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Microtubules Have Opposite Orientation in Axons and Dendrites of Drosophila Neurons

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In vertebrate neurons, axons have a uniform arrangement of microtubules with plus ends distal to the cell body (plus-end-out), and dendrites have equal numbers of plus- and minus-end-out microtubules. To determine whether microtubule orientation is a conserved feature of axons and dendrites, we analyzed microtubule orientation in invertebrate neurons. Using microtubule plus end dynamics, we mapped microtubule orientation in *Drosophila* sensory neurons, interneurons, and motor neurons. As expected, all axonal microtubules have plus-end-out orientation. However, in proximal dendrites of all classes of neuron, ~90% of dendritic microtubules were oriented with minus ends distal to the cell body. This result suggests that minus-end-out, rather than mixed orientation, microtubules are the signature of the dendritic microtubule cytoskeleton. Surprisingly, our map of microtubule orientation predicts that there are no tracks for direct cargo transport between the cell body and dendrites in unipolar neurons. We confirm this prediction, and validate the completeness of our map, by imaging endosome movements in motor neurons. As predicted by our map, endosomes travel smoothly between the cell body and axon, but they cannot move directly between the cell body and dendrites.

INTRODUCTION

Many differentiated cells have highly polarized arrays of microtubules that likely play a large role in establishing their specialized architecture and function. Neurons are strikingly polarized and initially seemed that they would be the clearest example of cells in which microtubule orientation formed the basis of directional transport and cell polarity (Black and Baas, 1989). Most neurons have a cell body in which the bulk of proteins are synthesized, dendrites that are specialized to receive signals, and axons that are specialized to send them. Where examined, microtubules in vertebrate dendrites have mixed orientation, and in axons they have uniform orientation with all plus ends distal to the cell body. Thus, the simplest model for selective transport from the cell body to dendrites is use of a minus end-directed motor. However, current models of transport into dendrites rely on plus end-directed motors (Setou *et al*., 2004; Hirokawa and Takemura, 2005; Kennedy and Ehlers, 2006; Levy and Holzbaur, 2006). These models raise the question: are minus-end-out microtubules important for directional transport or neuronal polarity?

Axonal microtubule orientation has been examined in a variety of neurons, all with the same result: >95% of plus ends are oriented away from the cell body (plus-end-out). Original studies on axonal microtubule orientation relied on decoration of microtubules with exogenous tubulin, which forms curved hooks on the sides of existing microtubules, and analysis by electron microscopy. The direction of hook curvature indicates microtubule polarity. This method was used to determine axonal microtubule orientation in many different types of vertebrate neurons (Burton and Paige, 1981; Heidemann *et al*., 1981; Baas *et al*., 1987, 1988; Troutt and Burnside, 1988). More recently, the direction of movement of proteins that bind to growing microtubule plus ends was used to analyze axonal microtubule orientation in cultured mouse hippocampal and Purkinje neurons (Stepanova *et al*., 2003) and cultured *Aplysia* neurons (Erez *et al*., 2007). Using both assays, in sensory and central neurons, in organisms ranging from the invertebrate *Aplysia* to mammals, >95% of axonal microtubules have been found to be plus-end-out. Additionally, second harmonic generation microscopy has confirmed axonal microtubules in vivo and in vitro have uniform microtubule orientation (Dombeck *et al*., 2003). Uniform plus-end-out microtubule orientation thus seems to be a universal and evolutionarily conserved signature of axons.

Similarly, mixed orientation of microtubules has been considered a signature of dendrites (Alberts *et al*., 2002). However, dendrites are generally much more difficult to study, and their microtubule organization has been examined much less than that of axons. The hook method has been used to analyze dendritic microtubule orientation in one type of neuron with branched dendrites in vivo: frog mitral cells, which are interneurons. In these dendrites, approximately equal numbers of microtubules had plus and minus ends distal to the cell body throughout the length of the dendrite (Burton, 1988). Both hook labeling and microtubule plus end-binding protein dynamics have been used to analyze microtubule orientation in dendrites in cultured rodent interneurons. In proximal dendrites, both methods showed mixed microtubule orientation, with roughly equal numbers pointing in each direction. Close to dendrite growth cones, most microtubules had plus ends out (Baas *et al*., 1988; Stepanova *et al*., 2003). Thus, the prevailing model of micro-

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Abbreviations used: da, dendritic arborization.

tubule orientation in vertebrate neurons is mixed in proximal dendrites, and plus-end-out in distal dendrites (Figure 1).

Dendrites contain organelles and proteins, including rough endoplasmic reticulum, the Golgi complex, and neurotransmitter receptors, that are rare in axons (Craig and Banker, 1994). One simple way to generate this asymmetry would be to selectively transport proteins and organelles into dendrites with a minus end-directed motor, because minus-end-out microtubules are only present in dendrites (Black and Baas, 1989). Selective transport of several types of vesicles from the cell body into dendrites has been observed previously (Burack *et al*., 2000; Rosales *et al*., 2005). Dendrites also lose dendritic shape and organelles when minus-end-out microtubules are reduced (Yu *et al*., 2000). However, the role of minus-end-out microtubules in selective transport into dendrites has been called into question for several reasons. First, most organelles can move bidirectionally and so seem to have both plus- and minus-end–directed motors associated with them (Welte, 2004). Second, cultured neurons have a region at their tip in which minus-end-out microtubules are rare (Baas *et al*., 1988). Third, plus-end–directed kinesins have been found in association with neurotransmitter receptors (Setou *et al*., 2000, 2002). Thus, current models of directional transport into dendrites rely on specific targeting of kinesins or kinesin-cargo pairs to plus-end-out dendritic microtubules (Setou *et al*., 2004; Hirokawa and Takemura, 2005; Kennedy and Ehlers, 2006; Levy and Holzbaur, 2006), and the role of minus-end-out microtubules in polarized transport and neuronal polarity is not clear.

