Processes Induced by Tau Expression in Sf9 Cells Have An Axon-like Microtubule Organization

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Abstract. We have indirectly analyzed the role of tau in generating the highly organized microtubule (MT) array of the axon. Axons contain MT arrays of uniform polarity orientation, plus ends distal to the cell body (Heidemann, S. R., J. M. Landers, and M. A. Hamborg. 1981. *J. Cell Biol.* 91:661–673). Surprisingly, these MTs do not radiate from a single discrete nucleating structure in the cell body (Sharp, G. A., K. Weber, and M. Osborn. 1982. *Eur. J. Cell Biol.* 29: 97–103), but rather stop and start at multiple sites along the length of the axon (Bray, D., and M. B. Bunge. 1981. *J. Neurocytol.* 10:589–605). When Sf9 ovarian cells are induced to express high levels of tau protein, they develop cellular processes which are similar in appearance to axons and which contain dense arrays of MTs (Knops, J., K. S. Kosik, G. Lee, J. D. Pardee, L. Cohen-Gould, and L. McConlogue. 1991. *J. Cell Biol.* 114:725–734). We have analyzed the organization of MTs within these arrays, and determined it to be similar, but not identical, to the organization of MTs within the axon. The caliber, MT number, and MT density vary significantly from process to process, but on average are manyfold higher in the tau-induced processes than typically found in axons. Greater than 89% of the MTs in the processes are oriented with their plus ends distal to the cell body, and this proportion is even higher in the processes that are most similar to axons with regard to caliber, MT number, and MT density. Similar to the situation in the axon, MTs are discontinuous along the length of the tau-induced processes, and do not emanate from any observable nucleating structure in the cell body. We have also identified bundles of MTs throughout the cell bodies of the Sf9 cells induced to express tau. Similar to the MT arrays in the processes, these MT bundles are not visibly associated with any other cytological structures that might regulate their polarity orientation. Nevertheless, these bundles consist of MTs most (>82%) of which have the same polarity orientation. Collectively, these results suggest that tau may play a fundamental role in generating MT organization in the axon. In particular, a key property of tau may be to bundle MTs preferentially with the same polarity orientation.

THE microtubule (MT)[1] array of the axon is highly organized. The MTs within the axon are uniform in their polarity orientation, with their plus ends distal to the cell body (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987*a*, 1988, 1989, 1991). However, unlike MT arrays of uniform polarity orientation in nonneuronal cells, the MT array of the axon does not emanate from a discrete centralized nucleating structure such as the centrosome (Lyser, 1968; Sharp et al., 1982). In fact, the organization of MTs in the axon is not dependent upon any cytological structure or information specific to the cell body (Baas et al., 1987*a*). Instead, MTs appear to be free in the axon, stopping and starting at multiple sites along its length (Zenker and Hohberg, 1973; Chalfie and Thompson, 1979; Bray and Bunge, 1981; Tsukita and Ishikawa, 1981; Stevens et al., 1988). In light of the significance of MT organization in determining the specific composition and cytoarchitecture of the axon (for reviews see Lasek et al., 1988; Black and Baas, 1989), there is great interest in elucidating the cellular and molecular mechanisms underlying the organization of MTs in the axon.

There are virtually no data currently available which address the genesis of uniform MT polarity orientation in the axon. However, recent studies have established that the unique assembly properties of axonal MTs contribute directly to the maintenance of this organization once it has been established. Especially stable MTs in the axon provide the exclusive sites for new MT assembly in the axon (Baas and Heidemann, 1986), and as such serve as individual nucleating structures that spatially regulate the dynamics of the MTs within the axon (Baas and Black, 1990). By limiting MT assembly in the axon to the elongation of these existing stable MTs, haphazard MT assembly is suppressed, and the high degree of MT organization in the axon is maintained. The capacity of stable MTs in the axon to act as nucleating

1. *Abbreviations used in this paper*: MAP, microtubule-associated protein; MT, microtubule.

structures is dependent upon their assembly competence, a property not generally shared by stable MTs in nonneuronal cells (Webster et al., 1987). Thus the assembly competence of stable MTs in the axon is a specialization that is essential to the regulation of MT organization in the axon. The factors which stabilize MTs in the axon without rendering them assembly incompetent remain completely unknown. In addition, it is unknown how the stable MTs in the axon originally obtain their uniform polarity orientation.

An intriguing possibility is that certain microtubule-associated proteins (MAP) endow axonal MTs with specialized properties important to their organization. A likely candidate in this regard is tau, a MAP abundant in the neuron, and highly enriched in the axon (Binder et al., 1985; Peng et al., 1986; for review see Matus, 1988). A growing body of evidence suggests that tau plays essential roles in MT nucleation, stability, and bundling (for reviews see Lee, 1990; Goedert et al., 1991). For example, when tau is microinjected into fibroblastic cells that normally do not express high levels of tau, the levels of stable MTs increase (Drubin and Kirschner, 1986). In addition, the transfection of tau into fibroblasts causes them to develop dense bundles of MTs in their cytoplasm (Kanai et al., 1989). Furthermore, in vitro analyses indicate that tau reduces the frequency of transitions between growing and shrinking phases of the MTs, and increases the number of MTs nucleated by the centrosome (Bre and Karsenti, 1990). Collectively, these results suggest that tau could play an essential role in establishing key features of MT organization in the axon.

Three additional lines of evidence provide strong support for this view. First, in PC12 cells, the levels of tau increase during neurite outgrowth, and this increase corresponds precisely with an increase in the levels of polymerized tubulin (Drubin et al., 1985). Second, the introduction of tau antisense into developing cerebellar neurons prevents the differentiation of their short primordial processes, one of which would otherwise have developed into an axon (Caceres and Kosik, 1990). This observation indicates that tau is a necessary component of the axonal MT system without which it does not differentiate. Finally, the infection of normally rounded insect ovarian Sf9 cells with a virus containing a tau cDNA insert results in the extension from these cells of long processes which are similar in appearance to axons, and which contain dense arrays of MTs (Knops et al., 1991). It is unclear why this more dramatic response was not observed in the transfected fibroblasts of Kanai et al. (1989) or in the fibroblasts microinjected with tau (Drubin and Kirschner, 1986). However, this result on the ovarian cells brings up the intriguing possibility that the addition of tau to a system which is normally deficient in tau can enable the system to elaborate a MT array like that characteristic of the axon. If this is correct, then it seems reasonable to conclude that tau may play an essential role in establishing MT organization in the axon.

In the present study, we have examined the organization of MTs within these tau-induced processes. Our results indicate that MT organization in these processes is similar to that in the axon; the MTs do not arise from a discrete nucleating structure in the cell body, are discontinuous along the length of the processes, and yet are nearly uniform in their polarity orientation, plus ends distal to the cell body. In addition, we have identified bundles of MTs throughout the cell body of the infected cells, somewhat similar to those observed by Kanai et al. (1989) in their transfected fibroblasts. These bundles, like those in the processes, also show no apparent association with any other cytological structures in the cell body, and yet consist of MTs most of which are aligned with the same polarity orientation. Collectively, these results suggest that tau may play a fundamental role in generating MT organization in the axon. In particular, a key property of tau may be to bundle MTs preferentially with the same polarity orientation.

Materials and Methods

Insect ovarian Sf9 cells were infected with a virus containing the transcript for either the three or four repeat form of tau, and cultured in 35-mm tissue culture dishes as previously described (Knops et al., 1991). These normally rounded cells, when infected in this manner, extend long cellular processes that are similar in appearance to axons. Ultrastructural analyses were performed after roughly 2 d in culture, by which time most of the cells had extended long processes, but had not yet begun to suffer from the toxic effects of the viral infection.

