Disorders of the Nervous System

Reduced TUBA1A Tubulin Causes Defects in Trafficking and Impaired Adult Motor Behavior

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Abstract

Newly born neurons express high levels of TUBA1A α-tubulin to assemble microtubules for neurite extension and to provide tracks for intracellular transport. In the adult brain, Tuba1a expression decreases dramatically. A mouse that harbors a loss-of-function mutation in the gene encoding TUBA1A (Tuba1aND/1) allows us to ask whether TUBA1A is important for the function of mature neurons. α-Tubulin levels are about half of wild type in juvenile Tuba1aND/1 brains, but are close to normal in older animals. In postnatal day (P)0 cultured neurons, reduced TUBA1A allows for assembly of less microtubules in axons resulting in more pausing during organelle trafficking. While Tuba1aND/1 mouse behavior is indistinguishable from wild-type siblings at weaning, Tuba1aND/1 mice develop adult-onset ataxia. Neurons important for motor function in Tuba1aND/1 remain indistinguishable from wild-type with respect to morphology and number and display no evidence of axon degeneration. Tuba1aND/1 neuromuscular junction (NMJ) synapses are the same size as wild-type before the onset of ataxia, but are reduced in size in older animals. Together, these data indicate that the TUBA1A-rich microtubule tracks that are assembled during development are essential for mature neuron function and maintenance of synapses over time.

Key words: ataxia; microtubule network; movement disorder; trafficking; TUBA1A; tubulin

Significance Statement

Defects in axonal trafficking have been reported in models of movement disorders, but it has been unclear whether trafficking defects are coincident or causative. Our results suggest that deficits in TUBA1A α-tubulin can cause trafficking defects that impair synaptic maintenance leading to a movement disorder without axon degeneration or impacting myelination or neuron survival.

Introduction

Microtubules are the tracks on which proteins, RNA, and organelles are trafficked. Microtubule polymers are composed of αβ-tubulin heterodimers. In humans, nine α-tubulin and 10 β-tubulin isotypes are differentially expressed and could influence properties of specific cellular microtubule networks (Gadadhar et al., 2017). In developing neurons, the most highly expressed α-tubulin is encoded by Tuba1a (Lewis et al., 1985; Miller et al., 1987; Gloster et al., 1994, 1999; Zhang et al., 2014). Heterozygous mutations that disrupt human TUBA1A are associated with severe brain malformations, termed tubulinopathies, suggesting that TUBA1A is critical for brain development (Keays et al., 2007; Kumar et al., 2010; Cushion et al., 2013; Aiken et al., 2017). The most common human TUBA1A variants dominantly disrupt microtubule function in neurons (Tian et al., 2010; Aiken et al., 2019b). No deletions, frameshift, or nonsense mutations have been identified in association with TUBA1A tubulinopathy patients (Fallet-Bianco et al., 2008; Cushion et al., 2013; Oegema et al., 2015). We recently proposed TUBA1A mutations that impair brain development dominantly disrupt microtubule function, while...
heterozygous TUBA1A loss-of-function mutations are tolerable for brain development and are not clinically reported (Aiken et al., 2019a). How TUBA1A contributes to adult neuron function has not been reported. TUBA1A constitutes ~95% of neuronal α-tubulin mRNA developmentally (Miller et al., 1987). While Tuba1a expression is reduced overall in the adult brain, some regions including the hippocampus, cerebellum, basal ganglia, and certain regions of the cerebral cortex retain TUBA1A expression in adulthood (Barnji and Miller, 1996). Over time, neuronal microtubule networks acquire post-translational modifications (PTMs) and associations with microtubule-associated proteins (MAPs) that stabilize neuronal microtubules (Brady et al., 1984; Yan et al., 1985; Audebert et al., 1994; Bonnet et al., 2001; Song et al., 2013; Natarajan et al., 2017). TUBA1A proteins may incorporate into microtubules during neurodevelopment and remain within the microtubule network of adult neurons. Indeed, TUBA1A, and other tubulins, have a half-life on the order of weeks to months in rodents (Heo et al., 2018). TUBA1A may be important in the adult neuronal microtubule network. However, mechanistic understanding of TUBA1A function in vivo has been limited by the high degree of sequence similarity between α-tubulin isoforms, as iso-type-specific α-tubulin antibodies are not available.

To overcome the technical limitations to studying the specific impact of TUBA1A in adult neurons, we use the Tuba1aND mutant mouse which harbors an asparagine to aspartic acid substitution at amino acid residue 102 (Gartz Hanson et al., 2016). Homozygous Tuba1aND/ND mice are neonatal lethal and have severe brain malformations consistent with homozygous Tuba1aND/null and Tuba1aqtx mutant mice (Bittermann et al., 2019) and similar to phenotypes observed in human patients with heterozygous TUBA1A mutations (Gartz Hanson et al., 2016; Aiken et al., 2017). The corresponding ND substitution in the primary α-tubulin in yeast decreased α-tubulin protein levels, altered microtubule dynamics, reduced incorporation of the mutant α-tubulin into the lattice, and impaired mitotic division (Gartz Hanson et al., 2016). Together, these data demonstrate that Tuba1aND substitution results in less stable microtubules. In contrast to the severe impact of Tuba1aND/ND homozygous substitution on neuronal development, heterozygous Tuba1aND+/− mice survive to adulthood (Gartz Hanson et al., 2016). The Tuba1aND+/− heterozygous mice can be used to interrogate the requirement for TUBA1A neuronal microtubules within the mature brain.

Here, we show that reduced function of TUBA1A, a tubulin isoform that has historically been characterized only in a developmental context, impacts the ability to maintain synapses over time and ultimately results in an adult-onset movement disorder. Microtubule tracks are not adequately assembled when there is a deficit in TUBA1A tubulin early in development. Insufficient microtubule tracks result in organelle trafficking deficits that are apparent in Tuba1aND+/− heterozygous neurons early in development. Despite microtubule track abnormalities and resulting changes in trafficking, Tuba1aND+/− microtubules are sufficient to support survival and morphologically normal neuromuscular junction (NMJ) synapses. However, NMJ synapse morphology and animal behavior deteriorate in an age-related manner in Tuba1aND+/− animals, without evidence of neuronal cell death or degeneration. Together, these data indicate that both developing and adult neurons require functional TUBA1A, and suggest that the “developmental” tubulin TUBA1A is required for mature neuronal function.

Materials and Methods

Mice

All animal research was performed in accordance with the Institutional Animal Care and Use Committee at the University of Colorado School of Medicine. The Tuba1aND mouse model was generated via an ENU-induced mutagenesis forward genetic screen and is described in more detail in Gartz Hanson et al. (2016). All mice used were maintained on a 129S1/C57Bl6 genetic background. Mice were kept on a 12/12 h light/dark cycle with ad libitum access to food and water. Tuba1aND and wild-type littermate mice were maintained on water supplemented with 0.2 g/l MgSO4 to promote Tuba1aND+/− survival and ability to reproduce. Male and female mice were represented in all studies. All mice were genotyped by PCR amplification of tail DNA followed by Sanger sequencing to differentiate homozygous or heterozygous Tuba1aND+/− mice from wild type.

