γ-Tubulin controls neuronal microtubule polarity independently of Golgi outposts

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ABSTRACT

Neurons have highly polarized arrangements of microtubules, but it is incompletely understood how microtubule polarity is controlled in either axons or dendrites. To explore whether microtubule nucleation by γ-tubulin might contribute to polarity, we analyzed neuronal microtubules in Drosophila containing gain- or loss-of-function alleles of γ-tubulin. Both increased and decreased activity of γ-tubulin, the core microtubule nucleation protein, altered microtubule polarity in axons and dendrites, suggesting a close link between regulation of nucleation and polarity. To test whether nucleation might locally regulate polarity in axons and dendrites, we examined the distribution of γ-tubulin. Consistent with local nucleation, tagged and endogenous γ-tubulins were found in specific positions in dendrites and axons. Because the Golgi complex can house nucleation sites, we explored whether microtubule nucleation might occur at dendritic Golgi outposts. However, distinct Golgi outposts were not present in all dendrites that required regulated nucleation for polarity. Moreover, when we dragged the Golgi out of dendrites with an activated kinesin, γ-tubulin remained in dendrites. We conclude that regulated microtubule nucleation controls neuronal microtubule polarity but that the Golgi complex is not directly involved in housing nucleation sites.

INTRODUCTION

In all neurons in which the issue has been examined, axons and dendrites are distinguished by their arrangement of microtubules. Microtubules have a stable minus end, which is where they initiate, and dynamic plus ends that serve as sites of tubulin addition and loss. Axons always have minus-end-out microtubules, whereas at least 50% of dendritic microtubules have minus ends out (Baas and Lin, 2011; Rolls, 2011). In fact, in vivo imaging in Drosophila and Caenorhabditis elegans demonstrates that dendrites in these animals contain almost exclusively minus-end-out microtubules (Stone et al., 2008; Goodwin et al., 2012). Because differences in microtubule polarity can be used to direct different cargoes from the cell body to axons or dendrites (Kapitein et al., 2010), they are likely to be at the core of compartmental identity in neurons. It is not well understood, however,

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M.M.N. performed all γ-tubulin-related experiments, except those noted in what follows, as well as microtubule orientation assays in class I da neurons with kinesin-Golgi fusions, Apc2-GFP localization assays, and Golgi marker comparison studies. C.J.M. cloned the kinesin-Golgi fusions into fly vectors, generated transgenic fly lines from these vectors, tested for expression of the fusions in larvae, and performed the Golgi localization experiments. E.S.M. performed microtubule orientation assays in the class IV da neurons with the kinesin-Golgi fusions and analyzed dendrite morphology in class IV neurons with the fusions. D.J.G. generated kymographs for fusions and analyzed dendrite morphology in class IV neurons with the fusions. M.M.R. supervised the planning of experiments and interpretation of results. M.M.N. prepared the figures and wrote the article with M.M.R. The authors declare that they have no competing interests.

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Abbreviation used: RFP, red fluorescent protein.

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Microtubules arise primarily by γ-tubulin-mediated nucleation. γ-Tubulin serves as the core of a complex that acts as a platform for addition of α- and β-tubulin dimers (Raynaud-Messina and Merdes, 2007). In many mitotic and some interphase animal cells, γ-tubulin is localized at the centrosome and organizes microtubules into a plus-end-out array. Neurons, however, do not conform to this simple arrangement, and the site of neuronal microtubule nucleation is central to two different mechanisms that have been proposed to contribute to neuronal microtubule polarity.

The microtubule transport model posits that microtubules are nucleated in the cell body; then, small, stable microtubule seeds are released from the nucleation site and transported in a polarized way into neuronal processes (Baas and Yu, 1996; Baas and Lin, 2011). This model accounts for plus-end-out axonal microtubule polarity through the action of dynein anchored at the axon initial segment. Anchored dynein would have its motor domain free to slide plus-end-out oriented pieces of microtubules into axons and minus-end-out pieces back into the cell body. Mixed polarity in dendrites could be accounted for by the action of kinesin-6 (CHO1/MKLP1) sliding minus-end-out microtubules along plus-end-out ones (Figure 1A, model 1). The transport model was originally suggested by studies on dissociated and cultured rodent neurons (Yu et al., 1993; Sharp et al., 1995; Baas and Yu, 1996). The role of dynein in transporting plus-end-out microtubules into axons has some in vivo experimental support as well. For example, in Drosophila neurons, an increase in minus-end-out microtubules is seen in axons of dynein-subunit mutants (Zheng et al., 2008), consistent with a role for dynein in pushing these microtubules back into the cell body. However, a recent study suggested that kinesin-1 rather than dynein might drive microtubules into newly growing axons (Lu et al., 2013). There is also experimental support for transport of minus-end-out microtubules into dendrites by kinesin-6; depletion of this motor reduces numbers of dendritic minus-end-out microtubules in primary cultures of rodent neurons (Sharp et al., 1997; Lin et al., 2012).