For standard transmission EM, cultures were prepared as previously described (Baas and Heidemann, 1986; Baas et al., 1987*b*). Briefly, cultures were fixed in 2% glutaraldehyde for 10 min, rinsed in a solution containing 0.1 M cacodylate and 5% sucrose, stained with 2 mg/ml tannic acid for 5 min, rinsed again, postfixed for 5 min with 1% osmium tetroxide, dehydrated with increasing concentrations of ethanol, and embedded using one of the available Epon clones. After curing of the resin, cells of interest were located by phase-contrast microscopy, circled with a diamond marker objective, and sectioned either parallel to the substratum to obtain longitudinal sections, or perpendicular to the substratum to obtain cross-sections.

For MT polarity determination, cultures were treated as previously described (Baas et al., 1989). Briefly, cultures were lysed in the presence of a special MT assembly buffer containing exogenous brain tubulin. Under these conditions, the exogenous tubulin adds onto existing MTs in the form of lateral protofilament sheets. These sheets appear as hooked appendages on the MTs when they are observed in cross-section with the electron microscope. A clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indicates the opposite (Heidemann and McIntosh, 1981).

Results

Expression of Tau Causes Sf9 Cells to Extend Processes

In a previous report, Knops et al. (1991) demonstrated that when normally rounded insect ovarian Sf9 cells are induced to express tau by infecting them with a virus containing a human tau cDNA insert, the cells respond by extending processes. These processes grow to be up to ~300 μm in length, and are similar to neuronal axons in their light microscopic appearance. Process elongation occurs principally over the second and third days postinfection, after which the cells begin to die from toxic effects of the virus. However, during the second and third days, the cells appear to be quite healthy based on their light microscopic appearance. All of the cells within the population become infected; however, the levels of tau vary significantly among the cells in the infected population, and accordingly, there is variability among cells with regard to the vigor with which they extend processes. The vast majority (>90%) of cells extending processes appear to extend only a single process, but caliber varies significantly from process to process, ranging from <0.15 μm to >1.5 μm. These same results are obtained when using viruses containing either the 3 or 4 repeat form of human tau (see Knops et al., 1991 for additional details). Likewise, in the present

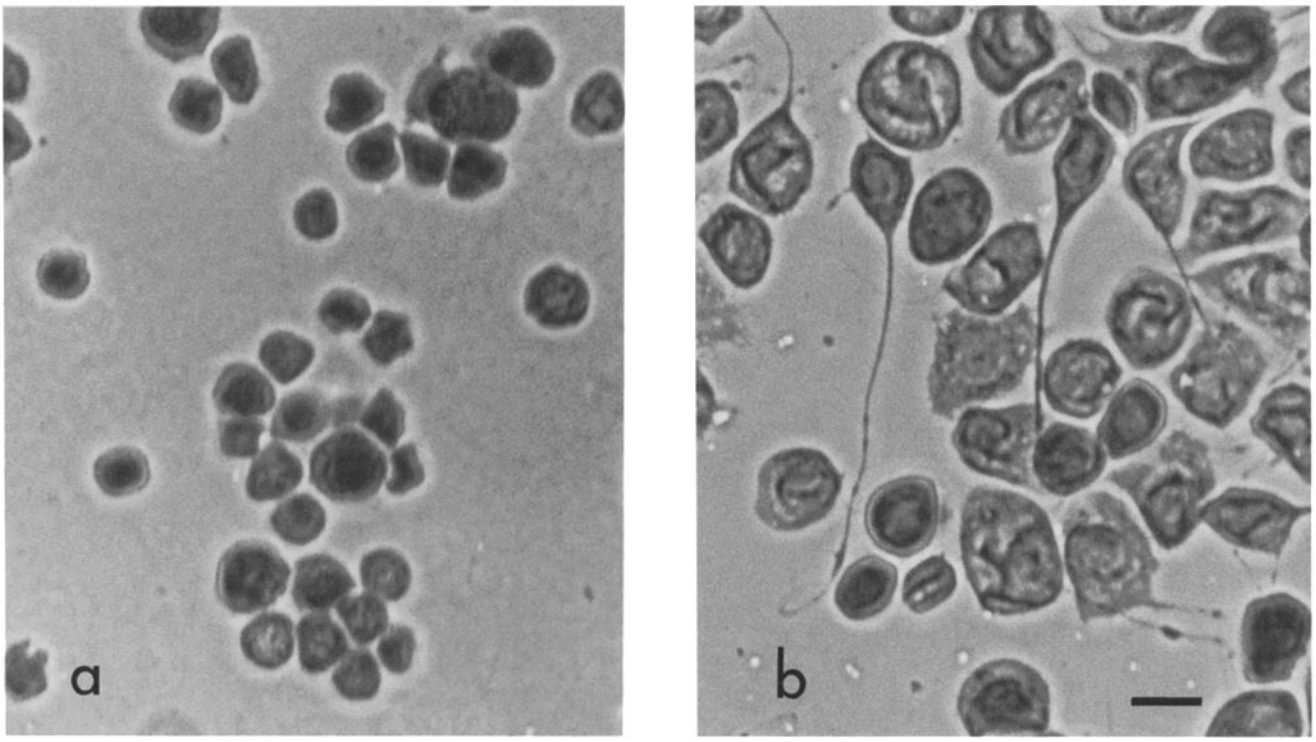

Figure 1. Phase–contrast micrographs of insect ovarian Sf9 cells. *a* shows wild type cells, while *b* shows cells 2-d postinfection with a virus containing a tau human cDNA insert (4 repeat form). The wild type cells are rounded, while the cells induced to express tau in this manner have extended processes that are similar in appearance to neuronal axons. See text and Knops et al. (1991) for more details. Bar, 20 μm.

study on MT organization in these cells, entirely similar results were obtained in parallel experiments using both forms of tau. For consistency, all of the figures presented in this article are derived from studies on the 4 repeat form.

Microtubule Organization in the Tau-induced Processes

Knops et al. (1991) previously reported that the processes extended by Sf9 cells induced to express tau (hereinafter referred to as tau-induced processes) contain dense arrays of MTs. In the present study, we have extended this observation by analyzing more comprehensively the organization of MTs within the Sf9 cells induced to express tau. Specifically, we used EM to determine whether the tau-induced processes share essential features of MT organization with the axon. For each of the electron microscopic studies reported in this study, unless otherwise noted, a minimum of 10 different cells infected with the 4 repeat form of tau and a similar number infected with the 3 repeat form were examined. Fig. 1 shows a phase–contrast micrograph of Sf9 cells fixed with glutaraldehyde and embedded for EM as described in Materials and Methods. Panel *a* shows the wild-type cells, while panel *b* shows cells 2 d after they were induced to express the 4 repeat form of tau. The wild-type cells are rounded with no processes, while most of the tau-induced cells have extended long processes. Prominent in the figure are at least two very robust processes with diameters greater than 1.0 μm. In addition, many processes are much finer than this, and these are barely visible at the light microscopic level. Unless otherwise noted, similar results on the broader and finer processes were obtained throughout our studies.