Behavioral phenotyping

Mice were assessed for abnormal motor function between two and 10 months of age. Mice were allowed to acclimate to the testing room for 30 min before behavioral assessment. Gait analysis testing was performed in an enclosed raised chamber with a strip of paper lining the bottom. Fore and hind paws were painted differing colors just before testing, after which mice were placed at the chamber entrance and allowed to run through. Distances between footprints taken from a running gait were analyzed in ImageJ (ImageJ; National Institutes of Health). Rotarod performance was assessed using a Rota-Rod Treadmill (Med Associates, Inc) apparatus with one mouse per lane and a maximum of four mice tested simultaneously. Mice received no prior training on the rotarod task. The rod initially began rotating at 3 RPM and increased until reaching a terminal rotational speed of 30 RPM after 3 min. The time until each mouse fell off or lost its grip such that it could not remain upright on the rod.
was measured. A cutoff time of 4 min was established for any mice that remained on the rod after peak rotational speed was reached. Mice performed three subsequent trials on the rotarod per testing session, with a 15-min rest period in between trials. Grip strength was tested in mouse forelimbs using a grip strength meter (Stoelting Co). Three subsequent forelimb grip strengths were recorded per testing session.

Mouse hind-paw sensory function was tested between three and six months.

For both Von Frey and Hargreaves analyses, individual mice were contained in 8 × 8 × 17 cm chambers and were allowed to acclimate to the testing chamber for 30 min. Mice were only tested when resting on all four paws in a non-grooming awake state. Von Frey analysis of mechanical stimulus sensation was performed in left and right hind-paws using Touch Test Sensory Probes (Stoelting Co). Von Frey performance score was analyzed using the SUDO method established in Bonin et al. (2014). Hargreaves analysis of thermal pain sensation was assessed using a paw thermal stimulator instrument (University of California, San Diego). Time until withdrawal from thermal stimuli was recorded for each animal in both left and right hind-paws. Three subsequent trials were performed for each hind-paw per testing session.

Histology

Mice were anesthetized and transcardially perfused with 0.1 M NaCl and 4% paraformaldehyde (PFA) for histology. Tissues of interest were dissected and postfixed in 4% PFA. Serial tissue sectioning was performed on a CM1520 cryostat (Leica) and 30-μm PFA. Serial tissue sectioning was performed on a CM1520 tissue microtome and postfixed in 4% glutaraldehyde 4% PFA overnight at 4°C. Following post-fixation, spinal cords were dissected from the spinal column, and a 2-mm region of the cervical spinal cord between C2 and C4 was dissected and sent for further processing and imaging by the CU School of Medicine Electron Microscopy Core facility. Myelin thickness, axon density, and axon diameter were measured from electron micrographs in the lumbar spinal cord. G-ratio was calculated using the formula $G = r_i/R_i$ where $r_i = \text{radius of axon and myelin}$. At least three sections per animal were analyzed, measuring 100 axons per animal. The same total area was quantified between wild-type and TubulinαND/+/ mice.

Electron microscopy

Mice used for electron microscopy were perfused with 0.1 M NaCl and 2.5% glutaraldehyde 4% PFA, after which the spinal column was dissected and postfixed in 2.5% glutaraldehyde 4% PFA overnight at 4°C. Following post-fixation, spinal cords were dissected from the spinal column, and a 2-mm region of the cervical spinal cord between C2 and C4 was dissected and sent for further processing and imaging by the CU School of Medicine Electron Microscopy Core facility. Myelin thickness, axon density, and axon diameter were measured from electron micrographs in the lumbar spinal cord. G-ratio was calculated using the formula $G = r_i/R_i$ where $r_i = \text{radius of axon and myelin}$. At least three sections per animal were analyzed, measuring 100 axons per animal. The same total area was quantified between wild-type and TubulinαND/+/ mice.

Cell culture and transfection

Dissociated neurons were cultured from post-natal day 0 (P0) to P2 mouse cortices. Brains were removed and placed into HBSS (Life Technologies) supplemented with 1 M HEPES (Life Technologies) and 1 mM kynurenic acid (Tocris Bioscience). Meninges were removed and cortices were dissected and cut into ~1-mm pieces. Cortical pieces were triturated to a single-cell suspension using glass Pasteur pipettes. Cortical neurons were plated onto 35-mm Poly-D-Lysine coated glass-bottom culture dishes at a density of 350,000 cells/35 mm. Neurons were maintained in a 37°C humidified incubator with 5% CO2 in phenol-free Neurobasal-A medium (Life Technologies) supplemented with B-27 (Thermo), pen/strep (Thermo), GlutaMax (Thermo), 5 ng/ml β-FGF (Invitrogen), and sodium pyruvate (Thermo). For GFP-MACF43 puncta analysis, neurons were nucleofected with 2 μg of plasmid DNA using a mouse neuron nucleofector kit (Lonza) and Nucleofector 2b Device (Lonza), then plated normally.

GFP-MACF43 and organelle transport assays

Primary cortical neurons nucleofected with GFP-MACF43 plasmid DNA were imaged at day in vitro (DIV) 1 to estimate the number of microtubule (+) ends per μm of axon. Cells were placed in a 37°C imaging chamber and imaged using a 40× oil objective on a Zeiss 780 confocal microscope using ZEN imaging software (Zeiss). GFP-MACF43 images were acquired every 2 s for a total duration of 2 min. Multiple 2-min videos were acquired for each neuron imaged, and data were used to generate a cell average, which was reported. Lysosomal and mitochondrial trafficking was assessed in cortical neurons at DIV7. Cortical neurons were incubated in either LysoTracker Red DND-99 or MitoTracker Red FM (Invitrogen) dye for 20 min at 37°C, after which normal culture media was replaced and cells were transferred to a 37°C imaging chamber and imaged on either a Zeiss 780 confocal (MitoTracker) or a Nikon Ti-E microscope equipped with a 1.3 NA 40× CFI60 objective.
Plan Fluor objective, piezo electric stage (Physik Instrumente), spinning disk confocal scanner unit (CSU10; Yokogawa), 488 nm and 561 nm lasers (Agilent Technologies), and an EMCCD camera (iXon Ultra 897; Andor Technology) using NIS Elements software (Nikon). Cells were imaged every 1 s or every 2 s for lysosomes and mitochondria, respectively.

