

REVIEW ARTICLE**Hereditary spastic paraplegia SPG4: what is known and not known about the disease****Joanna M. Solowska and Peter W. Baas**

Mutations in more than 70 distinct loci and more than 50 mutated gene products have been identified in patients with hereditary spastic paraplegias, a diverse group of neurological disorders characterized predominantly, but not exclusively, by progressive lower limb spasticity and weakness resulting from distal degeneration of corticospinal tract axons. Mutations in the *SPAST* (previously known as *SPG4*) gene that encodes the microtubule-severing protein called spastin, are the most common cause of the disease. The aetiology of the disease is poorly understood, but partial loss of microtubule-severing activity resulting from inactivating mutations in one *SPAST* allele is the most postulated explanation. Microtubule severing is important for regulating various aspects of the microtubule array, including microtubule number, length, and mobility. In addition, higher numbers of dynamic plus-ends of microtubules, resulting from microtubule-severing events, may play a role in endosomal tubulation and fission. Even so, there is growing evidence that decreased severing of microtubules does not fully explain HSP-SPG4. The presence of two translation initiation codons in *SPAST* allows synthesis of two spastin isoforms: a full-length isoform called M1 and a slightly shorter isoform called M87. M87 is more abundant in both neuronal and non-neuronal tissues. Studies on rodents suggest that M1 is only readily detected in adult spinal cord, which is where nerve degeneration mainly occurs in humans with HSP-SPG4. M1, due to its hydrophobic N-terminal domain not shared by M87, may insert into endoplasmic reticulum membrane, and together with reticulons, atlastin and REEP1, may play a role in the morphogenesis of this organelle. Some mutated spastins may act in dominant-negative fashion to lower microtubule-severing activity, but others have detrimental effects on neurons without further lowering microtubule severing. The observed adverse effects on microtubule dynamics, axonal transport, endoplasmic reticulum, and endosomal trafficking are likely caused not only by diminished severing of microtubules, but also by neurotoxicity of mutant spastin proteins, chiefly M1. Some large deletions in *SPAST* might also affect the function of adjacent genes, further complicating the aetiology of the disease.

Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, USA

Correspondence to: P. W. Baas, Ph.D.,
Department of Neurobiology and Anatomy,
Drexel University College of Medicine,
2900 Queen Lane, Philadelphia, PA 19129 USA
E-mail: pbaas@drexelmed.edu

Keywords: hereditary spastic paraplegia; *SPAST*; spastin; microtubule endoplasmic reticulum; endosome

Abbreviations: AAA = ATPase associated with various cellular activities; HSP = hereditary spastic paraplegia

Introduction

Hereditary spastic paraplegia (HSPs) are inherited disorders with a prevalence of 1.8/100 000 in most populations (Ruano *et al.*, 2014). Progressive lower limb spasticity

and weakness are the predominant but not exclusive manifestations of the disease. Limited post-mortem studies on HSP patients have consistently revealed distal-end degeneration of ascending sensory fibres and corticospinal tract axons. Corticospinal axons descending from the large

pyramidal neurons in layer V of motor cortex can include some of the longest CNS axons and tend to be vulnerable to upper motor neuron diseases. While some corticospinal axons synapse directly with lower motor neurons, most establish synapses with spinal interneurons that form connections with lower motor neurons innervating skeletal muscles. Even late in the progression of HSPs, there is generally very little neuronal cell death, which is consistent with the pathology being one of nerve degeneration. Mutations in more than 70 distinct loci (*SPG1–72*) and more than 50 mutated gene products have been identified in patients with HSPs (Lo Giudice *et al.*, 2014; Novarino *et al.*, 2014). While they have diverse functions, proteins encoded by HSP genes cluster within a small number of predicted cellular activities such as membrane traffic and organelle shaping, mitochondria regulation, myelination and lipid/sterol modification, axonal path-finding and axonal transport. Whether or not mutations in these various genes produce HSP pathology by a common, similar or different mechanism remains unknown. The clinical presentations and cellular pathways of HSPs have been previously reviewed (Salinas *et al.*, 2008; Blackstone, 2012; Fink, 2013; Lo Giudice *et al.*, 2014).

Mutations in the *SPAST* gene (located on 2p22.3), which encodes for an enzyme called spastin, are the most common cause of HSP and, depending upon the ethnic background of patients, account for 15–40% of all HSP cases (Hazan *et al.*, 1999; Fonknechten *et al.*, 2000; Shoukier *et al.*, 2009). Autosomal-dominant HSP-SPG4 in most cases is considered a prototypical ‘pure’ or ‘uncomplicated’ HSP with gait impairment due to spasticity and weakness of the lower extremities (each of variable degree and age-of-onset), but without loss of function in the upper limbs or diminished life expectancy. In some cases, HSP-SPG4 patients also manifest cognitive impairment (Orlacchio *et al.*, 2004; Shoukier *et al.*, 2009), cerebellar ataxia (Nielsen *et al.*, 2004), thin corpus callosum (Orlacchio *et al.*, 2004) or lower motor neuron dysfunction (McDermott *et al.*, 2006), and therefore their syndromes are classified clinically as ‘complicated.’ The key characteristics of HSP-SPG4 are summarized in Table 1.

Structure and function of spastin, the protein encoded by *SPAST*

The *SPAST* gene spans the region of ~90 kb of genomic DNA and contains 17 exons. Human spastin, encoded by *SPAST*, is a member of the AAA (ATPase associated with various cellular activities) protein family. The domain organization of spastin is presented in Fig. 1. The spastin AAA cassette contains three conserved ATPase domains, including Walker motif A (amino acids GPPGNGKT in positions 382–389) corresponding to the ATP-binding domain, Walker motif B (amino acids IIFIDE in positions

437–442), and the AAA minimal consensus sequence in position 480–498 (Hazan *et al.*, 1999). Spastin open reading frame (1848 base pairs) flanked by 5’UTR and 3’UTR has two initiation codons (Claudiani *et al.*, 2005). A weak Kozak sequence tgaATGa surrounding the M1 initiation codon deviates considerably from a good consensus sequence g(a)ccATGg and leads to leaky scanning of the first AUG. A better Kozak sequence ctcATGg is present at the M87 initiation codon (Kozak, 2002; Claudiani *et al.*, 2005). As a result, a 616 amino acid (68 kDa) isoform called M1 and a 530 amino acid (60 kDa) isoform called M87 are synthesized simultaneously but at different levels, as depicted in Fig. 1C. In rodents, the shorter isoform is the predominant spastin isoform in all tissues at all stages of development, whereas M1 is only detectably present in adult spinal cord (Solowska *et al.*, 2008). Similarly, analysis of spastin expression in adult human CNS has revealed the presence of M87 both in spinal cord and cerebral cortex, whereas significant levels of M1 were detected only in spinal cord and not in brain (Solowska *et al.*, 2010). Two additional spastin isoforms might be generated as a result of alternative mRNA splicing of exon 4 (Fig. 1C) (Svenson *et al.*, 2001; Claudiani *et al.*, 2005).

