The Microtubule Regulatory Protein Stathmin Is Required to Maintain the Integrity of Axonal Microtubules in Drosophila

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Abstract

Axonal transport, a form of long-distance, bi-directional intracellular transport that occurs between the cell body and synaptic terminal, is critical in maintaining the function and viability of neurons. We have identified a requirement for the stathmin (stai) gene in the maintenance of axonal microtubules and regulation of axonal transport in Drosophila. The stai gene encodes a cytosolic phosphoprotein that regulates microtubule dynamics by partitioning tubulin dimers between pools of soluble tubulin and polymerized microtubules, and by directly binding to microtubules and promoting depolymerization. Analysis of stai function in Drosophila, which has a single stai gene, circumvents potential complications with studies performed in vertebrate systems in which mutant phenotypes may be compensated by genetic redundancy of other members of the stai gene family. This has allowed us to identify an essential function for stai in the maintenance of the integrity of axonal microtubules. In addition to the severe disruption in the abundance and architecture of microtubules in the axons of stai mutant Drosophila, we also observe additional neurological phenotypes associated with loss of stai function including a posterior paralysis and tail-flip phenotype in third instar larvae, aberrant accumulation of transported membranous organelles in stai deficient axons, a progressive bang-sensitive response to mechanical stimulation reminiscent of the class of Drosophila mutants used to model human epileptic seizures, and a reduced adult lifespan. Reductions in the levels of Kinesin-1, the primary anterograde motor in axonal transport, enhance these phenotypes. Collectively, our results indicate that stai has an important role in neuronal function, likely through the maintenance of microtubule integrity in the axons of nerves of the peripheral nervous system necessary to support and sustain long-distance axonal transport.

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Introduction

The organized transport of organelles, vesicles and macromolecular protein complexes is necessary to support cellular growth, function, and viability. The requirement for an efficient transport system is pronounced in neurons because their architecture typically comprises a long, narrow axon that extends to the synaptic terminal, and is orders of magnitude longer than the diameter of the cell body. The axon serves to transmit action potentials between the cell body and synaptic terminal, but also acts as a conduit for the long-distance transport of materials between these two discrete cellular compartments.

In axons, the motor proteins cytoplasmic dynein and kinesin bidirectionally transport cargo along tracks of microtubules (MTs), the main cytoskeletal component of axons. MTs are hollow polymers formed by the lateral association of linear, polarized protofilaments of heterodimers of α and β-tubulin joined head-to-tail. MTs are dynamic structures, the assembly-favored ‘plus’ ends stochastically cycling between phases of growth and shrinkage, in a process known as ‘dynamic instability’ [1]. MTs are subject to intense regulation by a vast array of factors including MT-stabilizing proteins, MT-polymerizing and depolymerizing proteins, and MT-severing proteins that act coordinately to modulate MT dynamics by directly interacting with and modifying MTs (reviewed in [2]).
Impairment of the axonal transport system is believed to cause or dramatically contribute to the development of human neurodegenerative diseases (reviewed in [3,4]). Indeed, mutations in genes that encode MT motor proteins that power axonal transport have been directly linked to human neurodegenerative diseases [5–8]. However, maintenance of the MT network that supports axonal transport is also critical. Mutations in genes encoding microtubule-associated proteins (MAPs) or proteins that regulate MT dynamics also impair axonal transport and cause human neurodegenerative disease. The neuronal MAP tau is the major constituent of insoluble intracellular inclusions called neurofilament tangles, a pathological hallmark of neurodegenerative tauopathies including Alzheimer’s disease. Pathogenic forms of tau have been demonstrated to impair kinesin-dependent axonal transport [9,10], and reductions in kinesin transport exacerbate neurodegeneration in animal models of tauopathies [11]. In addition, mutations in the gene spastin, which encodes a MT severing protein, are the cause of the most common form of hereditary spastic paraplegia in humans [12]. Deletion or mutation of the mouse spastin gene impairs axonal transport, resulting in progressive axonal degeneration [13,14].

We have carried out genetic screens to identify novel genes required for axonal transport. Among the candidate genes isolated, we have identified mutant alleles in the gene that encodes the MT regulatory protein stathmin. Stathmin (also known as Oncoprotein18 (Op 18), Phosphoprotein p19, metablastin, Proasin, and Leukemia Associated Protein 18) is a cytosolic phosphoprotein that regulates MT dynamics by partitioning tubulin dimers from pools of soluble tubulin thus preventing their assembly into polymerized MTs, and by directly binding to MTs and promoting their disassembly [15,16]. These functions are attributable to different regions of the stathmin protein. The N-terminus of stathmin promotes MT catastrophes whereas the C-terminus exhibits tubulin-sequestering activity [17]. Four genes encode the vertebrate stathmin family of proteins; STMN1 encodes the ubiquitous, cytosolic stathmin protein, while STMN2, STMN3 and STMN4 encode the neuron-specific, membrane-associated stathmin-like proteins SCG10, SCLIP and RB3/RB3* respectively [18]. All share a homologous tubulin-binding stathmin-like domain, while SCG10, SCLIP and RB3 contain an N-terminal membrane-targeting domain [19]. The vertebrate stathmin proteins can bind two tubulin heterodimers and are subject to regulatory phosphorylation on four conserved serine residues that reduce their ability to bind tubulin and regulate MT polymerization [20,21].

In Drosophila, a single stathmin (stai) gene encodes four protein isoforms, representative of the entire vertebrate stathmin family [22]. The stai gene is differentially expressed during Drosophila embryogenesis, with transcripts encoding staiA isoforms maternally deposited to the embryo, whereas the staiB encoding isoforms are expressed at high levels in early neuroblasts, and later in the developing embryonic central and peripheral nervous system [22]. Neuronal enriched staiB isoforms have an N-terminal domain that has conserved cysteine amino acids that are potential palmitoylation sites thought to play a role in subcellular targeting of the protein [22].

Analysis of loss of stai function in Drosophila has allowed us to circumvent potential complications encountered in studies in vertebrate systems in which mutant phenotypes may be compensated by genetic redundancy of other member of the stai gene family.

In this study, we show through loss of stai activity, an essential function for stai in the maintenance of axonal MTs necessary to support axonal transport. The architecture of the axonal MT network appears severely disrupted in our stai mutants, implicating a critical role for stai in its regulation. Loss of stai activity results in a posterior paralysis and ‘tail-flip’ phenotype in third instar larvae, hallmarks of impaired axonal transport. Immunohistochemistry reveals aberrant accumulation of synaptic vesicles in the axons of segmental nerves of stai mutant larvae. Despite the disruption in axonal transport, a significant number of mutant animals survive to the adult stage but have a reduced lifespan. In addition, stai mutant adults exhibit a progressive, late-onset bang-sensitive seizure in response to mechanical stimulation. This epilepsy-like behavioral deficit is characteristic of the class of mechanical shock-sensitive Drosophila mutants that have altered neuronal excitability and are used to model human epilepsy [23]. We demonstrate by mutant analysis and transgene rescue that the observed phenotypes are the result of loss of stai function. We also show that expression of the human stathmin gene, STMN1, can rescue these defects indicating that the Drosophila and human proteins are functional orthologs. Genetic reduction in the levels of Kinesin-1, the primary anterograde motor of axonal transport, enhances stai mutant phenotypes. Collectively, our results indicate that stai has an important function in the maintenance of the integrity of MTs in the axons of nerve cells of the peripheral nervous system necessary to support axonal transport.

Materials and Methods

Fly Stocks

Drosophila were raised on standard culture media at 25°C. Strains used in these studies include OregonR, CantonS, w1118, staiB200, PBac[5HPw] staiB200, Df(2L) BSC5, Df(2L) ED384, Df(2L) ED385, Df(2L) BSC239, Df(2L) Exel1015, KhcB20, w1118, CyO P[FRT( w )] Tub-PBac{T} 2/wg31M, tub-Gal4, and tubulin-staiB2 (gift of Pernille Rorth, IMCB, Singapore), tubulin-STMN1. PBac[5HPw] staiB200 is a stai mutant Drosophila strain obtained from the Bloomington Drosophila Stock Center and is derived from a progenitor line containing a 5’ half-P construct generated by Brian Ring and Dan Garza [24].

The piggyBac insertion in PBac[5HPw] staiB200 was precisely excised by crossing PBac[5HPw] staiB200/Cyo against w1118, CyO P[FRT( w )] Tub-PBac{T} 2/wg31M and generating individual balanced stocks that were determined by eye color to have the piggyBac transposon excised. The nature of the excision for each was confirmed by PCR across the original insertion site and subsequent sequence analysis.

The staiB200 Khc20 chromosome was generated by meiotic recombination, and balanced over CyO Act GFP. Recombinant staiB200 Khc20 chromosomes were identified by outcrossing each potential recombinant line against w1118 and following the
inheritance of the mini w+, associated with staip200, in straight-winged progeny. The presence of Khc20 was inferred by the absence of straight-winged flies in each recombinant stock, and confirmed by lethality in heterozygous combination with Df(2R) Jp8.