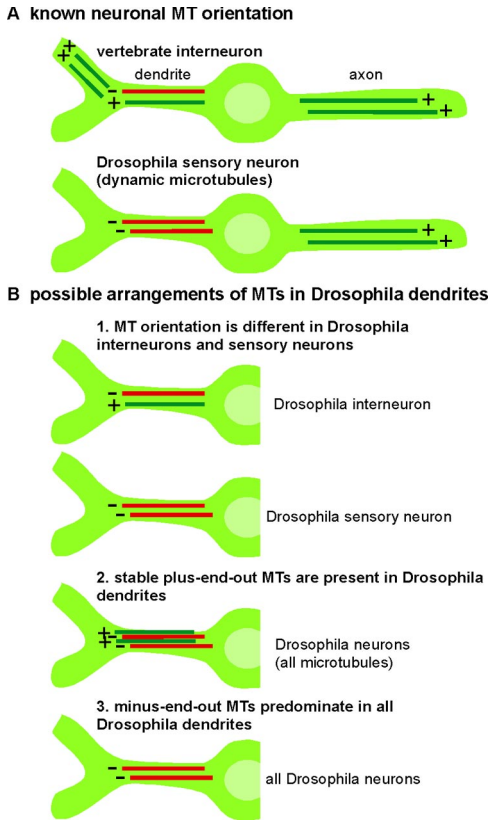


Figure 1. Known microtubule orientation in vertebrate and *Drosophila* da neurons, and possible scenarios for the arrangement of microtubules in fly dendrites. (A) In frog mitral cells and cultured rodent interneurons, microtubules in dendrites have mixed orientation, whereas in *Drosophila* da neurons ~95% have minus ends distal to the cell body based on EB1-GFP dynamics. In all neurons examined, plus-end-out microtubules predominate in axons. (B) The failure to find a significant population of plus-end-out microtubules in da neuron dendrites can be accounted for by several explanations. 1) The arrangement of microtubules in sensory da dendrites could be different from the arrangement in interneuron dendrites. 2) Analysis of microtubule orientation by EB1-GFP dynamics could have missed a significant population of stable plus-end-out microtubules. 3) Minus-end-out microtubules could predominate in all *Drosophila* neurons.

However, recent analysis of microtubule orientation in branched *Drosophila* sensory neurons raised the possibility that minus-end-out microtubules may actually be the most important component of the dendritic microtubule cytoskeleton. Using EB1-green fluorescent protein (GFP) dynamics to infer microtubule orientation, >95% of microtubules in dendritic arborization (da) dendrites were found to have minus ends distal to the cell body (Rolls *et al*., 2007). Several possible explanations exist for the difference between these results and those in vertebrate neurons: 1) dendrites of sensory neurons (examined in flies) have different arrangements of microtubules than interneurons (examined in vertebrates); 2) EB1-GFP dynamics revealed the orientation of a special subset of microtubules, and stable plus-end-out microtubules are present in *Drosophila* dendrites; or 3) all *Drosophila* dendrites have mostly minus-end-out microtubules (Figure 1).

To distinguish between these possibilities, we used EB1-GFP dynamics to generate a complete map of microtubule orientation in all major classes of *Drosophila* neurons: sensory neurons, interneurons, and motor neurons. We found that minus-end-out microtubules predominate in dendrites from all three types of neurons, and we propose that minus-end-out microtubules are a conserved signature of dendrites. Our map of microtubule orientation makes very specific predictions about the layout of microtubule tracks at dendrite branch points: that microtubules run between the cell body and dendrites, but not from one dendrite to the other. To test the completeness of our map, we analyzed the layout of stable microtubules and the paths taken by endosomes at dendrite branch points. Results from both methods agreed with predictions of the map based on EB1-GFP dynamics, and they were inconsistent with a set of stable plus-end-out microtubules in dendrites.

An even more striking test of our microtubule map was offered by unipolar neurons. Our microtubule map predicts that there are no continuous tracks for cargo transport between the cell body and dendrites of unipolar neurons. We confirmed this prediction by tracking endosomes in unipolar neurons. The absence of a direct route between the cell body and dendrites makes sense only in the context of our microtubule map.

MATERIALS AND METHODS

Fly Stocks

Gal4 driver lines including elav-Gal4 on the second and third chromosomes, RN2-Gal4, and P{GawB}109(2)80 as well as upstream activation sequence (UAS)-Rab4-red fluorescent protein (RFP) were provided by the Bloomington Drosophila Stock Center (Department of Biology, Indiana University, Bloomington, IN). P{Wee}tau[304]was generated for this study in a protein trap screen (Clyne *et al*., 2003). UAS:EB1-GFP lines were provided by Tadashi Uemura (Kyoto University, Kyoto, Japan).

Live Imaging of Da Neurons

Da neurons were imaged in whole, live second instar larvae. Larvae were either placed on a slide with halocarbon 27 oil (Sigma-Aldrich, St. Louis, MO), then covered with a coverslip and imaged within 10 min of mounting, or they

were placed in a chamber slide (Kiehart *et al*., 1994) in halocarbon oil with gas-permeable membrane (YSI, Yellow Springs, OH) on one side and a coverslip on the other side. Images were collected on an LSM510 confocal microscope Carl Zeiss (Thornwood, NY). Z stacks were collected for larvae homozygous for tau-GFP to image stable microtubules. Single frames were collected every 2 s to image EB1-GFP dynamics at dendrite tips. Larvae heterozygous for the 109(2)80 Gal4 and UAS:EB1-GFP were used for this experiment.

Live Imaging of Central Neurons

Embryos either heterozygous for the RN2-Gal4 and UAS:EB1-GFP, or homozygous for both transgenes, and RN2-Gal4, UAS:EB1-GFP; UAS-Rab4-RFP homozygous embryos, were collected for 5 h on apple caps with yeast paste and aged for 2 d on the cap at room temperature. Larvae were then transferred to standard fly media for 1 d at room temperature. Brains were then dissected from these larvae and cultured in imaging chambers as described previously (Siller *et al*., 2005). Images in a single plane were collected every 1 or 2 s with a Zeiss LSM510 confocal microscope. Movies were made with ImageJ (http://rsbweb.nih.gov/ij/) and QuickTime.

