Motor Neuron Synapse and Axon Defects in a C. elegans Alpha-Tubulin Mutant

Renee Baran1*, Liliana Castelblanco1, Garland Tang1, Ian Shapiro1, Alexandr Goncharov2,3, Yishi Jin2,3

1 Biology Department, Occidental College, Los Angeles, California, United States of America, 2 Section of Neurobiology, Division of Biological Sciences, University of California San Diego, San Diego, California, United States of America, 3 Howard Hughes Medical Institute, University of California San Diego, San Diego, California, United States of America

Abstract

Regulation of microtubule dynamics underlies many fundamental cellular mechanisms including cell division, cell motility, and transport. In neurons, microtubules play key roles in cell migration, axon outgrowth, control of axon and synapse growth, and the regulated transport of vesicles and structural components of synapses. Loss of synapse and axon integrity and disruption of axon transport characterize many neurodegenerative diseases. Recently, mutations that specifically alter the assembly or stability of microtubules have been found to directly cause neurodevelopmental defects or neurodegeneration in vertebrates. We report here the characterization of a missense mutation in the C-terminal domain of C. elegans alpha-tubulin, tba-1(ju89), that disrupts motor neuron synapse and axon development. Mutant ju89 animals exhibit reduction in the number and size of neuromuscular synapses, altered locomotion, and defects in axon extension. Although null mutations of tba-1 show a nearly wild-type pattern, similar axon outgrowth defects were observed in animals lacking the beta-tubulin TBB-2. Genetic analysis reveals that tba-1(ju89) affects synapse development independent of its role in axon outgrowth. tba-1(ju89) is an altered function allele that most likely perturbs interactions between the alpha-tubulin subunit and specific microtubule-associated proteins that control microtubule dynamics and transport of components needed for synapse and axon growth.

Introduction

Microtubules play multiple essential roles in cells including forming the mitotic spindle, transporting vesicles and organelles, providing structural support as part of the cytoskeleton, and serving as platforms for the assembly of signaling complexes. In neurons, the transport functions of microtubules are especially crucial for establishing neuron morphology and maintaining synapses distant from the cell body. Evidence from genetic model systems has revealed that microtubules also play key roles in synapse growth and plasticity [1–4], although these mechanisms are not fully defined.

Microtubules are composed of alpha- and beta subunits that are assembled into heterodimers with the help of cofactors and are then incorporated into microtubule polymers [5–6]. Like actin, microtubules are dynamic polymers, and their assembly and disassembly is highly regulated. Organization into complex microtubule arrays and integration with the actin cytoskeleton adds additional layers of complexity that are still poorly understood. Misregulation of microtubules, loss of synapses and breakdown of transport mechanisms have long been recognized as common denominators in the pathology of neurodegenerative diseases. The presence of hyper-phosphorylated tau in the neurofibrillary tangles of Alzheimer’s patients is one of the most intensely studied examples, but an increasing number of studies have established that perturbing any of the major mechanisms that regulate microtubule dynamics and transport can impact neural development or contribute to neuronal disease. Examples include mutations in tubulin folding co-factor E [7], the dynactin complex [8–9] and the microtubule severing protein, spastin [10], which have all been implicated in motor neuron degeneration.

Early studies of microtubule dynamics and function focused on the beta-tubulin subunit because of its role in hydrolyzing GTP during microtubule polymerization and position on the exposed, plus-ends of microtubules [3]. Subsequent research has uncovered important roles for the alpha-tubulin subunit in synaptic plasticity and neuronal disease. Alpha-tubulin mRNA is enriched at Aplysia synapses and translated locally in response to serotonin signaling [11]. In vertebrates, alpha-tubulin is a target of the E3 ligase and Familial Parkinson’s Disease protein Parkin [12–13]. Perturbations of plus-end microtubule binding proteins of the ebp l family, Familial Parkinson’s Disease protein Parkin [12–13]. Perturbations of plus-end microtubule binding proteins of the ebp l family, Familial Parkinson’s Disease protein Parkin [12–13]. Perturbations of plus-end microtubule binding proteins of the ebp l family, Familial Parkinson’s Disease protein Parkin [12–13]. 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We describe here the isolation and characterization of a novel gain-of-function allele of the *C. elegans* alpha-tubulin, *tba-1*. *tba-1(ju89)* mutants exhibit a reduction in the number and size of GABAergic and cholinergic motor neuron synapses, altered locomotion and axon extension defects. We show similar axon outgrowth defects also occur in mutants in which the *C. elegans* beta-tubulin TBB-2 is deleted. Genetic analysis suggests that many of the axon phenotypes observed in *ju89* mutants are likely mediated by altered or reduced function of microtubules composed of TBA-1 and TBB-2 subunits. We propose that *ju89* alters synapse and axon growth by interfering with specific microtubule-associated proteins that control microtubule dynamics and stability and regulate transport of structural components of axons and the presynaptic active zone.

**Results**

**Isolation of *ju89* and characterization of synapse and axon defects**

The GABAergic D motor neurons function as cross-inhibitors of body wall muscle contractions in *C. elegans* and share a similar unipolar morphology (Figure 1A). Each D neuron extends an anterior process along the ventral cord, branches to form a commissure and then branches again at the dorsal side and extends in both directions along the dorsal nerve cord. Reconstruction of adult synaptic connectivity from electron micrographs show the six dorsal D neurons (DDs) receive synaptic input from cholinergic motor neurons on the ventral side and form chemical synapses en passant with dorsal muscles. The 13 ventral