PCR Amplification and Sequencing of the stai Genomic Region

Genomic DNA was isolated from homozygous third instar staiapo larvae according to standard protocol [25]. The coding region of the stai gene was amplified in four separate PCR reactions using HotStar HiFidelity DNA Polymerase (Qiagen, Valencia, CA) according to manufacturer’s recommended protocol. PCR primers used were stai1 5'-GCTAATCAACGTGCTTAAAGCGAATT-3', stai2 5'-GTTCGGAATCGTGATGAGAATAAT-3', stai3 5'-GCATTACGGAATCTCAAGTTGCTA-3', stai4 5'-GTATCCACATTGTGATATGAGTACT-3', stai5 5'-GCCAGCAATCAAACACTTCAACAATG-3', stai6 5'-GCATTATGCTTCTAGCTGATAGT-3', stai7 5'-GCTACCAAACCTATTGCATCCCATAGT-3', and stai8 5'-CTCAGCTAAGCCGTATCCGCTTATTT-3', and stai9 5'-GCTTACAAACCTATTGCACTCCTAGT-3' and stai10 5'-GACTGGACTAGCAAACTGGTTAACATT-3'. All PCR products were resolved by agarose gel electrophoresis and purified using the QiAquick Gel Extraction Kit (Qiagen). PCR products were sequenced by Eurofins MWG Operon (Huntsville, AL)

qRT-PCR

mRNA purified from third instar larvae using the Oligotex Direct mRNA Mini Kit (Qiagen) was used to synthesize cDNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). RT-PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 Real Time PCR System (Applied Biosystems). In order to distinguish between stai transcripts with different transcriptional start sites, stai message was quantified with 'forward' oligonucleotides complementary to coding sequence within exon 2 (5'-GGTGAACAACACTGGTATCTG-3') shared by staiB isoforms, and sequence downstream of the copy insertion site in staiapo within exon 1 (5'-GATTAGTACCGACTGCTGTA-3') shared by staiA isoforms, in separate amplifications with a 'reverse' oligonucleotide complementary to sequence in exon 3 (5'-CTCAATCTCTCGACGCTAAC-3'), shared by all stai isoforms. Results were normalized against the signal from the GAPDH gene product (5'-AATTAAGGCAAGGTTCAGGA-3', 5'-ACCAAGAGATCAGGTTACGA-3'). All RT-PCR experiments were repeated five times for each genotype, and analyzed and quantified using the comparative C_t method (ΔΔC_t method). The results are presented as mean ± standard deviation (SD). Significance was determined by ANOVA of ΔC_t values [26,27].

Quantification of the Posterior Paralysis 'Tail-Flip’ Phenotype

The posterior paralysis phenotype of late third instar larvae was analyzed as described previously [28]. Briefly, third instar larvae of each genotype were collected, rinsed in ddH_2O and placed in 60 x 15 mm culture dishes, and the crawling behavior of each larva observed. A minimum of one hundred larvae was analyzed for each genotype. The severity of the posterior paralysis phenotype was scored and quantified using the following criteria; larvae were determined to exhibit a robust tail-flip if the tail was raised greater than 40° above horizontal when crawling; a mild tail-flip if the tail was raised less than 40° above horizontal when crawling, and no-tail flip if the larva exhibited a normal crawling behavior. The crawling behavior of third instar larvae was recorded with a Canon PowerShot G10 coupled to an Olympus SZ61 stereomicroscope and the resultant movies processed in iMovie and QuickTime Pro.

Larval Preparation, Immunohistochemistry and Microscopy

Immunostaining of larvae was performed according to standard protocol [29]. In brief, wandering third instar larvae were dissected in Ca²⁺ free HL3.1 buffer [30] and fixed for 30 minutes in freshly prepared 4% paraformaldehyde in PBS, pH 7.4. The larvae were washed and permeabilized in PBT (PBS + 0.2% Triton X-100), blocked in 1% BSA in PBT, and incubated overnight at 4°C with primary antibody in PBT. Following removal of the primary antibody, the larvae were washed in PBT and incubated for 2 hours at room temperature with fluorescently conjugated secondary antibody in PBT. Following removal of the secondary antibody and PBT washes, larval preparations were mounted on glass slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Antibodies were used at the following concentrations; mouse monoclonal anti-Drosophila cysteine string protein (DCSP-2 mAb 6D6) 1:200, anti-Futsch (mAb 22c10) 1:5 (Developmental Studies Hybridoma Bank, IA), anti-α-tubulin (mAb DM1A) 1:1000 (Sigma, St. Louis, MO), anti-acetylated α-tubulin (mAb L00) 1:200, Alexa Fluor 488 goat anti-mouse 1:250, and Alexa Fluor 568 goat anti-mouse 1:250 (Invitrogen). Nuclei were visualized by co-staining with Syto24 (Invitrogen) at a 1:1000 dilution during secondary antibody treatment.

Confocal Imaging and Analysis

All images were acquired on a Zeiss LSM-710 laser scanning confocal microscope. Imaging parameters, including signal gain, for a given experimental treatment were set such that the brightest treatment group produced images that were non-saturating. All imaging parameters were kept constant across a given experimental treatment.

Images of segmental nerve axons represent three-dimensional maximum intensity projections of serial stacks, ~10.4-14.2 μm thickness, acquired from the four to six medial segmental nerves spanning abdominal segments A4-A6. Disruptions in axonal transport were quantified from maximal intensity projection images of segmental nerve axons as they passed through abdominal segments A2 and A4 using the threshold and particle analysis functions in ImageJ (ver 1.47k).
with a size detection minimum of 0.5 µm². Results are reported as number of CSP accumulations/50 µm segmental nerve axon. Futsch and acetylated α-tubulin staining intensity was quantified using the histogram function of the Zen 2010 image analysis software package that reports a distribution of pixel intensities, as well as average pixel intensity, in a selected region of interest of an image. A lower threshold for the histogram analysis was applied to all images analyzed. Statistical analysis across the genotypes analyzed was performed using ANOVA of average pixel intensity of all segments analyzed within each experimental group, followed by Fisher’s LSD post hoc analysis.

Images of larval muscle represent three-dimensional maximum intensity projections of serial stacks, ~14.8-16.2 µm thickness, through muscle 6 at abdominal segment A4. Perinuclear MT density was quantified with ImageJ 1.44o by measuring the ratio of tubulin positive: negative staining signal in an area that extends 5 µm around the nucleus. Average perinuclear MT density was determined for each genotype by measuring MT density surrounding at least twenty nuclei, recorded from six larvae. The average perinuclear MT density for each genotype was normalized against wildtype.

Analysis of Lifespan and Bang-Sensitive Phenotypes

The life span of male Drosophila was measured at 25°C. Females were excluded from the life span analysis due to observed sex differences regarding the effect of dietary restriction on lifespan [31]. Adult flies were collected 24-hours post eclosion and maintained in standard culture vials (25 mm x 95 mm) with a maximum of 10 flies per vial. Flies were counted daily and transferred to fresh vials every other day. A minimum of one hundred flies per genotype was analyzed. The mean lifespan for each genotype was calculated by averaging individual lifespans for flies within a cohort. Significance was determined by ANOVA followed by Fisher’s and Scheffe’s post hoc analysis.

The bang-sensitive phenotype was quantified by the methodology of Ganetzky and Wu [32]. Briefly, male Drosophila of each genotype were aged at 25°C. Forty-eight hours prior to testing, the aged flies were CO₂ anaesthetized and a maximum of five flies were placed into fresh vials. The flies were mechanically stimulated by vortexing at full-speed for 5 minutes. The supernatant was decanted into new microcentrifuge tubes and the protein concentrations determined using the RC-DC Protein Assay (Bio-Rad, Hercules, CA). For each genotype, 25 µg of protein was resolved on NuPAGE 4-12% Bis-Tris Polyacrylamide Gels and transferred to nitrocellulose membrane (0.45 µm) (Bio-Rad) at 300 mA for 120 minutes in Tris-Glycine transfer buffer (25 mM Tris-Base, 190 mM Glycine). The efficiency of transfer was confirmed by Ponceau S stain (0.1% w/v Ponceau S, 5% v/v acetic acid). The blot was incubated in blocking buffer (5% Carnation dry milk, 50 mM Tris, 150 mM NaCl, 0.5% Tween 20) for one hour at room temperature prior to overnight incubation with primary antibody in antibody dilution buffer (1% Carnation dry milk, 50 mM Tris, 150 mM NaCl, 0.5% Tween 20) at 4°C. Following washes in wash buffer (0.2% Carnation dry milk, 50 mM Tris, 150 mM NaCl, 0.05% Tween 20), the blot was incubated with secondary antibody for two hours at room temperature. The SuperSignal Chemiluminescent Substrate Kit for Western Blotting (Pierce, Rockford, IL) was used for detection and the blot was exposed to X-ray film and developed for signal visualization. Primary antibodies included anti-α-tubulin 1:5,000 (DM1A T-9026, Sigma), anti-acetylated α-tubulin 1:5,000 (T-6793, Sigma), anti-actin 1:10,000 (mAb1501, Chemicon), anti-GADPH (IMG-3073, Imgenex Corp, San Diego, CA) anti-Kinesin heavy chain 1:5,000 (AKIN01, Cytoskeleton, Denver, CO) and anti-kinesin light chain 1:1,000. Secondary antibodies included HRP conjugated Goat anti-Mouse IgG (81-6520) and HRP conjugated Goat anti-Rabbit IgG (65-6120) used at 1:20,000 dilutions (Zymed Laboratories, Invitrogen Immunodetection). Western blots were quantified and data corrected for load using anti-GADPH antibody as a loading control. Quantitation was performed using a BioRad ChemiDoc XRS+ chemiluminescence detection system with a 16-bit CCD camera and ImageJ.