The major source of microtubules for transport in this type of model is the centrosome (Yu et al., 1993). However, in mammalian neurons, as they mature, the centrosome becomes less active for microtubule nucleation (Stiess et al., 2010), and in Drosophila neurons, nucleation does not occur at the centrosome (Nguyen et al., 2011). These studies suggest that either nucleation complexes are found at

how neuronal microtubule polarity is established or maintained. One factor likely to be important to generate a comprehensive model of microtubule polarity is the source of new microtubules.

FIGURE 1: Proposed positions of nucleation sites in neurons and changes in number of growing microtubules in different γ-tubulin mutants. (A) Left, key features of microtubule-sliding models, including nucleation of microtubules in the cell body, filtering of correctly polarized microtubule pieces at the start of the axon, and sliding minus-end-out microtubule seeds along plus-end-out ones in dendrites. Right, alternate model in which microtubules are generated locally in axons and dendrites. (B) Effect of mutations in γ-tubulin on microtubule dynamics analyzed in ddaE neurons expressing EB1-GFP under control of 221-Gal4. An example of a ddaE neuron. The dendritic analysis was performed in the main trunk (dotted line) of the dorsal comb dendrite of this cell. Axonal analysis was performed in the proximal axon. (C) Kymographs showing EB1-GFP comets over time. They were generated from time series of ddaE neurons expressing EB1-GFP. In all images the cell body is positioned at the right. (D) Quantitation of EB1-GFP comets in different genotypes. Number of EB1-GFP comets was analyzed by counting the EB1-GFP comets present in 10-μm lengths of axon in every 40th frame of movies. For dendrites, the complete number of comets was determined in the ddaE main trunk for an entire time series and then normalized to comets per 10-μm length and per 100 s. The number of animals from which the averages were quantitated is shown above the graph bars for each genotype. Error bars represent SDs, and a t test was used to determine significance.
different sites in the cell body in mature neurons or the cell body becomes less important as a source for new microtubules.

An alternate model is that microtubules are generated locally at nucleation sites in dendrites and axons rather than in the cell body (Figure 1A, model 2). In this case, motor transport of microtubule pieces would not be required to seed microtubules, although it could still be used as a source of tubulin subunits to extend microtubules.

The Golgi complex has been suggested as the local source of microtubule minus ends in Drosophila dendrites (Ori-McKenney et al., 2012), and there is precedent from other cell types for the Golgi to serve as a microtubule-organizing center (Efimov et al., 2007; Rivero et al., 2009). However, it has not been demonstrated that γ-tubulin localizes to neuronal Golgi in dendrites, and it is difficult to perform rigorous nucleation assays in neurons, as their microtubules are too stable to depolymerize. In the case of axons, the existence of local microtubule nucleation has not been intensively investigated. If microtubules are locally nucleated in axons or dendrites rather than initiated from transported seeds, then regulation of nucleation rather than action of motor proteins is likely to be critical for correct microtubule polarity.

To determine whether microtubule nucleation plays a role in axonal or dendritic microtubule polarity, we analyzed neurons in animals with different types of γ-tubulin mutations. We found that both loss- and gain-of-function γ-tubulin alleles altered axonal and dendritic microtubule polarity. This result suggests a close tie between nucleation and polarity, perhaps by local nucleation sites in axons and dendrites; changes in nucleation occurring in the cell body would not be expected to alter polarity in axons and dendrites, as motors would still filter only correctly oriented microtubule seeds into neurites.

To further probe the possibility of local nucleation, we assayed localization of endogenous and tagged γ-tubulin in axons and dendrites; both were present in distinctive spots at dendrite branch points and presynaptic terminals. Because the Golgi complex was previously suggested to be the site of dendritic nucleation (Ori-McKenney et al., 2012), we used several different approaches, including dragging the Golgi out of dendrites, to test the link between the Golgi complex and microtubule nucleation. Our data suggest that nucleation sites are not localized to the Golgi in dendrites. We therefore propose that tight regulation of microtubule nucleation in both axons and dendrites controls microtubule polarity without localization of nucleation sites to the Golgi complex.

RESULTS
Both loss of γ-tubulin and overactive γ-tubulin affect microtubule orientation in axons and dendrites of class I ddaE neurons

To determine whether γ-tubulin plays a role in controlling polarity of microtubules in axons and dendrites, we assayed microtubule orientation in neurons from animals with mutant versions of the γ-tubulin23C gene. In Drosophila, there are two γ-tubulin genes: γ-tubulin37C encodes a protein that is maternally expressed and involved in early embryonic development, and γ-tubulin23C encodes the major somatic γ-tubulin (Wiese, 2008). Microtubule orientation was assessed using EB1-green fluorescent protein (GFP) dynamics; EB1-GFP comets mark growing microtubules, and so the direction of comet movement indicates polarity of the microtubule (Stepanova et al., 2003). In normal Drosophila da neurons, axonal microtubules are oriented with almost 100% of their plus ends distal to the soma, whereas the opposite orientation is seen in dendrites, which have >90% of microtubule minus ends distal to the soma (Rolls et al., 2007; Stone et al., 2008). To assay the effects of changes in γ-tubulin on microtubule orientation, we looked at the class I ddaE neuron. The branches of the dorsal ddaE dendrite emerge at close to right angles from the main trunk of the dendrite, making this cell particularly sensitive to changes in regulation of microtubule polarity (Mattie et al., 2010).