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**Figure 1. GABAergic Motor Neuron Synapses are Defective in *tba-1(ju89)* Mutants.** (A) Diagram of *en passant* neuromuscular synapses in *C. elegans*. Dots represent synapses; dorsal D neurons (DDs) form synapses onto dorsal body-wall muscles, and ventral D neurons (VDs) synapse with ventral muscles. Presynaptic termini of the inhibitory GABAergic type D motor neurons are visualized with the synaptic vesicle marker *P*unc-25SNB-1::GFP. Each GFP puncta visible by fluorescence microscopy corresponds to the cumulative signal from all GFP-tagged vesicles at an individual synapse. (B) Morphology of the six DD motor neurons. Cell bodies are located in the ventral nerve cord. Each D neuron extends a ventral process, branches to form a commissure, and bifurcates at the dorsal nerve cord to form a dorsal process along the dorsal nerve cord. At the distal tip of each process, gap junctions are formed with adjacent DD neurons [17]. Expression pattern of SNB-1::GFP in wild-type (C) and mutant (D) dorsal nerve cord. Irregular size puncta and reduced numbers of puncta are evident in *ju89* mutants. An arrow designates the position of a DD commissure. (E) SNT-1 (synaptotagmin) expression in the dorsal nerve cord of wild-type and (F) *ju89* mutant animals. UNC-10::GFP expression along the dorsal nerve cord of (G) wild-type and (H) *ju89* worms. (I) Expression of the GABA-B receptor subunit, UNC-49::GFP, along dorsal muscles of wild-type animals and (J) *ju89* mutant worms. Gaps in UNC-49::GFP expression demonstrate the lack of post-synaptic structures in these regions. Wild-type *C. elegans* (K) have a larger body size than *tba-1(ju89)* (L). *C. elegans* move in a wavelike pattern. *ju89* mutants are uncoordinated, and the amplitude of the wave pattern is severely reduced compared to wild-type animals.

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D neurons (VDs) receive their input from dorsal cholinergic neurons and synapse onto the ventral muscles [17].

Synapses can be visualized in the 19 D type GABAergic motor neurons by driving expression of the synaptic vesicle marker synaptobrevin::GFP (SNB-1::GFP) with the promoter of the C. elegans GAD (glutamic acid decarboxylase) gene, unc-25 [18,19]. The P_{unc-25}SNB-1::GFP marker (ju89) appears as puncta of mostly uniform size and spacing along the ventral and dorsal nerve cords of wild-type animals (Figure 1C), corresponding to the GABAergic synapses defined by EM analysis [17]. ju89 was isolated from a genetic screen for mutations that alter the P_{unc-25}SNB-1::GFP expression pattern [20]. This approach successfully identified synapse defective (syd) genes that function in active zone formation and synapse morphology [21–22], neuronal polarity [20,23] and vesicle transport [24].

ju89 worms exhibited multiple defects in the SNB-1::GFP pattern along both the ventral and dorsal nerve cords that suggested a failure to properly form or maintain synapses. The number of SNB-1::GFP puncta was greatly reduced, and the SNB-1::GFP expression pattern was also observed along commissures (Figure 1D), a phenotype sometimes also observed along the dorsal cord. Weak diffuse GFP expression was also observed along the ventral and dorsal nerve cords (Figure 1G). The pattern of UNC-49::GFP puncta is expressed by both ventral and dorsal body wall muscles and appears as diffuse puncta adjacent to the ventral and dorsal nerve cords (Figure 1I & J). Large gaps in UNC-49::GFP expression were evident along both the dorsal and ventral muscles of adult ju89 mutants, indicating that postsynaptic structures were not present in these regions. In some ju89 mutant animals the GFP-tagged receptor appeared diffused throughout the muscle tissue, consistent with the failure of the receptor to completely localize to synapses.

To assess if presynaptic specializations were still present in these regions of the motor neuron axons, we examined the expression of a GFP-tagged active zone protein, UNC-10(RIM). In the presynaptic matrix, UNC-10 binds the scaffolding protein ELKS/ERC and interacts with the RAB-3 GTPase to recruit synaptic vesicles to the active zone [26]. Because the C. elegans cholinergic motor neurons run closely parallel to the GABAergic D motor neurons in the ventral and dorsal nerve cords, the synapses of the two classes of motor neurons cannot be distinguished from each other by immunohistochemistry and light microscopy. To view UNC-10 expressed by only the GABAergic neurons we crossed P_{unc-25}UNC-10::GFP (hp61) [27] into ju89 mutants, and compared the localization of UNC-10::GFP puncta in ju89 mutants to wild-type worms. Puncta for UNC-10::GFP appeared as an orderly row along the dorsal and ventral nerve cords in wild-type animals (Figure 1G). The pattern of UNC-10::GFP puncta in ju89 mutants (Figure 1H) was similar to the

| Table 1. SNB-1::GFP Expression by D Motor Neurons. |
|---|
| Genotype | Ventral Nerve Cord (VDs) | (n) | Dorsal Nerve Cord (DDs) | (n) |
|---|---|---|---|---|
| +/+ | 189±14 | 30 | 166±10 | 30 |
| tba-1/ju89 | 125±22* | 30 | 94±14* | 30 |
| tba-1/ju89/+ | 169±15* | 30 | 148±8* | 30 |
| qD9/ju89 | 148±15* | 10 | 122±12* | 10 |
| qD9/+ | 187±6 | 15 | 168±12 | 15 |
| tba-1(ok1135) | 192±6 | 30 | 167±7 | 30 |
| tba-1(ok1135)/+ | 180±9 | 30 | 164±9 | 30 |
| tba-1(ok1135)/ju89 | 155±7* | 15 | 130±8* | 15 |
| +; Extba-1(Extba-1) | nd | 107±3* | 25 |
| +; Extba-1 | nd | 166±7 | 25 |
| tbb-2(gk130) | 153±10* | 30 | 131±14* | 30 |
| tba-1/ju89; tbb-2(gk130) | 148±16* | 30 | 117±13* | 30 |
| tbb-1(gk207) | 173±8* | 30 | 158±7* | 30 |
| tba-1(ju89); tbb-1(gk207) | 76±12** | 30 | 65±14** | 30 |

