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A Composite Model for Establishing the Microtubule Arrays of the Neuron

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Abstract

Neurons generate two distinct types of processes, termed axons and dendrites, both of which rely on a highly organized array of microtubules for their growth and maintenance. Axonal microtubules are uniformly oriented with their plus ends distal to the cell body, whereas dendritic microtubules are nonuniformly oriented. In neither case are the microtubules attached to the centrosome or any detectable structure that could establish their distinct patterns of polarity orientation. Studies from our laboratory over the past few years have led us to propose the following model for the establishment of the axonal and dendritic microtubule arrays. Microtubules destined for these processes are nucleated at the centrosome within the cell body of the neuron and rapidly released. The released microtubules are then transported into developing axons and dendrites to support their growth. Early in neuronal development, the microtubules are transported with their plus ends leading into immature processes that are the common progenitors of both axons and dendrites. This sets up a uniformly plus-end-distal pattern of polarity orientation, which is preserved in the developing axon. In the case of the dendrite, the plus-end-distal microtubules are joined by another population of microtubules that are transported into these processes with their minus-ends leading. Implicit in this model is that neurons have specialized machinery for regulating the release of microtubules from the centrosome and for transporting them with great specificity.

Index Entries: Neuron; axon; dendrite; microtubule; microtubule polarity; microtubule transport; microtubule assembly; centrosome; microtubule-associated protein.

Introduction

Neurons are highly polarized cells consisting of a rounded cell body and several elongate processes. One of these processes, termed the axon, is specialized to transmit information over long distances. The other processes, termed dendrites, are specialized to receive and process information. Axons and dendrites are very different from one another in terms of

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their morphology and composition, and these differences are collectively referred to as the polarity of the neuron *(1–4)*. Axons and dendrites both contain dense arrays of microtubules that extend along their lengths. These microtubules are essential for providing the architectural support needed for the growth and maintenance of the processes and for regulating the transport of cytoplasmic organelles within them. The axonal and dendritic microtubule arrays differ from one another in many respects, and the differences between them are fundamental in defining many of the morphological and compositional differences that distinguish axons and dendrites from one another. Because of the importance of the axonal and dendritic microtubule arrays, there is great interest in elucidating the mechanisms by which they are established.

At present, two principal differences between axonal and dendritic microtubules have been documented. The first of these concerns their complement of microtubule-associated proteins (MAPs), such as τ and MAP2. Because of many factors, including the binding affinity of these MAPs to microtubules in each compartment of the neuron, axonal microtubules are generally richer in τ, whereas dendritic microtubules are generally richer in MAP2 *(5–8)*. MAPs are proteins that bind along the length of the microtubule and are generally thought to stabilize it against disassembly. In addition, MAPs are thought to bundle neighboring microtubules together and to regulate the spacing between microtubules within the bundle. Axonal microtubules are spaced more closely together than dendritic microtubules almost certainly because τ has a shorter projection domain than MAP2 *(9–12)*. A growing body of evidence suggests that differences in the MAP composition of axonal and dendritic microtubules also contribute, in ways that we do not yet understand, to features of neuronal morphology, such as the shape and number of each type of process extended by the neuron *(13)*.

The other main difference between axonal and dendritic microtubules concerns their polarity orientation. Microtubules are intrinsically polar structures, with the "plus" end favored for assembly and disassembly over the "minus" end *(14,15)*. The polarity of the microtubule is relevant not only to its assembly properties but also to its transport properties, because different classes of molecular motors transport organelles specifically toward either the plus or minus end of the microtubule *(16–19)*. In the axon all of the microtubules are oriented with their plus ends distal to the cell body *(20–25)*, whereas in the dendrite microtubules are of both orientations *(23–26)*. These distinct microtubule polarity patterns can explain many of the cytoplasmic differences between axons and dendrites *(19)*. Both processes contain such organelles as mitochondria that translocate toward the plus ends of microtubules, but only dendrites contain such organelles as ribosomes and Golgi elements that translocate toward minus ends of microtubules. In addition to providing an attractive mechanism for defining the unique cytoplasmic composition of each type of process, we suspect that these distinct microtubule polarity patterns also contribute to other features of neuronal polarity, such as the fact that axons achieve far greater lengths than dendrites.

Despite the importance of the axonal and dendritic microtubule arrays, the cellular and molecular mechanisms by which they are elaborated remain poorly understood and a matter of some controversy. In particular, there is disagreement concerning the specific roles and relative contributions of microtubule assembly events and microtubule transport events to the establishment and elaboration of these arrays. In the present article, we provide a historical perspective on the issue and review some of the pertinent findings that have been used to support differing models. Our main goal is to review recent studies from our laboratory that we believe support a composite model in which the neuronal microtubule arrays are established via the coordinated efforts of several different kinds of microtubule behaviors.