We first looked at the effects of different γ-tubulin alleles on the number of growing microtubule ends marked with EB1-GFP. The number of microtubule plus ends reflects overall levels of microtubule nucleation in both cultured cells (Pielh et al., 2004) and Drosophila neurons (Chen et al., 2012). The null allele of γ-tubulin, γTub23CAT15-2 (stop codon at amino acid 104; Vazquez et al., 2008), is homozygous lethal, so we used transheterozygotes of γTub23CAT15-2 with the hypomorphic allele, γTub23CA14-9 (amino acid substitution R217H; Vazquez et al., 2008). In addition to the loss-of-function mutants, we tested the γTub23Cosp1 mutant, which has dominant eye and wing defects (Vazquez et al., 2008). The original isolate of γTub23Cosp1 contained a suppressor mutation (Vazquez et al., 2008), which we removed for this analysis. Although γTub23Cosp1 homozygous adults do not survive, homozygotes do survive into larval life. We observed no difference in number of growing microtubule plus ends in γTub23CAT15-2/γTub23CA14-9 neurons (Figure 1, C and D); however, we did notice some changes in microtubule polarity in the trunk of the dendrite used for analysis (Figure 1, B and C). We previously showed, however, that fewer growing microtubules can be induced by axon injury in γTub23CAT15-2/+ animals, so we hypothesize that in unjured neurons, a very small amount of functional γ-tubulin can support normal microtubule growth. In animals homozygous for γTub23Cosp1, the number of growing microtubules was increased in axons and dendrites (Figure 1D). This γ-tubulin allele has not been extensively characterized, but it is dominant and contains a single amino acid substitution (M382I; Mahoney et al., 2006). In these larvae, we observed an increase in the number of EB1-GFP comets in axons and dendrites (Figure 1, C and D). This increase is consistent with the γTub23Cosp1 allele acting in a gain-of-function manner to increase microtubule nucleation. To confirm that this phenotype was due to the mutation in γ-tubulin, we added a GFP-tagged γ-tubulin transgene that we previously demonstrated to be capable of rescuing mutant phenotypes (Chen et al., 2012). This transgene suppressed the comet increase in γTub23Cosp1 neurons (Figure 1D), consistent with the phenotype being due to the point mutation in γ-tubulin. Because γ-tubulin acts in a multimeric complex, overexpressed γ-tubulin23C-GFP may outcompete the mutant allele for incorporation into the active nucleation complex restoring normal function. γ-Tubulin activity has also been associated with dendrite branching (Ori-McKenney et al., 2012). We therefore also assayed arbor complexity in both genetic backgrounds. In the loss-of-function background, class I ddaE neurons had no change in number of dendrite branch points (Supplemental Figure S1). However, in the γTub23Cosp1 background, branch point number was significantly increased (Supplemental Figure S1), again consistent with increased nucleation activity in this allele. We conclude that the γTub23Cosp1 allele is likely to be an overactive mutant of γ-tubulin.

We next tested whether microtubule polarity was altered in the γTub23C loss- or gain-of-function genetic backgrounds. In control neurons, axonal microtubule polarity was ∼95% plus end out; however, in transheterozygous loss-of-function mutant axons, EB1-GFP comets traveled away from the cell body only 85% of the time (Figure 2, A and B, and Supplemental Movies S1 and S3). In control dendrites, EB1-GFP comets traveled away the cell body <10% of the time. However, loss-of-function mutant dendrites were significantly more mixed, with ∼25% of EB1-GFP comets moving toward the soma (Figure 2, A and C, and Supplemental Movies S2 and S4).
When we assayed microtubule orientation in homozygous γTub23Cbmps1 larvae, we found that, as in the loss-of-function mutants, microtubules became more mixed in polarity in axons and dendrites (Figure 2, B–D, and Supplemental Movies S5 and S6). In addition, animals with only one copy of the γTub23Cbmps1 allele exhibited mixed microtubule polarity, consistent with a gain of function of this allele (Figure 2D). In either type of mutant background, microtubule polarity was rescued by overexpression of γ-tubulin23C-GFP (Figure 2E and Supplemental Movies S7–S12). Because either decreased or increased activity of γ-tubulin disrupted microtubule polarity, we conclude that regulated microtubule nucleation is required for axonal and dendritic microtubule polarity.