Genetic analysis of tubulin mutant synaptic defects based on expression pattern of the synaptic vesicle marker Synaptobrevin:GFP (SNB-1::GFP). Young adult worms were scored for the number of SNB-1::GFP puncta in the ventral and dorsal nerve cords. Animals carrying the ju89 mutant chromosome in trans to a wild-type (+/ju89) or deficiency (09F/ju89) chromosome or the tba-1 deletion allele (ok1135/ju89) exhibit partial defects. Deficiency/+ and ok1135/+ heterozygotes appear wild-type. Animals that express an extrachromosomal array containing tba-1 amplified from ju89 animals, Extba-1(Extba-1) resemble ju89 mutants. Animals homozygous for the null tbb-2 allele (gk207) have a similar decrease in total SNB-1::GFP as single mutants and in combination with tba-1/ju89). Mean ± sd. *p<.001, two-tailed T test. **Synapse loss is strongly enhanced in tba-1/ju89; tbb-1 (gk207) animals due to loss of axons in the dorsal and ventral nerve cord.
mutant SNB-1 pattern: UNC-10::GFP puncta were frequently smaller than wild-type and irregularly spaced, and diffuse UNC-10::GFP was visible in the commissures of adult ju89 animals. Again, the most distal tips of the DD axons appeared to be thinner and lacked full size UNC-10::GFP puncta. Thus, the presynaptic regions of GABAergic synapses were defective in both synaptic vesicles and the machinery required to recruit them.

To determine if all synapses were generally defective in ju89 mutants, we also examined mutant worms for the endogenous expression of a second synaptic vesicle marker synaptotagmin (SNT-1) (Figure 1E & F) and UNC-10/RIM. SNT-1 and UNC-10 antibodies detect all chemical synapses in the C. elegans nervous system. In the most severe cases, anti-SNT-1 Ab revealed occasional gaps and irregular size or position of SNT-1 puncta in ju89 mutants (Figure 1F), but expression of SNT-1 in mutant dorsal nerve cords appeared similar to wild-type SNT-1 (Figure 1E) expression in most animals, indicating that cholinergic motor neuron synapses are not globally disrupted in the mutants. Similar results were obtained using antibodies against UNC-10/RIM (data not shown).

Previous studies have shown that significant changes in expression of SNB-1::GFP often reflect underlying morphological changes at synapses [21–22]. Our GFP transgene expression data indicated that synapse formation and size could be altered in ju89 mutants, and that GABAergic neurons were most severely affected. To determine if the small SNB-1::GFP puncta in ju89 mutants correlated with morphological defects at synapses, we analyzed ju89 animals by electron microscopy. Compared to wild-type synapses (Figure 2A & B), a reduction in the number of synaptic vesicles is apparent in electron micrographs of both GABAergic (Figure 2C) and cholinergic synapses of the mutants (Figure 2D). An average of 20–30% fewer vesicles/synapse and an equivalent decrease in active zone size was measured in the cholinergic synapses of one animal, (Figure 2E & F), but the proportion of vesicles/active zone size was similar in wild-type and mutant animals (Figure 2G). The loss of GABAergic synapses was severe in the regions examined: the number of dorsal synapses in a section of the dorsal cord in one animal was reduced by 1/3 compared to a similar sized region in the wild-type control (2 synapses in ju89 vs. 6 synapses in the wild-type animal). When we examined the total number of GABAergic and cholinergic motor neuron axons present in the dorsal and ventral nerve cords, the number of axons in the ventral nerve cord was reduced by 8% compared to wild-type worms (49 versus 53 axons), and the number of axons in the dorsal nerve cord was reduced by 16% (9 versus 12 axons). This observation indicated that some of the mutant motor neuron axons failed to reach or extend normally along the dorsal nerve cord.

To determine if the decreased number of GABAergic synapses in ju89 mutants was due solely to defects in initial axon outgrowth and extension along the nerve cords, we examined the morphology of the dorsal D (DD) motor neurons in more detail. The six DD motor neurons are born and extend axons during the early larval stage and the adult stage, C. elegans nervous C. elegans chromosome I by standard two- and three-factor mapping analysis.

Lastly, we asked if the severity of synapse and axon loss increases as mutant animals grow to adulthood. Between the L1 larval stage and the adult stage, C. elegans increases in size by fourfold. During this period the DD motor neurons add new membrane and grow in size but do not extend growth cones and undergo new axon outgrowth. The dorsal synapses of the DD neurons first form at the L1/L2 larval transition, after the initial outgrowth of these neurons [17,29]; addition of new synapses and membrane to enlarge the neurons occurs during subsequent larval stages [27]. When we compared young adult wild-type and ju89 mutant worms, we observed an increase in the number of DD axons with gaps in the dorsal nerve cord, and 7% of the DD axons in adults exhibited defects in branching where the commissures meet the dorsal cord that were not observed in the same animals at the L1 stage (Table 2, Figure 3F).

The loss of GABAergic synapses in ju89 young adult worms thus appears more severe than can be accounted for solely by defects in initial axon outgrowth, suggesting that ju89 may disrupt synapse formation and function independently of an earlier role in neuron morphogenesis or guidance. The increases in DD branching and gaps in the dorsal cord observed in adult ju89 animals may represent failure of the axon to grow (inserting new membrane and synaptic components) as mutant animals mature to their adult size. Alternatively, the DD motor neuron morphology may be unstable in adults due to defective differentiation of the axon during the earlier outgrowth phase.

ju89 alters a conserved residue between helix 11 and helix 12 in alpha-tubulin TBA-1