Historical Perspective: The Microtubule Transport Controversy

The fundamental dilemma of the neuron is that most protein synthesis is limited to the cell body. Axons are generally ribosome deficient and therefore do not synthesize proteins. Dendrites contain ribosomes but do not contain the mRNAs for many molecules, including the tubulins. The fact that tubulin and other cytoskeletal proteins are synthesized only in the cell body presents a special challenge for the neuron given the long lengths that the processes can achieve. Particularly in the case of the axon, these lengths can be quite extraordinary, far exceeding the distances that tubulin molecules could effectively diffuse *(27)*.

The Microtubule Transport Model

Over two decades ago, Lasek and collaborators began to address how tubulin is moved from the cell body down the axon *(27–32)*. In these kinetic 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Levels of radioactivity were then 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. 1A). In its original form, the model held that microtubules are transported as a highly crosslinked network, but this element of the model was subsequently refined to hold that individual microtubules translocate independently or relative to one another *(27)*.

The Distal Assembly Model

Although these early kinetic studies were not performed on growing axons, it was proposed that the elaboration of the microtubule array during axon growth occurs via the transport of preassembled microtubules from the cell body down the axon. A challenge to this idea came in 1986, when Bray and collaborators evaluated the potential for different regions of the neuron to participate in elaborating the axonal microtubule array *(33)*. 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 micropipet to different regions of cultured chick sensory neurons in the presence of a flowing stream of culture medium. As a result of this experimental regimen, 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, they concluded that new microtubule assembly occurs locally within the axon itself, and specifically at the distal tip of the growing axon

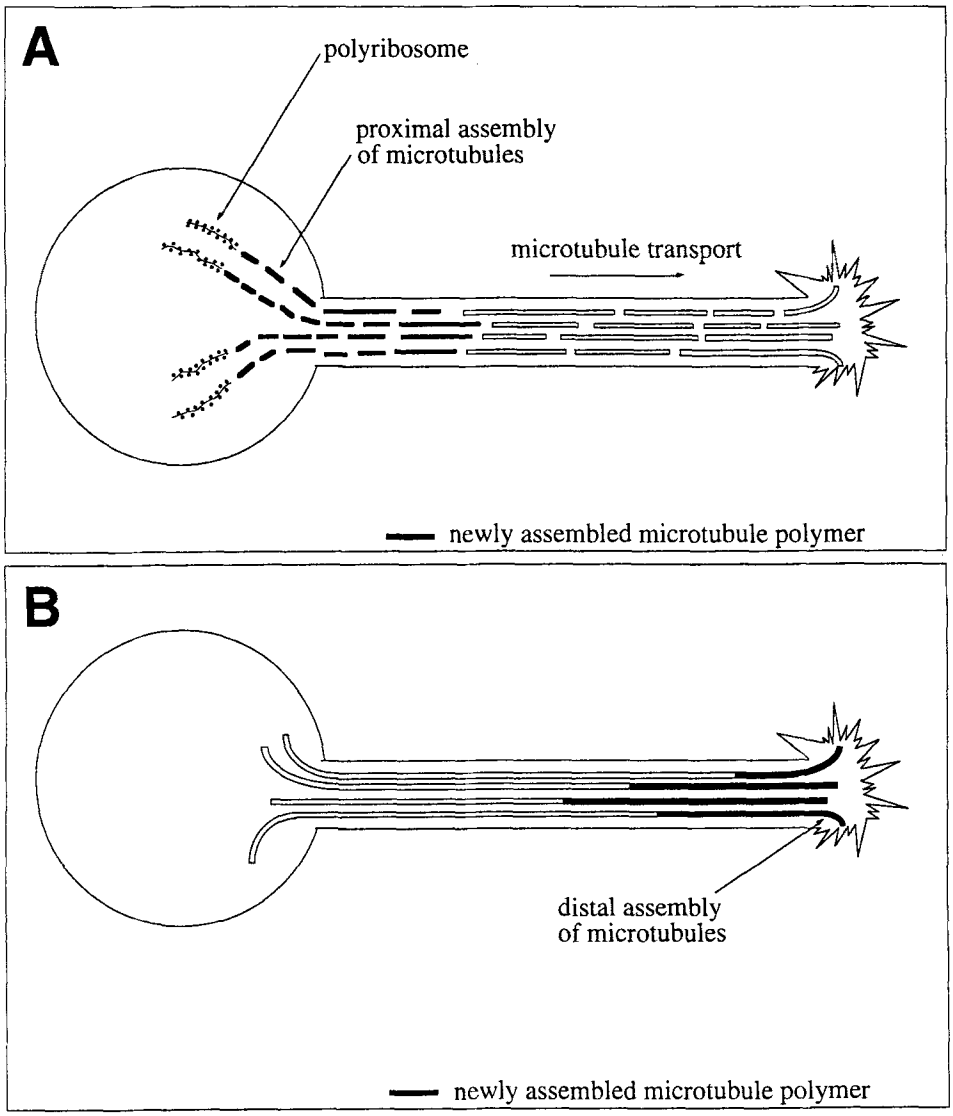

Fig. 1. Schematic representations of two models for the elaboration of the axonal microtubule array. In the model shown in **(A)**, it was proposed that newly synthesized tubulin subunits assemble to form microtubules within the cell body that are transported down the axon. In the model shown in **(B)**, it was proposed that the microtubule array of the axon is elaborated by net assembly of tubulin subunits onto the ends of the microtubules at the distal tip of the axon. In both schematics, newly assembled polymer is shown in black.

