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Modelling Hereditary Spastic Paraplegias using Human Pluripotent Stem Cells

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Modelling Hereditary Spastic Paraplegias using Human Pluripotent Stem Cells

Kyle R. Denton
University of Connecticut, [2015]

Abstract: Hereditary spastic paraplegias (HSP) comprise a large, heterogeneous group of genetic neurodegenerative disorders. The unifying symptom present among HSP patients is lower limb spasticity, which is caused by the degeneration of cortical spinal motor neuron (CSMN) axons. Research on various HSP subtypes has identified a number of common cellular pathways that are weak points for long projection neurons, particularly CSMNs. The focus of this work has been to generate novel cellular models of several HSP subtypes using human pluripotent stem cells (hPSCs). Stem cell models were chosen because of their ability to use cells that have the same genetic background as patients generate affected neuronal subtypes. The most common HSP subtype SPG4, which is caused by dominant mutations in the SPAST gene affecting the microtubule-severing enzyme spastin, was first analyzed. This revealed that SPG4-derived telencephalic glutamatergic neurons possess a number of abnormalities, including axonal swellings, reduced fast axonal transport, and increased microtubule stabilization. Treatment with the microtubule targeting drug vinblastine rescued the axonal swelling phenotype in neurons with reduced spastin. Next SPG3A was analyzed using patient-specific iPSC-derived telencephalic neurons. SPG3A cells with a novel P342S mutation in atlastin-1 had reduced endoplasmic reticulum (ER) complexity, and neurons had reduced neurite outgrowth and fast axonal transport. Similar to SPG4, vinblastine was able to rescue the neurite outgrowth phenotype in SPG3A neurons. Lastly, two rare, autosomal recessive forms of HSP, SPG15 and SPG48, which lack spastizin and AP5Z1 expression respectively, were examined. Telencephalic glutamatergic and midbrain dopaminergic neurons showed a number of defects, including reduced neurite outgrowth, increased apoptosis, abnormal mitochondrial...
morphology and membrane potential. Inhibition of mitochondrial fission with the small molecule mdivi-1 rescued mitochondrial and neurite outgrowth defects, offering a potential therapeutic avenue in the future. All of these studies emphasize the utility of hPSC-derived neurons to study human monogenic disorders and for testing therapeutic compounds. In the future, these models will allow further investigation of the mechanisms that cause the degeneration of a subset of neurons, which will be valuable not just for HSP, but for other neurodegenerative disorders.
Modelling Hereditary Spastic Paraplegias using Human Pluripotent Stem Cells

Kyle R. Denton

B.A., Clark University, [2010]
M.A., Clark University, [2011]

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
at the
University of Connecticut

[2015]
APPROVAL PAGE

Doctor of Philosophy Dissertation

Modelling Hereditary Spastic Paraplegias using Human Pluripotent Stem Cells

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1. Chapter 1:
1.1 Introduction

Hereditary spastic paraplegias (HSP) are a heterogeneous group of genetic disorders that result in progressive lower limb spasticity. The symptoms are caused by a length-dependent degeneration of axons, most severely affecting corticospinal motor neurons (CSMNs) and those of the dorsal columns. Affected neurons display “dying back” axonopathy, particularly in the spinal cord, resulting in lost lower motor neuron innervations which produce lower limb spasticity and weakness. Over the past decade much work has succeeded in increasing the understanding of the genetic causes of the disorder and the function of the genes involved. To date, there have been over 71 different gene loci linked, denoted SPG 1-71, of which over 54 genes have been shown to lead to HSP when mutated. These studies have provided insight into several common pathways whose disruption predominantly affects highly polarized projection neurons, revealing weaknesses in these cells due to their impressive morphology. CSMNs, which are the main neurons affected in HSP, can have axons that reach up to 1 meter in length, with axoplasm making up over 99% of the total cells’ volume in some cases [1]. This striking polarity makes these cells sensitive to perturbations to many cellular processes, including axonal transport, cytoskeletal dynamics, endoplasmic reticulum morphogenesis, and endocytic recycling.