Fig. 2 shows electron micrographs of longitudinal sections through four different tau-induced processes. In these, and in all of the other processes examined, dense MT bundles were prominent. The number of MTs and the spacing between MT profiles (that is, the tightness of the bundle) observed in the longitudinal sections varied among the processes we observed. In most processes, the MTs within the array appeared to fill the available space in the process together with other organelles and cytoplasm (Fig. 2 *a*), with an appearance roughly similar to what has been observed in the axons of cultured neurons (see for example Yamada et al., 1971; Bartlett and Banker, 1984; Baas and Heidemann, 1986; Baas et al., 1987*b*; Baas and Black, 1990). In other processes, the MT bundle filled the available space, but with a packing density substantially higher than in axons, to the near exclusion of other organelles (Fig. 2 *b*). In still other processes, we observed a very tight bundle of MTs occupying one portion of the process, surrounded by regions containing somewhat less tightly packed MTs (Fig. 2 *c*). A small number of processes contained a single, unusually tight bundle of MTs surrounded by regions almost completely devoid of MTs. Fig. 2 *d* shows a particularly dramatic example of one of these latter processes; the process is relatively straight, and yet contains in its central region a tight bundle of MTs which curves through the length of the process without loosing the integrity of the MT bundle.

Because longitudinal sections provide only a qualitative sense of the density of MTs in the tau-induced processes, we undertook cross-sectional analyses so that MT number and spacing in the processes could be quantified. MT numbers were counted in the cross-sections, and MT–MT distances

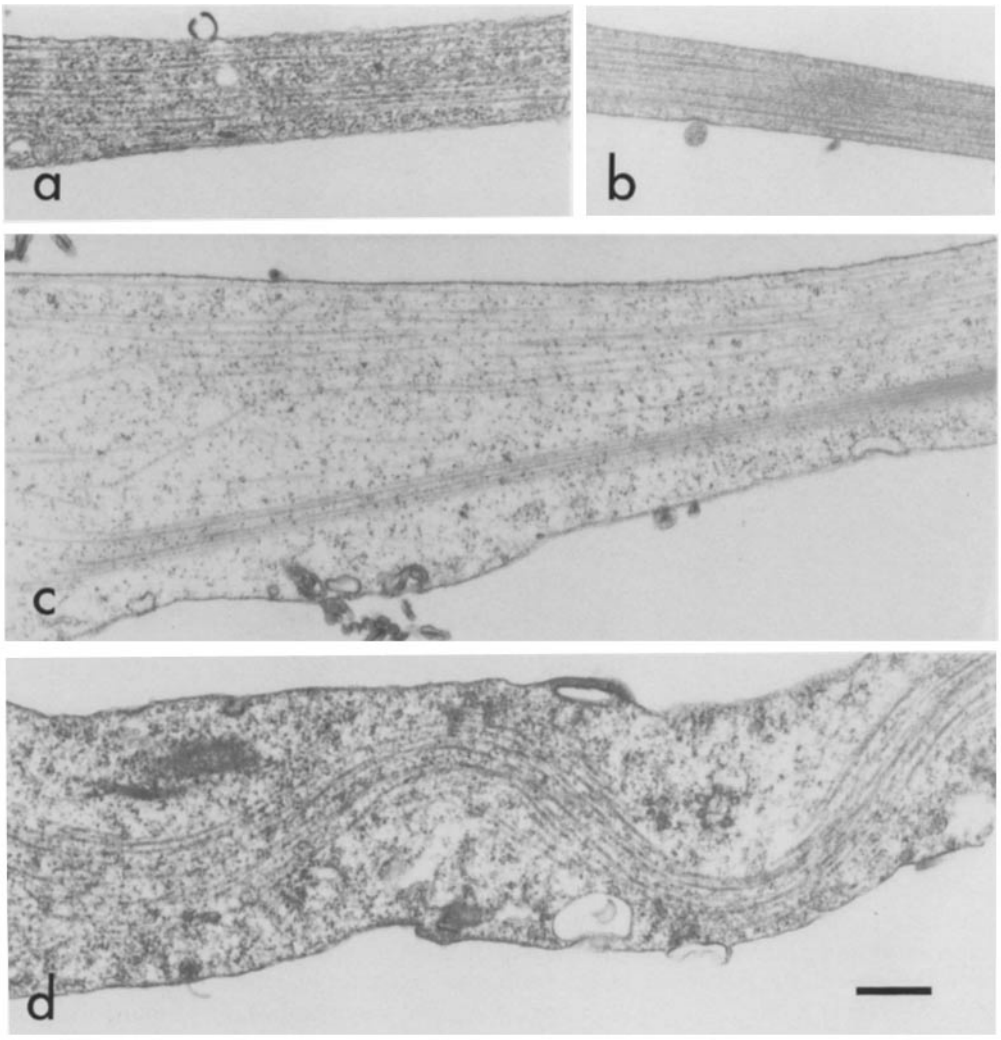

Figure 2. Transmission electron micrographs of longitudinal sections through tau-induced processes. Caliber, MT number, and MT spacing vary among processes. *a* shows a process in which the MT array fills the available space with an appearance roughly similar to the situation in the axon. *b* shows a process of similar caliber, but with a substantially higher packing density of MTs. *c* shows a process with a tight bundle of MTs occupying one portion of the process, surrounded by regions containing less tightly packed MTs. *d* shows a process with a very tightly packed bundle of MTs surrounded by regions almost devoid of MTs. The process is relatively straight, and yet contains a tight bundle of MTs which curves through the length of the process without losing the integrity of the MT bundle. Bar, 0.5 μm.

were calculated by a method similar to that of Sasaki et al. (1983; see also Stevens et al., 1988), except that our measurements were taken edge-to-edge as opposed to center-to-center. The distances between neighboring MTs were measured and averaged for each of the processes, and from these averages, a mean ± SD was calculated for the group. Consistent with our findings on longitudinal sections, we observed variability in both MT number and spacing in the tau-induced processes. However, there was a much higher degree of variability in MT number than in packing density, with the thinner processes possessing significantly fewer MTs than the broader processes. In random cross-sections, the thinner processes contained as few as three to four MTs, while the broader processes contained hundreds of MTs. By comparison, the axons of cultured sympathetic neurons contain on average ~10 MT profiles per cross-section (see Baas et al., 1991). The average spacing between MTs was 20 ± 6 nm for the 4 repeat form of tau and 21 ± 4 nm for the 3 repeat form (no statistical difference between them; $n = 10$). By comparison, axonal MTs are generally spaced about twice this distance apart (see Stevens et al., 1988), although this varies somewhat among different kinds of axons (see also Wuerker and Kirkpatrick, 1972; Bray and Bunge, 1981; Bartlett and Banker, 1984). These considerations indicate that the tau-induced processes contain MT bundles that are at least as tightly packed as the MT bundles found in axons, and that are most typically, and on average, significantly more tightly packed than those in the axon.

Microtubule Organization At the Tips of the Tau-induced Processes

In the course of our studies on the tau-induced processes, we analyzed the tips of these processes for specializations similar to those observed on the tips of axons. The distal tips of growing axons are marked by specializations called growth cones, which are essential for the growth and guidance of the axon (for review see Bray and Hollenbeck, 1988). Growth cones generally have a flattened palmate morphology, and extend many fine filopodia. However, depending on the type of neuron and the type of substrate on which it is growing, the morphology of the growth cone can be more or less dramatic. Thus while the distal tips of the tau-induced processes do not have dramatic growth cones judged by light microscopic criteria (Fig. 1 *b*; see also Knops et al., 1991), this does not rule out the possibility that they may have less dramatic but nevertheless functional growth cones at their tips. Ultrastructural observations provide some support for this