(Fig. 1B). Over the next few years since the publication of this work, a new model for axon growth emerged and gained popular favor. This model, sometimes referred to as the "distal assembly model," held that the net addition of new microtubule polymer to the growing axon occurs by local assembly at its distal tip rather than by transport of preassembled polymer.

Assaying Microtubule Transport in Living Cells

The difficulty with the distal assembly model in its original form is that it did not address the fundamental dilemma of the axon, namely how tubulin subunits are moved from the cell body down the axon. Proponents of the model subsequently acknowledged that tubu-

lin must be actively transported down the axon, but remained skeptical whether tubulin is transported in the form of preassembled microtubules. To address this issue, several laboratories 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 for potential movement 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 created 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 *(34)*. These studies suggested proximo-distal movement of the bleached zone but were controversial because the images were rather noisy. Later studies on PC12 cells and on avian and mammalian neurons failed to show microtubule movement *(35–37)*. The first photoactivation studies, performed on *Xenopus* axons, showed very clear proximo-distal movement, which occurred at roughly the same rate as the rates of slow transport previously reported by Lasek and collaborators *(38)*. For a short time it appeared that the issue was resolved and that the lack of movement observed in the previous studies owed to shortcomings in the bleaching technology. However, it was then 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 *(39,40)*, indicating that the differing results could not be attributed to the use of photoactivation vs photobleach. Two more recent studies, one using photoactivation on grasshopper axons *(41)* and the other using photobleach on zebrafish axons *(42)*, have also failed to detect movement. One possibility is that microtubules move in *Xenopus* axons but not in the axons of other animals. However, this view seems dissatisfying in that it would demand very different mechanisms for axon growth in different animals.

At present, the reasons for the differing results concerning microtubule movement are unclear and have been the source of additional controversy. The simplest possibility relates to the fact that living cells and biological molecules, particularly those conjugated to fluorescent probes, are highly susceptible to photodamage *(38,43–45)*, and may even break up under certain conditions *(44)*. 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 *(45)*. It may be, for example, that *Xenopus* axons are less susceptible to photodamage than mammalian or avian axons. 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 either to normal subunit turnover or to sensitivity. With regard to subunit turnover, individual microtubules constantly exchange subunits with the free tubulin pool, which occurs whether or not the microtubules are moving. Thus, depending on how rapidly this occurs, the marked regions on many of the microtubules may dissipate as a result of this exchange, masking any movements that might be occurring simultaneously. With regard to the issue of sensitivity, it is also relevant that microtubule movements may be highly asynchronous. For the marked microtubules to be detected it may be necessary for two or more marked microtubules to be 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 caused entirely by 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. This interpretation is supported by recent analyses on the lengths at which diffusion would fail to provide sufficient tubulin for different kinds of axons, depending on their growth characteristics *(41)*. These analyses strongly suggest that *Xenopus* axons are the only type of axon studied thus far that grow sufficiently fast that diffusion would fail over the relatively short lengths over which the photobleach/photoactivation approach is practical.

Interpreting these results is problematic for the various technical reasons outlined above. In addition, it is troubling that the studies that fail to show microtubule movement also fail to show the movement of tubulin in any form. Although the photobleach/photoactivation studies are certainly interesting, it is clear that additional studies will be required to better understand the results and that new approaches will be required to better address the controversial issue of microtubule transport in the axon.

Where Do Axonal and Dendritic Microtubules Come from?

Over the past few years we have been persuaded to believe that microtubule transport and local microtubule assembly both play important roles in the elaboration of the axonal and dendritic microtubule arrays. We were initially persuaded in this direction on the basis of the organization and structural features of these microtubule arrays and considerations on the strategies that other cells use to regulate microtubule structure and organization. In typical nonneuronal cells, organized microtubule arrays are established by limiting nucleation of new microtubules to discrete centralized structures, such as the centrosome. Nucleation from these structures helps regulate the number, length, lattice structure, and polarity orientation of the microtubules within the array *(46–48)*. The minus end of each microtubule is attached to the nucleating structure whereas the plus end emanates away from it, resulting in a microtubule array with a distinct polarity pattern. In axons and dendrites, the microtubules are not attached to the centrosome or any comparable nucleating structure, but rather stop and start at multiple sites along the length of the processes *(49–52)*. Nevertheless, the microtubules are tightly regulated with regard to polarity orientation, number, length, and lattice structure. How is this achieved?