1.1.1 Symptoms

Hereditary spastic paraplegia, also called Strumpell-Lorrain disease, was first described by the German neurologist Adolph Strumpell in 1883 [2] and later by the French doctor Maurice Lorrain in 1888 [3]. HSP is classified as a rare disorder by the Office of Rare Diseases (ORD) of the National Institutes of Health, and although the prevalence varies between studies, a similar
range of 2 – 9.6 in 100,000 individuals is observed [4, 5]. In the United States it is estimated that between 10,000 and 20,000 individuals suffer from one of the HSP subtypes, making it comparable to the more well-known neurodegenerative disease amyotrophic lateral sclerosis (ALS). Several methods of categorizing HSP are used. HSP can be classified based on its mode of inheritance, with all possible forms, dominant, recessive and X-linked reported [6-8]. Historically HSP has been classified into two broad categories based on the presence or absence of additional symptoms other than lower limb spasticity and weakness. HSP is classified as “pure” when spasticity is the only clinical observation, or “complex” when it coincides with other clinical features [9]. Common additional neurological features that accompany complex HSP include peripheral neuropathy, cataracts, muscle atrophy, ataxia, cognitive impairment, ichthyosis, epilepsy, dementia, and thinning of the corpus callosum [10, 11]. Most forms of complex HSP display autosomal recessive inheritance and therefore very few patients exist, making it unclear if the additional deficits are due to HSP or are from multiple inherited disorders [11]. An additional classification for HSP depends on the age of onset, with early onset forms presenting during infancy and late onset forms becoming noticeable as late as the eighth decade. More recently, with the identification of many causative genes, HSP is classified based on the particular gene that is affected. These classifications are currently not very useful to patients since there are still no treatments available for any forms of HSP, however they could help inform a patient of the likelihood of passing on the disorder to the next generation. In the future as new therapies emerge, the differences in HSP forms may play a more important role in guiding treatment course. In addition, the identification of causative genes has provided valuable insight into cellular pathways that are critical for axonal maintenance and the vulnerabilities displayed by the most morphologically impressive cells in the human body.
All forms of HSP display an insidious onset that can take many years before symptoms become obvious. Interestingly, when symptoms begin in early childhood, after the initial onset of spastic gait or toe walking, the progression usually levels off and does not worsen or improve during the duration of the patient’s life [10]. This is quite different than the disease course for patients with onset after the age of 6, where the gait deficits usually become progressively worse over many years and most will lose the ability to walk later in life [10, 12]. Muscle spasticity is more evident in the quadriceps, hamstrings and ankles due to hypertonicity. Often the lower limb spasticity is more severe than muscle weakness, which is exemplified in wheelchair bound patients who display normal individual muscle strength [12]. Common features other than lower limb spasticity include hyperreflexia, and subsets of patients display sensory deficits, mainly decreased vibration sensitivity around the ankles [10]. Urinary urgency is also frequently associated with both pure and complex HSP. The upper limbs of HSP patients generally do not display spasticity, but may show hyperreflexia [6]. This suggests that the longest axons are preferentially affected in HSP.

1.1.2 Neuropathology in HSP

Most postmortem studies have focused on pure HSP, and the major observation was distal axon degeneration of the longest descending motor fibers, mainly the corticospinal tract [13]. The most affected region was the thoracic spinal cord where, depending on the patient’s height, these axons can reach up to 1 meter in length. Some axons in the longest ascending fibers, the fasciculus gracilis tract, also display degeneration, although to a lesser degree than the corticospinal tract, which may explain the sensory deficits found in some patients [14]. Demyelination was observed in regions of axon degeneration, but it is generally believed that this is a secondary effect that follows axonal problems for most forms of HSP. Thinning of the thoracic spinal cord can be observed in HSP patients by MRI [15]. While there is significant
axon loss, neuron death is not easily detected in HSP patients, although there are some reports of decreased Betz cells (pyramidal neurons).

### 1.1.3 Prognosis and Treatments

Although HSP can cause significant long term disability for affected individuals, it is not believed to alter the lifespan of those individuals. Currently no therapies exist that can prevent, slow the progression, or cure HSP. Most therapies are aimed at alleviating muscle spasticity and urinary urgency in affected patients. Muscle spasticity treatments, which aim to maintain muscle functionality and prevent contractures, include the GABA$_B$ receptor agonist baclofen, and the muscle relaxants tixanidine or dantrolene [16]. Intrathecal botulimun toxin injection is occasionally used for more severe cases [17]. Urinary urgency is generally well controlled through treatment from drugs such as oxybutyinin [10]. Orthotic devises are often used to help patients walk with more stability, and supportive devices such as canes or wheelchairs may be required as the disease progresses. Physical therapy is recommended for all HSP patients to help preserve muscle strength and flexibility.

### 1.1.4 Cellular Pathogenesis

Breakthroughs in basic science research into the genetic causes and pathogenic mechanisms of HSP have dramatically accelerated in the past decade (Table 1). Work in this field has uncovered many cellular processes that are affected by multiple forms of HSP, which have greatly aided in the understanding of the importance of axonal maintenance in human disease (Fig. 1-1). Through the rest of this review, some highlights of the recent work on HSP will be covered, with a focus on the four forms investigated here, SPG4, SPG3A, SPG15, and SPG48. Next, background on the common pathways affected between these four HSP subtypes will be discussed, followed by an introduction to the model system we employed.
Table 1: HSP loci and known genes, divided into functional groups (compiled from OMIM and [1, 11, 18])

