GENERAL ARTICLE

Hereditary spastic paraplegia: gain-of-function mechanisms revealed by new transgenic mouse

Liang Qiang^{1,†}, Emanuela Piermarini^{1,†}, Hemalatha Muralidharan¹, Wenqian Yu¹, Lanfranco Leo¹, Laura E. Hennessy², Silvia Fernandes¹, Theresa Connors¹, Philip L. Yates¹, Michelle Swift¹, Lyandysha V. Zholudeva¹, Michael A. Lane¹, Gerardo Morfini³, Guillermo M. Alexander², Terry D. Heiman-Patterson² and Peter W. Baas^{1,*}

¹Department of Neurobiology and Anatomy and ²Department of Neurology, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA, USA and ³Department of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, IL, USA

*To whom correspondence should be addressed at: Department of Neurobiology and Anatomy and Department of Neurology Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA, USA. Tel: 215 991 8298; Fax: 215 843 9082; Email: pwb22@drexel.edu

Abstract

Mutations of the *SPAST* gene, which encodes the microtubule-severing protein spastin, are the most common cause of hereditary spastic paraplegia (HSP). Haploinsufficiency is the prevalent opinion as to the mechanism of the disease, but gain-of-function toxicity of the mutant proteins is another possibility. Here, we report a new transgenic mouse (termed *SPAST*^{C448Y} mouse) that is not haploinsufficient but expresses human spastin bearing the HSP pathogenic C448Y mutation. Expression of the mutant spastin was documented from fetus to adult, but gait defects reminiscent of HSP (not observed in spastin knockout mice) were adult onset, as is typical of human patients. Results of histological and tracer studies on the mouse are consistent with progressive dying back of corticospinal axons, which is characteristic of the disease. The C448Y-mutated spastin alters microtubule stability in a manner that is opposite to the expectations of haploinsufficiency. Neurons cultured from the mouse display deficits in organelle transport typical of axonal degenerative diseases, and these deficits were worsened by depletion of endogenous mouse spastin. These results on the *SPAST*^{C448Y} mouse are consistent with a gain-of-function mechanism underlying HSP, with spastin haploinsufficiency exacerbating the toxicity of the mutant spastin proteins. These findings reveal the need for a different therapeutic approach than indicated by haploinsufficiency alone.

Introduction

The hereditary spastic paraplegias (HSPs) comprise a group of heritable diseases in which patients suffer from progressive weakness and spasticity of lower limbs that are typically adult

onset (1). These hallmark clinical symptoms are associated with dying-back degeneration of corticospinal motor neurons whose cell bodies map to the cerebral cortex (2). Defining pathogenic mechanisms by which mutations in the *SPAST* gene, accounting for the majority of HSP cases (HSP-SPG4), cause

[†]Co-first authors (equal contributions).

Received: August 19, 2018. Revised: October 31, 2018. Accepted: December 2, 2018

© The Author(s) 2018. Published by Oxford University Press. All rights reserved.

For Permissions, please email: journals.permissions@oup.com

degeneration of these axons is critical for the development of effective therapeutic strategies. To date, haploinsufficiency of the spastin protein has been the prevalent explanation for HSP-SPG4 (3–10). A case for haploinsufficiency has been made mainly on the basis of the genetics, but also on the fact that mutant spastin proteins have not been detected in cells and tissues derived from human patients (11,12). Consistent with a mechanism based on haploinsufficiency, several lines of evidence indicate that the dosage of spastin, a microtubule-severing protein, is tightly regulated to ensure the normal vitality of the axon (13–16). However, the haploinsufficiency model fails to explain why there are no developmental abnormalities in HSP-SPG4 patients, why axonal degeneration is mostly confined to the corticospinal tracts and why several SPAST mutations cause HSP despite not affecting the enzymatic activity of the spastin protein (17). In addition, two independently generated spastin knockout mouse models do not recapitulate major HSP hallmarks (18,19), suggesting that haploinsufficiency does not suffice to explain HSP-SPG4 pathogenesis. While it is conceivable that corticospinal axons in humans are especially sensitive to lower spastin levels because of their great length, we have posited that mutant proteins with toxic properties may be the primary mechanism of the disease (17,20–24), as is the case with many other neurodegenerative diseases (25,26).

SPAST has two start codons that produce a longer isoform called M1 and a slightly shorter isoform called M87 (in humans) or M85 (in rodents) (27,28). The shorter isoform of spastin is widely distributed, while M1 is only detectably present in the adult spinal cord, the location of the corticospinal tracts that degenerate during HSP (24). Earlier studies on various different kinds of mutations indicate that truncated or mutated spastin proteins, even at extremely low levels, can elicit toxicity by affecting axonal transport, at least in part due to abnormal activation of a kinase that can suppress the function of molecular motor proteins (20,21). This effect is specific to mutant M1. A variety of studies comparing the effects of M1 and M87 counterparts bearing the same mutation consistently indicate toxicity of the M1 isoform with relatively little effect of the M87 isoform (20–22,24). To date, these experimental studies have been performed on cultures of fibroblasts, primary rodent neurons, and on *Drosophila*, as an animal model. There is a clear need for a good rodent model in which disease mechanisms and potential therapies can be studied.

To specifically address potentially toxic effects of mutant spastin *in vivo*, we have generated a novel HSP-SPG4 mouse model in which a human mutant SPAST transgene bearing the C448Y mutation (SPAST^{C448Y}) was inserted into the mouse ROSA26 locus. These animals were crossed with a ubiquitous Cre mouse line to enable ubiquitous tissue expression of mutant spastins. Despite normal levels of endogenous mouse spastin, both heterozygous and homozygous SPAST^{C448Y} mice display locomotor phenotypes reminiscent of HSP. Collectively, phenotypic studies on SPAST^{C448Y} mice support a toxic gain-of-function mechanism operating in HSP-SPG4, a finding with important implications for the eventual development of therapeutic strategies.

Results

SPAST^{C448Y} Rosa26 transgenic mouse model for studying gain-of-function aspects of HSP-SPG4

To investigate gain-of-function contributions of mutated human spastin to the disease symptoms of HSP-SPG4, a mouse model

Figure 1. Generation of SPAST^{C448Y} Rosa26 transgenic mouse model. (A) Represents a schematic of the strategy used to generate the Rosa26 knock-in SPAST^{C448Y} mouse model. The numerals identify the human SPAST exons. pA = human growth hormone polyA signal, SPAST^{C448Y} = human mutated SPAST CDS, STOP-neo = STOP neomycin resistance cassette, pCAG = strong fusion promoter of CMV immediate early enhancer and chicken α -actin promoter, DTA = Diphtheria toxin A negative selection marker. (B) gDNA products from qPCR were run on 1% agarose gel to visualize the SPAST band among the three genotypes.

of the disease with a C57BL/6 background was generated. Full-length human spastin (M1) with a missense mutation (C448Y) located at exon 11 within the AAA domain was inserted into the Rosa26 locus. The C448Y mutation is located in the second pore loop of the AAA domain of spastin and destroys the microtubule-severing activity of the protein (29). Since M1 and M87 harbor the identical AAA domain, C448Y is present in both spastin isoforms. A floxed (loxP-flanked) transcriptional STOP cassette was incorporated between the transgene and the promoter to allow expression to be dependent upon Cre recombinase (Fig. 1A). Ubiquitous Cre mice obtained from genOway were used to unlock the expression of the mutant spastin (Fig. 1A). All mice in these studies were unlocked mice that express either one copy of human mutated spastin (SPAST^{C448Y}/-) or two copies (SPAST^{C448Y}/SPAST^{C448Y}). No alterations were made in the gene for expression of the endogenous mouse spastin proteins. Genomic DNA (gDNA) obtained from ear punches was used to carry out quantitative real-time polymerase chain reaction (qRT-PCR) to identify genotypes. PCR products were visualized on agarose gels (Fig. 1B).

SPAST^{C448Y} mice display behavioral deficits underscored by gait impairment

Symptoms of HSP patients include lower limb spasticity (stiffness) and gait disturbances (stumbling and tripping), both of which are typically adult onset (6,30–32). Like the human patients, SPAST^{C448Y}/- and SPAST^{C448Y}/SPAST^{C448Y} mice showed no obvious defects in development compared to wild-type mice

and no behavioral abnormalities until adulthood. Males and females were separately analyzed in order to reveal potential sex differences in all of the behavioral examinations included in this study. We focused first on males, because they showed a stronger phenotype than females. A beam walk assay, widely used to test gait impairment (33–36), revealed deficits in the transgenic mice compared to wild-type. Figure 2A shows representative images of the mice walking along a narrow beam (see also Supplementary Material, Videos 1, 2 and 3 for comparison). Both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice displayed deficits in gripping the beam (Fig. 2A). We ranked the scores for the beam walk test by 0, 1 and 2, where 0 stands for no difficulty in walking along the beam with no stops, 1 stands for a minor defect in walking with one to two stops and 2 stands for a severe defect in walking with more than two stops or not being able to finish the test. The frequency of the mice manifesting no deficits (score 0) significantly decreased in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y}, as shown in Figure 2B. Consecutive (continuously positive for at least three times over the course of 1 week) scores of 2 were only present in SPAST^{C448Y}/– (52%, 11 out of 21 mice) and SPAST^{C448Y}/SPAST^{C448Y} (50%, 7 out of 14 mice). No wild-type mice showed consecutive scores of 2 through their life spans. The age of onset for consecutive scores of 2 in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} was 119 days and 80 days, respectively (Fig. 2C). No significant difference was found for consecutive scores of 1 across the groups. For detailed numbers, refer to Supplementary Material, Table 1.

Resting tremor in mice is often associated with muscle spasticity (37,38). In the wild-type animals, only 1 out of 15 mice (6.7%) that were tested appeared to be tremor positive (37) at the age of 247 days. However, all of the SPAST^{C448Y}/– (100%) and SPAST^{C448Y}/SPAST^{C448Y} (100%) mice showed resting tremor. The onset ages in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} were 83 days old and 70 days old, respectively, which indicates that the tremor observed in the mutant mice is not associated with aging (Fig. 2D). Hindlimb clasping, a behavioral test examining locomotor functions of the hindlimbs (37), was assessed by observing hindlimb splay of the mice lifted on their own tails (Fig. 2E). Mice were scored as 0, 1 or 2, with 0 representing a perfect splay of hindlimbs raised up to the horizontal line, 1 representing hindlimbs of the mice splayed below the horizontal line or a compromised capability to spread their hindlimbs and 2 representing a complete inability to spread their hindlimbs. The average scores for SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} were significantly increased compared to the wild-type (Fig. 2F). Many more mice in SPAST^{C448Y}/– (72%, 16 out of 22 mice) and SPAST^{C448Y}/SPAST^{C448Y} (58%, 7 out of 12 mice) showed consecutive scores of 1 compared to the wild-type (33%, 5 out of 15 mice). The ages of onset for consecutive scores of 1 in both SPAST^{C448Y}/– (80 days old) and SPAST^{C448Y}/SPAST^{C448Y} (73 days old) groups were much earlier than that in the wild-type (140 days old) (Fig. 2G). However, there was no significant difference in the onset age for consecutive scores of 2 across the three groups. Nevertheless, the splayed angles were significantly decreased in both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} compared to the wild-type (Fig. 2H). At age P90 and younger, no weight differences were observed among all three genotypes analyzed. However, a significant decrease in weight was observed for SPAST^{C448Y}/SPAST^{C448Y} mice older than age P150 (Fig. 2I). See also Fig. 2A). For detailed numbers, refer to Supplementary Material, Table 1.

