Cold temperature improves mobility and survival in Drosophila models of autosomal-dominant hereditary spastic paraplegia (AD-HSP)

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ABSTRACT
Autosomal-dominant hereditary spastic paraplegia (AD-HSP) is a crippling neurodegenerative disease for which effective treatment or cure remains unknown. Victims experience progressive mobility loss due to degeneration of the longest axons in the spinal cord. Over half of AD-HSP cases arise from loss-of-function mutations in spastin, which encodes a microtubule-severing AAA ATPase. In Drosophila models of AD-HSP, larvae lacking Spastin exhibit abnormal motor neuron morphology and function, and most die as pupae. Adult survivors display impaired mobility, reminiscent of the human disease. Here, we show that rearing pupae or adults at reduced temperature (18°C), compared with the standard temperature of 24°C, improves the survival and mobility of adult spastin mutants but leaves wild-type flies unaffected. Flies expressing human spastin with pathogenic mutations are similarly rescued. Additionally, larval cooling partially rescues the larval synaptic phenotype. Cooling thus alleviates known spastin phenotypes for each developmental stage at which it is administered and, notably, is effective even in mature adults. We find further that cold treatment rescues larval synaptic defects in flies with mutations in Flower (a protein with no known relation to Spastin) and mobility defects in flies lacking Kat60-L1, another microtubule-severing protein enriched in the CNS. Together, these data support the hypothesis that the beneficial effects of cold extend beyond specific alleviation of Spastin dysfunction, to at least a subset of cellular and behavioral neuronal defects. Mild hypothermia, a common neuroprotective technique in clinical treatment of acute anoxia, might thus hold additional promise as a therapeutic approach for AD-HSP and, potentially, for other neurodegenerative diseases.

KEY WORDS: Spastin, Hereditary spastic paraplegia, AD-HSP, Microtubule severing, Cold treatment, Therapeutic hypothermia, Drosophila disease model

INTRODUCTION
Hereditary spastic paraplegias (HSPs) are a group of neurodegenerative disorders marked by lower-limb spasticity (stiffness) and weakness, leading to progressive difficulty walking (Fink, 2013; http://www.sp-foundation.org/). Supportive treatments exist, but are of mixed efficacy and do not restore mobility. The most common form, pure autosomal dominant HSP (AD-HSP), accounts for 70-80% of HSP-affected families. AD-HSP pathology is characterized primarily by degeneration of the longest descending axons of the central nervous system (CNS). These originate from the upper motor neurons in the cortex and terminate in the lumbar spine, innervating the α1 motor neurons that control leg movement. The relative specificity of the affected neuronal population is striking, but still not understood.

Over 50% of cases of pure AD-HSP are caused by mutations in spastin (Hazan et al., 1999), which encodes one of a small family of microtubule-severing proteins—hexameric ATPases that disassemble microtubules along their length (Roll-Mecak and McNally, 2010; Sharp and Ross, 2012). Although it is not yet clear why Spastin is important in neurons, one thought is that its severing activity shortens microtubules for efficient transport into axons (Errico et al., 2002; Yu et al., 2008). Other models suggest that Spastin is required for net microtubule loss (Trotta et al., 2004) or growth (Sherwood et al., 2004) at motor neuron synapses, axon guidance (Wood et al., 2006; Butler et al., 2010) and axon transport (Kasher et al., 2009; Fassier et al., 2013). Most recently, data has emerged supporting roles for Spastin in membrane regulation, promoting tubular endoplasmic reticulum (Park et al., 2010) and endosomal tubule formation (Allison et al., 2013). Much progress has been made, but Spastin’s relevant functions, regulatory pathways and the specific mechanisms by which its mutations lead to axonal degeneration remain unclear.

Drosophila melanogaster is an effective model system for study of a wide variety of neurodegenerative diseases because of its high conservation of neuronal gene function with humans, short generation time, well-characterized features and the availability of a wide range of genetic and experimental tools (Bilen and Bonini, 2005). In regards to providing a model for AD-HSP, Drosophila Spastin, like its vertebrate orthologs, severs purified microtubules and those in Drosophila S2 cells (Roll-Mecak and Vale, 2005). Knocking down fly Spastin using a RNA-interference (RNAi) transgene (Trotta et al., 2004) or deletion of the endogenous gene (Sherwood et al., 2004) both cause synaptic defects at the Drosophila larval neuromuscular junction (NMJ), supporting a role for spastin in regulating synaptic morphology and function. Orthologs of several other HSP causative genes studied in Drosophila also exhibit progressive neurodegeneration, supporting the relevance of flies in providing insights into mechanisms underlying this disease (Wang and O’Kane, 2008; Ozdowski et al., 2014).