Generation of Antisera against Drosophila Kinesin Light Chain

Rabbit polyclonal antisera was produced against recombinant Drosophila kinesin light chain protein following PCR amplification of the Klc gene from cDNA clone NM_079325.3 with primers Klc(Xho1) 5’-ATCCGAGCTCGAGACGCAAATGCTGCAAG-3’ and Klc(Kpn1) 5’-CGAATTCCATGGTACCTTATGGTTTCG-3’. The resultant PCR product was cut with Xho1 and Kpn1 and subcloned into the Xho1/Kpn1 site of the pRSET-A plasmid to generate pRSET-A-Klc. pRSET-A-Klc was sequenced to confirm a complete Klc open reading frame, transformed into BL21(DE3) pLysS, and expression of the His-tagged Klc protein was induced in a 100 ml culture at OD₆₀₀=0.5 with IPTG to a final concentration of 1 mM. The cells were harvested four hours post induction and the recombinant Klc protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen), electrophoretically isolated and used to raise antisera in rabbits (Covance Research Products, Denver, PA). The specificity of the Klc antibody was confirmed by western blot against total protein extract isolated from third instar larvae. In wildtype Drosophila, the antibody recognized a single band of approximately 60 kDa, and a reduction in the intensity of the signal from this 60 kDa band was observed in protein from a
heterozygous strain harboring the chromosomal deletion Df(3L) 8ex94 that removes the Klc gene [33].

**Generation of Transgenic Drosophila for Ubiquitous Expression of Human Stathmin**

PCR was used to introduce a 5′ Not1 and 3′ Xho1 restriction site flanking the full-length open reading frame of the cDNA clone encoding human stathmin 1 (STMN1) transcript variant 1 (Accession No. NM_203401.1, gift of Lynne Cassimeris, Lehigh University, PA) with PCR primers hstai(Not1) 5′- GACTCGAGTTAGTCAGCTTCAGTCTC-3′. The PCR cytological region 26B5-26C1 on the left arm of the second Drosophila chromosome by its failure to complement gene mutation. Heteroallelic combination of additional mutation responsible for the homozygous lethality (n=1000). This was confounding given that greater allele causes semi-lethality, with approximately 40% of the expected homozygous progeny from a heterozygous cross surviving to the adult stage (n=1900), suggesting that stai function is not essential for adult viability. These results are supported by the previous observation that stai null Drosophila are adult viable [45]. Unexpectedly, homozygous staiB200 larvae were inviable past the third instar stage with no homozygous adults recovered among the progeny of a heterozygous cross (n=1000). This was confounding given that greater stai message was detected in homozygous staiB200 larvae than staiB200 larvae and message encoding both staiA and staiB isoforms in staiB200/Df(2L) Exel6015 larvae are reduced to undetectable levels (Figure 1C).

**Lethal Phase Analysis of stai Mutants**

Despite the dramatic reduction in stai message, the staiB200 allele causes semi-lethality, with approximately 40% of the expected homozygous progeny from a heterozygous cross surviving to the adult stage (n=1900), suggesting that stai function is not essential for adult viability. These results are supported by the previous observation that stai null Drosophila are adult viable [45]. Unexpectedly, homozygous staiB200 larvae were inviable past the third instar stage with no homozygous adults recovered among the progeny of a heterozygous cross (n=1000). This was confounding given that greater stai message was detected in homozygous staiB200 larvae than staiB200 larvae and message encoding both staiA and staiB isoforms in staiB200/Df(2L) Exel6015 larvae are reduced to undetectable levels (Figure 1C).

**Expression of stai is Reduced in staiBdp and staiB200 Larvae**

We used qRT-PCR to determine if the copia insertion in staiBdp and the piggyBac insertion in staiB200 altered the expression level of the stai gene in third instar larvae. Amplification with primer pairs specific for detection of staiA and staiB mRNA isoforms revealed significantly reduced levels of both transcripts in homozygous staiBdp third instar larvae (P<0.01) (Figure 1C). Transcripts encoding staiA isoforms were more strongly affected, reduced to as little as 12.0 ± 1.0% (mean ± SD) of wild type levels, compared to staiB encoding transcripts, which were reduced to 41.0 ± 4.0% of wild type levels (Figure 1C). As expected, heterozygous combination of staiBdp with Df(2L) Exel6015, a 170 kb chromosomal deletion that removes 14 genes including stai, reduces the abundance of staiA and staiB derived transcripts to 4.0 ± 1.0% and 15.0 ± 5.0% respectively, approximately 50% of the levels observed in staiBdp homozygous larvae (Figure 1C). The level of staiB mRNA is also reduced in homozygous staiB200 mutant third instar larvae, to 1.0 ± 0.1% of wildtype (P<0.0001) (Figure 1C). The levels of staiA encoding transcript in homozygous staiB200 larvae and message encoding both staiA and staiB isoforms in staiB200/Df(2L) Exel6015 larvae are reduced to undetectable levels (Figure 1C).
Figure 1. Identification of Mutations in the stathmin (stai) Locus. (A) Genomic structure of the Drosophila stai locus and the positions of the mutagenic copia retrotransposon stai^{rdtp} and the piggyBac element stai^{B200}. Exons are boxed, noncoding portions of exons are white, exons 3, 4, 5, shared by all stai proteins, are light grey (after Lachkar et al, 2010). Transcripts encoding staiA isoforms include exons 1 and 2 with alternative splicing that either includes (staiA1) or excludes (staiA2) exon 6. Transcripts encoding staiB isoforms include exons 1’ and 2’ with alternative splicing that either includes (staiB1) or excludes (staiB2) exon 6. The copia retrotransposon stai^{rdtp} is inserted in the open reading frame of the stai gene in exon 1’, ten base pairs downstream of the translational start site used to produce nervous system enriched staiB encoding transcripts. The piggyBac element PBac(5HPw ^{+})stai^{B200} is inserted in the 2.8 kb intron separating exons 1’ and 2’, 1.3kb downstream of the splice junction of exon 1’. (B) The copia insertion in the stai gene was identified by PCR amplification across the open reading frame of stai exon 1’ that resulted in an unexpectedly large 5.6 kb product from genomic DNA isolated from stai^{rdtp} homozygous larvae. The black arrowheads in Figure 1A represent the relative position of PCR primers used. (C) qRT-PCR of staiA and staiB mRNA derived from third instar larvae is shown. The expression of staiA and staiB is significantly reduced in all stai mutant genotypes analyzed compared to wild type expression levels (P<0.01). The red and blue arrowheads in Figure 1A represent the relative position of primers used for qRT-PCR of staiA and staiB message. Results are normalized against the expression of the GAPDH gene product and are presented as mean ± SD.

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to other loci, resulting in complex phenotypes [46,47], the focus of this study is the staïB200 mutant allele and the use of the staïCh chromosome is limited to heterozygous analysis or in heteroallelic combination with staïB200 and Df(2L) Exel6015.

**Loss of staï Function Results in a Larval Posterior Paralytic Phenotype**

Homzygous staïCh and staïB200 third instar larvae exhibited phenotypes characteristic of mutants with impaired axonal transport. Although staï mutant larvae were of comparable size to wild type larvae, their posterior regions were often weakly tapered and reduced in diameter as reported in other axonal transport mutants (data not shown) [48]. When homozygous staïCh and staïB200 larvae crawled along the surface of a substrate, they exhibited a crawling behavior in which the posterior body segments sharply flipped upward after each peristaltic wave of muscle contraction during the crawling cycle (compare Figure 2A with 2B and 2C, Movies S1-S3). Heterozygous combination of staïB200 with staïCh recapitulated the tail-flip phenotype (Movie S4). The larval posterior-paralysis or tail-flip phenotype is a hallmark of defective axonal transport in Drosophila and has been previously described for mutations in genes that encode components of MT motor protein complexes, such as kinesin and cytoplasmic dynein, as well as motor accessory proteins [28,29,33,35–37]. The phenotype is thought to result from a dorsal-ventral gradient in posterior muscle paralysis that causes an imbalance in contraction of the larval body wall muscles [29]. Interestingly, staï null third instar larvae also exhibited a posterior paralysis phenotype (gift of Pernille Rorth, IMCB, Singapore) (Movie S5).

We quantified the posterior paralysis and tail-flip phenotype of staï mutant larvae as described in the Materials and Methods. The posterior paralytic phenotype was incompletely penetrant, with 76.7% (n=92/120) of staïB200 third instar larvae examined exhibiting a tail-flip (Table S1). Of the staïB200 larvae exhibiting a posterior paralysis, 69.6% (n=64/92) had a robust tail-flip while 30.4% (n=28/92) had a mild tail-flip. The posterior paralysis phenotype was not observed in heterozygous staïB200/+ larvae (n=0/130) or larvae heterozygous for the chromosomal deletion Df(2L) Exel6015 (n=0/120). Heterozygous combination of the staïB200 allele with Df(2L) Exel6015 increased the penetrance of third instar larvae exhibiting the posterior paralytic phenotype to 89.1% (n=90/101), with 75.6% (n=68/90) exhibiting a robust tail-flip and 24.4% (n=22/90) a mild tail-flip (Table S1). Thus, despite having almost undetectable levels of staï expression, as quantified by qRT-PCR (Figure 1C), the staïB200 allele maintains some residual staï function and behaves like a strong hypomorphic mutation.