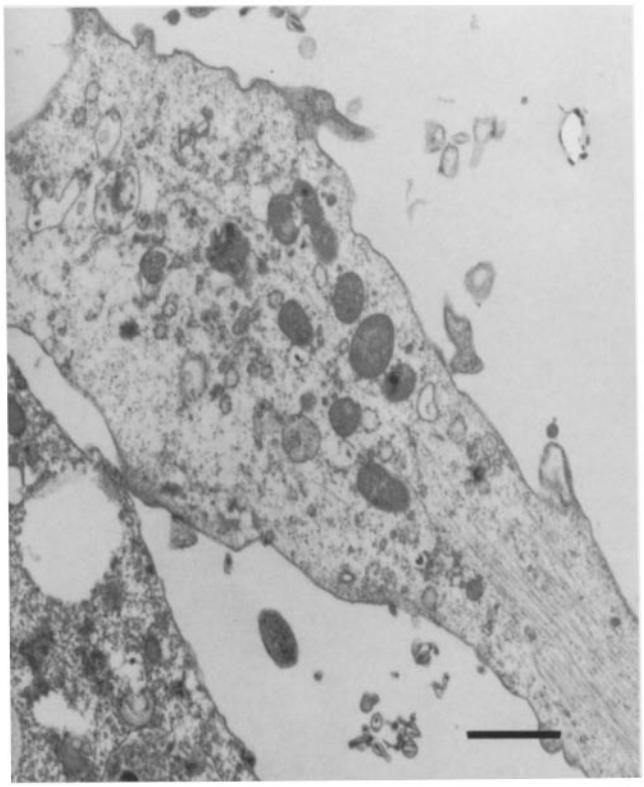

Figure 3. Transmission electron micrograph of the distal tip of a tau-induced process. Similar to the situation in axons, the vast majority of the MTs end short of the tip, leaving an expanded distal region filled with membranous elements. Bar, 1.0 μm.

conclusion. Axonal MTs generally terminate at the base of the growth cone, with only a very few MTs extending into the more flattened spread region (Bunge, 1973; Cheng and Reese, 1985; Sinclair et al., 1988; Bridgman and Daily, 1989). This paucity of MTs is thought to be important in permitting the motile functions of the growth cone (Bray et al., 1978; Joshi et al., 1986). Similar to the situation in axons, the MTs in the distal region of the tau-induced processes end short of the tip, leaving an expanded distal region filled with membranous elements (Fig. 3). However, as with light microscopy, no unambiguous filopodia or lamellipodia could be observed on the tips of the processes observed at the electron microscopic level. Thus, based on the present observations, it is impossible to conclude with confidence whether or not the tau-induced processes have modest growth cones at their tips.

Microtubules Are Discontinuous along the Tau-induced Processes

To determine whether MTs stop and start along the length of the tau-induced processes, we used an abbreviated version of the method used by Bray and Bunge (1981) in their studies on the axon. For these studies, we analyzed cross-sections along the length of tau-induced processes at distances roughly 20 μm apart. We reasoned that if the MTs were continuous from the cell body into the processes, then the number of MTs observed in each cross-section should be the same or decrease with distance from the cell body. However, if the MTs were discontinuous, stopping and starting along the length of the processes, we should be able to detect increases and decreases in the number of MTs at different sites along the length of the process. We observed decreases but not increases in MT number along the length of processes that were shorter than 100 μm. Fig. 4 *a* summarizes our findings on one such process, in which we found, proximal-to-distal, 190, 174 (shown in Fig. 4 *b*), 133, 78 (Fig. 4 *c*), 59, 30, and 19 MTs over a distance ranging from near the cell body to near the distal tip. In processes over 100 μm in length, we clearly observed both stopping and starting. Fig. 4 *d* summarizes our data on one of these longer processes, in which we found, proximal-to-distal, 205, 198, 192 (shown in Fig. 4 *e*), 209, 192, 209 (Fig. 4 *f*), 244 (Fig. 4 *g*), and 23 MTs. This means that between the first two sections, at least 7 MTs end; between the second and third sections, at least 6 MTs end; between the third and fourth sections at least 17 MTs start; between the fourth and fifth sections, at least 17 MTs end; and so forth. Because we did not perform complete serial reconstructions, we cannot resolve whether or not the discontinuity of the MT array is even greater (more stopping and starting) than indicated by these data. Nevertheless, our results clearly indicate that MTs stop and start at multiple sites along the lengths of the tau-induced processes, at least after they grow to exceed 100 μm in length.

Microtubule Polarity Orientation in the Tau-induced Processes

Of particular importance to the present studies is the polarity orientation of MTs in the tau-induced processes. In the axon, MT polarity orientation is uniform, with the plus ends of the MTs directed away from the cell body (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987*a*, 1988, 1989, 1991). To determine whether this feature of MT organization is shared by the tau-induced processes, we used the standard "hook" procedure for MT polarity determination (Heidemann and McIntosh, 1981; see Baas et al., 1989 and Materials and Methods for more details). In this procedure, the existing MTs are decorated with lateral protofilament sheets, the curvature of which, when observed in cross-section, 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. Because of some error in this assay, MT arrays of uniform polarity orientation may show a proportion of hooks of the same curvature ranging from 90–100%, such that, for diagnostic purposes, a proportion >90% is considered an accurate indicator of uniform polarity orientation (See Heidemann and McIntosh, 1981). The extremely tight packing density of MTs in the processes (see above) resulted in more ambiguity in interpreting hooks than is typical in the axon. That is, hooks from neighboring MTs oftentimes touched, rendering their MT of origin and direction of curvature uninterpretable. In addition, the frequency of hook formation was low in many processes, presumably also a result of insufficient space between MTs. On average, <40% of the MTs in the processes were hooked, compared to ~77% in the axons of cultured neurons similarly treated for MT polarity determination (Baas et al., 1987*a*, 1988, 1989, 1991). However, it has been established that reliable results on MT polarity orientation are provided even with a very low frequency of interpretable hooks (Heidemann et al., 1984).

