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STUDIES ON HEREDITARY SPASTIC PARAPLEGIA PROTEINS

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Man saknar inte kon förrän båset är tomt.

ABSTRACT

The hereditary spastic paraplegias (HSPs) are a clinically and genetically diverse group of inherited neurological disorders that primarily cause progressive spasticity and weakness in the lower limbs due to a length-dependent, retrograde degradation of the corticospinal motor neurons. Purely spastic paraplegia is also known as uncomplicated HSP, but complicated forms of HSP exist as well, with symptoms such as mental retardation, dementia, seizures, and optic, cortical, and cerebellar atrophy. Twenty gene products have been identified from over 40 different known SPG spastic gait loci (*SPG1-46*), which may be inherited in autosomal dominant, autosomal recessive, or X-linked manners.

The work presented in this thesis focuses on two different HSP proteins: atlastin-1, a member of the dynamin superfamily of large GTPases through sequence similarity, and maspardin – Mast syndrome, spastic paraplegia, autosomal recessive with dementia. Mutations in the atlastin-1 gene, *SPG3A*, are the second most common cause of autosomal dominant HSP – around 10% of all cases – many of which are quite early in onset (in childhood) when compared to other forms of HSP. Disease-causing point mutations are distributed throughout the coding region, although most are clustered in known domains – GTP binding/GTPase functional areas, a coiled coil region in the middle of the protein product, and in the transmembrane areas at the C-terminal end. On the other hand, the only causative mutation in *SPG21*/*MAST* (maspardin) that has been found is a frameshift-producing alteration after the second third of the gene, which induces a premature truncation of the gene product and loss of the last 95 amino acids of the wild type protein. This mutation is only inherited in an autosomal recessive manner, and causes a complicated HSP with additional symptoms such as dementia, white matter abnormalities, and cerebellar and extrapyramidal signs.

Atlastin-1 is localized to the ER and *cis*-Golgi apparatus in the adult brain, and appears to exist natively as oligomers, most likely tetramers. Wild-type atlastin-1 is a functional GTPase, but in paper I we found that several missense atlastin-1 mutations have impaired GTPase activity. We also found that atlastin-1 is highly enriched in vesicular structures within growth cones, varicosities, and axonal branch points. Knockdown of atlastin-1 using small hairpin RNAs impairs axon formation and elongation during neuronal development and reduces the total number of neuronal processes. In paper II we examined a novel SPG3A mutation causative for HSP that did not affect GTPase activity or interactions between atlastin and spastin, the gene most mutated in HSP. However, immunoblots from patient lymphoblasts showed a reduction in atlastin-1 protein levels, indicating that mutant atlastin-1 may cause disease pathogenesis through a dominant-negative, loss-of-function manner through protein destabilization.

Mast syndrome is likely caused by a loss of protein function. In paper IV we generated *SPG21-/-* transgenic mice as a possible model for SPG21. Though *SPG21-/-* mice appeared normal at birth, within several months they developed a mild but progressive hind limb dysfunction. Cultured cerebral cortical neurons from *SPG21-/-* mice exhibited significantly more axonal branching than neurons cultured from wild-type animals, although a comprehensive neuropathological analysis did not reveal any abnormalities consistent with those observed in human HSP.

While a unifying mechanism for all the genes and proteins known to be involved in HSP has yet to be found, our data support the idea that axonal trafficking and proper neurite branching may represent a common cellular pathogenic theme.

LIST OF PUBLICATIONS

I. Zhu PP, **Soderblom C**, Tao-Cheng JH, Stadler J, Blackstone C. SPG3A protein atlastin-1 is enriched in growth cones and promotes axon elongation during neuronal development. *Hum Mol Genet*. 2006 Apr 15;15(8):1343-53.

II. Meijer IA, Dion P, Laurent S, Dupré N, Brais B, Levert A, Puymirat J, Rioux MF, Sylvain M, Zhu PP, **Soderblom C**, Stadler J, Blackstone C, Rouleau GA. Characterization of a novel SPG3A deletion in a French-Canadian family. *Ann Neurol*. 2007 Jun;61(6):599-603.

III. Rismanchi N*, **Soderblom C***, Stadler J, Zhu PP, Blackstone C. Atlastin GTPases are required for Golgi apparatus and ER morphogenesis. *Hum Mol Genet*. 2008 Jun 1;17(11):1591-604.
*These authors contributed equally.

IV. **Soderblom C**, Stadler J, Jupille H, Blackstone C, Shupliakov O, Hanna MC. Targeted disruption of the Mast syndrome gene SPG21 in mice impairs hind limb function and alters axon branching in cultured cortical neurons. *Submitted manuscript under revision*.

Review:
Soderblom C, Blackstone C. Traffic accidents: molecular genetic insights into the pathogenesis of the hereditary spastic paraplegias. *Pharmacol Ther*. 2006 Jan;109(1-2):42-56.

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LIST OF ABBREVIATIONS

AD	Autosomal dominant
ALS	Amyotrophic lateral sclerosis
AR	Autosomal recessive
CAM	Cell adhesion molecule
CBP	Calmodulin-binding peptide
CMT	Charcot-Marie-Tooth disease
CNS	Central nervous system
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
GDP	Guanosine diphosphate
GJC	Gap junction protein
GTP	Guanosine triphosphate
HA	Hemagglutinin
Hsp	Heat shock protein
HSP	Hereditary spastic paraplegia
KIF	Kinesin family member
NIPA	Non-imprinted in Prader-Willi/Angelman syndrome region protein
NTE	Neuropathy target esterase
PBS	Phospate-buffered saline
PCR	Polymerase chain reaction
PLP	Proteolipoprotein
PNS	Peripheral nervous system
REEP	Receptor expression-enhancing protein
RNA	Ribonucleic acid
SCA	Spinocerebellar ataxia
SPG	Spastic gait locus
TM	Transmembrane

1 INTRODUCTION

1.1 NEURODEGENERATION AND THE MOTOR SYSTEM

Our brains control almost everything we do – from higher thought, learning, memory, and emotion to how these elements affect our actions. Some simpler behaviors, such as locomotion, are "encoded" by central pattern generators in the spinal cord, below the brain (1). However, before these programs can be used, higher order understanding and planning must happen, primarily in the motor cortex, which accepts inputs from a variety of sources that are then relayed through the spinal cord to the lower motor neurons and the muscles. These elements and connections can be thought of as the motor system – from the M1 primary motor area to the corticospinal tract to the alpha motor neurons that synapse on our muscles – which allows us to control movements that require skill. M1 is uniquely filled with large corticospinal neurons – about 30% of the corticospinal neurons that project from M1 are considered large, and these in turn represent almost 80% of all large corticospinal neurons (2). The majority of these large neurons decussate in the lower brainstem pyramids (around 75%) as the lateral corticospinal tract, but the rest either cross in the spinal cord or do not cross at all – the ventral corticospinal tract. It is the neurons that decussate earlier that terminate in the lateral motor nuclei and innervate distal-forelimb muscles; uncrossed corticospinal neurons innervate shoulder proximal-forelimb muscles after terminating in the medial motor nuclei or spinal cord intermediate zones (3, 4). In humans and other primates, these terminations are also monosynaptic connections to the alpha motor neurons of the ventral horn (5) (see **Figure 1**).

The complete loss or partial destruction of neurons in the central nervous system, either through acute trauma or gradual degeneration, has frightening implications. While acute trauma is often unforeseen, the loss of neurons due to environmental or hereditary factors occurs insidiously until disease manifests itself. The first symptoms, such as the loss of control of an otherwise "normal" function, can be disarmingly mild until disability and possibly death occur. A dearth of neurons can occur in any area of the brain or spinal cord, potentially affecting memory, emotions, and voluntary actions. Dementia caused by Alzheimer's disease, the inability to produce movement in Parkinson's disease, or the overactive movements of Huntington's disease are all caused by damage to controlling centers in the brain. The spinal cord, too, can be affected, as in amyotrophic lateral sclerosis (ALS), a motor neuron disease, or spinocerebellar ataxia (SCA). Demyelinating diseases such as some forms of Charcot-Marie-Tooth neuropathy (CMT) and multiple sclerosis can also be devastating. In addition to severely decreasing a patient's quality of life, a diagnosis of one of these disorders also often implies a drastically shortened lifespan.