Female SPAST^{C448Y} mice showed less severe motor defects compared to males (see Supplementary Material, Fig. 1A–F). This gender difference is consistent with evidence from clinical examinations of HSP-SPG4 patients, which show males with

much more severe walking difficulties than females (39). Therefore, all of the subsequent studies, except for the studies on primary neuronal cultures, were performed with the adult male mice (3 months or 6 months or older than 6 months). For detailed numbers, refer to the legend for Supplementary Material, Figure 1.

Abnormal axonal morphology in the spinal cords of SPAST^{C448Y} mice

No gross morphological defect was observed via hematoxylin and eosin staining in either SPAST^{C448Y}/– or SPAST^{C448Y}/SPAST^{C448Y} compared to wild-type mice (Supplementary Material, Fig. 2A–L). HSP-SGP4 is thought to be primarily caused by degeneration of the upper motor neurons whose cell bodies reside in the motor cerebral cortex with their long axons projecting into the spinal cord to form the corticospinal tracts (2,23,40). Based on the clinical manifestation of the SPAST^{C448Y} mice, we sought to determine whether degeneration of corticospinal tracts could be detected in these mice. Typical axonal degeneration can be reflected by axonal dieback. Therefore, we first examined cross sections from the spinal cord at the lumbar level when the animals reached 3 months old. We used immunofluorescence staining for SMI312 to visualize the axons in these sections. SMI312 is a mixture of monoclonal antibodies against highly phosphorylated epitopes on neurofilament proteins M and H. It is also well known that enhanced SMI312 immunoreactivity is associated with axonal degeneration (41,42). Interestingly, a much more elevated SMI312 immunoreactivity was detected within the white matter of both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice compared to the wild-type, whereas no difference was identified in the grey matter among the three groups (Fig. 3A–E). We further discovered that the increase in SMI312 fluorescence intensity in the white matter from SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} is not due to the increase in the number of axons labeled with SMI312, suggesting that escalated phosphorylation of neurofilaments M and H is present in the mutant axons (Fig. 3F).

Next, we evaluated axonal morphology in three distinct regions within the white matter—dorsal, lateral and ventral columns—within the lumbar spinal cord. In mice, the dorsal column contains more than 90% of the corticospinal tracts; the lateral column contains all of the rubrospinal tracts, less than 10% of the corticospinal tracts and half the reticulospinal tracts and the ventral column contains half the reticulospinal tracts and all of the vestibulospinal tracts (43). In the cross sections of the wild-type mice, axons in all of the three regions mostly appear with a ‘regular’ cross-sectional morphology (diameter < 1 µm, Fig. 3Aa’–a’’) and very few of them present with ‘irregular’ morphology (irregular elongated threads, > 1 µm, Fig. 3Aa’–a’’). The former are axons sectioned in near-perfect cross section (relative to the long axis of the spinal cord), while the latter suggest contortions of the axon which may be associated with axonal degeneration and dieback. In the cross sections of SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice, morphological changes of the axons were detected in the dorsal and ventral columns. In these regions, there were significantly decreased numbers of regular axons and significantly increased numbers of irregular axons, which is consistent with axonal dieback (Fig. 3Bb’–b’’, 3Cc’–c’’, G and I). No such differences were identified in the lateral column from the mutant mice (Fig. 3H). For detailed numbers, refer to the legend for Figure 3.

To examine whether axonal degeneration is progressive, we analyzed axon numbers from the spinal cord (1 year old) at both cervical and lumbar levels by carrying out toluidine

Figure 2. Gait impairment in SPAST^{C448Y} mice assessed by behavioral assays. (A) Representative pictures of mice while performing the beam walk assay, highlighting a prominent defect in normal gripping of the beam (compare the red dashed circle in wild-type with the yellow dashed one) in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice compared to wild-type. (B) Percentage of SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice showing a normal beam walk phenotype is significantly decreased compared to wild-type. (C) Age of disease onset in days for mice with consecutive beam walk scores of 2. No wild-type mice displayed consecutive scores of 2. (D) Onset ages for resting tremor. Only one wild-type mouse displayed a positive tremor. (E) Mice tested for the hindlimb clasp assay are shown. The dashed light blue triangles highlight differences in the hindlimb splayed angle. (F) Average hindlimb clasp score was evaluated in mice > 60 days of age. (G) Age of disease onset was also evaluated for mice with consecutive hindlimb clasp scores of 1. (H) The splayed hindlimb angle was quantified for the three groups, with a significantly decreased angle in both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} compared to wild-type. (I) Decreased body weight was observed in adult mice (>150 days). The number of mice used for the beam walk assay was $n = 15$ mice for wild-type, $n = 21$ mice for SPAST^{C448Y}/– and $n = 14$ mice for SPAST^{C448Y}/SPAST^{C448Y}. The number of mice for the hindlimb clasp assay was $n = 15$ mice for wild-type, $n = 22$ mice for SPAST^{C448Y}/– and $n = 12$ mice for SPAST^{C448Y}/SPAST^{C448Y}. The number of mice for the resting tremor assay was $n = 13$ mice for wild-type, $n = 18$ mice for SPAST^{C448Y}/– and $n = 15$ mice for SPAST^{C448Y}/SPAST^{C448Y}. Data are represented as mean $\pm$ S.E.M. For statistical tests, one-way analysis of variance (ANOVA) with Tukey post hoc analysis was conducted. * $P < 0.05$, ** $P < 0.002$, *** $P < 0.001$. For details on scoring system, see Results. For additional information related to this figure, see [Supplementary Material, Table 1](#).

blue staining on semi-thin plastic-embedded cross sections. Significantly reduced axon numbers were observed from the dorsal columns at the lumbar levels in both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y}. However, such reduction was not detected at the cervical levels in those animals (Fig. 4). These results indicate that progressive axonal degeneration takes place in the dorsal column of the SPAST^{C448Y} Rosa26 transgenic animals. For detailed numbers, refer to [Supplementary Material, Table 2](#).

Although irregular axonal morphology was detected within some regions of the white matter from the mutant mice, the specificity of the axons that were affected remained unknown. To determine whether the afflicted axons observed with SMI312 staining includes descending motor tracts, we labeled the descending tracts using biotinylated dextran amines (BDA). Three injections of BDA were delivered to the aforementioned three columns at the cervical level in the spinal cord (C1/2) of either wild-type mice or SPAST^{C448Y}/SPAST^{C448Y} mice at 5

Figure 3. Changes in axon shape at the lumbar level of the spinal cord. (A–C) Cross sections of the lumbar spinal cord stained for SMI312 show increased fluorescence intensity per 100 μm^2 in the white matter. (D) Quantification shows increased white matter SMI312 fluorescence intensity in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice compared to wild-type. The average $\pm$ S.E.M. is 12.31 $\pm$ 1.31, 25.91 $\pm$ 2.43, and 29.54 $\pm$ 1.67 for wild-type, SPAST^{C448Y}/–, and SPAST^{C448Y}/SPAST^{C448Y}, respectively. (E) No significant changes were observed in the grey matter. (F) No significant changes were observed in the total axon number per 100 μm^2 in the dorsal column, lateral column and ventral column (a'–c"). (G) Quantification of axons in the dorsal column shows that axon shape is significantly different among the groups, and a more irregular shape is found in both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y}, with the percentage of irregular axons as 24% $\pm$ 3.51% in SPAST^{C448Y}/– and 43% $\pm$ 3.48% in SPAST^{C448Y}/SPAST^{C448Y} compared to 12% $\pm$ 3.52% in wild-type. (H) No significant changes in axon shape were observed in the lateral column. (I) The percentage of irregular axons is also increased in the ventral column in both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y}, quantified as 9.91% $\pm$ 2.42% in wild-type, 21.61% $\pm$ 6.22% in SPAST^{C448Y}/– and 36.83% $\pm$ 2.36% in SPAST^{C448Y}/SPAST^{C448Y}. Scale bar = 200 μm for A–C, and scale bar = 100 μm for (a'–c"). Histology was quantified in three mice for each genotype. Data are represented as mean $\pm$ S.E.M. For statistical tests, one-way ANOVA with Tukey post hoc analysis was conducted. * P < 0.05, ** P < 0.002, *** P < 0.001.

months of age. One month after the injections, we examined both the cervical and lumbar spinal cords by double labeling of BDA and SMI312 in order to evaluate axonal degeneration (Fig. 5A). Theoretically, compared to the normal condition, decreased numbers of double-labeled axons at the lumbar level would suggest potential axonal degeneration or retraction (Fig. 5B). Interestingly, a significantly decreased number of double-labeled axons was detected in the dorsal column at the lumbar level of the SPAST^{C448Y}/SPAST^{C448Y} mouse (Fig. 5G), whereas no such difference was detected in the lateral or ventral

columns at the lumbar level of the SPAST^{C448Y}/SPAST^{C448Y} mouse (Fig. 5H and I).