Axonal and Dendritic Microtubules Originate Within the Cell Body

As a first measure toward investigating this issue, we sought to determine the sites where axonal microtubules originate, which is to say that we sought to identify and localize their nucleating structures. The classic method for identifying microtubule-nucleating structures within cells is to depolymerize existing microtubules with nocodazole, a potent but reversible microtubule depolymerizing agent, and then remove the drug so that microtubules can reassemble from their sites of origin. This method, first used to identify the centrosome as a microtubule-nucleating structure in nonneuronal cells *(53)*, was used in our laboratory to identify potential microtubule-nucleating structures within the axons of cultured sympathetic neurons *(54)*. After drug removal, all new microtubule polymers arose specifically from the plus ends of the short microtu-

bule fragments that resisted depolymerization. No microtubules arose independently of these microtubule fragments, suggesting that the plus ends of pre-existing microtubules are the exclusive sites of microtubule assembly in the axon. These findings are consistent with previous work on cultured sensory neurons demonstrating that when all microtubule polymer is pharmacologically depolymerized from isolated axons, no microtubules reassemble after removing the drug *(55)*. Together, these studies demonstrate that no entirely new microtubules arise within the axon itself. Thus, we concluded, by the process of elimination, that new microtubules destined for the axon must be nucleated within the cell body. Although similar studies were not performed on dendrites, the observation on cultured hippocampal neurons that minus-end-distal microtubules appear in dendrites later in their development than plus-end-distal microtubules caused us to wonder whether dendrites may acquire the unique capacity, not shared by the axon, to locally nucleate oppositely oriented microtubules *(23,24)*.

A Centrosomal Origin for Axonal and Dendritic Microtubules?

To explore the issue further, we determined the distribution of γ-tubulin within cultured sympathetic neurons *(56)*. γ-Tubulin is a member of the tubulin superfamily that is present within cells at very low levels compared to the levels of α- or β-tubulin. Unlike α- and β-tubulin, γ-tubulin is not a component of the microtubule itself, but is localized to the sites of microtubule nucleation within a cell and has been shown to be required for microtubule nucleation in all cell types examined *(57)*. Using both biochemical and immunoelectron microscopic assays, we demonstrated that there is no γ-tubulin within the axon, which is consistent with our previous conclusion that no new microtubules are nucleated within the axon. In addition and initially somewhat surprisingly, we found no γ-tubulin in the dendrite, indicating that no new microtubules are nucleated in these processes. In fact, we found no γ-tubulin anywhere in the neuron except at the centrosome, suggesting that the centrosome is the sole site for the generation of new microtubules in the neuron. On the basis of these findings, we proposed that all microtubules destined for axons and dendrites are nucleated at the centrosome within the cell body of the neuron, released into the cytoplasm, and then transported into these processes.

If our reasoning is correct, the neuronal centrosome must be an extremely potent microtubule-nucleating structure, capable of generating hundreds of microtubules for the growth and maintenance of elaborate axonal and dendritic arbors. In addition, it is implicit in our proposal that the neuronal centrosome has the capacity to rapidly release the microtubules it nucleates, so that they can be exported into and down the length of the processes. Electron microscopic analyses of different kinds of neurons at different developmental stages vary with regard to the appearance of the centrosome, but most studies reveal relatively few microtubules directly attached to the centrosome. In our studies on cultured sympathetic neurons, generally fewer than ten and often no microtubules were attached to the centrosome *(56,58)*. These observations raised the possibility that axonal and dendritic microtubules may not originate at the centrosome, and that the neuronal centrosome may actually be relatively inactive. Alternatively, however, the nucleation and release of microtubules from the neuronal centrosome may be so rapid that there is insufficient time for substantial numbers of attached microtubules to accumulate at the centrosome before they are released.

To address this issue, we tested the capacity of the neuronal centrosome to act as a microtubule-nucleating structure using the same drug-recovery regimen used in our previous work on the axon *(58)*. Within a few minutes of drug removal, hundreds of microtubules reassembled in the region of the centrosome, and most of these microtubules were

clearly attached to the centrosome. A portion of the microtubules were not attached to the centrosome, but were aligned side-by-side with the attached microtubules, suggesting that the unattached microtubules had been released from the centrosome after their nucleation. In addition, unattached microtubules were present in the cell body at decreasing levels with increasing distance from the centrosome. By 30 min, the microtubule array was indistinguishable from that of control neurons, suggesting that the hundreds of microtubules nucleated from the centrosome after the first few minutes were subsequently released and translocated away from the centrosome. These results demonstrate that the neuronal centrosome is a highly potent microtubule-nucleating structure, and provide strong indirect support for the idea that microtubules nucleated from the centrosome are released for translocation into other regions of the neuron.