Worldwide, Alzheimer's disease affects more than 35 million people, making it the most common form of dementia. The pathology of the disease is due to lesions or plaques that form ubiquitously throughout the brain, most likely due to damage from the accumulation of misfolded proteins. This damage results in inflammation and

subsequent dysfunction of normal healthy processes, especially in synaptic transmission (6).

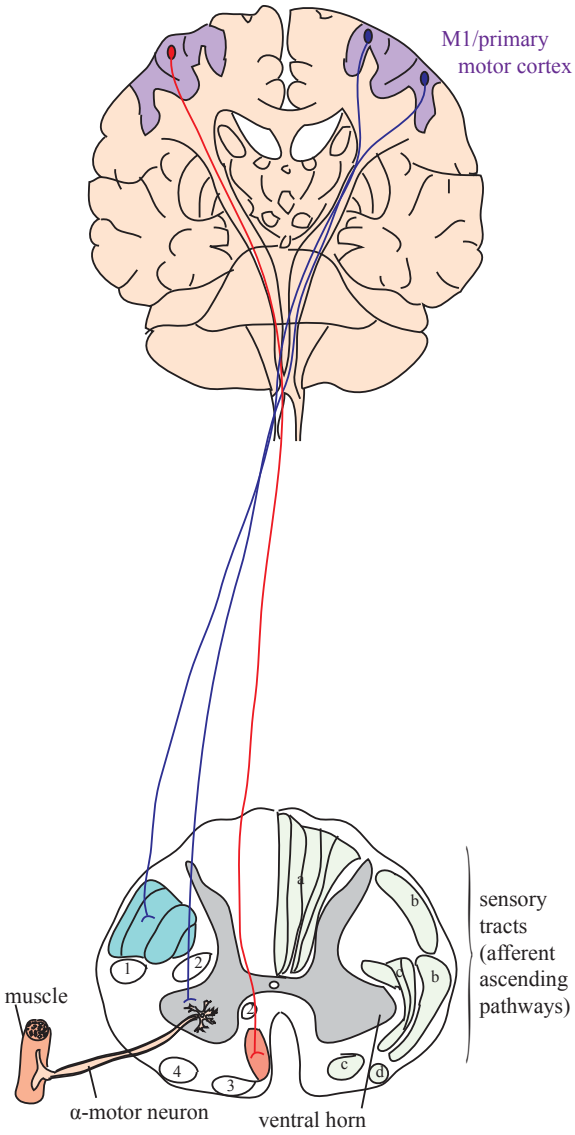

Figure 1. Schematic illustrating the pyramidal motor system in humans. Of particular interest are the primary motor cortex/M1, the descending lateral (blue, crossing in brainstem pyramids) and anterior (red, uncrossed) corticospinal tracts, and the ventral horn containing the cell bodies of the alpha motor neurons. Other descending tracts on the left side of the lumbar spinal cord slice include 1) Rubrospinal tract 2) Reticulospinal tract 3) Vestibulospinal tract 4) Olivospinal tract. Sensory and ascending tracts are indicated in light green on the right: a) Dorsal column medial lemniscus system b) Spinocerebellar tracts c) Anterolateral system d) Spino-olivary fibers.

Parkinson's and Huntington's diseases both result from degeneration of the basal ganglia, which plans voluntary actions and has roles in learning and memory. It is buried deep within the forebrain and consists of four structures – the striatum receives input from the rest of the brain (mostly the neocortex), the pallidum is the only output structure (to the thalamus) in the basal ganglia, the substantia nigra is the main source

of dopaminergic neurons, and the subthalamic nucleus serves as an internal relay. Output to the thalamus is processed through either the direct (which facilitates movement) or indirect (which inhibits movement) pathways. Normally, the opposing pathways regulate each other, but when the direct pathway is disturbed, the indirect pathway "takes over" and parkinsonism results, and when the opposite occurs, cholera, the hyperactive movements characteristic of Huntington's disease, occurs (7).

The spinal cord is made up of various fibers that directly or indirectly synapse with or are part of one of the tracts that group functionally similar elements together. Sensory pathways ascend with information gathered from the peripheral nervous system (PNS) headed to the brain, while control of basic functions and voluntary movement descend in fibers that are located either ventromedially towards the interior of the spinal cord or dorsolaterally, to the exterior side of the ventromedial areas. The ventromedial pathways – the interstitiospinal and tectospinal tracts, the lateral and medial vestibulospinal tracts, and the reticulospinal and bulbospinal tracts – originate in the brainstem and control basic functions such as respiration. The dorsolateral pathways – the rubrospinal tract, the pontospinal tract, and the corticospinal and corticobulbar pathways – are responsible for fine control of voluntary movements (8). In humans, the corticospinal tract synapses directly on spinal motor neurons including those in the lumbar regions – the neurons that are often destroyed, damaged, or otherwise impaired in motor neuron diseases such as ALS and some forms of SCA.

SCA, however, primarily affects the cerebellum and the brainstem and the peripheral nerves along with the spinal cord. There are more than 30 unique genetic causes for different types of SCA that fall into three categories: conventional mutations, expanded CAG/polyQ repeats, and non-protein coding repeats. The disease comprises a group of dominantly inherited ataxias with classic cerebellar symptoms such as difficulty with gait; patients are usually first diagnosed when a gait imbalance is first noticed, followed by problems with vision: difficulty focusing and abnormal saccades or rapid eye movements. These problems are caused by non-discriminatory degeneration of the cerebellum that affects all layers – the deep cerebellar nuclei along with the molecular, Purkinje, and granule cell layers. Differences in symptoms are presumably due to the different types of mutations detected in patients, with expansion repeats present in varying sizes depending on the individual, which, like Huntington's, can increase in size in successive generations, causing earlier onset and more severe disease (9). The prevalence of SCA worldwide has been estimated to be 1-5 per 100,000.

Another disease affecting a neuronal descending pathway descending through the spinal cord is hereditary spastic paraplegia (HSP), caused by mutations in a heterogeneous group of over 40 different genes. As the name suggests, HSP is characterized by spasticity and paralysis that usually become apparent later in life, at least past childhood, although the age of onset depends on the genes mutated (10). The hereditary component may be inherited in an autosomal dominant, autosomal recessive, or X-linked fashion; novel genetic loci are constantly being found.

The symptoms of HSP are caused by a length-dependent degeneration of some of the longest neurons in the central nervous system – the corticospinal tract. In an unaffected individual, these neurons normally run from the layer V pyramidal cells in

the motor cortex, through the brainstem, and down to synapse on the alpha motor neurons in the lumbosacral spinal cord, which then send directions to our muscles via neuromuscular junctions. However, in diseased patients, the brain's directions are no longer properly relayed to the alpha motor neurons, and correct movements do not occur. In some cases, spasticity and paraplegia can be complicated by other symptoms such as mental retardation and emotional abnormalities due to effects on other neurons.

Unfortunately, no part of the motor system – the motor cortex, the basal ganglia, the cerebellum, and the spinal cord – is immune from damage. Interestingly, in addition to sharing a hereditary component, the above named disorders may also share a common mechanism leading to disease – an inherited dysfunction in axonal transport leading to life-changing consequences.