M1 spastin only detectably accumulates in the cerebral cortex and lower spinal cords of SPAST^{C448Y} mice

The human sequence of SPAST inserted in the SPAST^{C448Y} mouse retained the two original start codons. The first one is weak, which leads to a full-length isoform named M1. The second

Figure 4. Progressive loss of axons in the dorsal column of the spinal cord in both SPAST^{C448Y/+} and SPAST^{C448Y/SPAST^{C448Y}} mice. Higher magnification toluidine blue-stained images of the dorsal columns at the lumbar level showing that compared to (A) wild-type, (B) SPAST^{C448Y/+} and (C) SPAST^{C448Y/SPAST^{C448Y}} mice show decreased axon numbers. The total numbers of axons per 100 μm² were quantified at both cervical and lumbar levels for the dorsal, lateral and ventral columns. (D) No significant differences in axon numbers among the three regions at the cervical level were observed. (E) Significantly reduced axon numbers were identified in the dorsal columns from the mutant animals (both SPAST^{C448Y/+} and SPAST^{C448Y/SPAST^{C448Y}}) at the lumbar levels. Data are represented as mean ± S.E.M. For statistical tests, one-way ANOVA with Tukey post hoc analysis was conducted. Scale bar = 20 μm. **P < 0.002, ***P < 0.001. For additional information related to this figure, see [Supplementary Material, Table 2](#).

one is the more dominant start codon, promoting production of a shorter isoform called M87 (27,28). Previously, in studies on rats, we found that M85 (the short form of spastin in rodents) is dominantly present in various organs with relatively high levels, whereas M1 is undetectable in most tissues except at a very low expression level in the adult spinal cord. Our unlocked SPAST^{C448Y} mouse was designed and generated to express both isoforms of the human mutated spastin ubiquitously without any disturbance of their original start codons. To examine the expression pattern of M1 and M87 spastin isoforms in SPAST^{C448Y} mice, western blot analyses were performed on the cerebral cortex, cerebellum, spinal cord, heart, liver and kidney at three different ages: P0 (neonatal, except the spinal cord due to the technical challenge to obtain enough tissue for the assay), P80 (postnatal day 80) and P200 (postnatal day 200). No antibody can distinguish between human spastin and rodent spastin. Therefore, in western blot analyses of our SPAST^{C448Y} mice, endogenous mouse M1 spastin resides in the same band as exogenous human M1 spastin, and endogenous mouse M85 spastin is together with exogenous human M87 spastin. In wild-type mice, we identified moderate expression levels of M85 in the cerebral cortex and spinal cord without detecting obvious M1 expression (Fig. 6A–D). Compared to the wild-type in the cerebral cortex, at P0 there was no difference in the M85/M87 level in SPAST^{C448Y/+}, but there was a 2.8-fold increase in SPAST^{C448Y/SPAST^{C448Y}}; at P80 there was a 2.5-fold and a 3-fold increase in SPAST^{C448Y/+} and SPAST^{C448Y/SPAST^{C448Y}},

respectively, and at P200 the level increased by 3-fold and 9-fold in SPAST^{C448Y/+} and SPAST^{C448Y/SPAST^{C448Y}}, respectively (Fig. 6A and C). For the spinal cord analyses, we separated the cord into upper spinal cord and lower spinal cord by cutting the cord at thoracic level T10–11. Increased levels of M85/M87 were also detected in upper and lower spinal cord tissues from the mutant mice compared to the wild-type at both P80 (1.3- and 1.6-fold increase in the upper and lower spinal cord of SPAST^{C448Y/+}, respectively; 3.9- and 2.1-fold increase in the upper and lower spinal cord of SPAST^{C448Y/SPAST^{C448Y}}, respectively) and P200 (0.4- and 0.8-fold increase in the upper and lower spinal cord of SPAST^{C448Y/+}, respectively; 3.5- and 2.4-fold increase in the upper and lower spinal cord of SPAST^{C448Y/SPAST^{C448Y}}, respectively) (Fig. 6B and D). However, M1 accumulation was only detected in the cerebral cortex of SPAST^{C448Y/+} mice at P200 and in SPAST^{C448Y/SPAST^{C448Y}} mice at P80 and P200 (Fig. 6A and E). Interestingly, in the spinal cord, M1 accumulation only appeared in the lower spinal cord section of SPAST^{C448Y/+} mice and SPAST^{C448Y/SPAST^{C448Y}} mice at P200 (Fig. 6B and F), which is reminiscent of the phenotypical change in axonal morphologies identified in lumbar spinal cord (Fig. 3). Taken together, these results support the hypothesis that mutated M1 spastin tends to accumulate over time, which potentially contributes to the gain-of-function mechanism for HSP-SPG4. No M1 was detected in any other tissues at any age (Supplementary Material, Fig. 3A–H). For detailed numbers, refer to [Supplementary Material, Tables 3 and 5](#).

Figure 5. Anterograde tracing highlights degenerating axons in the spinal cord of SPAST^{C448Y}/SPAST^{C448Y} mice. (A) The schematic shows that mice 5 months old were used for the anterograde tracing studies. BDA dye was injected at the C1/2 level and then tissues were collected 4 weeks later. (B) The cartoon illustrates the injection approach for the analyses. (C–F) Magnifications of the dorsal column at cervical and lumbar levels for both wild-type and SPAST^{C448Y}/SPAST^{C448Y} highlight decreased numbers of double-labeled axons in the SPAST^{C448Y}/SPAST^{C448Y} mice. Percentage of double-labeled axons was quantified as the ratio between the cervical and lumbar levels. (G) The quantification for the dorsal column shows only 56 ± 20% of double-labeled axons in SPAST^{C448Y}/SPAST^{C448Y} mice relative to control. (H–I) Quantifications of the ventral (H) and lateral (I) columns do not show significant differences in double-labeled axons between wild-type and SPAST^{C448Y}/SPAST^{C448Y}. The histology was quantified in three mice for each genotype. Data are represented as mean ± S.E.M. For statistical analysis, Student's t-test (two-tailed) was performed. Scale bar = 100 µm. *P < 0.05.

Significant reduction of microtubule stability in spinal cords of SPAST^{C448Y} mice

If haploinsufficiency is the only contributor to the etiology of HSP-SPG4, an increase in microtubule stability would be expected, as previously reported of spastin knockout mice and flies, as well as cultured rat neurons depleted of spastin by siRNA (15,18,19,44–49). However, we have consistently detected the opposite effect, namely a decrease in microtubule stability, in various experimental models involving expression of pathogenic spastin mutants (20–22). Since M1 (the more toxic of the two isoforms when mutated) is specifically accumulated in lower spinal cords in both SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice, we examined the stability of the microtubules in the spinal cords from these mice. Tissue lysates from upper and lower spinal cords in wild-type, SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} P200 mice were used for this study. Western blot analysis revealed no difference in the amount of β III-tubulin among the three genotypes (Fig. 7A and B), while the levels of acetylated

tubulin and detyrosinated tubulin were both significantly decreased in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice, particularly at the lower spinal cords (Fig. 7A, C and D). Lower levels of acetylated tubulin and detyrosinated tubulin indicate decreased microtubule stability. Thus, the animal with the more representative HSP phenotype (our gain-of-function mouse) has the opposite microtubule stability defect as the animals with the less representative HSP phenotype (the knockout mice). For detailed numbers, refer to the legend for Figure 7.

C448Y human mutant spastin proteins do not behave in dominant-negative fashion in SPAST^{C448Y} mice

Like other microtubule-severing enzymes, spastin forms hexamers to cut microtubules (29,50). In the presence of a mutant spastin, the spastin hexamers formed will be composed of mutant as well as wild-type spastin proteins, rendering a functionally compromised complex (51). Therefore, the mutant

Figure 6. Evaluation of M1 and M85/M87 spastin isoform expression in the cortex and spinal cord. (A–B) Representative western blots of lysates from the cortex (A) and spinal cord (B) from wild-type, SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice at ages P0, P80 and P200. (C) Quantifications of spastin intensity were normalized with Coomassie staining and compared to wild-type. Data are represented as mean ± S.E.M., with wild-type animal values normalized to 1. (D) Quantifications of M85/M87 spastin expression in upper and lower spinal cord levels at P80 and at P200 for wild-type, SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} mice. (E) M1 isoform is also detected in SPAST^{C448Y}/– at P200 and in SPAST^{C448Y}/SPAST^{C448Y} at both P80 and P200, as highlighted by the green arrowheads in (A). Analyses were conducted by normalizing the M1 band intensity with Coomassie staining and expressed as mean ± S.E.M. Bar graph shows the quantifications of cortex lysates at P80 and at P200. (F) At P200, M1 is also detected in spinal cord tissues of SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y}. Western blot lysates were collected from three mice for each genotype for all ages. For statistical tests, one-way ANOVA with Tukey post hoc analysis was conducted. **P* < 0.05, ***P* < 0.002. For additional information related to this figure, see [Supplementary Material, Table 3](#).

human spastin proteins could theoretically elicit a dominant-negative activity to inhibit the physiological functions of the wild-type rodent spastin proteins. However, to our initial surprise, the C448Y mutant did not show dominant-negative properties in our previous studies (22), which is one of the reasons why we chose it for our transgenic mouse. In our mice, a dominant-negative effect would elicit a loss-of-function phenotype which could have been confused for a gain-of-function

phenotype. The microtubule stability studies described above do not support a loss-of-function scenario for our mouse, but we wanted to delve deeper into this matter because it is so important to the interpretation of our observations. We previously reported that depletion of spastin reduces axonal length and the number of axonal branches in developing rat hippocampal neuronal cultures (52). To confirm that the mouse spastin retains its physiological functions in SPAST^{C448Y}/SPAST^{C448Y}

Figure 7. Western blot analysis reveals changes in microtubule stability. (A) Representative western blots of upper and lower spinal cord immunoblotted for acetylated tubulin, deetyrosinated tubulin and β III-tubulin. (B–D) All western blot analyses were normalized to GAPDH. (B) Analysis of β III-tubulin revealed no significant changes in the expression level at upper and lower spinal cords. (C) Analysis for acetylated tubulin revealed a decrease in microtubule stability at the lower spinal cord for SPAST^{C448Y}/SPAST^{C448Y} and SPAST^{C448Y}/SPAST^{C448Y}, with expression levels at 1 ± 0.1 , 0.739 ± 0.006 and 0.565 ± 0.116 for wild-type, SPAST^{C448Y}/SPAST^{C448Y} and SPAST^{C448Y}/SPAST^{C448Y}, respectively. (D) Deetyrosinated tubulin also revealed a decrease in microtubule stability at the lower spinal cord for SPAST^{C448Y}/SPAST^{C448Y} and SPAST^{C448Y}/SPAST^{C448Y}, with expression at 1 ± 0.01 , 0.605 ± 0.020 and 0.358 ± 0.091 for wild-type, SPAST^{C448Y}/SPAST^{C448Y} and SPAST^{C448Y}/SPAST^{C448Y}, respectively. Western blot lysates were collected from three mice for each genotype. For statistical tests, one-way ANOVA with Tukey post hoc analysis was conducted. * $P < 0.05$, ** $P < 0.002$, *** $P < 0.001$.

mice, we designed siRNA to knock down mouse spastin, without affecting human spastin, in order to ascertain whether those deficits in axonal development in the cortical cultures could be recapitulated. Only if the mouse spastin retains its physiological functions should we detect the alterations in axonal morphology previously identified when spastin is depleted. Figure 8A shows that we were able to knock down mouse spastin, and Figure 8B shows that we were also able to knock down human spastin (by human-specific spastin siRNA) in the cortical neuronal cultures derived from newborn wild-type and SPAST^{C448Y}/SPAST^{C448Y} mice. The spastin level in the cultured cortical neurons treated with the siRNA targeting mouse spastin was about 40% in the wild-type and 30% in SPAST^{C448Y}/SPAST^{C448Y} (compared to their own controls), while the spastin level became 10–20% when the neurons from SPAST^{C448Y}/SPAST^{C448Y} were treated with the siRNA targeting human spastin. Consistent with our previous findings, the cortical neurons from both wild-type and SPAST^{C448Y}/SPAST^{C448Y} displayed shorter axons and fewer axonal branches when the mouse spastin was knocked down, whereas no obvious changes in those two parameters were found when human spastin was diminished (Fig. 8C–I). Thus, the human mutated spastin proteins in the SPAST^{C448Y} mouse do not act in a dominant-negative fashion, at least not with regard to the functions involved in these morphological phenotypes (or microtubule stability, as described above). For detailed numbers, refer to Supplementary Material, Table 4.