We mapped the ju89 mutation between egl-33 and lin-11 on C. elegans chromosome I by standard two- and three-factor mapping (Figure 5A) (see Experimental Procedures). Both the SNB-1::GFP defects and uncoordinated movement of ju89 mutants were rescued with fosmid H01117 and cosmid F26E4 (Figure 5B). We subsequently narrowed the minimal rescuing region to a 10.5 kb genomic clone that contained the two-gene operon encoding tba-1(F26E4.10), and drsh-1 (F26E4.8). Genomic subclones that contained only tba-1, the first gene of the operon, were sufficient to rescue the ju89 mutant defects. Deletions in tba-1, but not drsh-1, abolished the rescuing activity (Figure 5B).
tba-1 encodes one of nine predicted alpha-tubulins in C. elegans [30]. TBA-1 and its close parologue, TBA-2, are both required maternally and are expressed by all cells of the early embryo [31]. TBA-1 continues to be expressed by neurons in adults [32]. Microtubules are composed of heterodimers of alpha-tubulin and beta-tubulin that are preassembled before addition to the growing plus ends of microtubule polymers. All alpha-tubulin isoforms are highly homologous in sequence, with the greatest variation occurring in the C terminal domain that binds motor proteins and other microtubule-associated proteins. The crystal structure for a human alpha-tubulin is defined [33]. Sequence analysis of tba-1 in ju89 mutant worms identified a point mutation that changes a conserved glycine to arginine (G414R) (Figure 5C & D).

Based on the crystal structure of the human tubulin dimer [33–34], the ju89 mutation is located in the C-terminal domain of alpha-tubulin in a loop near the final alpha-helix, H12 (Figure 5C & D). The mutation is predicted to alter the external architecture of the microtubule polymer and can thereby influence the binding of motor proteins and other microtubule-associated proteins.

The tba-1(ju89) allele has altered gene function

Dominant mutations in a closely related human alpha-tubulin, TUBA1A (TUBA-3), are associated with defects in neuron migration and axon tract formation and are proposed to be due to haploinsufficiency of TUBA1A/TUBA3 [16]. Because the ju89 mutation may cause a structural change in TBA-1, we asked if it...
**Table 2. DD Neuron Outgrowth and Stability.**

| Animals affected | Axons affected | % Dorsal cord missing | Commissure outgrowth defects | Commissure branching defects |
|------------------|---------------|-----------------------|-----------------------------|-----------------------------|
| **Axon outgrowth L1 larvae** |               |                       |                             |                             |
| wild-type        | 8/33 (24%)    | 10/198 (5%)           | 0/198 (0%)                  | 0/198 (0%)                  |
| tba-1(ju89)      | 44/52 (85%)*  | 62/211 (29%)*         | 7/302 (2%)                  | 0/312 (0%)                  |
| tbb-2(gk130)     | 54/57 (95%)*  | 59/342 (17%)*         | 2/342 (<1%)                 | 0/342 (0%)                  |
| tba-1(ju89);tbb-2(gk130) | 47/54 (87%)*  | 55/324 (17%)*         | 3/324 (<1%)*                | 1/324 (<1%)                 |
| tbb-1(gk207)     | 27/50 (54%)*  | 34/250 (13.6%)*       | 4.5                         | 1/250 (<1%);                |
| tba-1(ju89);tbb-1(gk207) | 43/50 (86%)*  | 79/250 (32%)*         | 20.5                        | 1/250 (<1%)                 |
| **Axon stability Young Adult** |               |                       |                             |                             |
| wild-type        | 6/100 (6%)    | 10/500 (2%)           | nd                          | 2/500 (<1%)                 |
| tba-1(ju89)      | 11/45 (24%)*  | 18/225 (8%)*          | nd                          | 6/225 (3%)*                 |

The axon morphology of the six dorsal D (DD) motor neurons was visualized with P_{flp-13}:GFP (juIs145) and P_{unc-25}:GFP (juIs76) transgenes in L1 larvae and with P_{flp-13}:GFP in young adults (see Methods). All 6 DD axons were evaluated in animals expressing juIs76, and DD1-DD5 axons were examined in worms that expressed the juIs145 reporter. Animals were scored for gaps along the dorsal nerve cord, commissures that stopped short of the dorsal nerve cord, and abnormal branching morphology where commissures join the dorsal nerve cord (Figure 3). tbb-2(gk130) mutants and tba-1(ju89);tbb-2(gk130) animals exhibit similar DD axon defects, whereas axon defects are increased in tba-1(ju89);tbb-1(gk207) double mutants.

*Two-tailed Z-test, *p* <.005.

**p* <.05.

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**Figure 3. Axon Outgrowth Defects in Tubulin Mutants.** (A) Diagrams of defects in axon extension and commissure outgrowth observed in newly hatched mutant worms (see Table 2). The morphology of the DD neurons in wild-type and mutant worms was visualized with the cytoplasmic axon markers P_{unc-25}:GFP or P_{flp-13}:GFP. Examples of axon defects in tubulin mutants: (B), (C) short regions (arrows) where axons fail to extend to their full length along the dorsal nerve cord of tba-1(ju89) worms; (D) a commissure branches prematurely and fails to reach the dorsal nerve cord in a tbb-1(gk207) mutant animal; (E) axon outgrowth or extension defects in all six DD neurons of a newly hatched L1 larvae. The DD6 commissure stalls prematurely (arrow), and the DD2 cell body is also displaced anterior. (F) abnormal commissure branch (arrow) in a tba-1(ju89) mutant. Scale bar 20 μM.

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behaved as a dominant, altered function mutation or exhibited haploinsufficiency. Wild-type C. elegans move in a sinusoidal wave pattern, whereas ju89 homozygotes were uncoordinated, and the amplitude of the wave pattern was greatly reduced when animals move forward (Figure 1K & L). This visible locomotion phenotype differs from the shrinker phenotype of worms that have lost all GABAergic synaptic function [18,35], suggesting that GABAergic synaptic transmission is only partially affected in ju89 mutants or that alterations in the synaptic transmission of cholinergic motor neurons or interneurons may also contribute to the locomotion phenotype. We found that ju89 heterozygotes (ju89/+ ) moved in a pattern intermediate between wild-type and homozygous mutant worms and also exhibited weak variable defects in SNB-1::GFP expression, including irregular spaced puncta and a reduction in the number of dorsal puncta that varied between 10 and 20% (Table 1). The tba-1(ok1135) deletion removes almost 900 bp (70%) of the tba-1 coding sequence and is a likely null allele (Figure 5B), ok1135 animals have wild-type locomotion and SNB-1::GFP expression patterns (Table 1).