Spastin is a microtubule-severing ATPase that breaks longer microtubules into shorter ones and thereby regulates the number and mobility of microtubules and the distribution of their dynamic plus-ends (Errico *et al.*, 2002; Evans *et al.*, 2005; Roll-Mecak and Vale, 2005; Baas *et al.*, 2006). To sever microtubules, spastin assembles into hexamers that dock on microtubules and break them by tugging the negatively charged C-terminal of tubulin through the central pore of the hexamer (White *et al.*, 2007; Roll-Mecak and Vale, 2008). Polyglutamylation of tubulin stimulates spastin-mediated severing most likely because of an increase in negative charge of the microtubule tails (Lacroix *et al.*, 2010). Because modifications such as polyglutamylation tend to correspond to microtubule stability, spastin has a stronger proclivity for severing in the stable region of axonal microtubules as opposed to the more labile/dynamic region situated toward the microtubule’s plus-end. Consistent with this, knockdown of spastin tends to shift the microtubule mass of the axon toward a higher proportion of the more stable regions (Riano *et al.*, 2009).

Figure 2 illustrates schematically the interactions of spastin functional domains with other proteins involved in different cellular activities. Functional analyses of truncated spastin cDNAs have revealed that the microtubule-binding domain (MTBD) situated between residues 270–328, and ATPase AAA domain, spanning residues 342–599, are sufficient for hexamerization and microtubule severing (White *et al.*, 2007). The microtubule interacting and trafficking (MIT) domain, spanning residues 116–194, is required for interaction with two endosomal sorting complex required for transport III (ESCRT-III) proteins, charged multivesicular body protein 1B (CHMP1B) and increased sodium tolerance (IST1) (Reid *et al.*, 2005; Yang *et al.*, 2008;

Table 1 Clinical characteristics of HSP-SPG4

Affected gene	<i>SPAST</i> (previously known as <i>SPG-4</i>) mapped to 2p22.3
Protein	Spastin, a microtubule-severing ATP-ase
Mutations	Missense, nonsense, splice site, insertions, small and large deletions
Inheritance	Autosomal dominant
Clinical presentation	Uncomplicated HSP, in some cases late onset of cognitive impairment. No genotype–phenotype correlation.
Genetic penetrance	Age dependent, as high as 80–90%
Age of onset	Infancy through senescence. Average age of onset 29 ± 17 years.
Functional impairment	Highly variable from slight stiffness in the legs to wheelchair bound.
Progression	Faster in patients with late-onset of the disease

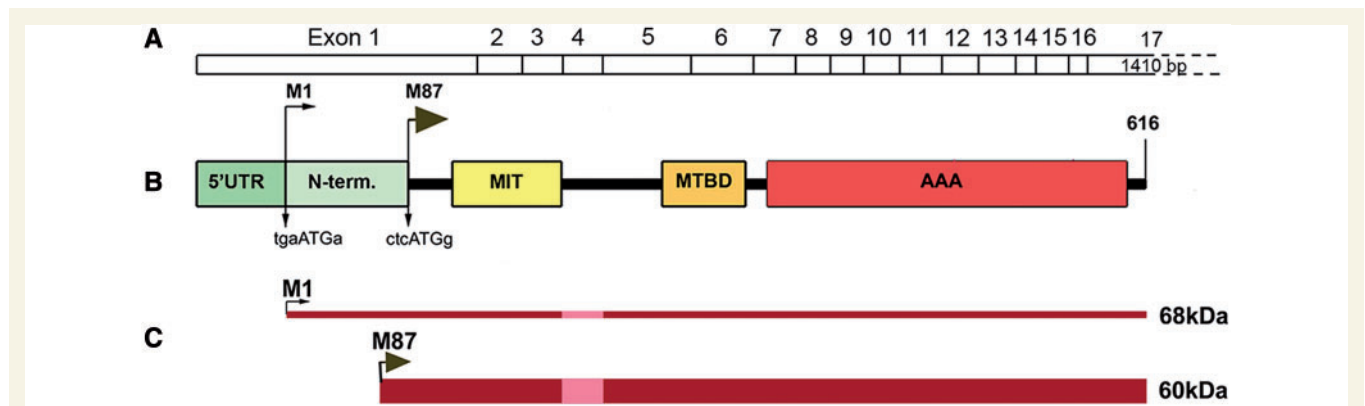


Figure 1 Schematic representation of spastin structure. (A) Spastin exons 1–17. (B) Spastin functional domains: N-term = N-terminal sequence present only in M1 spastin isoform; MIT = microtubule interacting and trafficking domain; MTBD = microtubule-binding domain; AAA = ATPase associated with various cellular activities. The Kozak's sequence tgaAUGa surrounding M1 start codon deviates considerably from a good consensus sequence g(a)ccAUGg. A better Kozak sequence ctcAUGg is present at the M87 initiation codon. (C) A leaky scanning of the first initiation codon with a poor Kozak's sequence leads to a preferred initiation of translation at the second AUG. As a result, both 68 kDa M1 and 60 kDa M87 spastin isoforms are expressed simultaneously but at different levels. A thin dark red line represents low levels of M1 expression and a dark red bar represents considerably higher levels of M87. Light red represents the M1 and M87 regions that are not present in spastin isoforms encoded by alternatively spliced mRNA lacking exon 4.

Connell *et al.*, 2009; Blackstone *et al.*, 2011; Guizetti *et al.*, 2011; Allison *et al.*, 2013).

ESCRT-III proteins are required for completion of abscission at the end stage of cytokinesis, when the two daughter cells are physically severed from one another. CHMP1B recruits spastin to the midbody, presumably to sever microtubules and enable full constriction at the abscission site. Depletion of spastin from HeLa cells results in delayed abscission because the microtubule disruption that normally accompanies abscission does not occur (Yang *et al.*, 2008; Connell *et al.*, 2009; Guizetti *et al.*, 2011). However, this function may be redundant with another protein or pathway, as spastin depletion *in vivo* does not result in any defects in cell division.

Recruitment of spastin into the ESCRT-III complex facilitated by IST1 might promote fission of recycling tubules from the endosome, one of the steps in a process that controls the balance between receptor degradation and recycling (Blackstone *et al.*, 2011; Allison *et al.*, 2013). Depletion of spastin by siRNA from HeLa cells leads to increased tubulation of the early endosomal compartment

and results in defective receptor sorting through endosomal tubular recycling compartments. An increase in complex tubular structures has also been observed in axonal growth cones of motor neurons cultured from spastin-depleted zebrafish embryos (Allison *et al.*, 2013).

Analyses of endogenous spastin distribution in HeLa cells and rat cortical neurons have revealed co-localization of M87 spastin with the centrosomal protein NA14, suggesting that NA14 might act as a molecular adaptor involved in targeting spastin to the centrosome and midbody, to facilitate microtubule severing in these specific locations (Errico *et al.*, 2004; Goyal *et al.*, 2014). Spastin activity is also implicated in downregulation of bone morphogenic protein (BMP) signalling (Tsang *et al.*, 2009), which regulates axonal growth, guidance and differentiation during mammalian development (Charron and Tessier-Lavigne, 2007).