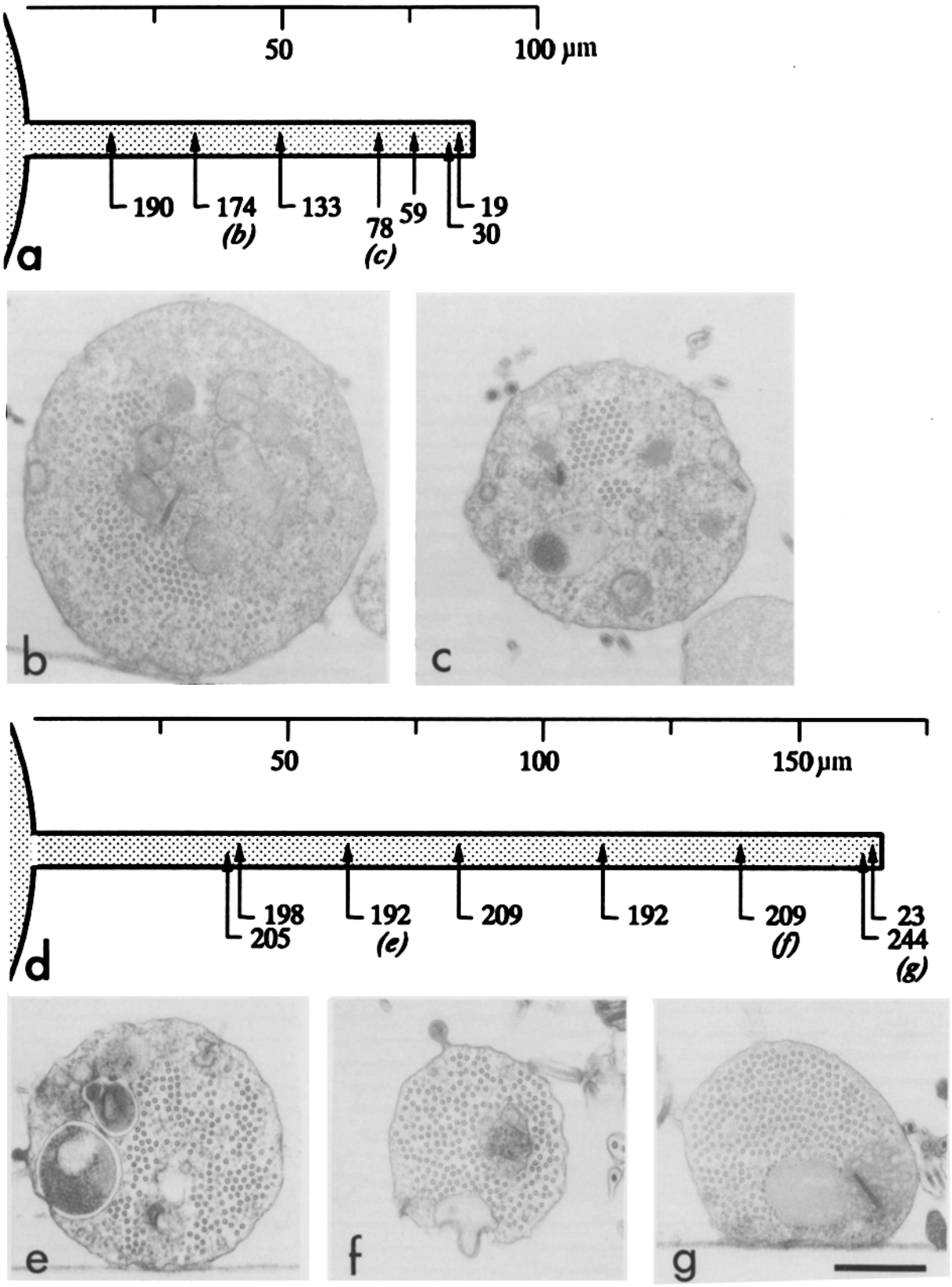

Figure 4. Analyses on MT continuity along the lengths of tau-induced processes. Cross-sections were taken at various points along the lengths of individual processes, and the numbers of MTs appearing in the cross-sections were scored. *a* and *d* schematically depict the data taken from processes shorter and longer than 100 μm, respectively. The arrows mark points at which cross-sections were taken, the numbers accompanying the arrows indicate the numbers of MTs scored at each of these points, and the scale bars accompanying the schematic indicate the distance from the cell body of the points. The electron micrographs corresponding to the points marked by letters *b*, *c*, *e*, *f*, and *g* are shown in the panels indicated by these letters, respectively. In the processes shorter than 100 μm, the data indicate that MTs stop along the length of the process, but do not resolve whether MTs start along the process as well (see text). In processes longer than 100 μm, MTs clearly stop and start at multiple sites along the length of the processes. See text for additional details. Bar, 0.5 μm.

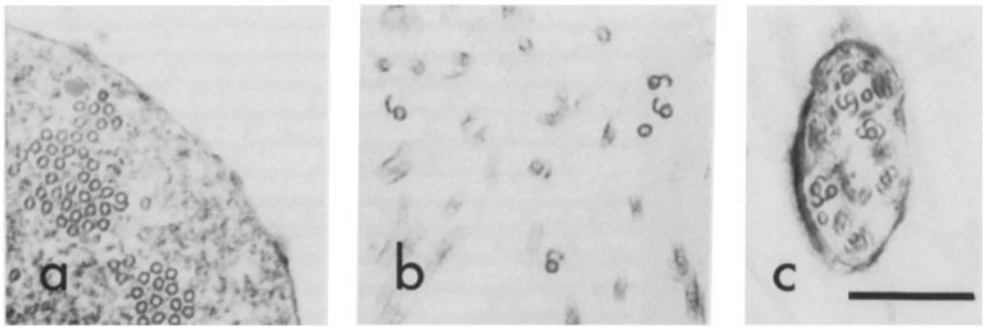

Figure 5. MT polarity orientation in tau-induced processes. MT polarity orientation was determined using the standard hooking protocol (see Materials and Methods and Results for details). *a* shows a region of a typical process with densely packed MTs. The spacing between MTs is so close that most protofilament appendages join a neighboring MT, and appear as a noncurving link between the MTs, rather than as hooks. Many other MTs show no protofilament appendages. In this region of the process, only one MT shows an interpretable hook. *b* shows a region from a process whose cell membrane was disintegrated during the lysis protocol. The MTs within the process have been moved apart during the lysis, resulting in a high proportion of interpretable hooks. *c* shows a process whose caliber, MT number, and MT spacing are similar to the axon (at least after lysis), with a high proportion of interpretable hooks. The hooks are predominantly clockwise from the vantage point of the distal tip, indicating uniform or near uniform MT polarity orientation in these processes, plus ends distal to the cell body (see text and Table I for more details). Bar, 0.25 μm.

Nevertheless, in light of the low hooking frequency, we analyzed a high number of processes (25 each for the 4 and 3 repeat forms of tau), to ensure confidence in our results. Fig. 5 *a* shows a region of a typical process with a broad caliber, tight packing density of MTs, and only a single MT with an interpretable hook in the region shown. In other processes, the plasma membrane was largely disintegrated by the lysis, and the MTs were scattered apart, resulting in a higher proportion of interpretable hooks (see Fig. 5 *b*). Finally, some processes were more similar to axons with regard to MT spacing (at least after lysis if not before), and these processes generally consisted of a rather high proportion of interpretable hooks (see Fig. 5 *c*).

Table I. Microtubule Polarity Orientation in Sf9 Cells Induced to Express Tau

	CW#	CCW#	AMB#	%HK*	%CW*
Processes (3 repeat)	161	25	352	34 ± 25	89 ± 8
Processes (4 repeat)	175	31	305	39 ± 23	89 ± 6
Cell body (3 repeat)	95	24	100	23 ± 10	83 ± 5
Cell body (4 repeat)	75	17	78	27 ± 15	82 ± 6

CW, microtubules with clockwise hooks. In the processes, curvature is viewed from the tip of the process looking toward the cell body. In the cell body, there was no common vantage point from which to view the hooks. Therefore, the hooks of common curvature that were most numerous were termed clockwise. CCW, microtubules with counterclockwise hooks. AMB, microtubules with ambiguous hooks. HK, microtubules with hooks. The expressions 3 repeat and 4 repeat refer to the specific form of tau that the cells were induced to express (see text).

# Indicates sum of all MTs for all 25 samples of each type analyzed.

* Mean ± standard deviation for all 25 samples of each type scored.

MT polarity orientation was determined using the standard hook technique (see Materials and Methods and Results). Because, in this technique, ≥90% of hooks curving in a common direction indicates uniform MT polarity orientation (Heidemann and McIntosh, 1981), these results indicate that MT polarity orientation is uniform or nearly uniform in the processes, plus ends distal to the cell body. Interestingly, when the 10 processes that were, on a qualitative level, most similar to axons in terms of caliber, MT number, and MT spacing were considered apart from the others, the %CW hooks was slightly higher (91 ± 5%), unequivocally indicating uniform MT polarity orientation as judged by the hook technique. In the MT bundles present in the cell body, MTs are of near, but certainly not absolute, uniform polarity orientation. See Text for details.

