Perspective

A New Perspective on Microtubules and Axon Growth

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NEURONAL cells have a complex morphology, extending most of their cytoplasm over long distances in the form of axons. The protein synthetic machinery is excluded from the axon, making the axon dependent upon the cell body for the tremendous levels of proteins required for its growth (Lasek and Brady, 1981). As a result, axon growth requires an efficient and sophisticated machinery to transport proteins from the cell body into and over great distances down the axon. In addition, axon growth is dependent upon mechanisms that permit the generation and maintenance of a highly exaggerated anisotropic morphology. These transport and architectural needs are fulfilled by the cytoskeletal elements within the axon. In particular, microtubules are prominent components of the cytoskeleton which provide structural support for the axon, and direct the transport of organelles and proteins through the axoplasm (for reviews see Black and Baas, 1989; Brady et al., 1991). For this reason, there is great interest in elucidating the mechanisms by which the axonal microtubule array is elaborated.

Addressing this issue has been challenging, due to the many complex features of the axonal microtubule array. Like microtubules in other cell types, axonal microtubules are uniformly oriented with their plus ends toward the periphery (Heidemann et al., 1981; Baas et al., 1988). However, unlike the situation in other cell types, axonal microtubules are not attached to a discrete nucleating structure such as the centrosome (Lyser, 1968), but rather are free in the cytoplasm (Bray and Bunge, 1981). These observations indicate that highly specialized mechanisms exist to establish and maintain microtubule polarity orientation in the axon, and that these mechanisms are active at significant distances from the cell body. In addition, the great lengths that axons achieve result in other unique demands on the axonal microtubule array. For example, local mechanisms in the axon must exist to ensure that sufficient numbers of microtubules can be made available to accommodate rapid morphological changes such as collateral branching or remodeling of synapses that occur in response to physiological stimuli.

Despite widespread interest and intensive study over the past several years, the mechanisms by which axonal microtubules are generated and organized remain a matter of great controversy. In fact, the wealth of available data has given rise to two rather polarized models. One model stresses the role of the cell body in generating the axonal microtubule array, whereas the other emphasizes the role of local mechanisms within the axon itself. Recent work suggests that events significant to the generation of the axonal microtubule array occur within both of these compartments, and that axon growth is dependent upon the coordinated efforts of several types of microtubule behavior. Our goal in this article is to evaluate the existing models in light of more recent data, and provide new perspectives on the manner by which the axonal microtubule array is generated.

Evidence Supporting a Role for the Cell Body

The first comprehensive model for axon growth was put forth by Lasek and collaborators over a decade ago, and was based on the kinetics of tubulin transport from the cell body into and down the axon. In these experiments, radiolabeled amino acids were injected into the cell bodies of neurons to radioactively tag the newly synthesized proteins. The movement of these proteins into and down the axon was then analyzed and quantified using a biochemical method. After various time intervals, the axon was cut into segments and the protein composition of each segment was resolved using SDS-PAGE. Then, levels of radioactivity were measured in bands corresponding to specific proteins, one of which was tubulin. These experiments showed that the movement of newly synthesized tubulin down the axon occurs as a discrete wave traveling at a rate (≈1 mm/d) that is inconsistent with diffusive movement. Moreover, the radiolabeled tubulin was not extractable under conditions that removed unpolymerized tubulin, suggesting that tubulin is transported in an assembled form. Based on these data, Lasek and collaborators proposed that microtubules destined for the axon are preassembled in the cell body, and then translocated by an active transport mechanism into and down the axon (Fig. 1 *a*). In its original form, the model held that microtubules are transported as a highly cross-linked network (Lasek, 1982), but this element of the model was subsequently refined to hold that individual microtubules translocate independently or relative to one another (Lasek, 1988).

This model is attractive because it explains how tubulin can be transported from the cell body over great distances through the ribosome-deficient axoplasm, an accomplishment inconsistent with the laws of diffusion (Lasek, 1988). In addition, the notion that microtubules themselves are the transport form of tubulin is consistent with more recent work on the molecular basis of intracellular transport. These