Localization of γ-tubulin in axons and dendrites

Disruption of microtubule polarity in axons and dendrites by different types of γ-tubulin alleles suggested a close relationship between microtubule polarity and microtubule nucleation and was consistent with local nucleation mediated by γ-tubulin in these compartments. Although γ-tubulin localization is easy to detect when it is concentrated on the centrosome, it can be much more elusive when distributed to other sites in the cell. Nevertheless, we tried to determine the localization of both endogenous and GFP-tagged γ-tubulin. In the cell body, γ-tubulin23C-GFP has a diffuse pattern (Nguyen et al., 2011). Diffuse tagged and endogenous γ-tubulin was seen throughout axons of da neurons. In addition, motor neuron axons are present in the body wall under the da neurons, and we observed concentrations of γ-tubulin in their large presynaptic boutons (Figure 3, A–C). In dendrites, endogenous and tagged γ-tubulin was concentrated at dendrite branch points (Figure 3, D–G). For comparison, we examined a soluble fluorescent protein in the same neurons and calculated the ratio of the fluorescence intensity at the branch points and between branch points (Supplemental Figure S2). This ratio was close to 1 for the soluble protein and >2 for γ-tubulin23C-GFP, indicating that this marker is significantly concentrated at the branch point. Although staining for endogenous γ-tubulin was quite faint and somewhat variable, more signal was present within branch points than between branch points in dendrites (Figure 3F and Supplemental Figure S3). Relative branch point fluorescence was lower in the

**FIGURE 2:** γ-Tubulin mutants have defects in neuronal microtubule polarity. EB1-GFP was expressed in larval class I da neurons using 221-Gal4 in different mutant backgrounds. The direction of EB1-GFP comet movement was quantified in the dorsal comb dendrite of ddaE neurons. n, number of EB1-GFP comets analyzed for each genotype. (A) γ-Tubulin loss-of-function alleles analyzed as heterozygotes or transheterozygotes for axonal polarity (left) and dendritic polarity (right). (B, C) Examples of EB1-GFP comets in axons (B) and dendrites (C). Comets moving away from the cell body are indicated with red arrowheads, and comets moving toward the cell body are indicated with green arrowheads. In all images dorsal is up. Supplemental Movies S1–S6 show the EB1-GFP movement illustrated by the stills. (D) Both heterozygous and homozygous γTub23Cbmps1 mutants were analyzed for microtubule polarity in axons (left) and dendrites (right). (E) γTub23C-GFP was expressed under Gal4-UAS control in different genetic backgrounds, and microtubule polarity was analyzed in axons (left) and dendrite (right). Supplemental Movies S7–S12 illustrate the EB1-GFP movement in each genetic background. A Fisher’s exact test was used to calculate p values.
nucleation outside the cell body. In particular, the concentration at branching points of γ-tubulin were fixed and stained with antibodies to GFP, to the microtubule binding protein Futsch (22C10 antibody), and with anti-horseradish peroxidase (HRP), which stains neuronal membranes in Drosophila. (C) Larvae without tagged γ-tubulin were fixed and stained with 22C10, anti-HRP, and anti–γ-tubulin23C antibodies. In B and C, arrows indicate synaptic boutons. (D–G) Localization of γ-tubulin in dendrites of da neurons. (D, E) γ-Tubulin23C-GFP and mCD8-RFP were expressed in class I da neurons with 221-Gal4, and cells were either visualized in whole live animals (D) or fixed and stained (E). (F) Animals without overexpressed RFP were expressed in class I da neurons with 221-Gal4, and cells were either visualized in whole live animals (D) or fixed and stained (E). (G) γ-Tubulin23C-GFP and mCD8-RFP expressed in class IV da neurons with 477-Gal4 and imaged in living animals. In D–G, arrows indicate dendrite branch points.

γTub23C<sup>AT5</sup>-γTub23C<sup>AT14</sup> neurons, consistent with reduced but not absent γ-tubulin in this background, whereas branch point concentration was similar to control neurons in γTub23C<sup>omp17</sup> animals (Supplemental Figure S3). Overall the localization of tagged and endogenous γ-tubulin at axons and dendrites is consistent with it controlling nucleation outside the cell body. In particular, the concentration at dendrite branch points suggested that nucleation activity might be concentrated at these junctions.

Localization of nucleation activity in neurons is very challenging. Nucleation assays typically rely on depolymerizing microtubules completely and then assaying the site where microtubules first start regrowing. Neuronal microtubules are incredibly stable, and we have not been able to depolymerize them in neurons in vivo in Drosophila or in tissue explants and thus have not been able to perform traditional nucleation assays. Although the number of EB1-GFP comets is a rough readout for overall level of microtubule nucleation activity, in neurons with long, stable microtubules, individual plus ends may be far away from the nucleation site. Similarly, although appearance of new EB1-GFP comets was previously counted as a readout of microtubule nucleation (Ori-McKenney et al., 2012), most new EB1-GFP comets in neurons are likely to represent catastrophe rescue rather than nucleation events. However, we reasoned that the sites where appearance of comets was preferentially increased in the overactive γTub23C<sup>omp17</sup> allele would be at least loosely indicative of nucleation sites. We therefore assayed appearance of new EB1-GFP comets (or comet spawning) in neurons with different γTub23C alleles (Figure 4 and Supplemental Movies S13–S15). We found that comet spawning was increased more at branch points than between branch points when nucleation was up-regulated in the γTub23C<sup>omp17</sup> background (Figure 4 and Supplemental Movie S15). Surprisingly, branch point spawning was also preferentially increased in the loss-of-function background, perhaps because remaining nucleation sites were activated as much as possible to make up for the reduction in γ-tubulin (Figure 4 and Supplemental Movie S14). The preferential increase in spawning at branch points when γ-tubulin activity was altered suggests a link between dendrite branch points and microtubule nucleation activity, which is consistent with the concentration of endogenous and tagged γ-tubulin at branch points.