Lysosomal transport studies reveal mechanistic information for HSP-SPG4 as well as potential therapies

We previously showed that M1 spastin bearing a variety of different HSP pathological mutations promotes deficits in organelle transport through a mechanism involving aberrant activation

of casein kinase 2 (CK2), a protein kinase that phosphorylates molecular motor proteins and inhibits their function (21). With this in mind, we investigated whether haploinsufficiency has any effect on this phenomenon. As with the morphological studies discussed above, the cortical neurons were cultured from newborn mice because there are no successful methods for culturing these neurons from adult mice. Kymographs in Figure 8J represent three different movement statuses of axonal lysosomes, namely retrograde, halted and stationary. By 'halted', we mean lysosomes that were moving but stopped moving during the bout of imaging. Interestingly, compared to the cortical neurons derived from P0 wild-type mice, cortical neurons from P0 SPAST^{C448Y}/SPAST^{C448Y} and SPAST^{C448Y}/SPAST^{C448Y} mice showed a significant decrease in retrograde movements, with a corresponding increase in movements that were halted or stationary (Fig. 8K). However, no instantaneous velocity differences in lysosomal movement were observed among any of the experimental groups (data not shown). Thus, even though the transgenic mouse is not HSP-symptomatic until adulthood, deficits at the cellular level could be detected in neurons from newborn animals. Mouse spastin siRNA resulted in a reduction of the retrograde lysosomal transport by 10–15% in neurons derived from all of the three genotypes (Fig. 8K), which is consistent with the haploinsufficiency exacerbating the gain-of-function deficits induced by the human mutant spastins. For detailed numbers, refer to Supplementary Material, Table 4.

The next question was whether the lysosome transport defects could be rescued by human spastin siRNA. Curiously, human spastin siRNA applied to cultures from either SPAST^{C448Y}/SPAST^{C448Y} or SPAST^{C448Y}/SPAST^{C448Y} mice did not rescue their lysosomal movement defects (Fig. 8K). While this result initially seemed baffling, we suspect that it reflects the fact that only vanishingly small amounts of mutant M1 are required

Figure 8. Functional analysis reveals morphological changes and lysosomal transport alterations in primary cortical neurons derived from SPAST^{C448Y} mice. (A) Representative western blot on primary cortical neurons from wild-type and SPAST^{C448Y}/SPAST^{C448Y} P0 mice transfected with control (ctrl), mouse spastin or human spastin siRNA. (B) Quantifications of spastin expression in cell lysates with ctrl, mouse, or human siRNA were normalized to GAPDH and compared to wild-type. Data are represented as mean ± S.E.M., with wild-type ctrl siRNA taken as 1. (C–G) Representative images show the morphology of primary cortical neurons after transfection of cells with ctrl, mouse or human spastin siRNA. (H) Quantifications of axonal length are shown as mean ± S.E.M. for wild-type or SPAST^{C448Y}/SPAST^{C448Y} with ctrl, mouse or human siRNA. (I) Primary branch numbers of the axon have been quantified as shown. (J) Representative kymographs showing retrograde, halted and stationary movements during lysosomal transport. (K) Quantification of lysosomal movement in cortical neurons from wild-type or SPAST^{C448Y} mice. Neurons were transfected with ctrl, mouse spastin or human spastin siRNA and exposed to lysotracking dye for 5 h, after which they were imaged for 2 min. Percent of retrograde (red), halted (orange) and stationary (green) movement of lysosomes was quantified as mean ± S.E.M. (L) Quantifications of retrograde, halted, stationary lysosomal transport in SPAST^{C448Y}/SPAST^{C448Y} with ctrl, mouse or human siRNA after treatment with 20 μM TBCA for 24 h are expressed as percentage ± S.E.M. The total number of events for this experiment is n = 21 for the ctrl siRNA, n = 25 for the mouse spastin siRNA and n = 22 for the human spastin siRNA; while in the presence of TBCA, n = 23 for ctrl siRNA, n = 24 for mouse spastin siRNA and n = 26 for human spastin siRNA. Scale bar = 50 μm. For statistical tests, one-way ANOVA with Tukey post hoc analysis was conducted. *P < 0.05, **P < 0.002, ***P < 0.001. For additional information related to this figure, see [Supplementary Material, Table 4](#).

to elicit axonal transport defects (21,24), and hence the small amount of mutant proteins remaining after siRNA treatment were sufficient to elicit these lysosomal transport defects. To verify this speculation, we examined lysosomal movements in SPAST^{C448Y}/SPAST^{C448Y} cultured cortical neurons treated with Tetrabromocinnamic acid (TBCA), a competitive casein kinase 2 (CK2) inhibitor that was already used in our previous work (21).

As shown in [Figure 8L](#), TBCA restored the retrograde lysosomal movements in cultured neurons from SPAST^{C448Y}/SPAST^{C448Y} treated with control siRNA, mouse spastin siRNA and human spastin siRNA. Consistent with experiments in isolated squid axoplasm showing that nanomolar levels of mutant M1 spastin inhibit axonal transport (21,24), these results suggest that very small amounts of mutant M1 (such as those remaining after

siRNA treatment) are sufficient to elicit the lysosomal transport deficits. For detailed numbers, refer to [Supplementary Material, Table 4](#).

Discussion

Preclinical studies on human diseases require a vertebrate model so that pathogenic mechanisms and potential therapies can be studied at the systems and behavioral levels. Mainly driven by the assumption of haploinsufficiency as the mechanism underlying HSP-SPG4, two spastin knockout mouse models have been generated, but phenotypic analyses of these mice failed to reveal locomotor deficits such as those observed in HSP patients (18,19). Recently, a mouse model that better reflects the genetic aspect of HSP-SPG4 was generated, with the adult homozygotes displaying a mild gait deficit. For this model, a loss-of-function mutation (N384K) was introduced into the endogenous mouse spastin gene using CRISPR-Cas9-based techniques (53). While the results on this mouse were touted as supporting the haploinsufficiency model, we disagree because if that were the case, the knockout mice should display an even more aggressive phenotype. A more likely scenario is that the gait deficiencies were caused by toxicity of the mutant mouse spastin. Of potential importance to the very mild phenotype is the fact that human and mouse spastin sequences differ within the M1-specific domain, which is the likely culprit for the toxicity of the mutant protein. Precedent exists for mouse counterparts of pathogenic mutant human genes being non-pathogenic (54), and hence the possibility is very real that the mutated mouse gene in the CRISPR-Cas9-based mouse is less toxic than its human counterpart.

Our mouse was created for the specific purpose of testing the merits of a gain-of-function mechanism for the disease versus haploinsufficiency. The fact that our mouse is not haploinsufficient and expresses the human version of the mutated SPAST gene are both keys to achieving this purpose, as is the fact that the mutation we chose does not produce a protein with dominant-negative properties (22). A dominant-negative mechanism is not haploinsufficiency but is similar in the sense of starving the system of functional protein. Our mouse displays no observable disease phenotype until it reaches adulthood, after which a spastic-like tremor and gait deficiency become apparent, both of which are more severe in homozygotes than heterozygotes and more severe in males than females. The behavioral symptoms are accompanied by corticospinal degeneration, manifested as progressive dieback of axons within the tracks, which is what occurs in the human disease (6,23,30,31). This HSP-like phenotype was achieved after crossing the locked mouse with a ubiquitous Cre mouse, without the need for a Cre mouse that targets expression to corticospinal motor neurons or even to the CNS, thus providing confidence that the phenotype of the unlocked mouse reflects the innate vulnerability of the corticospinal tracts.

Understanding the mechanism of the disease is the key to developing appropriate therapies. If the cause of the disease is cytotoxic mutant spastin within corticospinal neurons, then the best strategy would be to rid the neurons of that cytotoxic protein, as well as inhibit the pathogenic pathways activated by it. A model based on haploinsufficiency would call for a very different strategy, presumably restoring the levels of functional spastin to normal. Downstream of the primary cause of the disease are cellular effects, for example, on microtubules. As indicated by post-translational tubulin modifications, haploinsufficiency results in an increase in microtubule stability (15,45,46,55–60),

whereas in HSP-SPG4 patient-derived neurons, a consistent decrease in microtubule stability has been observed (12,48,49). Our previous studies revealed that expression of pathogenic mutants of SPAST, especially the M1 isoforms, substantially reduce microtubule stability (22). Consistent with these latter findings as well as the studies on patient-derived cells, our mouse displays the opposite effect on microtubules as haploinsufficiency, and yet better aligns phenotypically with the symptoms of the disease. This is important therapeutically if the plan is to treat patients with microtubule-active drugs designed to restore the microtubule array to normal. Proponents of haploinsufficiency are using drugs such as vinblastine at very low dosage as treatment, with the idea that the drugs will 'destabilize' the microtubules that are presumed to be overly stable (45,47–49). Ironically, the drugs used at these levels are actually kinetic stabilizers of microtubules (61,62), and therefore might actually help alleviate the disease symptoms, but for the opposite reason that the investigators presume. Of course, the effects on microtubules are not the only potential mechanism of axonal degeneration, as spastin deficiencies and abnormalities have also been implicated in malfunctioning of the endoplasmic reticulum (63,64), lipid droplets (65), endosomes (66) and molecular motor proteins (67,68).

One of the best arguments in favor of a loss-of-function mechanism comes from work on *Drosophila*, in which it was demonstrated that flies partially depleted of spastin have seemingly normal axons, until those axons are amputated (16). Relative to controls, which regenerate fairly well, the spastin-deficient axons do not regenerate. Thus, even though the axons appear normal, they are more vulnerable to insult when spastin levels are reduced. If the same is true of vertebrate neurons, then the reduced functional spastin levels in HSP patients might make their corticospinal axons particularly vulnerable to the toxic effects of the mutant spastin proteins. Our present studies on cortical neurons cultured from our transgenic mouse demonstrate lysosomal transport deficiencies that are made worse, not better, by depletion of mouse spastin using siRNA. This result supports the view that haploinsufficiency exacerbates the cellular defects resulting from the gain-of-function toxicity of the mutant spastins. We are eager to explore other cellular defects to ascertain whether this same conclusion holds true, and we are also eager to cross our transgenic mouse with a knockout mouse, in order to ascertain whether the HSP-like symptoms are worsened.