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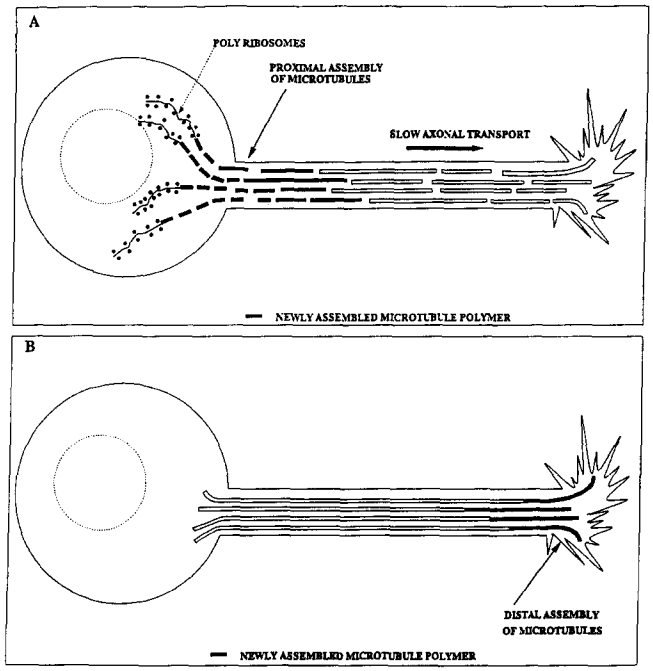

Figure 1. Schematic representations of the cell body-based and axon-based models for the elaboration of the axonal microtubule array shown in *A* and *B*, respectively. In the cell body-based model, it was proposed that newly synthesized tubulin subunits assemble to form microtubules in the cell body, and these microtubules are conveyed down the axon together with several other proteins by a process called slow transport. In the axon-based model, it was proposed that the microtubule array of the axon is elaborated by extending the distal ends of microtubules as the axon grows.

studies demonstrate that nondiffusive movements within the cell are the result of molecular motors that attach to assembled structures and move them relative to other assembled structures. Moreover, these motors have been shown to interact directly with microtubules, and create movement relative to the polarity of the microtubule (for reviews see Black and Baas, 1989; Brady, 1991). In fact, it is now well accepted that membranous organelles such as synaptic vesicles and Golgi elements are transported through the cytoplasm along microtubules specifically toward the plus or minus end, respectively. Given that all motion is relative, it is not difficult to imagine how similar mechanisms could result in movement of the microtubules themselves. Moreover, there is precedent for the capacity of molecular motors to translocate microtubules both in vitro and in vivo. For example, isolated microtubules combined with transport motors have been shown to translocate on glass coverslips (see for example Vale et al., 1985). In addition, evidence for microtubule sliding has been documented in other cell types (Satir, 1982; Koonce and Schliwa, 1986; Burnside, 1988), and it now appears that molecular motors and microtubule movements are essential for the formation of the mitotic spindle and for force generation during mitosis (for review see Fuller and Wilson, 1992).

Evidence Indicating that the Axonal Microtubule Array Is Generated Locally

A challenge to the preoccupation with the cell body came in 1986, when Bray and collaborators evaluated the potential for different regions of the neuron to participate in elaborating the axonal microtubule array (Bamburg et al., 1986). These authors reasoned that axon growth should be arrested if microtubules could be prevented from assembling at their sites of origin. To accomplish this, antimicrotubule drugs were applied with a micropipette to different regions of cultured neurons in the presence of a flowing stream of culture medium. As a result of this experimental regime, the site of interest was continuously exposed to the drug, but the drug was rapidly carried away, along with the streaming medium, before it could diffuse to other regions of the neuron. When the drugs were applied locally to the cell body, axon growth was not visibly affected over a 30-min time frame. By contrast, axon growth was immediately arrested when the drugs were applied to the growing tip of the axon. Based on these results, the authors concluded that the cell body may not be the principal site of interest with regard to the generation of the axonal microtubule array. Instead, it appeared that new microtubules may be assembled locally within the axon itself (Fig. 1 *b*).

Since these studies were reported, other evidence has emerged which supports a role for a local contribution to the generation of the axonal microtubule array. For example, immunological studies indicate that over half of the microtubule mass of the axon is rich in unmodified alpha-tubulin variants that identify newly assembled polymer within living cells (Baas and Black, 1990). This view is reinforced by observations that neurons microinjected with tagged tubulin rapidly incorporate this tubulin into microtubules along the entire length of the axon as well as within the cell body (Keith, 1987; Okabe and Hirokawa, 1988, 1989, 1992, 1993; Lim et al., 1989, 1990), a result which is consistent with previous indications that microtubules and free tubulin are in equilibrium with one another within the axon (Morris and Lasek, 1984). Finally, and perhaps most provocatively, the importance of local microtubule assembly in the axon is inescapable in light of the observation that axonal microtubules achieve lengths far exceeding the diameter of the cell body. Coupled with recent evidence that microtubule assembly in the axon occurs specifically from the plus ends of microtubules (Okabe and Hirokawa, 1988; Baas and Ahmad, 1992), and the fact that microtubule plus-ends are directed away from the cell body, this latter observation on microtubule length indicates that substantial microtubule assembly must occur within the axon itself.