<table>
<thead>
<tr>
<th>Locus</th>
<th>Inheritance</th>
<th>Locus</th>
<th>Protein</th>
<th>Clinical features</th>
<th>Frequency</th>
<th>Models</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPG3A (Atlastin-1)</td>
<td>AD</td>
<td>14q12-q21</td>
<td>Atlastin-1</td>
<td>Early-onset pure, slow progression HSP</td>
<td>&lt;10% AD HSP</td>
<td>C. elegans [19], Zebrafish [20], drosophila [21, 22], mouse [23], iPSC-derived neurons [24]</td>
</tr>
<tr>
<td>SPG4 (SPAST)</td>
<td>AD</td>
<td>2p22</td>
<td>Spastin</td>
<td>Variable-onset mainly pure HSP</td>
<td>40% of AD HSP</td>
<td>Zebrafish [25, 26], drosophila [27, 28], mouse [29], iPSC-derived neurons [30]</td>
</tr>
<tr>
<td>SPG6 (NIPA1)</td>
<td>AD</td>
<td>15q11.2-q12</td>
<td>Non-imprinted in Prader-Willi/Angelman syndrome region protein 1) NIPA1</td>
<td>Adult-onset pure HSP</td>
<td>~10 families</td>
<td>C. elegans [31], drosophila [32]</td>
</tr>
<tr>
<td>SPG8 (KIAA0196)</td>
<td>AD</td>
<td>8q24</td>
<td>Strumpellin</td>
<td>Adult-onset pure HSP, marked spasticity</td>
<td>&lt;10 families</td>
<td>Drosophila [33], Zebrafish [34]</td>
</tr>
<tr>
<td>SPG10 (KIF5A)</td>
<td>AD</td>
<td>12q13</td>
<td>Kinesin family member 5A</td>
<td>Early-onset pure HSP, can be complicated with distal amyotrophy</td>
<td>&lt;10 families</td>
<td>Drosophila [35], Mouse [35]</td>
</tr>
<tr>
<td>SPG11 (KIAA1840)</td>
<td>AR</td>
<td>15q</td>
<td>Spatacsin</td>
<td>Childhood to early adult onset, thin corpus callosum, cognitive impairment, neuropathy</td>
<td>Many families</td>
<td>Zebrafish [36, 37], iPSC-derived neurons [38]</td>
</tr>
<tr>
<td>SPG12 (RTN2)</td>
<td>AD</td>
<td>19q13</td>
<td>Reticulon-2</td>
<td>Early-onset pure HSP</td>
<td>&lt;10 families</td>
<td></td>
</tr>
<tr>
<td>SPG15 (2FYVE26)</td>
<td>AR</td>
<td>14q</td>
<td>Spastizin</td>
<td>Kjellin syndrome: adolescent onset, pigmented retinopathy, cerebellar signs, mental retardation</td>
<td>&lt;10 families</td>
<td></td>
</tr>
<tr>
<td>SPG17 (BSCL2)</td>
<td>AD</td>
<td>11q12-q14</td>
<td>Seipin</td>
<td>Silver syndrome: variable onset, distal amyotrophy in hands more than in feet</td>
<td>&lt;20s families</td>
<td></td>
</tr>
<tr>
<td>SPG18 (ERLIN2)</td>
<td>AR</td>
<td>8p11.23</td>
<td>Endoplasmic reticulum, lipid raft associated protein 2</td>
<td>Severe complex HSP, psychomotor retardation, thin corpus callosum, intellectual disability</td>
<td>5 families</td>
<td></td>
</tr>
<tr>
<td>SPG20</td>
<td>AR</td>
<td>13q</td>
<td>Spartan</td>
<td>Troyer syndrome: childhood onset, amyotrophy, cerebellar signs, developmental delay</td>
<td>&gt;10 families</td>
<td></td>
</tr>
<tr>
<td>SPG21 (ACP33)</td>
<td>AR</td>
<td>15q</td>
<td>Maspardin</td>
<td>Mast syndrome: early adult onset, thin corpus callosum, cognitive decline, extrapyramidal features, cerebellar signs</td>
<td>&gt;10 families</td>
<td>Mouse [39]</td>
</tr>
<tr>
<td>SPG33 (2FYVE27)</td>
<td>AD</td>
<td>10q24.2</td>
<td>Protrudin</td>
<td>Uncomplicated HSP</td>
<td>1 family</td>
<td>Zebrafish [40]</td>
</tr>
<tr>
<td>SPG30 (KIF1A)</td>
<td>AR</td>
<td>2q37.2</td>
<td>KIF1A</td>
<td>Adolescent-onset pure HSP, sensory neuropathy</td>
<td>1 family</td>
<td>C. elegans [41], Mouse [42]</td>
</tr>
<tr>
<td>SPG31 (REEP1)</td>
<td>AD</td>
<td>2p12</td>
<td>Receptor expression-enhancing protein 1</td>
<td>Variable-onset pure HSP</td>
<td>8% of AD pure HSP</td>
<td>Mouse [43]</td>
</tr>
<tr>
<td>SPG51 (AP4E1)</td>
<td>AR</td>
<td>15q21.2</td>
<td>Adaptor protein complex 4 eta 1</td>
<td>Complicated, microcephaly, hypotonia, white matter loss, cognitive impairment</td>
<td>2 families</td>
<td></td>
</tr>
<tr>
<td>Inheritance</td>
<td>Locus</td>
<td>Protein</td>
<td>Clinical features</td>
<td>Frequency</td>
<td>Models</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>SPG53</strong> (VPS37A)</td>
<td>AR 8p22</td>
<td>Vacuolar protein sorting 37A</td>
<td>Complicated early-onset HSP, cognitive impairment, impaired vibration sensitivity</td>
<td>2 families</td>
<td>Zebrafish [44]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG58</strong> (KIF1C)</td>
<td>AR chr17:49 04143</td>
<td>Kinesin family member 1C</td>
<td>Complicated and uncomplicated, early childhood onset, increased deep tendon reflexes, white matter abnormalities</td>
<td>2 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG59</strong> (USP8)</td>
<td>AR chr15:50 769124</td>
<td>Ubiquitin specific peptidase 8</td>
<td>Complicated, early childhood onset, increased deep tendon reflexes, positive clonus</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG60</strong> (WDR48)</td>
<td>AR chr3:391 35498</td>
<td>WD repeat domain 48</td>
<td>Complicated, early onset, increased patellar and absent Achilles tendon Reflexes, peripheral neuropathy</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG61</strong> (AR L6IP1)</td>
<td>AR chr16:804609</td>
<td>ADP-ribosylation factor-like 6 interacting protein 1</td>
<td>Complicated, early onset, increased patellar and absent Achilles tendon reflexes</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG62</strong> (ERLIN1)</td>
<td>AR chr10:10 1914679</td>
<td>ER lipid raft associated 1</td>
<td>Uncomplicated, early onset, increased deep tendon reflexes</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mitochondrial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG13</strong> (HSPDI)</td>
<td>AD 2q24-q34</td>
<td>Heat shock protein 60</td>
<td>Adult-onset pure HSP</td>
<td>&lt;10 families</td>
<td>Mouse [45]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG17</strong></td>
<td>AR 16q</td>
<td>Paraplegin</td>
<td>Variable onset, cerebellar signs, optic atrophy, neuropathy</td>
<td>~30 families</td>
<td>Mouse [46]</td>
<td></td>
</tr>
<tr>
<td><strong>Lipid/Myelination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG2</strong> (PLP1)</td>
<td>X- linked Xq21</td>
<td>Proteolipoprotein 1</td>
<td>Quadriplegia, nystagmus, mental retardation, seizures</td>
<td>&lt;100 familial cases</td>
<td>Mouse [47]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG5</strong> (CYP7B1)</td>
<td>AR 8p</td>
<td>Cytochrome P450-7B1</td>
<td>Variable-onset pure and complicated HSP</td>
<td>~20 families</td>
<td>Mouse [48]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG28</strong> (DDHD1)</td>
<td>AR 14q22.1</td>
<td>Phospholipase DDHD1</td>
<td>Early-onset pure HSP</td>
<td>3 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG35</strong></td>
<td>AR 16q21-q23</td>
<td>Fatty acid 2-hydroxylase</td>
<td>Childhood onset, intellectual decline, seizures</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG66</strong> (ARS1)</td>
<td>AR chr5:149 676845</td>
<td>Arylsulfatase family member 1</td>
<td>Complicated, early onset, absent deep tendon reflexes</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG54</strong> (DDHD2)</td>
<td>AR chr8:381 03473</td>