In the 25 tau-induced processes from experiments with each of the 4 and 3 repeat forms of tau, 89 ± 6% and 89 ± 8%, respectively, of the hooks were clockwise as viewed from the vantage point of the distal tips of the processes (see Table I). The fact that the proportion of clockwise hooks is very close to 90% indicates that MT polarity orientation in the tau-induced processes is either uniform or nearly uniform, with plus ends distal to the cell body. The 50 processes sampled were chosen randomly and consisted of processes with a wide range of calibers, MT numbers, and MT densities. Interestingly, when we analyzed data taken from the 10 processes that, on a qualitative level, were most similar to axons with regard to these criteria (see Fig. 5 *c*), the proportion of clockwise hooks was somewhat higher, 91 ± 5%, sufficiently high to conclude that these processes contain MTs of uniform polarity orientation. The remaining 40 processes, considered apart from these 10, contained 88 ± 5% clockwise hooks, still indicating uniform or near uniform MT polarity orientation in these processes.

Microtubules in the Tau-induced Processes Are Not Organized by a Centralized Nucleating Structure

Studies on the axon hillock region of the neuron indicate that MTs in the axon do not emanate from a single discrete nucleating structure in the cell body (see Introduction). Instead, they either begin fairly abruptly in the proximal region of the axon (Baas et al., 1987*b*) or coalesce into the axon from multiple points in the cell body (Bartlett and Banker, 1984; Stevens et al., 1988). To determine whether the MT bundles in the tau-induced processes emanate from any cytological structure in the cell body that might influence their organization, we analyzed longitudinal sections in the hillock region between the processes and the cell bodies. Because potential nucleating structures may be present, but not apparent in each thin section, multiple thin sections (>90% of the sections containing the hillock) were examined for each region analyzed. Fig. 6 shows a region of a wild-type Sf9 cell, while Fig. 7 shows four examples of cell bodies of Sf9 cells induced to express tau, each with a hillock region of a process. The wild-type cells contain MTs, but they do not appear in tight bundles; they are separated by distances greater than many MT diameters, although they sometimes run roughly parallel to one another near the cell membrane.

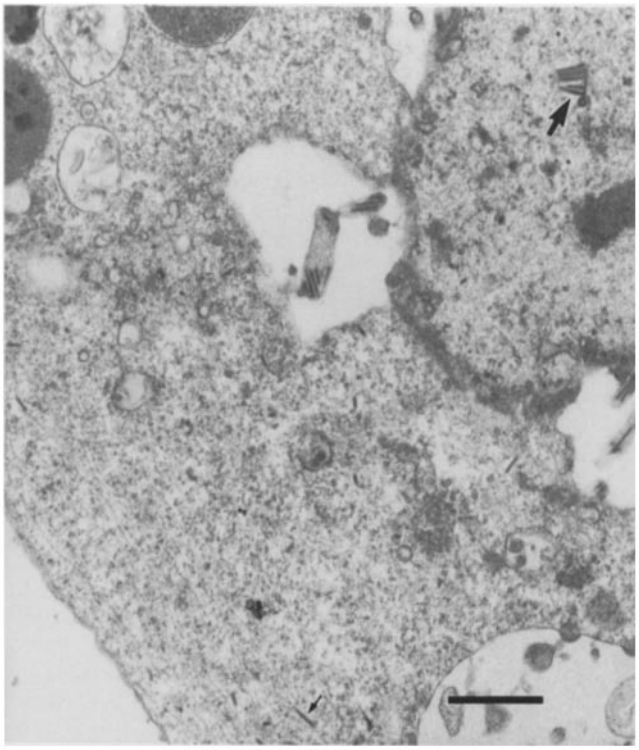

Figure 6. Transmission electron micrograph of a region of a wild-type Sf9 cell infected with the virus not containing the cDNA insert for tau. MTs are present, and some are aligned roughly parallel to one another near the cell periphery, but they do not appear in tight bundles. That is, the MTs are separated by distances greater than many MT diameters. Also apparent, and indicated by arrows, are electron-dense organelles similar in appearance to a type of secretory organelle found in oocytes (Adams and Hertig, 1964). The origin and function of these organelles are unknown. Bar, 1.0 μm.

In contrast, in the infected cells, distinct MTs bundles are prominent, not only in the hillock region extending into the processes, but also throughout the cell body.

We initially examined the MT bundles in the hillock region of the infected cells. In each cell examined, MTs were continuous between the processes and the cell body. In all cases, the integrity of the MT bundle was at least partially maintained in this region of the cell body near the hillock. In some cases, thick bundles of MTs continued directly into the cell body without curving (Fig. 7 *a*), while in other cases, the MT bundle curved either in one direction (Fig. 7 *b*) or two (Fig. 7 *c*). However, in no case did the MT bundle fan out in the cell body such that the bundle lost its integrity. These observations indicate that the bundling of MTs in the hillock region of these cells is dramatically tighter than the bundling of MTs in the axon–hillock region of the neuron. Nevertheless, similar to the MT bundles in the axon, in no case could the MT bundles in the tau-induced processes be traced to any cytological structure in the cell body that might serve as a MT-nucleating structure.

As noted above, the cell bodies of the infected cells also contained MT bundles which seemed to bear no particular relationship to the MT bundles in the processes. This observation suggests that expression of high levels of tau protein results directly in the bundling of MTs, independent of their transport into cellular processes, and confirms previous results of this type on fibroblastic cells (Kanai et al., 1989). Like the MT bundles in the processes, the MT bundles in the cell bodies varied in their appearance. Most bundles were rather short relative to the diameter of the cell body, stopping and starting abruptly in the cytoplasm (see especially Fig. 7 *a*). Sometimes, two bundles crossed in the region of the hillock, one bundle leading into the process and the other simply terminating in the cytoplasm (Fig. 7 *d*). Like the MT bundles in the processes, in no case could any of the bundles in the cell body be traced to any cytological structure which might serve as a MT-nucleating structure. Collectively, our observations indicate that MT bundling in the infected cells is accentuated above and beyond that typically observed in nerve cells.

Microtubule Polarity Orientation in the Cell Bodies of Sf9 Cells Induced to Express Tau

The MT bundles present in the cell bodies of the infected cells offered a fortuitous avenue for us to begin to address whether the bundling properties of tau can directly account for the genesis of uniform MT polarity orientation. In the processes, it seems plausible that other factors such as the directional preference of MT transport may play a role in determining MT polarity orientation. These factors may not play a role in orienting MTs in the cell body bundles because these MTs are not being transported down a process (but see Discussion for further considerations). Because of our inability to visualize the MT bundles in the cell body at the light microscopic level, it was impossible to orient our block in such a way as to intentionally section cell body bundles in cross-section. Therefore, for these studies, we analyzed sections of cells chosen at random taken perpendicular to the culture dish, searching for bundles of hooked MTs taken in cross-section. Although the precise effects of the lysis on MT spacing are unclear (the lysis itself probably moving MTs apart and the hypertonicity of the hooking buffer having the opposite effect on the cell bodies, whose membranes are only partially lysed in our mild extraction protocol), it was our strong impression that the spacing between MTs was greater in lysed cells than in unlysed cells. Thus, we defined a bundle as including MTs spaced no greater than 150 nm from their nearest neighbor, several times the distance between MTs in unlysed cells. Although somewhat arbitrary, the selection of this broad distance permitted a particularly conservative test of uniformity among the MTs with regard to polarity orientation because neighboring MTs outside of the bundle would not necessarily be expected to have the same polarity orientation as MTs within the bundle. Using this method, we located 25 bundles each from cells induced to express the 3 or 4 repeat forms of tau, and scored the number of hooks turning in one direction versus the number turning in the other direction. Similar to our studies on the processes, the frequency of interpretable hooks was low, prompting us to score this relatively large number of samples. Because there was no common vantage point from which to label the hooks as clockwise or counterclockwise, the hooks of common curvature that were most numerous were arbitrarily termed clockwise.