Real-time Imaging of Microtubule Behavior During Axon Growth

Two main conclusions can be drawn from the observations described thus far. First, it is clear that axonal microtubules can and do elongate locally within the axon. Second, to accommodate this assembly, there must be active transport of tubulin subunits into the axon from their site of synthesis in the cell body. The suggestion by Lasek and collaborators that tubulin is transported in the form of microtubules is, however, controversial, and not the only form in which tubulin could conceivably be transported. Direct observation of microtubule movement in the axon is problematic for technical reasons; axonal microtubules are extremely long, averaging 100 μm, and tightly bundled. As a result, it is virtually

impossible to image both ends of a single microtubule, to determine whether or not it is moving. To circumvent this problem, several laboratories have recently utilized a very clever real-time imaging approach in which a narrow mark is made across the microtubule array of the axon. The behavior of this mark is then monitored over time. Two variations of this approach have been used. The initial studies used a photobleach technology, in which the microtubules were made to incorporate fluorescent tubulin after which the mark was made by bleaching with an intense beam of light. Later studies used a photoactivation technology, in which the neuronal microtubules were made to incorporate tubulin conjugated to a caged-fluorescein, which does not fluoresce until it has been activated by a beam of light. The two methods differ in contrast, with the former providing a dark mark on fluorescent microtubules, and the latter providing a glowing mark on nonfluorescent microtubules. In addition, photoactivation requires a less intense beam of light than does photobleaching, and thus may reduce the potential for photodamage.

The first photobleach studies were performed on the axon-like neurites of PC12 cells (Keith, 1987). These studies suggested proximodistal movement of the bleached zone, but were controversial because of the poor quality of the images. Later studies on PC12 cells and on avian and mammalian neurons failed to show microtubule movement (Lim et al., 1989; 1990; Okabe and Hirokawa, 1989). The first photoactivation studies, performed on *Xenopus* axons, showed very clear proximo-distal movement, and this movement occurred at roughly the same rate as the rates of slow transport previously reported by Lasek and collaborators. More recently, Okabe and Hirokawa (1992, 1993) have shown that the photoactivation and photobleach approaches both reveal microtubule movement in the axons of *Xenopus* neurons but not in the axons of mouse neurons, indicating that the differing results cannot be attributed to the use of photoactivation versus photobleach. These authors have proposed that microtubules move in *Xenopus* axons but not in mammalian axons. However, this view seems unlikely and intellectually dissatisfying in that it would demand very different mechanisms for axon growth in these animals. It is pertinent to note that in all of the experiments using this approach, whether or not microtubule movement was observed, the marked bands gradually recovered, and this was interpreted as confirmation that local microtubule assembly dynamics are active within the axon.

At present, the reasons for the differing results concerning microtubule movement are unclear, and have been the source of additional controversy, and much good-natured disagreement. The simplest possibility relates to the fact that living cells and biological molecules, particularly those conjugated to fluorescent probes, are highly susceptible to photodamage (Vigers et al., 1988; Reinsch et al., 1991). It may be that microtubule transport normally occurs in all of the axons that have been studied, but that experimental artifacts stopped the movement in those cases in which no movement was detected. It may be, for example, that *Xenopus* axons are less susceptible to photodamage than mammalian or avian axons. This possibility is supported by recent evidence showing that whether or not the photobleach technology reveals microtubule movement in the neurites of PC12 cells depends on the intensity of the beam used for bleaching (Keith and Farmer, 1993). Another possibility is that microtubule movement occurs in all cases and is not arrested by photodamage, but that the movement is simply not detected for reasons relating to contrast. For example, in some cases microtubule movement may be highly asynchronous. For the marked microtubules to be detected, it may be necessary that two or more marked microtubules are aligned with one another, and if the movement is highly asynchronous, this alignment may never occur. However, the marked zone would gradually recover as marked microtubules move away from the zone. If this is correct, then the recovery that is consistently observed would not be due entirely to microtubule assembly dynamics, but also in part because of this asynchronous microtubule movement. Still another possibility is that microtubules may be moving or stationary within the axon at different stages of its development, or depending on local demands for additional tubulin subunits or microtubule polymer. That is, the differing results may reflect different but normal stages in axon growth where microtubule movement may be more or less active.

