Drosophila Spastin Regulates Synaptic Microtubule Networks and Is Required for Normal Motor Function

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The most common form of human autosomal dominant hereditary spastic paraplegia (AD-HSP) is caused by mutations in the SPG4 (spastin) gene, which encodes an AAA ATPase closely related in sequence to the microtubule-severing protein Katanin. Patients with AD-HSP exhibit degeneration of the distal regions of the longest axons in the spinal cord. Loss-of-function mutations in the Drosophila spastin gene produce larval neuromuscular junction (NMJ) phenotypes. NMJ synaptic boutons in spastin mutants are more numerous and more clustered than in wild-type, and transmitter release is impaired. spastin-null adult flies have severe movement defects. They do not fly or jump, they climb poorly, and they have short lifespans. spastin hypomorphs have weaker behavioral phenotypes. Overexpression of Spastin erases the muscle microtubule network. This gain-of-function phenotype is consistent with the hypothesis that Spastin has microtubule-severing activity, and implies that spastin loss-of-function mutants should have an increased number of microtubules. Surprisingly, however, we observed the opposite phenotype: in spastin-null mutants, there are fewer microtubule bundles within the NMJ, especially in its distal boutons. The Drosophila NMJ is a glutamatergic synapse that resembles excitatory synapses in the mammalian spinal cord, so the reduction of organized presynaptic microtubules that we observe in spastin mutants may be relevant to an understanding of human Spastin’s role in maintenance of axon terminals in the spinal cord.

Introduction

“Pure” autosomal dominant hereditary spastic paraplegia (AD-HSP) is an inherited disease characterized by bilateral spasticity in the absence of other phenotypes (reviewed in Fink 2003; Reid 2003). Afflicted patients experience difficulty in walking and have a distinctive gait. Degeneration of the lateral corticospinal tracts, which contain the axons of cortical neurons that innervate primary limb motoneurons, is observed in the lumbar regions of the spinal cord in patients with AD-HSP. The distal segments of long dorsal root ganglion axons also display degeneration. No evidence is seen for cell death or for primary myelination defects, and the axons of primary motor neurons do not degenerate (Miaa and Behan 1974; Wharton et al. 2003). AD-HSP thus appears to selectively affect the distal regions of the longest axons within the spinal cord.

Because pathology is usually confined to long spinal cord axons, it has been suggested that the primary defect in pure AD-HSP is in axonal transport or some other process required for maintenance of axon terminals. Perturbation of anterograde or retrograde axonal transport might selectively affect the longest axons, because they would be most vulnerable to a reduction in efficiency of transport of material to or from their terminals.

About 40% of cases of pure AD-HSP are caused by mutations in the SPG4 gene, which encodes an AAA ATPase called Spastin (Hazan et al. 1999). AAA ATPases are a large and diverse set of proteins that include an approximately 250–amino acid (aa) conserved domain containing Walker A and B ATP-binding motif sequences (reviewed in Confalonieri and Duguet 1995; Patel and Latterich 1998; Neuwald et al. 1999). They use energy obtained from ATP hydrolysis to catalyze assembly or disassembly of a variety of protein complexes. AAA proteins are involved in many cellular processes, including vesicle trafficking, protein degradation, and microtubule dynamics. Many AAA ATPases form hexameric rings, and it is thought that the ring structures are required for catalytic activity (Vale 2000).

Spastin is a member of the “meiotic” subgroup of AAA ATPases (Frohlich 2001; Frickey and Lupas 2004), which contains proteins involved in vesicle trafficking and microtubule dynamics.

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Abbreviations: aa, amino acid; AD-HSP, autosomal dominant hereditary spastic paraplegia; CNS, central nervous system; Dlg, Discs-large; EJP, excitatory junction potential; GS, GeneSwitch; LOF, loss of function; mAb, monoclonal antibody; mEJP, “mini” excitatory junction potential; NMJ, neuromuscular junction; QC, quantal content; scg, scabrous; spin, spintet; Syt, Synaptotagmin; VNC, ventral nerve cord; WCS, Canton S w–

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tubule dynamics. The only member of the subgroup whose activities have been biochemically characterized is Katanin-60, which is the catalytic subunit of a microtubule-severing protein (McNally and Vale 1993; Hartman et al. 1998). Katanin-60 and Spastin are homologous only within their AAA domains. However, cell culture studies have provided evidence that Spastin is also involved in microtubule dynamics. Expression of wild-type human Spastin in transfected cell lines and cortical neurons caused disassembly of the microtubule cytoskeleton, while a mutant Spastin lacking catalytic activity colocalized with tubulin (Errico et al. 2002; McDermott et al. 2003).

The mechanisms by which spastin mutations produce dominant spasticity phenotypes in humans are controversial. A wide variety of nonsense and frameshift mutations, but no complete gene deletions, have been found in families with AD-HSP. It has been suggested that dominance arises from haploinsufficiency (Charvin et al. 2003). This model, however, would require that the processes in which Spastin participates are vulnerable to a 50% decrease in its enzymatic activity. Another possibility is that truncated or missense mutant Spastins function as dominant negatives. Hexameric AAA ATPase ring complexes might be especially vulnerable to the presence of nonfunctional ("poison") subunits that assemble into rings but lack catalytic activity. Consistent with this idea, expression of a mutant Spastin that associates with microtubules but cannot catalyze severing altered organelle distribution in transfected cells (McDermott et al. 2003). In the dominant negative model, AD-HSP might only occur when Spastin activity is eliminated or greatly reduced.

In this study, we describe the phenotypes arising from mutation of the Drosophila ortholog of human spastin. We initially identified this gene in a gain-of-function screen, in which we found that its overexpression in neurons causes axons in the embryonic central nervous system (CNS) to converge onto the midline (Sun 2000). Overexpression of Spastin in muscles erases their microtubule networks, consistent with the idea that Spastin is a microtubule-severing protein.

We made loss-of-function (LOF) spastin mutations, and found that they produce recessive phenotypes affecting the larval neuromuscular system. The Drosophila neuromuscular junction (NMJ) uses glutamate as its neurotransmitter and employs ionotropic glutamate receptors homologous to vertebrate AMPA receptors (Schuster et al. 1991; Petersen et al. 1997; Marrus et al. 2004). It is organized into presynaptic boutons that are surrounded by a postsynaptic scaffold, and its synapses exhibit plastic behavior during development. These properties make the fly NMJ a useful genetic model system for the study of glutamatergic synapses in the mammalian brain and spinal cord (Keshishian et al. 1996; Koh et al. 2000).

During the period from larval hatching through the third instar stage, the number of boutons at each NMJ increases by up to 10-fold in order to keep pace with the growth of its muscle target. New boutons are added by a process of budding (Zito et al. 1999). As these boutons mature, their microtubule cytoskeleton is thought to progress through a regulated series of alterations (Roos et al. 2000; Pennetta et al. 2002).