1.2 PATHOGENIC MECHANISMS OF HEREDITARY SPASTIC PARAPLEGIA – AN INTERRUPTION IN INTRACELLULAR TRAFFICKING

The pathogenesis of HSP is characterized by the degeneration of the lateral corticospinal tract, primarily in the lumbar region (11), with significant negative changes in axonal densities of both small and large diameter axons in the corticospinal tract and in sensory tracts at the upper cervical level. As the areas of corticospinal and posterior white matter columns were also reduced, it has been estimated that there is a loss in total axon number in these tracts of more than 60% (12), indicating that the degeneration is both length- and neuron subtype-dependent, with relatively few other pathological changes seen in other brain areas in patients suffering from pure HSP. How mutations in presumably over forty different proteins (see **Table 1**) cause the pathogenesis of hereditary spastic paraplegia and why certain types of cells are preferentially damaged are not entirely known, although it is likely that these very large neurons with some of the longest axons in the central nervous system are especially susceptible to disorders that affect processes that ensure the health of the axon such as correct myelination and intracellular transport, both anterograde and retrograde. Indeed, although protein products of genes known to be affected in HSP are scattered throughout the cell (see **Figure 2**), many tend to fall within these functional groups or are involved in cell adhesion and mitochondrial stability. Most mutations and the subsequent protein defects they cause can be considered to affect normal trafficking and transport, whether the HSP is inherited in an X-linked or an autosomal – dominant or recessive – fashion.

Table 1: Known HSP genes and protein products

Spastic gait locus	Mode of inheritance	Chromosomal location	Additional clinical symptoms, if any	Gene product	Protein function
SPG1	X-linked (13)	Xq28	Hydrocephalus, mental retardation	L1 CAM	Axonal glycoprotein involved in neuronal migration & differentiation
SPG2	X-linked (14)	Xq22	Seizures, cerebellar syndrome	PLP1, DM20	Transmembrane proteolipid component of myelin
SPG3A	AD (15)	14q12-q21	Uncomplicated	Atlastin-1	Golgi GTPase
SPG4	AD (16)	2p22		Spastin	AAA microtubule-severing protein
SPG5A	AR (17)	8q11.1-q21.2	Uncomplicated	Cytochrome P450-7B1	ER synthesis of cholesterol
SPG6	AD (18)	15q11.2-q12	Uncomplicated	NIPA1	Membrane transporter/receptor
SPG7	AR (19)	16q24.3	Optic, cortical, cerebellar atrophy	Paraplegin	Mitochondrial ATPase – proteolytic & chaperone activities at inner mitochondrial membrane
SPG8	AD (20)	8q24	Uncomplicated	Strumpellin	
SPG9	AD (21)	10q23.3-q24.2	Cataracts, gastroesophagel reflux		
SPG10	AD (22)	12q13		KIF5A	Microtubule motor in intracellular organelle transport
SPG11	AR (23)	15q13-15	Thin corpus callosum	Spatacsin	TM protein phosphorylated upon DNA damage
SPG12	AD (24)	19q13	Uncomplicated		
SPG13	AD (25)	2q24-q34	Uncomplicated	Hsp60	Mitochondrial chaperonin
SPG14	AR (26)	3q27-q28	Mild mental retardation		
SPG15	AR (27)	14q22-q24	Mental retardation, pigmented maculopathy, distal amyotrophy, dysarthria	Spastizin	Targeted to membrane lipids
SPG16	X-linked	Xq11.2			
SPG17	AD	11q12-q14		Seipin	ER protein implicated in control of lipid droplet morphology
SPG18	AD				

Spastic gait locus	Mode of inheritance	Chromosomal location	Additional clinical symptoms, if any	Gene product	Protein function
SPG19	AD (28)	9q33-q34	Uncomplicated		
SPG20	AR (29)	13q12.3	Developmental delay, short stature	Spartin	Endosomal trafficking
SPG21	AR (30)	15q22.31		Maspardin	
SPG23	AR (31)	1q24-q32	Hypopigmentation, cognitive impairment		
SPG24	AR	13q14	Uncomplicated		
SPG25	AR	6q23.3-q24.1			
SPG26	AR (32)	12p11.1-q14	Dysarthria, distal amyotrophy, mild intellectual impairment		
SPG27	AR (33)	10q22.1-q24.1	Uncomplicated		
SPG28	AR (34)	14q21.3-q22.3	Uncomplicated		
SPG29	AD (35)	1p31-p21	Hearing impairment, persistent vomiting		
SPG30	AR (36)	2q37	Uncomplicated		
SPG31	AD (37)	2p12	Uncomplicated	REEP1	Mitochondrial protein
SPG32	AR (38)	14q12-q21	Mild mental retardation, cerebellar atrophy		
SPG34	X-linked (39)	Xq24-q25	Uncomplicated		
SPG35	AR (40)	16q21-q23	Intellectual disability, seizures		
SPG36	AD (41)	12q23-q24	Sensory & motor neuropathy		
SPG37	AD (42)	8p21.1-q13.3	Uncomplicated		
SPG38	AD (43)	4p16-p15	Temporal lobe epilepsy, cognitive impairment		
SPG39	AR (44)	19p13		NTE	Hydrolyzes membrane phospholipids
SPG41	AD	11p14.1-p11.2			
SPG42	AD (45)	3q24-26	Uncomplicated	Acetyl-CoA transporter	Carries acetyl-CoA into lumen of Golgi apparatus
SPG43	AR (46)	19p13.11-q12	Atrophy of distal upper extremities		
SPG44	AR (47)	1q42.13	Ataxia, dysarthria, seizures	GJC2	Central & peripheral myelination
SPG45	AR (48)	10q24.3-q25.1	Mental retardation		
SPG46	AR				