Western blotting

Protein was isolated from brains of P0–P2 and adult mice by dounce homogenization and ultra-centrifugation, using a modified version of the protocol described by Vallee (1982). Protein concentrations were assessed using a BCA assay (Thermo), and relative concentration was determined using a Synergy H1 microplate reader (Bio-Rad Instruments). For tubulin Western blotting, 0.25 μg of brain lysate was loaded per lane, for all other blots 10 μg of tissue lysate was used. Protein was run on 4–20% gradient acrylamide gels (Bio-Rad Laboratories) at 150 mV for 1 h. Proteins were transferred to PVDF blotting membranes (Bio-Rad) in transfer buffer containing 25 mM Tris-base, 192 mM glycine, and 15% methanol for 1 h at 75 V at 4°C. Gels were dyed with Coomassie Blue (Bio-Rad) post-transfer to assess transfer efficiency for each blot. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST) with 5% BSA for 1 h and incubated in primary antibody overnight at 4°C. Primary antibodies were diluted in TBST containing 1% BSA at 4°C overnight. Blots were incubated in secondary antibodies diluted in TBST containing 0.5% BSA with streptavidin-HRP (Bio-Rad, 1:10,000) for 1 h at room temperature. Primary antibodies for Western blotting were as follows: mouse anti-DM1A (Sigma-Aldrich T6199; 1:10,000), rabbit anti-GAPDH (Cell Signaling 14C10; 1:1000), mouse anti-acetylated tubulin (Sigma-Aldrich T7451; 1:1000), mouse anti-tyrosinated tubulin (Sigma T9028; 1:5000), rabbit anti-detyrosinated tubulin (Abcam ab48389; 1:1000), mouse anti-polyglutamylated tubulin (GT335, Adipogen AG-20B-0020; 1:2000), and mouse anti-SMI32 (BioLegend 801702; 1:500). Secondary antibodies used were HRP conjugated and diluted 1:5000 (Santa Cruz Biotechnology). Blots were developed in ECL solution (Bio-Rad) and imaged using a ChemiDoc MP imager (Bio-Rad).

RNA isolation + RT-PCR

RNA was isolated from flash-frozen mouse brains dissected from P0 or adult mice using an SV Total RNA Isolation system (Promega). RNA concentration and purity were determined using a spectrophotometer, then cDNA was synthesized using the RT2 First Strand kit (Qiagen). RT–PCR reactions were prepared with SYBR Green RT-PCR Master mix (Thermo) and run with a CFX Connect Real-Time System (Bio-Rad). Samples were run in triplicate, results were analyzed in Excel. All qPCR data presented in this manuscript was normalized to expression of the housekeeping gene Cyclin A. Tuba1a expression is dramatically decreased, however certain brain regions including the neocortex and hippocampus retain Tuba1a expression into adulthood (Barnji and Miller, 1996). Many studies have focused on the role of Tuba1a during brain development, but it is unclear how much Tuba1a contributes to the α-tubulin pool in adult neurons of the brain and spinal cord. Although TUBA1A is ubiquitously expressed in post-mitotic neurons, the relative abundance of different α-tubulin isotypes in specific neuronal subtypes over time has not been well characterized. To understand how the neuronal α-tubulin isotype blend changes over time, we assessed mRNA for Tuba1a, Tuba1b, Tuba1c, and Tuba4a in the cortex and spinal cord of post-natal day 0 (P0) and adult wild-
Postnatally, normalized to housekeeping gene Cyclin A. Bars represent mean ± SEM.

Figure 1. Changes in α-tubulin isotype expression in cortex (CTX) and spinal cord (SC) between post-natal day 0 (P0) and adult. A, Bar graph representing relative quantity of TUBA1A mRNA in P0 (left) and adult (right) mouse cortex and spinal cord. B, Bar graph representing relative quantity of TUBA1B mRNA in cortex and spinal cord of P0 (left) and adult (right) mice. C, Bar graph representing relative quantity of TUBA4A mRNA in cortex and spinal cord of P0 (left) and adult (right) mice. D, Bar graphs representing the α-tubulin isotype mRNA expression data from A–C as a percentage of the total α-tubulin isotype composition. Three animals per genotype were analyzed for each time point, with three technical replicates per animal. Data are plotted as relative mRNA quantity normalized to housekeeping gene Cyclin A. Bars represent mean ± SEM.

Type mice (Fig. 1A–C; N = 3). Unfortunately, levels of Tuba1c mRNA were too low to be reliably detected in our assays, thus we have omitted those data. Although the relative quantity of mRNA for each isoform differed between the cortex and spinal cord, we found that the ratio of α-tubulin isotypes was comparable between spinal cord and cortex, in both newborn and adult animals (Fig. 1D; N = 3). Intriguingly, although Tuba1a expression is downregulated between development and adulthood, we found that in wild-type adult mice, Tuba1a comprises 32% of all cortical and 47% of all spinal cord α-tubulin mRNA (Fig. 1D). These data demonstrate that although Tuba1a mRNA abundance decreases postnatally, Tuba1a is the most abundant α-tubulin isoform in adult spinal cord and the second-most abundant α-tubulin in adult cortex. Because Tuba1a constitutes a large percentage of the total α-tubulin in both cortical and spinal neurons over time, we predict that Tuba1a plays an important role in the adult neuronal microtubule network.

Tuba1aND is a loss-of-function mutation in mice

While it is known that Tuba1a contributes significantly to development, it has been difficult to determine how this isoform contributes to adult neuronal function due to the lack of available tools to study Tuba1a protein. Previous data in yeast (Gartz Hanson et al., 2016) suggested that heterozygous Tuba1aND+ mouse brains compared with wild-type at birth (Fig. 2A,B; N = 4, p < 0.0001) consistent with the molecular impact of the ND substitution in yeast reported in Hanson et al., (2016). This reduction in α-tubulin protein was not due to a change in brain size, as weights between wild-type and Tuba1aND+ brains were comparable (Extended Data Fig. 2-1; N = 10, p = 0.68). Adult Tuba1aND+ α-tubulin protein levels were comparable to wild type (Fig. 2A,B; N = 6, p = 0.38). The observed reduction in developmental α-tubulin indicates that the availability of α-tubulin protein is decreased in Tuba1aND+ neurons during a critical period for neuronal microtubule network establishment. As total α-tubulin protein levels normalized in the brains of adult Tuba1aND+ mice, we assessed brain tubulin PTMs because PTMs influence binding interactions with MAPs and overall microtubule function. Western blotting of whole-brain lysates from adult wild-type and Tuba1aND+ mice revealed no genotypic difference in the abundance of acetylated (N = 5; p = 0.66), tyrosinated (N = 3; p = 0.52), detyrosinated (N = 5; p = 0.81), or polyglutamylated (N = 3; p = 0.31) α-tubulin relative to the total α-tubulin (Fig. 2C,D). Therefore, we conclude that Tuba1aND substitution reduces the availability of α-tubulin protein developmentally, but does not impact the PTM landscape of adult brain microtubules.