Several lines of evidence from our laboratory indicate that mutant M1 is the cytotoxic culprit of the disease (20–22). Whether we are using cultured vertebrate neurons, cultured vertebrate non-neuronal cells, *Drosophila* neurons or squid axoplasm, introduction of mutant M87 is relatively harmless compared to introduction of mutant M1, which produces cytotoxic results. For both truncating mutations and C448Y mutation, the mutant M87 is as stable as its wild-type counterpart while the mutant M1 is more stable than its wild-type counterpart (20), suggesting that, at least in the case of these mutations, mutant M1 is not only more toxic than its M87 counterpart, but also less prone to degradation. Consistent with previous studies conducted in cell cultures, in our mouse, there is a higher proportion of M1 to M87 relative to wild-type. The question arises as to why no behavioral phenotype is observed in the mice (or most human patients) until adulthood, if even vanishingly low amounts of mutant M1 are sufficient to elicit cytotoxic effects, at least on organelle transport (21,24). In fact, we did observe organelle deficits in neurons from newborn SPAST^{C448Y} mice, which is consistent with our previous work. We suspect that

the severity of the cellular deficits is not sufficient to elicit a phenotype at the behavioral or anatomical levels until the animal reaches a certain age, at which time the cellular deficits achieve a level sufficient to elicit nerve degeneration, at least in the case of the corticospinal tracts. No other behavioral defects beyond those reported here were obvious, but we made no efforts to seek out other deficits, for example in cognition or memory (44) because our primary interests for now are anatomical and movement behavioral correlates of HSP.

A potential criticism of our previous studies is that the cytotoxic effects we documented in other model systems represent an overexpression phenotype that is not reflective of the true disease mechanism. The present results with the new transgenic mouse argue against that view. First of all, compared to many well-accepted murine disease models (69–77), our mouse expresses the human mutant protein at relatively modest levels. Moreover, the fact that the expression levels do not produce cellular defects strong enough to elicit obvious behavioral phenotypes until adulthood, and apparently not sufficient to produce disease in most tissues or organs of the body, argues against a non-specific overexpression phenotype. The present results are consistent with our previous studies indicating that vanishingly small amounts of mutant protein are sufficient to produce toxic effects at the cellular level (21). An accumulation of mutant protein in the distal region of the spinal cord has been observed in a human patient (17) and recapitulated in our mouse, in which the microtubule stability deficit is especially pronounced in the distal region of the cord. Collectively, our results are consistent with an accumulation of mutant M1 in the distal axons of the corticospinal tract causing the axonal degeneration observed in SPAST^{C448Y} mice, with haploinsufficiency contributing to the vulnerability of these axons to the toxicity of the mutant protein. Our mouse will be useful for further testing this conclusion and for screening potential treatments.

Materials and Methods

Animals

All animal experiments were performed in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011) and were reviewed and approved by the Institutional Animal Care and Use Committee at Drexel University College of Medicine. The animal work for this study was carried out under the project license 1045165, protocol no. 20601.

Transgenic mouse generation, breeding and genotyping

An illustration of the strategy used to generate the HSP-SGP4 mouse is shown in Figure 1A (for additional details, see figure legend and Results). A transgenic SPAST^{C448Y} mouse model that carries a point mutation in exon 11 of human full-length SPAST [c.1343g>a (p.Cys448Tyr), NM_014946.3, CCDS_1778.1] on a C57BL/6 genetic background was developed by genOway Inc. A CAG promoter-driven floxed (loxP-flanked) STOP cassette followed by a human SPAST coding sequence (CDS) with a C448Y mutation was inserted into the Gt(ROSA)26Sor locus to allow the expression of human SPAST to be dependent upon Cre recombinase. These mice were considered 'locked' SPAST^{C448Y} mice. In this study, we obtained 'unlocked' SPAST^{C448Y} heterozygous mice by mating a ubiquitous Cre mouse strain with the 'locked' SPAST^{C448Y} mice. The ubiquitous Cre line was developed by pronuclear injection of the cytomegalovirus (CMV)-Cre con-

struct by genOway. Therefore, all of the mutant mice used in this study were Cre-unlocked mice that expressed the mutant human spastin. We had initially planned to cross the locked mice with Cre mice that would specifically allow expression in the central nervous system, but this became unnecessary when the ubiquitous Cre mouse produced unlocked mice with behavioral and anatomical deficits remarkably consistent with HSP.

Mice were housed in their own cages in a mouse room that is on a reversed light/dark cycle (lights off 7 p.m. to 7 a.m. and lights on 7 a.m. to 7 p.m.) with free access to drinking water and food. Heterozygous constitutive SPAST^{C448Y}/- expressing mice were further interbred to generate wild-type, heterozygous (named SPAST^{C448Y}/-) and homozygous (named SPAST^{C448Y}/SPAST^{C448Y}) mice. The cohort of mice analyzed in the behavioral studies was made up of groups of wild-type, SPAST^{C448Y}/- and SPAST^{C448Y}/SPAST^{C448Y} littermates. P0 mice used for primary culture were sacrificed by decapitation (tail tissues were used for genotype analyses), and adult mice used for histological analyses, tracing experiments and western blot analyses were sacrificed by intraperitoneal injection of Euthasol solution 0.1 ml (catalog 50989056912, VEDCO, Greeley, Colorado) and dissected for tissue collection.

In order to identify the genotypes of the wild-type, SPAST^{C448Y}/- and SPAST^{C448Y}/SPAST^{C448Y} mice, a qRT-PCR was conducted. Briefly, small amounts of tissues were collected by one to two ear punches from littermates as they were weaned, or 1 mm long tail tips were used for genotyping newborn pups. The tissues were digested to obtain mouse gDNA using the EZ Tissue DNA Isolation Kit (catalog M1003, EZ BioResearch, St Louis, Missouri). For each mouse, 7.5 ng of gDNA was used to run a qRT-PCR, using iTaq Universal SYBR Green Supermix (catalog 1725120, BioRad, Hercules, California). qRT-PCR was carried out with a pair of primers that are specific for the human spastin (FW: 5'-AGCACAACTTGCTAGAATGACTG-3'; RV: 5'-AAGTTTGAGGGCTGACGCTG-3'), and IL-2 (FW: 5'-CTAGGCCACAGAATTGAAAGATCT-3'; RV: 5'-GTAGGTGGAAATTCTAGCATCATCC-3') was used as a control gene with two copies, in order to identify genotypes based on relative DNA content. The PCR reaction was carried out using the StepOne™ Real-Time PCR System (catalog 4376357, Applied Biosystems, Foster City, California). Genotypes were detected using LinRegPCR software version 5.1.1. A 1% agarose gel was then run at 80 V in order to visualize the bands relative to each genotype.

Behavioral examinations

For all of the behavioral examinations, mice were tested three times per week after they were 30 days old. Clinical status of animals was evaluated by scoring deficits in beam walk and hindlimb clasping (including measuring the splayed angles), examining resting tremors and measuring their weights.

Beam walk assay. Beam walk was ranked on a scale between 0 to 2, where mice were scored 0 when they walked across the beam without any pauses or foot faults, a score of 1 was given when they had one or two stops or foot faults when walking across the beam, and a score of 2 was given when they stopped more than twice or had more than two foot faults when walking across the beam or if they could not finish the walk at all. If the mice were scored as 2 for three consecutive trials over the course of 1 week, the age of the mice on the first trial was considered as the age of disease onset.

Resting tremor assay. For the resting tremor assay, mice were placed on the palm of the hand and allowed to acclimate to reduce tremor due to fear, and then scored as a YES if tremor

vibrations were felt or as a NO in the absence of tremor. Only the mice that exhibited resting tremor for 10 consecutive trials were considered positive due to the nature of variability for this assay. The onset age of the resting tremor was recorded as the age of the mice on the first trial of 10 consecutive trials that manifested resting tremor.

Hindlimb clasping assay. Hindlimb clasping was performed by lifting the mouse by its tail and examining the posture of the hindlimbs. Mice were ranked as 0 when they showed a perfect splay by raising the hindlimbs up to the horizontal line; they were ranked as 1 when their hindlimbs splayed below the horizontal line and ranked as 2 when they showed a complete inability to spread their hindlimbs outwards.

The age of disease onset was reported as the age of the mice on the first trial of three consecutive trials with scores of 1. In addition, hindlimb splayed angle values were determined by drawing two straight lines from the anus to each paw. Note that all of the onset ages were presented as average days $\pm$ S.E.M. **Weight.** We measured the weights of all of the mice once a week. The values were presented as the average weight $\pm$ S.E.M.

Immunohistochemistry on spinal cord cross sections

For histological studies, mice 180 days (90 days old mice were used for axonal morphological analyses using SMI312, see Fig. 3) or older were sacrificed via euthanasia by intraperitoneal injection of Euthasol solution at a dose of 0.1 ml. Transcardial perfusion was performed and 0.9% NaCl (catalog BP358-212, Fisher Bioreagent) was used to rinse out blood, followed by 4% paraformaldehyde (4% PFA, catalog 19202, Electron Microscopy Sciences, Hatfield, Pennsylvania) in 0.1 M phosphate buffered saline (PBS; catalog BP399-20, Fisher Bioreagent) for tissue fixation. Spinal cords were dissected out and left in 4% PFA overnight post-fixation followed by immersion in 15% and 30% sucrose (catalog BP220-212, Fisher Bioreagent) solutions sequentially before freezing the cords. Spinal cords were then embedded horizontally in M1 (catalog 1310, Thermo Scientific, Waltham, Massachusetts), cross-sectioned in 20 μ m thick sections and mounted on positively charged slides (catalog 12-550-15, Fisher Brand). Immunohistochemistry (IHC) was carried out on cross sections derived from lumbar levels. Briefly, sections for IHC were subjected to a quenching step for 2 h at room temperature to reduce the reaction of endogenous peroxidases, followed by a blocking step in 10% goat serum with 0.1% Triton X-100. Cross sections were stained for the neurofilament marker SMI312 (catalog 837904, BioLegend, San Diego, California) (1:500) and then Alexa488-conjugated secondary antibody (1:1000; catalog A11029, Life Technologies, Carlsband, California). Glass cover slips were mounted on microscope slides with Fluoro-Gel (catalog 17985-10, Electron Microscopy Sciences) to reduce fading of fluorescence, and then the samples were examined by a Leica TCS SP8 confocal microscope using a 63x oil-immersion objective. Quantifications were made by evaluating the fluorescence intensity per 100 μ m² in white and grey matter. Moreover, numbers of regular and irregular axons (representing axons sectioned in perfect cross section or skewed, respectively) were counted in three regions of interest: dorsal, ventral and lateral columns. The total number of axons per 100 μ m² in each region, as well as the percentages of regular and irregular axons within the total axons per 100 μ m² was quantified in each region. Hematoxylin and eosin staining was performed to evaluate basic histological structures in tissues from different organs. Tissues derived from the hearts, lungs, kidneys and livers in the mice of the three genotypes were collected after perfusion (see the

above procedure). Sections were air-dried to remove moisture and then stained with 0.1% Mayer's hematoxylin and 0.5% eosin sequentially. Glass cover slips were mounted on microscope slides using Cytoseal XYL (catalog 8312-16E, Thermo Fisher) and examined by a DM5500 B Leica microscope using a 10X objective.