**staï Deficient Axons Exhibit Defects in Axonal Transport**

Mutations in genes required for axonal transport not only cause a similar posterior paralysis and tail-flip phenotype in third instar larvae, but they also result in the accumulation of anterograde and retrograde membranous axonal cargo in the segmental nerves of these animals [29]. To determine if the posterior paralysis phenotype observed in staï mutant larvae resulted from impaired axonal transport, the distribution of cysteine string protein (CSP) in the axonal compartment was assayed by immunohistochemistry. CSP is a synaptic vesicle protein transported in a microtubule-dependent manner within the axons of larval segmental nerves [49,50]. In wildtype segmental nerve axons, CSP exhibits a uniform, punctate staining pattern throughout the axoplasm (Figure 2D). Heterozygous staïB200/+ larvae exhibited an average of 1.64 ± 2.95 (mean ± SD) CSP accumulations/50 µm segmental nerve axon examined (n=71 axon segments examined, p>0.05) (arrowheads Figure 2E, 2H). staïB200/+ larvae exhibit a normal crawling behavior and the absence of a posterior paralysis phenotype, indicating axonal transport is not significantly compromised in peripheral nerve axons. In contrast, homozygous staïB200 third instar larvae exhibited 13.55 ± 5.56 aggregations of CSP/50 µm segmental nerve axon (n=52, p<0.0001, Figure 2F, 2H), consistent with an impairment in axonal transport. The CSP aggregations were not significantly enhanced in staïB200/Df(2L) Exel6015 larvae (15.10 ± 6.07 CSP accumulations/50 µm segmental nerve axon, n=57, p=0.06, Figure 2G, 2H). These results confirm that staï activity is required for efficient axonal transport in peripheral nerves.

To determine if there was an anterior to posterior gradient in the phenotypic severity of CSP aggregations in segmental nerve axons of staï mutant larvae, we quantified and compared the extent of axonal transport defects in regions of segmental nerve axons that passed through the more anterior abdominal segment A2 and the posterior abdominal segment A4 (Figure 2H). The only regional difference in the abundance of axonal clogs in segmental nerve axons was observed in axons from staïB200/Df(2L) Exel6015 larvae (p<0.0001).

**Adult Viable staï Mutants Have Reduced Lifespans**

Our observations indicate that Drosophila can tolerate significant reductions in staï levels. Despite a large percentage of third instar larvae exhibiting a posterior paralysis due to impairment of axonal transport, a significant percentage of staï mutant animals manage to survive to the adult stage but exhibit significant reductions in average adult lifespan at 25°C (Figure 3). The staïB200/+ heterozygote has an average adult lifespan of 81.0 ± 1.2 days, not significantly different from the 77.5 ± 1.6 days observed in the wildtype (P ≥0.05) (Figure 3A, 3B) or the 80.2 ± 1.6 days in Df(2L) Exel6015/+ adults (P≥0.05) (data not shown). In contrast, a significant reduction in average adult lifespan was observed for staïB200 adults, averaging 46.6 ± 2.3 days (P<0.0001) (Figure 3A, B). The average adult lifespan was reduced even further, to 34.8 ± 2.3 days (P<0.0001) in staïB200/Df(2L) Exel6015 adult males (Figure 3A, 3B).

**Adult Viable staï Mutants Exhibit a Progressive Bang-Sensitive Seizure Phenotype**

While performing the lifespan analysis on adult viable staï mutants, we observed they exhibit a temporary paralysis in response to the mechanical activity of being transferred to new food vials. This phenotype is similar to that observed in the "bang-sensitive" class of genes, mutant allelic of which cause neurological defects characterized by a paralysis or seizure in response to mechanical stimulation. Mutant alleles of a number...
of genes have been identified that exhibit a bang-sensitive phenotype including bang-sensitive (bas), bang-senseless (bss), technical knockout (tko), slam dance (sda) and easily shocked (eas). These genes are involved in diverse cellular processes including ion transport, Ca^{2+} release and mitochondrial metabolism [51,52]. Typically, the seizures become easier to trigger, and the subsequent paralysis increases in duration, with increasing age [53].

We quantified the response of stai mutant animals to mechanical stimulation by performing a bang-sensitive assay on adult male flies of different ages. We observed a normal response to, and recovery from, mechanical stimulation for all genotypes tested at 1, 7 and 14 days post eclosion (Tables S2 and S3). However, 20.9% of sta^{B200} males tested at 21 days post eclosion (n=67) exhibited bang-sensitive paralysis with a mean recovery period of 48.6 ± 26.7 seconds (mean ± SD). During the bang-sensitive paralysis, flies laid motionless on the media at the bottom of the vial for periods ranging from 10 seconds to 3 minutes, depending on age. Upon recovery, flies exhibited a hyperactive phase, known as a recovery seizure, in which they vigorously flapped their wings and moved their legs for approximately 4-6 seconds, prior to righting themselves to a standing position and walking away. By 42 days, 75.8% of sta^{B200} adults exhibited bang-sensitive paralysis, phenotype...
Figure 3. **Loss of stai Function Reduces Adult Drosophila Lifespan.** The lifespan of adult male *Drosophila* of seven different genotypes were measured at 25°C. A minimum of one hundred flies for each genotype were analyzed and the number of surviving flies were counted daily and survivorship curves generated (A). Adult wildtype *Drosophila* had an average lifespan of 77.5 ± 1.6 days (mean ± SE), not significantly different from the average lifespan of 81.0 ± 1.2 days observed in stai\textsuperscript{B200/+} heterozygous adults (p≥0.05) or the 80.2 ± 1.6 day average lifespan of Df(2L)\textsubscript{Exel6015/+} adults (p≥0.05) (data not shown). In contrast, the average lifespan of homozygous stai\textsuperscript{B200} adults was 46.6 ± 2.3 days, significantly less than the average lifespan of wildtype, and heterozygous stai\textsuperscript{B200/+} and Df(2L)\textsubscript{Exel6015/+} adults (p<0.001). Adult males of the genotype stai\textsuperscript{B200/Df(2L)Exel6015} had an even shorter average adult lifespan of 34.8 ± 2.3 days, significantly less than stai\textsuperscript{B200} homozygous adult males (p<0.001). Introduction of an exogenous copy of a ubiquitously expressed transgene encoding the neuronal specific stai\textsuperscript{B2} isoform, tub-stai\textsuperscript{B2}, rescued the adult average lifespan of both stai\textsuperscript{B200} homozygous adults to 68.5 ± 2.3 days (p<0.001) and stai\textsuperscript{B200/Df(2L) Exel6015} adults to 64.8 ± 2.3 days (p<0.001).

**Table**

| Genotype                                      | Average Lifespan (days) |
|-----------------------------------------------|-------------------------|
| wildtype                                      | 77.5 ± 1.6              |
| stai\textsuperscript{B200/+}                 | 81.0 ± 1.2              |
| stai\textsuperscript{B200}                   | 46.6 ± 2.3              |
| stai\textsuperscript{B200/Df(2L)Exel6015}    | 34.8 ± 2.3              |
| stai\textsuperscript{B200; tub-stai\textsuperscript{B2}/+} | 68.5 ± 2.3              |
| stai\textsuperscript{B200/Df(2L)Exel6015; tub-stai\textsuperscript{B2}/+} | 64.8 ± 2.3              |
| stai\textsuperscript{excision}               | 74.6 ± 1.2              |

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with an increase in the mean recovery period to 76.6 ± 41.4 seconds (n=62) (Movie S9). By 56 days, 91.7% of staiB200 adults exhibited the bang-sensitive response with a mean recovery period of 145.4 ± 63.8 seconds (n=144).

Interestingly, the progressive bang-sensitive phenotype was also observed in heterozygous staiB200/+ and staiB2/+ adults, but with a delayed onset and a marked reduction in the recovery period following paralysis compared to staiB200 adults (Tables S2 and S3). A single copy of the tub-staiB2 transgene also significantly increased average adult lifespan to 68.5 ± 2.3 days in staiB200; tub-staiB2/+ adults, and to 64.8 ± 2.3 days in staiB200; DR(2L) Exel6015; tub-staiB2/+ adults compared to the average lifespan of staiB200 and staiB200; DR(2L) Exel6015 adults (P<0.001) (Figure 3B). While the ectopic expression of staiB2 rescued the posterior paralysis and aberrant accumulation of CSP in segmental nerve axons of staiB2 homozygous larvae (data not shown), it did not rescue lethality, supporting the idea that the staiB2 chromosome carries a linked, lethal mutation.