In this paper, we show that synaptic growth and function are altered in spastin mutant larval NMJs. Boutons are more numerous than in wild-type larvae, and synaptic transmission is impaired. These changes could result from alterations in synaptic microtubule dynamics, because we find that microtubule bundles are depleted from the distal boutons of NMJs in spastin-null mutants. This is surprising, because the fact that Spastin overexpression destroys microtubule networks might lead one to expect that its removal would increase the number of microtubules. Morphological and microtubule phenotypes are seen only for a total gene deletion, indicating that complete loss of Spastin function is required to alter synaptic microtubules in the fly system. The phenotypes we see are quite different from those described in a recently published study of perturbation of Drosophila spastin using RNAi methods (Trotta et al. 2004). In particular, the changes in synaptic microtubules that occur in spastin LOF mutants are opposite to those reported in the RNAi perturbation paper.

spastin is not an essential gene, but mutant adults have severely compromised motor behavior. Null mutants cannot fly or jump, they climb slowly, and they often drag their hind legs. While it is intriguing that spastin mutant flies display such movement phenotypes, further work will be required to determine whether Drosophila can provide a useful organismal model system for human AD-HSP. Nevertheless, insights into the cellular functions of Drosophila Spastin obtained from our work should be relevant to an understanding of Spastin’s functions in human neurons.

Results

The Drosophila spastin Gene

We identified spastin in an “EP” screen for genes involved in embryonic CNS development. EPs are P element derivatives with a block of “UAS” sites recognized by the yeast transcription factor GAL4 near one end (Rorth 1996). An EP element inserted in the proper orientation upstream of a gene will drive its expression in a cell-specific manner when the insertion line is crossed to the appropriate promoter-GAL4 “driver” line (Brand and Perrimon 1993). We generated approximately 6,000 new EP insertion lines and screened them by crossing to pan-neuronal (Elav-barα) and pan-muscle (24B) GAL4 driver lines (Lin and Goodman 1994; Luo et al. 1994). Those lines for which crosses to either driver generated reduced numbers (<20% of expected) of viable adult progeny containing both the EP element and the driver were saved.

About 2% of lines (131) exhibited lethality or reduced viability with one of the drivers, and 62 of these were lethal or semilethal with both drivers. The T32 insertion on the
close relatives in human and fly. Dm CG3326 is the counterpart of the human fidgetin/fidlik gene pair, while CG1193 probably encodes a second fly ortholog of human Katanin-60. In the mouse, fidgetin mutations produce inner ear defects that cause head-shaking and circling behaviors (Cox et al. 2000).

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third chromosome conferred complete lethality when crossed to either driver, and produced a neuronal-driver-dependent axonal phenotype (see below).

To identify the gene driven by the T32 element, we cloned a genomic DNA fragment adjacent to the insertion site and used it to identify a full-length cDNA encoding a 758-aa protein that is a member of the AAA ATPase family (Figure 1A). The T32 EP element is inserted into the 5’ UTR, 222 nucleotides upstream of the predicted ATG start codon (Figure 1B; Sun 2000).

The gene driven by T32 is orthologous to the human SPG4 (spastin) gene that is mutated in the most common form of AD-HSP (Hazan et al. 1999). Mammalian Spastins are the only proteins that are homologous to both the C-terminal AAA domain and the N-terminal region of fly Spastin (Figure 1A). Spastin exhibits homology to all other AAA proteins only within its AAA domain (approximately aa 460-754 of fly Spastin). There are about 30 AAA proteins encoded in the Drosophila genome.

The Drosophila Spastin sequence from aa 233 to the C terminus is 49% identical to that of human Spastin (616 aa). The AAA domains of the two proteins are 67% identical. The other region that is conserved between the Spastins (34% identity) corresponds to aa 233-404 of the fly sequence. The same region is also weakly related (26% identity) to human Spastin, the product of the SPG20 gene mutated in Troyer syndrome, a form of “complicated” HSP (Patel et al. 2002; Ciccarelli et al. 2003). Spastin is not an AAA ATPase. The AAA protein with a known biochemical function that is most closely related to Spastin (41% identity in the AAA domain) is Katanin-60 (Figure 1A and 1C; McNally and Vale 1993; Hartman et al. 1998).

Drosophila Spastin Localizes to the Cytoplasm

Human Spastin is thought to be a cytoplasmic protein expressed in many cell types, based on localization of epitope-tagged proteins expressed in transfected cells, and antibody staining of human tissue and neuronal cell lines (Errico et al. 2002, 2004; McDermott et al. 2003; Wharton et al. 2003). However, antibodies generated against Spastin are also reported to stain nuclei in several cell lines and mouse spinal cord neurons (Charvin et al. 2003; Errico et al. 2004). This dual subcellular localization has been proposed to reflect a role for human Spastin in processes involving highly dynamic microtubule states, such as during cell division (reflected in Spastin’s nuclear localization) and in axon outgrowth, suggested by the distal cytoplasmic staining of growing axons in culture (Errico et al. 2004).

To investigate the subcellular localization of Drosophila Spastin, we generated a variety of antibodies against different regions of the protein (see Materials and Methods). We evaluated these antibodies, by staining stage 16 embryos overexpressing Spastin in the striped Engrailed pattern from an engrailed-GAL4 driver. Two different antibodies revealed Spastin expression in the expected striped pattern, which includes a subset of CNS neurons (Figure 2A). Anti-Spastin antibodies stained both the cell bodies and the axons of these neurons (Figure 2B). In the epithelial portions of the Engrailed stripes, where the cells are flat and spread out, we observed that Spastin is expressed uniformly in the cytoplasm, but did not detect any nuclear staining (Figure 2C; see also Figure 7E, showing cytoplasmic expression in muscles).

spastin mRNA is expressed at low levels within the embryonic ventral nerve cord (VNC) in wild-type embryos (Sun 2000; Kammermeier et al. 2003). Endogenous Spastin
appears to be a very rare protein, and we have not been able to define a staining pattern in wild-type embryos or larvae that disappears in null mutants. An independently generated anti-*Drosophila* Spastin antibody was reported to stain the cytoplasm of both neurons and muscles in wild-type larvae, and staining was also detected at NMJ boutons (Trotta et al. 2004). Taken together, these results suggest that fly Spastin, like human Spastin, is likely to be a widely expressed protein that is primarily localized to the cytoplasm.

**Spastin Overexpression in Neurons Causes Collapse of the Embryonic CNS**

Crossing the T32 insertion line to scabrous (sca)-GAL4, which is expressed in neuronal precursors and neurons (Klaes et al. 1994), produced very strong CNS phenotypes. In Figure 3, these are visualized by staining with monoclonal antibody (mAb) 1D4 (Van Vactor et al. 1993), which labels a set of three longitudinal axon bundles. At 23 °C, embryos displayed abnormal midline crossing of the inner 1D4 bundle, and the entire VNC was narrowed at these crossing sites (Figure 3B). When crosses were performed at 29 °C, a temperature at which GAL4 transactivation is stronger, the VNC collapsed onto the midline and discrete longitudinal bundles were no longer apparent (Figure 3C). We also made transgenic lines bearing a full-length spastin cDNA driven by a UAS-containing promoter. After crossing to *sea-GAL4*, such lines produced even stronger phenotypes, in which the VNC collapsed at 23 °C (Figure 3D). Consistent with this observation, we found that more Spastin protein was made in driver × UAS-spastin embryos than in driver × T32 insertion embryos.