The spastin gene is completely deleted in the spastin null Drosophila model of AD-HSP (Sherwood et al., 2004). Spastin larvae are homozygous-viable and have no obvious behavioral defects, but electrophysiological analysis at the NMJ reveals that...
TRANSLATIONAL IMPACT

Clinical issue
Autosomal dominant hereditary spastic paraplegia, or AD-HSP, is an inherited neurodegenerative disease that manifests as early as toddlerhood, causing progressive loss of mobility. The primary symptoms, leg spasticity and weakness, arise from localized degeneration of the longest central nervous system axons. The identification of several causative genes has made the study of AD-HSP using model systems eminently feasible, and the fruit fly Drosophila has been used extensively to explore the underlying pathology. Spastin, the gene most commonly mutated in AD-HSP, encodes a member of the microtubule-severing protein family in both humans and flies, and flies that lack spastin also exhibit compromised mobility. Despite significant progress since its first report, a clear understanding of the role of Spastin in the nervous system is lacking, and there are still no reliable therapies for AD-HSP. This study explores a fortuitous observation that cold temperatures alleviate symptoms in Drosophila models of spastin-mediated AD-HSP, in order to investigate whether cooling could provide a therapeutic approach to this disease in humans.

Results
The authors test their hypothesis that cold treatment mitigates behavioral and cellular spastin mutant defects by examining eclosion (emergence of adult flies from the pupal case), mobility, lifespan and synapse morphology in mutant and wild-type flies reared in cold conditions during discrete developmental periods. They find that in spastin null animals, as well as in mutant flies that model AD-HSP through the expression of pathogenic human spastin, cooling alleviates the reduced lifespan, slowed mobility and aberrant synapse morphology caused by spastin loss. Cold-induced alleviation of phenotypes was most effective during the developmental periods, when spastin is required, consistent with the effects being mediated through a mechanism linked with spastin loss. The effects were not seen in wild-type animals, demonstrating specific mitigation of mutant phenotypes. The authors further show that cooling alleviates neuronal defects due to mutations in a related microtubule-severing protein, Kat-60L1, and in Flower, which regulates synaptic vesicle endocytosis. These results provide evidence that moderate hypothermia could be broadly effective in alleviating neurodegenerative phenotypes.

Implications and future directions
Therapeutic hypothermia is commonly employed in clinical settings to prevent anoxia-induced neurological damage following stroke and cardiac arrest, although the mechanisms for its efficacy remain unknown. This work implicates cooling as a novel therapeutic approach for neuronal dysfunction in AD-HSP, and potentially in other neurodegenerative diseases. Future studies should address the effects of localized cold treatment in vertebrate models of AD-HSP, and utilize the Drosophila model system to systematically investigate the underlying molecular mechanisms and spatiotemporal requirements for the neuroprotective effects of hypothermia. The work also highlights the finding that neuronal phenotypes are potentially very sensitive to variations in temperature, which should be taken into account when designing studies of neurodegenerative phenotypes in model systems.

RESULTS
Pupal stage cold treatment improves spastin mutant eclosion
Most Drosophila lacking spastin survive into metamorphosis but fail to emerge from the pupal case. However, spastin5.75 nulls reared at 18°C rather than 24°C were considerably more successful at reaching adulthood. We quantified this cold-induced alleviation of pupal lethality and tested for a developmental period(s) during which it is effective. Cooling could be required throughout development, or alternatively, be necessary only at a particular stage. The former would support a broad effect, such as general metabolism or developmental rate. The latter, if coinciding with the temporal requirements for Spastin, would support a mechanism relevant to the loss of Spastin function in the nervous system.