Analysis of EB1-GFP and Rab4-RFP Dynamics

Moving structures were manually tracked in movies. Only structures that could be clearly observed in three consecutive frames were included. For statistical analysis, data were divided into sets based on order of acquisition; so, for example, if 16 brains were imaged as for Figure 7, the first five animals would be one group, the second five would be another group, and the last six would be the final group. This grouping allowed us to calculate standard deviations. The numbers of objects from single animals was too low to consider each animal separately. Information about numbers of animals in sets, and total numbers of fluorescent objects counted, is in the figure legends.

RESULTS

Microtubules Are Predominantly Minus-End-Out in Proximal Branched Sensory Dendrites, and Mixed Orientation in Sensory Dendrite Tips

We previously examined microtubule orientation in *Drosophila* sensory neurons with branched dendrites (Rolls *et al*., 2007). In that study, we analyzed microtubule orientation in proximal dendrites, and we grouped data from different subclasses of dendritic arborization neurons. To complete and refine the map of microtubule orientation in *Drosophila* sensory neurons, we analyzed EB1-GFP dynamics in both the tips, and main trunk, of dendrites in a single type of class I dendritic arborization neuron, ddaE (Figure 2 and Supplemental Movie 1). We expressed EB1-GFP in da neurons by using the pan-neuronal elav-Gal4 line and performed live imaging of EB1-GFP in whole larvae. EB1-GFP dots moved in one direction, and then disappeared, consistent with binding only to growing microtubule plus ends. Dots moving away from the cell body therefore indicated plus-end-out microtubules, and dots moving toward the cell body indicated minus-end-out microtubules.

In the main dendrite trunk (Figure 2, B and C, arrowhead) 94 ± 3.5% of EB1-GFP dots, moved toward the cell body, representing minus-end-out microtubules. This is similar to our previous results in mixed classes of da neurons (Rolls *et al*., 2007). However, distal to the last branch point, EB1-GFP dynamics indicated a more mixed population of microtubules, with only 55 ± 15% minus-end-out (Figure 2, B and C, arrows). This increase in plus-end-out microtubules at dendrite tips is very similar to that seen in dendrites of cultured vertebrate neurons.

Most Microtubules in Drosophila Interneuron Dendrites Have Minus Ends Distal to the Cell Body

Sensory dendrites are not postsynaptic; so, they could have a different arrangement of microtubules than central dendrites. To determine the arrangement of dendritic microtubules in central neurons, we imaged EB1-GFP dynamics in an interneuron in the larval brain. Live imaging of subcellular structures has not been performed previously in *Drosophila* central neuron dendrites. Central dendrites are generally embedded deep within the nervous system, and they have complex three-dimensional structures. To identify a central neuron with dendrites amenable to live imaging, we screened through Gal4 lines that drive expression in subsets of neurons. The RN2-Gal4 line expresses Gal4 under control of part of the *eve* enhancer (Fujioka *et al*., 2003). In 3-d-old larvae, it reproducibly expressed UAS-controlled EB1-GFP in a single isolated neuron per brain lobe. In larvae with one

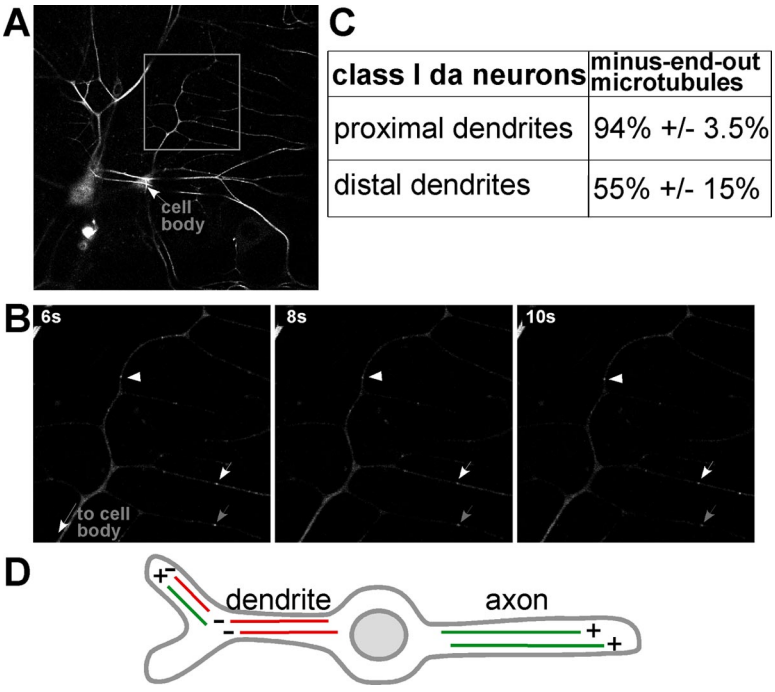


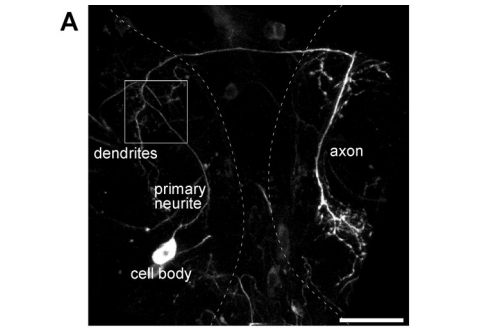
Figure 2. Microtubule orientation in dendrites of a type I da sensory neuron. (A) An overview of EB1-GFP in the region of the dorsal cluster of the peripheral nervous system in a live larva is shown. The cell body of ddaE is indicated with an arrow. Cell bodies of other peripheral neurons are to the left. The boxed region of ddaE dendrites was chosen for higher power imaging, and frames were acquired every 2 s. Three frames are shown in B. Two EB1-GFP dots that move toward the cell body are indicated with a white arrow and arrowhead, and a dot in a distal dendrite that moves away from the cell body is indicated with a gray arrow. (C) Microtubule orientation derived from this type of data is summarized in the table. Data were derived from four groups of larvae (3 groups of 9 larvae and 1 group of 10 larvae). A single da neuron was analyzed in each larva. In total, 143 EB1-GFP dots were counted in the main dendrite trunk and 54 dots in distal dendrites. (D) Diagram of microtubule orientation in da neurons. Bar, 20 μm (A) and 10 μm (B).

copy of RN2-Gal4 and UAS-EB1-GFP, expression was often seen in only one lobe (Figure 3A), whereas with two copies of each transgene, expression was seen in both lobes (data not shown). We could identify dendrites and axons of this neuron based on morphology: dendrites branched off the primary neurite on the same side of the brain as the cell body, and axons continued to the other brain lobe where they branched and often had large bulb-like terminals that likely house presynaptic specializations (Figure 3A). Unlike the da neurons, this neuron is unipolar. This arrangement of axons and dendrites branching from a single process, or primary neurite, that arises from the cell body is frequently seen in invertebrates (Craig and Banker, 1994; Sanchez-Soriano *et al*., 2005), but it can also be present in familiar systems used for neuronal polarity including cultured rodent hippocampal neurons (see, for example, Figure 4 in Burack *et al*., 2000).