A Functional Centrosome Is Important for Axon Growth

These studies indicated that the centrosome could in theory act as a generator of microtubules for redistribution into the axon, but did not test whether centrosomal microtubules are essential for the initiation and growth of axons and dendrites. To begin to explore this issue, we next determined the effects on axon growth of experimentally disabling the neuronal centrosome *(59)*. Our strategy was to microinject into cultured sympathetic neurons the same antibody to γ-tubulin previously shown to recognize the neuronal centrosome *(56)* and previously shown to arrest microtubule nucleation at the centrosome when microinjected into nonneuronal cells *(60)*. If centrosomally derived microtubules are required for the growth of the axon, we would expect inhibition of centrosome function to compromise or inhibit axon growth. To assess the effects of centrosomal inhibition over a 2-h time period (the time period over which the antibody is effective), it was also necessary to deplete the neuron experimentally of pre-existing microtubules. These microtubules, presumably already nucleated and released from the centrosome, are assembly-competent and capable of supporting substantial levels of axon growth in the absence of further microtubule assembly. After depolymerizing microtubules with nocodazole, the antibody was microinjected into neurons and then the drug was rinsed from the cultures. Reassembly of microtubules over the next 2 h was severely diminished under these conditions, and axon growth was either compromised or completely abolished. These results indicate that a functional centrosome is important for axon growth. Because of the short time period over which the antibody is active, we have not been able to take a similar approach in studies on dendrites, which grow more slowly and require several days to develop.

New Data on Microtubule Transport in Axons and Dendrites

In reconsidering the issue of microtubule transport, two issues came to our minds. First, if microtubules destined for axons and dendrites do in fact originate within the cell body, then the transport of these microtubules is a logistical necessity. Second, the regulated transport of microtubules into these processes offers an attractive means by which the distinct polarity patterns of axons and dendrites might be established. It is well recognized that different classes of microtubule-based transport motors move organelles specifically toward either the plus or the minus end of the microtubule, and it is not difficult to imagine how similar motors could move a microtubule specifically with either its plus or minus end leading. Given the myriad complications with available live-cell techniques, we sought to develop a novel strategy for addressing the contribution, if any, of microtubule transport to the elaboration of the axonal and dendritic microtubule arrays. To accomplish this, we

used a pharmacologic approach to experimentally dissect apart the contribution of microtubule transport from that of microtubule assembly. We exposed cultured neurons to nanomolar levels of the antimicrotubule drug vinblastine, a treatment that inhibited further microtubule assembly but did not depolymerize the existing microtubules. Under these conditions, any changes in the organization or distribution of the microtubules could only be attributed to their transport from one location in the neuron to another.

The Transport of Axonal Microtubules Establishes Their Uniform Polarity Orientation

Our first study using this approach was on the axons of sympathetic neurons *(61)*. These neurons were exposed to the drug shortly after plating, before the outgrowth of any processes. After an overnight bout of axon outgrowth, we quantified the levels of microtubule mass in the cell body and in the newly grown axons. We also measured the lengths of individual microtubules. Both were the same or lower than in freshly plated neurons, confirming the efficacy of the drug treatment. Notably, there was a net transfer of microtubules from the cell body into the axon as it grew, indicating that neurons have potent mechanisms for transporting preassembled microtubules from one compartment into the other. In addition, the microtubules within the experimental axons were uniformly plus-end-distal, strongly suggesting that it is indeed the transport properties of axonal microtubules that establish their characteristic polarity pattern.

Centrosomal Microtubules Are Transported into the Axon

Our second study on sympathetic neurons was similar to the first, except that we sought to determine very specifically whether centrosomal microtubules are transported into axons *(62)*. For these studies, we plated sympathetic neurons and treated with high doses of nocodazole to depolymerize most of the existing microtubules, rinsed out the drug, permitted a few minutes of microtubule reassembly at the centrosome, and then added nanomolar levels of vinblastine to curtail any further assembly. Within minutes, unattached microtubules began to appear in the cytoplasm, and by 10 min many of these had reached the periphery of the cell body. By 1 h, few or no microtubules were attached to the centrosome and the vast majority of the microtubules were concentrated at the cell periphery. In the case of the neurons that were able to grow axons under these conditions, microtubules appeared progressively farther down the axons with increasing time. These results indicate that centrosomal microtubules are released, transported to the periphery of the cell body, and then transported into developing axons.

Transport of Dendritic Microtubules Establishes Their Nonuniform Polarity Orientation

In our third study using the vinblastine strategy, we sought to determine the contribution of microtubule transport to the elaboration of the dendritic microtubule array *(63)*. For these analyses, we turned to cultured hippocampal neurons because of the stereotyped manner by which axons and dendrites develop. Initially, these neurons extend several essentially similar immature processes that are the common precursors of both axons and dendrites. These immature processes contain microtubules that are uniformly oriented with their plus-ends distal to the cell body, a pattern that is preserved in the developing axon *(24)*. In contrast, developing dendrites gradually acquire nonuniform microtubule polarity orientation resulting from the addition of a subpopulation of oppositely oriented microtubules *(24)*. In theory, these minus-end-distal microtubules could be locally nucleated and assembled within the dendrite itself, or could be transported into the dendrite after their nucleation within the cell