White-CantonS (WCS) controls and spastin5.75 mutant Drosophila were raised in parallel at 24°C ("untreated") or moved to 18°C during either their larval or pupal stage of development, and differences in eclosion rates determined for each condition (Fig. 1A,B). Compared with the 86% eclosion rate of WCS flies, only 18% of homozygous spastin5.75 flies maintained at 24°C eclosed (Table 1). Cold treatment specifically during the larval stage resulted in slightly, but not significantly, higher mutant eclosion (Table 1; Fig. 1B). Only pupal stage cold treatment produced a significant increase to levels that, although still well below wild type, were 70% greater than those for untreated mutants (Fig. 1B). This effect was specific to the spastin mutation, as cold did not affect WCS eclosion.

Both pupal and adult cold treatment improve adult mobility
We next looked at whether cold also improves the dramatically weakened mobility of spastin mutant flies, by measuring adult
climbing rates (Table 2; Fig. 2A). All mutants that eclosed following larval stage cold treatment did not climb, dying shortly after eclosion. However, spastin5.75 flies cold-treated as pupae climbed 46% faster than mutants maintained at 24°C, and when cold-treated as adults, climbed 85% faster. By contrast, WCS climbing was unaffected by pupal and adult cold treatment, and also impaired by larval-stage treatment (Fig. 2A).

**Pupal stage cold treatment extends lifespan**

Besides compromised mobility, spastin5.75 flies are short-lived compared with wild type (Sherwood et al., 2004). Whereas WCS flies at 24°C lived 3–4 weeks on average, mutants survived only 1 week (Fig. 2B,C, Table 3). As in the climb rate experiments, larval cold treatment was deleterious to mutant fly survival. Adult-stage cold treatment extended the average lifespan 2.5-fold, but this was not different from WCS flies, which lived 2.3-fold longer when cold-treated as adults (Fig. 2B,C). However, whereas pupal cold treatment left WCS flies unaffected, spastin5.75 flies lived twice as long as untreated mutants (Fig. 2B). The survival curve for pupal cold-treated spastin5.75 flies (dark blue solid line) showed a clear right shift of the entire population relative to untreated mutant flies (red long dashed line; Fig. 2C). Notably, about 10% of treated mutants had considerably extended lifetimes, which were threefold longer than untreated mutants and comparable to the maximal lifespan of control WCS flies.

These measurements indicate that pupal development is an effective period for cold to enhance eclosion, mobility and lifespan of adult spastin5.75 mutants, although it has little effect on wild-type flies. Additionally, cooling significantly improves mobility even when applied in spastin5.75 adults, when the nervous system has already matured. Larval cooling, by contrast, exacerbates spastin mutant phenotypes in adults. Taken together, these data indicate that cooling-induced rescue of adult phenotypes arises from the effects of mild hypothermia on the adult nervous system, which is assembled de novo during pupal stages as part of metamorphosis.

**Pupal stage cold treatment improves eclosion and mobility of AD-HSP genotype flies**

We sought further evidence for the specificity of cold rescue to loss of Spastin function by performing the previous experiments in a Drosophila AD-HSP model that recapitulates spastin gene dosage and allelic severity of the human disease (Du et al., 2010). We used two fly strains designed to genocopy humans at the spastin locus. In the first, denoted HWT,HWT, flies lacking endogenous spastin (i.e. spastin5.75 flies) instead express wild-type human Spastin, encoded by two copies of a human spastin transgene, in all neurons. The second genotype, HL44,HR388, mimics the most severe form of spastin-mediated AD-HSP in humans (Svenson et al., 2004). Instead of wild-type human spastin, these flies carry one copy each of transgenes encoding the S44L or K388R human Spastin mutations, however, are severely compromised in eclosion, mobility and survival. Thus, the HL44,HR388 flies provided a genetic control group (because, like WCS, these express wild-type spastin) and the HL44,HL44 flies modeled severe AD-HSP.

As further evidence of the therapeutic effect of cold, HL44,HL44 flies cold-treated as pupae eclosed nearly twice as often as those kept at 24°C, whereas HWT,HWT eclosion was unaffected by cold (Fig. 3A; Table 4). HL44,HR388 flies also climbed 47% and 60% faster, respectively, if cold-treated as pupae or adults (Fig. 3B, Table 5), but cold treatment of HWT,HWT at these stages yielded adults that climbed only half as quickly. Although spastin transgene expression might have been reduced at 18°C, our previous studies showed that a single copy of HWT rescues the null mutants as effectively as two copies.