**Golgi outposts are positioned at proximal dendrite branch points in neurons with large arbors**

Golgi outposts have been suggested to be sites of acentrosomal microtubule nucleation at dendrite branch points of Drosophila da neurons (Ori-McKenney et al., 2012). The Golgi is thus a good candidate for housing dendritic γ-tubulin. However, because the Golgi is found only in dendrites and not axons (Craig and Banker, 1994; Horton and Ehlers, 2003; Ye et al., 2007), it would be unlikely to house any γ-tubulin involved in controlling axonal microtubule organization.
surprisingly, we noticed a difference in dendritic Golgi outpost localization between class I and class IV (ddaC) da neurons. Previous descriptions of Golgi outposts, we analyzed the localization of Golgi markers in class I (ddaE) and class IV (ddaC) da neurons. To test whether the Golgi might colocalize with γ-tubulin in vivo, we expressed trans-Golgi tagging proteins. We expressed GalT-YFP, a trans-Golgi marker, and ManII-GFP, a medial/trans-Golgi marker, in da neurons (Ye et al., 2007; Zheng et al., 2008; Ori-McKenney et al., 2012). Both Golgi markers labeled the larger Golgi outposts seen in proximal dendrite branch points of large class IV neurons (Supplemental Figure S4, A–C). Smaller, dimmer spots of fluorescence were seen in both axons and dendrites, but because axons do not contain Golgi, we considered that these spots were more likely due to leakage of overexpressed markers into some other compartment. When counting Golgi outposts in dendrites, we included only puncta that were larger and brighter than these fluorescent spots seen throughout the neuron. For most analyses we used GaIT-YFP because it had less background expression, including complete exclusion from the nucleus.

Surprisingly, we noticed a difference in dendritic Golgi outpost localization between class I and class IV da neurons. Compared to class IV da neurons, class I da neurons have simple dendrite arbors. Although prominent Golgi complexes were seen in the cell body in class I neurons, most had no dendritic Golgi outposts identified (Gillingham and Munro, 2003). GMAP-210 localizes to the cis-Golgi using a region near its C-terminus (Chen et al., 1999), and golgin-97 localizes to the trans-Golgi through its GRIP domain (Barr, 1999; Kjer-Nielsen et al., 1999; Munro and Nichols, 1999). We attached the Golgi-binding domains from these two proteins to truncated kinesin heavy chain because truncated forms are constitutively activated (Verhey et al., 1998). To test these fusion proteins, we expressed them in cultured mammalian cells that have the Golgi complex positioned adjacent to the centrosomal microtubule-organizing center. In these cells, microtubule minus ends are found in the middle of the cell, and plus ends are near the cell periphery. In control cells without the kinesin-Golgi fusions, a Golgi marker formed a tight clump in the middle of the cell, whereas in cells that expressed kinesin-Golgi fusions, the Golgi was fragmented and moved to the cell periphery, presumably by being dragged out toward microtubule plus ends (Supplemental Figure S6). The fusion to the GMAP-210 domain colocalized with a cis-Golgi marker as expected but was able to relocalize both cis- and trans-Golgi compartments (Supplemental Figure S6). Overexpression of a mutant version of the golgin-97-binding domain that does not target the Golgi had no effect on the Golgi complex, and the effect of the kinesin-G97 fusion was specific to the Golgi complex, as lysosomes and endoplasmic reticulum were unaffected (Supplemental Figure S6). We next generated transgenic flies expressing these constructs (Figure 5A), since the
Golgi-binding domains are well conserved. To determine whether these transgenes were being expressed, we stained larvae with anti-myc antibodies; myc staining was seen in a punctate pattern in neurons expressing these transgenes were being expressed, we stained larvae with anti-myc antibody. (C) mCD8-RFP and GalT-YFP were expressed with the kinesin-Golgi fusions in larvae using 477-Gal4. Specific transgene insertions are indicated with designations JJ.5 and V.3. Arrows indicate Golgi punctae. Dendrites had severely reduced arbors in neurons expressing either kinesin-Golgi fusion. (D) Left, Golgi outposts counted in ddaC dendrites and proximal axons. Average numbers of Golgi in each compartment. Right, numbers of Golgi normalized to the number of proximal branch points. p values were calculated with a t test. Error bars show SD; n, number of cells analyzed in each genotype.