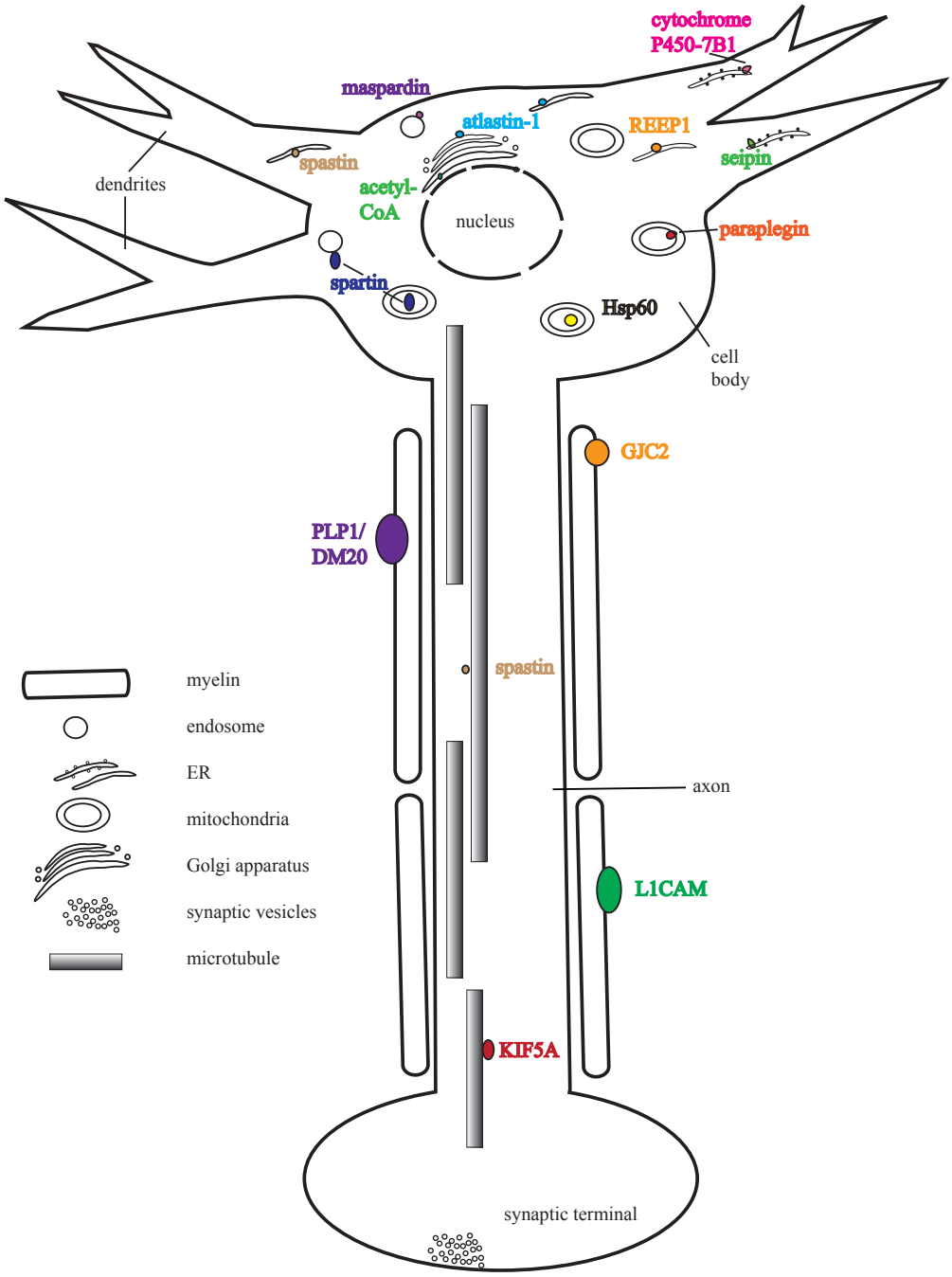

Figure 2. Schematic illustrating the placement of known HSP proteins in a neuron.

The SPG3A gene locus was first identified in a HSP screen almost a decade ago (15) as a large GTPase related to the dynamin superfamily through sequence similarity (see **Figure 3**). HSP patients with mutations in SPG3A often observe an earlier age of onset of symptoms, sometimes even in infancy (49), but mostly in late childhood and during the teenage years. A total of 39 missense and frameshift mutations in SPG3A have been reported (see **Table 2**), which accounts for about 10% of all AD-HSP cases. A later functional characterization showed that atlastin-1 was present mostly in the brain, enriched in the pyramidal neurons of the hippocampus and the cerebral cortex. Intracellularly, it colocalizes with Golgi apparatus markers, and can self-associate, most

likely forming a tetramer natively. It was additionally found to be a transmembrane protein with both its N- and C-terminal ends exposed to the cytoplasm (50). Also, atlastin can associate with spastin, a AAA (ATPases associated with various cellular activities) protein that is able to both bundle and sever microtubules *in vivo* (51) and the protein product of the gene most mutated in AD-HSP, by yeast two-hybrid and co-immunoprecipitation (52).

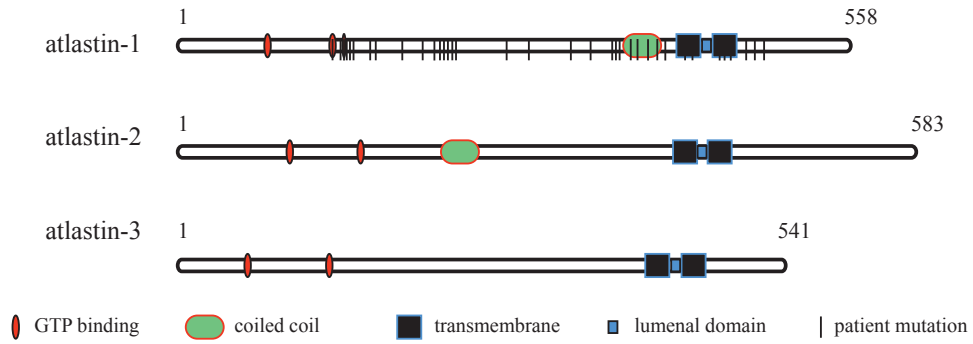

Figure 3. Schematic illustrating domains of interest in the Atlastin family of large GTPases. Black vertical lines in atlastin-1 indicate published patient mutations.

In contrast, there is only one HSP-causative mutation in maspardin known – a single nucleotide insertion (601insA) leading to a frameshift (fs201-212x213) and the truncation of the last 95 amino acids. This form of complicated autosomal recessive HSP is found primarily in Old Order Amish populations in central Pennsylvania. Mast syndrome was first described in the 1960s as a "recessively inherited form of presenile dementia with motor disturbances;" patients were observed to suffer from emotional lability along with spasticity, hyperreflexia, hypertonicity, and various other abnormal motor signs (53). Molecularly, maspardin was first described as an interaction partner of CD4 in T cells, specifically as an intracellular ligand (54). The previously unknown protein, named ACP33 for acid cluster protein 33, was found to have an α/β hydrolase domain, although it lacks the catalytic activity of other known α/β hydrolases. A Northern blot of human tissue and a Western blot of different cell lines indicated that ACP33/maspardin is expressed ubiquitously throughout the body and in a variety of cultured cell lines. Subcellular localization with antibody tagging in HUT 78 cells showed both endogenous and overexpressed maspardin in the cytosol and on intracellular vesicles, especially early endosomes. Maspardin was then found to be mutated in Mast syndrome, a form of HSP complicated by dementia (30). Patients suffering from the disease first presented learning difficulties in junior high school, and motor problems like incoordination were sometimes noticed in early childhood, although by their 20s most were "normal," married with children and a job. However, during their late 20s to early 40s, declines in speech were noticed, along with personality changes, seizures, and difficulty swallowing. At the time of examination, clear motor problems, enough to cause some to be bedridden, were evident, along with deficiencies in mental function, cerebellar dysfunction, and the suggestion of peripheral neuropathy in advanced cases. Magnetic resonance imaging (MRI) brain scans for three representative patients showed demyelination, a thin corpus callosum, and cerebellar

and cerebral atrophy. Additionally, co-immunoprecipitation experiments have established the aldehyde dehydrogenase ALDH16A1 as a binding partner of maspardin (55), although the functions of both proteins are still unknown.

This thesis will discuss the functional characterization of the HSP proteins mentioned above. We have examined the protein product of SPG3A, atlastin-1, and a couple of related family members, atlastin-2 and -3, in mostly *in vitro* systems. We also studied maspardin (Mast syndrome, spastic paraplegia, autosomal recessive with dementia) in a transgenic knockout mouse model.

Table 2: Known *SPG3A/atlastin-1* mutations

Exon	Mutation	Effect on protein	Domain affected	Ref
4	c.452T>C	p.Phe151Ser	GTPase	(56)
4	c.467C>T	p.Thr156Ile		(57)
4	c.470T>G	p.Leu157Trp	GTPase	(58)
4	c.481G>C	p.Ala161Pro	P* site	(59)
4	c.484A>C	p.Thr162Pro		(60)
4	c.493G>A	p.Ala165Thr		(61)
5	c.565C>G	p.His189Asp		(62)
5	c.572A>G	p.Gln191Arg	GTPase	(63)
7	c.650G>A	p.Arg217Gln	GTPase	(64)
7	c.715C>T	p.Arg239Cys	GTPase	(15)
7	c.716G>T	p.Arg239Leu	GTPase	(61)
8	c.740A>C	p.His247Pro		(59)
8	c.740A>G	p.His247Arg		(61)
8	c.749T>C	p.Leu250Pro	GTPase	(63)
8	c.751C>A	p.Gln251Lys	GTPase	(65)
8	c.757G>A	p.Val253Ile	GTPase	(65)
8	c.773A>G	p.His258Arg	GTPase	(15)
8	c.776C>A	p.Ser259Tyr	GTPase	(15)
8	c.776C>T	p.Ser259Phe	GTPase	(61)
9	c.944T>G	p.Ile315Ser		(56)
10	c.1006C>T	p.Tyr336His		(63)
12	c.1123T>C	p.Cys375Arg		(60)
12	c.1193C>A	p.Ser398Tyr		(56)
12	c.1220A>G	p.Lys407Arg	GTPase	(66)
12	c.1222G>A	p.Met408Val		(49)
12	c.1223T>C	p.Met408Thr		(63)
12	c.1239T>C	p.Phe413Leu	Coiled coil	(65)
12	c.1243C>T	p.Arg415Trp	Coiled coil	(67)
12	c.1247G>A	p.Arg416His	Coiled coil	(68)
12	c.1306_1308delAAT	p.del436Asn	Coiled coil	(69)
12	c.1319A>C	p.Asn440Thr	GTPase	(65)
12	c.1376A>G	p.Tyr459Cys	TM	(70)
12	c.1406G>C	p.Gly469Ala	TM	(63)
12	c.1445G>T	p.Gly482Val	TM	(63)
12	c.1474_1475insG	p.Ala492fsX522	TM	(63)
12	c.1483C>T	p.Arg495Trp	TM	(65)
12	c.1504_1505insG	p.Glu502fsX522		(71)
12	c.1520_1521insA	p.Ile507fsX522		(72)
13	c.1556G>A	p.Ser519Asn	C-term cytoplasmic	(65)