body. To distinguish between these possibilities, we exposed cultured hippocampal neurons to nanomolar levels of vinblastine after one of the immature processes had developed into the axon but before the others had become dendrites. This treatment did not abolish dendritic differentiation, which occurred in timely fashion over the next 2–3 d. The growth of these dendrites was accompanied by a diminution of microtubules from the cell body, indicating a net transfer of microtubules from one compartment into the other. During this time, minus-end-distal microtubules arose in the experimental dendrites, indicating that new microtubule assembly is not required for the acquisition of nonuniform microtubule polarity orientation in the dendrite. Minus-end-distal microtubules predominated in the more proximal region of experimental dendrites, indicating that most of the microtubules at this stage of development are transported into the dendrite with their minus-ends leading. These observations indicate that transport of microtubules from the cell body is an essential feature of dendritic development that establishes the nonuniform polarity orientation of microtubules in the dendrite.

The Controversy Continues

In summary, our studies using this pharmacologic approach strongly suggest that microtubules are actively transported into axons and dendrites, and that it is the specific transport properties of the microtubules that establish their distinct patterns of polarity orientation. Although these conclusions are compelling, we acknowledge that our strategy is indirect, and hence that conclusions on microtubule transport are inferential. Certain workers remain skeptical and are seeking to identify other nonpolymeric forms in which tubulin might be transported down the axon *(42)*. We speculate that the development of new live cell strategies will confirm that tubulin is transported as preassembled microtubules, as our indirect analyses suggest.

New Data on Microtubule Assembly in Axons and Dendrites

Much of the controversy in the field derives from what we feel is a mistaken impression by proponents of the distal assembly model that microtubule transport and local assembly are mutually exclusive. In fact, considerations on the lengths of microtubules in axons and dendrites indicate that any model invoking microtubule transport from the cell body must also invoke microtubule assembly within the processes. Microtubules in the cell body are relatively short, a few microns in length, whereas microtubules in axons and dendrites can achieve lengths well over 100 μm, many times the length that could fit within the cell body *(51,52)*. Thus, it is clear that at least some of the microtubules originating within the cell body and moving down these processes must elongate substantially as they move. Any model suggesting that microtubules move without elongating within the processes clearly cannot be correct, but to the best of our knowledge, no one has ever proposed such a model.

The point of controversy, as we see it, concerns whether or not local microtubule assembly events actually increase the total microtubule mass within the processes. In other words, do local assembly events result in a net increase in microtubule mass? We have argued that during their transit down axons and dendrites, many of the microtubules shorten or completely depolymerize to provide the subunits needed for others to elongate *(58,61–64)*. Thus, microtubule transport is required to increase the total polymer mass, but local assembly and disassembly events are required for axonal and dendritic microtubules to achieve their characteristic lengths. This view is supported by recent studies from our laboratory in which serial reconstruction of electron micrographs was used to determine changes in microtubule number and length during axon differentiation *(65)*. Also compelling is the fact that such a shift in microtubule lengths is precisely what would be expected on

the basis of the modern dynamic instability model for microtubule behavior in living cells *(66)*. Thus, the myriad reports on local microtubule assembly events in axons and dendrites are consistent with microtubule transport and demonstrate other microtubule behaviors that must accompany microtubule transport in order for the axonal and dendritic microtubule arrays to be elaborated.

Worth mentioning in this context is a recent study from our laboratory in which we reexamined the results obtained from the original study that gave birth to the distal assembly model. As discussed earlier, the foundation for the distal assembly model was a study by Bamburg and collaborators in 1986 in which antimicrotubule drugs were applied topically for 30 min to discrete regions of cultured sensory neurons *(33)*. The axon continued to grow when the drugs were applied to the cell body, but stopped growing when the drugs were applied to the distal tip of the axon. Assuming that the sole action of the drug was to inhibit microtubule assembly, subsequent workers interpreted these findings as indicating that the growth of the axon requires net microtubule assembly at its distal tip. However, this interpretation is valid only if the drug treatment inhibits microtubule assembly without depolymerizing existing polymer. If the drug substantially depolymerizes microtubules in the distal region of the axon, the logic breaks down in that the axon would stop growing regardless of how the microtubules normally arrive in this region. We repeated the experiments using a broader range of drug treatments and evaluated using electron microscopy the effects of these treatments on microtubule levels *(67)*. Our results indicate that the drug treatments used in the original study went far beyond inhibiting microtubule assembly and also caused substantial microtubule disassembly. Notably, when the drug regimen was altered to inhibit microtubule assembly while inducing lower levels of microtubule disassembly in the distal region of the axon, the axon continued to grow. These results indicate that, contrary to popular interpretations of the original study, the growth of the axon is not dependent on net microtubule assembly at its distal tip.