To determine if this weak dominant effect of ju89 was due to gene dosage, we examined the SNB-1::GFP expression in worms that were heterozygous for a deficiency chromosome, (qDj9) or heterozygous for the ok1135 deletion. The number of synapses based on SNB-1::GFP in qDj9/+ heterozygotes and ok1135/+ heterozygotes was the same as in wild-type worms (Table 1), indicating that tba-1 is not haploinsufficient. Moreover, the SNB-1::GFP defects of either ju89/qDj9 heterozygotes or ju89/ok1135 heterozygotes were less severe than ju89/ju89 homozygotes, revealing that ju89 behaves genetically as an altered function mutation. As further evidence that the ju89 allele of tba-1 can dominantly disrupt synapse and axon development, we amplified tba-1 genomic DNA from wild-type and mutant animals, and injected it in parallel into wild-type worms (see Experimental Methods). Worms from fourteen of twenty-seven extragenic lines derived from tba-1 DNA amplified from ju89 mutants (Exba-1R414) exhibited SNB-1::GFP defects and uncoordinated movement similar to homozygous ju89 mutants (Table 1, Figure 6B & D). We quantified the reduction in synapses for one ExMtba-1 line and determined that the number of dorsal SNB-1::GFP puncta was reduced by an average of 35%. In comparison, all lines that expressed arrays of wild-type tba-1 DNA injected at the same concentration were wild-type (Table 1; Figure 6A & C). Thus, the presence of the ju89 tba-1 allele was sufficient to alter motor neuron development and function.

Figure 4. Synapse Loss in DD Axons of tba-1(ju89) Animals. The DD neuron axon marker P

Pflp-13::GFP was coexpressed with a presynaptic marker P

mCherry::RAB-3 in wild-type (A, C, E) and mutant (B, D, F) tba-1(ju89) animals (see Methods). Axon extension was similar in both the wild-type (A) and mutant (B) axons depicted. mCherry::RAB-3 puncta were reduced or missing in the tba-1(ju89) mutant axon (D, F) compared to the wild-type worm (C, E). The bracket in (F) delineates a region in which mCherry::RAB-3 puncta are missing or greatly reduced in size in the mutant. Scale bar 20 μM.

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Genetic interactions with loss-of-function tbb-1 and tbb-2 tubulin mutants

tba-1 is one of two C. elegans alpha-tubulins and two beta-tubulins expressed maternally during early embryogenesis [31]. RNAi knockdown of pairwise combinations of tba-1, tba-2, tbb-1 and tbb-2 and analysis of deletion alleles demonstrated that each set of tubulins have overlapping functions for embryonic viability [36–37]. However, these tubulins are not completely redundant in the embryo. The microtubule severing protein MEI-1 (katanin) acts preferentially on microtubules containing alpha-tubulin tba-2 and the beta-tubulin tbb-2 [38–39]. Gene-specific RNAi targeting of tbb-2 also results in defects in centrosome positioning and ability of microtubules to interact properly with the cell cortex in early embryos that were not observed in single gene RNAi knockdown of the other three embryonic tubulins [26]. In later larval and adult stages, the expression pattern of these tubulins is more varied. tba-1 and tbb-2 are widely expressed throughout the C. elegans nervous system [31,38], whereas neuronal expression of tba-2 is restricted to a subset of cholinergic motor neurons (DB and VB neurons), sensory neurons and interneurons [40].

When we examined deletion alleles of tba-1, tbb-1 and tbb-2 generated by the C. elegans Gene Knock-out Consortium for
viability and defects in D neuron development we found similar results. We were unable to recover viable double mutant combinations of any of these three genes (tba-2 deletion alleles were not available to include in this analysis). By contrast, 72% (169/235) of tba-1(ju89); tbb-2(gk130) and 68% (204/301) of tba-1(ju89); tbb-1(gk207) animals were viable at 20°C and were evaluated for expression of the SNB-1::GFP marker and DD morphology (Tables 1 & 2). This result supports that TBA-1-G414R subunits produced in ju89 mutants can be incorporated into microtubules and retain essential functions in embryos.

Our analysis revealed that L1 larvae deficient for TBB-2 or TBB-1 exhibited DD axon outgrowth defects similar to, but weaker than, tba-1(ju89) mutants (Table 2):17% of tbb-2(gk130) axons were defective compared to 13.6% of defective axons in tbb-1(gk207) animals and 20% of axons that terminated prematurely in tba-1(ju89) mutants (Table 2). The frequency of axon defects was similar in the tbb-2(gk130) single and the tba-1(ju89); tbb-2(gk130) double mutant. The lack of a strong additive axon phenotype in the tba-1(ju89); tbb-2(gk130) double mutants suggested that axon outgrowth defects in tba-1(ju89) may involve loss of TBB-2 function. Indeed, when TBB-1 function was removed in the tba-1(ju89); tbb-1(gk207) double mutant, increased synthetic embryonic lethality was observed at 20°C, and DD motor neuron development was more severely affected in the surviving L1 larvae. 32% did not extend completely along the dorsal nerve cord (Tables 1 & 2), and DD neurons were mispositioned or missing in 10% (5/50) of the tba-1(ju89); tbb-1(gk207) double mutant animals (Figure 3E). Unlike the more complex phenotype of the dominant ju89 single mutants, the severe loss of synapses in the ju89; tbb-1(gk207) double mutant and the decrease in synapses in tbb-2(gk130) loss-of-function animals (Table 1) appears to be caused by defective axon outgrowth.