<td>DDHD domain-containing protein 2</td>
<td>Complicated, early onset, intellectual disability</td>
<td>6 families</td>
<td>Drosophila [49]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG67</strong> (PGAP1)</td>
<td>AR chr2:197 712670</td>
<td>Post-GPI attachment to proteins 1</td>
<td>Complicated, early onset, increased deep tendon reflexes,</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Axon guidance/synapse related</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG1</strong> (L1CAM)</td>
<td>X- linked Xq28</td>
<td>L1 cell adhesion molecule</td>
<td>Mental retardation, hypoplasia of corpus callosum, adducted thumbs, hydrocephalus</td>
<td>Over 100 familial cases</td>
<td>C. elegans [50], mouse [46]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG68</strong> (FLRT1)</td>
<td>AR chr11:63 885762</td>
<td>Fibronectin leucine rich transmembrane protein 1</td>
<td>Complicated, early onset, increased deep tendon reflexes, patellar clonus, peripheral neuropathy</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG69</strong> (RAB3GAP 2)</td>
<td>AR chr1:220 357421</td>
<td>RAB3 GTPase activating protein subunit 2</td>
<td>Complicated, early onset, increased deep tendon reflexes, positive clonus, intellectual disability, deafness, cataract</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locus</td>
<td>Protein</td>
<td>Clinical features</td>
<td>Frequency</td>
<td>Models</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Nucleotide metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG63 (AMPD2)</td>
<td>AR</td>
<td>chr1:110 167989 Adenosine monophosphate deaminase 2</td>
<td>Complicated, early childhood onset, increased deep tendon reflexes</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG64 (ENTPD1)</td>
<td>AR</td>
<td>chr10:97 605168 Ectonucleoside triphosphate diphosphohydrolase 1</td>
<td>Complicated, early onset, increased deep tendon reflexes</td>
<td>2 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG65 (NT5C2)</td>
<td>AR</td>
<td>chr10:10 4899162 5'-nucleotidase, cytosolic II</td>
<td>Complicated and uncomplicated, both early and adult onset, increased deep tendon reflexes</td>
<td>5 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG9</td>
<td>AD</td>
<td>10q23.3-q24.2 Unknown</td>
<td>Cataracts, motor neuropathy, skeletal abnormalities, gastro-oesophageal reflux</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG14</td>
<td>AR</td>
<td>3q27-q38 Unknown</td>
<td>Variable onset, motor neuropathy, mental retardation</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG16</td>
<td>X-linked</td>
<td>Xq11.2 Unknown</td>
<td>HSP with onset in infancy, aphasia, sphincter disturbance, mental retardation</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG19</td>
<td>AD</td>
<td>9q33-q34 Unknown</td>
<td>Adult-onset pure HSP</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG23</td>
<td>AR</td>
<td>1q24-q32 Unknown</td>
<td>Lison syndrome: childhood onset, pigmentation abnormalities, facial and skeletal dysmorphism, cognitive decline, tremor</td>
<td>4 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG24</td>
<td>AR</td>
<td>13q14 Unknown</td>
<td>Childhood-onset pure HSP, pseudobulbar signs</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG25</td>
<td>AR</td>
<td>6q23-q23 Unknown</td>
<td>Adult onset, cataracts, prolapsed intervertebral discs</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG26</td>
<td>AR</td>
<td>12p11.1-q14 Unknown</td>
<td>Complicated HSP, adult onset, neuropathy and distal wasting, intellectual impairment</td>
<td>6 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG27</td>
<td>AR</td>
<td>10q22.1-q24.1 Unknown</td>
<td>Variable onset, cerebellar signs, neuropathy, mental retardation, microcephaly</td>
<td>2 families</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG29</td>
<td>AD</td>
<td>1p31-p21 Unknown</td>
<td>Early adolescence onset, sensorineural deafness, hiatus hernia, pes cavus, hyperbilirubinaemia</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG32</td>
<td>AR</td>
<td>14q12-q21 Unknown</td>
<td>Childhood onset, mental retardation, thin corpus callosum, pontine dysraphism</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG34</td>
<td>X-linked (R)</td>
<td>Xq24-q25 Unknown</td>
<td>Pure HSP, late childhood onset</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG36</td>
<td>AD</td>
<td>12q23-q24 Unknown</td>
<td>Complicated HSP, lower limb sensory deficits</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG37</td>
<td>AD</td>
<td>8p21.1-q13.3 Unknown</td>
<td>Pure HSP, variable onset, hyperreflexia of the upper limbs, decreased vibration sensitivity</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPG38</td>
<td>AD</td>
<td>4p16-p15 Unknown</td>
<td>Distal amyotrophy (Silver syndrome)</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inheritance</td>
<td>Locus</td>
<td>Protein</td>
<td>Clinical features</td>
<td>Frequency</td>
<td>Models</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>SPG39</strong></td>
<td>AR 19p13</td>
<td>Neuropathy target esterase</td>
<td>Childhood onset, marked distal wasting in all four limbs</td>
<td>2 families</td>
<td>Drosophila [51], Mouse [52]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG41</strong></td>
<td>AD 11p14.1-p11.2</td>
<td>Unknown</td>
<td>Pure HSP, urinary urgency, adolescent onset</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG42</strong></td>
<td>AD 3q25.31</td>
<td>acetyl-CoA transporter</td>
<td>Early onset</td>
<td>1 family</td>
<td>Zebrafish [53]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG43</strong></td>
<td>AR 19p13.11-q12</td>
<td>C19orf12</td>
<td>Associated with amyotrophy</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG44</strong></td>
<td>AR 1q42.13</td>
<td>Gap junction protein 12</td>
<td>Seizures and spasticity</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG45</strong></td>
<td>AR 10q24.32-q24.33</td>
<td>5'-nucleotidase, cytosolic II</td>
<td>Very early onset, mental retardation and ocular signs</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SPG70</strong></td>
<td>AR chr12:57 908741</td>
<td>Cytosolic methionyl-tRNA synthetase</td>
<td>Complicated, early onset, increased deep tendon reflexes</td>
<td>1 family</td>
<td>Zebrafish [18]</td>
<td></td>
</tr>
<tr>
<td><strong>SPG71</strong></td>
<td>AR chr5:324 06955</td>
<td>ZFR</td>
<td>Uncomplicated, early onset, increased deep tendon reflexes, positive clonus, thin corpus callosum</td>
<td>1 family</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 1-1: Major pathways affected in HSP.** Proteins involved in HSP have a wide range of cellular functions, however many of them cluster into several common cellular pathways. Those examined here are shown in red. Modified from [54].
1.1.5 SPG4 (Spastin)