FIGURE 5: Expression of kinesin-Golgi fusions affects Golgi localization, Golgi morphology, and neuronal morphology. (A) Diagram of kinesin fusion constructs expressed in Drosophila neurons. (B) Larvae expressing GalT-YFP and myc-tagged kinesin-Golgi fusions in ddaC neurons with the 477-Gal4 driver were stained with anti-myc antibody. Myc staining was seen in a punctate pattern in neurons. (C) mCD8-RFP and GalT-YFP were expressed with the kinesin-Golgi fusions in larvae using 477-Gal4. Specific transgene insertions are indicated with designations JJ.5 and V.3. Arrows indicate Golgi punctae. Dendrites had severely reduced arbors in neurons expressing either kinesin-Golgi fusion. (D) Left, Golgi outposts counted in ddaC dendrites and proximal axons. Average numbers of Golgi in each compartment. Right, numbers of Golgi normalized to the number of proximal branch points. p values were calculated with a t test. Error bars show SD; n, number of cells analyzed in each genotype.

Golgi disruption results in small changes in microtubule polarity in dendrites but not axons of class I and class IV da neurons

If the Golgi houses microtubule nucleation sites, then we might expect to see similar phenotypes from Golgi disruption and γ-tubulin23C mutants. To examine microtubule polarity in neurons expressing kinesin-Golgi fusions, we analyzed EB1-GFP comet movement. In control axons of class IV da neurons expressing only the kinesin motor domain, microtubules were primarily plus end out, as expected (Figure 6A). This plus-end-out orientation did not significantly change with expression of the kinesin-Golgi fusions. In dendrites, we found a significant increase in the percentage of plus-end-out microtubules with two of the three kinesin-Golgi fusion
insertions tested (Figure 6, A and B). This effect was much more variable between lines than the effect on dendrite morphology, perhaps indicating that microtubule polarity is less sensitive to Golgi disruption than other dendritic features.

To probe the relationship between the Golgi and polarity further, we assayed microtubule polarity in class I ddaE neurons that do not have obvious Golgi outposts. We saw a subtle yet significant mixing of microtubule orientation in these dendrites (Figure 6, C and D). On the basis of these results, we think it most likely that disruption of the Golgi affects microtubule polarity in dendrites indirectly, perhaps by altering trafficking of a microtubule regulator. Unlike mutations in \( \gamma \)-tubulin23C, disruption of the Golgi never altered axonal microtubule orientation.

Golgi disruption alters localization of Apc2-GFP in Drosophila da neurons

To probe whether Golgi disruption might alter identified regulators of dendritic microtubule polarity, we examined the distribution of Apc2-GFP in the presence of the kinesin-Golgi fusions. Maintenance of dendrite microtubule orientation in Drosophila da neurons has been found to require kinesin-2, EB1, Apc, and Apc2, which are proposed to work together to direct growing microtubules through branch points toward the cell body (Mattie et al., 2010). Apc2-GFP localizes very specifically to dendrite branch points, and knockdown of Apc2 alters microtubule orientation in da neurons (Mattie et al., 2010). To test whether the changes in dendrite microtubule orientation seen in the kinesin-Golgi fusions might be a result of mislocalization of Apc2, we looked for changes in Apc2-GFP distribution in the presence of the fusions. Control neurons that expressed only the kinesin motor domain had Apc2-GFP at dendrite branch points (Figure 7A and Supplemental Figure S9A). In neurons expressing kinesin-Golgi fusions, Apc2-GFP was much less concentrated at the branch point in ddaE and ddaC neurons (arrowheads, Figure 7A and Supplemental Figure S9A). Mistargeting of Apc2-GFP could account for the subtle change in microtubule polarity induced by expression of kinesin-Golgi fusions.

**Localization of \( \gamma \)-tubulin23C-GFP to dendrite branch points is not affected by Golgi disruption**

To test whether disruption of the Golgi affected localization of \( \gamma \)-tubulin23C-GFP in a similar manner to Apc2-GFP, we expressed both \( \gamma \)-tubulin23C-GFP and mCD8-mRFP in
γ-Tubulin controls microtubule polarity

DISCUSSION

To investigate the relationship between microtubule nucleation and polarized organization of microtubules in neurons, we examined the effects of different γ-tubulin mutants on axonal and dendritic microtubule polarity. Surprisingly, loss- and gain-of-function γ-tubulin alleles altered both axonal and dendritic microtubule polarity. Because this suggested a close link between microtubule nucleation and polarity control, we examined localization of endogenous and tagged γ-tubulin. Whether in fixed or living animals, γ-tubulin was observed in axons and dendrites and was particularly concentrated at dendrite branch points and axon termini.