In brain explants, we could image EB1-GFP movements in the primary neurite, dendrites, and axons of this cell (Figure 3B and Supplemental Movie 2). In axons and the primary neurite, all dots moved away from the cell body, confirming that plus-end-out microtubules are a general feature of *Drosophila* axons. In dendrites 87.6 ± 2.5% of EB1-GFP dots moved toward the cell body (Figure 3C). Thus, as in peripheral neurons, almost all microtubules in dendrites of an interneuron in the larval brain are minus-end-out.

Minus-End-Out Microtubules Predominate in Drosophila Motor Neuron Dendrites

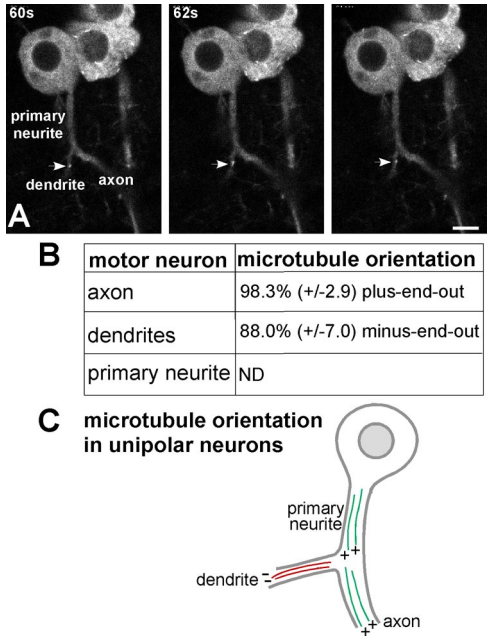
To determine whether all *Drosophila* neurons are likely to have a similar arrangement of microtubules, we extended our analysis of microtubule orientation to one more type of neuron. Motor neurons have been well characterized in *Drosophila*, and they have dendrites that are in many ways similar to vertebrate dendrites (Sanchez-Soriano *et al*., 2005). RN2-Gal4 drives expression of UAS-controlled transgenes in three neurons per hemisegment in the *Drosophila* embryonic ventral nerve cord. These cells have been identified as the motor neurons RP2 and aCC and the interneuron pCC (Fujioka *et al*., 2003). We were not able to image EB1-GFP dynamics in embryos, but expression in these cells persisted in larvae that were homozygous for RN2-Gal4 and UAS-EB1-GFP. In larvae, motor neuron dendrites were much longer and more branched than in embryos, and we could use brain explants that included the ventral ganglion to image EB1-GFP dynamics in them (Figure 4 and Supplemen-



C

interneuron	microtubule orientation
axon	100% plus-end-out
dendrites	87.6% (+/-2.5) minus-end-out
primary neurite	100% plus-end-out

Figure 3. Microtubule orientation in an interneuron. (A) Overview of the single neuron in larval brains that expresses bright EB1-GFP in response to the RN2-Gal4. The image is a projection of a confocal stack. Dendrites branch from the primary neurite on the same side of the brain as the cell body. An axon crosses to the other brain lobe and makes terminal branches with large synaptic boutons. Approximate outline of brain lobes is indicated with dotted line. Image was rotated from original and placed on a black background. (B) Three frames from a movie of a different brain are shown. The area shown in the frames is similar to the boxed area in A, with the dendrite at the left. Two EB1-GFP dots, indicated by arrows, move toward the point at which the dendrite branches from the primary neurite, and thus toward the cell body. (C) Quantitation of EB1-GFP movements. Dots were tracked in the axon just beyond the point at which the dendrites branch off, in proximal dendrites, and in the primary neurite. Two groups of six and one group of five brains were analyzed, with a total of 64 EB1-GFP dots. Bar, 25 μm (A) and 10 μm (B).



B

motor neuron	microtubule orientation
axon	98.3% (+/-2.9) plus-end-out
dendrites	88.0% (+/-7.0) minus-end-out
primary neurite	ND

C

Figure 4. Microtubule orientation in motor neurons. (A) Three consecutive frames from a movie of EB1-GFP dynamics in the ventral ganglion of a brain explant are shown. EB1-GFP expression is controlled by RN2-Gal4. The motor neuron axons were easily identifiable as they leave the ventral ganglion in a motor nerve. Dendrites branch from the primary neurite and make complex arborizations in the ventral ganglion. We focused mostly on the proximal region of the dendrite to avoid ambiguities from crossing dendrite branches. Arrows indicate an EB1-GFP dot in a dendrite that moves toward the cell body and thus is at the tip of a minus-end-out microtubule. (B) Quantitation of microtubule orientation in motor neuron axons and dendrites derived from EB1-GFP dynamics. We were unable to quantitate data in the primary neurite due to overlapping structures. Axon data were acquired from two groups of six and one group of seven brains, with a total of 45 EB1-GFP dots counted. Dendrite data were acquired from two sets of 12 and one set of 13 brains, with a total of 69 dots counted. (C) Diagram of microtubule orientation in unipolar neurons, based on data from the interneuron in Figure 3 and motor neurons. Bar, 5 μm (A).

tal Movie 3). In axons of RP2 or aCC motor neurons, most microtubules were plus-end-out as expected (Figure 4B). In dendrites, results were similar to those in da neurons and interneurons, with 88.0 ± 7% minus-end-out microtubules (Figure 4). We were unable to image EB1-GFP dynamics in the primary neurite, as other structures overlapped this part of the cell.