For light microscopy, semi-thin sections were prepared from 1-year-old mice that had been sacrificed via euthanasia as described above. For this study the perfusion step was performed using a mixture of 2% PFA and 1.5% glutaraldehyde (catalog 16314, Electron Microscopy Sciences) in 0.1 M PBS. Fixed spinal cords from perfused animals were left at least one night in fixative solution before being processed for plastic embedding. Cords were then washed in 0.1 M phosphate buffer and post-fixed in 1% OSO₄ for 2 h, followed by sequential washes in 70%, 95% and 100% ethanol and two washes in propylene oxide (PO). At this point, cords were incubated for 1 h in a mixture of Epon:PO with a 1:1 dilution, and subsequently incubated for another hour in Epon:PO mixture at 2:1 dilution. Cords were finally embedded in capsules and allowed to polymerize for 72 h in a 60°C oven. 1 μ m sections from wild-type, SPAST^{C448Y}/- and SPAST^{C448Y}/SPAST^{C448Y} were stained with toluidine blue to identify the axons on the cross sections. Cross sections from cervical and lumbar level were both taken into consideration for the analyses and total numbers of axons within 100 μ m² were counted using Stereo Investigator software version 11 in dorsal, lateral and ventral columns.

Anterograde anatomical tracing to evaluate axonal degeneration

The strategy to label descending tracts in the spinal cord via rhodamine-labeled biotinylated dextran amine (BDA) (catalog 1956, Thermo Fisher) is shown in Figure 5A and B. Briefly, animals were subjected to 4% isoflurane to induce anesthesia and maintained at 2% isoflurane throughout the surgery. Spinal cords at the upper cervical level (C1/2) were surgically exposed in both wild-type and SPAST^{C448Y}/SPAST^{C448Y} mice that were 5 months old and were injected with BDA. Three injections of BDA with 0.5 μ l each were carried out for labeling dorsal, lateral and ventral columns using stereotaxic coordinates. Once the BDA was delivered, the muscles were sutured in layers and the skin was closed with wound clips. Animals were allowed to recover with daily care and close monitoring for 1 week. Four weeks after BDA injections, mice were sacrificed as described above, and 20 μ m thick cross sections from the cervical and lumbar levels were stained for SMI312. The analysis was conducted by counting the number of double-labeled axons (red for BDA and green for SMI312) in three regions of interest: the lateral, dorsal and ventral columns at both the cervical and lumbar levels. Graphs were then made depicting the ratio between the lumbar level counts to that of the cervical level and presented as the percentage relative to wild-type mice, which is taken as 100% $\pm$ S.E.M.

Western blot analysis to evaluate spastin levels and microtubule stability

Animals were euthanized as described followed by dissection of the cerebral cortices, cerebella, spinal cords, hearts, livers and kidneys at three different ages: P0 (except the spinal cord), P80 and P200. Dissected tissues were homogenized in ice-cold 1X RIPA buffer (catalog 89901, Thermo Fisher) in the presence of a phosphatase/protease inhibitor cocktail (catalog A32953 and A32957 for protease and phosphatase inhibitors respectively,

Pierce). Typically, 20–40 µg of tissues lysates was run on 4–15% C Bis/Tris gels (catalog 456-1084, BioRad) to perform an SDS-PAGE electrophoresis. Proteins were then transferred to a polyvinylidene difluoride membrane (catalog 1620177, BioRad), blocked with 5% non-fat dry milk (catalog M0841, LabScientific, Highlands, New Jersey) in 1X TBS containing 0.025% Tween-20 (TTBS) for 1 h at room temperature. Primary antibodies were diluted in 5% milk and incubated overnight at 4°C. After rinsing with TTBS three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies 1:5000 (Jackson Laboratory, Bar Harbor, Maine) at room temperature for 2 h, and proteins were visualized using enhanced chemiluminescence (catalog 34580, Thermo Fisher).

To examine the levels of spastin, which included both M1 and M85/M87, tissue lysates from cerebral cortices, cerebella, spinal cords, hearts, livers and kidneys from P0 (except the spinal cord), P80 and P200 mice for all three genotypes were homogenized and processed as described above, and then probed with spastin antibody 1:100 (catalog ab77144, Abcam, Cambridge, United Kingdom). Coomassie brilliant blue (catalog 1860957, Thermo Scientific) staining was used to normalize the amount of protein loaded. Protein samples from HEK293 cells that stably overexpressed full-length human spastin were used as indications of the molecular weights of M1 and M85/87. Graphs were made to show the amount of protein $\pm$ S.E.M. relative to the wild-type samples, taken as 1.

For microtubule stability studies, spinal cord lysates from P200 mice were divided into upper spinal cord (above T10–11) and lower spinal cord (below T10–11) segments and probed with β III-tubulin (1:1000; catalog 801202, BioLegend), acetylated tubulin (1:10,000; catalog T6793, Sigma Aldrich, St Louis, Missouri) and detyrosinated tubulin (1:500; catalog AB3201, Millipore, Burlington, Massachusetts). GAPDH (1:10,000; catalog ab8245, Abcam) was used as a loading control. Analyses were performed by evaluating the band intensities using Image Lab software version 5.2.1 provided by BioRad. Graphs were then made depicting the amount of β III-tubulin relative to control, taken as $1 \pm$ S.E.M. The levels of acetylated tubulin and detyrosinated tubulin were calculated as ratios to the level of β III-tubulin in the same sample. We considered the ratios in wild-type animals as 1, and the ratios in SPAST^{C448Y}/– and SPAST^{C448Y}/SPAST^{C448Y} were presented relative to those in the wild-type animals.

Primary cortical neuronal cultures and immunocytochemistry

Primary cortical neurons were isolated from P0 and P1 mouse pups and plated on poly-L-lysine (PLL; catalog P7280, Sigma Aldrich)-coated dishes in neurobasal medium supplemented with 5% fetal bovine serum (catalog SH30070.02, Hyclone, South Logan, Utah), 1% B27 (catalog 17504-044, Gibco), 1% glucose (catalog G8769-100ML, Sigma Aldrich) and 1% glutamax (35050-061, Gibco) (the procedure was adapted from Qiang et al. 2006 with some modifications) (78). Cortical neurons from each pup were plated as one cortex per dish followed by immediate genotyping. Neurons from the same genotype were grouped together after they were dissociated from the dishes by Accutase (catalog 07920, STEMCELL Technologies, Vancouver, Canada). A pool of four different siRNAs targeting mouse spastin (5'-UGUCUAAUGGUUGUGUAUCdTdT-3'; 5'-UGCUACCACAGAAUUAUCCdTdT-3'; 5'-AAUUGGCCUACAUCUACdTdT-3'; 5'-UAAACUAAUGGCAAAUUCdTdT-3'; custom designed by Sigma Aldrich), three siRNAs targeting human spastin (5'-AUUAAAGAAGGUUGCAUUCdTdT-3'; 5'-UGUACUAGGUGCUCUAU

GdTdT-3'; 5'-UAGUACUGUCAUUUAUAGACdTdT-3'; custom designed by Sigma Aldrich) or scramble siRNA (catalog SIC001, Sigma Aldrich), was delivered by electroporation into the corresponding genotypical groups using Amaxa Nucleofector II (catalog AAB-1001, Lonza, Basel, Switzerland). Neurons transfected with different siRNA were re-plated on plastic dishes for 4 days in order to reach the maximum effect of spastin knockdown in each group. Then, those cultured neurons from each group were either re-plated onto glass-bottomed dishes coated with PLL for morphology and vesicle transport experiments or collected and lysed in 1X RIPA buffer for western blotting analysis. Neurons that were re-plated were cultured for 2 days before being fixed in 4% PFA, 0.2% glutaraldehyde (catalog 50-262-19, Electron Microscopy Sciences) and 0.1% Triton X-100 (catalog X100-100ML, Sigma Aldrich) in 0.1% PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.9). The fixed cortical neurons were immunostained with mouse monoclonal β III-tubulin and goat anti-mouse secondary antibodies for morphological analyses. All of the images were acquired by a Zeiss inverted microscope with a 63X oil-immersion objective.

Lysosomal transport assay

The lysosomal transport study was performed on the final re-plated cortical neuronal cultures with various siRNA-treated groups in different genotypes, as well as neuronal cultures from SPAST^{C448Y}/SPAST^{C448Y} animals treated with a competitive casein kinase II inhibitor III, TBCA (catalog 218710, Calbiochem) at 20 µM for 24 h before live-cell imaging. LysoTracker Green DND-26 (100 µM; catalog L7526, Thermo Fisher) was used for bath incubation for 5 h prior to imaging. Cells were imaged on a Z1 Zeiss observer microscope using a 100X oil-immersion objective with 1.46 numerical aperture. Images were acquired every second for a total of 2 min to visualize lysosomal movements. Kymographs were generated using ImageJ software for quantification of retrograde and halted movements, as well as stationary lysosomes.

Statistical analyses

Behavioral tests were conducted as blinded experiments. During the scoring, the researcher did not know the genotype of the mice until the final analysis of the data.

GraphPad (Prism 7, GraphPad software) or MS Excel was used to perform statistical tests and plot results. Data are presented as mean $\pm$ S.E.M. Whisker box plot represents the distribution of the data. The middle line indicates the median. The box extends from 25th to 75th percentiles. Upper and lower Whiskers represent the maximum and the minimum of the data. All data sets were tested for normality using Shapiro–Wilk's test. Statistical significance was evaluated by analysis of variance (ANOVA), Tukey honestly significance difference (HSD) post hoc test or by the Student's t-test (two-tailed). A $P < 0.05$ or a $P < 0.001$ was considered significant.

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We thank Dr Joanna M. Solowska (of Drexel University) for invaluable input during the early stages of planning the new

transgenic mouse and Dr Kenny Simansky (of Drexel University) for his unfailing support of the project since its inception.

Conflict of Interest statement. None declared.

Funding

National Institutes of Health (NS28785 to P.W.B.); Spastic Paraplegia Foundation (to P.W.B.); Pennsylvania Department of Health CURE program via Drexel University College of Medicine (4100062203 to P.W.B.); Craig H. Neilsen Foundation (381793 to L.Q., 338432 to M.A.L.); ALS Hope Foundation (to T.D.H.); Spastic Paraplegia Foundation (to G.M.); National Institute of Health (NS081112 to M.A.L.); Tom Wahlig Foundation (2017 Advanced Scholarship for Research into Hereditary Spastic Paraplegia and Related Diseases to P.W.B.); Drexel Dean's Fellowship for Excellence in Collaborative Research Training (to L.L., L.V.Z.).