**spastin LOF Mutations Produce Larval NMJ Phenotypes**

To evaluate Spastin’s functions during development, we generated several deletion mutations from the T32 insertion by imprecise excision. We mapped their breakpoints by sequencing, and these data are displayed in Figure 1B. In line 10-12, about half of the first exon is deleted. The 17-7 deletion ends within the second intron and thus removes the entire first exon (encoding sequence up to aa 251). In both of these lines, DNA encoding the protein region conserved between human and *Drosophila* Spastin is still present. Deletion 5.75 removes the entire spastin gene, as well as the intergenic region and 129 bp at the 3′ end of the sequence of the adjacent predicted gene Rox8. Rox8 contains RRM RNA-binding domains. The 5.75 deletion removes the C-terminal 43 aa of the Rox8 protein, but does not delete into the RRM domains. The function of Rox8 is unknown, and there are no existing *Rox8* mutations (Brand and Bourbon 1993). Because the only null spastin mutation also affects Rox8, we relied on rescue experiments (see below) to demonstrate that the phenotypes we describe for the null mutant are due to loss of Spastin.

Flies homozygous for *spastin*10-12 and *spastin*17-7 have behavioral phenotypes, but they eclose at normal frequencies and are fertile (see below). In contrast, most homozygous spastin5.75 pupae do not eclose. spastin5.75 adults have very
Figure 4. Synaptic Boutons Are Smaller, More Numerous, and Clustered in spastin LOF Mutants

(A–F) Representative A3 NMJs on muscles 6/7 (A–C) or muscle 4 (D–F) stained with antibodies against Dlg (green) and Syt (magenta) are shown for control larvae (WCS; A and D), spastin5.75 larvae (B and E), and larvae expressing Spastin from the spin-GAL4 driver in a spastin5.75 mutant background (Rescue; C and F). Boutons are arranged in a linear pattern in WCS larvae, whereas in spastin5.75 larvae their distribution is more clustered and individual boutons are smaller. These phenotypes are rescued by Spastin expression via the spin-GAL4 driver. Scale bars, 10 μm.

(G) Quantitation of bouton numbers in spastin mutants relative to wild-type and rescued larvae demonstrates complete rescue of the null
phenotype by spin- or Elav-GS-GAL4-driven expression of Spastin. spastin-null mutants have on average 1.6-fold more type Ib boutons on muscle 4 compared to WCS control larvae. Similarly, spastin-null mutants (of genotype spin-GAL4;CyOKr-GFP; spastin-5.75) have 1.7-fold more boutons compared to their sibling rescued larvae (genotype spin-GAL4/UAS-spastin; spastin-5.75). Boutons are also 1.6-fold more numerous in spastin-null larvae from a neuronal rescue cross (genotype +;CyOKr-GFP; Elav-GS-GAL4, spastin-5.75;spastin-5.75) compared to their siblings in which UAS-spastin is expressed in neurons postembryonically (genotype UAS-spastin;CyOKr-GFP; Elav-GS-GAL4; spastin-5.75;spastin-5.75).

To confirm that loss of Spastin produced the observed NMJ phenotype, we counted the numbers of boutons at the muscle 4 NMJs of segments A2 and A3, where boutons typically form on the internal surface of the muscle and are thus easily imaged. Dlg is expressed at much higher levels at Ib compared to Is boutons, allowing the two types of bouton to be distinguished and counted. Because of the larger variability in Is bouton number between NMJs, we focused our quantitative analysis on the type Ib boutons. However, the numbers of both bouton types were similarly affected in spastin-5.75 larvae.

The number of Ib boutons per muscle 4 NMJ was increased by 1.6-fold relative to WCS in spastin mutants at room temperature (approximately 23 °C) (Figure 4G), and the boutons often formed dense clusters, particularly at the ends of NMJ branches (Figure 4E). This morphology was rarely observed in wild-type muscle 4 NMJs, where boutons were arranged more linearly (Figure 4D). The clustered boutons resemble the “satellite” boutons described by other investigators (Torroja et al. 1999; Franco et al. 2004; Koh et al. 2004; Marie et al. 2004). Hypomorphic spastin10-12 and spastin5.77 mutants had bouton numbers that did not differ significantly from controls.

To confirm that loss of Spastin produced the observed NMJ alterations, and to determine whether Spastin is required presynaptically or postsynaptically, we needed to evaluate rescue of the phenotype by expression of Spastin from a UAS-spastin cDNA insertion. This was difficult because of the early lethality produced by expression of Spastin from most drivers. UAS-spastin animals bearing pan-neuronal (Elav-GAL4), motoneuronal (OK6-GAL4), or pan-muscle (24B-GAL4 or G14-GAL4) drivers did not survive to larval stages at 23 °C, and few larvae appeared even at 18 °C. However, third instar larvae in which Spastin expression from the cDNA was conferred by spinster (spin)-GAL4, a weak driver that functions in both neurons and muscles (Sweeney and Davis 2002), did survive at 23 °C. We were also able to obtain larvae in which Spastin expression was induced in neurons postembryonically. This was done by crossing UAS-spastin to Elav-Geneswitch (GS)-GAL4, a driver line bearing a neurally expressed GAL4 derivative that is only active in the presence of the progesterone analog RU486 (Osterwalder et al. 2001; McGuire et al. 2004). Newly hatched larvae from this cross were maintained on RU486-containing food until the third instar stage.

To assay rescue, we combined the spin-GAL4 and Elav-GS-GAL4 drivers and UAS-spastin insertions separately with spastin-5.75, crossed the driver and UAS-spastin lines together, and assayed NMJ phenotypes in the F1 driver-GAL4/UAS-spastin; spastin-5.75 larvae. In each cross, we compared the rescued larvae to their unrescued spastin mutant siblings (driver-GAL4; spastin-5.75), because the presence of the driver chromosome had effects on the absolute number of Ib boutons (see Materials and Methods). We observed that the ratio of muscle 4 Ib bouton numbers in spastin mutant controls versus rescued larvae was 1.7 for spin-GAL4, and 1.6 for Elav-GS-GAL4 (Figure 4G). Since the ratio of bouton numbers for spastin-5.75 versus WCS was 1.6, this indicated that rescue was essentially complete in both cases. We also observed that the abnormal bouton clustering was eliminated in rescued larvae using either driver (Figures 4E and S1). These results demonstrate that loss of Spastin from neurons during larval development causes the NMJ bouton phenotypes seen in spastin-5.75 mutants.

To examine the consequences of driver-dependent postembryonic neuronal expression of Spastin in a wild-type background, we also counted boutons in UAS-spastin; Elav-GS-GAL4 larvae grown on RU486 food (see Materials and Methods). We observed that these larvae had fewer boutons than their siblings (0.83 ± 0.07 fold change), and some of their boutons appeared larger (Figure S1C). This phenotype is very mild, but it does suggest that loss and increased expression of Spastin can produce opposite effects on the NMJ.