The localization of γ-tubulin to axons and dendrites, as well as the close tie between microtubule polarity and γ-tubulin activity, argues that many microtubules in mature neurons are likely to be generated locally rather than transported from the cell body. Although transport of microtubule pieces from the cell body is important for initial axon outgrowth (Yu et al., 1993; Lu et al., 2013) and early dendritic development (Sharp et al., 1995, 1997; Yu et al., 2000; Lin et al., 2012), its role in more-mature neurons is not as clear. Indeed, kinesin-6, the motor that is involved in microtubule transport into dendrites, is down-regulated as neurons mature (Lin et al., 2012), and kinesin-1–mediated microtubule sliding is important only in the earliest stages of axon initiation in Drosophila (Lu et al., 2013).

Local nucleation of microtubules in dendrites was recently proposed to play a role in complex branching of class IV dendritic
arborization neurons and was suggested to occur at Golgi outposts (Ori-McKenney et al., 2012). Although our results support an important role for microtubule nucleation in dendrites, we were unable to find any evidence that nucleation sites were housed on the Golgi complex. First, changes in microtubule nucleation also affected axonal polarity even though Golgi outposts are not found in axons. Second, using well-established Golgi markers, including the one used in the previous dendritic nucleation study (Ori-McKenney et al., 2012), we found that Golgi outposts were only present in large dendrite arbors, although minus-end-out microtubules and a link between nucleation and polarity were found in small arbors (ddaE) as well as large arbors (ddaC). Third, whereas dragging the Golgi out of dendrites with kinesin-Golgi fusions disrupted branch point localization of Apc2-GFP, it did not alter γ-tubulin23C-GFP concentration at dendrite branch points. Thus, although we favor a model in which regulated nucleation locally controls microtubule polarity in axons and dendrites, we do not find support for the idea that this regulated nucleation occurs at the Golgi complex. The Golgi is only one of several cellular membranes known to house nucleation sites; in muscle cells, γ-tubulin nucleates microtubules at the nuclear envelope (Bugnard et al., 2005), and in the C. elegans gonad, γ-tubulin–mediated nucleation occurs at the plasma membrane (Zhou et al., 2009). It is therefore possible that γ-tubulin in dendrite branch points is associated with either the plasma membrane or some other internal membrane structure such as the endoplasmic reticulum.

How γ-tubulin might control microtubule polarity in axons and dendrites will be extremely interesting to determine. On the basis of our γ-tubulin mutant results and the previous finding that microtubule dynamics in axons and dendrites was massively upregulated by axon injury or stress through nucleation (Stone et al., 2010; Chen et al., 2012), we propose a model in which γ-tubulin in axons and dendrites is tightly negatively regulated (Figure 8). The proposed negative regulator not only would control number of active nucleation sites, but it also would need to link nucleation to polarity in some way to account for the polarity changes in loss- and gain-of-function γ-tubulin mutant backgrounds. Loss of interaction with the negative regulator could explain both the increase in dynamics and polarity disruption seen in the γTub23Cbmps1 overactive mutants. Involvement of a negative regulator to control polarized use of nucleation sites could also explain why reduction of γ-tubulin affects microtubule orientation. When γ-tubulin levels are decreased, the negative regulator may be inactivated in order for the remaining γ-tubulin to become active (Figure 8). As a trade-off, the newly activated γ-tubulin would be dysregulated. Of course, the existence of such a regulator is speculative at this point. However, it is clear that understanding how microtubule nucleation is regulated in neurons will be critical for generating complete models of neuronal microtubule polarity.

MATERIALS AND METHODS

Drosophila stocks

The Bloomington Drosophila Stock Center (Bloomington, IN) provided all Gal4 driver lines, all γ-tubulin mutant lines, and all fluorescently tagged marker lines except those noted further. For γ-tubulin mutant studies, we took out the l(l)dd4suph suppressor from the fly stock to obtain γTub23Cbmps1/CyO flies. The Golgi markers

| Mixed |Minus-end-out | Plus-end-out | I | Mixed |
|---|---|---|---|---|
| plus-end-out MT | Golgi | γ-tubulin |negative regulator |
| minus-end-out MT | | | |

FIGURE 8: Model for the regulation of γ-tubulin in Drosophila neurons. On the basis of localization and mutant phenotypes, we propose that γ-tubulin localizes throughout axons and dendrites, with concentrations at specific places, including dendrite branch points. Under normal circumstances, a negative regulator of nucleation could ensure that new microtubules are generated in the correct orientation in axons and dendrites. When levels of γ-tubulin are reduced (left), the negative regulator is inactivated so that microtubules can still be generated, but polarity control is disrupted. The overactive γ-tubulin mutant may have increased activity because it fails to interact with the negative regulator (right). In many cells in which γ-tubulin activity is linked to microtubule polarity in axons and dendrites, the Golgi complex is found only in the cell body.
UAS-GalT-YFP and UAS-ManII-EGFP were obtained from Bing Ye (University of Michigan, Ann Arbor, MI) and Kassandra Ori-McKenney (University of California, San Francisco, San Francisco, CA). UAS-EB1-GFP lines were previously described (Rolls et al., 2007), as was generation of UAS-γ-tubulin23C-GFP (Nguyen et al., 2011).