Because axonal and dendritic microtubules have a similar arrangement in all major types of *Drosophila* neurons (summarized in Figures 2D and 4C) based on EB1-GFP dynamics, we can eliminate neuron subtype differences (Figure 1B) as an explanation for the difference between mammalian and *Drosophila* dendritic microtubule orientation. We next tested whether EB1-GFP dynamics might have missed a population of stable plus-end-out dendritic microtubules.

Organization of Stable Microtubules at Dendrite Branch Points Is Consistent with a Predominantly Minus-End-Out Microtubule Orientation

Our map of microtubule orientation makes specific predictions about the layout of microtubules at dendrite branch points. We tested whether our map, which is based on dynamic microtubules, also represents the organization of stable microtubules in dendrites by using two methods: imaging stable microtubules at dendrite branch points, and imaging movements of cargo along microtubule tracks at dendrite branch points.

If almost all microtubules in dendrites have a minus-end-out orientation, then very few microtubules should extend from one dendrite to the other at a branch point; they should mostly extend between the cell body and dendrites (Figure 5A). To visualize stable microtubules clearly, we used a GFP protein trap line that contains the GFP coding sequence inserted into the genomic copy of the *Drosophila* tau gene (see Clyne *et al*., 2003 for a description of how these lines were generated). This insertion results in expression of tau-GFP from the endogenous promoter, and in larvae fluorescence is seen in da neurons, but not the surrounding tissues. Unlike mammalian tau, which binds only axonal microtubules, Drosophila tau is found on microtubules in both axons and dendrites, although in some neurons it is enriched in the first part of the axon (Rolls *et al*., 2007). This difference may be because tau is the only *Drosophila* member of the family of proteins that also includes the broadly expressed microtubule-associated protein (MAP) 4 and dendritic MAP2 in mammals.

We assayed the path of tau-labeled microtubules at dendrite branch points in live larvae (Figure 5B). Like many vertebrate neurons, da neurons are multipolar, so that one axon and several dendrites arise directly from the cell body. If microtubules were seen to cross from one branch to another at triangular branch points, then one of the dendrites distal to that point must contain a plus-end-out microtubule, and the other a minus-end-out microtubule (Figure 5A, thick red line). We classified large dendrite branches near the cell body into three groups: dendrite-to-dendrite microtubule present, absent, or not determined (ND). We examined larvae in three sets, each time trying different imaging conditions to reduce the number of blurry branches we could not classify into "present" or "absent." The absent-to-present ratio was quite constant, independently of how many blurry branches were in the images. Most of the clear branches did not contain a microtubule that extended from one distal dendrite to the other (Figure 5B), consistent with predictions from our map of dynamic microtubules.

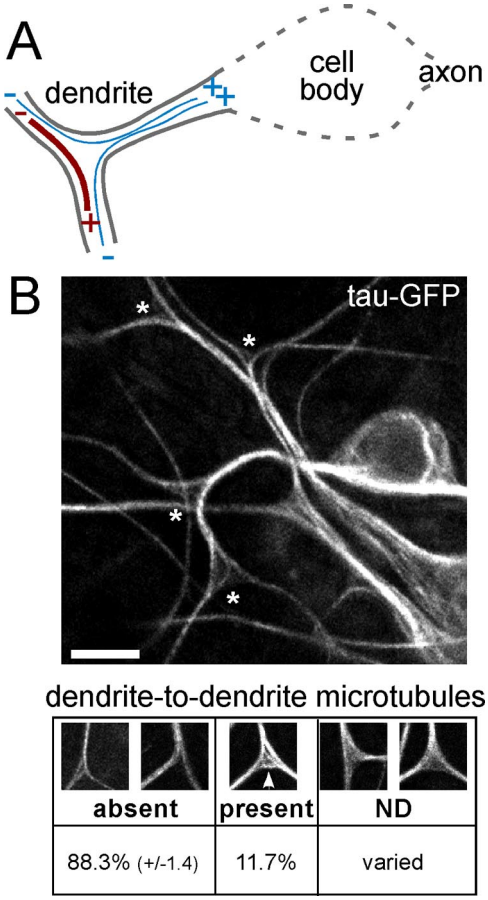


Figure 5. The arrangement of stable microtubules at da dendrite branch points is consistent with most microtubules in dendrites having the same orientation. (A) EB1-GFP dynamics indicated that minus-end-out microtubules are present in da dendrites (thin blue lines). To determine whether plus-end-out microtubules are also abundant, we assayed dendrite branch points for microtubules that form dendrite-to-dendrite bridges (thick red line). (B) A tau-GFP protein trap line was used to visualize microtubules in da dendrites in whole, living larvae. A single plane from an image stack acquired with a confocal microscope is shown. Stars indicate triangular branch points with clear microtubules that run between the cell body and dendrite branches, but not from one dendrite branch to another. Examples of different classes of branch points are shown: first two images are examples of branches in which a dendrite-to-dendrite microtubule is absent, third image is a branch in which a dendrite-to-dendrite microtubule is present (arrow points to dendrite-to-dendrite microtubule), and last two images were not determined (ND) because the image was not clear at the branch. In each panel, the top of the image is closest to the cell body. Branches were classified into these groups in three experiments with slightly different imaging conditions. The number of ND branches varied depending on the imaging conditions, and so was not included in the percentage. The percentage of branches without dendrite-to-dendrite microtubules varied very little. We analyzed 117 images, with 194 branches in the first two categories, and 88 ND. Bar, 5 μm (B).

Endosome Movements at Dendrite Branches Follow Predicted Tracks

Like EB1-GFP, it is possible that tau-GFP gives an incomplete picture of dendritic microtubules; so, we also tested our predictions about microtubule layout with another method. We reasoned that tracking the movement of cargo along microtubules would give an additional readout of their arrangement. We labeled endosomes in neurons ex-

pressing EB1-GFP with Rab4-RFP and followed their movement at dendrite branch points (Figure 6 and Supplemental Movie 4). The Rab4-RFP transgene has not been extensively characterized, but its localization to punctate structures throughout neurons in our study and a previous study (Sweeney, 2006) is consistent with endosomal localization. Like other organelles (Welte, 2004), Rab4-RFP endosomes moved bidirectionally along microtubules. Overall, more moved toward than away from the cell body, consistent with net movement of endosomes from the periphery to the cell center. Endosomes that traveled completely through the branch were classified as moving between the dendrite and cell body (97.2 ± 1.7%) or from dendrite to dendrite (1.7 ± 2.9%). Consistent with our predictions about the layout of microtubule tracks, very few endosomes moved from dendrite to dendrite. This result suggests (although does not completely rule out) that there is not a stable population of plus-end-out microtubules that is invisible in experiments with EB1-GFP.