Neurotransmitter Release Is Impaired in spastin Mutants

To evaluate whether spastin mutations cause alterations in the electrophysiological properties of the NMJ, we evaluated synaptic transmission at the muscle 6 NMJ in WCS, mutant, and rescued larvae raised at 18 °C. In spastin-5.75 larvae, there was a reduction in the amplitudes of evoked responses...
Figure 5. NMJs in spastin Mutant Larvae Display Reduced QC

(A) Representative EJP (upper) and mEJP (lower) traces are shown for control (WCS), spastin-null mutant (spastin5.75), and spin-GAL4/UAS-spastin; spastin17-7 (Rescue) larvae. All recordings were from the A3 or A4 muscle 6 NMJ.

(B) The average EJP amplitude is decreased by about 20% in spastin-null mutants (37 ± 2.0 mV, n = 26) relative to control (48 ± 2.4 mV, n = 14) and Rescue (42.4 ± 1.2 mV, n = 28) larvae, and is intermediate between control and null levels in hypomorphic spastin5.75/spastin17-7 transheterozygotes (41.9 ± 1.5 mV, n = 22).

(C) The average amplitude of spontaneous events (mEJPs) is increased slightly in spastin nulls relative to control and Rescue larvae.

(D) The average frequency of spontaneous events is not affected in spastin mutants compared to control. Rescue larvae had a slightly higher mEJP frequency.

(E) Average QC, a measure of the amount of neurotransmitter released per action potential, is significantly lower in transheterozygotes (28 ± 1.9 mV, n = 8) compared to WCS (30 ± 1.9, p = 0.1 compared to WCS).

(F) Average QC is temperature dependent in spastin5.75/spastin17-7 transheterozygotes, but not in homozygous spastin-null or control larvae. QC measured in transheterozygotes raised at 18°C (light gray bars) is intermediate between that of control and nulls. At room temperature (dark gray) and 29°C (black bars), similar QC values are measured in transheterozygotes and null mutants.

*p < 0.05; **p < 0.005.

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(excitatory junction potentials [EJPs]) to depolarization of the innervating nerves. Average EJP amplitudes in the null were reduced to 78% of the levels in control (WCS) larvae (Figure 5A and 5B; p < 0.003). We also examined the average amplitude and frequency of responses to single vesicles of spontaneously released neurotransmitter (“mini” EJPs [mEJPs]). mEJP amplitude was increased slightly, to 117% of WCS levels (Figure 5A and 5C; p < 0.03). There was no significant change in mEJP frequency (Figure 5D; p = 0.3).

Quantal content (QC), a measure of the number of vesicles released per evoked event, was calculated by dividing the EJP amplitude by the average mEJP amplitude. Because the evoked EJP was decreased and the mEJP increased in spastin5.75 mutants, QC was reduced to 67% of WCS levels in these larvae (Figure 5E; p < 3 × 10−6). spastin5.75/spastin17-7 larvae had EJP amplitude, mEJP amplitude, and QC values intermediate between those of spastin-null and control larvae. QC in these transheterozygotes was also decreased significantly, to 78% of WCS levels (Figure 5E; p < 0.002). The changes in EJP amplitude and QC observed in spastin5.75 mutants were completely rescued by spin-GAL4-driven Spastin expression. Average QC in rescued larvae was not significantly different from wild-type (p > 0.1), and was 30% greater than in spastin5.75 mutants (p < 0.005; Figure 5E).

We also observed that the electrophysiological phenotypes of the hypomorphic spastin17-7/spastin5.75 larvae were temperature sensitive. While the average QC in transheterozygotes was reduced to 78% of controls at 18°C, this effect was exacerbated at higher temperatures. At 29°C, QC was 54% of wild-type (Figure 5F). However, QC in control or in spastin5.75 null larvae was unaffected by temperature. These results suggest that the N-terminally truncated Spastin protein that is probably made from the spastin17-7 allele is temperature sensitive. At 29°C, this hypomorphic allele behaves as a null with respect to NMJ electrophysiology. Finally, we examined escaper larvae (Elav-GAL4UAS-FGRV; T32/+; R804) overexpressing Spastin in neurons, and found that synaptic transmission in these larvae was not significantly different from wild-type (data not shown).

spastin Mutant Adults Have Severe Behavioral Phenotypes

We observed that only approximately 20% of homozygous spastin5.75 pupae were able to eclose at room temperature compared to 94% of heterozygotes, and the adults that emerged had severe movement defects (see Video S1). They could not fly at all, and did not even appear to move their wings, although the wings inflated and straightened in a normal manner immediately after eclosion. They also did not jump spontaneously, but would jump if persistently prodded in the abdomen. Their legs were weak; when standing, the metathoracic legs often slipped out from underneath them, and during walking they often dragged these legs (see Video S1). They also had difficulty holding on to surfaces when they were upside down. These phenotypes were temperature dependent. Null mutant flies that developed at 18°C eclosed at much higher rates (56%) than at higher temperatures and moved more normally. Flies homozygous for the hypomorphic mutations, spastin10-12 and spastin17-7, eclosed at normal frequencies at all temperatures.

To evaluate these movement defects, we assayed flight and climbing ability in spastin-null and hypomorph flies (Figure 6). The flight assay could only be used for hypomorphs since null mutants were flightless. In this assay, flies were released into the top of a vertical cylinder that had been coated on the inside with oil (Benzer 1973; Atkinson et al. 2000). Poor fliers who took longer to fly fell to the bottom or collided with the lower walls of the cylinder, while good fliers who responded rapidly to being dropped collided with the upper walls. A histogram of the distribution of oil-trapped flies along the height of the cylinder showed that more than half of the spastin hypomorphs did not fly in time to avoid falling to the bottom of the cylinder (Figure 6A). In contrast, the majority (approximately 75%) of the controls, including w1118 (another Canton S-derived w− control) flies and flies homozygous for T32 (in the absence of a GAL4 driver; these have orange eyes), flew well enough to distribute themselves along the sides of the column. Interestingly, for those hypomorphs that did fly out to the sides, their distribution paralleled that of the controls, suggesting that flight responses in the column were relatively normal in this subpopulation of the mutants (Figure 6B).

In the climbing assay, flies were tested for their ability to climb up the side of a vial within a limited time period. All WCS and almost all homozygous spastin10-12 and spastin17-7 flies, but only about 40% of spastin5.75 flies, climbed to the top of the vial within 30 s (Figure 6C). This difference did not reflect a loss of geotactic behavior, since spastin5.75 flies were typically found at the tops of their vials after several minutes. Overall, mean climbing velocity was approximately 9-fold slower for spastin-null mutants than for wild-type flies, while the hypomorphs were about 2-fold slower than controls (Figure 6D).

We also measured the lifespan of the flies. Under our conditions, WCS flies lived an average of 46 d at 25°C.
spastin$^{10-12}$ and spastin$^{17-2}$ flies had somewhat shorter lifetimes, surviving an average of 35 d. Lifespan was dramatically reduced in spastin$^{5.75}$ flies, which lived an average of only 8 d (Figure 6E and 6F). Examination of mortality curves (Figure 6E), however, revealed that, as in the case of flight ability in the hypomorphs, these flies had a bimodal lifespan distribution. Only 55% of flies were still alive at 4 d post-eclosion, but most of these (37% of the total) then remained alive until about 25 d post-eclosion. After this time, they rapidly died off, and no flies remained alive more than 33 d.