Collateral Branching: A Special Challenge for the Axonal Microtubule Array

A centrosomal origin for each and every microtubule in the axon is a satisfying idea for many reasons, but also presents a dilemma of its own. Many axons achieve great lengths and require rapid increases in microtubule numbers at distances far from the cell body, for example, during the formation of collateral branches. It is difficult to imagine how the great numbers of new microtubules required for collateral branch formation could derive directly from the centrosome and translocate at necessary rates over such great distances. It seems more likely that local mechanisms exist within the axon itself to account for the rapid and local increases in microtubule number required for the formation of collateral branches. In studies on axon branch formation in cultured hippocampal neurons, we have demonstrated that existing microtubules in the axon can locally fragment *(68)*. This fragmentation transforms one microtubule into many, all of which are assembly competent and capable of rapidly elongating, and all of which inherit the centrosomally derived characteristics of their predecessor microtubules. In this manner, fragmentation of microtubules within the axon provides a mechanism to increase the number of "centrosomal" microtubules without having to nucleate new microtubules at the centrosome. Our studies demonstrate that microtubule fragmentation occurs specifically at the branch point, after which a portion of the fragments are partitioned into the branch while the others remain within the parent axon. It may be that microtubule fragmentation is active in axons and dendrites under other circumstances as well, permitting the processes to be less dependent on the centrosome for the creation of "new" microtubules. It should be

noted, however, that microtubule fragmentation only increases the number of individual microtubules and does not increase the levels of polymer. For this reason, microtubule transport is still required to increase the microtubule polymer mass, regardless of how active microtubule fragmentation may be.

Establishing the Microtubule Arrays of the Neuron

On the basis of the studies described thus far, we have proposed a composite model for the elaboration of the axonal and dendritic microtubule arrays. We prefer to use the word "composite" because our model incorporates data previously used to support earlier models based either on microtubule transport or distal microtubule assembly. In our model, microtubules destined for axons and dendrites are nucleated within the cell body of the neuron, specifically at the centrosome. Following their nucleation, the microtubules are rapidly released into the cytoplasm of the cell body. At this point, specific molecular motors transport the microtubules into developing processes specifically with the plus ends of the microtubules leading. This sets up a uniformly plus-end-distal pattern of polarity orientation in the immature processes, which is preserved in the immature process that develops into the axon. As the other processes develop into dendrites a second population of microtubules is transported into these processes. These microtubules are transported specifically with their minus ends leading, thus establishing a nonuniform pattern of microtubule polarity orientation in developing dendrites. Concomitantly with these transport events, a portion of the microtubules shorten to yield subunits for other microtubules to elongate. This results in a shift from high numbers of very short microtubules to lower numbers of much longer microtubules. In this manner, axonal and dendritic microtubules inherit their lattice structure from centrosomal nucleation, their characteristic polarity patterns from their transport properties, and their characteristic lengths from local dynamic events. Local microtubule fragmentation provides additional flexibility in the system, permitting rapid increases in microtubule number at sites distal to the centrosome. Figure 2 is a schematic illustration of our model.

There are two main challenges for the future. The first is to rigorously test our model, and in particular to develop new higher-resolution live-cell approaches for visualizing microtubule transport. The second is to elucidate the molecular machinery by which the various aspects of the model are regulated. In particular, it will be important to identify the factors that regulate microtubule release from the centrosome and to identify the specific molecular motors that transport microtubules into axons and dendrites. One possibility is that terminally postmitotic cells, such as the neuron, utilize essentially the same or similar molecular machinery for process outgrowth that undifferentiated cells utilize for mitosis. Both involve centrosomal microtubules and microtubule transport events, but in the case of the undifferentiated cells, most of the microtubules remain attached to the centrosome so that the motors can drive movements of the mitotic apparatus. In neurons, mitosis no longer occurs and the microtubules are rapidly released so the motors can drive the microtubules away from the centrosome and into developing processes. It is possible that neurons express higher levels or differently regulate such proteins as centrin, which may induce the severing of microtubules from the centrosome *(69)*. After their release, the microtubules have to be sufficiently stable that they do not depolymerize and must be drawn together into paraxial bundles. Several lines of evidence suggest that these functions are served by neuron-specific MAPs, such as τ and MAP2 *(5–13,70–72)*. However, recent studies involving transgenic mice call into question the specific roles that individual MAPs may play during the establishment of the axonal and dendritic microtubule arrays *(73)*. Is the