Finally, we were also able to rescue the stai mutant phenotypes with the ubiquitous ectopic expression of the human stathmin gene, STMN1. A single copy of the tub-STMN1 transgene significantly reduced the aberrant accumulation of CSP in the segmental nerve axons of staiB200 larvae (3.92 ± 3.87 CSP accumulations/50 µm segmental nerve axon, n=84, p<0.0001, compare Figure 4A and 4D Figure 4E). The tub-STMN1 transgene also reduced the percentage of staiB200 larvae that exhibited a posterior paralysis phenotype from 76.7% (n=92/120) to 39.6% (n=44/111) (Table S1). In addition, the severity of the posterior paralysis phenotype was reduced to only 9.1% (n=4/44) of staiB200; tub-STMN1 larvae that exhibited a robust tail-flip from 69.6% (n=64/92) of staiB200 larvae (Table S1 Movie S8). These data support previous observations that the Drosophila and human stathmin proteins are functionally equivalent orthologs.

Although all observed stai mutant phenotypes could be rescued with both Drosophila staiB2 and human STMN1 transgenes, rescue was incomplete. This was likely due to the fact that the rescuing Drosophila staiB2 transgene expresses only one of the four known stai protein isoforms, which may have been insufficient for complete rescue. In addition, expression of both transgenes was under the control of the tubulin promoter that may not have provided the correct spatial and temporal expression of the transgenes.

**Loss of stai Function Reduces Tubulin and Kinesin Heavy Chain Protein Levels**

Since stai functions as a tubulin-binding protein and is known to regulate MT dynamics, we used western blot analysis to examine and quantify tubulin levels in total protein extracts isolated from stai mutant third instar larvae and adults. Levels of α-tubulin were significantly reduced in staiB200+/+ larvae compared with wild type controls (p<0.0001, Figure 5A, 5B). A more pronounced reduction in the levels of α-tubulin protein was observed in homozygous staiB200 larvae (Figure 5A). Third instar staiB200 larvae had levels of α-tubulin that were 15.0 ± 16.6% (mean ± SD) of that observed in wild type larvae (p<0.0001, Figure 5A, 5B). In order to assess the stability of polymerized MTs, we also assayed for levels of acetylated α-tubulin in stai mutant larvae. The levels of acetylated α-tubulin were also significantly reduced in staiB200/+ mutant larvae (p<0.0001, Figure 5A, 5B), and more greatly reduced in homozygous staiB200 mutant larvae to 36.9 ± 12.9% of the levels observed in wildtype (p<0.0001, Figure 5A, 5B). The posterior paralysis displayed by stai homozygotes is reminiscent of the ‘tail flip’ phenotype first described in Kinesin.
heavy chain (Khc) mutant larvae [29]. We therefore examined protein extracts from staI mutant animals to see if Khc levels were affected. We observed a significant reduction in the level of Khc, the force generating subunit of the MT motor protein kinesin-1, in staI mutant larvae (Figure 5A). Heterozygous staI+/+ larvae had levels of Khc protein that were 91.2 ± 4.2% of that observed in wildtype (p<0.05) whereas staI larvae had levels that were 53.8 ± 2.3% of wildtype (p<0.0001, Figure 5A, 5B). Collectively, these data suggest a general impairment of the MT-based transport system following loss of staI function in Drosophila.

Given the observed effect of loss of staI function on the levels of α-tubulin, acetylated α-tubulin and Khc protein in third instar larvae (Figure 5A), and the observation that ectopic expression of staI rescued the shortened lifespan of staI adults (Figure 3A), we analyzed the levels of α-tubulin and kinesin-1 motor components in adult staI mutant animals at 1 day and 21 days post-eclosion (Figure 5C). These ages were chosen because they corresponded to the age at which a bang-sensitive response to mechanical stimulation was first observed in staI deficient animals (Table S2). At 1 day post-eclosion, the levels of α-tubulin, Khc and Kinesin light chain (Klc) subunits were equivalent across all genotypes analyzed (Figure 5C). However, by 21 days post-eclosion, there was a marked reduction in the levels of α-tubulin, Khc and Klc proteins in staI adults. Surprisingly, ectopic expression of staI not only restored the levels of α-tubulin in staI larvae, tub-staI+/+ juveniles that had 0.91 ± 1.37 CSP accumulations/50 µm segmental nerve axon, p<0.01) compared to staI third instar larvae. In panels A-D, the scale bar = 10 µm.

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Figure 4. Reversion and Genetic Rescue of Fast Axonal Transport Defects in staI Deficient Axons. Confocal micrographs of segmental nerves from (A) staI homozygous third instar larvae stained with antibodies against synaptic vesicle protein cysteine string protein (CSP). Note the abundant accumulation of CSP throughout the segmental nerve, indicative of a disruption in fast axonal transport. (B) In contrast, precise excision of the mutagenic piggyBac element PBac(5HPw), responsible for the loss of staI function in staI homozygous third instar larvae, completely restores axonal transport and reverts the CSP staining pattern in the axon to the punctate, uniform distribution observed in wildtype axons (compare Figure 4B with Figure 2D) (Movie S6). (C) Introduction of a single copy of a ubiquitously expressed transgene encoding the neuronal specific tub-STMN1 transgene, completely restores axonal transport and reverts the CSP staining pattern in the axon to the punctate, uniform distribution observed in wildtype axons (compare Figure 4B with Figure 2D) (Movie S6). (D) Introduction of a single copy of a ubiquitously expressed human transgene encoding the staI gene, tub-staI, also diminishes the accumulation of CSP in the segmental nerves of staI deficient axons of staI larvae, indicating the Drosophila and human staI proteins are functional orthologs. (E) The severity of axonal transport defects was quantified by averaging the number of accumulations of CSP immunopositive anterograde and retrograde membranous axonal cargos observed in the segmental nerve axons of staI deficient third instar larvae. Axonal transport defects are reverted to wildtype in the axons of staI larvae that had 0.91 ± 1.37 CSP accumulations/50 µm segmental nerve axon (p<0.0001). Axonal transport defects are reduced in the segmental nerve axons of staI larvae expressing a single copy of the Drosophila rescue transgene tub-staI, 6.79 ± 6.43 axonal clogs/50 µm segmental nerve axon (p<0.0001), and the human transgene tub-STMN1, 3.92 ± 3.87 axonal clogs/50 µm segmental nerve axon (p<0.0001). Results are reported as the average number of axonal clogs (mean ± SD) per 50 µm segmental nerve axon. A small but significant increase in the severity of axonal clogs was observed in the segmental nerve axons of staI/+, Khc+/staI larvae (15.89 ± 3.80 CSP accumulations/50 µm segmental nerve axon, p<0.01) compared to staI third instar larvae. In panels A-D, the scale bar = 10 µm.

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Genetic Reductions in the Kinesin Heavy Chain Gene Enhance staI Phenotypes

Because loss of staI function resulted in decreased levels of Khc protein in third instar larvae, and ectopic expression of staI restored reduced levels of conventional kinesin motor protein subunits in adult flies, we assayed for a genetic interaction between staI and kinesin by combining a genetic reduction in the gene that encodes the conventional kinesin heavy chain protein, Khc, with mutations in the staI gene.
Figure 5. stai Mutants Have Reduced Levels of α-Tubulin, Acetylated α-Tubulin, and Kinesin Heavy Chain Protein. (A) Representative western blots of total protein extracted from stai mutant third instar larvae. Heterozygous stai/+ mutant larvae exhibit mild reductions in the levels of α-tubulin and acetylated α-tubulin compared with wild type larvae. Homozygous stai mutant larvae have dramatic reductions in the levels of α-tubulin, acetylated α-tubulin and the heavy chain subunit of the microtubule motor protein kinesin compared to wildtype. Df(2L) BSC239 represents a second chromosomal deficiency that excludes the stai gene. GAPDH is used as a loading control. (B) Quantification of western blots of total protein extracts isolated from third instar larvae probed with antibodies against α-tubulin (mAB DM1A), acetylated α-tubulin (mAB 611B1), and kinesin heavy chain (AKIN01). Quantification was performed as described in the Materials and Methods. Results are normalized against wildtype and presented as mean ± S.D. Statistical comparison reveals that the levels of α-tubulin, acetylated α-tubulin, and kinesin heavy chain proteins for all genotypes examined differ significantly from wildtype (p<0.001), with the exception of levels of acetylated α-tubulin in staiB20/+/ larvae (**p<0.01) and kinesin heavy chain in staiB20B20/+/ (*p<0.05). (C) Western blot analysis of total protein extracted from stai mutant adult Drosophila aged 1 day and 21 days post eclosion. The levels of α-tubulin, and conventional kinesin motor heavy and light chain subunits are present at equal levels in wildtype, staiB20B20 and staiB20B20; tub-staiB2 adults 1 day post eclosion. At 21 days post eclosion, the age we observe staiB20 adults exhibiting a bang-sensitive response to mechanical stimulation, the levels of α-tubulin, and conventional kinesin motor heavy and light chain subunits are noticeably reduced. Introduction of an exogenous copy of the rescuing transgene tub-staiB2, not only increases the levels of α-tubulin, but also increases levels of the conventional kinesin motor heavy and light chain subunits.