**Table 1. Pupal stage cold treatment significantly increases the eclosion rate of spastin5.75 flies**

| Treatment stage | WCS | spastin5.75 |
|-----------------|-----|-------------|
| Eclosion rate (%) | n | Eclosion rate (%) | n |
| Untrained | 86.3±6.3 | 10 vials, 143 larvae | 18.2±3.1 | 20 vials, 571 larvae |
| Larval | 76.7±6.1 | 3 vials, 70 larvae | 25.9±8.1 | 6 vials, 70 larvae |
| Pupal | 80.2±6.2 | 14 vials, 170 larvae | 30.8±3.7 | 26 vials, 578 larvae |

Values shown are mean ± s.e.m. Averages that differ significantly (P<0.05) from the non-cold-treated (untreated) condition of the same genotype are highlighted in bold.
Table 2. Both pupal and adult stage cold treatment increase the climb rate of spastinF5.75 flies

| Treatment stage | WCS Climb rate (cm/s) | n | spastinF5.75 Climb rate (cm/s) | n |
|-----------------|-----------------------|---|-------------------------------|---|
| Untreated Larval | 2.33±0.08             | 58 | 0.65±0.09                     | 25 |
| Pupal 2.33±0.06 | 95                    |   | 0.95±0.08                     | 85 |
| Adult 2.51±0.18 | 23                    |   | 1.20±0.19                     | 17 |

Values shown are mean ± s.e.m. Averages that differ significantly (P<0.05) from the non-cold-treated (untreated) condition of the same genotype are highlighted in bold.

Table 3. Pupal and adult stage cold treatment both increase the lifespan of spastinF5.75 flies

| Treatment stage | WCS Lifespan (days) | n | spastinF5.75 Lifespan (days) | n |
|-----------------|---------------------|---|-------------------------------|---|
| Untreated Larval | 25.0±1.6            | 29 | 7.0±1.4                      | 7  |
| Pupal 25.5±1.4  | 33                   |   | 13.9±1.9                     | 18 |
| Adult 57.0±2.1  | 13                   |   | 17.2±2.0                     | 6  |

Values shown are mean ± s.e.m. Averages that differ significantly (P<0.05) from the non-cold-treated (untreated) condition of the same genotype are highlighted in bold.

making it unlikely that temperature-based alterations in expression could account for the alleviatory effects seen here (Du et al., 2010). Overall, the parallels between these results and those for spastinF5.75 strongly support the specificity of cold alleviation to defects caused by Spastin dysfunction in the nervous system.

Cooling rescues synapse morphology defects in spastin larvae

We next looked at whether cooling rescues the cell biology of neurons affected in spastin mutants. We were unable to address this issue in adults because the cellular loci of adult defects in spastin mutants remain unknown. We thus investigated spastin defects at the larval NMJ, which are well characterized (Sherwood et al., 2004). Although previous experiments showed that synaptic transmission in mutants is reduced relative to wild type at both normal and low temperatures, we examined whether other cellular defects at this stage could be alleviated by cold. At room temperature, spastin mutants have a distinctive NMJ morphology, with greater numbers of smaller synaptic boutons, which are sometimes arrayed in grape-like ‘bunches’ and contain only sparse microtubules (Fig. 4A) (Sherwood et al., 2004; Du et al., 2010; Ozdowski et al., 2011). These bunched terminal arrangements are not observed at wild-type NMJs, which consist of large, round and linearly arrayed boutons penetrated by a clear microtubule bundle. Comparison of synaptic terminals in 18°C- versus 24°C-reared larvae showed that cooling mitigated the spastin mutant morphology (Fig. 4A1,A2), both with respect to the total number of boutons per muscle and the number of terminal boutons (a measure of synaptic arbor branching). WCS synapse morphology was unaffected. However, we did not detect a change in stable microtubule penetration into terminal boutons, measured by the 22C10 antibody against the Drosophila MAP1b ortholog, Futsch (Fig. 4A3). Larval cooling thus partially rescues the cellular defects resulting from spastin loss, mitigating bouton morphology but not stable microtubule distribution or synaptic function.