While additional efforts will be required to resolve the controversy surrounding these results, there is an additional line of reasoning that argues that the studies which fail to show movement are problematic. As noted above, there is no question that tubulin must be actively transported down the axon, whether in the form of microtubules or in some as yet unidentified form. Results that fail to show any movement whatsoever therefore fail to show movement that is known to exist. Thus, the usefulness of the negative results in addressing whether or not microtubules move in the axon is questionable.

The Origins of Axonal Microtubules

All of the studies described in the previous sections indicate that the mechanism by which the axonal microtubule array is elaborated is complex, and involves the coordinated efforts of several types of microtubule behavior. To better understand how the axonal microtubule array is elaborated, we have attempted to dissect apart these various behaviors, study their individual contributions, and then incorporate available data into a composite model. A principal focus of our efforts has been to determine the sites of origins for axonal microtubules. As one step toward accomplishing this, we determined the distribution in the neuron of gamma-tubulin, a newly discovered member of the tubulin superfamily (Oakley and Oakley, 1989) shown to be essential for the formation of microtubules in all eucaryotic cell types examined (see for example Joshi et al., 1992). If gamma-tubulin is also required for the formation of new microtubules in the neuron, then its distribution will reveal the sites where microtubule formation occurs. Our studies indicate that, in neurons, this protein is restricted to the pericentriolar region within the cell body, and is not present anywhere else in the cell body, nor in the axon (Baas and Joshi, 1992). Thus, if neuronal microtubules require gamma-tubulin for their formation, the centrosome is the site where new microtubules form for the entire neuron. If this is correct, then microtubules destined for the axon must be released from the centrosome within the cell body and then transported into and down the axon.

Interestingly, a centrosomal origin for axonal microtu-

bules is not a new hypothesis, and was originally proposed long before gamma-tubulin was discovered. As early as 1965 before "spindle tubules" and "neurotubules" were both identified as "microtubules," Gonatas and Robbins (1965) examined the lattice structure of neurotubules in chick embryo retina, found it to be indistinguishable from that of spindle tubules, and concluded that "neurotubules probably arise from the centrioles." Similarly, in ultrastructural studies on rabbit embryo dorsal root ganglion neuroblasts, Tennyson (1965) concluded that neurotubules "probably originate from the centriole . . ." and "migrate into the neurite." These early speculations based on microtubule lattice structure are supported by more recent studies on microtubule protofilament number and centrosomal nucleation. In studies performed in vitro, microtubules assembled de novo tended to vary in protofilament number, while those nucleated from the centrosome consistently displayed a 13-protofilament lattice (Evans et al., 1985), the same number of protofilaments as axonal microtubules (Tilney et al., 1973). Although there is some question as to whether these results can be applied to living cells (see for example Mogensen et al., 1989), these observations on microtubule lattice structure provide an independent line of evidence favoring a centrosomal origin for axonal microtubules.

The notion that the cell body is the site of origin for axonal microtubules is also supported by studies on the effects of reversible microtubule depolymerization in the axon. When reversible antimicrotubule drugs are used to partially depolymerize axonal microtubules, the depolymerized polymer rapidly repolymerizes upon removal of the drug. However, when all of the microtubule polymer is depolymerized, no microtubule reassembly occurs after drug removal (Baas and Heidemann, 1986). The results of more recent studies offer a clear explanation for these observations. Using markers of newly assembled microtubule polymer, Baas and Ahmad (1992) demonstrated that all newly assembled microtubule polymer in the axon elongates directly from the plus ends of preexisting polymer, and that no new polymer assembles de novo. These results explain not only how axons recover polymer, but also maintain and recover their correct plus-end-distal orientation after an episode of depolymerization. Taken together, the results of these drug studies and our observations on gamma-tubulin distribution in the neuron indicate that new microtubules required for axon growth arise within the cell body, most probably at the centrosome, and hence, provide new and provocative support for the view that mechanisms within the cell body are key to the elaboration of the axonal microtubule array.