Mice have nine α-tubulin genes and mammalian neurons express at least four distinct α-tubulin genes (Miller et al., 1987; Bamji and Miller, 1996; Gloster et al., 1999; Zhang et al., 2014). Mutations that disrupt the major isoform, Tuba1a, could potentially induce compensatory changes to the expression of other α-tubulin mRNAs. We
found that mRNA expression of Tuba1a and other brain α-tubulin isotypes was not significantly changed in heterozygous Tuba1aND/1 P0 brain lysates (Fig. 2E; p > 0.9 for all comparisons by two-way ANOVA). Additionally, the relative ratio of each α-tubulin isotype mRNA was unchanged in Tuba1aND/1 P0 brain lysates, with Tuba1a constituting ~98% of all α-tubulin mRNA in both wild-type and Tuba1aND/1 brains (Fig. 2F; N = 3, p = 0.92). We conclude
from these results that alternative α-tubulin isotypes are not upregulated to compensate for the loss of TUBA1A protein in the developing brain. We examined the abundance of α-tubulin isotype mRNAs in the cortex of adult wild-type and Tuba1aND/1 mice and found that Tuba1a remains one of the predominant α-tubulins for both genotypes (Fig. 2G; N = 3; p > 0.7 for all by two-way ANOVA). The modest, but statistically insignificant increase in Tuba1a mRNA in the adult Tuba1aND/1 cortex slightly shifted the ratio of α-tubulin isotype mRNAs in the adult cortex, with an increased percentage of Tuba1a α-tubulin mRNA compared with the other isotypes (Fig. 2H; p < 0.0001 in Tuba1aND/1 cortex compared with wild-type by two-way ANOVA). Together, these data demonstrate that Tuba1aND/+ transiently depletes α-tubulin protein during a critical developmental window, without inducing compensatory upregulation of other α-tubulin isotypes.

Reduced TUBA1A function results in fewer microtubule tracks in neurites

To determine how reduced TUBA1A impacts the development of a neuronal microtubule network, we used primary neurons from wild-type and Tuba1aND/1 P0–P2 cortices to examine microtubule organization in vitro. Wild-type and Tuba1aND/1 primary cortical neurons were nucleofected with membrane-bound Myr-TdTomato and GFP-MACF43, which binds to polymerizing microtubule (+)-end-binding proteins (EBs) without affecting microtubule function (Yau et al., 2016). Kymograph plots generated from GFP-MACF43 videos allowed us to interrogate the number of growing microtubule (+)-ends per micrometer of neurite at day in vitro (DIV) 1 (Fig. 3A–C). We found that Tuba1aND/1 neurites have approximately half the number of GFP-MACF43 puncta per micrometer of neurite as wild-type neurites [2.610 + 0.20 growing microtubule ends in Tuba1aND/1 vs 4.636 ± 0.34 in wild type (Fig. 3D); n = 24 neurites; p < 0.0001]. These data suggest the approximate 50% reduction in α-tubulin protein in observed in developmental Tuba1aND/1 mice causes functional loss of microtubule track density within developing Tuba1aND/1 neurons.

TUBA1A is required for functional intracellular transport

One critical function of neuronal microtubules is to facilitate intracellular trafficking of proteins, mRNAs, and organelles between the cell body and distal neurites. Thus,
we explored the possibility that reduced TUBA1A function and a reduced density of microtubule tracks impairs microtubule-based intracellular transport. To evaluate how loss of TUBA1A impacts the dynamics of microtubule-based transport, we examined transport of lysosomes in cultured primary neurons from P0–P2 wild-type and Tuba1aND/1 cortices.

Lysosomes facilitate enzymatic degradation of intracellular components and are highly motile organelles that are transported bidirectionally on microtubules through all compartments of the neuron, making them an ideal organelle in which we could examine microtubule-based transport efficiency. Time-lapse images of lysosome movement in wild-type or Tuba1aND/1 cortical neurons were used to generate kymograph plots of lysosome movement over time within single wild-type and Tuba1aND/1 axons and dendrites (Fig. 4A, B), from which we could measure velocity, dynamics of movement, and total distance traveled. From these analyses we found that during the 3-min imaging period, ~70% of wild-type lysosomes were in motion, whereas ~30% of Tuba1aND/1 lysosomes were stationary (Fig. 4C).

Figure 4. Tuba1aND/1 neurons have deficits in organelle transport caused by increased stationary cargoes. A, Still images from time-lapse microscopy of DIV3 cortical neurons labeled with LysoTracker dye to mark lysosomes in wild-type (WT) and Tuba1aND/1 neurons. B, Representative kymograph plots of lysosome movement over time within select neurites for wild type (top) and Tuba1aND/1 (bottom). C, Scatter plot representing the percent of stationary lysosomes per kymograph in wild-type and Tuba1aND/1 neurons. Data points represent individual neurons (N = 3 mice, n = 25 neurons, p = 0.001). D, Scatter plot representing lysosome velocities in μm/s for wild-type and Tuba1aND/1 neurons. Data points represent individual lysosome events (n = 635 lysosomes, p = 0.27). E, Scatter plot representing pause duration (s), for moving lysosomes in wild-type and Tuba1aND/1 neurons. Data points represent individual pause events (n = 338 events, p = 0.56). F, Scatter plot representing total distance traveled (μm) by moving lysosomes in wild-type and Tuba1aND/1 neurons. Data points represent individual lysosomes (n = 635 lysosomes, p = 0.003). G, Scatter plot representing the percentage of stationary mitochondria in wild-type and Tuba1aND/1 neurons. Data points represent individual neurons (N = 3 mice, n = 30 neurons, p = 0.34 by). H, Scatter plot the velocity of short mitochondrial movements in wild-type and Tuba1aND/1 neurons. Data points represent individual mitochondria (N = 3 mice, n = 301 mitochondria, p = 0.45). I, Scatter plot representing pause duration (s), for moving mitochondria in wild-type and Tuba1aND/1 neurons. Data points represent individual pause events (n = 417 events, p = 0.79). J, Scatter plot representing total distance traveled (μm) by moving mitochondria in wild-type and Tuba1aND/1 neurons. Data points represent individual mitochondria (n = 301 mitochondria, p = 0.01). Cortical neurons from three animals per genotype were analyzed. Lysosomes were imaged once every second for 3 min, mitochondria were imaged once every 2 s for 2 min. Statistical differences between groups were assessed by Student’s t test; **p < 0.01; ***p < 0.001. In all plots, line designates mean with error bars indicating SEM.
lysosomes were moving, while the remainder were stationary for the duration of the video (Fig. 4C; N = 3 mice, n = 25 neurons). A significantly higher number of Tuba1a^ND/− lysosomes were stationary compared with wild type, with only 46% of Tuba1a^ND/− lysosomes moving during the 3-min window (Fig. 4C; p = 0.001). Of those lysosomes that were moving, the velocity of each movement was unchanged between wild-type and Tuba1a^ND/− neurons, with average velocity of 0.56 ± 0.02 and 0.53 ± 0.02 μm/s for wild-type and Tuba1a^ND/− neurons, respectively (Fig. 4D; n = 635 lysosomes, p = 0.27). During active transport, cargoes exhibit phases of movement and pausing, thus we wanted to evaluate the dynamics of lysosome movement by measuring pause duration for moving lysosomes. There was no difference in pause duration of moving lysosomes in Tuba1a^ND/− neurons, which paused on average 17.51 ± 1.0 s, compared with a 16.19 ± 0.81-s pause duration in wild-type neurons (Fig. 4E; n = 337 events, p = 0.56). Finally, we observed a significant decrease in the total distance traveled by lysosomes over the 3-min imaging window, from 24.77 ± 1.84 μm in wild-type neurons to 17.19 ± 1.66 μm in Tuba1a^ND/− neurons (Fig. 4F; n = 100 neurons, p = 0.003). These data demonstrate that Tuba1a^ND/− substitution causes a deficit in neuronal intracellular transport by which Tuba1a^ND/− lysosomes do not travel as far as wild type within the same time window.