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of a single copy of Khc did not cause a posterior paralysis phenotype or enhance the axonal transport defects observed in staI/Df(2L) Exel6015 larvae. However, introduction of the Khc20 null allele into the homozygous staI genetic background increased the penetrance of the posterior paralysis phenotype observed in staI homozygous larvae to 97.1% (n = 101/104) compared with only 76.7% (n = 92/120) of staI homozygous larvae (Table S1). In addition, we observed a small, but significant, increase in the severity of aberrant accumulation of CSP in the segmental nerve axons of staI homozygous larvae (Figure 6A). In contrast, the distribution of Futsch protein appeared to be disrupted and less consistent along the length of segmental nerve axons of staI mutant larvae, intense and prominent in some axonal regions (arrows Figure 6B), yet noticeably reduced and absent in other regions of the same axon (small arrowheads Figure 6B). We interpret this observation as a disruption in Futsch-stabilized MTs within staI mutant larvae. We quantified and compared the average pixel intensity of Futsch-stabilized MTs within staI mutant larvae to that observed in wildtype axons (WT 19000 ± 5200 arbitrary units (a.u.), n = 43; staI/Df(2L) Exel6015 larvae (7600 ± 2200 a.u, n = 42, p < 0.0001, Figure 6G, Table S4). The average intensity of Futsch staining was further reduced in the axons of staI homoyzygous third instar larvae (9000 ± 3000 a.u, n = 32 p < 0.0001, compare Figure 6A and B) and greater yet in the axons of staI/Df(2L) Exel6015 larvae (7600 ± 2200 a.u, n = 30, p < 0.0001, compare Figure 6A and C) compared to wildtype.

Wild type larvae have a well-defined MT network in segmental nerve axons, as determined by the even distribution of Futsch protein along the length of individual axons within segmental nerve bundles (data not shown). However, introduction of the Khc20 null allele into the homozygous staI genetic background increased the penetrance of the posterior paralysis phenotype observed in staI homozygous larvae to 97.1% (n = 101/104) compared with only 76.7% (n = 92/120) of staI homozygous larvae (Table S1). In addition, we observed a small, but significant, increase in the severity of aberrant accumulation of CSP in the segmental nerve axons of staI homozygous larvae (Figure 6A). In contrast, the distribution of Futsch protein appeared to be disrupted and less consistent along the length of segmental nerve axons of staI mutant larvae, intense and prominent in some axonal regions (arrows Figure 6B), yet noticeably reduced and absent in other regions of the same axon (small arrowheads Figure 6B). We interpret this observation as a disruption in Futsch-stabilized MTs within staI mutant larvae. We quantified and compared the average pixel intensity of Futsch-stabilized MTs within staI mutant larvae to that observed in wildtype axons (WT 19000 ± 5200 arbitrary units (a.u.), n = 43; staI/Df(2L) Exel6015 larvae (7600 ± 2200 a.u, n = 42, p < 0.0001, Figure 6G, Table S4). The average intensity of Futsch staining was further reduced in the axons of staI homoyzygous third instar larvae (9000 ± 3000 a.u, n = 32 p < 0.0001, compare Figure 6A and B) and greater yet in the axons of staI/Df(2L) Exel6015 larvae (7600 ± 2200 a.u, n = 30, p < 0.0001, compare Figure 6A and C) compared to wildtype.

Loss of staI Function Alters the Integrity of Axonal MTs

MTs are the main cytoskeletal component of axons. The motor proteins kinesin and cytoplasmic dynein mediate organelle transport on MT tracks within the axon. Given the known function of staI as a regulator of MT dynamics and our demonstration that loss of staI function disrupts axonal transport and reduces the levels of α-tubulin and acetylated α-tubulin in third instar larvae, we assayed the integrity of MTs in segmental nerve axons by immunostaining staI mutant larvae with antibody against the neuronal specific MAP Futsch (mAb 22c10), the Drosophila homolog of the vertebrate MAP1B protein that reveals stabilized MTs in neurons [55].

Wild type larvae have a well-defined MT network in segmental nerve axons, as determined by the even distribution of Futsch protein along the length of individual axons within the compound nerve (Figure 6A). In contrast, the distribution of Futsch protein appeared to be disrupted and less consistent along the length of segmental nerve axons of staI mutant larvae, intense and prominent in some axonal regions (arrows Figure 6B), yet noticeably reduced and absent in other regions of the same axon (small arrowheads Figure 6B). We interpret this observation as a disruption in Futsch-stabilized MTs within staI mutant larvae. We quantified and compared the average pixel intensity of Futsch-stabilized MTs within staI mutant larvae to that observed in wildtype axons (WT 19000 ± 5200 arbitrary units (a.u.), n = 43; staI/Df(2L) Exel6015 larvae (7600 ± 2200 a.u, n = 42, p < 0.0001, Figure 6G, Table S4). The average intensity of Futsch staining was further reduced in the axons of staI homoyzygous third instar larvae (9000 ± 3000 a.u, n = 32 p < 0.0001, compare Figure 6A and B) and greater yet in the axons of staI/Df(2L) Exel6015 larvae (7600 ± 2200 a.u, n = 30, p < 0.0001, compare Figure 6A and C) compared to wildtype.

Loss of staI Function Alters the Integrity of Axonal MTs

Figure 6. Loss of staI Function Reduces Futsch (MAP1B) Protein Levels in Axons and Alters the Integrity of Axonal Microtubules. (A–F) Confocal micrographs of segmental nerve axons from third instar larvae immunostained with antibody against the MAP Futsch (mAb 22c10). (A) Wild type larvae have well defined Futsch staining in individual axons within segmental nerve bundles. (B) In contrast, loss of staI function results in an inconsistent distribution of Futsch protein along the length of individual axons within segmental nerve bundles. In some regions of an axon, there is prominent Futsch staining (arrows) whereas Futsch staining is noticeably reduced in other areas of the same axon (small arrowheads). In addition, loss of staI function causes gross perturbations in axon morphology (large arrowheads). (C) The perturbations in axon morphology are more profound in segmental nerve axons from staI/Df(2L) Exel6015 larvae. (D) Precise excision of the piggyBac element FBac(SHPw)5HPw reverts the observed phenotypes to wildtype. The reduction in Futsch staining intensity and perturbations in axon morphology observed in staI deficient segmental nerve axons can be ameliorated with the ubiquitous ectopic expression of Drosophila staI2 (E) or human STMN1 (F). (G) Quantification of average pixel intensity of Futsch immunostained axons of third instar larval segmental nerves. Results are presented in arbitrary units (a.u.) of fluorescence intensity (mean ± S.D.). In panels A–F the scale bar = 10 µm.

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STMN1 (the axon, MTs also provide internal structural support that acetylated α-tubulin immunostained segmental nerves of wild type larvae are arranged in linear, ordered bundles manner. (Figure 6A). In contrast, deficient segmental nerve axons is significantly restored with the ubiquitous ectopic expression of 

Individual axons that comprise the compound segmental nerve tainsting intensity detected in the axons of 

In addition to acting as tracks to support transport through the axon, MTs also provide internal structural support that influences and maintains the morphology of the axon. Individual axons that comprise the compound segmental nerve of wild type larvae are arranged in linear, ordered bundles (Figure 6A). In contrast, stai deficient larvae exhibited alterations in the morphology of individual axons within the segmental nerve that are not observed in wildtype or stai/+ larvae (large arrowheads Figure 6B). Individual axons were often bent and non-linear in stai deficient segmental nerves. This observed alteration in axon morphology was more pronounced and affects a greater number of segmental nerve axons of (10800 ± 4100 a.u., n=24, p=0.12) and stai/+ (10800 ± 1800 a.u., n=22, p=0.14) larvae compared with homozygous larvae (9000 ± 3000 a.u., n=32). However, the rescue transgenes did significantly increase the average pixel intensity of acetylated α-tubulin staining detected in the axons of both stai/+ (13400 ± 4500 a.u., n=80, p<0.0001, compare Figure 7A and E) and stai/+ (12200 ± 3300, n=115, p<0.05, compare Figure 7A and F) third instar larvae.

**Loss of stai Function Alters the Integrity of Muscle MTs**

The observed phenotypes following loss of stai function are consistent with a reduction in the level of polymerized MTs in the axons of stai mutant animals, contradictory to the function of stai as a MT depolymerizing protein. Because the integrity of MTs within individual segmental nerve axons is difficult to assess by direct visualization, we also looked at the effect of loss of stai function on the architecture of the MT network in body wall muscle 6 from abdominal segment A4 of our stai mutant larvae.

The MT network in muscle cells from wild type larvae is very well defined, enriched around the nucleus and extending into the cytoplasm (Figure S1 A). The MT network of stai/+ muscle cells appears indistinguishable from wildtype (data not shown). The MT network is noticeably less dense, however, both around the nucleus and within the cytoplasm of muscle cells from stai mutant larvae (compare Figure S1 A and B). The reduction in polymerized MTs is even more pronounced in stai/+ larvae. (compare Figure 6C with Figure 6E, F). The rescue transgenes improved, but did not significantly increase, the average pixel intensity of Futsch staining detected in the axons of stai/+ (10800 ± 4100 a.u., n=24, p=0.12) and stai/+ (10800 ± 1800 a.u., n=22, p=0.14) larvae compared with homozygous larvae (9000 ± 3000 a.u., n=32). However, the rescue transgenes did significantly increase the average pixel intensity of acetylated α-tubulin staining detected in the axons of both stai/+ (13400 ± 4500 a.u., n=80, p<0.0001, compare Figure 7A and E) and stai/+ (12200 ± 3300, n=115, p<0.05, compare Figure 7A and F) third instar larvae.
confirming that the stafr000 allele retains residual staI activity. This observation directly supports our results from western blot data regarding the observed reduction in levels of acetylated α-tubulin in staI and staID(2L) Exel6015 third instar larvae. Interestingly, we detect an abundance of fragmented MTs within the cytoplasm of staI deficient cells (arrowheads Figure S1 B, C).