Additional considerations indicate that mechanisms relevant to the origins of axonal microtubules may also occur within the axon itself. If no entirely new microtubules are generated within the axon itself, what accounts for the local increases in microtubule number required for rapid responses of the axon to physiological stimuli at sites distal to the cell body? It is difficult to imagine how the additional microtubules required for the creation of a collateral branch could originate within the cell body and traverse many hundreds of microns to supply the immediate need for new microtubules required for this kind of activity. Additional observations on experimental microtubule depolymerization speak to this issue. Increases in microtubule number can be experimentally induced in the axon by cold treatment (Heideman et al., 1984; Joshi et al., 1986), with the additional microtubules arising by fragmentation of preexisting microtubules (Mandelkow and Mandelkow, 1985). These resulting microtubules share the same polarity orientation, and would be expected to also share the same centrosomally-derived characteristics of their predecessor-microtubules. More recent evidence indicates that the process of microtubule fragmentation is a physiological event, and that specific microtubule severing proteins exist within living cells (Sanders and Salisbury, 1989; Vale, 1991). Such severing would be expected to result in MT fragments with relatively stable minus ends, as has been shown to be the case in experimentally severed MTs at the centrosome (Nicklas et al., 1989) and within the axon (White et al., 1987). In addition, it is interesting to note that a decrease in microtubule mass in the axon results in an increase in the motile activity of the otherwise quiescent surface of the axon shaft (Bray et al., 1978; Joshi et al., 1986). Thus, available evidence indicates a tight coupling between changes in the microtubule array of the axon and other events required for local alterations in axonal morphology such as the production of collateral branches.

Assembly and Transport are Both Important Events for Axonal Microtubules

If our hypothesis is correct, and microtubules destined from the axon are released from the centrosome, there would be a clear need for an active transport mechanism to translocate them into and down the axon. In addition, during transit, these microtubules would have to undergo substantial elongation to achieve the great lengths characteristic of axonal microtubules, many-fold the diameter of the cell body. Thus, a centrosomal origin for axonal microtubules demands coordinated efforts between microtubule transport and elongation within the axon. To study this further, we have developed an experimental approach that dissects apart the contribution of microtubule transport from that of assembly, and thus permits us to analyze their separate contributions to the elaboration of the axonal microtubule array.

In this approach, neurons are cultured in the presence of nanomolar levels of vinblastine, a treatment which arrests microtubule assembly without substantially disassembling existing microtubules in the cell body (Baas and Ahmad, 1993). Thus, while no further microtubule assembly occurs under these conditions, the pool of microtubules within the cell body can be transported into the axon as it grows. Under these conditions, there is a progressive increase in microtubules in the growing axon, and a corresponding depletion of microtubules from the cell body. This result indicates that highly efficient mechanisms exist in the neuron to transport microtubules from the cell body into and down the axon. As predicted, the microtubules within the axon are short, a fraction of the cell body's diameter, dramatically illustrating the need for local microtubule assembly to generate the microtubule lengths normally found in the axon. It should be noted that these studies, which indicate the need for both microtubule transport and assembly, were performed in mammalian neurons, a type of neuron in which the photobleach/photoactivation approach has been unable to detect microtubule movement. The results of these vinblastine studies strongly argue that tubulin is transported in the form of microtubules,

and suggest that conclusions to the contrary drawn from the photobleach/photoactivation technology must be re-evaluated.

In addition, these studies provide another kind of information relevant to the generation of the axonal microtubule array. We have determined that the microtubules within axons grown in the presence of vinblastine are uniformly oriented with their plus ends distal to the cell body (Baas and Ahmad, 1993). Thus, in the absence of microtubule assembly, the transport properties of the microtubules alone can establish their characteristic organization. Based on these data, we conclude that microtubules are transported from the cell body into the axon exclusively with their plus ends leading, and that it is this unidirectional transport that establishes the plus-end-distal microtubule polarity orientation characteristic of the axon.

These data and considerations bring to the fore several interesting questions concerning microtubule transport in the axon. For example, what is the transport motor that moves microtubules into and down the axon, and what are the structures against which the microtubules move? At present, the answers to these questions are unknown. As noted in a previous section of this article, there is growing knowledge about microtubule-based motility events in living cells. At least one motor, dynein, has the correct directionality to move microtubules with plus ends leading. However, the rate at which motors such as dynein move organelles relative to microtubules is generally far faster (by at least 10-fold) than the rate at which the transport studies suggest that microtubules move. A slower rate of microtubule transport could be explained by a greater drag on the microtubules, which on average are significantly longer than the diameter of the organelles that move along the microtubules. With regard to the structures against which microtubules move, possible candidates include the long stretches of ER within the axon, neurofilaments in the axon, or other microtubules (see Baas and Ahmad, 1993 for more discussion). Identification of the molecular motor for microtubule transport in the axon and elucidation of the other features of this transport will be matters of great interest for future studies.