As shown in Fig. 2, the MIT, MTBD and AAA domains are present in both M1 and M87 spastin isoforms, whereas the 86-amino acid N-terminal domain is present only in M1 spastin. The M1 hydrophobic region spanning amino acids 49–80 has been suggested to form a hairpin that can

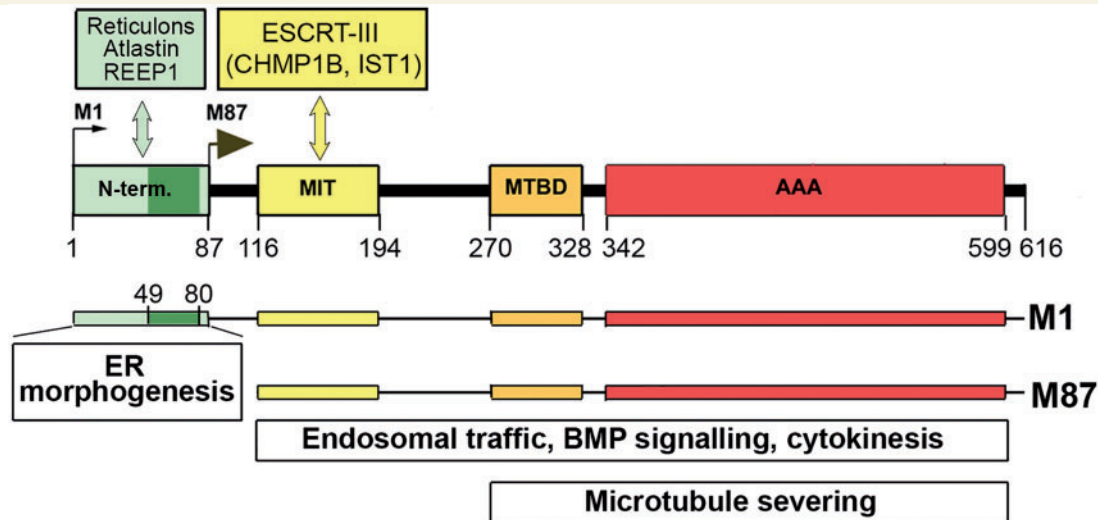


Figure 2 M1 and M87 interacting proteins and involvement of spastin isoforms in cellular functions. Insertion of hydrophobic region (dark green, amino acids 49–80) of the M1-specific N-terminal domain (amino acids 1–87) into endoplasmic reticulum (ER) membrane and interaction with reticulons, atlastin and REEP1 is responsible for endoplasmic reticulum morphogenesis. Interaction of MIT domain (amino acids 116–194) with ESCRT-III complex proteins is required for endosomal trafficking and cytokinesis. Microtubule-binding domain (MTBD) (amino acids 270–328) and AAA domain (amino acids 342–599) are essential for microtubule severing as well as endosomal trafficking and cytokinesis. BMP = bone morphogenic protein.

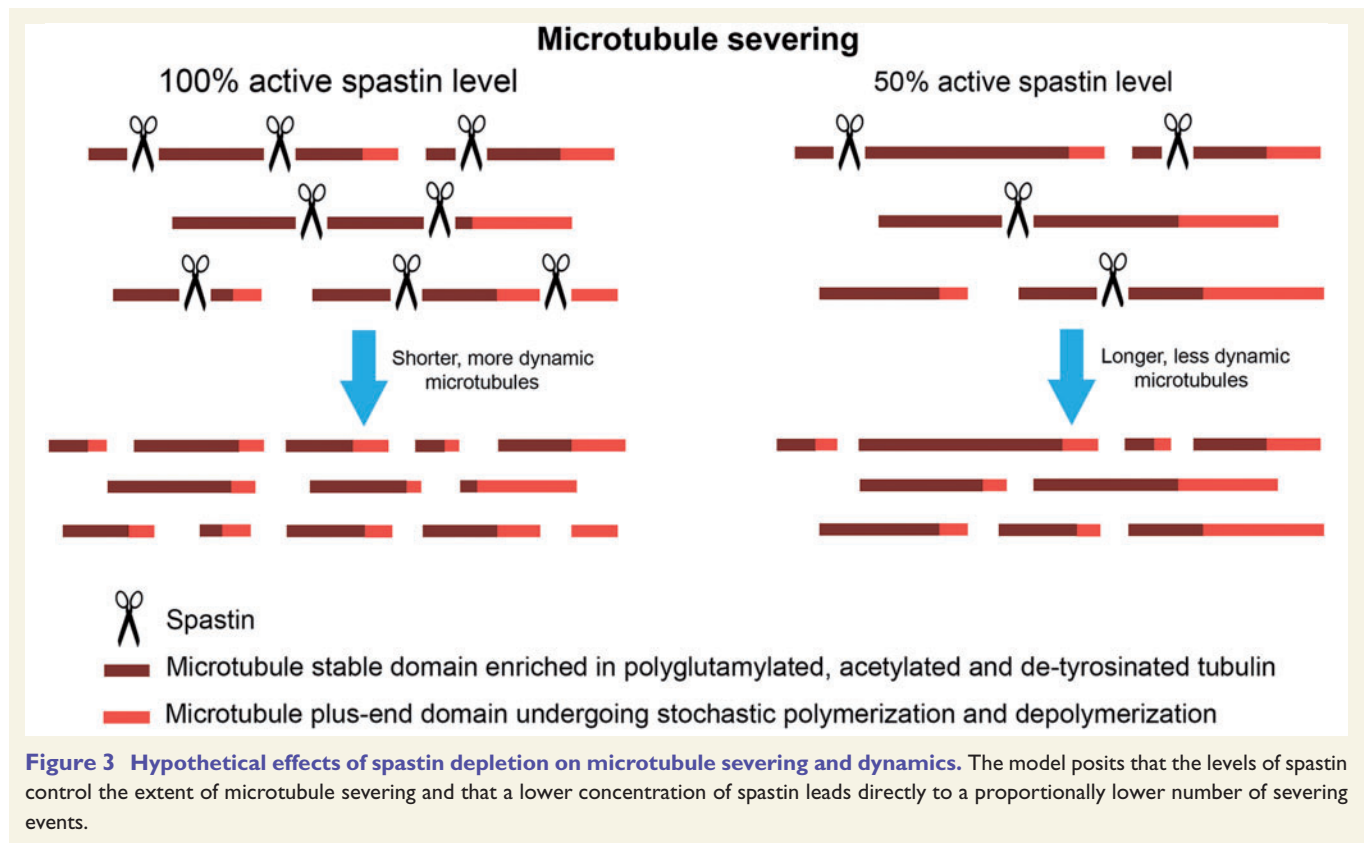
partially insert into endoplasmic reticulum membrane. Spastin hydrophobic hairpins can interact with hydrophobic hairpins of atlastin 1 (encoded by *ATL1*) and REEP1 (Evans *et al.*, 2006; Sanderson *et al.*, 2006; Park *et al.*, 2010; Blackstone *et al.*, 2011). Atlastin 1 is a large integral-membrane GTPase that mediates homotypic fusion of endoplasmic reticulum tubules (Hu *et al.*, 2009). Mutations of atlastin 1 are the cause of HSP-SPG3A, the second most common HSP (Zhao *et al.*, 2001). REEP1 (receptor expression enhancing protein 1) is an endoplasmic reticulum morphogen that links endoplasmic reticulum membranes to microtubules. Mutations in REEP1 have been found in patients with HSP-SPG31 (Beetz *et al.*, 2008). Interaction of spastin with the endoplasmic reticulum-shaping protein reticulon 1 (*RTN1*) has also been reported (Mannan *et al.*, 2006). These findings suggest that coordinated interaction of atlastin 1, M1 spastin and REEP1 might be important for shaping of the endoplasmic reticulum tubules through hydrophobic wedging and for endoplasmic reticulum–microtubule interactions that build the tubular endoplasmic reticulum network (Park *et al.*, 2010; Blackstone *et al.*, 2011, 2012; Lumb *et al.*, 2012). It is unclear whether M1 inserted into endoplasmic reticulum membrane would at the same time form hexamers with M87 to sever microtubules.