Another spastin phenotype observed in adults was male-specific lethality (Figure 6G). For WCS and spastin hypomorphs, more than 40% of eclosed adults were males. However, only approximately 10% of eclosed spastin$^{5.75}$ flies were male. We do not understand the origins of this phenotype.

In summary, spastin-null adult flies had severely compromised movement behavior and were short-lived, while spastin hypomorphs displayed weaker movement and lifespan phenotypes. We also examined rescue for these behavioral phenotypes. When compared to their non-rescued siblings (spin-GAL4/CyOKr-GFP; spastin$^{5.75}$) from the same cross, spin-GAL4/UAS-spastin; spastin$^{5.75}$ flies climbed better, were more coordinated, and lived longer (Figure S2; Video S1), indicating that partial rescue was achieved. These flies were still very slow, and it is clear that spin-GAL4-driven Spastin expression did not restore behavior to the levels characteristic of control flies such as WCS. However, genetic background effects made the precise efficacy of rescue achieved in this experiment difficult to determine (the non-rescued spastin$^{5.75}$ sibling flies bearing the driver and balancer chromosomes used in the rescue cross were much more unhealthy and slow-moving than spastin$^{5.75}$ flies without these chromosomes).

Spastin Overexpression Erases Microtubule Networks In Vivo

To investigate whether Drosophila Spastin affects microtubule networks, we overexpressed it in embryonic muscles using the G14-GAL4 or 24B-GAL4 drivers, and then visualized muscle microtubules in late stage 16 embryos with an anti-β3-
tubulin antibody that preferentially stains polymerized tubulin (Buttgereit et al. 1996). In wild-type embryos, a complex network of microtubules aligned along the muscle axes was observed (Figure 7A; two segments are shown). This was clearly seen in both vertically oriented (18 and 21–24) and diagonally oriented (5,11, and 19) muscles.

When Spastin was overexpressed in muscles, the muscle microtubule network completely disappeared (Figure 7B). The muscles themselves appeared rounded, and were partially or totally detached from their insertion sites. This detachment may have been a consequence of the dissolution of the microtubule network. Oriented microtubule networks could still be seen within the cap cells of the chordotonal organs in each segment (Figure 7B, brackets); these cells did not express the GAL4 driver and therefore did not overexpress Spastin. Dissolution of the microtubule network was therefore specific to cells in which Spastin was overexpressed.

We also overexpressed Spastin in larval muscles by crossing the muscle-specific MHC-GS-GAL4 driver line to UAS-spastin flies. MHC-GS-GAL4 is RU486-inducible, but we could not feed the larvae with RU486 as this was lethal. However, MHC-GS-GAL4 also confers late RU486-independent expression in third instar larvae, and we were able to obtain escaper larvae from the cross and double-stain these for Spastin and α-tubulin. As shown in Figure 7C–7E, larvae that lacked detectable Spastin expression had a dense network of muscle microtubules. In contrast, MHC-GS-GAL4/UAS-spastin larvae that had high levels of muscle Spastin displayed a dramatic reduction in microtubules, so that only faint and sparse microtubules were observed in the muscle fibers (Figure 7F–7H). The strongly staining microtubules still visible in these larvae are those of the neurons and tracheae, which do not overexpress Spastin (Figure 7G–7H). These results also show that this α-tubulin antibody preferentially recognizes polymerized tubulin, since the total amount of tubulin dimers would be the same in both sets of muscles (tubulin dimers are very stable proteins and are unlikely to be proteolyzed after severing).

Interestingly, when we overexpressed Spastin in the embryonic or larval CNS, we did not observe an obvious alteration of the axonal microtubule architecture (see also Figure 8). This suggests that Spastin may be unable to disassemble stable axonal microtubule bundles. Nevertheless, the dramatic effects of Spastin overexpression on muscle microtubules suggest that the embryonic CNS collapse phenotype conferred by neuronal overexpression (see Figure 3) may also arise from breakdown of key neuronal microtubules during the axonal growth phase.

Microtubule Bundles Are Depleted in Distal NMJ Boutons of spastin LOF Mutants

The finding that Spastin overexpression erases the microtubule network in muscles suggested that the spastin LOF NMJ phenotypes could arise from alterations in microtubule networks. To investigate this, we first examined the distribution of Futsch, a microtubule-associated protein related to vertebrate MAP1B (Hummel et al. 2000; Roos et al. 2000). Futsch staining is restricted to stable neuronal microtubule bundles. Because Futsch is not expressed in the underlying muscle, Futsch antibody staining provides the optimal method for quantitatively evaluating stable microtubules within NMJ boutons.

At muscle 4 NMJs in wild-type larvae (Figure 8A) we observed continuous microtubule bundles stained by anti-Futsch (mAb 22C10; green) within axons and along the axis of each branch of the NMJ (delineated by anti-HRP, which labels neuronal membranes; magenta). The intensity of Futsch staining weakens in the distal portions of the branches. Consistent with earlier findings, we also observed distinctive “loops” of Futsch staining within some boutons (Roos et al. 2000; Packard et al. 2002; Pennetta et al. 2002). Loops were typically observed in terminal boutons at the ends of branches. In some terminal boutons, however, we detected only punctate staining or no staining at all. This last case may reflect the limits of detection rather than the complete absence of Futsch protein in a bouton.

We quantified Futsch distribution by dividing the patterns of Futsch staining in boutons into three classes: continuous (bundles or splayed bundles), looped, and diffuse or undetectable. In spastin5.75 larvae (Figure 8B), there was a shift in the Futsch pattern toward less organized morphologies (i.e., diffuse/undetectable). At the muscle 4 NMJ of spastin mutant larvae, 74% and 54% as many boutons contained continuous and looped Futsch, respectively, as compared to WCS. In contrast, 63% more boutons in spastin mutants displayed only diffuse or no staining (Figure 8D). These differences were most pronounced at the distal ends of the synaptic branches (Figure 8B, arrows and arrowheads). 65% of terminal boutons in mutants had no detectable Futsch staining, as compared to 8% in WCS (Figure 8E; $p < 2 \times 10^{-5}$).

The Futsch distribution phenotypes were rescued by expression of Futsch from the spin-GAL4 or the RU486-induced, neural-specific Elav-GS-GAL4 drivers. Rescued larvae had Futsch staining patterns very similar to those seen in WCS (Figure 8C–8E). These results show that the reduction in stable synaptic microtubules seen in spastin LOF mutants is due to loss of Spastin from neurons during larval development. We also examined Futsch staining in Elav-GS-GAL4/UAS-spastin larvae grown on RU486 food, but saw no difference from the pattern in wild-type controls. Thus, stable microtubules at the NMJ do not break down when Spastin is overexpressed at the levels induced by this driver.