2 AIMS

1. To further examine the localization and functional roles of the *SPG3A* protein atlastin-1, a dynamin-like GTPase.

2. To characterize the cellular function(s) of the related non-neuronal GTPases atlastin-2 and -3.

3. To generate a *SPG21* knockout mouse as a model of human HSP and to examine and describe any abnormalities present.

3 METHODS

3.1 MOLECULAR BIOLOGY

DNA constructs The full coding sequence of the human atlastin-1 GTPase (GenBank™/EBI accession number NM_015915), atlastin-2a (GenBank accession number NM_022374), atlastin-2b (GenBank accession number AAM97342), and atlastin-3 (GenBank accession number AK097588) were amplified by PCR using *Pfu Turbo* (Stratagene, La Jolla, CA) from a Marathon human brain (cerebral cortex) cDNA library (Clontech) and confirmed by DNA sequencing. The full-length atlastin-1, -2a, -2b, and -3 (preceded by a Kozak consensus sequence) cDNAs were cloned into the *Xma*I site of the eukaryotic expression vector pGW1 with Myc or hemagglutinin (HA) epitope tags at the N terminus (73). The full-length atlastin cDNAs were also cloned into the *Xma*I site of pRK5 (Genentech, South San Francisco, CA) for expression of the untagged protein. Atlastin deletion constructs were generated by PCR using *Pfu Turbo*, and site-directed mutagenesis was performed using the QuikChange method (Stratagene). The YFP-Golgi expression construct was purchased from BD Biosciences Clontech.

Yeast two-hybrid Yeast two-hybrid tests were performed using the L40 yeast strain, harboring the reporter genes *HIS3* and ß-*galactosidase* under the control of upstream LexA binding sites. Strength of interaction was assessed by nutritional selection on histidine-lacking media.

3.2 BIOCHEMISTRY

Interaction studies COS-7 cells co-transfected with HA- and Myc-atlastin-1 or transfected with Myc-atlastin-1 alone were washed twice with PBS and then harvested in 0.5% Triton X-100 and PBS and clarified by centrifugation at 130,000 × *g* for 30 min. Extracts (100 μg of protein) were incubated for 1–2 h at 4 °C with 5 μg of rabbit polyclonal anti-HA probe antibodies (Y-11) pre-coupled to protein A-Sepharose CL-4B (Amersham Biosciences). Beads were washed three times with 0.5% Triton X-100 and PBS. Bound proteins were resolved by SDS-PAGE and immunoblotted with mouse monoclonal anti-Myc antibodies.

For chemical cross-linking studies, COS-7 cells over-expressing wild-type atlastin-1 or SPG3A mutants were washed twice with phosphate-buffered saline (PBS) and lysed; the post-nuclear supernatant was then incubated with 0.7 mM BS^3 (Pierce) in PBS for 2 h on ice. After the reactions were quenched with 1 M Tris–HCl buffer (pH 7.5), proteins were resolved by SDS–PAGE (6% acrylamide gels) and immunoblotted with anti-Myc antibodies.

Antibodies Affinity-purified polyclonal antibodies were generated commercially (Quality Controlled Biochemicals) in rabbits and goats against atlastin-2b (No. 6955; residues 561–578; acetyl-CIKAGLTDQVSHHARLKTD-OH) and atlastin-3 (No.

7053; residues 550–567; acetyl-CATVRDAVVGRPSMDKKAQ-OH) as well as to a region common to atlastin-2a and -2b (No. 6327; residues 1–17; MAEGDEAARGQQPHQGLC-OH) and a region identical in atlastin-1, -2 and -3 (No. 6944; residues 341–358 in atlastin-1; acetyl-LPHPKSMLQATAEANNLAC-OH), with terminal cysteine residues added to facilitate coupling to keyhole limpet hemocyanin. Other antibodies used include mouse monoclonal anti-GM130 (IgG_1, clone 35; BD Transduction Laboratories), anti-Myc-epitope (IgG_1, clone 9E10; Santa Cruz Biotechnology), PLCγ-1 (mixed IgGs, #05-163, Millipore), and anti-BiP/Grp78 (IgG_{2a}, clone 40; BD Transduction Laboratories) as well as rabbit polyclonal anti-calnexin (H-70; Santa Cruz Biotechnology).

GTPase activity assays Wild-type and SPG3A mutant atlastin-1 cDNA were subcloned into pCAL-n-EK for the production of calmodulin-binding peptide (CBP) fusion proteins (Stratagene). Expression of CBP-atlastin-1 in *Escherichia coli* BL21(DE3) was induced by 100 μm isopropyl-β-d-thiogalactopyranoside for 4.5 h at 25 °C. After pelleting, cells were resuspended in 50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 5 mm $MgCl_2$, 2 mm $CaCl_2$, 10% glycerol, 10 mm β-mercaptoethanol, 1.0% Triton X-100, and 0.5 mm phenylmethylsulfonyl fluoride and ruptured by two passages through a French pressure cell at 10,000 p.s.i. The extract was clarified by centrifugation at 50,000 × *g* for 30 min and then applied to calmodulin affinity resin (Stratagene). After washing with 50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 5 mm $MgCl_2$, 2 mm $CaCl_2$, 10 mm β-mercaptoethanol, and 0.1% Triton X-100, bound fusion proteins were eluted with 50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 10 mm β-mercaptoethanol, 2 mm EGTA, and 0.1% Triton X-100. Affinity-purified CBP-atlastin-1 fusion protein was dialyzed against assay buffer (20 mm HEPES (pH 7.2), 2 mm $MgCl_2$, and 1 mm dithiothreitol). The reaction mixture for the GTPase assay included dialyzed CBP-atlastin-1 with 0.05% bovine serum albumin and 0.825 μm [α-^{32}P]GTP (3000 Ci/mmol; ICN Biomedicals, Irvine, CA) in assay buffer. Samples of the reaction mixture at various time points (0–60 min) were spotted onto polyethyleneimine cellulose on polyester TLC plates (Sigma). Guanine nucleotides were separated by ascending chromatography in 1 m LiCl and 1.2 m formic acid. The [^{32}P]GDP and [^{32}P]GTP spots were identified, and intensities were quantified using PhosphorImager and ImageQuant software (Amersham Biosciences). GTPase activity was expressed as a ratio of GDP to total guanine nucleotides (GTP + GDP) at each time point. Data were compared using Student's *t*-test, and differences were considered to be significant if $P<0.05$.