The mean $\pm$ SD of clockwise hooks for the 4 and 3 repeat forms of tau were 82 $\pm$ 6% and 83 $\pm$ 5%, respectively (see Table I; Fig. 8), indicating near but certainly not absolute uniformity in MT polarity orientation. Examination of the hooking pattern in individual MT bundles revealed no directional preference of plus or minus ends of MTs toward either

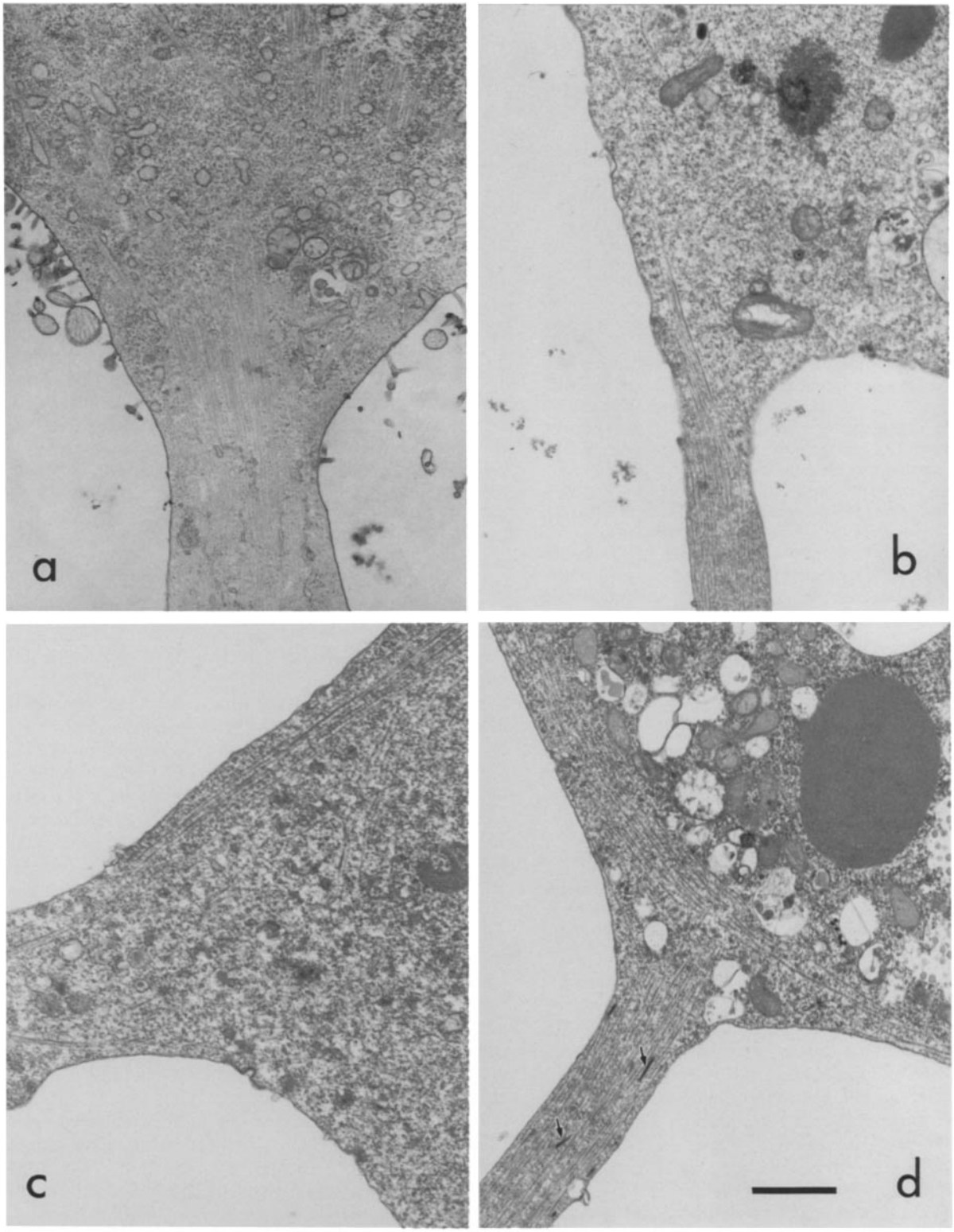

Figure 7. Transmission electron micrographs of hillock regions of Sf9 cells induced to express tau. Distinct MT bundles are present in the processes, and also in the cell bodies. In *a*, the MT bundle of the process continues into the cell body without curving. In *b*, the MT bundle curves in one direction as it enters the cell body, while in *c*, the bundle curves in two directions. In *d*, a second MT bundle appears in the hillock region, crossing roughly perpendicular to the MT bundle entering the process. Interestingly, in *d*, the electron-dense organelles discussed in Fig. 6 are present in the process, aligned relative to its long axis, as if they may be translocating down the process (*arrows*). Bar, 1.0 μm.

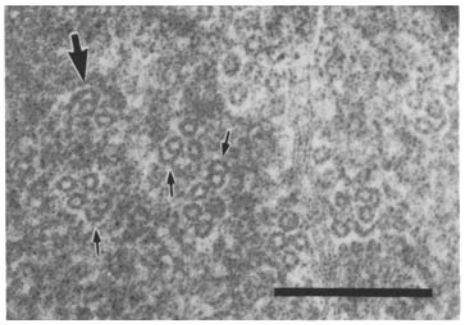

Figure 8. Polarity orientation of MTs within the MT bundles present in the cell bodies of Sf9 cells induced to express tau. MT polarity orientation was determined by the standard hook protocol (see Materials and Methods and Results). Because the precise effects of lysis on MT spacing are unclear, and in order to be particularly conservative in the treatment of our data, MT bundles in these preparations were defined as groups of MTs spaced no greater than 150 nm from their nearest neighbor (see Results for more details). Because there was no common vantage point from which to judge MTs as clockwise or counterclockwise, the highest number of hooks turning in a common direction were arbitrarily termed clockwise. In the region shown, three MTs have clockwise hooks (*small arrows*), one MT has a counterclockwise hook (*large arrow*), and the remaining hooked MTs are ambiguous. The mean ± SD for the 3 and 4 repeat forms of tau were 83 ± 5% and 82 ± 6%, respectively, indicating near, but certainly not absolute, uniform polarity orientation of MTs within the bundles (see Table I and text). Bar, 0.25 μm.

the cell center or periphery. Collectively, these results suggest that tau bundles MTs preferentially, but probably not exclusively, with the same polarity orientation. However, additional factors are apparently required to orient MTs with their plus ends specifically pointing away from the cell body during process elongation (see Discussion).

Discussion

We have used an indirect approach to investigate the role of tau in the establishment and maintenance of MT organization in the axon. Tau is a MAP which is concentrated in the axon, and which has been implicated in the nucleation, assembly, stability, and bundling of MTs (see Introduction). When normally rounded insect ovarian Sf9 cells are induced to express tau, they extend long cellular processes which are similar in appearance to axons, and which contain dense bundles of MTs (Knops et al., 1991). Our results demonstrate that the organization of MTs within these processes is similar, but not identical, to the organization of MTs within the axon. On average, the MTs within the tau-induced processes are more numerous and more tightly packed than the MTs within the axon. However, similar to MTs in the axon, the MTs in the tau-induced processes do not emanate from a discrete nucleating structure in the cell body, and are discontinuous along the length of the processes. In addition, MTs in the tau-induced processes are uniform or nearly uniform in their polarity orientation, plus ends distal to the cell body. Collectively, these results indicate that under the cellular conditions present in Sf9 cells, the addition of tau is sufficient to induce the generation of MT arrays with an organization similar to those in the axon. Based on this observation, it seems reasonable to conclude that tau may play a central role in the establishment and maintenance of MT organization in the axon.