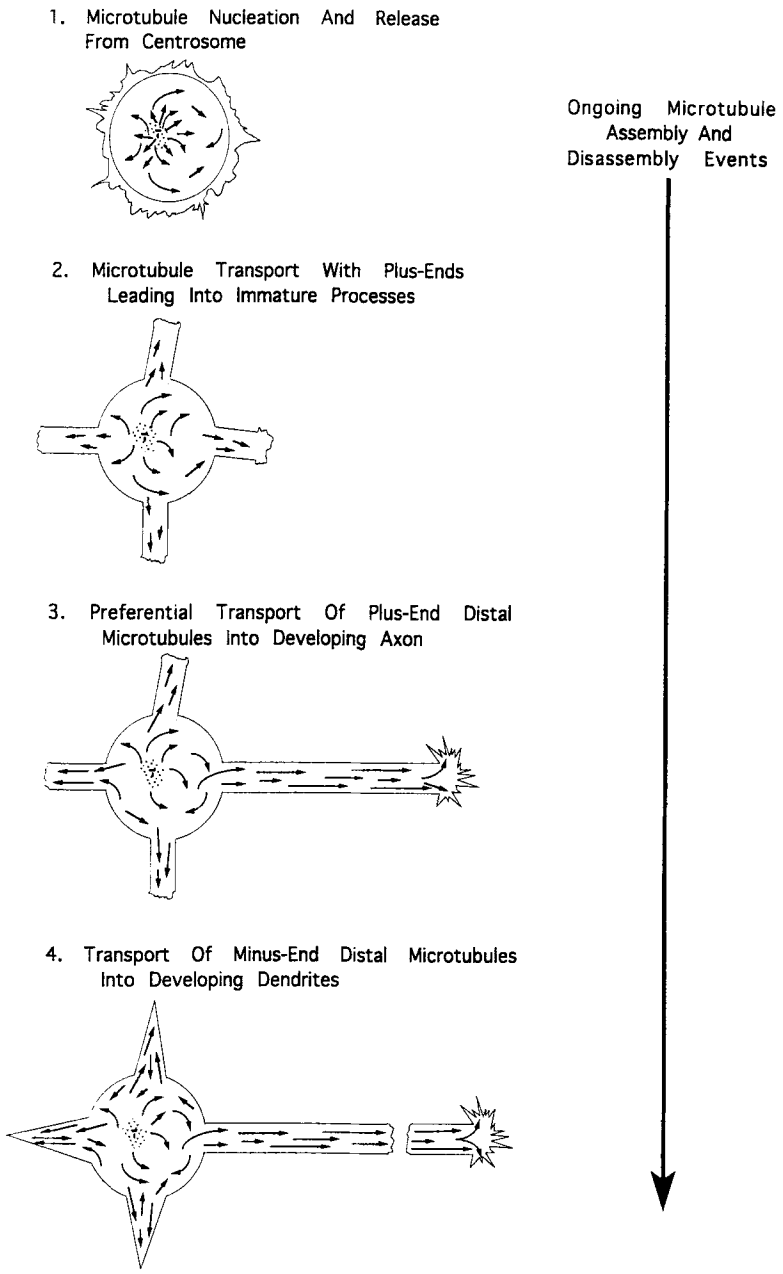

Fig. 2. Composite model for the elaboration of the axonal and dendritic microtubule arrays. In this model, microtubules destined for axons and dendrites are nucleated within the cell body of the neuron, specifically at the centrosome. Following their nucleation, the microtubules are rapidly released into the cytoplasm of the cell body. At this point, specific molecular motors transport the microtubules into developing processes specifically with the plus ends of the microtubules leading. This sets up a uniformly plus-end-distal pattern of polarity orientation in the immature processes that is preserved in the immature process that develops into the axon. As the other processes develop into dendrites, a second population of microtubules is transported into these processes. These microtubules are transported specifically with their minus ends leading, thus establishing a nonuniform pattern of microtubule polarity orientation in developing dendrites. Concomitantly with these transport events, a portion of the microtubules shortens to yield subunits for other microtubules to elongate. This results in a shift from high numbers of very short microtubules to lower numbers of much longer microtubules. Microtubules are shown in this schematic as solid black line segments with an arrowhead indicating the plus end of the microtubule.

segregation of τ and MAP2 essential for establishing the specific microtubule arrays of axons and dendrites, or is this segregation secondary? Perhaps the most confounding mystery concerns the transport machinery. Are the motors that transport axonal and dendritic microtubules the same motors that transport microtubules during mitosis, or are entirely novel motors expressed during neuronal differentiation? How is it that microtubules with plus-ends leading are transported into all processes of the neuron, but microtubules with minus-ends leading are transported only into dendrites? Resolution of these issues will be essential for understanding the complex mechanisms by which the microtubule arrays of the neuron are established.

Note Added in Proof

Two recent studies provide additional strong evidence for the transport of preassembled microtubules down the axon. In the first study, fluorescent stable microtubule fragments were injected into living squid axons. These microtubule fragments were observed to move anterogradely down the axon at the rate of slow transport, demonstrating the directional transport of preassembled microtubule polymer *(74)*. In the second study, we microinjected biotinylated tubulin into rat sympathetic neurons that had already grown short axons, after which the axons were permitted to grow longer. We reasoned that any polymer that assembled or turned over subunits after the introduction of the probe should label for biotinylated tubulin in immunoelectron microscopic analyses, whereas any polymer that was already assembled but did not turn over should not label. Therefore, the presence in the newly grown region of the axon of any unlabeled microtubule polymer would be indicative of microtubule transport. The newly grown region of the axon contained both labeled and unlabeled polymer, indicating that microtubule assembly and transport are both active during axon growth *(75)*.

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