3.3 CELL CULTURES AND TRANSFECTIONS

HeLa cells were maintained in Dulbecco's MEM supplemented with 10% fetal bovine serum (Gibco). For immunostaining experiments, HeLa cells plated on coverslips in 6-well plates were transfected with 1 μg of plasmid DNA using Lipofectamine (Invitrogen). Twenty-four hours later, cells were washed in phosphate-buffered saline [PBS (phosphate-buffered saline); pH 7.4] and fixed using 4% formaldehyde. Cells to be used for immunoblot analysis were washed with PBS and lysed with 0.1% Triton X-

100 in PBS. For siRNA transfections, HeLa cells were plated at 50% confluency and transfected the next morning with 100 nM siRNA oligonucleotides using Oligofectamine (Invitrogen) for 4 h. Cells in the double knockdown conditions were treated with 150 nM of each siRNA oligonucleotide duplex. Cells were then scraped for immunoblot analysis or fixed for immunocytochemical analysis 48 and 72 h after transfection. Specific siRNA oligonucleotides (Invitrogen) for atlastin-2 were targeted against the following sequences:
#1, GGAGCUAUCCUUAUGAACAUUCAUA;
#2, UCCUGGUCUUAAAGUUGCAACUAAU; and
#3, GAGAGCUUCGAAAUCUGGUUCCAUU.
Atlastin-3-specific siRNAs (Invitrogen) were as follows:
#1, GCCCUGACUUUGAUGGGAAAUUAAA;
#2, GGGCUACAUCAGGUAUUCUGGUCAA;
#3, GGUUAGAGAUUGGAGUUUCCCUUAU.
The control siRNA oligonucleotide was obtained from Ambion.

3.4 INTRACELLULAR TRAFFICKING – BFA TREATMENT AND VSVG ASSAY

HeLa cells plated in two-well glass bottom chambers were transfected with 0.5 µg of YFP-Golgi DNA, and the following day they were placed in growth media containing 5 µg/ml BFA (Epicentre). An image was acquired before addition of the BFA and subsequent images were taken every 30 s after addition of BFA.
For VSVG-GFP trafficking studies, HeLa cells grown on coverslips were transfected with the ts045 VSVG-GFP construct and immediately placed in a 40°C incubator. Sixteen hours after transfection, HeLa cells were moved to 32°C to allow for VSVG trafficking from ER to Golgi, and then fixed and processed for immunocytochemical analysis 0- 180 min after the temperature change.

3.5 TRANSGENIC MICE

Generation and breeding of knock out mice *SPG21-/-* mice were generated commercially (Caliper Life Sciences). For construction of the targeting vector, the mouse chromosome 9 sequence (nt # 65,660,000~65,740,000) was retrieved from the Ensembl database (http://www.ensembl.org) and used as reference in this project. BAC clone RP24-285H11 was used for generating homologous arms and southern probes by PCR or the RED cloning/gap-repair method. The 5' (4.1 kb) and 3' (5.8 kb) homologous arms were generated by RED cloning/gap repair. They were cloned into the LloxNwCD vector, with fidelity confirmed by restriction digestion and end-sequencing. The final vector was obtained by standard molecular cloning methods. Aside from homologous arms, the final vector also contained a LoxP-flanked Neo expression cassette (for positive selection of ES cells), and a DTA expression cassette (for negative selection of ES cells). Final vector construction was confirmed by both restriction digestion and end sequencing analysis. *Not*I digestion was used to linearize the final vector for electroporation. 5' and 3' external probes were generated by PCR

using proofreading TaKaRa LA Taq (Takara Bio) and tested using genomic Southern blot analysis for ES screening. They were cloned into the pCR4.0-TOPO backbone and confirmed by DNA sequencing (see **Figure 4**).

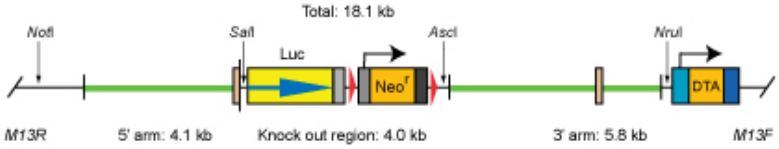

Figure 4. Deletion construct for the generation of* SPG21-/- *mice.

For genotyping, genomic DNA was isolated from tail snips using standard procedures. Gene-specific PCR was carried out in an MJ Research PTC-200 DNA Engine Thermal Cycler (Bio-Rad) using Taq DNA polymerase (Invitrogen) and primers specific for exon 3 of *SPG21* (Forward: 5' CGTGGATGACGATGACAGTA-3'), Neo cassette (Forward: 5'-GCCAGCTCATTCCTCCCACTCAT -3'), and genomic reverse (5'-AAAACTAAAGGCTAGCCGGG-3'). Reactions were subjected to an initial denaturing step of 2 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 90 s at 60 °C, and 60 s at 72 °C, with a final extension of 72°C for 10 min.

Behavioral studies For the beam-walking test, male mice of the indicated ages (2-12 months) were placed at one end of an 80 cm long, 1 cm-square wooden rod elevated 30 cm above the bench with wood supports. They were allowed to walk to the goal box at the other end, with the time to traverse recorded. The number of foot slips (one or both hind limbs slipped from the beam) was recorded using a tally counter.

Histology Mice were perfused transcardially with 5% paraformaldehyde (PFA). Brains and spinal cords were isolated and post-fixed in 5% PFA for at least 48 h. Whole brains and large sections of spinal cord were then Golgi-stained by incubation in 3% potassium dichromate in 5% PFA for up to one week, followed by two washes in freshly-prepared 0.75% silver nitrate and subsequently incubation in 0.75% silver nitrate for up to one week. Sections (50 µm thick) were then cut in distilled water on a sliding Leica vibratome and subsequently Nissl-stained.

Immunocytochemistry of tissue sections Mice were transcardially perfused with 4% PFA and skeletal muscles, spinal cords, and brains were isolated, post-fixed in 4% PFA overnight, and cryoprotected sequentially in 15% and 30% sucrose. Brains were cut coronally and spinal cords and some muscles were cut transversely on a cryostat (14 µm thick sections); the other muscle sections (30 µm thick) were cut longitudinally. Polyclonal rabbit anti-synaptotagmin I antibodies (gift of Dr. Peter Löw, KI) were used to stain all section types, while polyclonal rabbit anti-GluR2/3 (3 µg/ml) (gift of Dr. Katherine Roche, NINDS, NIH), chicken anti-neurofilament-H (Clontech; 1:50,000), and rat anti-MBP (Millipore; 1:300) were

applied to brain and spinal cord sections. Additionally, polyclonal rabbit anti-neurofilament-M (Millipore; 1:300) was used to label muscle sections. After washing, each primary antibody was followed by an appropriate Alexa Fluor-conjugated secondary antibody, or else α-bungarotoxin (Invitrogen) where indicated for muscles.

For whole-mount analysis of adult hind limb muscles, mice were transcardially perfused with 4% PFA. Lateral gastrocnemius muscles were isolated, post-fixed in 4% PFA overnight, and partitioned into four longitudinal sections using a scalpel. The tissue was rinsed in PBS and incubated in 0.1 M glycine/phosphate-buffered saline (PBS) at 4°C overnight. Subsequently, tissue was permeabilized in 1% Triton X-100/PBS at room temperature for 8 h. Antibody staining was performed with anti-neurofilament-M antibody in 5% normal goat serum (NGS)/PBS at 4°C for 40 h. Samples were washed 3 times for 20 min each in PBS and incubated at 4°C overnight using Alexa Fluor-conjugated secondary antibody (Invitrogen, 1:100) and α-bungarotoxin in 5% NGS in PBS. Muscles were washed four times for 20 min in 5% NGS/PBS, rinsed in PBS, and mounted. Images were obtained with an inverted Zeiss LSM 510 META confocal microscope. Z-stack projections were made from serial scanning every 1 μm to reconstruct the NMJ using the tool available in the LSM software. Measurements of average cross sectional areas and number of muscle fibers per NMJ and relative fluorescent intensities of brain and spinal cord markers were performed using ImageJ (NIH).