To further understand the impact of reduced TUBA1A function on neuronal microtubule-based transport, we examined mitochondrial trafficking in cultured primary Tuba1a^ND/− neurons compared with wild type. Transport of mitochondria and lysosomes is conducted by a different set of kinesins (Hurd and Saxton, 1996; Tanaka et al., 1998; Hendricks et al., 2010; Maday et al., 2014). Therefore, examining transport of multiple cargoes provides mechanistic insight into the intracellular trafficking disruption in Tuba1a^ND/− neurons. The analyses described above were repeated in DIV7 wild-type and Tuba1a^ND/− cortical neurons that had mitochondria labeled (Extended Data Fig. 4-1). Over the course of a 2-min imaging period, we observed that the number of moving mitochondria was similar between wild-type (36%) and Tuba1a^ND/− (40%) neurons, and the remainder were stationary for the duration of the video (Fig. 4G; N = 3 mice, n = 30 neurons, p = 0.38). Importantly, the relative number of stationary mitochondria was more than double the number of stationary lysosomes in wild-type neurons (64.78 ± 2.9% and 30.01 ± 5.0%, respectively; Fig. 4C, G). Similar to what was observed for lysosomal trafficking, we found that the velocity of moving mitochondria was unchanged between wild-type (0.45 ± 0.02 μm/s) and Tuba1a^ND/− (0.43 ± 0.02 μm/s) neurons (Fig. 4H; n = 301 events, p = 0.51). These mitochondrial movements were interrupted by differential periods of pause, with a significant increase in Tuba1a^ND/− mitochondrial pausing compared with wild type, with average pause durations of 28.36 ± 1.6 and 24 ± 1.4 s, respectively (Fig. 4I; n = 251 events, p = 0.02). The total distance traveled by motile mitochondria during the 2-min imaging window was found to be significantly decreased in Tuba1a^ND/− neurons, with Tuba1a^ND/− mitochondria traveling 9.46 ± 1.3 μm on average compared with 15.8 ± 1.7 μm in wild-type neurons (Fig. 4J; n = 301 events, p = 0.01). Combined, these analyses indicate that a TUBA1A deficit impairs transport of multiple organelle types in cortical neurons, resulting in a significant reduction in the total distance of organelle movement.

**Diminished TUBA1A causes late-onset behavioral deficits**

Having established that neurons from young Tuba1a^ND mutant animals exhibit deficits in microtubule density and organelle trafficking, we next asked whether these lead to impairments at the level of behavior. The Tuba1a^ND mutant allele was identified due to locomotor deficits apparent in homozygous mutant embryos. Tuba1a^ND/ND homozygous substitution induced severe neurodevelopmental abnormalities that impaired motor neuron function and caused perinatal lethality (Gartz Hanson et al., 2016). In contrast, Tuba1a^ND/− embryos survive to adulthood and were indistinguishable from wild-type siblings at the age of weaning (P21). Tuba1a^ND/− embryos exhibit normal presynaptic and postsynaptic motor development within the diaphragm and respond to touch stimulation (Gartz Hanson et al., 2016). However, as Tuba1a^ND/− mice aged, we noticed differences in locomotion that were quantified with gait analysis. While the gait of two-month-old Tuba1a^ND/− mice is not significantly different from wild type (N = 2), by three months of age, Tuba1a^ND/− mice develop an ataxic gait, as evidenced by a wider rear stance (Fig. 5A,B; N = 5, p = 0.02). Tuba1a^ND/− mice developed impaired motor coordination by five months of age, as assessed by rotarod performance (Fig. 5C; N = 10, p = 0.03). After the initial onset of behavioral phenotypes, Tuba1a^ND/− mice performed worse than wild type at all time points examined (p < 0.05 for all). Heterozygous Tuba1a^ND/− mice did not display deficits in motor learning as assessed by the difference in rotarod performance between trials (Fig. 5D; N = 10, wild-type R² = 0.98, Tuba1a^ND/− R² = 0.89, p = 0.31 by linear regression). Locomotor impairment of these mice impeded more rigorous testing of cognitive function. Importantly, forelimb function, and all other parameters of gait were similar to that measured in wild-type animals (Extended Data Fig. 5-1). Body weights of Tuba1a^ND/− mice were comparable to that of control mice (Fig. 5F; N = 7, p = 0.24), and forelimb muscular strength was not impaired (Fig. 5E; N = 7, p = 0.19). These results indicate that neither altered muscle content nor gross muscle function is responsible for the observed motor deficits, indicating that neither altered muscle content nor gross muscle function is responsible for the observed motor deficits, indicating Tuba1a^ND/− induced neuronal dysfunction is a likely cause. The hindlimb-specificity of this behavioral phenotype further suggests that neurons with extremely long axons, such as those that innervate the hindlimbs, may be uniquely sensitive to perturbations of Tuba1a.

Tuba1a is expressed in all postmitotic neurons (Paden et al., 1995; Bamji and Miller, 1996), therefore we predicted that behavioral impairments induced by Tuba1a^ND/− substitution would not be restricted to motor neurons. To assess whether Tuba1a^ND/− adult mice had deficits in sensory behavior, we examined hind-paw sensory...
Figure 5. Tuba1a<sup>ND</sup>/ mice exhibit adult-onset motor and sensory behavioral deficits. A, Images depicting gait abnormalities in Tuba1a<sup>ND</sup>/ adult mice compared with wild-type (WT). Still images were taken from video of Tuba1a<sup>ND</sup>/ and wild-type mice mid-stride. B, Line graph quantifying changes in rear stance width at two months (p = 0.61), three months (p = 0.02), five months (p = 0.04), eight months (p = 0.03), and 10 months (p < 0.0001) of age in wild-type and Tuba1a<sup>ND</sup>/ mice. C, Line graph representing latency to fall on rotarod task at two months (p = 0.31), three months (p = 0.82), five months (p = 0.03), eight months (p = 0.004), and 10 months (p = 0.04) of age in wild-type and Tuba1a<sup>ND</sup>/ mice. Points depict the mean stance width or latency to fall by genotype with SEM. The same mice were analyzed throughout, and a two-way ANOVA was used to assess statistical significance between groups. Number of mice per genotype is as follows: N = 2 at two months, N = 5 at three months, N = 8 at five months, N = 12 at eight months, N = 8 at 10 months. D, Line graph representing performance over three subsequent rotarod trials in a single testing session at five months of age for Tuba1a<sup>ND</sup>/ compared with wild type. Slopes of the lines for wild type and Tuba1a<sup>ND</sup>/ were compared by linear regression and were not found to be significantly different (p = 0.32, wild-type R<sup>2</sup> = 0.98, Tuba1a<sup>ND</sup>/ R<sup>2</sup> = 0.89). E, Scatter plot of grip strength assessed in the forelimbs of female five-month-old wild-type and Tuba1a<sup>ND</sup>/ mice (N = 7, p = 0.19). F, Bar graph of total body weight for eight- to 10-month-old Tuba1a<sup>ND</sup>/ and wild-type female mice (N = 7, p = 0.24). G, Bar graph of SUDO scores Bonin et al. (2014) for Von Frey mechanical sensation testing in three- to six-month-old wild-type and Tuba1a<sup>ND</sup>/ mice (N = 7, p = 0.04). H, Bar graph of time to withdraw from thermal stimulus in Hargreaves behavioral analysis on three-month-old wild-type and Tuba1a<sup>ND</sup>/ mice (N = 7, p = 0.19). Sex as a variable did not significantly impact rear stance width or rotarod performance at any time point examined (N = 7 females, N = 5 males; p > 0.05 for all comparisons of male vs female). Sex was also not found to significantly influence sensory behavior (N = 7 males, N = 2 females; p > 0.05 for all comparisons); *p < 0.05, **p < 0.01, ****p < 0.0001. For line graphs, points indicate mean by genotype at each time point ± SEM. For Bar graphs, bars represent mean ± SEM. *p < 0.05; **p < 0.01; ****p < 0.0001.
function using two separate assays in mature mice. Response to hind-paw stimulation with Von Frey filaments revealed an increased threshold for retraction in Tuba1aND/1 compared with wild-type mice, indicating reduced sensation of mechanical stimuli (Fig. 5G; N = 7, p = 0.04). Additionally, adult Tuba1aND/1 animals displayed a modest, but not statistically significant decrease in thermal pain sensation of the hind-paws, as measured by a Hargreaves assay (Fig. 5H; N = 7, p = 0.19). These results support our prediction that Tuba1aND/1-induced behavioral deficits are not restricted to the motor system, but rather appear to preferentially impact the far-reaching neurons that innervate the hindlimbs. The degenerative behavioral phenotypes in Tuba1aND/1 mice indicate that Tuba1a deficiency has long-lasting consequences for neuronal function.