References

- McDermott, C., White, K., Bushby, K. and Shaw, P. (2000) Hereditary spastic paraparesis: a review of new developments. *J. Neurol. Neurosurg. Psychiatry*, **69**, 150–160.
- Denton, K.R., Xu, C., Shah, H. and Li, X.J. (2016) Modeling axonal defects in hereditary spastic paraplegia with human pluripotent stem cells. *Front. Biol. (Beijing)*, **11**, 339–354.
- Hazan, J., Fonknechten, N., Mavel, D., Paternotte, C., Samson, D., Artiguenave, F., Davoine, C.S., Cruaud, C., Durr, A., Wincker, P. et al. (1999) Spastin, a new AAA protein, is altered in the most frequent form of autosomal dominant spastic paraplegia. *Nat. Genet.*, **23**, 296–303.
- Fonknechten, N., Mavel, D., Byrne, P., Davoine, C.S., Cruaud, C., Bonsch, D., Samson, D., Coutinho, P., Hutchinson, M., McMonagle, P. et al. (2000) Spectrum of SPG4 mutations in autosomal dominant spastic paraplegia. *Hum. Mol. Genet.*, **9**, 637–644.
- Burger, J., Fonknechten, N., Hoeltzenbein, M., Neumann, L., Bratanoff, E., Hazan, J. and Reis, A. (2000) Hereditary spastic paraplegia caused by mutations in the SPG4 gene. *Eur. J. Hum. Genet.*, **8**, 771–776.
- Depienne, C., Stevanin, G., Brice, A. and Durr, A. (2007) Hereditary spastic paraplegias: an update. *Curr. Opin. Neurol.*, **20**, 674–680.
- Boone, P.M., Yuan, B., Campbell, I.M., Scull, J.C., Withers, M.A., Baggett, B.C., Beck, C.R., Shaw, C.J., Stankiewicz, P., Moretti, P. et al. (2014) The Alu-rich genomic architecture of SPAST predisposes to diverse and functionally distinct disease-associated CNV alleles. *Am. J. Hum. Genet.*, **95**, 143–161.
- Julien, C., Lissouba, A., Madabattula, S., Fardghassemi, Y., Rosenfelt, C., Androschuk, A., Strautman, J., Wong, C., Bysice, A., O'Sullivan, J. et al. (2016) Conserved pharmacological rescue of hereditary spastic paraplegia-related phenotypes across model organisms. *Hum. Mol. Genet.*, **25**, 1088–1099.
- Newton, R., Allison, R., Edgar, J.R., Lumb, J.H., Rodger, C.E., Manna, P.T., Rizo, T., Kohl, Z., Nygren, A.O.H., Arning, L. et al. (2018) Mechanistic basis of an epistatic interaction reducing age at onset in hereditary spastic paraplegia. *Brain*, **141**, 1286–1299.
- Kawarai, T., Montecchiani, C., Miyamoto, R., Gaudiello, F., Caltagirone, C., Izumi, Y., Kaji, R. and Orlacchio, A. (2017) Spastic paraplegia type 4: a novel SPAST splice site donor mutation and expansion of the phenotype variability. *J. Neurol. Sci.*, **380**, 92–97.
- Svensson, I.K., Ashley-Koch, A.E., Gaskell, P.C., Riney, T.J., Cumming, W.J., Kingston, H.M., Hogan, E.L., Boustany, R.M., Vance, J.M., Nance, M.A., Pericak-Vance, M.A. and Marchuk, D.A. (2001) Identification and expression analysis of spastin gene mutations in hereditary spastic paraplegia. *Am. J. Hum. Genet.*, **68**, 1077–1085.
- Abrahamsen, G., Fan, Y., Matigian, N., Wali, G., Bellette, B., Sutharsan, R., Raju, J., Wood, S.A., Veivers, D., Sue, C.M. and Mackay-Sim, A. (2013) A patient-derived stem cell model of hereditary spastic paraplegia with SPAST mutations. *Dis. Model. Mech.*, **6**, 489–502.
- Abrahamsen, G., Fan, Y., Matigian, N., Wali, G., Bellette, B., Sutharsan, R., Raju, J., Wood, S.A., Veivers, D., Sue, C.M. and Mackay-Sim, A. (2015) A patient-derived stem cell model of hereditary spastic paraplegia with SPAST mutations. *Dis. Model. Mech.*, **8**, 1339.
- Havlicek, S., Kohl, Z., Mishra, H.K., Prots, I., Eberhardt, E., Denguir, N., Wend, H., Plotz, S., Boyer, L., Marchetto, M.C. et al. (2014) Gene dosage-dependent rescue of HSP neurite defects in SPG4 patients' neurons. *Hum. Mol. Genet.*, **23**, 2527–2541.
- Riano, E., Martignoni, M., Mancuso, G., Cartelli, D., Crippa, F., Toldo, I., Siciliano, G., Di Bella, D., Taroni, F., Bassi, M.T., Cappelletti, G. and Rugarli, E.I. (2009) Pleiotropic effects of spastin on neurite growth depending on expression levels. *J. Neurochem.*, **108**, 1277–1288.
- Stone, M.C., Rao, K., Gheres, K.W., Kim, S., Tao, J., La Rochelle, C., Folker, C.T., Sherwood, N.T. and Rolls, M.M. (2012) Normal spastin gene dosage is specifically required for axon regeneration. *Cell Rep.*, **2**, 1340–1350.
- Solowska, J.M., Garbern, J.Y. and Baas, P.W. (2010) Evaluation of loss of function as an explanation for SPG4-based hereditary spastic paraplegia. *Hum. Mol. Genet.*, **19**, 2767–2779.
- Kasher, P.R., De Vos, K.J., Wharton, S.B., Manser, C., Bennett, E.J., Bingley, M., Wood, J.D., Milner, R., McDermott, C.J., Miller, C.C., Shaw, P.J. and Grierson, A.J. (2009) Direct evidence for axonal transport defects in a novel mouse model of mutant spastin-induced hereditary spastic paraplegia (HSP) and human HSP patients. *J. Neurochem.*, **110**, 34–44.
- Tarrade, A., Fassier, C., Courageot, S., Charvin, D., Vitte, J., Peris, L., Thorel, A., Mouisel, E., Fonknechten, N., Roblot, N. et al. (2006) A mutation of spastin is responsible for swellings and impairment of transport in a region of axon characterized by changes in microtubule composition. *Hum. Mol. Genet.*, **15**, 3544–3558.
- Solowska, J.M., Rao, A.N. and Baas, P.W. (2017) Truncating mutations of SPAST associated with hereditary spastic paraplegia indicate greater accumulation and toxicity of the M1 isoform of spastin. *Mol. Biol. Cell*, **28**, 1728–1737.
- Leo, L., Weissmann, C., Burns, M., Kang, M., Song, Y., Qiang, L., Brady, S.T., Baas, P.W. and Morfini, G. (2017) Mutant spastin proteins promote deficits in axonal transport through an isoform-specific mechanism involving casein kinase 2 activation. *Hum. Mol. Genet.*, **26**, 2321–2334.
- Solowska, J.M., D'Rozario, M., Jean, D.C., Davidson, M.W., Marendra, D.R. and Baas, P.W. (2014) Pathogenic mutation of spastin has gain-of-function effects on microtubule dynamics. *J. Neurosci.*, **34**, 1856–1867.
- Solowska, J.M. and Baas, P.W. (2015) Hereditary spastic paraplegia SPG4: what is known and not known about the disease. *Brain*, **138**, 2471–2484.
- Solowska, J.M., Morfini, G., Fahnrikar, A., Himes, B.T., Brady, S.T., Huang, D. and Baas, P.W. (2008) Quantitative and functional analyses of spastin in the nervous system: impli-