Mutants in flower and kat-60L1 are also rescued by cold treatment

We have shown that cooling elicits effects specifically on spastin mutant phenotypes in comparison with controls. To help in understanding whether cold exerts a general rescuing effect on defective synaptic growth or whether these effects are also specific to mutations in spastin, we examined animals with mutations in flower (five), a putative Ca²⁺ channel that regulates synaptic vesicle

Fig. 2. Pupal or adult-stage cooling increases mobility and lifespan of spastinF5.75 flies.

(A) SpastinF5.75 flies cold-treated as pupae or adults climb faster than untreated mutants kept at 24°C (P<0.05 or P<6x10⁻³, respectively). Cold has no effect on WCS mobility except when administered during the larval stage, which decreases climb rates by almost 20% (P<3x10⁻³). Larval cooling is even more deleterious to mutants, which are unable to climb after eclosion. (B) Average adult mutant lifespan is also considerably reduced by larval cooling, but is nearly doubled by pupal cooling (P<0.05). Average WCS lifespan is affected only by adult-stage cold treatment, which affects mutants to a similar degree (P<2x10⁻³). (C) Left: Survival curves overlap between untreated WCS flies (red triangles, long dashed line) and WCS flies cooled as larvae (light blue circles, short dashed line) or pupae (dark blue squares, solid line). Adult stage cooling (green boxes, dotted dashed line) right-shifts the population as a whole, doubling average lifespan. Right: The population of cold-treated spastinF5.75 adults is similarly right-shifted relative to untreated mutants. However, pupal cooling also right-shifts the spastinF5.75 survival curve and extends maximum lifespan over threefold compared with untreated mutants. *P<0.05; **P<0.005.
endocytosis and has no known link to microtubule regulation (Yao et al., 2009). Similar to spastin mutants, however, fwe<sup>DB25/DB56</sup> larval NMJs have smaller and more numerous boutons, often arrayed in bunches, and are compromised in synaptic transmission. Rearing fwe<sup>DB25/DB56</sup> larvae at 18°C significantly reduced synaptic bouton number and arrangement to resemble wild-type morphologies (Fig. 4B), suggesting that the effects of cooling are not limited to mutations in spastin function.

We also examined adult behavioral phenotypes in mutants of a gene closely related to spastin, kat-60L1. Like Spastin, Kat-60L1 severs microtubules and is required during larval and pupal neuronal development, although the precise roles of each protein are distinct (Lee et al., 2009; Stewart et al., 2012). We examined the effect of cold on adult fly mobility in kat-60L1<sup>F8Bac</sup>, a partial loss of function genotype associated with milder pathogenesis (Du et al., 2010; Fang Du and N.T.S., unpublished results). In comparison, pupal cooling of spastin<sup>5.75</sup> null mutants increased eclosion by 70%.

Importantly, cooling during the pupal and adult stages did not affect eclosion or motor behavior in wild-type flies. This suggests that cooling not only compensates for defects in neuronal function caused by lack of Spastin (or other mutations), but is also innocuous to properly functioning neurons. Although cooling administered at the larval stage was ultimately deleterious to both control and spastin mutant adults, mutant larval synapses were effectively restored to wild-type morphologies. This suggests that cold was beneficial for some spastin-mediated defects at this stage, but also had nonspecific, toxic effects on a cell population required later, in adults.

What is the mechanism(s) underlying the rescuing effect of cold? Our demonstration that cold alleviates not just spastin mutant phenotypes, but also mutant phenotypes in fwe and kat-60L1, indicates that that rescuing effects of cold on nervous system function might be quite broad. All three genes are important in synapse formation, although kat-60L1 has been shown to act post-rather than pre-synaptically at larval and pupal stages. Reduced temperature could thus be generally beneficial to synaptic dysfunction, perhaps by reducing activity or metabolic load. Alternatively, fwe, spastin and kat-60L1 might share a common pathway component(s), as yet undiscovered, that is directly affected by cold. For example, cold itself is well known to destabilize microtubules, particularly at temperatures below 20°C (Delphin et al., 2012), and is often used in experiments to depolymerize microtubules (Baaas et al., 1994; Cottam et al., 2006). Cold could thus substitute directly for the microtubule-severing function of