Loss-of-function model of HSP-SPG4

Over 200 different mutations have been found in *SPAST* (Hazan *et al.*, 1999; Fonknechten *et al.*, 2000; Lindsey

et al., 2000; Shoukier *et al.*, 2009). Missense mutations are clustered mainly in the AAA domain, while nonsense, splice site mutations and insertions/deletions have been found in all locations in *SPAST*. Interestingly, no mutations have been detected in *SPAST* exon 4, which might be alternatively spliced (Svenson *et al.*, 2001; Shoukier *et al.*, 2009). It has been also demonstrated that 18–20% of patients carry large deletions in the spastin gene (Beetz *et al.*, 2006; Depienne *et al.*, 2007; Boone *et al.*, 2014). The spectrum of mutations found in HSP-SPG4 has prompted a loss-of-function explanation for the disease. As most pathogenic mutations in *SPAST* affect spastin domains necessary for its hexamerization, microtubule binding, or ATP hydrolysis, inadequate microtubule severing resulting from inactivation of one spastin allele (haploinsufficiency) has become a postulated explanation for the symptoms of HSP-SPG4 (Hazan *et al.*, 1999; Burger *et al.*, 2000; Fonknechten *et al.*, 2000). Figure 3 schematically depicts the hypothetical consequences of the partial loss of spastin activity on microtubule severing and dynamics. This model posits that spastin expression levels are the limiting factor in microtubule severing. Loss-of-function is a plausible mechanism for HSP-SPG4 if indeed the number of severing events is directly proportional to the concentration of spastin and if a decrease in the number of severing events is detrimental specifically to a subset of corticospinal axons.

In neurons, severing of long microtubules into shorter ones is critical for efficient microtubule transport because only short microtubules are able to move in a rapid and concerted fashion within the axon (Wang and Brown, 2002; Baas *et al.*, 2006). Microtubule severing also creates greater numbers of free, dynamic microtubule plus-ends



that can interact with plus-end associated proteins and cellular cortical structures (Roll-Mecak and Vale, 2006). An abundance of short mobile microtubules and free microtubule ends, as well as a greater fraction of labile/dynamic microtubule mass, is important for axonal growth and for the formation of new axonal branches, all of which are compromised in cultured rodent neurons depleted of spastin (Yu *et al.*, 2008; Riano *et al.*, 2009; Qiang *et al.*, 2010).

Drosophila model of HSP-SPG4

Negative effects of spastin depletion on axon outgrowth and branching in cultured rodent neurons suggest the importance of microtubule severing during development. Indeed, morpholino-based knockdown of spastin from developing zebrafish embryos results in impaired outgrowth of axons from spinal and branchiomotor neurons (Wood *et al.*, 2006). In *Drosophila*, however, loss of spastin function does not result in such developmental deficits of axonal outgrowth. Instead, ubiquitous depletion of spastin (encoded by *spas*) with RNAi or deletion of the entire *SPAST* gene (homozygous null spastin mutation) resulted in low eclosion rates of ~20% and decreased neuromuscular junction synaptic area with an increased number of synaptic buttons in third instar larvae. The effects of lack of spastin on neuromuscular junction synaptic area were likely due to inhibition of microtubule severing, as the levels of acetylated tubulin at the neuromuscular junction

presynaptic terminals become elevated while the total tubulin levels are reduced, indicating a shift toward a greater fraction of stable microtubules (Sherwood *et al.*, 2004; Trotta *et al.*, 2004; Orso *et al.*, 2005). *Spas*-null flies that survived to adulthood were short-lived and could not fly or jump. Interestingly, partial or complete deletion of the first exon of *Drosophila SPAST*, which should abolish protein synthesis due to a removal of translation initiation codon(s), resulted in a milder phenotype than removal of the entire gene. Homozygous flies lacking the first spastin exon eclosed at normal frequencies and exhibited less severe motor defects than flies with the entire spastin gene deleted (Sherwood *et al.*, 2004).

When depletion of spastin with RNAi was limited to the CNS, newly eclosed flies exhibited normal motor functions and their brains had normal anatomical and histological organization. Over time, however, locomotor performance declined more rapidly in experimental than in control flies, and signs of neurodegeneration began to appear in their brains (Orso *et al.*, 2005). It has been also reported that loss of one copy of the spastin gene severely disrupted the capacity of *Drosophila* axons to regenerate (Stone *et al.*, 2012).

Mouse model of HSP-SPG4

For the purpose of evaluating the significance of spastin loss in mammals, two spastin knockout mouse models

have been generated and analysed (Tarrade *et al.*, 2006; Kasher *et al.*, 2009). In the first model, spastin exons 5–7 were deleted via homologous recombination. As a result of the accompanying frame-shift, a termination codon was created 29 base pairs downstream from the new exon 4–8 junction. Interestingly, truncated spastin transcripts lacking exons 5–7 were readily detected in various tissues including brain, indicating that nonsense-mediated mRNA decay did not completely eliminate the mRNA with a premature termination codon. Truncated spastin protein, however, was not detected in brain (Tarrade *et al.*, 2006). In the second mouse model, exon 7 of spastin was deleted as a result of a point mutation in the splice donor site. Sequencing revealed that the mutated transcription product would encode truncated spastin with 50 novel C-terminal amino acids followed by a termination codon. Again, truncated mRNA was detected but truncated protein was not detected in mouse brain (Kasher *et al.*, 2009). Neither of these spastin mutations generated in mice led to developmental abnormalities, and compared to many human HSP-SPG4 patients carrying just one copy of mutated *Spast*, homozygous *Spast* knockout mice had only mild motor defects and heterozygous mice did not exhibit any detectable gait abnormalities.

Human neuronal model of HSP-SPG4

To generate human models of HSP-SPG4, fibroblasts from one patient with G>T substitution located in *SPAST* exon 4 (Denton *et al.*, 2014) and from two patients carrying *SPAST* R562X mutation (Havlicek *et al.*, 2014) were infected with lentiviruses carrying pluripotency genes *SOX2*, *POU5F1* (also known as *OCT3/4*), *KLF4* and *MYC* (also known as *c-MYC*). Subsequently, the induced pluripotent stem cells were grown either in neural differentiation medium and differentiated into telencephalic glutamatergic neurons expressing TBR1⁺ (Denton *et al.*, 2014) or underwent spontaneous undirected differentiation into mostly glutamatergic neurons, expressing CTIP2 (encoded by *BCL11B*) protein found *in vivo* in layer 5/6 neurons (Havlicek *et al.*, 2014). Identically treated fibroblasts from healthy individuals were used as controls. SPG4 patient-derived neurons show decreased expression of wild-type spastin and axonal swellings filled with loosely arranged, fragmented microtubules and accumulated mitochondria. The number of swellings in heterozygous SPG4 patient-derived neurons was significantly higher than the number of swellings in cortical neuron cultures from homozygous *Spast* knockout mice. One of the two studies found that the levels of acetylated tubulin in SPG4 patient-derived neurons were no higher than in control neurons with the authors suggesting that microtubule dynamics were relatively normal due to the increase in expression of katanin (another microtubule-severing protein) (Havlicek *et al.*, 2014). The other study, however, reported a dramatic increase in acetylated tubulin levels in SPG4-derived neurons (Denton *et al.*, 2014). In addition, SPG4 patient-derived neurons

had lower numbers of shorter and less branched primary neurites, which is similar to the phenotype observed when rat hippocampal neurons in culture were depleted of spastin by siRNA (Qiang *et al.*, 2010). How these observations apply to the nerve degeneration in HSP remains unclear, as such deficits in neurite outgrowth have not been reported in HSP-SPG4 patients. As mutated spastin proteins were not detected, the phenotypes observed in SPG4 patient-derived neurons were attributed to the loss of spastin microtubule-severing activity that cannot be fully substituted by other severing enzymes.