Having demonstrated statistically significant differences in Futsch localization between control and spastin mutant NMJs, we then directly examined tubulin in muscle 4 NMJ boutons using fixation conditions that reduce muscle microtubule stability (see Materials and Methods). The pattern of NMJ microtubules is complex, and is difficult to quantitatively analyze because of residual signal from microtubules in the underlying muscle. However, using the α-tubulin antibody described above, we were able to clearly visualize looping microtubule structures (Figure 9A, green) within anti-HRP-labeled boutons (magenta). These loops were present both along the branches and in the terminal boutons (inset). spastin5.75 NMJs exhibited weaker α-tubulin staining than wild-type controls, particularly in terminal boutons (Figure 9B). Looped microtubule structures could be seen in some boutons in mutants. However, boutons at the ends of NMJ branches or at the outer edges of the bouton clumps that are characteristic of spastin NMJs often lacked any tubulin staining (inset). Thus, our results indicate that microtubule bundles are selectively depleted from the distal boutons of NMJs in larvae lacking Spastin protein.
Figure 7. Spastin Overexpression in Muscles Erases the Microtubule Network

(A) An antibody against β3-tubulin stains body wall muscles and chordotonal cap cells in stage 16 wild-type embryos. Two abdominal hemisegments are shown; muscle fiber numbers are labeled in one. The cap cells (brackets) are difficult to distinguish in this panel because of high levels of muscle tubulin staining. They extend diagonally from about the middle of muscle 18 to muscle 22. Anterior is to the left, and dorsal is up.

(B) When Spastin is overexpressed in muscles (genotype: G14-GAL4/þ; T32/þ), β3-tubulin staining is very weak and has a disorganized pattern in
most muscle fibers, but an intact microtubule network is still present in the cap cells, which do not express this driver (brackets). The muscle fibers are misshapen and partially (arrowhead) or completely (arrow) detached from their insertion sites.

(C–H) Similarly, the microtubule network (recognized by antibodies to β-tubulin) is almost eliminated by high-level Spastin expression in third instar larval muscles. Larvae of genotype UAS-spastinMHC-GS-GAL4213-3; spastin5.75/TM3Ser-ActGFP overexpress Spastin protein specifically in muscles to varying degrees. Wild-type larval muscles had undetectable levels of Spastin using pAb 1239 (C) and displayed a dense network of microtubule bundles in the muscle (D and E), as well as in trachea (D, arrow) and neurons (D, arrowhead denotes a terminal arbor). In contrast, larval muscles expressing high levels of Spastin (F) show only faint muscle microtubule staining (G and H), while tracheal (G, arrow) and neuronal (G, arrowhead) staining remain robust.

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Discussion

Mutations in the human spastin gene, which encodes an AAA ATPase, are the most common cause of pure AD-HSP. We identified the Drosophila Spastin ortholog (see Figure 1) in a gain-of-function screen (see Figure 3). Drosophila Spastin is a cytoplasmic protein that can also localize to axons (see Figure 2). spastin-null larvae have altered NMJs in which presynaptic boutons are more numerous and smaller than in wild-type, and are organized in dense clusters (see Figure 4). These changes in bouton number and organization are rescued by expression of Spastin in neurons (see Figures 4 and S1). QC, a measure of the number of vesicles of neurotransmitter released in response to an action potential, is reduced at NMJs in both null and hypomorphic spastin mutants (see Figure 5).

spastin-null flies have severe movement defects. They cannot fly at all, and do not jump. They climb and walk very slowly, often drag their hind legs when walking (see Video S1), and have greatly reduced lifespans. spastin hypomorphs have milder phenotypes, displaying flying defects and a decrease in climbing speed (see Figure 6).

Regulation of Synaptic Microtubule Networks by Spastin

The AAA domain of Spastin is quite similar to that of Katanin-60, which is a microtubule-severing protein. To determine whether Spastin might also sever or otherwise alter microtubules in vivo, we overexpressed the protein in embryonic and larval muscles. Strikingly, this overexpression erases or greatly reduces the microtubule network (see Figure 7). These data are consistent with the finding that overexpression of human Spastin in transfected mammalian cells causes microtubule disassembly (Errico et al. 2002; McDermott et al. 2003).

Having demonstrated that Spastin can cause disassembly of microtubules in vivo, we then examined how its absence affects the synaptic microtubule cytoskeleton. Based on the overexpression phenotype, one might have expected that microtubules would be more stable or more numerous in spastin LOF mutants. However, our observations indicate the opposite: microtubule bundles are depleted in NMJ boutons when Spastin is absent.

At the wild-type muscle 4 NMJ, boutons are arranged along linear axes. Continuous microtubule bundles run along the axes and connect to larger bundles within the innervating axon. Microtubules within boutons are typically arranged in loops and swirls. In spastin-null mutants, boutons are arranged in clumps, and the distal boutons of these clumps often lack any detectable tubulin staining (see Figure 9). Looped microtubule structures are present within some proximal boutons, however, and the bundles connecting the NMJ to the axon are still present. These results suggest that the absence of Spastin selectively affects the construction of the presynaptic microtubule cytoskeleton, and that the severity of the microtubule defects in a bouton are correlated with its distance from the NMJ’s axonal branchpoint.

We quantitated these defects using an antibody against the microtubule-associated Futsch protein, which defines a subpopulation of stable neuronal microtubule bundles. In wild-type larvae, Futsch staining forms continuous lines along the main branches of the NMJ. Some individual boutons have Futsch loops, while others display only diffuse staining. A comparison of wild-type and spastin-null larvae shows that the distribution of Futsch within boutons shifts from organized structures (bundles and loops) toward diffuse patterns or the absence of detectable staining. This effect is most pronounced at terminal boutons, and is rescued by neuronal expression of Spastin (see Figure 8).

If Spastin’s function in vivo is to disassemble microtubules, as suggested by our overexpression experiments (see Figure 7), why does its absence produce a paradoxical reduction in microtubules within the NMJ (see Figures 8 and 9)? One possibility is that microtubule severing is required for movement of microtubules into or within the presynaptic region. Some evidence for this idea has been published. In one study, injection of function-blocking anti-Katanin-60 antibody into cultured sympathetic neurons reduced process outgrowth, and microtubules were 4- to 5-fold longer in antibody-injected neurons than in control cells (Ahmad et al. 1999). More recent work demonstrated that expression of dominant-negative Katanin-60 reduces axonal outgrowth (Karabay et al. 2004). These results were interpreted as indicating that Katanin is required for severing microtubules to a length that allows their transport along the axon to its growing tip. When Katanin is inhibited, microtubule segments may be too long to be efficiently transported, and this results in a reduction in axon outgrowth.

Based on these findings, we suggest that the depletion of microtubules in the distal boutons of spastin mutant NMJs arises because severing of axonal microtubules by Spastin is necessary to generate microtubule polymers that are short enough to be efficiently moved into and through the presynaptic terminals. Perhaps Spastin normally excises sections of microtubules at branchpoints where NMJ branches leave the axon trunk, and these severed microtubule segments (or individual tubulin dimers) are then moved distally into the boutons of the NMJ as it grows.