A Direct Track between the Cell Body and Dendrites Is Absent in Unipolar Motor Neurons

To test our map of microtubule orientation more rigorously, we used the Rab4-RFP motility assay to test a surprising and nonintuitive prediction of the map. In dendrites, the prediction that most microtubule tracks run between the cell body and dendrite, rather than from dendrite to dendrite, makes sense based on the necessity of transporting cargo between the site of most protein synthesis, the cell body, and dendrites. In unipolar neurons our orientation map (Figure 4C) predicts very few tracks between the cell body and dendrites (Figure 7A).

To determine whether cargo follows tracks predicted by our microtubule map, or the more logical direct route between cell body and dendrites in unipolar neurons, we imaged Rab4-RFP-labeled endosomes in motor neurons at the primary neurite–dendrite–axon junction (Figure 7 and Supplemental Movie 5). Of endosomes that moved through the branch (as opposed to stopping in it), we found that 41.1 ± 8.4% traveled between cell body to axon (movement in both directions was pooled). However, only 5.6 ± 5.1% moved between the cell body and dendrite, whereas 53.3 ± 13.3% moved between the dendrite and axon. Thus, consistent with our prediction, many unipolar neurons in *Drosophila* do not seem to have tracks for direct transport between the cell body and dendrites. Instead, cargo must follow an indirect route, either via the axon, or a fusing and budding event with other membranes in the dendrite branch. This result would be very surprising in a context other than our microtubule map.

DISCUSSION

By examining microtubule orientation with EB1-GFP dynamics in axons and dendrites of all major classes of fly neurons, we have generated a map of neuronal microtubule polarity in flies. As in all neurons so far examined, axonal microtubules were oriented with plus ends distal to the cell body. Unlike vertebrate neurons, in which dendritic microtubules were analyzed previously, all major classes of *Drosophila* neurons had the majority of dendritic microtubules oriented with minus ends distal to the cell body. In da dendrites, we were able to analyze microtubule orientation at the distal tip, and we found that in the last dendritic segment microtubules had mixed orientation. We also used two other methods to try to identify a stable population of plus-end-out dendritic microtubules, and we could not find any evidence for these.

Our general picture of microtubule orientation in *Drosophila* neurons (Figures 2D and 4C) is thus a more polarized version of vertebrate neuronal microtubule orientation (Figure 1). The predominance of minus-end-out microtubules in

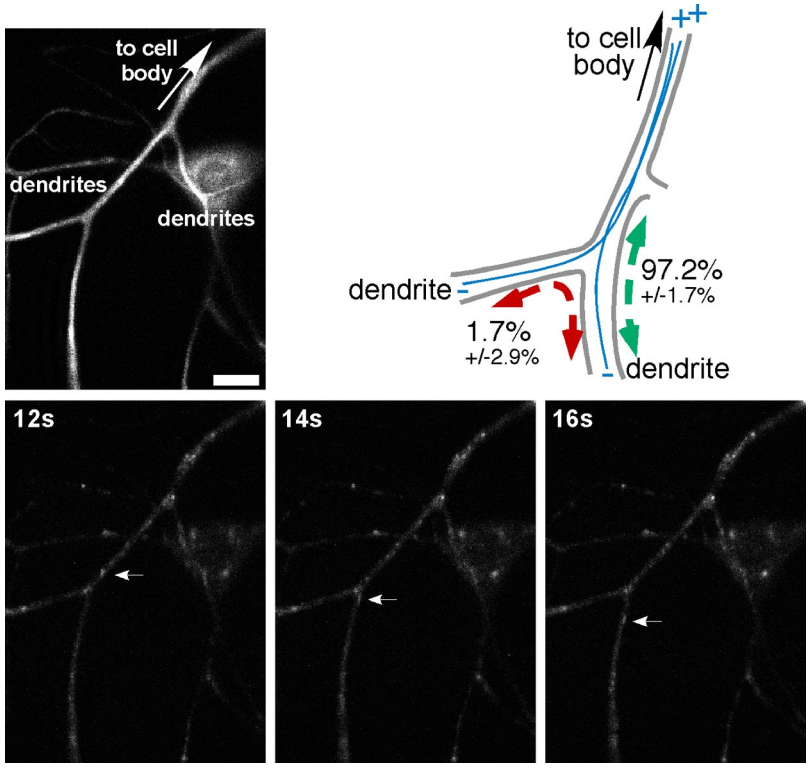


Figure 6. Routes of endosome transport in da neuron dendrites. Rab4-RFP was imaged in da neurons in larvae heterozygous for the 109(2)80 Gal4 driver, UAS-EB1-GFP, and UAS-Rab4-RFP. Movies were acquired at 2-s intervals. For the example shown, the first image from the EB1-GFP track shows the layout of the dendrites (top left). The cell body is out of the frame at the top. These neurons are multipolar, so all processes in the image are dendrites. Three frames from the Rab4-RFP track are shown. An endosome moving from the cell body side of the branch out into a distal dendrite is indicated with an arrow. Movements of endosomes through dendrite branch points are summarized in the diagram. Predicted microtubule tracks are drawn in blue. Endosomes that moved through a branch were categorized as traveling between cell body and distal dendrite (green double headed arrow), or from dendrite to dendrite (red double arrow). Two groups of six larvae and one group of five were analyzed, and the total number of endosomes counted was 43. Bar, 5 μm.