Perturbation in axonal transport in HSP-SPG4

The most prominent phenomenon observed in HSP-SPG4 mammalian models has been the presence of focal axonal swellings. In homozygous mice, such swellings present in both descending and ascending tracts of the spinal cord are filled with organelles and filaments. Axonal swellings were also found in SPG4 patient-derived neurons and sections of spinal cord from two HSP-SPG4 patients (Kasher *et al.*, 2009). These findings might be best explained by perturbations of axonal transport. In cultured neurons derived from one of the mouse models of HSP-SPG4, selective reduction of anterograde transport was found (Kasher *et al.*, 2009). On this basis, it was proposed that spastin depletion results in misregulation of microtubule dynamics, leading to increased cargo stalling, possibly by disturbing cargo loading on microtubules (Fassier *et al.*, 2013). This explanation, however, is not entirely satisfactory for various reasons. First, axonal swellings have not always been observed in cultured neurons depleted of spastin by siRNA (Yu *et al.*, 2008; Qiang *et al.*, 2010) and were not reported in *Drosophila* models (Sherwood *et al.*, 2004; Trotta *et al.*, 2004). Second, it has been shown that kinesin 1, the motor protein responsible for most anterograde organelle transport in the axon, preferentially moves on stable microtubules rich in acetylated and detyrosinated α -tubulin (Cai *et al.*, 2009), and such modifications are not less but rather more numerous on the microtubules of spastin-depleted cells (Sherwood *et al.*, 2004; Trotta *et al.*, 2004; Orso *et al.*, 2005; Riano *et al.*, 2009; Fassier *et al.*, 2013). It has also been shown that endoplasmic reticulum extension (sliding) along microtubules is facilitated by tubulin acetylation (Friedman *et al.*, 2010). In SPG4 patient-derived neurons, retrograde transport was decreased and anterograde transport seemed to be only marginally affected (Denton *et al.*, 2014; Havlicek *et al.*, 2014).

Finally, while severing increases the number of microtubule plus-ends that could then undergo polymerization and depolymerization, there is no indication that the rate of microtubule polymerization or depolymerization, within itself, is affected by wild-type spastin. Indeed, no decrease in microtubule polymerization has been found in spastin-depleted cultured neurons (Qiang *et al.*, 2010; Fassier *et al.*,

2013). It is, therefore, unclear why the use of vinblastine at nanomolar concentrations resulted in improved motor function in *Drosophila* (Orso *et al.*, 2005) as well as decreased axonal swellings in spastin-depleted neuronal cells (Fassier *et al.*, 2013) or SPG4 patient-derived neurons (Denton *et al.*, 2014). At this concentration vinblastine is a ‘kinetic stabilizer’ of microtubules that inhibits both the frequency and the rate of microtubule polymerization and depolymerization (Yang *et al.*, 2010; Baas and Ahmad, 2013), not a destabilizer that would increase microtubule dynamics as has been proposed in these studies. As microtubule-modifying drugs have been suggested as a potential therapy for HSP-SPG4, further studies and clarification on these issues is of great practical importance. Interestingly, sub-stoichiometric concentrations of either vinblastine or taxol restored neurite outgrowth in SPG3A patient-derived forebrain neurons expressing mutated atlastin 1 protein (Zhu *et al.*, 2014), suggesting that microtubules might be a therapeutic target in various HSPs.

Loss of spastin activity in HSP-SPG4 patients

Most (but not all) human patients carrying a single mutated *SPAST* allele exhibit a phenotype characteristic of HSP, indicating high but not complete, age-dependent genetic penetrance of 50% at age 27 and 80% at 50 years. Approximately 6% of individuals with *SPAST* mutations are completely asymptomatic (Dürr *et al.*, 2012). The mean age at onset, reported to be 29 ± 17 years, encompasses a broad range of ages from early childhood to late adulthood onset (1 to 74 years). The disability levels vary from patients being able to walk but not run or walk with aid, to patients being wheelchair-bound. Typically, the level of disability notably increases with the duration of the disease. Despite that different *SPAST* mutations presumably decrease spastin activity to different degrees, no clear correlation has been identified between the type of mutation and the severity of the phenotype (Fonknechten *et al.*, 2000; Yip *et al.*, 2003; McDermott *et al.*, 2006; Shoukier *et al.*, 2009).

Theoretically, full-length spastins carrying missense mutations could have dominant-negative activity and thereby further decrease activity of normal spastin encoded by the wild-type allele. As a result, patients carrying such mutations would have less than half of normal spastin activity. On the other end of the spectrum, some *SPAST* splice site mutations result in only slight reduction in wild-type spastin mRNA expression (Svenson *et al.*, 2001), and there are patients with typical HSP-SPG4 symptoms who carry missense mutations that are localized outside the MTBD and AAA domains and do not affect spastin microtubule-severing activity (Sauter *et al.*, 2002; Patrono *et al.*, 2005; Crippa *et al.*, 2006; Solowska *et al.*, 2010). Some of these mutations localized in the MIT domain might,

however, encode spastins unable to interact with ESCRT-III proteins and hence unable to participate in endosomal dynamics.

The compound heterozygosity for the missense S44L and P361L or D470V *SPAST* mutations causes a rare severe infantile HSP (Chinnery *et al.*, 2004; Svenson *et al.*, 2004). P361L or D470V mutations are localized in the AAA domain and most likely inactivate spastin. The S44L mutation is located outside of the DNA region encoding the M87 isoform and does not affect the severing activity of M87 (Solowska *et al.*, 2010). The S44L mutation found in ~0.6–3% of the population is asymptomatic in the overwhelming majority of carriers and therefore is considered a polymorphism that might act as a phenotypic modifier (Svenson *et al.*, 2004; Erichsen *et al.*, 2007). It has been suggested, however, that the S44L mutation might affect a cryptic M87 promoter in exon 1 and thereby downregulate the expression of the M87 isoform (Mancuso and Rugarli, 2008), or it might increase the stability of the M1 isoform (Schickel *et al.*, 2007). Infantile onset of HSP was also diagnosed in cases of heterozygous missense *SPAST* mutations such as G471D (Blair *et al.*, 2007) and D613H (but not D613A) (Aulitzky *et al.*, 2014), and there are cases in which the compound heterozygosity (e.g. S44L and R503W) did not result in infantile onset of HSP-SPG4 (Shoukier *et al.*, 2009), indicating once again that the severity of the HSP-SPG4 cannot be predicted on the basis of *SPAST* mutation type. Significant clinical variability is also typically observed in families where all individuals have the identical mutation in the *SPAST* gene, and therefore presumably experience the same loss of spastin function (Meijer *et al.*, 2002). Such apparent lack of correlation between the severity of HSP-SPG4 phenotype and the degree to which spastin activity is lost due to a particular *SPAST* mutation suggests that factors other than loss of active spastin contribute to the aetiology of the disease.