**Table 4. Pupal stage cold treatment increases the eclosion rate of H<sup>−44</sup> fwe<sup>R388</sup> transgenic flies**

| Treatment stage | H<sup>HWT</sup>,H<sup>HWT</sup> | H<sup>−44</sup>,fwe<sup>R388</sup> |
|-----------------|----------------|-----------------------------|
| Untreated       | 35.1±23.2; 2 vials, 54 larvae | 11.5±5.0; 3 vials, 89 larvae |
| Pupal           | 34.6±4.2; 8 vials, 244 larvae | 22.4±3.1; 9 vials, 293 larvae |

Values shown are mean ± s.e.m. Averages that differ significantly (P<0.05) from the non-cold-treated (untreated) condition of the same genotype are highlighted in bold.
Spastin by promoting microtubule destabilization. Cold-mediated rescue of Kat-60L1 mutants supported this idea; however, we did not observe obvious differences in stable microtubule distribution at cold-treated spastin5.75 synapses or in Drosophila S2R+ cells (data not shown), and fwe mutants, which have not been implicated in microtubule dysregulation, were also rescued by cooling.

In humans, cooling has been shown to be generally neuroprotective, and mild or moderate therapeutic hypothermia (e.g. 33-35°C) has long had clinical applications, including reducing neurological injury in patients following cardiac arrest, traumatic brain injury, epilepsy and stroke (Hartemink et al., 2004; Yenari and Han, 2012). Furthermore, Yang and colleagues found that exposure to even near-freezing temperatures results in minimal neuropathology in rat and cat neocortex and hippocampus (Yang et al., 2006). Although commonly administered in situations involving acute brain injury, the mechanism by which cooling confers neuroprotection or therapeutic improvement is unknown, multifactorial and context-dependent (Choi et al., 2012; Yenari and Han, 2012).

Table 5. Pupal stage cold treatment increases the climb rate of H44,H388 transgenic flies

| Treatment stage | Climb rate cm/s | n | Climb rate cm/s | n |
|-----------------|-----------------|---|-----------------|---|
| Untreated       | 1.57±0.07       | 48 | 0.72±0.06      | 44 |
| Pupal           | 0.95±0.05       | 33 | 1.06±0.07      | 23 |
| Adult           | 0.76±0.23       | 7  | 1.15±0.11      | 20 |

Values shown are mean ± s.e.m. Averages that differ significantly (P<0.05) from the non-cold-treated (untreated) condition of the same genotype are highlighted in bold.

Fig. 4. Cooling rescues synapse morphology in both spastin and flower mutants, as well as climb rates in kat-60L1 mutants. (A1, A2) Synaptic terminals of spastin5.75 larvae raised at 25°C have smaller, more numerous and bunched (*) boutons, unlike linearly arrayed WCS boutons. Rearing at 18°C restores mutant synapse morphology towards wild type, as measured by total number of synaptic boutons per muscle (P<0.02 compared with 25°C mutants) and terminal bouton number, a measure of arbor branching (P<5×10⁻³). Bunches are not observed in cold-treated larvae (P<0.03). (A3) Cooling does not rescue the distribution of stable microtubules within synaptic boutons, as seen by the increased number of boutons without Futsch immunostaining in spastin5.75 mutants, even with cold treatment. (B1) Mutants in flower, which mediates synaptic vesicle endocytosis, also exhibit small, bunched synaptic boutons at the larval NMJ that are rescued to more wild-type morphologies by cooling. (B2) Both total and terminal bouton numbers are significantly reduced in fwe larvae reared at 18°C compared with 25°C (P<4×10⁻⁷). (C) Pupal stage cooling increases climb rate in flies lacking Kat-60L1, a microtubule-severing protein related to Spastin. Flies from cold-treated pupae expressing low (kat-60L1PBac) or no kat-60L1 (kat-60L1BE6) climb faster than untreated flies of the same genotype (P<8×10⁻⁴ and P<1×10⁻⁸, respectively). *P<0.05; **P<0.005.
It will be important to characterize the in vivo effects of cold in mouse models of AD-HSP (Kasher et al., 2009; Fassier et al., 2013). The specificity of the effect of cold on mutant and not wild-type animals in our experiments, together with the spatially localized neurodegeneration in AD-HSP, suggest that moderate hypothermia could be applied in a highly targeted manner in this disease context, with minimal negative effects. Future studies should furthermore elucidate the underlying cellular mechanisms and potentially broader applications of cold in alleviating neuronal dysfunction in neurodegeneration. Because Drosophila are ectothermic, with body temperatures that vary with their environment, they provide a straightforward system in which the cell biological effects of temperature change can be studied in vivo. Finally, our data highlight the potential sensitivity of neuronal phenotypes to variations in temperature and thus its importance as a consideration in studies of neuronal function, neurodegeneration and behavior.