Our genetic analysis indicates that the function of microtubules containing TBA-1-TBB-2 heterodimers is compromised in ju89.
mutants. DD neuron development may be less severely impaired in ju89 single mutants because microtubules containing TBA-1/TBB1 heterodimers are still able to compensate for most alterations in TBA-1-TBB2 heterodimer function during axon and synapse growth, or TBB1 may form functional microtubules with another partially redundant alpha-tubulin expressed by the DD neurons. Interestingly, although the axon defects of tba-1 ju89 mutants were similar to ju89 homozygotes. All seven lines expressing wild-type tba-1 (Exta-1) were wild-type. Expression of the juls1 SNB-1::GFP transgene in the dorsal nerve cord of wild-type N2 worms expressing (A) Exta-1; or (B) Exta-1(R414). The body size and locomotion of transgenic animals expressing wild-type tba-1 arrays (C) was the same as wild-type worms, whereas N2 worms expressing arrays of tba-1 amplified from the ju89 mutant (D) exhibited the smaller body size and uncoordinated movement characteristic of ju89 mutants. Scale bar 5 μM. doi:10.1371/journal.pone.0009655.g006

Discussion

Over the past decade, many structural components of the presynaptic active zone have been identified by biochemical or genetic strategies. One unresolved question in axon and presynaptic terminal differentiation is how the different components of the presynaptic specialization are regulated and transported to synaptic sites. Dynamic formation of nascent presynaptic structures occurs during axon outgrowth prior to synapse formation independent of contact with post-synaptic partners and is an integral step in establishing the connectivity pattern of a neuron [41]. Transport packets containing preassembled components of the active zone cytomatrix have also been detected in differentiating axons, and these transport packets localize independently of neurotransmitter vesicles [42–44]. Extension of the axon and transport of synaptic components are both dependent on the intricate regulation of microtubule dynamics and interactions with retrograde and anterograde motor proteins.

Maintenance of existing synapses and growth of new synapses are thought to rely on similar transport mechanisms. Between the late hatching and young adult stage, C. elegans goes through four larval molts and increases in size four-fold. As the animal grows, new membrane is added to existing motor neuron axons, and the neurons must either add new synapses or increase the size of existing synapses to maintain effective synaptic transmission. This transition has been documented in detail for the C. elegans DD neurons by Yeh and Zhen [27]. Using GFP markers and electron microscopy, they determined that the number of new GABAergic synapses increases by less than 30%, while each existing synapse increases in size by at least threefold. The distance between synapses also increases as new membrane is added to the axon.

Our analysis of tba-1(ju89) defects suggests that defects in microtubule structure or dynamics in the mutants compromises motor neuron axon and synapse growth. Our data indicates that the tba-1(ju89) mutation perturbs initial axon outgrowth, and that the mutants exhibit additional synapse defects. Many of the synaptic phenotypes we have documented in ju89 mutants are consistent with defects in the biogenesis or trafficking of vesicles containing structural components of motor neuron synapses and axons, rather than a defect solely in synaptic vesicle transport. In Drosophila imac mutants, for example, synaptic boutons completely disappear from motor neurons due to loss of a kinesin-3 motor that is implicated in transport of active zone cytomatrix proteins as well as synaptic vesicles [45]. When imac function is removed, the distal tips of the axons also remain thin and undifferentiated, and synaptic vesicles are mislocalized. We identified quite similar, but weaker, phenotypes in the tba-1(ju89) mutants. Both the synaptic vesicle marker, SNB-1::GFP and the active zone marker, UNC-10::GFP, have reduced or absent expression at the distal tips of the D neurons, even when a thin neurite is still visible by epi-fluorescence microscopy. Indeed, the expression pattern of the synaptic vesicle marker SNB-1::GFP and active zone marker UNC-10::GFP in the GABAergic DD motor neuron were identical in ju89 mutants. A small decrease in size of active zones and the vesicle pool detectable at these synapses was also observed in the cholinergic motor neurons by EM, while the proportion of synaptic vesicles/size of active zone was similar in wild-type and mutant animals. All of these data are consistent with a failure of the axons and synapses to grow properly as animals mature. The aberrant branching that increases with the age of the mutants may also reflect defects in microtubule stability and transport that slowly become more severe as axons grow to their full adult size.

Our genetic analysis demonstrates that the ju89 mutation dominantly alters tba-1 function. Heterozygous ju89 animals display locomotion and SNB-1::GFP phenotypes intermediate between wild-type and ju89 homozygotes. We were also able to induce the mutant defects by expressing mutant TBA-1 protein in wild-type animals. By contrast, the loss-of-function tba-1 allele appears nearly wild-type, likely due to overlapping function with other alpha-tubulins. Similar results have been observed during early cell divisions, where altered function alleles of tubulin mutations often have a more severe impact on microtubule function than null alleles [39,46]. The genetic data that ju89; tbb-2
and ju89; tbb-1 double mutants are viable, while loss-of-function tba-1 allele, ok1135 is lethal in combination with tbb-1 or tbb-2 null alleles, argues that the mutant TBA-1 protein is incorporated into microtubules and able to perform part of its wild-type functions. The absence of synaptic TBA-2 expression by the GABAergic D motor neurons and anterior cholinergic motor neurons [40] may explain why the tba-1(ju89) allele has a more deleterious affect on these neurons. Our genetic analysis indicates that TBA-1(R414) interacts with both TBB-1 and TBB-2 during neuronal development, but that MTs formed by TBA-1 and TBB-2 may be more severely affected for some functions. This result is consistent with another dominant allele of TBA-1, m346f, that disrupts mitotic spindle positioning and causes severe embryonic lethality [37]. However, the relative contribution of each beta-tubulin to the ju89 phenotypes may be more complex. Autoregulation and feedback between tubulins has been observed in C. elegans embryos, and could be a factor in later stages of development [46].