Mutated spastin proteins in HSP-SPG4

The overwhelming majority of mutations found in HSP-SPG4 patients would abolish microtubule-severing activity of spastin encoded by the mutated *SPAST* allele and theoretically result in microtubules that are lower in number but more stable (Fig. 3). In human patients, such loss of microtubule severing does not, however, lead to abnormalities during nervous system development, which is when greater microtubule mobility and a higher fraction of labile/dynamic microtubule mass are particularly important to accommodate the growth of axons (Baas *et al.*, 2006). One possibility is that reducing active spastin levels has no functional impact because the total levels of spastin and other microtubule-severing proteins such as katanin are higher during development than in the adult (Solowska *et al.*, 2008). Another possibility, however, is that insufficient

microtubule severing, within itself, does not adequately explain the disease and that a toxic gain-of-function mechanism contributes to HSP-SPG4 pathology. A comparison of the phenotypes observed in the spastin knockout mouse with that of cattle with bovine spinal dysmyelination strongly suggests that both mutated spastin proteins and the loss of spastin function might play a role in the aetiology of HSP-SPG4. Motor defects observed in homozygous knockout mice are generally mild compared to those suffered by many human patients carrying just one mutated *SPAST* allele (Tarrade *et al.*, 2006; Kasher *et al.*, 2009). In cattle with bovine spinal dysmyelination, a naturally occurring neurodegenerative disease caused by the missense inactivating R560Q spastin mutation, heterozygous carriers are asymptomatic (Thomsen *et al.*, 2010). As the genetic penetrance of HSP-SPG4 is highly age-dependent in humans, it is possible that the animals carrying heterozygous R560Q mutation simply did not live long enough to develop symptoms. Bovine natural lifespan is ~25 years, but in modern dairy farming, lifespans usually do not exceed 5–7 years. Interestingly, in humans heterozygous *SPAST* mutations R562Q (Meijer *et al.*, 2002), R562G (Svenson *et al.*, 2001) or R562X (Fonknechten *et al.*, 2000; Meijer *et al.*, 2002) that correspond to bovine R560Q result in HSP.

Calves homozygous for the R560Q spastin mutation, unlike knockout mice, manifest clinical signs immediately after birth with complete penetrance of the phenotype. Newborns are alert to their surroundings but cannot raise or move their limbs. Microscopic examination indicated bilateral symmetrical dysmyelination of axons in the cervical and thoracic spinal cords of affected animals. Such myelination defects were not observed in any other parts of the nervous system. Ultrastructural examination showed the presence of slightly swollen axons with accumulation of disoriented microtubules and intermediate filaments as well as the presence of highly swollen axons filled with organelles such as mitochondria and lysosomal bodies (Thomsen *et al.*, 2010). Interestingly, some loss of myelin has also been observed in spinal cords of heterozygous HSP-SPG4 patients and accompanying decreases in phosphorylated epitopes of neurofilament and tubulin proteins has suggested that the dysmyelination occurred in the context of axonal degeneration (Wharton *et al.*, 2003).

The question arises as to why phenotypes observed in the absence of active spastin are so profoundly different in two mammalian species. One possibility is that mice are particularly resistant to spastin inactivation. That, however, would make mice an unsuitable model for human HSP-SPG4. The other and more likely explanation lies in different expression of mutated spastin proteins in those two models. In mouse models carrying truncating mutations, despite the presence of corresponding truncated transcripts, proteins have not been detected, suggesting that such proteins are efficiently degraded. The R560Q missense mutation, however, would produce full-length inactive spastin, and experimental results indicate that such spastin is metabolically as stable as wild-type spastin (Solowska *et al.*,

2010, 2014). Therefore the major difference between mice and bovine, each equally depleted of spastin microtubule-severing activity but exhibiting significantly different phenotypes, likely lies in the presence or absence of mutated spastin proteins (Table 2).

Missense *SPAST* mutations

Neurotoxic proteins are a central feature of many neurodegenerative diseases, but toxicity of spastins has gone relatively unstudied mainly because it is often argued that most mutated spastins are expressed at levels that are too low to be toxic (Burger *et al.*, 2000). There are, however, compelling reasons why neurotoxicity of mutated spastins should not be dismissed. About 30% of *SPAST* mutations are missense mutations, which are expected to produce stable mutated spastin mRNAs and proteins. Full-length spastins with missense mutations could act in dominant-negative fashion to lower the activity of spastin encoded by the wild-type allele. In studies on *Drosophila*, RNAi knock-down or expression of spastin carrying the missense K467R mutation resulted in similar phenotypes, suggesting that K468R mutated protein acting through a dominant-negative mechanism interferes with the function of endogenous spastin (Orso *et al.*, 2005). In human patients, however, missense *SPAST* mutations do not lead to more severe symptoms that would be expected if wild-type spastin activity were further lowered by dominant-negative inhibition of function. *In vitro* experiments have shown that while mutated spastin may increase the length of the so-called pre-severing phase (time from the addition of spastin and ATP to microtubules immobilized on microscopic coverslips and the start of severing) the actual severing of microtubules by wild-type spastin was not affected by the presence of mutated spastin (Eckert *et al.*, 2012). Also, the M1 or M87 isoform carrying the pathogenic C448Y mutation did not diminish severing activity of wild-type M87 when the two were co-expressed in fibroblasts (Solowska *et al.*, 2014). Therefore, not all *SPAST* missense mutations generate proteins with dominant-negative properties.

Truncating *SPAST* mutations

Many of the splice site mutations and insertions/deletions in *SPAST* (with the exception of in-frame exon deletions) could generate premature termination codons. Premature termination codons are also created by missense point mutations. While the majority of mRNAs with premature termination codons do undergo a rapid decay, some escape degradation and are stable. In fact, truncated mRNAs have been readily detected in mouse models with premature termination codons generated in their *SPAST* gene (Tarrade *et al.*, 2006; Kasher *et al.*, 2009). During mRNA splicing, a multi-subunit protein complex termed the exon junction complex is deposited 20–24 nucleotides upstream of the exon–exon junction and then removed from the mRNA by the elongating ribosome. Generally, premature

Table 2 Animal models for spastin mutations

Organism	Mutation	Predicted spastin inactivation	Predicted level of mutated spastin	Onset/severity
Mouse	Homozygous deletion exons 5–7	100%	Not detected	Late onset, mild
Mouse	Homozygous splice site mutation	100%	Not detected	Late onset, mild
Bovine	Homozygous R560Q mutation	100%	Wild-type level	Early onset, very severe

termination codons located more than 50–55 nucleotides upstream of the exon-exon junction prevent the removal of exon junction complex and such leftover exon junction complex(es) serve as an anchoring point for the assembly of the nonsense-mediated decay complex and subsequent degradation of mRNA. As the removal of exon junction complexes requires a pioneer round of translation, the truncated proteins are synthesized, but inefficiently (Matsuda *et al.*, 2008; Rebbapragada and Lykke-Andersen, 2009; Schweingruber *et al.*, 2013). There are also some premature stop codons in *SPAST* located <50–55 nucleotides upstream of the 3'-most exon-exon junction, and therefore unlikely to produce mRNAs undergoing degradation (Beetz *et al.*, 2006; Aridon *et al.*, 2007; Shoukier *et al.*, 2009).