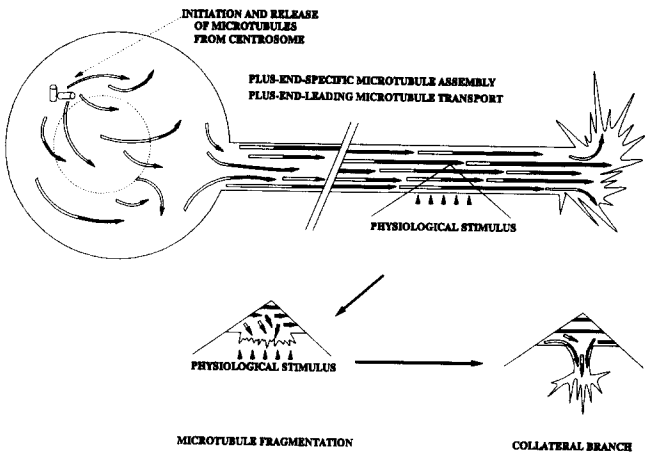

Figure 2. Schematic representation of our model for the elaboration of the axonal microtubule array. Microtubules destined for the axon are initiated at the centrosome, and then released for translocation. Released microtubules are transported through the cytoplasm with their plus ends leading, and many of these are transported into the axon. In the schematic, the white portions of the microtubules represent the part assembled from the centrosome, while the black portions represent the part assembled after release from the centrosome. The lengths are not to scale, as in the neuron, the latter would be significantly longer than the former. Arrows represent the plus ends of the microtubules. The space between the slanted lines through the axon represents hundreds of microns of axon growth. During transit, the microtubules elongate specifically from their plus ends. At sites distal to the cell body, local requirements for additional microtubules are fulfilled by the fragmentation of these existing microtubules in response to physiological stimuli. The microtubules that result from fragmentation inherit the centrosomally derived characteristics of their predecessor microtubules, and as well as their plus-end-distal polarity orientation. In addition, the resulting microtubules continue plus-end-led translocation as well as plus-end-specific assembly into new collateral branches as well as down the mainshaft of the axon.

A New Model for Microtubules and Axon Growth

Our recent observations on the origins of axonal microtubules and the relative contributions of microtubule transport and assembly provide new insights into the mechanisms by which the axonal microtubule array is elaborated and expanded during axon growth. Based on these observations and the large bodies of data used to support each of the two previous models for the elaboration of the axonal microtubule array, we have developed a composite model that is consistent with all of these bodies of data (see Fig. 2). We envision a scenario in which microtubules destined for the axon are initiated at the centrosome within the cell body, after which they are released from this structure and transported into the axon. The transport of these microtubules is unidirectional with regard to their polarity, plus ends leading, thus establishing the characteristic microtubule polarity orientation of the axon. In addition, these microtubules have the regular 13 protofilament lattice that results from centrosomal nucleation. The microtubules released from the centrosome are short, no longer than the diameter of the cell body, and many of them elongate during their transit into and down the axon. Many of the microtubules also shorten in transit down the axon, providing a source of tubulin subunits for the elongation of the others. In this way, large numbers of short microtubules give way to smaller numbers of longer microtubules (see Ahmad et al., 1993, for more discussion). Such a length redistribution is a hallmark feature of the dynamic instability model for microtubule behavior (for review see Kirschner and Mitchison, 1986), and as such the acquisition of data on the specific lengths of individual microtubules along the axon will be of great interest. Microtubule length changes occur exclusively from their plus ends, and we postulate that length changes occur all along the length of the axon. The most distal region of the axon where the microtubule array terminates is particularly rich in microtubule plus ends, and hence could be a significant site of microtubule assembly. Along the length of the axon, individual microtubules have the capacity to fragment in response to physiological stimuli, thus, locally and rapidly increasing the supply of microtubules as needed for events such as collateral branching. All of the resulting microtubules inherit the centrosomal characteristics of their predecessor microtubules. In addition, the resulting microtubules continue plus-end-led

translocation as well as plus-end-specific assembly into the new collateral branches as well as down the mainshaft of the axon. In this manner, data previously used to support both the cell body-based model as well as data used to support the local control-based model are accounted for, and come together into a highly satisfactory composite model for the elaboration of the axonal microtubule array.

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