**MATERIALS AND METHODS**

**Drosophila strains**

Flies were reared on standard cornmeal/agar/molasses medium at either 24-25°C (untreated) or 18°C (cold treatment). Genetic controls were white-CantonS (WCS), the CantonS wild-type strain backcrossed to white ten times (gift of Anne Simon, Western University, Ontario, Canada) (Sherwood et al., 2004). Homozygous spastin null (spastin10/spastin10) animals were obtained by picking non-Tubby larvae from spastin10/TM6B stocks. Transgenic line H96.59/H96.58 carried one copy each of transgenes encoding S44L and K388R human Spastin mutations, expressed via the inducible geneswitch elav-GAL4 driver (Osterwalder et al., 2001), in the spastin10 null background. H97.514/H97.514 flies had two copies of wild-type human Spastin in the same background (Du et al., 2010). Kat-60L1 mutants were generated as described previously (Stewart et al., 2011). Trans-heterozygous flowerDB25/DB56 larvae were maintained at 25°C or 18°C after collecting non-Tubby larvae from each vial. After eclosing, each adult fly was moved to an individual food vial. All tests were done at 24°C; adults maintained at 18°C per fly and the results averaged. Flies were then transferred back into their original food vial for testing. Mobility was measured using flies aged 0-27 days of adult flies from each vial divided by the number of larvae originally placed into that vial. After eclosing, each adult fly was moved to an individual food vial.

**Assessment of cold treatment on behavioral phenotypes**

Third-instar larvae of the appropriate genotype were picked with a cotton swab and placed in a clean vial. Eclosion rate was calculated as the number of adult flies from each vial divided by the number of larvae originally placed into that vial. After eclosing, each adult fly was moved to an individual food vial.

Adult mobility was assessed via a climb rate test: single flies were transferred to an empty vial, tapped to the bottom, and the rates at which they climbed up to an 8-cm mark measured. This was repeated three times per fly and the results averaged. Flies were then transferred back into their individual food vial and replaced at either 24°C or 18°C depending on experimental group. All tests were done at 24°C; adults maintained at 18°C were first equilibrated for 30 minutes at 24°C prior to being transferred to the empty vial for testing. Mobility was measured using flies aged 0-27 days. The average age of flies tested was not correlated with the average climb rate.

Adults were transferred to new food vials every 1-2 weeks to maintain healthy living conditions. Lifespan was the number of days from eclosion until death.

The Student’s t-test was used to measure statistical significance. Graphs are presented as percentage changes due to the large differences between wild-type and spastin mutant controls; raw values (mean ± s.e.m.) and number of experiments are listed in the tables and figures.

**Neuromuscular junction immunohistochemistry**

Third-instar WCS and homozygous spastin10/spastin10 larvae were dissected, fixed and immunostained using standard methods (e.g. Ozdowski et al., 2011). Briefly, larvae were dissected in room temperature PBS and fixed for 5 minutes in 4% paraformaldehyde, immunostained at 4°C overnight using the neuronal membrane marker rabbit anti-HRP (1:100; Jackson ImmunoResearch, PA, 323-005-021) alone or with mAb 22C10 to label microtubules (mouse anti-TuA, 1:50; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Secondary antibodies (Alexa Fluor 488 goat anti-rabbit A-11070 and Alexa Fluor 568 goat anti-mouse A-11031; 1:400; Life Technologies, Grand Island, NY) were incubated for 2-3 hours at room temperature. Fillets were mounted in H-1000 (Vector Laboratories, Burlingame, CA) and z-series images of muscle 4 synapses from larval segments 2-4 acquired on a Zeiss LSM 510 inverted confocal microscope using 63× 1.4 N.A. or 100× 1.2 N.A. Planapo objectives (Oberkochen, Germany).

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