As discussed earlier, the lack of detectable levels of truncated spastins in the brain of mouse models of HSP-SPG4 (Tarrade *et al.*, 2006; Kasher *et al.*, 2009; Fassier *et al.*, 2013), in lymphoblastoid cell lines derived from human HSP-SPG4 patients (Riano *et al.*, 2009) and in neuronal cells derived from pluripotent stem cells generated from fibroblast of HSP-SPG4 patients (Denton *et al.*, 2014; Havlicek *et al.*, 2014) has led to the view that a partial loss of spastin microtubule-severing activity, not the presence of neurotoxic spastin proteins, best explains the observed HSP pathologies. However, none of these models truly mirrors the human disease, as HSP-SPG4 in most cases develops over many years and affects mainly a subset of long upper motor neuron axons. The absence of mutated spastins in short-term cultured cells does not preclude the possibility that mutated spastins are present in the affected corticospinal axons of HSP-SPG4 patients. Moreover, as both mRNAs carrying premature termination codons and truncated proteins could be unstable, detection of such proteins may require immunoprecipitation and stringent extraction, and these methods were not used to detect mutated spastins in HSP-SPG4 patient-derived lymphoblastoid or neuronal cell lines. Studies of mutant forms of Cu, Zn-superoxide dismutase (SOD1) that contribute to the cases of familial amyotrophic lateral sclerosis (ALS) revealed, for example, that very minute levels of a C-terminally truncated version of SOD1 expressed in transgenic mice, induce late-onset ALS with rapid progression to death. Analyses of spinal cord and brain of a patient with ALS carrying the same mutation showed that the mutated protein was expressed below 0.5% of the SOD1 levels in controls (Jonsson *et al.*, 2004). In another

study, pathogenic SOD1 was detected only after immunoprecipitation that allowed for concentration of small amounts of mutated protein expressed in transgenic mice (Watanabe *et al.*, 2005). To the best of our knowledge, the spinal cord of only one patient carrying a *SPAST* splice mutation resulting in in-frame skipping of exon 11 (Svenson *et al.*, 2004) has been examined for the presence of mutant spastins using western blotting with an anti-human spastin antibody, a method that allows M1 to be distinguished from M87. In this case, a prominent band corresponding to the truncated mutant M1 was detected in thoracic but not in cervical spinal cord (Solowska *et al.*, 2010). Interestingly, post-mortem studies in HSP patients consistently report the most severe axon degeneration in the thoracic spinal cord (Fink, 2013).

Large deletions of *SPAST*

Another category of mutations in spastin gene are large deletions varying from 1.3 kb to 1283.9 kb. Such deletions are most likely facilitated by Alu-rich genomic architecture of *SPAST* that render the locus susceptible to a variety of genomic Alu-mediated rearrangements (Beetz *et al.*, 2006; Depienne *et al.*, 2007; Boone *et al.*, 2014). Of 50 different combinations of *SPAST* deleted exons, none has been a deletion of the entire spastin gene (Boone *et al.*, 2014), indicating that in many cases synthesis of truncated spastins cannot be ruled out, particularly because correspondingly shortened transcripts have been found in patient lymphocytes (Beetz *et al.*, 2006). Furthermore, almost half of the deletions extended beyond the boundaries of *SPAST* and some of them interrupted adjacent genes, potentially affecting their expression. Twelve deletions extending the 3' end of *SPAST* might form chimeric genes that yield fusion transcripts with possible phenotypic consequences. Chimeras between *SPAST* and *SLC30A6* (solute carrier Family 30, member 6) encoding zinc transporter ZnT6 might be of particular interest, as alterations in zinc and zinc transporters dynamics have been found in the brain of humans with Alzheimer's disease (Smith *et al.*, 2006). In rare HSP-SPG4 cases, deletions of the entire *SPAST* gene that would preclude synthesis of any mutated spastin protein were reported (Depienne *et al.*, 2007). It is unclear, however, how such large DNA deletions of at least 90 kb affected the integrity of *SPAST* neighbouring genes (or if any other HSP genes were mutated in these patients), and therefore it is difficult to judge whether HSP in these cases

developed solely as the result of the inactivation of one *SPAST* allele.

Pathogenic M1 and M87 spastin isoforms

Determining the role of the spastin isoforms M1 and M87 in HSP-SPG4 is likely to be crucial for understanding the aetiology of the disease. Analysis of spastin expression pattern in rodents revealed the presence of appreciable levels of M1 spastin only in adult spinal cord (Solowska *et al.*, 2008). Also in adult human CNS, levels of M1 in spinal cord are significantly higher than in cerebral cortex (Solowska *et al.*, 2010). M1 expression level seems to be strictly controlled by GC-rich 5'UTR, the upstream AUG sequence and a weak Kozak consensus sequence at the first initiation codon (Fig. 1). Such translation-reducing measures are often used to prevent harmful overproduction of toxic proteins (Kozak, 2002). As a result, wild-type M87 isoform is always more abundant in both neuronal and non-neuronal tissues. Experimental expression of truncated spastins revealed, however, that the levels of M87 were often significantly lower than the levels of the simultaneously expressed M1, despite a weak Kozak sequence at the M1 initiation codon (our unpublished observations), indicating that truncating mutations might significantly decrease the stability of M87 and at least to some extent explain why truncated M87 spastins were not detected in lymphocytes of HSP-SPG4 patients (Riano *et al.*, 2009) or in human induced pluripotent stem cell-derived neuronal cells generated from fibroblasts of HSP-SPG4 patients (Denton *et al.*, 2014; Havlicek *et al.*, 2014).

Removal of 5'UTR from spastin cDNA and creation of a good Kozak sequence at the M1 initiation codon prevented leaky translation from the M87 start codon. Moreover, the synthesis of both isoforms under control of identical promoter and Kozak sequence resulted in significantly higher expression of wild-type or mutated M1 than of the corresponding M87, suggesting that the degradation of M87 is more efficient than that of M1 (Solowska *et al.*, 2010, 2014). Toxicity of individual spastin isoforms carrying missense C448Y mutation found in HSP-SPG4 patients (Hazan *et al.*, 1999; Fonknechten *et al.*, 2000) was tested in rat primary cortical neurons and in transgenic *Drosophila* (Solowska *et al.*, 2014). The high levels of mutated M1 with pathogenic C448Y mutation might at least partly explain why M1 C448Y was significantly more detrimental to neurite outgrowth in cultured neurons and caused notably more severe motor defects in transgenic *Drosophila* than M87 C448Y. The flies expressing pathogenic M1 also experienced greater progressive decline of climbing activity than flies expressing pathogenic M87 (Solowska *et al.*, 2014). Thus, the specificity of the disease for adult corticospinal tracts might relate directly to the elevated levels of mutated M1 not M87, particularly given that truncating

mutations significantly decrease stability of many mutated M87 but not M1. The motor defects observed in *Drosophila* and reduced neurite outgrowth in cultured cortical neurons expressing spastins with the C488Y mutation did not result from dominant-negative activity, suggesting that at least some *SPAST* mutations produce neurotoxic proteins (Solowska *et al.*, 2014). Interestingly, experiments in which cultured primary cortical neurons were transfected with truncated, GFP-tagged mouse spastins showed that only M1 but not M87 isoform had detrimental effects on neurite outgrowth, and even low (1–10 nM) concentrations of truncated M1 significantly inhibited fast axonal transport in squid axoplasm (Solowska *et al.*, 2008).