- cations for hereditary spastic paraplegia. *J. Neurosci.*, **28**, 2147–2157.
25. Ugalde, C.L., Finkelstein, D.I., Lawson, V.A. and Hill, A.F. (2016) Pathogenic mechanisms of prion protein, amyloid- β and α -synuclein misfolding: the prion concept and neurotoxicity of protein oligomers. *J. Neurochem.*, **139**, 162–180.
 26. Winklhofer, K.F., Tatzelt, J. and Haass, C. (2008) The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *EMBO J.*, **27**, 336–349.
 27. Claudiani, P., Riano, E., Errico, A., Andolfi, G. and Rugarli, E.I. (2005) Spastin subcellular localization is regulated through usage of different translation start sites and active export from the nucleus. *Exp. Cell Res.*, **309**, 358–369.
 28. Kozak, M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. *Gene*, **299**, 1–34.
 29. White, S.R., Evans, K.J., Lary, J., Cole, J.L. and Luring, B. (2007) Recognition of C-terminal amino acids in tubulin by pore loops in Spastin is important for microtubule severing. *J. Cell Biol.*, **176**, 995–1005.
 30. Salinas, S., Proukakis, C., Crosby, A. and Warner, T.T. (2008) Hereditary spastic paraplegia: clinical features and pathogenic mechanisms. *Lancet Neurol.*, **7**, 1127–1138.
 31. Stevanin, G., Ruberg, M. and Brice, A. (2008) Recent advances in the genetics of spastic paraplegias. *Curr. Neurol. Neurosci. Rep.*, **8**, 198–210.
 32. Fink, J.K., Heiman-Patterson, T., Bird, T., Cambi, F., Dube, M.P., Figlewicz, D.A., Fink, J.K., Haines, J.L., Heiman-Patterson, T., Hentati, A. et al. (1996) Hereditary spastic paraplegia: advances in genetic research. Hereditary spastic paraplegia working group. *Neurology*, **46**, 1507–1514.
 33. Abou-Donia, M.B., Goldstein, L.B., Jones, K.H., Abdel-Rahman, A.A., Damodaran, T.V., Dechkovskaia, A.M., Bullman, S.L., Amir, B.E. and Khan, W.A. (2001) Locomotor and sensorimotor performance deficit in rats following exposure to pyridostigmine bromide, DEET, and permethrin, alone and in combination. *Toxicol. Sci.*, **60**, 305–314.
 34. Capoccia, S., Maccarinelli, F., Buffoli, B., Rodella, L.F., Cremona, O., Arosio, P. and Cirulli, F. (2015) Behavioral characterization of mouse models of neuroferritinopathy. *PLoS One*, **10**, e0118990.
 35. Carter, R.J., Morton, J. and Dunnett, S.B. (2001) Motor coordination and balance in rodents. *Curr. Protoc. Neurosci.*, **Chapter 8**, Unit, **8**, 12.
 36. Luong, T.N., Carlisle, H.J., Southwell, A. and Patterson, P.H. (2011) Assessment of motor balance and coordination in mice using the balance beam. *J. Vis. Exp.*, **10**.
 37. Heiman-Patterson, T.D., Blankenhorn, E.P., Sher, R.B., Jiang, J., Welsh, P., Dixon, M.C., Jeffrey, J.I., Wong, P., Cox, G.A. and Alexander, G.M. (2015) Genetic background effects on disease onset and lifespan of the mutant dynactin p150Glued mouse model of motor neuron disease. *PLoS One*, **10**, e0117848.
 38. Puschmann, A. and Wszolek, Z.K. (2011) Diagnosis and treatment of common forms of tremor. *Semin. Neurol.*, **31**, 65–77.
 39. Starling, A., Rocco, P., Passos-Bueno, M.R., Hazan, J., Marie, S.K. and Zatz, M. (2002) Autosomal dominant (AD) pure spastic paraplegia (HSP) linked to locus SPG4 affects almost exclusively males in a large pedigree. *J. Med. Genet.*, **39**, e77.
 40. Lo Giudice, T., Lombardi, F., Santorelli, F.M., Kwarai, T. and Orlandi, A. (2014) Hereditary spastic paraplegia: clinical-genetic characteristics and evolving molecular mechanisms. *Exp. Neurol.*, **261**, 518–539.
 41. Dale, J.M. and Garcia, M.L. (2012) Neurofilament phosphorylation during development and disease: which came first, the phosphorylation or the accumulation? *J. Amino Acids*, **2012**, 382107.
 42. Nagaraja, T.N., Gourie-Devi, M., Nalini, A. and Raju, T.R. (1994) Neurofilament phosphorylation is enhanced in cultured chick spinal cord neurons exposed to cerebrospinal fluid from amyotrophic lateral sclerosis patients. *Acta Neuropathol.*, **88**, 349–352.
 43. Sengul, G. and Watson, C. (2012) In Watson, C., Paxinos, G. and Puelles, L. (eds), *The Mouse Nervous System*. Academic Press, Elsevier Science & Technology, pp. 424–458.
 44. Chelban, V., Tucci, A., Lynch, D.S., Polke, J.M., Santos, L., Jonvik, H., Groppa, S., Wood, N.W. and Houlden, H. (2017) Truncating mutations in SPAST patients are associated with a high rate of psychiatric comorbidities in hereditary spastic paraplegia. *J. Neurol. Neurosurg. Psychiatry*, **88**, 681–687.
 45. Fassier, C., Tarrade, A., Peris, L., Courageot, S., Mailly, P., Dalard, C., Delga, S., Roblot, N., Lefevre, J., Job, D. et al. (2013) Microtubule-targeting drugs rescue axonal swellings in cortical neurons from spastin knockout mice. *Dis. Model. Mech.*, **6**, 72–83.
 46. Orso, G., Martinuzzi, A., Rossetto, M.G., Sartori, E., Feany, M. and Daga, A. (2005) Disease-related phenotypes in a Drosophila model of hereditary spastic paraplegia are ameliorated by treatment with vinblastine. *J. Clin. Invest.*, **115**, 3026–3034.
 47. Denton, K.R., Lei, L., Grenier, J., Rodionov, V., Blackstone, C. and Li, X.J. (2014) Loss of spastin function results in disease-specific axonal defects in human pluripotent stem cell-based models of hereditary spastic paraplegia. *Stem Cells*, **32**, 414–423.
 48. Fan, Y., Wali, G., Sutharsan, R., Bellette, B., Crane, D.I., Sue, C.M. and Mackay-Sim, A. (2014) Low dose tubulin-binding drugs rescue peroxisome trafficking deficit in patient-derived stem cells in hereditary spastic paraplegia. *Biol. Open*, **3**, 494–502.
 49. Wali, G., Sutharsan, R., Fan, Y., Stewart, R., Tello Velasquez, J., Sue, C.M., Crane, D.I. and Mackay-Sim, A. (2016) Mechanism of impaired microtubule-dependent peroxisome trafficking and oxidative stress in SPAST-mutated cells from patients with hereditary spastic paraplegia. *Sci. Rep.*, **6**, 27004.
 50. Roll-Mecak, A. and Vale, R.D. (2008) Structural basis of microtubule severing by the hereditary spastic paraplegia protein spastin. *Nature*, **451**, 363–367.
 51. Eckert, T., Link, S., Le, D.T., Sobczak, J.P., Gieseke, A., Richter, K. and Woehlke, G. (2012) Subunit interactions and cooperativity in the microtubule-severing AAA ATPase spastin. *J. Biol. Chem.*, **287**, 26278–26290.
 52. Yu, W., Qiang, L., Solowska, J.M., Karabay, A., Korulu, S. and Baas, P.W. (2008) The microtubule-severing proteins spastin and katanin participate differently in the formation of axonal branches. *Mol. Biol. Cell*, **19**, 1485–1498.
 53. Connell, J.W., Allison, R. and Reid, E. (2016) Quantitative gait analysis using a motorized treadmill system sensitively detects motor abnormalities in mice expressing ATPase defective spastin. *PLoS One*, **11**, e0152413.
 54. Pouladi, M.A., Morton, A.J. and Hayden, M.R. (2013) Choosing an animal model for the study of Huntington's disease. *Nat. Rev. Neurosci.*, **14**, 708–721.
 55. Baas, P.W., Vidya Nadar, C. and Myers, K.A. (2006) Axonal transport of microtubules: the long and short of it. *Traffic*, **7**, 490–498.

56. Errico, A., Ballabio, A. and Rugarli, E.I. (2002) Spastin, the protein mutated in autosomal dominant hereditary spastic paraplegia, is involved in microtubule dynamics. *Hum. Mol. Genet.*, **11**, 153–163.
57. Evans, K.J., Gomes, E.R., Reisenweber, S.M., Gundersen, G.G. and Luring, B.P. (2005) Linking axonal degeneration to microtubule remodeling by spastin-mediated microtubule severing. *J. Cell Biol.*, **168**, 599–606.
58. Lacroix, B., van Dijk, J., Gold, N.D., Guizetti, J., Aldrian-Herrada, G., Rogowski, K., Gerlich, D.W. and Janke, C. (2010) Tubulin polyglutamylation stimulates spastin-mediated microtubule severing. *J. Cell Biol.*, **189**, 945–954.
59. Roll-Mecak, A. and Vale, R.D. (2005) The Drosophila homologue of the hereditary spastic paraplegia protein, spastin, severs and disassembles microtubules. *Curr. Biol.*, **15**, 650–655.
60. Trotta, N., Orso, G., Rossetto, M.G., Daga, A. and Broadie, K. (2004) The hereditary spastic paraplegia gene, spastin, regulates microtubule stability to modulate synaptic structure and function. *Curr. Biol.*, **14**, 1135–1147.
61. Castle, B.T., McCubbin, S., Prah, L.S., Bernens, J.N., Sept, D. and Odde, D.J. (2017) Mechanisms of kinetic stabilization by the drugs paclitaxel and vinblastine. *Mol. Biol. Cell*, **28**, 1238–1257.
62. Baas, P.W. and Ahmad, F.J. (2013) Beyond taxol: microtubule-based treatment of disease and injury of the nervous system. *Brain*, **136**, 2937–2951.
63. Blackstone, C., O’Kane, C.J. and Reid, E. (2011) Hereditary spastic paraplegias: membrane traffic and the motor pathway. *Nat. Rev. Neurosci.*, **12**, 31–42.
64. Park, S.H., Zhu, P.P., Parker, R.L. and Blackstone, C. (2010) Hereditary spastic paraplegia proteins REEP1, spastin, and atlastin-1 coordinate microtubule interactions with the tubular ER network. *J. Clin. Invest.*, **120**, 1097–1110.
65. Papadopoulos, C., Orso, G., Mancuso, G., Herholz, M., Gumeni, S., Tadepalle, N., Jungst, C., Tzschichholz, A., Schauss, A., Honing, S. et al. (2015) Spastin binds to lipid droplets and affects lipid metabolism. *PLoS Genet.*, **11**, e1005149.
66. Allison, R., Edgar, J.R., Pearson, G., Rizo, T., Newton, T., Gunther, S., Berner, F., Hague, J., Connell, J.W., Winkler, J. et al. (2017) Defects in ER-endosome contacts impact lysosome function in hereditary spastic paraplegia. *J. Cell Biol.*, **216**, 1337–1355.
67. Reid, E. (2003) Science in motion: common molecular pathological themes emerge in the hereditary spastic paraplegias. *J. Med. Genet.*, **40**, 81–86.
68. Reid, E., Kloos, M., Ashley-Koch, A., Hughes, L., Bevan, S., Svenson, I.K., Graham, F.L., Gaskell, P.C., Dearlove, A., Pericak-Vance, M.A., Rubinsztein, D.C. and Pericak-Marchuk, D.A. (2002) A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10). *Am. J. Hum. Genet.*, **71**, 1189–1194.
69. Sturchler-Pierrat, C., Abramowski, D., Duke, M., Wiederhold, K.H., Mistl, C., Rothacher, S., Ledermann, B., Burki, K., Frey, P., Paganetti, P.A. et al. (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proc. Natl. Acad. Sci. U. S. A.*, **94**, 13287–13292.
70. Hsiao, K.K., Borchelt, D.R., Olson, K., Johannsdottir, R., Kitt, C., Yunis, W., Xu, S., Eckman, C., Younkin, S., Price, D. et al. (1995) Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer amyloid precursor proteins. *Neuron*, **15**, 1203–1218.
71. Borchelt, D.R., Ratovitski, T., van Lare, J., Lee, M.K., Gonzales, V., Jenkins, N.A., Copeland, N.G., Price, D.L. and Sisodia, S.S. (1997) Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron*, **19**, 939–945.
72. Dyrks, T., Dyrks, E., Masters, C.L. and Beyreuther, K. (1993) Amyloidogenicity of rodent and human beta A4 sequences. *FEBS Lett.*, **324**, 231–236.
73. Brion, J.P., Trempe, G. and Octave, J.N. (1999) Transgenic expression of the shortest human tau affects its compartmentalization and its phosphorylation as in the pretangle stage of Alzheimer’s disease. *Am. J. Pathol.*, **154**, 255–270.
74. Gotz, J., Probst, A., Spillantini, M.G., Schafer, T., Jakes, R., Burki, K. and Goedert, M. (1995) Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. *EMBO J.*, **14**, 1304–1313.
75. Sotiropoulos, E., Vassilatis, D.K., Vila, M. and Stefanis, L. (2010) Selective noradrenergic vulnerability in α -synuclein transgenic mice. *Neurobiol. Aging*, **31**, 2103–2114.
76. Oaks, A.W., Frankfurt, M., Finkelstein, D.I. and Sidhu, A. (2013) Age-dependent effects of A53T α -synuclein on behavior and dopaminergic function. *PLoS One*, **8**, e60378.
77. Paumier, K.L., Sukoff Rizzo, S.J., Berger, Z., Chen, Y., Gonzales, C., Kaftan, E., Li, L., Lotarski, S., Monaghan, M., Shen, W. et al. (2013) Behavioral characterization of A53T mice reveals early and late stage deficits related to Parkinson’s disease. *PLoS One*, **8**, e70274.
78. Qiang, L., Yu, W., Andreadis, A., Luo, M. and Baas, P.W. (2006) Tau protects microtubules in the axon from severing by katanin. *J. Neurosci.*, **26**, 3120–3129.