The ju89 mutation is located near the beginning of helix 12 of the TBA-1 C-terminus near the alpha-beta tubulin heterodimer boundary, but it is not part of the intradimer interface. Cryo-EM studies indicate that H11 and H12 project to the outside of the microtubule and contribute to ridge and groove structures on the external face of microtubules, providing a binding surface for structural MAPs and motor proteins [47]. The ju89 mutation occurs in a critical location on the microtubule surface that could disrupt specific microtubule-associated proteins (MAPs). Motor proteins and other MAPs can selectively bind either the alpha or beta subunits of the microtubule polymer, and structural MAPs that stabilize protofilaments into the typical13-protofilament microtubule can potentially interact with both classes of tubulin. Some of the proteins identified to bind the alpha-tubulin subunit include the ebp family of microtubule plus-end binding proteins [15], tubulin destabilizing proteins op50/statmin [48] and spastin [10], tubulin folding cofactor E [7,49], doublecortin [50,51] and the kinesin-3 motor Kif1A [52]. Because the genetic analysis suggests MTs containing TBA-1 and TBB2 may be especially impaired in ju89 mutants, MAPs that specifically interact with TBB-2 could also be affected.

Models for how tba-1(ju89) may affect microtubule dynamics and axon transport
tba-1(ju89) mutants exhibit pleiotropic defects in synapse and axon axon transport that suggest TBA-1 interactions with several motors and structural MAPS may be altered that could alter microtubule dynamics or function. One likely candidate is the C. elegans homologue of Kif1A and IMAC: UNC-104. Similar to the phenotypes observed in Drosophila IMAC mutants, transport of synaptic vesicles is severely affected by loss-of-function mutations in unc-104 [53]. The decrease in synaptic vesicles at ju89 motor neuron synapses is consistent with partial loss of UNC-104-mediated transport. However, UNC-104 does not solely control localization of synaptic components such as UNC-10/RIM, UNC-15 and ELKS in C. elegans [26], and additional motors involved in transport of these proteins or other active zone proteins could also be perturbed in the tba-1 mutants.

A second possibility is that the kinetics of tubulin folding and heterodimer formation is compromised by the ju89 mutation. Mutations in tubulin-specific chaperonin E (TBCE), a key cofactor in tubulin dimer assembly and disassembly, have been shown to cause motor neuron axon defects and degeneration in mice [7]. The loss of distal microtubules and retrograde dying back of motor neuron axons in these mutants was subsequently shown to involve loss of TBCE function at the golgi apparatus where it regulates routing of axonal tubulin [49]. In a recent investigation of TBCE function in Drosophila, RNAi knockdown of either presynaptic or postsynaptic TBCE disrupted the distinctive microtubule loop structures present at the fly neuromuscular junction [54].

A third possibility is that ju89 disrupts TBA-1 interactions with a specific MAP, such as doublecortin, that can stabilize MTs and MT interactions with motor proteins. Mutations in human and mouse alpha-tubulin, TUBA1A (TUB3A) cause lissencephaly [16]. Both the human lissencephaly R402H TUBA1A mutation and tba-1(ju89) G414R mutations are predicted to alter the H11–H12 loop of alpha-tubulin. Lissencephaly results from the failure of vertebrate cortical neurons to migrate properly during development, and is frequently caused by mutations in doublecortin (DCX) [35]. DCX is a potent microtubule stabilizer expressed by post-mitotic neurons that binds between the protofilaments of the MT polymer, promoting polymerization and stable microtubules that can support kinesin and dynein activity [54–55]. Analysis of the binding of DCX to microtubules indicates that the H11–H12 loop of alpha-tubulin can form an interface with DCX or the closely related dclk(doublecortin related kinase) [55]. Vertebrate DCX and Dclk are also present at axon termini in developing neurons [56–57], and defects in axon extension and transport occur in mice heterozygous for mutations in the Mouse homologues of these proteins [58–59]. Axon outgrowth defects and disorganized axon tracts were also identified recently in studies of stillborn human TUBA1A embryos [60] and in a broad spectrum of developmental brain disorders newly characterized in mammalian beta-tubulin mutants [61]. It is noteworthy that dominant mutations in the mammalian beta-tubulin TUBB3 result both in reduced microtubule dynamics and perturbed MT binding to the kinesin Kif21A in in vitro experiments, and are associated with defects in axon maintenance in addition to an earlier developmental affect on axon outgrowth [61].

The C. elegans genome contains a single doublecortin family member, the dclk protein ZYG-8. zyg-8 is a maternal effect lethal gene in C. elegans; embryos from homozygous mutant hermaphrodites arrest due to defects in positioning the mitotic spindle [62]. It remains to be determined if TBA-1 interactions with TBCE, ZYG-8 or specific kinesins are altered in ju89 mutants; these questions can be addressed by direct biochemical experiments and genetic modifier screens in the future. The similarity between the tba-1(ju89) phenotypes and neurodevelopmental defects reported in mammalian motor neuron diseases make the C. elegans tba-1(ju89) mutant a useful genetic tool to study the microtubule-mediated functions altered in these disorders and identify mechanisms that can stabilize axons and reverse synapse loss.

Materials and Methods

C. elegans strains and genetics
All strains were cultured at 20°C as described by Brenner [63]. tba-1(ju89) was isolated from an ethylmethane sulfonate (EMS) mutagenesis screen of N2 animals carrying the integrated chromosomal array ju11 {P unc-25SNB::GFP; lin-15(+)}). The ju89 mutation was backcrossed multiple times. Linkage mapping placed ju89 to chromosome one based on the uncoordinated and syd (synapse defective) phenotype. Standard two and three factor questions can be addressed by direct biochemical experiments and genetic modifier screens in the future. The similarity between the tba-1(ju89) phenotypes and neurodevelopmental defects reported in mammalian motor neuron diseases make the C. elegans tba-1(ju89) mutant a useful genetic tool to study the microtubule-mediated functions altered in these disorders and identify mechanisms that can stabilize axons and reverse synapse loss.
5(za193)unc-13(e1091)/ht2; +/ht2 III; him-8(ae36) IV; MT151: gfp-33(n151); SI726; unc-29(h1) hp6 dpy-24(s71); VC364: tbb-1(eg207)III; VC167: tbb-2(gk130)III. The genotypes of other markers used are: od522 (UNC-49::GFP); ju1337 (P_{gk130}-SNB-1::GFP); ju4567 (P_{uv25}-GFP); ju1545 (P_{uv25}-GFP); hp6167 (P_{uv25}-UNG10-GFP, ju13698) [P_{unc-11::Cherry::RAB-3}] [53]. Strains carrying extragenic tba-1 arrays were generated as described below.