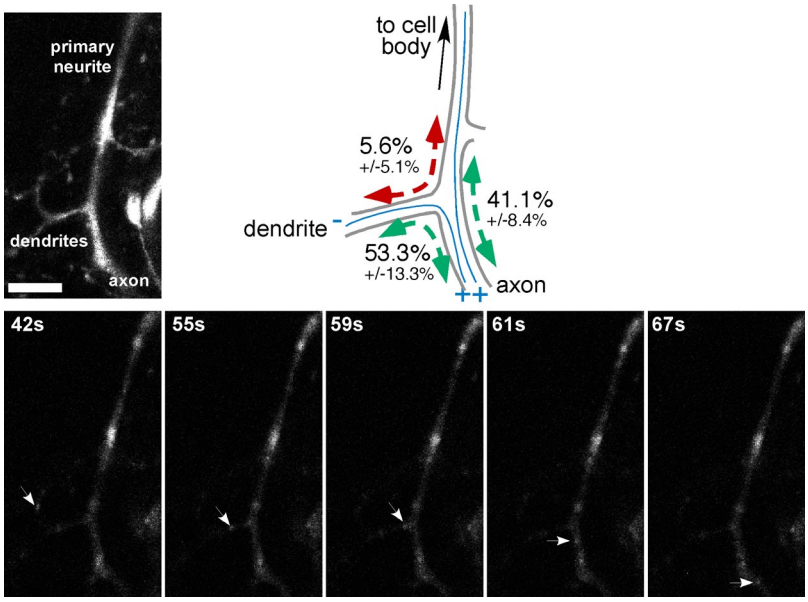


Figure 7. Routes of endosome transport in motor neurons. Endosomes in motor neurons were labeled with RFP in flies homozygous for RN2-Gal4, UAS-EB1-GFP, and UAS-Rab4-RFP. Movies were acquired at 1- or 2-s intervals: 1 s in the example shown. Two frames in different focal planes from the EB1-GFP track were summed to give an overview (top left). The cell body is out of the frame at the top, and the axon exits at the bottom. Five frames from the Rab4-RFP track are shown. An endosome that travels from the dendrites to the axon is indicated with arrows. The whole movie is available as Supplemental Movie 5. A summary of routes taken through the primary neurite–axon–dendrite branch is shown at top right. The predicted layout of microtubules is indicated by blue lines. Endosomes that moved through the branch were classified as moving between the cell body and dendrites (red double arrow), between cell body and axon (green double arrow at right), or between dendrite and axon (green double arrow at left). Two groups of five, and one group of six, larval brains were analyzed. Total number of endosomes counted was 34. Bar, 5 μm.

proximal dendrites, which ranged from 88 to 94% in different classes of neurons, suggests that minus-end-out microtubules, rather than mixed orientation microtubules, are the defining feature of the dendritic microtubule cytoskeleton.

In addition to analyzing microtubule orientation in axons and dendrites, we obtained results in the primary neurite of a unipolar interneuron. Models of neuronal compartmentalization generally divide neurons into two major regions: somatodendritic and axonal (Craig and Banker, 1994). Because axonal and dendritic cargoes must both pass through the primary neurite, the expected microtubule arrangement was not clear. We show that the primary neurite has the same arrangement of microtubules as the axon. This result generates the surprising prediction that cargo cannot take a direct route from the primary neurite into dendrites or back. We confirmed this prediction by imaging movements of endosomes at the branch point. In a previous study of vesicle movement in cultured rat hippocampal neurons, transport at a similar branch was analyzed. In these cells, vesicles could travel directly between the primary neurite and dendrite (Burack *et al*., 2000), consistent with the presence of mixed orientation microtubules in these dendrites.

The extreme polarity of microtubules in *Drosophila* neurons raises questions about how dendrite microtubule polarity is established, and it has important implications for models of polarized neuronal transport. One mechanism that contributes to dendritic microtubule organization has been described. The dendritic kinesin MKLP1 (Xu *et al*., 2006) is proposed to transport minus-end-out microtubules into dendrites along plus-end-out microtubules (Sharp *et al*., 1997; Yu *et al*., 2000). In *Drosophila*, other mechanisms must account for minus-end-out microtubules in dendrites because there are so few plus-end-out microtubules present. It will be extremely interesting to determine how a minus-end-out microtubule array is generated in proximal dendrites.

The predominance of minus-end-out microtubules in *Drosophila* dendrites suggests that, at least in this organism, minus-end–directed motors are the primary motors for anterograde dendritic traffic and that microtubule polarity could be key to polarized neuronal transport. The only essential minus-end–directed motor in *Drosophila* is dynein. If dynein is required for the bulk of transport into dendrites but not axons, one would expect mutations in dynein subunits to significantly affect dendrite, but not axon outgrowth. In fact, *Drosophila* neurons with mutations in cytoplasmic dynein or *Lis1* exhibit specific reduction of dendrite growth. In clones of neurons with mutations in *Lis1* or *Dhc64C*, dendrite growth was severely reduced, but axons were normal length (Liu *et al*., 2000). At the time these phenotypes were reported, the specific effect of dynein loss on dendrite growth was difficult to explain, and it was suggested to arise from sensitivity of a population of minus-end-out microtubules to loss of dynein, or greater overall structural changes of dendrites than axons during development (Liu *et al*., 2000). Our analysis of microtubule orientation in *Drosophila* dendrites offers an extremely simple interpretation for the selective effect of dynein mutations on dendrite growth: that dynein is the major motor for transport of membranes and proteins required for dendrite growth from the cell body into dendrites.

Cargo can be selectively transported into dendrites in cultured hippocampal neurons (Burack *et al*., 2000; Rosales *et al*., 2005), but the role of microtubule orientation in this, or any other, aspect of neuronal polarity is not clear. We expect that the very simple and highly polarized layout of microtubules in *Drosophila* neurons will allow the function and significance of microtubule orientation in neurons to be uncovered.

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REFERENCES

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell, New York: Garland Science.

Baas, P. W., Deitch, J. S., Black, M. M., and Banker, G. A. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. Proc. Natl. Acad. Sci. USA *85*, 8335–8339.

Baas, P. W., White, L. A., and Heidemann, S. R. (1987). Microtubule polarity reversal accompanies regrowth of amputated neurites. Proc. Natl. Acad. Sci. USA *84*, 5272–5276.