**Generation of kinesin-Golgi fusions and transgenic fly lines**

Mammalian vectors containing the motor domain of rat Kif5b (Khc) connected to either the C-terminal region of the cis-Golgi protein GMAP210 or the C-terminus of the trans-Golgi protein golgin-97 via a linker and myc tag were generated. A mutant version of the golgin-97 targeting domain that cannot localize to the Golgi was also used (G97-Y697A) for validation (Munro and Nichols, 1999). These fusions were tested in mammalian COS cells first. Kinesin fusions were transiently expressed in COS cells from plasmids GRIP22 (khc-G210), GRIP26 (khc-G97), and GRIP27 (khc-G97-Y697A). Cells were transfected, fixed, and processed for immunofluorescence as described previously (Levine and Munro, 1998).

Once tested, the fusions were placed into the fly vector pUAST::3YCT. Transgenic flies were obtained using standard genetic procedures. Five transgenic fly lines were generated that were used in this study: 1) khc, which is a control fusion without the Golgi-binding domain; 2) khc-G97 (JJ.2), which we also use as a control, as lack of c-myc staining indicates that the fusion is not being expressed; 3) khc-G97 (JJ.5); 4) khc-G210 (V.2); and 5) khc-G210 (V.3).

**Larval immunostaining**

Immunostaining of third-instar larvae was performed as previously described (Nguyen et al., 2011). Images of larval fillets were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), and the appropriate stacks were combined to make composite images. The following primary antibodies were used: mouse anti-c-myc (Developmental Studies Hybridoma Bank), 22C10 (Developmental Studies Hybridoma Bank), Alexa Fluor–conjugated goat anti-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA), rabbit anti-GFP (Abcam, Cambridge, MA), and R62 against γ-tubulin23C (a gift from Brigitte Raynaud-Messina, Institute of Pharmacology and Structural Biology, Toulouse, France; Raynaud-Messina et al., 2001). The appropriate secondary fluorescent antibodies were obtained from Jackson ImmunoResearch.

**Localization experiments**

In all in vivo localization and morphology experiments, a time series was taken for each cell, and the stage and focus were moved incrementally in order to capture all of the dendrites. The resulting time-series images were then broken into stacks for each area focused on and then pasted together to make a composite image of the cell.

For comparison of endogenous and GFP-tagged γ-tubulin concentrations at dendrite branch points versus non–branch points, an ImageJ (National Institutes of Health, Bethesda, MD) plug-in was created to measure fluorescence intensity. The plug-in averaged all of the pixels in a specified quantification area, except for any pixel lower than a specified background value. Only one frame per movie, where the branch point and non–branch point were most in focus, was analyzed, and the same quantification areas were used between the control and the endogenous γ-tubulin analyses.

For quantitation of Golgi outposts, larval neurons expressing UAS-GalT-YFP were imaged live using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan). The Golgi outposts were manually counted in the axons and dendrites of each cell.

**Analysis of dendrite morphology**

The class I da neurons of larvae expressing EB1-GFP were imaged in vivo using a Zeiss LSM 510 confocal microscope. Morphology of the proximal axon, cell body, and the entire dendrite arbor was imaged in the same manner as the localization experiments. To measure dendrite complexity, the total number of dendrite branch points in each class I neuron was determined, and the average number of branch points per class I neuron was calculated.

The class IV da neurons of larvae expressing EB1-GFP were imaged in vivo using a Zeiss AxioMager M2 microscope with Colibri LED light source (Carl Zeiss). To measure dendrite radius, the distance from the center of the soma to the most-distal dendrite tip was quantitated. To measure dendrite complexity, a circle with a 100 μm radius was drawn, with the center of the circle as the center of the soma. For each neuron analyzed, the dendrite branches that crossed through the border of each of these circles were counted.

**Analysis of microtubule orientation and dynamics**

EB1-GFP directionality experiments were performed as previously described (Nguyen et al., 2011). For analysis of EB1-GFP dynamics, larvae were imaged live using an Olympus FluoView FV1000. For axons, the visible comets were counted every 40 frames (2 s/frame) over a 10-μm length and the weighted mean calculated. For dendrites, the visible comets within the dendritic trunk were counted and normalized to comets per 10-μm length per 100 s. All images were analyzed using ImageJ.

**Analysis of EB1-GFP spawning at dendrite branch points**

For EB1-GFP comet-spawning analysis, image stacks were manually processed to include only those regions of the dendrite that remained in focus for the duration. Branch points were circled on an overlay, and the total branch point number was recorded, as well as the non–branch point dendrite length. EB1-GFP comets were then counted as they appeared over the course of the video. Any comet whose origination could not be seen was excluded from the analysis. The appearance of each new comet was classified as either a branch point comet or a non–branch point comet. Values were normalized by branch point number for branch point comets and by dendrite length for non–branch point comets and pooled for each subset.

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