Is Spastin also involved in axon outgrowth or guidance, as suggested by its embryonic gain-of-function phenotype (see Figure 3)? Clearly loss of Spastin activity in Drosophila does not strongly affect outgrowth, since the embryonic CNS axon ladder develops in a normal manner and motor axons reach their appropriate targets in spastin mutants. Furthermore, axonal and muscle microtubules are not detectably altered in spastin-null embryos. Severing of microtubules in vivo,
Figure 8. The Distribution of Stable NMJ Microtubule Bundles Marked by the MAP1B-like Protein Futsch Is Altered in spastin Mutant Larvae
(A–C) Anti-Futsch labels stable microtubule bundles in axons, NMJ boutons, and interbouton regions. The muscle 4 NMJs in segment A3 of third instar wild-type (A), spastin\(^{5.75}\) (B), and spin-GAL4/UAS-spastin; spastin\(^{5.75}\) (Rescue) (C) larvae were immunostained with anti-HRP (A and B) or anti-Syt antibodies (C) to label presynaptic boutons (magenta), and mAb 22C10 to label Futsch protein (A–C, green). Arrows and arrowheads mark the terminal boutons (those at the ends of synaptic branches); boutons marked by arrows in (A–C) are enlarged in insets in the middle and right panels to show examples of Futsch patterns. (A) In control (WCS) larvae, terminal boutons have both looped (arrowheads) and diffuse, punctate (right panel, arrows and inset) patterns of Futsch staining. (B) In spastin mutants, Futsch staining appears similarly strong in axon bundles (not shown) and along the main branches of the bouton arbor. More distal and terminal boutons, however, have diffuse or no Futsch staining (arrows and arrowheads). Note the absence of green staining in insets. (C) The distribution of Futsch staining is restored to the control pattern by spin-GAL4-driven expression of Spastin in the mutant background (arrows and arrowheads indicate loops). Scale bar, 5 \(\mu\)m.

(D and E) Quantitative assessment of Futsch staining data. Futsch staining at A2 and A3 muscle 4 NMJs was classified as continuous (bundles or splayed bundles), looped, or diffuse or undetectable (none) for each bouton. (D) The percentage of boutons exhibiting continuous or looped Futsch staining (relative to the total number of boutons for each NMJ) is decreased in spastin mutants relative to controls, while the percentage of boutons having diffuse or no staining is increased. In total, 58\% ± 4.2\% of boutons in controls have a continuous pattern of Futsch staining, while only 42\% ± 1.5\% do in mutants. Boutons in this class are predominantly along the major (more proximal) branches of the axon arbor. Similarly, 11\% ± 1.7\% of wild-type boutons have Futsch loops, but only 6.0\% ± 1.1\% do in mutants. Most mutant boutons show only diffuse or no Futsch staining (32\% ± 2.2\%, versus 32\% ± 3.2\% in controls). Futsch distribution is restored to the control pattern by spin-GAL4- or Elav-GS-GAL4-driven expression of Spastin. (E) The difference in Futsch distribution is most pronounced at terminal boutons. There is no detectable Futsch staining in the majority of terminal boutons (65\% ± 4.5\%) in spastin mutants, compared to only 7.8\% ± 5.8\% of terminal boutons in wild-type larvae (\(p < 2 \times 10^{-6}\)). Futsch staining is restored in most terminal boutons of spin-GAL4- or Elav-GS-GAL4-rescued larvae, with only 20\% ± 3.7\% and 19\% ± 5.9\% of boutons, respectively, showing no staining (\(p = 0.09\) compared to WCS). Terminal bouton staining in larvae overexpressing Spastin in neurons was unaffected relative to controls (\(p = 0.12\)). **, \(p < 0.005\); *, \(p < 0.03\) relative to WCS; \(n > 8\) NMJs scored in all cases.

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Figure 9. The Microtubule Network in NMJ Boutons Is Altered or Absent in spastin Mutant Larvae
(A) In wild-type (WCS) larvae, an antibody against \(\alpha\)-tubulin (green) reveals the distribution of the network of microtubule bundles within the A3 muscle 4 NMJ bouton arbor. Presynaptic bouton membranes are labeled by anti-HRP antibody (magenta), and mAb 22C10 to label Futsch protein (A–C, green). The microtubule network has a complex structure and extends into the terminal boutons (arrowheads and inset). Many proximal boutons have loops (arrows). Microtubules are also observed outside of the boundaries of the NMJ; these are within the muscle fiber, which also expresses \(\alpha\)-tubulin. Staining of these muscle microtubules is minimized by the use of Bouin’s fix.

(B) In spastin\(^{5.75}\) mutants, the microtubule network is much sparser than in controls, particularly in the distal boutons at the edges of the bouton clumps that are characteristic of spastin mutant NMJs (arrowheads and inset). Many of these distal boutons have little or no detectable \(\alpha\)-tubulin staining. More proximal boutons still have tubulin loops, however (arrows).

Scale bar, 5 \(\mu\)m.

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Implications of Studies of Drosophila Spastin for the Understanding of Human AD-HSP

spastin-null adult flies have severe movement defects. Their hind legs are particularly weak (see Video S1); this is interesting in light of the restriction of symptoms to the legs in most AD-HSP patients. Other aspects of the spastin mutant adult phenotypes also resemble observations made in human AD-HSP patients. The penetrance of the human spasticity phenotype is highly variable, so that some individuals carrying a spastin mutation appear unaffected, while others with the same mutation are confined to wheelchairs. In our experiments, we observed that some spastin hypomorphs exhibit normal flying behavior in a cylinder assay, while most fail to fly and crash into the cylinder base (see Figure 6A and 6B). Half of the spastin-null adults die within 4 d, but most of the survivors then live more than 25 d (see Figure 6E). The selective male lethality we observed (see Figure 6G) is also interesting in light of the discovery of a large SGP4 pedigree in which only males exhibit AD-HSP phenotypes (Starling et al. 2002).

Despite these apparent parallels, there is no evidence at present that the fly behavioral phenotypes arise through mechanisms related to those that cause human AD-HSP. The anatomy of the Drosophila nervous system is quite different from that of the mammalian spinal cord. Furthermore, AD-HSP is thought to be a neurodegenerative disease that progresses over a period of years, and it is unclear whether neurodegeneration as a result of spastin mutations could occur during the short lifespan of Drosophila. Further work will be required to determine to what extent the Drosophila system can provide an organismal model for AD-HSP pathology.

The clearest implications of our work for AD-HSP emerge from the analysis of the cellular phenotypes arising from loss of Spastin. We show that the absence of Spastin alters the microtubule network at nerve terminals. Microtubule bundles are depleted in the distal boutons of the NMJ, which is a glutamatergic synapse that resembles excitatory synapses within the mammalian spinal cord. These results suggest that microtubules within the terminals of neurons in the human spinal cord could also be disordered or absent in patients with AD-HSP. The terminals of these neurons might eventually degenerate as a consequence of these microtubule defects, leading to a selective loss of distal axon segments within the spinal cord.