Mutations to the *SPAST (SPG4)* gene were found to result in pure autosomal dominant HSP in 1999 and are by far the most common form of HSP, accounting for 40% of all dominant HSP cases [55]. The *SPAST* gene encodes the ubiquitously expressed microtubule-severing protein spastin. All types of possible mutations in *SPG4* have been identified, with 28% missense, 15% nonsense, 26.5% splice-site point, 23% deletions and 7.5% insertion mutations [56]. Spastin is a member of the ATPase associated with diverse cellular activities (AAA) family that also includes the microtubule-severing protein p60 katanin. AAA ATPase domains use the hydrolysis of ATP to drive a conformation change in the protein [57]. In the case of spastin, upon binding to microtubules, the AAA ATPase domain tugs on the C terminus of tubulin, causing an internal break [58]. Four main isoforms for spastin have been identified from the 17 exon protein, generated through combinations of alternative splicing, different promoters and different translational initiation sites [59, 60]. The two major protein products are the larger, full-length protein, termed M1 spastin, which is 616 amino acids in length, and the shorter 530 amino acid M87 protein that lacks 86 amino acids on its N-terminus (Figure. 1-2). Two less abundant isoforms exist, which are variations of M1 and M87, that lack exon 4, which would encode 32 amino acids [59]. Currently the effects of the presence or absence of this region are not understood since the role of these 32 amino acids is unknown. Interestingly, M87 spastin is more abundant in all cell types than M1, although M1 is more abundant in the brain and spinal cord compared to other locations [59, 61]. This observation hints at the possibility that M1 spastin is responsible for HSP development, since mainly the central nervous system is affected. More compelling evidence for the role of M1 spastin in HSP is the observation that two other HSP causative proteins, atlastin-1 and NA14, interact with a region on the N-terminus of spastin, which is only present in the full-length M1 isoform. In rats, it was found that M1 spastin was absent in developing neurons and became more abundant in mature neurons [61]. Of particular note was the abundance of M1 spastin in the adult spinal cord, which is the location of
degenerating axons in HSP patients. The importance of M1 spastin was recently called into question when a report examining SPG4 iPSC-derived neurons found overexpressing either M1 or M87 spastin could rescue neurite outgrowth defects [62]. This suggests that both M1 and M87 spastin are important for neurite outgrowth, but it remains unclear if both isoforms contribute equally to axonal maintenance in vivo.