Why mutated spastin proteins are toxic is unknown. Hypothetically, as shown in Fig. 4, mutated spastin proteins might affect various cellular activities. It has been shown that M1 with missense mutation C448Y decorates a subset of microtubules in cultured fibroblast and neuronal cells and decreases the rates of microtubule polymerization and depolymerization (Solowska *et al.*, 2014). As microtubule decoration has been reported for a number of spastins carrying inactivating missense mutations in the AAA domain (Errico *et al.*, 2002; McDermott *et al.*, 2003; Evans *et al.*, 2005), it is possible that many mutated spastin proteins decrease microtubule dynamics via a gain-of-function mechanism, and exacerbate the effects of the lost microtubule-severing activity. Interestingly, in the case of the pathogenic C448Y mutation, the M87 protein that does not bind to microtubules causes the inverse effect on microtubules, rendering them more dynamic rather than less dynamic (Solowska *et al.*, 2014).

A more permanent association of mutated M1 with microtubules might also cause a decrease of endoplasmic reticulum sliding along microtubules while lower rates of polymerization and depolymerization might decrease growth and retraction of endoplasmic reticulum tubules attached to microtubule plus-ends. Truncated M1 isoforms lacking the microtubule-binding domain could theoretically be inserted into endoplasmic reticulum membranes but would not be able to participate in the interaction of endoplasmic reticulum with microtubules. Mutant M1 inserted into endoplasmic reticulum membranes could also adversely affect endoplasmic reticulum morphogenesis.

Mutant M1 bound to microtubules could interfere with a variety of proteins that are involved in axonal transport. As truncated mouse M1 spastin inhibited fast axonal transport in squid axoplasm (Solowska *et al.*, 2008), it has been speculated that mutated M1, similarly to other neuropathogenic peptides including huntingtin, filamentous tau, prenilin 1 and oligomeric amyloid- β , promotes abnormal activation of selected protein kinases. Such activation might lead to aberrant phosphorylation and regulation of molecular motor proteins, kinesin 1 and cytoplasmic dynein, that are responsible for axonal transport (Morfini *et al.*, 2009). Finally, spastin mutations might result in synthesis of misfolded proteins and promote formation of

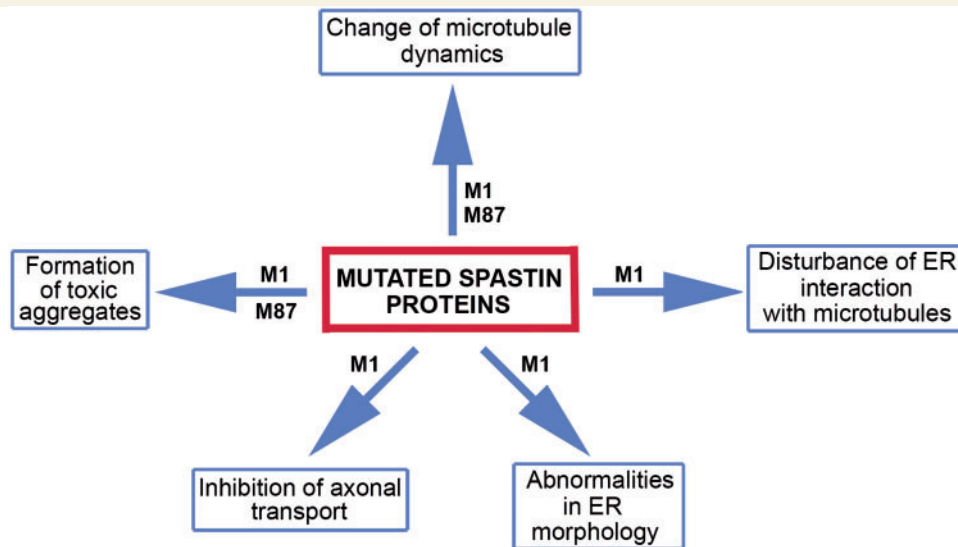


Figure 4 Cellular activities hypothetically affected by mutated spastin proteins. Mutated M1 protein might affect endoplasmic reticulum (ER) morphology and interaction with microtubules, as well as axonal transport. Mutated M1 and M87 might affect microtubule dynamics and form toxic aggregates.

toxic aggregates, particularly in the case of M1 with inappropriately exposed hydrophobic domain.

Concluding remarks

Mutations in the *SPAST* gene encoding spastin have long been known to exist in the most common form of HSP. A breakthrough in mechanistic understanding of the disease came when it was discovered that spastin is an enzyme that severs long microtubules into shorter ones. Because most pathogenic *SPAST* mutations destroy the enzymatic activity of spastin, the idea that insufficient microtubule severing is the cause of HSP-SPG4 became popular. However, this explanation, while appealing in its simplicity, cannot fully explain the disease. For example, it is unclear why a partial loss of microtubule-severing activity would be more damaging to adult axons than to developing axons, when the latter rely more than the former on increased microtubule number, mobility and dynamics. It is also unclear why the corticospinal tracts would suffer more than other long axons in the body. Some insights are provided by considering that a leaky scanning of the first initiation codon of *SPAST* and a preferential translation from the second initiation codon lead to a simultaneous synthesis of two spastin isoforms termed M1 and M87. There is far more M87 than M1, indicating that deficiencies in M87 might be mainly responsible for any aspects of the disease that are caused by insufficient microtubule severing, including deficits in endosomal trafficking. The M1 isoform, owing to the presence of the hydrophobic N-terminal domain, can be inserted into endoplasmic reticulum membranes and involved in endoplasmic reticulum morphogenesis and endoplasmic reticulum–microtubule interactions. M1

carrying inactivating missense mutations can persistently associate with microtubules, potentially causing deficits in axonal transport. Most truncating mutations result in M1 that is not able to interact with microtubules but such mutant spastins could promote activation of selected protein kinases leading to abnormal phosphorylation of motor proteins, and hence cause defects in axonal transport in an entirely different manner. The hydrophobic domain of M1 could also promote aggregation of the mutated spastin, and if this is the case, the capacity of a particular patient to dispose of such misfolded protein might affect the severity of the disease. M1 is only detectably present in adult spinal cord, which is consistent with a mechanism by which either loss of M1 function or toxicity of mutant M1 could be especially relevant to degeneration of the corticospinal tracts. In addition, some deletions of parts or all of *SPAST* may also include neighbouring genes and affect their function. Finally, it is relevant to note that endosome trafficking, endoplasmic reticulum morphogenesis, bone morphogenic protein signalling, and microtubule dynamics, all of which involve spastin in one way or another, are also affected by mutations in other HSP associated genes, none of which severs microtubules. In conclusion, while identifying spastin as a microtubule-severing protein was an important mechanistic breakthrough, it seems certain that insufficient microtubule severing alone is not an adequate explanation for HSP-SPG4.

Acknowledgements

We are thankful to past and present members of our laboratory who contributed to our work on hereditary spastic paraplegia, as well as our invaluable collaborators, Drs

Gerardo Morfini, Daniel Marena, and Terry Heiman-Patterson. The authors declare no financial or conflicting interests.

Funding

The relevant work in the Baas Laboratory was supported by National Science Foundation Grant IOS0841245 and National Institutes of Health Grants R01 NS28785, and also by grants from the Philadelphia Institute of Neurodegenerative Diseases, the Pennsylvania Department of Health CURE program to Drexel University College of Medicine, and the Spastic Paraplegia Foundation.

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