**TUBA1A deficit impairs hindlimb behavior without apparent neuronal cell death or axon degeneration**

Complex motor behaviors involve multiple types of motor neurons coordinated through multiple brain regions including the cortex, cerebellum, and spinal cord. We examined survival of neurons that are important for coordinated movement in each of these systems in Tuba1aND/1 and wild-type mice. Cortical lamination occurs normally in Tuba1aND/1 mice, and there was no evidence of heterotopic neurons in Tuba1aND/1 brains (Fig. 6A; N = 3). To assess cortical motor neuron number, we used the transcription factor ER81 as a marker of Layer V cortical neurons that project to the spinal cord (Yoneshima et al., 2006). We found no difference in the number of cortical motor neurons in Layer V of the motor cortex and no evidence of apoptosis before or after the onset of quantifiable motor deficits in Tuba1aND/1 and wild type (Fig. 6B, C; N = 3, p = 0.12). Quantification of motor neurons in serial sections through the lumbar spinal cord, which contains neurons that innervate the rear limbs, revealed that there was no alteration to the number of spinal motor neurons in Tuba1aND/1 animals compared with wild type (Fig. 6D;
Figure 7. No deficits in axon morphology, myelination, or survival in Tuba1aND/1 mice. A, Electron micrographs of spinal cord axons in cross-section for adult wild-type (left) and Tuba1aND/1 (right) mice. Six images per animal and two animals per genotype were assessed for EM analyses. B, Scatter plot displaying myelin thickness measure (G-ratio) for two wild-type and two Tuba1aND/1 mice (N=2 mice, n=100 axons, p=0.42). C, Scatter plot of axon density quantified in electron micrograph images for adult wild-type and Tuba1aND/1 mice (N=2 mice, n=6 fields, p=0.72). D, Scatter plot of axon diameter quantified in electron micrograph images.
for adult wild-type and Tuba1aND/+ mice (N = 2 mice, n = 100 axons, p = 0.63). E, Histogram representing the distribution of axon diameters from data in D, F. Anti-SMI-32 (non-phosphorylated neurofilament) immunofluorescence images in cortex of adult wild-type (left) and Tuba1aND/+ mice (right); 40× magnification. Scale bar: 10 μm. G, Bar graph representing SMI-32 intensity in a cortical region of interest for wild-type and Tuba1aND/+ mice. Data points represent regions of interest, with three animals per genotype assessed (p = 0.77). H, Western blotting for SMI-32 in whole-brain lysates from adult wild-type and Tuba1aND/+ mice. Bands for heavy (200 kDa), medium (160 kDa), and light (125 kDa) chain non-phosphorylated neurofilament shown (top). GAPDH used for normalization (37 kDa; bottom). Data points represent technical replicates with three biological replicates performed. I, Bar graphs representing quantification of SMI-32 medium (right, p = 0.61) and heavy (left, p = 0.70) expression in whole-brain lysates, normalized to GAPDH. For all graphs, bar or line indicates mean and error bars display SEM.

Discussion

Here, we show that significant reduction in developmental total α-tubulin resulting from a loss of function mutation in Tuba1a (Tuba1aND/+ ) causes an adult onset movement disorder. We show that assembly of the microtubule network is altered resulting in disrupted organelle trafficking early in development in cultured Tuba1aND/+ cortical neurons, suggesting that neuronal microtubule networks are not set up correctly when there is not adequate α-tubulin available (Figs. 1-4). While these aberrant neuronal microtubule networks are sufficient to establish juvenile NMJ synapses similarly to wild type, Tuba1aND/+ NMJ synapses deteriorate over time (Fig. 8), potentially contributing to adult-onset motor deficits. This work provides the first evidence that the developmental tubulin TUBA1A is necessary for adult neuronal function and suggests that mature neurons require TUBA1A for proper function throughout their lifetime.
Figure 8. Neuromuscular Junction (NMJ) synapses deteriorate over time in *Tuba1a*ND/- mice. A, Teased Extensor digitorum longus (EDL) muscle fibers labeled with α-bungarotoxin (cyan) and synaptophysin (magenta) for one-month-old (top) and one-year-old (bottom) wild-type and *Tuba1a*ND/- mice. Muscle fibers from three mice per genotype at each time point were assessed. B, Scatter plot of EDL synaptic (μm²) measured from α-bungarotoxin labeling in juvenile wild-type and *Tuba1a*ND/- mice (*N* = 3 mice, *n* = 69 synapses, *p* = 0.43). C, Scatter plot of postsynaptic acetylcholine receptors (AChRs) density measured as synaptic α-bungarotoxin intensity in arbitrary units (a.u.) divided by synaptic area in juvenile mice (*N* = 3 mice, *n* = 39 synapses, *p* = 0.89). D, Scatter plot of presynaptic vesicle density measured as synaptic synaptophysin intensity divided by synaptic area in juvenile mice (*N* = 3 mice, *n* = 39 synapses, *p* = 0.54). E, Scatter plot of the ratio of synaptophysin to AChR (presynaptic to postsynaptic) intensity in juvenile wild-type and *Tuba1a*ND/- mice (*N* = 3 mice, *n* = 39 synapses, *p* = 0.74). F, Scatter plot of EDL synaptic area measured from α-bungarotoxin labeling in adult wild-type and *Tuba1a*ND/- mice (*N* = 3 mice, *n* = 45 synapses, *p* < 0.0001). G, Scatter plot of postsynaptic AChR density measured as synaptic α-bungarotoxin intensity divided by synaptic area in adult mice (*N* = 3 mice, *n* = 45 synapses, *p* < 0.0001). H, Scatter plot of presynaptic vesicle density measured as synaptic synaptophysin intensity divided by synaptic area in adult mice (*N* = 3, *n* = 45, *p* < 0.0001). I, Scatter plot of the ratio of synaptophysin to AChR (presynaptic to postsynaptic) intensity in adult wild-type and *Tuba1a*ND/- mice (*N* = 3 mice, *n* = 45 synapses, *p* = 0.03). For all graphs, data points represent individual synapses with line representing mean of data ± SEM. Data were nested by animal and analyzed for statistical significance between genotypes by a two-way ANOVA. *p* < 0.05; ****p < 0.0001.
The microtubule cytoskeleton forms the tracks on which motor proteins bind to facilitate intracellular transport. Mitochondria and lysosomes are both transported by dynein motor proteins in the retrograde direction, but differ in the kinesin motors that primarily regulate their anterograde movement, with lysosome transport dominated by kinesin-2 and mitochondrial transport occurring primarily by kinesin-1 (Tanaka et al., 1998; Ligon and Steward, 2000; Hendricks et al., 2010). Thus, this is the first demonstration that reducing available TUBA1A affects organelle trafficking in living neurons.