The spastin protein contains four major domains (Figure 1-2) with the characteristic AAA ATPase domain located at the far C-terminus portion of the protein, and directly adjacent to this domain is a microtubule binding domain (MBD). Both the AAA ATPase and the MBD allow spastin to interact with microtubules and generate internal breaks in them. Towards the N-terminal region of spastin is a hydrophobic region (HR) that is believed to be involved in localizing the protein to specific subcellular membranes by interacting with other proteins present. Lastly, the microtubule interacting and trafficking (MIT) domain is an additional protein interaction domain. Because M87 lacks the first 86 amino acids of full length spastin, it does not possess the hydrophobic region, which seems to account for the differences in subcellular localization between M1 and M87 [63]. The M87 isoform is mainly a cytosolic protein that is important for cytokinesis through recruitment to the midbody between two still connected daughter cells after cell division. Here spastin cleaves the microtubules present and allows for the completion of abscission [64]. The M1 isoform is usually membrane associated, localizing mainly to the endoplasmic reticulum, the early secretory pathway and the early ER-to-Golgi intermediate compartment [65]. It is believed that the localization of M1 spastin is due to the hydrophobic domain at its N-terminus.

A number of studies have shown that spastin is capable of severing microtubules in vitro [66]. Cell culture studies were originally utilized to determine the function of spastin. When an ATPase-defective form of spastin (K388R) was overexpressed in Cos-7 cells, researchers noticed that the mutated protein localized with microtubules, suggesting that spastin could
interact with microtubules, but without the ability to hydrolyze ATP, could not dissociate from them [66]. Also, when wild-type spastin was overexpressed, microtubule disassembly was increased, providing the first evidence for the microtubule severing activity of spastin. Based on structural studies and comparison to other AAA ATPase proteins, spastin is thought to interact with microtubules through its MBD, allowing the AAA ATPase domain to interact with tubulin, and ATP hydrolysis provides energy needed for a conformational change that results in the destabilization of the microtubule. It has been shown that spastin can be present in both monomeric and hexameric form, with the hexamer relying on the N-terminus for assembly [67].

The large variety of different mutations present in the spastin gene of HSP patients (>250) has led to different hypotheses for the mechanism of these mutations. The majority of mutations are nonsense, deletions, or splice site mutations, which are believed to reduce the amount of spastin present in a cell, causing disease through a haploinsufficiency mechanism [63]. Recently, the observation that almost all of the missense mutations appeared in the AAA domain suggests the possibility that some of these mutations could function through a dominant negative mechanism, since the protein forms oligomers. In 2008, it was shown that at least one missense mutation, E442Q, acted in a dominant negative fashion [67]. Using co-localization studies, the researchers were able to show that the mutant spastin was able to interact with wildtype spastin, and perturb its localization.

Besides the role of spastin in endoplasmic reticulum morphogenesis, new lines of evidence suggest that it is involved in axonal transport [61, 68]. This fits with the role spastin plays in microtubule severing, as microtubule arrays are present through the entire length of axons and provide both structural support and serve as the railways for organelle transport. Axonal transport deficits also neatly match the observation that only the longest projection neurons are affected, since they would put the largest strain on transport systems to deliver cellular contents to the most distal portions of the cell. If proper materials are not delivered to
Figure 1-2: Spastin isoforms and domains. The N-terminus of the protein contains two domains important for protein-protein interactions, the hydrophobic region (HR) and the microtubule interacting and targeting (MIT) domains. The C-terminus contains a microtubule binding domain (MBD) and an AAA ATPase domain, which allows spastin to interact and sever microtubules. Modified from [1].
the distal regions, it could cause a dying back of the axon, which is seen in HSP. Some of the best lines of evidence linking spastin and transport come from two different HSP mouse models that possess different spastin mutations. The first generated strain had a deletion in spastin that resulted in a premature stop codon to study the effects of reduced levels of spastin [29]. These mutant mice displayed minor gait alterations compared to wild-type at 22 months. At as early as 4 months the mutant mice showed defects in axons located in the spinal cord, with axonal swellings evident in both ascending and descending tracts. In affected axons of the corticospinal tract, dramatic disorganization of cytoskeletal components was observed. These abnormalities were restricted to axons, and the neuronal cell bodies remained unaffected. By culturing cortical neurons from these mice, the researchers found that retrograde axonal transport was impaired. This was evident by the observation that the volume of the axon distal to a region of swelling was significantly larger than the proximal region of the axon. Both mitochondria and peroxisomes accumulated in the distal portion axon swellings, and not in other regions of the cell. In a second HSP mouse model from 2009 containing a splice site mutation that is expected to generate a premature stop codon, similar abnormalities to the first mouse model were also shown [68]. Gait abnormalities and axonal swellings were again present in these mice, but what made this study unique was the quantification of both anterograde and retrograde axonal transport. To do this, time-lapse microscopy was used to follow labeled mitochondria and APP as a marker for membrane bound organelles. Contrary to what was suggested in the first HSP mouse model, these researchers found anterograde axonal transport to be diminished, not retrograde. Also, swellings appeared to be in random locations along the axon, where in the previous mouse model, the swellings were more distally localized. These inconsistencies between the two mouse models suggest that different types of mutations could function in different ways even if both result in decreased spastin levels, and it remains to be seen if this is indeed the case in human neurons.
As mentioned earlier, M1 spastin localizes to the early secretory pathway in cells, interacting with these membranes through the hydrophobic domain at the N-terminus. The HD is predicted to form a hairpin loop capable of inserting itself into the outer leaflet of a lipid bilayer through hydrophobic wedging [69]. Three classes of proteins that display hydrophobic wedging have also been shown to insert themselves into membranes as a mechanism for generating the extreme membrane curvature that is seen in the tubular endoplasmic reticulum, including the reticulons, REEPs and atlastins [69]. Interestingly, members of all three classes are associated with additional forms of autosomal dominant pure HSP, suggesting a similar mechanism.