We quantified the density of perinuclear MTs in muscle cells of our staI mutant animals and found that MT density was significantly reduced in stafr000 (P<0.05) and stafr000/Df(2L) Exel6015 (P<0.01) larvae (Figure S1 G). The architecture of the MT network was restored to wildtype in stafrcoiD muscle cells (Figure S1 D) and rescued with expression of both the tub-stafB2 (Figure S1 E), and the human tub-STMN1 transgenes (Figure S1 F). Rescue of the MT network in stafr000 larvae with the tub-stafB2 transgene resulted in a perinuclear MT density greater than wildtype (P<0.01) (Figure S1 G).

**Discussion**

The development and function of the nervous system is dependent on a highly dynamic, yet tightly regulated, microtubule (MT) cytoskeleton (reviewed in [2,57]). The stathmin (staI) family of proteins comprise a group of MT destabilizing proteins that indirectly function to regulate microtubule dynamics by binding and sequestering free tubulin and stimulating MT depolymerization [16]. It has been previously demonstrated in Drosophila that staI is maternally contributed to the oocyte and is required for the development of the embryonic nervous system [58]. The recessive, hypomorphic alleles we have isolated in the Drosophila staI gene have allowed us to circumvent the requirement for staI during embryonic development, and identify a function in the maintenance of axonal MTs in the mature nervous system, necessary to support and sustain axonal transport.

Collectively, our results indicate that staI function is required for axonal transport. Phenotypes consistent with impaired axonal transport, including a posterior paralysis tail-flip and accumulation of synaptic proteins in peripheral segmental nerve axons, were observed in staI mutant larvae. Furthermore, genetic perturbations that independently affect axonal transport (such as a 50% reduction in Kinesin heavy chain (Khc) copy number) enhance the severity of the staI mutant phenotype including the accumulation of synaptic proteins in segmental nerve axons. We also observe reductions in the levels of tubulin, acetylated tubulin and kinesin heavy and light chain protein subunits in our staI mutants. Importantly, Khc levels were reduced by less than 50%. Since Khc hemizygotes are phenotypically normal, our data argue that the observed reduction in Khc levels contribute to the staI mutant phenotype, but are not its primary cause. These phenotypes are ameliorated by ectopic expression of a Drosophila staI transgene encoding a neuronal specific staI isoform. In addition, we are able to rescue the observed phenotypes with ectopic expression of the human gene STMN1. Mutations in cytoplasmic dynein subunits and motor protein accessory factors also result in axonal transport defects. A potential direction for future studies could be to comprehensively examine how microtubule motor protein expression is affected in Drosophila staI mutants. A comparison of STMN1<sup>++</sup> vs. STMN1<sup>-/-</sup> mouse embryonic fibroblasts identified motor proteins as the largest group of genes with altered expression profiles. Interestingly, while differential expression of 13 kinesin motor subunits was detected alteration in dynein motor subunit expression was not observed, and only 5 genes encoding dynein light or intermediate chains showed altered expression [59].

The severity of axonal transport blockages observed in staI mutant axons is significantly less than that observed in microtubule motor mutants. It has previously been reported that Drosophila third instar larvae null for the microtubule motor proteins Dynemin heavy chain (Dhc) and Khc exhibit 250 ± 25 clogs per 50 µm length of segmental nerve axon [60]. Third instar larvae of the genotype stafr000/Df(2L) Exel6015 exhibit on average 15.10 ± 6.07 clogs per 50 µm length of segmental nerve, significantly less than reported in Dhc and Khc null third instar larvae. The severity of axonal transport blockages observed in staI mutant larvae, however, is equivalent to levels observed in larvae null for the amyloid precursor protein-like gene Appl [61]. Interestingly, Appl null larvae are also homozygous viable, suggesting that the severity of the disruption of axonal transport may determine viability and in part explain why a significant percentage of stafr000 and stafr000/Df(2L) Exel6015 mutants are homozygous viable.

We routinely recover viable stafr000/Df(2L) Exel6015 adult flies, despite undetectable levels of staI transcript as measured by qPCR analysis, suggesting that staI function is not necessary for viability. This observation is consistent with a recent report that staI knockout flies are viable, indicating that staI is a non-essential gene [45]. The staI mutant adult flies exhibit a significantly reduced lifespan and a progressive, age-dependent epileptic-like seizure and paralysis in response to mechanical stimulation. To our knowledge, the bang-sensitive phenotype has not been previously reported in motor-protein mutants, and is not specific to defective axonal transport. However mutations in the Khc gene have been shown to exhibit a temperature-sensitive paralysis, a phenotype found in bang-sensitive paralytic (para) and maleless (mle) mutants, and interact with para and mle to cause lethality [62]. Khc mutants also suppressed the Shaker and ether-a-go-go leg shaking phenotype, which is similarly suppressed by para and mle alleles [62]. Hurd et al. conclude that Khc is likely required for anterograde transport of vesicles bearing sodium membrane channels and that reduced transport of these channels results in reduced sodium current. Likewise, the identification of a progressive bang-sensitive phenotype in the staI mutants we have analyzed, suggests that the transport of ion channels to the axon membrane is impaired.

Our observation of age-dependent phenotypes in Drosophila staI mutants parallels the observation that STMN1 knockout mice initially develop normally and display no overt phenotypes [63], but when aged exhibit a minor axonopathy [64] and behavioral deficits that include a lack of both learned and innate fear responses [65]. Interestingly, altered levels of STMN1 expression in human brain tissue has recently been linked to intractable temporal lobe epilepsy [66].
It has recently been reported that stai is required for stability of the neuromuscular junction in Drosophila, a phenotype that likely arises as a result of impaired axonal transport [67]. While Graf et al., present evidence of perturbed axonal transport, analysis of the integrity of axonal MTs did not reveal alterations in MT architecture. Our analysis supports the observation that stai function is necessary for axonal transport. In contrast to the studies of Graf et al., however, we also present evidence that the MT cytoskeleton of the axon is disrupted in stai mutant larvae and likely the cause of impaired axonal transport. We demonstrate this in three ways. First, we observe a reduction in the intensity of Futsch and acetylated α-tubulin staining in the segmental nerve axon that correlates with a reduction in stai dosage, and an increase in the severity of CSP accumulation in the axon. Second, we observe alterations in the organization of Futsch staining in individual segmental nerve axons lacking stai function. Finally, within the segmental nerve, individual axons exhibit an altered, distorted morphology in which they undulate along the length of the compound nerve bundle. We interpret this phenotype as a consequence of reduced axonal MTs that result in a loss of internal structural support for the axon. Interestingly, this phenotype is similar to the morphology of axons that are subjected to dynamic stretch injury, resulting in the breakage of MTs within the axon that correlate with undulations in axonal morphology [68]. One possible explanation for the observed differences in axonal MT morphology reported by each group is perdurance of maternally contributed stai product in differently aged third instar larvae examined in each study.

A wealth of evidence indicates that stai family members are negative regulators of MT dynamics. Decreasing the levels of stai in vivo increases polymerized MTs, as demonstrated by microinjection of neutralizing anti-stathmin antibody into newt lung cells [69], shRNA depletion of endogenous stathmin in human interphase cells [70,71], and knockout of stathmin function in MEFs [27], and amygdala slices from whole brains of stathmin knockout mice [65]. Conversely, microinjection of wildtype stathmin protein into newt lung cells results in massive loss of MT polymer [72]. Surprisingly, in contrast to vertebrate systems, loss of stai function results in dramatic reduction in the levels of both α-tubulin and acetylated α-tubulin. This reduction was visible on western blots as well as by immunohistochemical visualization that showed that polymerized MTs were reduced in our stai mutant Drosophila.

It is not clear why we see a reduction in the levels of polymerized tubulin in our stai mutant Drosophila. It has been previously demonstrated that the addition of recombinant Drosophila stai to purified bovine brain tubulin in vitro results in a decrease in MT polymerization in a dose dependent manner, while the expression of three different Drosophila stai isoforms, staiA1, staiB1 and staiB2 in HeLa cells in vivo depolymerizes the MT network in a subset of cells [22]. These observations indicate that Drosophila stai isoforms also act as MT depolymerizers in mammalian cells and mammalian cell extracts. It is possible that the MTs within the axonal compartment of a Drosophila nerve cell are subject to extrinsic signals, regulatory factors and MAPs that are distinct and not present in other subcellular regions of a neuron, or in different cell types. While this may be true, it is an unsatisfactory explanation because we also observe a reduction in polymerized MTs in muscle cells that comprise the body wall musculature.