Mitochondria and lysosomes move with similar average velocities in wild-type neurons, however the dynamics of movement for these two organelle types vary greatly. We observed a larger proportion of stationary mitochondria that were affected by depletion of TUBA1A function, suggesting that motors interact with the microtubule substrate normally (Fig. 4D,H). Importantly, we did not see differences in amounts of PTMs that are known to affect the activity of motors on their microtubule substrates (Fig. 2C, D). Thus, this is the first demonstration that reducing available TUBA1A affects organelle trafficking in living neurons.

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| Data structure                  | Type of test          | Power               |
|---------------------------------|-----------------------|---------------------|
| 1 Normal distribution          | Two-way ANOVA        | 0.4478 to 1.369     |
| 2 Normal distribution          | Unpaired t test       | –0.03822 to 0.02562 |
| 3 Normal distribution          | Two-way ANOVA        | –0.6247 to 0.1850   |
| 4 Normal distribution          | Unpaired t test       | –2.839 to 1.889     |
| 5 Normal distribution          | Unpaired t test       | –0.5567 to 0.9367   |
| 6 Normal distribution          | Unpaired t test       | –0.3997 to 0.3219   |
| 7 Normal distribution          | Unpaired t test       | –0.5390 to 1.306    |
| 8 Normal distribution          | Two-way ANOVA        | –1.079 to 1.932     |
| 9 Normal distribution          | Two-way ANOVA        | –1.272 to 1.272     |
| 10 Normal distribution         | Two-way ANOVA        | –0.5164 to 0.2583   |
| 11 Normal distribution         | Two-way ANOVA        | –0.6096 to 0.6096   |
| 12 Normal distribution         | Unpaired t test       | –2.814 to –1.238    |
| 13 Normal distribution         | Unpaired t test       | 1.294 to 26.44      |
| 14 Log-normal distribution     | Welch's t test       | –0.06284 to 0.02308 |
| 15 Log-normal distribution     | Mann-Whitney U        | –0.5000 to 1.500 (approximate) |
| 16 Normal distribution         | Unpaired t test       | –12.53 to –2.627    |
| 17 Normal distribution         | Unpaired t test       | –14.22 to 5.521     |
| 18 Log-normal distribution     | Welch's t test       | –0.06821 to 0.03392 |
| 19 Log-normal distribution     | Mann-Whitney U        | 0.5000 to 4.834 (approximate) |
| 20 Normal distribution         | Unpaired t test       | –10.51 to –2.180    |
| 21 Normal distribution         | Two-way ANOVA        | –0.6070 to –0.2015  |
| 22 Normal distribution         | Two-way ANOVA        | 51.50 to 237.4      |
| 23 Normal distribution         | Linear regression    | –18.51 to 65.11 (WT); –53.22 to 83.22 (Tuba1aND) |
| 24 Normal distribution         | Unpaired t test       | –6.520 to 1.782     |
| 25 Normal distribution         | Unpaired t test       | –0.07948 to 0.3509  |
| 26 Normal distribution         | Unpaired t test       | 0.1362 to 3.864     |
| 27 Normal distribution         | Unpaired t test       | –1.274 to 5.889     |
| 28 Normal distribution         | Two-way ANOVA        | –111.3 to 17.97 (3 months); –83.08 to 46.19 (10 months) |
| 29 Normal distribution         | Two-way ANOVA        | –9.574 to 6.240 (3 months); –12.80 to 3.018 (10 months) |
| 30 Normal distribution         | Two-way ANOVA        | –360.1 to 129.1 (3 months); –277.1 to 212.1 (10 months) |
| 31 Normal distribution         | Unpaired t test       | –0.03303 to 0.05303 |
| 32 Normal distribution         | Unpaired t test       | –0.1935 to 0.1596   |
| 33 Normal distribution         | Unpaired t test       | –0.5387 to 0.7019   |
| 34 Normal distribution         | Unpaired t test       | –608.3 to 741.8     |
| 35 Normal distribution         | Unpaired t test       | –0.8969 to 1.449 (NFT-M); –2.403 to 3.450 (NFT-H) |
| 36 Normal distribution         | Unpaired t test       | –113.7 to –37.30    |
| 37 Normal distribution         | Unpaired t test       | –0.04520 to 0.08451 (pre); –0.1550 to 0.1791 (post) |
| 38 Normal distribution         | Unpaired t test       | –0.1173 to 0.1639   |
| 39 Normal distribution         | Unpaired t test       | –116.1 to –56.69    |
| 40 Normal distribution         | Unpaired t test       | 0.1642 to 0.3719    |
| 41 Normal distribution         | Unpaired t test       | 0.1905 to 0.4305    |
| 42 Normal distribution         | Unpaired t test       | –0.3050 to –0.01338 |
mitochondrial population (Fig. 4G). The relatively subtle
deficit in pause duration that was observed in mitochondria
was not detectable in lysosomes, which had much shorter
pause durations on average (Fig. 4F). Despite the discrep-
ancies in how these abnormalities were detected between
different organelles, the overall distance traveled by mito-
chondria and lysosomes was consistently reduced in
Tuba1a<sup>ND1</sup> neurons compared with wild type (Fig. 4F, J).
The increase in pausing or stalling behavior (Fig. 4C, J),
combined with the reduced α-tubulin protein during develop-
ment, and normal neuronal survival (Figs. 2, 6, 7) sug-
gests that reduced availability of TUBA1A is detrimental to
adult neuronal function, but not neuronal survival.