Black, M. M., and Baas, P. W. (1989). The basis of polarity in neurons. Trends Neurosci. *12*, 211–214.

Burack, M. A., Silverman, M. A., and Banker, G. (2000). The role of selective transport in neuronal protein sorting. Neuron *26*, 465–472.

Burton, P. R. (1988). Dendrites of mitral cell neurons contain microtubules of opposite polarity. Brain Res. *473*, 107–115.

Burton, P. R., and Paige, J. L. (1981). Polarity of axoplasmic microtubules in the olfactory nerve of the frog. Proc. Natl. Acad. Sci. USA *78*, 3269–3273.

Clyne, P. J., Brotman, J. S., Sweeney, S. T., and Davis, G. (2003). Green fluorescent protein tagging Drosophila proteins at their native genomic loci with small P elements. Genetics *165*, 1433–1441.

Craig, A. M., and Banker, G. (1994). Neuronal polarity. Annu. Rev. Neurosci. *17*, 267–310.

Dombeck, D. A., Kasischke, K. A., Vishwasrao, H. D., Ingelsson, M., Hyman, B. T., and Webb, W. W. (2003). Uniform polarity microtubule assemblies imaged in native brain tissue by second-harmonic generation microscopy. Proc. Natl. Acad. Sci. USA *100*, 7081–7086.

Erez, H., Malkinson, G., Prager-Khoutorsky, M., De Zeeuw, C. I., Hoogenraad, C. C., and Spira, M. E. (2007). Formation of microtubule-based traps controls the sorting and concentration of vesicles to restricted sites of regenerating neurons after axotomy. J. Cell Biol. *176*, 497–507.

Fujioka, M., Lear, B. C., Landgraf, M., Yusibova, G. L., Zhou, J., Riley, K. M., Patel, N. H., and Jaynes, J. B. (2003). Even-skipped, acting as a repressor, regulates axonal projections in *Drosophila*. Development *130*, 5385–5400.

Heidemann, S. R., Landers, J. M., and Hamborg, M. A. (1981). Polarity orientation of axonal microtubules. J. Cell Biol. *91*, 661–665.

Hirokawa, N., and Takemura, R. (2005). Molecular motors and mechanisms of directional transport in neurons. Nat. Rev. Neurosci. *6*, 201–214.

Kennedy, M. J., and Ehlers, M. D. (2006). Organelles and trafficking machinery for postsynaptic plasticity. Annu. Rev. Neurosci. *29*, 325–362.

Kiehart, D. P., Montague, R. A., Rickoll, L., Thomas, G. L., and Foard, D. (1994). High-resolution microscopic methods for the analysis of cellular movements in *Drosophila* embryos. In: *Drosophila melanogaster*: practical uses in cellular and molecular biology, ed. L.S.B. Goldstein and E. A. Fyrberg, San Diego: Academic Press, 507–532.

Levy, J. R., and Holzbaur, E. L. (2006). Cytoplasmic dynein/dynactin function and dysfunction in motor neurons. Int. J. Dev. Neurosci. *24*, 103–111.

Liu, Z., Steward, R., and Luo, L. (2000). Drosophila Lis1 is required for neuroblast proliferation, dendritic elaboration and axonal transport. Nat. Cell Biol. *2*, 776–783.

Rolls, M. M., Satoh, D., Clyne, P. J., Henner, A. L., Uemura, T., and Doe, C. Q. (2007). Polarity and compartmentalization of *Drosophila* neurons. Neural Dev. *2*, 7.

Rosales, C. R., Osborne, K. D., Zuccarino, G. V., Scheiffele, P., and Silverman, M. A. (2005). A cytoplasmic motif targets neuroligin-1 exclusively to dendrites of cultured hippocampal neurons. Eur. J. Neurosci. *22*, 2381–2386.

Sanchez-Soriano, N., Bottenberg, W., Fiala, A., Haessler, U., Kerassoviti, A., Knust, E., Lohr, R., and Prokop, A. (2005). Are dendrites in *Drosophila* homologous to vertebrate dendrites? Dev. Biol. *288*, 126–138.

Setou, M., Hayasaka, T., and Yao, I. (2004). Axonal transport versus dendritic transport. J. Neurobiol. *58*, 201–206.

Setou, M., Nakagawa, T., Seog, D. H., and Hirokawa, N. (2000). Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. Science *288*, 1796–1802.

Setou, M., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M., and Hirokawa, N. (2002). Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. Nature *417*, 83–87.

Sharp, D. J., Yu, W., Ferhat, L., Kuriyama, R., Rueger, D. C., and Baas, P. W. (1997). Identification of a microtubule-associated motor protein essential for dendritic differentiation. J. Cell Biol. *138*, 833–843.

Siller, K. H., Serr, M., Steward, R., Hays, T. S., and Doe, C. Q. (2005). Live imaging of *Drosophila* brain neuroblasts reveals a role for Lis1/dynactin in spindle assembly and mitotic checkpoint control. Mol. Biol. Cell *16*, 5127–5140.

Stepanova, T., Slemmer, J., Hoogenraad, C. C., Lansbergen, G., Dortland, B., De Zeeuw, C. I., Grosveld, F., van Cappellen, G., Akhmanova, A., and Galjart, N. (2003). Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). J. Neurosci. *23*, 2655–2664.

Sweeney, N. T., Brenman, J. E., Jan, Y. N., and Gao, F. B. (2006). The coiled-coil protein shrub controls neuronal morphogenesis in Drosophila. Curr. Biol. *16*, 1006–1011.

Troutt, L. L., and Burnside, B. (1988). Microtubule polarity and distribution in teleost photoreceptors. J. Neurosci. *8*, 2371–2380.

Welte, M. A. (2004). Bidirectional transport along microtubules. Curr. Biol. *14*, R525–R537.

Xu, X., He, C., Zhang, Z., and Chen, Y. (2006). MKLP1 requires specific domains for its dendritic targeting. J. Cell Sci. *119*, 452–458.

Yu, W., Cook, C., Sauter, C., Kuriyama, R., Kaplan, P. L., and Baas, P. W. (2000). Depletion of a microtubule-associated motor protein induces the loss of dendritic identity. J. Neurosci. *20*, 5782–5791.