4 RESULTS

4.1 ATLASTIN-1, -2, AND -3 ARE MEMBERS OF THE ATLASTIN SUPERFAMILY OF LARGE GTPASES

In **Paper I**, we further characterized atlastin-1's location and function. While it was already known that it is present in the Golgi apparatus and the ER in adult neurons (50), and its sequence similarity to dynamin indicated that it was probably important in intracellular membrane trafficking, this had not previously been tested. Also, although new point mutations in the *SPG3A* gene are constantly being documented, their effects on the function of atlastin-1 had not been investigated. We examined these questions further in cultured neurons and *in vitro* assays.

Wild-type atlastin-1 had been shown to be an integral membrane protein that exists as functional oligomers, so we first tested whether several known missense mutations in the *SPG3A* gene would change this property (see **Figure 1** in paper). Using membrane association studies, we showed that the mutated atlastins were still present in the membrane fraction (see **Figure 1C** in paper). Additionally, each mutation could still associate with both itself and the wild-type protein, as shown in yeast-two hybrid studies and co-immunoprecipitation (see **Figures 1A & D** in paper). Finally, FPLC gel-exclusion chromatography and chemical cross-linking with BS^3 were used to show that the mutated atlastins were most likely present natively as tetramers (see **Figures 1E & F** in paper), indicating that the native structural properties of the protein had not been compromised by the respective mutations.

We also examined whether these point mutations might have caused a defect in the function of atlastin-1 with a GTPase activity assay. Not surprisingly, every mutation that we tested decreased the protein's GTPase effectiveness, though sometimes only modestly (see **Figure 2** in paper).

Since HSP caused by mutations in the SPG3A gene typically manifests itself a little earlier than other HSPs, we then examined the level of atlastin-1 protein expression in rat brain homogenates from E18 embryos, P8 pups, and adult rats, and found that atlastin-1 levels increased gradually though development (see **Figure 3** in paper).

Although atlastin-1 has been localized to the Golgi apparatus previously (50), we checked whether it might also be present in other structures. Indeed, in **Figure 3**, we used sucrose density gradient fractionation of atlastin-1 from cultured cortical neurons to show that some atlastin-1 is present in both *cis*-Golgi apparatus (p115) and endoplasmic reticulum (Grp78) fractions. We then co-localized atlastin-1 with antibodies specific for the *cis*-Golgi apparatus and the ER-Golgi intermediate complex (p115) and the endoplasmic reticulum (KDEL) in cultured cortical neurons (see **Figure 5** in paper) and with clathrin and AP-2 in axonal growth cones and varicosities (see **Figure 6** in paper), and confirmed this localization with electron microscopy (see **Figure 7** in paper).

Finally, to examine the effects of a loss of atlastin-1 expression in cultured neurons, we introduced shRNAs to knock down atlastin-1 (see **Figure 8** in paper). We found that knock down of atlastin-1 expression inhibits axon formation and elongation as well as dendrite formation, which we believe to be important in the pathogenesis of hereditary spastic paraplegia.

In **Paper II**, we examined a novel mutation in the *SPG3A* gene that was causative for hereditary spastic paraplegia. Overall, the age of onset of hereditary spastic paraplegia symptoms was quite young, on average at 1-2 years (see **Table** in paper). However, the in-frame deletion (p.del436N) resulted in a total lack of detectable atlastin-1 protein in cultured lymphoblast cell lines from patients without a corresponding change in total atlastin-1 RNA, as measured by qRT-PCR (see **Figure 1** in paper). Additionally, the mutant atlastin-1 could still interact normally with spastin (*SPG4*) via yeast two-hybrid, oligomerize, and hydrolyze GTP (see **Figure 2** in paper), indicating that disease pathogenesis might be caused by a loss of function.

In **Paper III**, we characterized two additional members of the atlastin family of GTPases. This was the first attempt to clarify the functions of other members of the atlastin family that might have pathogenic significance. However, while atlastin-1 is primarily present in brain tissue, atlastin-2 and -3 are ubiquitously expressed in all somatic tissues, with a small amount of atlastin-3 found in the brain as well (see **Figure 1d** in paper). We then confirmed that atlastin-2 and -3 have much of the same properties as atlastin-1 – they are all membrane-associated (see **Figure 2** in paper) and they form oligomeric complexes (see **Figure 3** in paper). However, atlastin-2 and -3 were not able to interact with spastin via yeast two-hybrid (see **Figure 11** in paper). We also showed that both atlastin-2 and -3 are present endogenously in the endoplasmic reticulum of HeLa cells (see **Figures 4 & 5** in paper), and then knocked down atlastin-2 and -3 expression with siRNA and found that lack of either protein caused Golgi apparatus fragmentation (see **Figure 6** in paper). However, this fragmentation was not functionally significant, as VSVG trafficking was not impaired in cells with both atlastin-2 and -3 expression knocked down (see **Figure 8** in paper). We then mutated residues in each of the atlastins, and found that overexpression of these mutated proteins caused a distinct change in endoplasmic reticulum morphology (see **Figure 9** in paper) but did not alter secretory trafficking, again measured by VSVG (see **Figure 10** in paper). However, the changes seen in intracellular trafficking pathways might still be significant in disease pathogenesis.

4.2 CHARACTERIZATION OF A *SPG21/MAST* KNOCKOUT MOUSE

In **Paper IV**, we characterized a transgenic mouse line missing the *SPG21* gene, which normally encodes maspardin/ACP33. These *SPG21-/-* mice were generated by homologous recombination with the goal of disrupting exon 3 the gene (see **Figure 2** in paper).

At birth, knockout pups appeared normal. However, at several months of age, most *SPG21-/-* mice started dragging their hind limbs, which worsened with increasing

age. When subjected to a narrow beam-walking test at 2-6 months of age, *SPG21-/-* mice slipped more often on the beam, and as they grew older exhibited difficulty in walking on the beam, quantified as a delay in the time taken to traverse the length of the beam (see **Figure 3** in paper).

We then examined cultured cortical neurons taken from *SPG21-/-* mice. Many of these cells appeared to have the morphology of pyramidal-type neurons. Under normal primary culture conditions, axons of *SPG21-/-* neurons appeared to have significantly more axon branches than axons from *SPG21+/+* neurons, although the length of the primary axon and the number of primary axons were unchanged (see **Figure 4** in paper). We also tested the cultured cells' responses to the BDNF growth factor, but found no significant effects 4 hours after stimulation.

A comprehensive pathological characterization of aged *SPG21-/-* mice at 18-19 months of age was conducted, with a focus on the central nervous system. Serum chemistries, hematology studies, and organ/body weights were all examined, and pathologic changes such as degeneration of the knee, mild degenerative disc disease of the spine, and loss of neurons from the spiral ganglia of the inner ear were seen, but in both groups of aged animals. We also examined central motor pathways – the motor cortex, spinal cord, and neuromuscular junctions – in more detail, but did not find appreciable differences between *SPG21-/-* and *SPG21+/+* animals at any level (see **Figures 5-8** in paper).

Finally, a gene chip analysis was conducted on mRNA isolated from both knockout and wild-type animals at 6 and 15 months of age (see **Table 1** in paper). The most interesting finding was an upregulation in the level of KIF5A (a kinesin family member motor protein that is important for the transport of cytoplasmic cargoes along microtubules (74)) mRNA in older animals, which could have indicated an abnormality in cargo trafficking. However, when protein levels were examined by Western blotting, no significant changes were noted (see **Figure 9** in paper).