The abundance of α-tubulin protein during brain develop-
ment is reduced in Tuba1a<sup>ND1</sup> brains (Fig. 2). This
deficit in α-tubulin reduced the number of growing micro-
tubule (+)-ends per micrometer of neurite in developing
Tuba1a<sup>ND1</sup> neurons, indicating the neuronal microtubule
network contains a lower density of microtubules in
Tuba1a<sup>ND1</sup> mice (Fig. 3). We did not observe differences
in neuronal survival, axon caliber, myelination, or markers
for axon degeneration in Tuba1a<sup>ND1</sup> compared with wild-
type siblings (Figs. 6, 7) suggesting that reduced TUBA1A
impacts neuron function rather than neuron survival or
axon morphology. Collectively, these results could sug-
gest that adult Tuba1a expression may be important for
maintenance of microtubule tracks in neurons and mutant
TUBA1A<sup>ND</sup> microtubules are not sufficient for that re-
quirement. We favor a second model, that the develop-
mental TUBA1A deficit does not allow a neuron to
correctly establish microtubule networks of the appropri-
ate density, and these defects cannot be sufficiently re-
paired to maintain functional NMJ synapses in adulthood
despite adequate levels of adult α-tubulin protein (Figs. 2,
3, 8).

Intracellular transport deficits in Tuba1a<sup>ND1</sup> neurons
provide a putative mechanism by which reduced TUBA1A
impairs neuronal function to cause adult-onset behavioral
deficits. However, as cortical neurons are not representa-
tive of the adult condition, follow-up studies are needed
to determine whether trafficking deficits persist into adult-
hood in the Tuba1a<sup>ND1</sup> model. Because developmental
α-tubulin protein is reduced in Tuba1a<sup>ND1</sup> brains without
detectable compensation from any other α-tubulin iso-
types (Fig. 2), we propose a model in which early α-tubulin
deficits cause formation of inadequate neuronal microtubu-
bule tracks that cannot be rescued by subsequent resto-
rati on of α-tubulin protein. Over time, the consequences
of early tubulin deficiency could become more pro-
nounced and result in impairment of synaptic function
and animal behavior. Microtubules in neurons are not sin-
gly nucleated from the centrosome, but rather exist in
short segments that tile the length of the axons and den-
drites (Stiess et al., 2010). When moving cargoes reach
the end of a microtubule segment, the motor protein
pauses to switch tracks (Yogev et al., 2016). Thus, the ob-
served increase in organelle pausing behavior paired with
developmental decreases in α-tubulin protein and micro-
tubule density could indicate that motor proteins in
Tuba1a<sup>ND1</sup> neurons encounter microtubule ends more
frequently, due to the presence of potential gaps in the
tiled microtubule network. Alternatively, while no compen-
sation for loss of TUBA1A was observed at the mRNA
level, decreased α-tubulin protein would likely shift the
α-tubulin isotype blend at the protein level. It has been
previously shown that incorporation of different tubulin
isotypes alters microtubule properties, whether directly or
through effects on MAP binding (Panda et al., 1994;
Siraajuddin et al., 2014; Gadadhar et al., 2017; Honda et
al., 2017). Although α-tubulin protein levels are similar to
wild type in the adult Tuba1a<sup>ND1</sup> brain, it is unclear
whether adult Tuba1a<sup>ND1</sup> microtubule polymers contain
normal α-tubulin isotype compositions.

Microtubules and intracellular transport are important
for synaptic signaling and maintenance (Kim and Lisman,
2001; Jaworski et al., 2009; Maas et al., 2009; Stephan et
al., 2015). Neurons are morphologically complex cells,
with processes often extending long distances from the
soma, and are thus highly dependent on functional intra-
cellular transport. Transport deficits, such as those ob-
served in the Tuba1a<sup>ND1</sup> model could impair both
delivery and clearance of cargoes if persistent over time,
leading to neuronal dysfunction. Functional intracellular
transport is essential for delivery of mitochondria and syn-
aptic components to the presynapse (Goldstein et al.,
2008). Thus, the transport deficits caused by Tuba1a<sup>ND1</sup>
substitution could alter synaptic transmission and thereby
cause deterioration of synapse morphology, potentially
explaining the observed NMJ deficits (Fig. 8). The in-
creased density of both presynaptic and postsynaptic
machinery that was observed in the Tuba1a<sup>ND1</sup> NMJ
(Fig. 8G, H) suggests a possible impairment in clearance
of vesicles from the presynapse and receptors from the
postsynapse. Moreover, the reduction in NMJ synaptic
area (Fig. 8F) and branching complexity indicates that the
increased density of AChRs and presynaptic vesicles may
be a form of compensation for improperly functioning
synapses. This hypothesis is further supported by the ob-
served dysregulation of presynaptic to postsynaptic com-
ponent ratios in adult Tuba1a<sup>ND1</sup> synapses (Fig. 8I).
NMJ synapses are generally quite stable over time, but are
known to undergo morphologic changes in response to
processes that alter synaptic transmission, such as dis-
eases of the neuromuscular system and aging (Murray et
al., 2008, 2010; Jones et al., 2017; Liu et al., 2017).
Decreased NMJ size explains impaired motor behaviors
in Tuba1a<sup>ND1</sup> mice (Fig. 5A–C). However, given that
Tuba1a is expressed in all neurons, and Tuba1a<sup>ND1</sup> mice
also exhibited degenerative sensory deficits (Fig. 5G, H),
we do not expect that the NMJ is the only synapse type
affected by deficiency in TUBA1A, but this idea remains
to be tested. The prominence of hindlimb-specific behav-
ioral phenotypes in the Tuba1a<sup>ND1</sup> mouse model sug-
gests that axon length may predict sensitivity to tubulin
deficiency.

Microtubule dysfunction, including intracellular traffick-
ing defects, have been described in nearly every neurode-
generative disease of the nervous system, and can
precede other signs of neuronal dysfunction (Reynolds et
al., 2004; De Vos et al., 2008; Cartelli et al., 2010;
Matamoros and Baas, 2016; Brady and Morfni, 2017. Although there is abundant evidence of microtubule dysfunction in neurodegenerative disease, it has been difficult to pinpoint how or whether the microtubule cytoskeleton contributes to disease etiology. Microtubule dynamics, PTMs, and MAP interactions become dysregulated in neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease, and amyotrophic lateral sclerosis (ALS); however, it has remained unclear as to whether cytoskeletal dysfunction is causative of disease or merely correlated with its progression (Cartelli et al., 2010; Rogowski et al., 2010; Brunden et al., 2011; Dubey et al., 2015; Matamoros and Baas, 2016). Mutations that disrupt the neuronal α-tubulin TUBA4A have been identified familial and sporadic ALS (Rademakers and van Blitterswijk, 2014; Smith et al., 2014; Pensato et al., 2015; Li et al., 2018). The mechanisms by which TUBA4A mutations may cause neurodegeneration remain unclear. Using the Tubα1ND+ model, we have demonstrated that loss-of-function in a major neuronal α-tubulin is sufficient to induce adult-onset behavioral degeneration. Further, we have shown that Tubα1ND+ does not induce neuronal death or degeneration, but rather causes synaptic abnormalities, potentially by impairing intracellular transport. We provide direct evidence that deficits in microtubule function can cause degenerative neuronal pathology at the molecular, cellular, and behavioral level.

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