1.1.6 SPG3A (Atlastin-1)

SPG3A is caused by mutations to the protein atlastin-1 and accounts for approximately 10% of HSP cases, making it the second most common cause of HSP [70]. Atlastin-1 mutations result in early onset pure HSP that displays autosomal dominant inheritance. Because deficits are seen at such a young age in SPG3A patients, it has been suggested that this form of HSP is neurodevelopmental, while other late onset forms are neurodegenerative. Atlastin-1 is the founding member of a small group of proteins (atlastin1-3) in the superfamily of dynamin-related GTPases. It is a 558 amino acid protein that possesses a large GTPase domain at the N-terminus, followed by a middle domain that serves an unknown function, and two transmembrane domains at the C-terminus [71] (Fig. 1-3). Atlastin-1 localizes to the Golgi apparatus and also to particular regions of the tubular endoplasmic reticulum network [71, 72]. The localization of atlastin-1 to the ER is due to the presence of an ER localization signal at the C-terminus. Early work into the function of atlastin-1 has relied on in vitro cellular models. In 2008, a group found that when a mutant or dominant negative atlastin-1 protein was overexpressed in HeLa cells, the endoplasmic reticulum was severely affected. Much less branching was present in the tubular endoplasmic reticulum, while there was an increase in the endoplasmic reticular sheets, associated with the rough ER [71]. This observation, along with
Figure 1-3: Atlastin-1 domains. Atlastin-1 consists of three main domains, the large GTPase domain, the middle linker domain, and two trans-membrane domains (TMDs). Each TMD partially inserts into ER lipid bilayers through hydrophobic wedging. At the C-terminus is a KDEL ER retention (ERR) signal.
subsequent studies, has led to the prevailing model that implicates atlastin-1 in the formation of the smooth endoplasmic reticulum network, which is present throughout the entirety of all cells, including axons and dendrites of neurons [73]. The tubular ER is important for lipid and cholesterol metabolism and serves as a store for calcium inside of the cell, which is important for synaptic transmission and plasticity [73]. The ER is a very dynamic organelle with two main subdivisions: the sheets where protein synthesis occurs and the vast network of tubular ER [74]. The tubular ER is present throughout the entirety of the cell and moves along microtubule tracts, allowing it to localize in even the most distal portions of a neuron’s axon [73]. Atlastin-1 controls ER network morphogenesis by driving the fusion of individual ER tubules, allowing the formation of three-way junctions. The reduction of atlastin-1 in rat cortical neurons resulted in decreased axonal length and the presence of cells that completely lacked an axon [71]. In vivo studies of atlastin-1 function have focused on model organisms such as Drosophila and zebrafish (Danio rerio). When the atlastin-1 homologue was knocked-down in zebrafish, researchers observed abnormal organization of spinal motor axons which reduced larval movement [20]. These abnormalities were credited to alterations in bone morphogenetic protein (BMP) signaling. The observation that atlastin-1 can partially localize to endosomes suggested that it may alter endocytosis of BMP receptors. There were no observed abnormalities in ER organization, which suggests a potential difference in the function of atlastin-1 between zebrafish and mammals, and further studies are needed to clarify these issues.

In addition to regulation of ER morphogenesis, atlastin-1 has been shown to regulate lipid droplet production. Lipid droplets consist of lipid ester cores that are surrounded by phospholipid monolayers and serve as the major fat storage organelles in eukaryotic cells [75]. The ER plays an important role in lipid droplet synthesis, as the two organelles are closely associated with one another, and lipid droplet growth requires interaction with the ER [76]. The role of atlastin family members in lipid droplet regulation was first identified in C. elegans, where mutations not only altered ER morphology, but also lipid droplet size [19]. In contrast,
overexpression of atlastin increased lipid droplet size. These observations were later confirmed in HeLa cells [77]. This work suggests that atlastin-mediated changes to ER morphology affects lipid droplet shape. In the future, it will be interesting to determine if alterations to lipid droplet size have any pathological consequences in SPG3A patients.