5 CONCLUSIONS

1. **The *SPG3A* protein atlastin-1 is enriched in vesicular structures within growth cones, varicosities, and at axonal branch points. Knockdown of atlastin-1 expression impairs axon formation and elongation during development.**

2. **Atlastin-2 and -3, other atlastin family member proteins, are expressed ubiquitously. Knockdown of either and both proteins' expression levels results in, among other things, fragmention of the Golgi apparatus and changes in ER morphology, indicating a role in organelle morphology and shaping.**

3. **Maspardin knockout mice exhibit deficiencies in locomotion reminiscent of human HSP, suggesting its utility as a model to study this disease. Cultured *SPG21-/-* neurons exhibit abnormally exuberant axonal branching, suggesting that a component of intracellular axonal trafficking is likely affected.**

6 DISCUSSION

Today, HSP is treated symptomatically, mostly with agents to relieve spasticity. GABA agonists (75) and derivatives (76-83) have been in use for the treatment of HSP and other disorders affecting spinal movement the longest. Patients receive intrathecal (directly through the theca of the spinal cord into the spinal fluid) dosages to reduce muscle tone and improve muscle performance and bladder function, but side effects such as headaches, nausea, dizziness, drowsiness, and weakness are common. In patients unresponsive to these therapies, targeted electrical stimulation has been successfully used, with recovery in gait patterns and standing stances noted (84). Other patients have recently been treated with injections of botulinum neurotoxin type A (BoNT-A), which resulted in subjectively measured reductions in spasticity (85). However, the mechanism(s) causing the degeneration leading to paraplegia in this case (and in similar diseases) has yet to be completely explained, which underscores the need for the development and utilization of reliable model systems for both the understanding and treatment of HSP. These systems could also give insights into the function and potential repair of the spinal cord following trauma or other degenerative disorders. There have been many attempts at modeling HSP in a variety of non-mammalian systems, including *Drosophila*, *C. elegans,* and zebrafish. More common HSP proteins – spastin, for example – have been studied extensively in all three systems (86-90) and successfully rescued with expression of the wild-type protein or agents such as the microtubule targeting and stabilizing drug vinblastine (91).

Mouse models are probably the closest we can justifiably get to studying HSP in a mammalian system. While further experiments are necessary to elucidate the pathogenesis underlying the movement deficiencies in our transgenic model, it may still prove useful as research using other models has shown, although finding the disease-causing mechanism(s) may prove difficult. In our case, our model probably has some compensatory mechanism that allows it to survive with a relatively mild phenotype and masks changes similar to what we observed in cultured cells grown outside of their *in vivo* environments. Also, while mice are certainly useful, no non-primate organism has a human-like corticospinal system, making it difficult to both create a model and to interpret changes, so it is not surprising that the cognitive defects often seen in complicated forms of HSP and other neurodegenerative diseases are hard to create and measure in rodents. However, depending on the gene involved, in some models, such as a complete knockout of the *Hspd1* gene that encodes the Hsp60 mitochondrial chaperone, are embryonic lethal (92), but others, such as the spastin knockout (93), the myelin *Plp* knockout (94), and the ALS2-deficient mouse (95) are viable but with axonal transport irregularities and progressive axonal degeneration. Other models such as the paraplegin-deficient mouse allow the mice to at least be born and in some cases survive to adulthood with both molecular and movement-related defects – in this case axonal swelling and degeneration progressively correlated with reduced performance levels in rotarod tests (96). However, these knockout mice showed wild-type motor abilities for up to 10 months following intramuscular

injections of paraplegin via an adenoassociated virus, suggesting a possible treatment direction (97).

In this thesis I attempted to better understand the functions of two classes of genes and their products seemingly connected only though a disease they can both cause when mutated – the atlastin superfamily of large GTPases and the maspardin Mast syndrome protein. We looked deeper into the function and locations of the brain-specific atlastin-1 protein, and found that in addition to being present in the Golgi apparatus as previously reported, it could also be found in vesicular structures at axonal branch points, growth cones, and varicosities along the axon. Knock-down of atlastin-1 using shRNA in cultured cortical neurons adversely affected neurite outgrowth and significantly shortened the length of the axon, if present. Also, some known *SPG3A* patient mutations do lead to proteins that are functionally deficient when compared to wild-type GTPase activity, depending on the mutation studied. We also characterized the related non-neuronal atlastin-2 and -3 proteins and found them present in the endoplasmic reticulum and in all tissues. Perturbing the expression levels of these proteins, both through siRNA knockdown and overexpression of dominant-negative constructs, contributed to protein changes such as Golgi apparatus fragmentation, indicating a possible collective role of all three proteins in protein sorting/secretion in a wild-type state.

Since papers I, II, and III were published, atlastin-1 has been shown to be required for formation of the structure of the endoplasmic reticulum; depletion of atlastin-1 with siRNA in HeLa cells or overexpression of atlastin-1 in COS-7 cells lead to a change in tubule interactions and a lack of ER tubule branching, which was then confirmed using the Sey1p atlastin ortholog in *S. cerevisiae* (98). Also, *Drosophila* Atlastin has been localized to ER membranes; when depleted by RNAi, ER fragmentation occurs. Atlastin can drive membrane fusion in a GTP-dependent manner *in vitro* on proteoliposomes; this ability disappears in GTPase-deficient Atlastin. However, the overexpression of Atlastin in flies causes enlargement of the ER, which could be due to excessive fusion of ER membranes (99) or perhaps some form of aggregation (100). Interestingly, *Drosophila* Atlastin has also been shown to regulate microtubule stability in muscles and to be required for proper synapse development (101); loss of Atlastin also caused dopaminergic neuron death in aged flies (102). Indeed, as Atlastin-1 interacts with spastin and REEP1, another HSP protein, maintenance of ER structure and function could be a key to the pathogenesis of HSP (103), and agents that promote stability of the ER and healthy organelle size could be useful in treating this disease. However, how atlastin-1 and the ER relate to other HSP proteins that normally reside on microtubules, in mitochondria, and on other organelles and are integral components of myelin is unknown, and much remains to be explained.

Understanding if and how known HSP proteins interact with each other might also provide suggestions for how a somewhat common set of symptoms might be related through so many different genes. For example, although further studies are required, in paper IV we found a relationship between abolishing the maspardin protein and increases in levels of KIF5A mRNA, which can also be mutated in a different type of HSP. Normally, KIF5A kinesin plays a role in many dynamic cellular functions such as vesicular and organelle transport, microtubule movement, morphogenesis, and

spindle formation and elongation. Mutations in the *SPG10* locus can cause both early- and adult-onset HSPs (104-110); *in vitro* studies indicate that these patient mutations can cause KIF5A to have a reduced affinity for microtubules and/or decreases in gliding velocity (111), indicating a change compared to wild-type functions. While the protein levels of KIF5A and pretty much everything else besides maspardin were normal in our mouse model, we noted that the mice did develop a steadily progressive movement inability starting from about half a year of age, although total lifespans of the mice were no shorter than their wild-type counterparts. We measured a significant increase in the amount of time required to cross a walking beam and also found that cultured cortical neurons from *SPG21-/-* branched significantly more exuberantly than wild type littermate neurons. We were unable to find any traces of what this caused in adult animals following comprehensive neuropathological studies, but we remain hopeful that the molecular basis will be found, as behaviorally this mouse can be considered a model for HSP.

Indeed, the most striking differences we found in studies of both proteins was in whether and how much neurite outgrowth occurred in culture following different treatments – knockdown of atlastin-1 levels using shRNAs in cultured rat cortical neurons significantly decreased the chances that a given neuron would form an axon and, if an axon was formed, significantly decreased its length when compared to shRNA vector treated neurons. Conversely, cultured cortical neurons from *SPG21-/-* mice exhibited increased branching when compared to neurons from wild-type littermates. It is therefore likely that these two proteins normally function in separate, possibly opposing, pathways, but these dramatic effects further underscore the importance of correctly functioning intracellular trafficking and transport in the continued health of the cell and the larger organism.

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