**Molecular biology.** Cosmids and fosmids were provided by the Sanger Centre, Hinxton, UK. A 10.5 kb PstI and PvuII fragment of genomic DNA derived from cosmid F26E4 was cloned into plBluescript to generate pCZ483. pCZ483 includes both genes of the tba-1 operon (tba-1 and dsh-1) and 2 kb of upstream genomic sequence. All other genomic subclones were deletion derivatives of pCZ483. pCZ483 was generated by digesting pCZ483 with BstZ171 and religating, resulting in a subclone in which exon 3 of tba-1 was deleted. pCZ486 contains a deletion only in the dsh-1 coding region and was constructed by digesting pCZ483 with SphI and religating. pCZ484 is a pBluescript subclone containing a PVU1::AcII fragment of pCZ483 that encodes only tba-1(F26E4.8).

The joy9 lesion was identified by determining the genomic sequence of all exons and introns of tba-1 and 1.2 kb of sequence upstream of the 5’ end of tba-1. Genomic DNA from joy9 mutants was generated by standard PCR techniques, and PCR products from three independent PCR reactions for each primer pair was used to obtain sequence from both strands (UC-Berkeley Sequencing Center). The following primer pairs were used to confirm tbb-1 and tbb-2 deletions: tbb-1: 5’-CATTGATATATTCCGGCTCGAC-G3’ and 5’GTAGAGATCGTTTCTC-TCCAGCTG3’; tbb-2: 5’-CTAGGAAGTTACCTGCCGCTGTA-3’ and 5’-GACAAGTCTAGCTCCTTGTGTG3’.

**Transgenic strains.** Germline transformation was performed following standard C. elegans procedures [63]. Cosmids and fosmid DNA and subclones were injected at 5–2 ng/ul of PCR product together with 20 ng/ul of P_{tet-gfp} as selectable markers. The Expand Long PCR system (Roche) was used to amplify the 4.5 kb tba-1 genomic region from N2 and joy9 worms. Strains expressing either the PCR product amplified from wild-type DNA {Extba-1} or from the mutant joy9 DNA {Exba1R414} were constructed by injecting wild-type worms with 1 ng/ul of PCR product together with 20 ng/ul of P_{tet-gfp} as a selectable marker [66].

**GFP and immunocytochemical analysis.** Live GFP observation and imaging was performed with an HQ-FITC filter setup (Chroma, VT, USA) and 63X objective on a Zeiss AxioplanII with a Zeiss Axioscan imaging system and a Zeiss Pascal Confocal Microscope. Additional images were recorded using HQ-FITC and HQ-rhodamine fillers and 60X objective on a Nikon E800 microscope equipped with a Coolscan ES camera and Metamorph software. For imaging, adult animals were anesthetized using phenox propanol or pretreated in 2% paraformaldehyde for 20 minutes. L1 larvae were imaged without anesthetic. Whole-mount staining for anti-SNT-1 was used to mark paradafmaldehyde fixation [67]. For anti-UNC-10 (Rim) staining, worms were prepared using Bouin’s fixative [68], Rabbit anti-SNT-1 and anti-UNC-10 antibodies were provided by Mike Nonet. Secondary mouse anti-rabbit antibodies were obtained from Jackson Laboratories.

**Analysis of axon outgrowth.** The six dorsal D (DD) motor neurons were visualized with P_{gk130}-GFP (juIs145); P_{gk130}-SNB-1-GFP (juIs137); and P_{uv25}-GFP (juIs76) transgenes. All six DD axons were examined in L1 larvae expressing juIs176, and DD-DE5 axons were examined in L1s and adult worms that expressed the juIs145 or juIs137 reporter transgenes. To quantify DD axon outgrowth defects along the dorsal nerve cord, images were collected using a Coolscan ES CCD camera (Roper) and Metamorph software. Metamorph or ImageJ software was used to measure the total dorsal nerve cord length and any detectable break or gap(s) present in the dorsal nerve cord for each animal. To assess changes in DD morphology between L1 and adult stages, L1s with no defects in juIs145 GFP expression were selected, grown for 2 days at 20–22°C, and then were examined a second time by epi-fluorescence microscopy.

**EM analysis.** Young adult juIs1 (wild-type) and tba-1(ju99); juIs11 animals were fixed in parallel using glutaraldehyde and osmium as described previously [21]. Serial thin sections (45 nm) were collected from the anterior of each animal, between the nerve ring and the vulva. A JEO1-1200 electron microscope equipped with a GATAN digital camera was used to photograph the ventral and dorsal nerve cords. GABAergic NMJs were identified as synapses made by the DD or VD neurons onto muscle arms. Cholinergic NMJs were identified as dyadic synapses onto both muscle arms and DD or VD neurons. The identity of the nerve processes in the nerve cord was determined by comparison to EM images from the C. elegans database [17]. Data was collected from one juIs1 animals (1,500 sections) and two ju99; juIs11 animals (400 sections each).

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**Author Contributions**

Conceived and designed the experiments: RB YJ. Performed the experiments: RB LC GT IS AG YJ. Analyzed the data: RB LC YJ. Wrote the paper: RB YJ.

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