The Role of Tau in Microtubule Organization

In cells induced to express tau, MT bundling is accentuated above and beyond that normally observed in neuronal or nonneuronal cells (see also Kanai et al., 1989). In the test tube, individual tau molecules can form cross-bridges along the lengths of neighboring MTs, with each cross-bridge consisting of a projection domain that is ~20 nm in length (Hirokawa et al., 1988; but also see Black, 1987). The MT bundles in cells induced to express tau also contain MTs spaced ~20-nm apart, suggesting that neighboring MTs in these cells may also be bound by cross-bridges consisting of single tau molecules. Notably, the average distance between MTs in the axon is substantially greater, roughly twice that observed in the tau-induced processes. This fact is not inconsistent with the notion that tau determines the spacing between axonal MTs. The tau molecule is highly dynamic in terms of its shape, and can, for example, elongate to almost twice its original length when phosphorylated (Hagestedt et al., 1989). Furthermore, a growing body of evidence suggests that the cross-bridges between cytoskeletal elements in the axon are transient, forming and breaking continuously at different points along the MTs (for discussion see Lasek, 1988). Collectively, these observations implicate tau in the formation of a coherent but spatially dynamic MT bundle in the axon, and in determining the spacing of MTs within this bundle.

Another fundamental issue with regard to the properties of tau is whether the molecule has a role in the establishment or maintenance of the uniform polarity orientation of MTs in the axon. For example, might there be a stereospecificity by which tau binds to a MT such that it exclusively or preferentially binds other MTs of the same polarity orientation? The observation that tau expression in Sf9 cells induces the formation of MT bundles of near uniform polarity orientation, both in cell bodies and processes, suggests that this might be the case. However, several considerations indicate a need for caution in interpreting this observation. First, our studies have dealt with living cells, rather than isolated tau molecules and MTs. Living cells contain a myriad of other factors which could also contribute to the regulation of MT polarity orientation. For example, one possibility is that an additional factor conspires with tau to generate uniform MT polarity orientation, and that this factor is present in Sf9 cells, but not in fibroblasts, accounting for the fact that fibroblasts induced to express tau do not extend processes (Kanai et al., 1989). The requirement for additional factors affecting MT polarity orientation is clear in the case of the processes, wherein MTs are not only nearly uniform with regard to polarity orientation, but their organization is consistently plus-end-distal as opposed to minus-end-distal. Thus, while tau may bundle MTs preferentially with the same polarity orientation, at least one additional factor is necessary to specifically orient the plus ends of the MTs toward the cell periphery during process elongation.

One possibility with regard to such a factor is that MT polarity orientation is, in part, determined by the transport machinery required to elaborate the MT array of the axon. A long-standing hypothesis has been that MTs are trans-

ported down the axon as the assembled polymer (Lasek, 1982), and recent experimental evidence strongly supports this view (Reinsch et al., 1991). Based on these findings, it seems reasonable that MT transport may be unidirectional with regard to the polarity of the MT. That is, MTs may be transported down the axon exclusively with plus ends leading. Thus, it is possible that the polarity orientation of the MTs within the axon results from their transport properties, and not the bundling properties of tau. However, our studies indicate that expression of tau in Sf9 cells also induces MT bundles of near uniform polarity orientation in cell bodies, and these MTs are clearly not moving unidirectionally down a process. Nevertheless, given the paucity of information on MT transport, one cannot dismiss the possibility that MT transport mechanisms might be functioning in cell bodies as well as processes. For example, MTs may be moving randomly through the cytoplasm of the cell body until they align with other MTs of the same polarity orientation, after which the MTs can be bundled by tau. One such bundle may translocate and elongate down a process, initiating an axon-like MT pattern in the growing process. The specialization of a single bundle with this capacity, although hypothetical at present, is consistent with our observation that Sf9 cells induced to express tau generally produce a single process, and may relate to the fact that neurons generally produce only a single axon (and several dendrites).

Another mechanism by which tau might regulate MT organization in the axon is via MT stabilization. As noted in the Introduction, especially stable MTs provide the exclusive sites for new MT assembly in the axon (Baas and Heidemann, 1986), and as such serve as individual nucleating structures that spatially regulate MT dynamics in the axon (Baas and Black, 1990). The capacity of stable MTs in the axon to perform in this manner is dependent upon their assembly competence, a property not generally shared by stable MTs in nonneuronal cells (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). The assembly competence of stable MTs in the axon (specifically at their plus ends; see Baas and Black, 1990) indicates that they are stabilized by a mechanism other than an end-cap, presumably the binding of accessory proteins along the length of the MT. The enrichment of tau in the axon, its ability to promote MT assembly and nucleation in the test tube, and its apparent capacity to stabilize MTs in living cells (see Introduction) render it an attractive candidate in this regard. Additional support is provided by our results indicating that expression of tau can permit nonneuronal cells to assemble relatively long MTs that are "free" at both ends, and yet are apparently not prone to catastrophic disassembly. Another possibility is that tau may not play a direct role in MT stabilization, but may function principally as a MT elongator, endowing stable MTs in axons with assembly competence. Current efforts are aimed at identifying the factor(s) which account for the unique stability properties of MTs in the axon.

The Role of Tau in Neuronal Polarity

The present studies on tau represent part of our continuing efforts to elucidate the factors which generate and maintain the fundamental polarity of the neuron. Vertebrate neurons typically extend two distinct types of processes, axons and dendrites, which differ in structure and function (for review, see Lasek, 1988; Black and Baas, 1989). Dendrites, like axons, contain a discontinuous MT array that does not emanate from a discrete nucleating structure in the cell body (Sasaki et al., 1983). However, unlike axons, dendrites contain roughly equal numbers of MTs of each polarity orientation (Baas et al., 1988, 1989, 1991; Burton, 1988). Axons and dendrites also differ in MAP composition; axons are enriched in tau, while dendrites are enriched in MAP-2 (for review see Matus, 1988). In light of our present results, it is intriguing to contemplate that MAP-2 may cross-link MTs of opposite polarity orientation, while tau bundles MTs of the same polarity orientation. However, available information indicates that the relationship between MAP composition and MT polarity orientation is more complex. This is exemplified by our previous discussion indicating that factors in addition to tau, regardless of its bundling properties, would be required to account for MT polarity orientation in the axon. In addition, it is pertinent to note that both MAP-2 and tau are present in the primordial processes that give rise to both axons and dendrites (Dotti et al., 1987; Kosik and Finch, 1987), and yet these primordial processes contain MTs of uniform polarity orientation (Baas et al., 1989). In a related study, Caceres and Kosik (1990) reported that the introduction of tau antisense prevents axonal development, but has no noticeable effect on the extension of the primordial processes, raising the possibility that MT polarity orientation in these processes may be uniform even in the absence of tau. Clearly, more information is needed to elucidate the relationship between MAP composition and MT polarity orientation. As next steps toward addressing this issue, we are currently analyzing the effects on MT organization of inducing Sf9 cells to express MAP-2, and plan to analyze MT polarity orientation in the primordial processes of neurons into which we have introduced tau antisense.

In conclusion, our studies provide strong indirect evidence favoring a role for tau in the elaboration and maintenance of MT organization in the axon. At present, the mechanisms by which tau functions as well as the co-factors with which tau interacts to accomplish this feat remain a matter of speculation. Future efforts, some of which we have mentioned here, are aimed at addressing these issues, with the larger goal of elucidating the mechanisms by which proteins such as tau contribute to defining the unique structural and functional properties of axons and dendrites.

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