavor in recent years because of the demonstration of local MT assembly within the axon itself (Bamburg et al., 1986; Baas and Heidemann, 1986; Baas et al., 1987; Okabe and Hirokawa, 1988, 1990; Robson and Burgoyne, 1988; Lim et al., 1989, 1990; Keith, 1990; Baas and Black, 1990; Arregui et al., 1991). However, in its truest form, the model of Lasek and collaborators accounts for both polymer movement and elongation within the axon (for more details, see Lasek, 1988). Our data support this view, suggesting that axonal MTs originate in the cell body, but are elongated principally within the axon. If this is correct, attention shifts to identifying the structures in the cell body that nucleate MTs for the axon. One possibility is that MTs destined for the axon are nucleated by a traditional MT nucleating structure such as the centrosome, after which they detach from this structure, and then move into the axon. In support of this possibility, there is some precedent for MT detachment from the centrosome in other cell types (McBeath and Fujiwara, 1990). Alternatively, however, the MT nucleating capacity of the centrosome may itself become dissociated from that structure during neuronal development, and take on a widespread distribution throughout the somatodendritic compartment of the neuron (Baas et al., 1989). In either view, the notion of MT movement into and along the axon is essential. The concept of MT movement and elongation occurring in concert with one another has recently garnered very elegant experimental support in studies tracing the progress of photoactivated bands of tubulin in the axon (for details see Reinsch et al., 1991).

In conclusion, our studies have provided direct information on the identity and distribution of MT nucleating structures in the axon. The plus ends of stable MTs are the exclusive nucleating structures for MTs in the axon, and they have a widespread distribution along the axon's length. As usual, the resolution of this issue has introduced a myriad of new questions. We have provided some discussion with regard to these questions, and our future efforts with be aimed at testing the merits of our speculations.

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