Materials and Methods

Genetics and molecular biology. For the overexpression screen, approximately 6,000 new EP insertion lines were generated by crossing an X chromosome EP line, EP55, to a Sh2-3 transposase line. EP insertions on Chromosome II or III were crossed to the pan-neuronal driver line Elav-GAL4 and the muscle driver line 24B-GAL4. Embryos from EP lines that exhibited less than 20% viability in combination with either driver were immunostained with mAb 1D4. Eighteen lines had embryonic CNS and/or motor axon defects when crossed to Elav-GAL4, including line T32. The flanking genomic region of T32 was cloned and sequenced by plasmid rescue. This sequence matched three overlapping EST’s from BDGP, one of which, CH11184, contained the complete ORF of the gene downstream of the T32 insertion. This cDNA was sequenced in its entirety. Unrooted trees for Spastin and its closest relatives were constructed using six different algorithms (Bitch, Kitsch, neighbor, upgma, protein maximum likelihood, and parsimony) from the Phylip package, based on alignment to the PFAM AAA consensus. For the spastin excision lines, alleles 10-12, 17-7, and 5.75 were
generated via imprecise excision of EP T32 using Sh-A2-3. All alleles were homozygous viable, and their deletions were mapped by PCR and sequencing of larval or adult genomic DNA. Allele 5.75 causes sterility in both sexes.

For the spastin rescue construct, the UAS-spastin cDNA construct was made by cloning a 2.9-kb BglII fragment from GH11184 into the BglII site of pUAST downstream from the CSW promoter. The resulting construct contains the spastin cDNA up to 350 bp after the stop codon (excluding 681 bp of the 3′ UTR) and including 28 bp of polylinker sequence from the po2T plasmid at the 5′ end. The construct was injected at approximately 300 ng into Kc127 embryos and several transgenic lines recovered; experiments described here used the Chromosome II insertion line 8-3-5.

Rescue of spastin-null phenotypes by spin-GAL4-driven expression was assayed by crossing UAS-spastin/CyOKr-GFP, spastin T32/TM3SerAct-GFP to spin-GAL4; CyOKr-GFP, spastin T32/TM3SerAct-GFP. The numbers of Ibboutons in rescued larvae (UAS-spastin; CyOKr-GFP; Elav-CyOKr-GFP, spastin T32/TM3SerAct-GFP) were compared to those in unrescued sibling mutants (spastin/CyOKr-GFP; spastin T32/TM3SerAct-GFP) to calculate the ratios used to determine the efficacy of rescue (see Figure 4G). This was done because we observed that the presence of the driver chromosone in the background increased the absolute number of Ib boutons, so that the appropriate control was to the sibling mutants also bearing this chromosome. Rescue of spastin-null phenotypes by postembryonic Elav-GS-GAL4-driven expression was assayed by crossing UAS-spastin/CyOKr-GFP, spastin T32/TM3SerAct-GFP to Elav-GS-GAL4; CyOKr-GFP, spastin T32/TM3SerAct-GFP and raising the larvae on RU486-containing food as described (Osterwalder et al. 2001; McGuire et al. 2004). The numbers of Ib boutons in rescued larvae (UAS-spastin+/++; Elav-GS-GAL4, spastin T32+/++; spastin T32−/−) were compared to those in unrescued sibling mutants (CyOKr-GFP++; Elav-GS-GAL4, spastin T32+/++; spastin T32−/−) as described above for UAS-driven expression of Spastin in a wild-type background was assayed by counting Ib bouton numbers in UAS-spastin++; Elav-GS-GAL4, spastin T32+/++; spastin T32−/−. The wild-type control lines were used Oregon R spastin+/+ and CSW, a Canton S line backcrossed ten times to white (gift of Anne Simons). For larval overexpression experiments, the MHC-GS-GAL4 driver was used to induce muscle-specific expression of UAS-spastin at 23°C in the absence of RU486. Expression induced by this driver was confirmed in a separate cross using UAS-EGFP.

Generation of Spastin antibodies. Regions of the spastin cDNA encoding aa 136–416 (pGEX-T32PvuII), 1–167 (pAcG2T-T32BamRL), and 380–708 (pAcG2T-T32BBA) were subcloned from GH11184 into bacterial (pGEX) or baculovirus (pAcG2T) expression vectors. Expressed protein (in the form of inclusion bodies for T32PvuII) was injected into guinea pigs (Covance, Princeton, New Jersey, United States) and the antisera tested on en-GAL4; T2/+ embryos. Anti-sera against T32PvuII (865E) and T32BBA (1297) both showed strong staining in the Engrailed pattern; thus, both were recognized over the range of splice variants. 96E5 was induced by incubation with membrane-bound pGEX-T32PvuII protein and subsequent elution with 100 mM glycine (pH 2.5) followed by neutralization with 3M Tris (pH 8.8). pAb1239 was affinity purified using the immunogen bound with 100 mM glycine (pH 8.8). pAb1239 was affinity purified using the immunogen bound with 100 mM glycine (pH 8.8). pAb1239 was affinity purified using the immunogen bound with 100 mM glycine (pH 8.8). pAb1239 was affinity purified using the immunogen bound with 100 mM glycine (pH 8.8). pAb1239 was affinity purified using the immunogen bound with 100 mM glycine (pH 8.8). pAb1239 was affinity purified using the immunogen bound with 100 mM glycine (pH 8.8).
show that they are reduced in number (83% of control; see text). Scale bar, 5 μm.

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**Figure S2.** Adult Behavior Is Partially Rescued by spin-GAL4-Driven Expression of Spastin in the spinster-Null Background

Behavioral tests were performed on flies from the four genotypes arising from the spin-GAL4 rescue crosses, raised at 18 °C. These genotypes were (1) spin-GAL4/UAS-spastin; spinster (spin Rescue), (2) spin-GAL4(CyO:OK); spinster (non-rescued spinster mutant, denoted 5.75[R]), (3) spin-GAL4/UAS-spinster; TM3SerAct-GFP (CyCtrl; heterozygous for the spinster mutation), and spin-GAL4(CyO:OK); spinster; TM3SerAct-GFP (CyCtrl; heterozygous for the spinster mutation).

(A) Climbing behavior. None of the spinster mutants (0%) from these crosses (5.75[R]: n = 21) reached the top of the vial in the prescribed 30 s time limit, compared to 8% for Rescue flies (n = 75), and 100% for both spinster/+ controls (n = 39 and 21). Twenty-seven percent of mutant, D. Pokhil, A. Renkawitz-Pohl (1996) Muscle development and attachment to the epidermis is accompanied by expression of beta 3 and beta 1 tubulin isoforms, Int J Dev Biol 40: 189–196


obvious, particularly for the mesothoracic and metathoracic legs, both when walking and standing still. She climbs poorly, and when rotated so that she is upside-down, is unable to maintain a hanging position. No wing movement or jumping is observed.

Segment 3: Rescue. In spin-GAL4/UAS-spastin; spinster flies, Spastin expression via the spin-GAL4 driver partially rescues the movement defects seen in spinster mutants. Two males are shown, followed by one female. Note their improved leg steadiness, velocity, and hanging ability. These flies can also jump spontaneously. The female appears to be less fully rescued; however, she is able to walk upside-down for prolonged periods, and exhibits wing movement.

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**Conflicts of interest.** The authors have declared that no conflicts of interest exist.

**Author contributions.** NTS, Q.S., and KZ conceived and designed the experiments. NTS, Q.S., and MX analyzed the data. BZ oversaw the electrophysiology experiments and analysis. NTS and KZ wrote the paper.


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