Another possibility is that reduction in stai could alter the expression of a gene that encodes a MT polymer stabilizer. However, a more likely explanation for the observed reduction of the MT network in stai mutant Drosophila, is that the phenotype is the consequence of a complete depletion of all stai protein activity. Unlike vertebrate model systems, the entire stai family of proteins are encoded by a single gene in Drosophila that produces four stai protein isoforms [22]. Therefore, mutation in the stai gene removes or greatly reduces the activity of all stai proteins. In contrast, in studies performed in vertebrate model systems the function of only one member of the stai family is altered, and genetic redundancy by the remaining stathmin-like proteins may compensate and account for the observed phenotypic differences. Indeed, it has been reported in STMN1 knockout MEFs that the expression of STMN2 is decreased and STMN4 is increased [59], while in STMN1 knockout mice, an increase in the expression of STMN3 and STMN4 is observed [64,73]. The stai protein has two known activities; a MT depolymerizing activity as well as a tubulin binding activity. Depleting stai not only removes the MT depolymerizing activity of the protein, which is expected to result in an increase in the polymerized MT network, but it also abolishes its tubulin binding activity as well, which may be required to maintain the polymerized MT network. The net effect of removing both functions entirely may be a gross reduction in the MT network.

Observation of the MT network in muscle cells of third instar larvae may provide a clue as to how the MT network becomes reduced in stai mutant larvae. The distinct architecture allowed us to observe an abundance of fragmented or severed MTs in the peripheral cytoplasm of cells lacking stai activity. The MT cytoskeleton of a cell is subject to both stabilizing and destabilizing factors that act coordinately to maintain the MT architecture. For example, an antagonistic relationship in maintaining the MT network has been demonstrated between tubulin-specific chaperone E (TBCE), required for the formation of tubulin heterodimers, and the microtubule severing protein spastin [74]. It is possible that stai acts downstream of TBCE, and its removal results in a diminished MT network, as observed in TBCE mutant larvae, suggesting an antagonistic relationship with MT severing proteins spastin and/or katanin. It is interesting to note that mutation of TBCE in mice results in a progressive motor neuropathy [75,76] while mutations in spastin are the cause of the most common form of hereditary spastic paraplegia in humans, likely through disruption of axonal transport [12–14].

Stai is a cytosolic phosphoprotein whose function is regulated by many different signaling pathways [77]. The ability of stai to bind tubulin is dependent on its phosphorylation state. In Xenopus metaphase extracts, the phosphorylation of stathmin is in part regulated by the serine/threonine type-2A phosphatase PP2A [78]. PP2A is known to associate with MTs and is thought to regulate MTs through phosphorylation of the MAP Tau [79,80]. Interestingly, PP2A is a component of the
striatin-interacting phosphatase and kinase (STRIPAK) complex, of which the Monopolar spindle-one-binder protein 3 (Mob3/Phocein) is a known component [81]. DMob4, the *Drosophila* Phocein homolog has recently been demonstrated to regulate microtubule organization and axonal transport [82].

Defects in axonal transport are known to cause or contribute to neurodegenerative disease and it is possible that *staI* may play a role in some cases. In mammals, loss of *staI* function results in mild, progressive phenotypes consistent with neurodegenerative disease [64]. In addition, a link has been established between the altered expression of stathmin and the abnormal architecture of MTs and organelle transport in motor axons of a mouse model of the motor neuron degeneration disorder spinal muscular atrophy [83]. Our in vivo analysis in *Drosophila* has identified a role for *staI* in regulating the architecture of MTs in the axon of peripheral nerves, necessary to support and maintain axonal transport.

**Supporting Information**

**Figure S1.** Loss of staI Function Alters the Integrity of Muscle Microtubules. (A–F) Confocal micrographs obtained from body wall muscle 6 from third instar larvae immunostained with antibody against the α-tubulin (DM1A) (green) and counterstained with nucleic acid stain Syto24 (red). (A) Wild type animals have an extensive, well-defined MT network in muscle cells. (B) The MT architecture, however, is greatly reduced in staI200 mutant larvae. (C) The MT architecture is more severely reduced in staI200/Df(2L) Exel6015 mutant larvae. Loss of staI function not only reduces the density of the MT architecture, but also results in fragmented MTs in the peripheral cytoplasm. (D) The MT architecture is reverted to wildtype in staImutant, a precise excision allele derived from staI200. The reduced density of the MT cytoskeleton is ameliorated with the ubiquitous expression of *Drosophila* staIB2 (E) or human STMN1 (F). In panels A–F the scale bar = 20 μm, n = nerve. (G) The density of perinuclear MTs was quantified in third instar larvae body wall muscle. Results are normalized against wildtype and are presented as mean ± SD.

(MOV)

**Movie S1.** Wildtype *Drosophila* third instar larvae exhibit a normal crawling behavior. (MOV)

**Movie S2.** *Drosophila* third instar larvae homozygous for staI200 exhibit a robust 'tail flip' phenotype during crawling. (MOV)

**Movie S3.** *Drosophila* third instar larvae homozygous for staI200 exhibit a robust 'tail flip' phenotype during crawling. (MOV)

**Movie S4.** *Drosophila* third instar larvae heterozygous for staI200 and staI200 recapitulate the robust 'tail flip' phenotype during crawling, observed in larvae homozygous for each staI allele. (MOV)

**Movie S5.** *Drosophila* third instar larvae homozygous for staI200 exhibit a robust 'tail flip' phenotype during crawling. (MOV)

**Movie S6.** *Drosophila* third instar larvae homozygous for staIexcision, a reversion allele generated from the precise excision of the mutagenic piggyBac element PBac(5HPw+) in staI200, exhibit a normal larval crawling behavior. (MOV)

**Movie S7.** *Drosophila* third instar larvae homozygous for staI200 and a single copy of the rescue transgene tub-staiB2 exhibit a rescued crawling behavior. (MOV)

**Movie S8.** *Drosophila* third instar larvae homozygous for staI200 and a single copy of the rescue transgene tub-STMN1 exhibit a rescued crawling behavior. (MOV)

**Movie S9.** Representative movie showing the bang-sensitive phenotype as observed in 42-day old staI200 adult *Drosophila*. (MOV)

**Table S1.** Penetrance of the Posterior Paralysis Phenotype Observed in Wandering Third Instar Larvae. The penetrance and severity of the posterior paralysis phenotype was scored and quantified by measuring the severity of the tailflip, as determined by the angle that the tail was raised above the substrate on which the larvae crawled. If the tail was raised greater than 40° above the horizontal crawling plane during the crawling cycle, larvae were scored as having a robust tail-flip. If the tail was raised less than 40° above horizontal during the crawling cycle, larvae were scored as having a mild tail-flip. If larvae exhibited a normal crawling behavior, they were scored as having no tail-flip. The crawling behavior of at least one hundred larvae was analyzed, for a minimum of one minute each, for each genotype tested.

(XLSX)

**Table S2.** Percentage of Adult *Drosophila* Exhibiting a Bang-Sensitive Phenotype. Adult male *Drosophila* were assayed for paralytic behavior in response to mechanical stimulation at six different age periods post eclosion. In wild type controls, a paralysis in response to mechanical stimulation was observed with a very low penetrance of 1.5% at 42 days of age, and 3.3% at 56 days of age. This response is attributed to the general effects of aging on the adult nervous system. A pronounced bang-sensitive phenotype is observed in 20.9% of homozygous staI200 mutant animals tested at 21 days of age, increasing to 75.8% by 42 days of age and 91.7% at 56 days of age. The bang-sensitive phenotype is also observed at appreciable levels in staI200/4 and staI200/ flies, but with a later onset than observed in staI200 homozygous adults. At 42 days of age, 25.2% of staI200/4 and 18.0% of staI200/ adults exhibit a bang sensitive phenotype, increasing to 58.0% of staI200/ and 52.9% of staI200/ flies by 56 days. Ubiquitous expression of a rescuing transgene, tub-staiB2, delays the onset of the bang-sensitive response to mechanical stimulation to 24.6% of staI200/; tub-staiB2/+ animals aged 56 days. A minimal response to mechanical stimulation, not significantly different
that wildtype, was observed in staB+/+; tub-staiB2/+ animals aged 56 days. The tub-staiB2 transgene also delayed and ameliorated the bang-sensitive phenotype in staB200 homozygous adults. The response to mechanical stimulation by genotype matched staB200 adults is not significantly different than the response of wild type control animals. A minimum of fifty adult Drosophila were tested at each age for each genotype analyzed. (XLSX)

Table S3. Average Recovery Period of Adult Drosophila Exhibiting a Bang-Sensitive Phenotype. The average time required for recovery from a bang-sensitive paralysis induced by mechanical stimulation as described in the Materials and Methods was assayed in adult male Drosophila. Time is represented as average recovery time (secs) ± standard deviation (SD).

Table S4. Average Pixel Intensity of Futsch Immunostained Segmental Nerve Axons of Drosophila Third Instar Larvae. The average pixel intensity of Futsch immunostained segmental nerve axons was determined as described in the Materials and Methods. Average pixel intensity is reported as mean ± S.D. in arbitrary units (a.u.) of fluorescence. (XLSX)

Table S5. Average Pixel Intensity of Acetylated α-Tubulin Immunostained Segmental Nerve Axons of Drosophila Third Instar Larvae. The average pixel intensity of acetylated α-tubulin immunostained segmental nerve axons was determined as described in the Materials and Methods.

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Conceived and designed the experiments: LSBG JED. Performed the experiments: JED NKL AZ. Analyzed the data: JED. Contributed reagents/materials/analysis tools: LSBG JED. Contributed reagents/materials/analysis tools: LSBG JED. Wrote the manuscript: JED. Wrote the manuscript: JED.
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