1.1.7 ER morphogen complex

In addition to atlastin-1 and spastin, two other HSP-related proteins were found to localize to the endoplasmic reticulum. The third most common cause of HSP involves mutations to the SPG31 gene, which encodes the receptor expression enhancing protein 1 (REEP1) [69]. Humans and other mammals have 6 different REEP family members (REEP1-6), which are highly conserved membrane proteins. Through phylogenetic analysis, REEP1 was found to be closely related to the DP1/Yop1p proteins that are involved in shaping tubular ER. In fact, REEP1 predominantly localized with tubular ER, and similar to what was seen in atlastin-1 mutant expressing cells, mutant REEP1 expression impaired tubular ER network formation. In cultured rat cortical neurons, REEP1 colocalized with both M1 spastin and atlastin-1 through its hydrophobic domains. The ER shaping protein reticulon-2 was identified as the causative gene in the autosomal dominant SPG12 [78]. Like REEP1, reticulon-2 also is a direct binding partner with M1 spastin.

This determination that four HSP proteins localize to the ER and interact with each other has led to the proposal that defects to the so called “ER morphogen complex” are central to the pathogenesis of over 60% of HSP [1]. The “ER morphogen complex” describes a complex formed by interacting proteins, including atlastin-1, spastin, REEP1 and reticulons, that functions to regulate the formation and localization of the endoplasmic reticulum. Atlastin-1 is needed for the formation of the tubule ER network, by regulating the fusion of tubules to form three-way junctions. REEP1 and reticulons are critical proteins involved in shaping the ER, and can generate the extreme curvature displayed in the tubular ER. Lastly, spastin is involved in linking the ER to cell's cytoskeleton, through microtubule interaction. This is believed to provide
structural support for the ER, and also allows transport of the ER throughout the cell. If any of these components of the “ER morphogen complex” are mutated, the resulting abnormality in ER morphology could negatively affect axons of long motor neurons, leading to loss of synaptic connection and lower limb spasticity [1].

1.1.8 SPG11, SPG15, and SPG48

Besides the more common autosomal dominant forms of HSP, there is a long list of recessive mutations that can lead to complex forms of HSP. SPG11 and SPG15 are the most common recessive forms of HSP, and are almost clinically identical [79, 80]. These two forms account for 25% of the autosomal recessive subtypes [81]. Both forms present with cognitive impairment, dementia, ataxia, and thinning of the corpus callosum, in addition to lower limb spasticity. SPG11 and SPG15 encode spatacsin and spastizin respectively, both of which are direct binding partners [82, 83]. These two proteins can also bind to the heterotetrameric adaptor protein complex 5 (AP5), which is affected in SPG48. SPG48 is the result of mutations to the \( AP5Z1 \) gene, which encodes the adaptor protein complex ζ subunit (AP5Z1) [84]. AP5Z1 is a subunit of the common heterotetrameric adaptor protein complex 5 (AP-5), an evolutionarily conserved complex thought to be involved in endosomal dynamics, although its precise localization and function are still unclear [85]. Adaptor proteins (APs) are a family of 5 related protein complexes, with AP-5 being the most recently discovered [86]. AP-1 and AP-2 are the most well-characterized, and have been shown to promote clathrin assembly \emph{in vitro} by providing a bride between clathrin and membranes [87]. APs are comprised of 5 subunits, β1-5, one of either \( \alpha, \gamma, \delta, \epsilon, \) or \( \zeta, \) and μ1-5, and σ1-5 [85]. In addition to AP5Z1, mutations to the AP4 subunit AP4E1 were shown in 2011 to result in an autosomal recessive form of HSP, SPG51 [88]. Another interesting aspect of SPG11, SPG15, and SPG48 is that they can present with prominent juvenile parkinsonism, which has shown improvement following dopaminergic therapy [62, 89, 90]. It is particularly intriguing that both muscle spasticity, due to cortical spinal
motor neuron degeneration, and parkinsonism, caused by dopaminergic defects, occur following the loss of one gene.

Although there is relatively little known about these three proteins, there is evidence that they may be involved in axonal outgrowth and targeting in zebrafish [91], autophagy [92-94], cytokinesis [95, 96], and potentially in axonal transport [81]. The evidence linking spastizin and spatacsin to autophagy is particularly convincing. Through a series of knockdown and overexpression experiments, Chang et. al. demonstrated that spastizin and spatacsin are required for autophagic lysosome reformation, a process that allows the replenishment of lysosomes [93]. Another report showed that spastizin knockdown fibroblasts had an impairment of autophagosome maturation and an accumulation of immature autophagosomes [97]. This link with autophagy is particularly interesting, since defects in this pathway have been associated with other forms of parkinsonism. Kufor-Rakeb syndrome, which has symptoms that are similar to SPG15, including pyramidal degeneration and early onset parkinsonism, is caused by loss-of-function mutations to the gene that encodes the lysosomal protein ATP13A2 (PARK9) [39]. In addition, it has been suggested that abnormal accumulation of α-synuclein inhibits the lysosomal enzyme glucocerebrosidase, potentially causing Parkinson’s disease [40, 41]. Importantly, loss-of-function mutations to the glucocerebrosidase gene results in Gaucher disease, which is associated with parkinsonism [42].

1.1.9 HSP Summary

As the causative genes are identified in the remaining HSP loci, more themes important to axonal maintenance besides endoplasmic reticulum morphogeneis, axonal transport, and microtubule dynamics are expected to be identified. This review presented the field's current understanding of the three most common causes of HSP, involving proteins that function together to form, localize, and maintain the tubular ER network in cells. It has become quite clear that proper functioning of the ER is needed not only for the formation and stability of the
longest axons in the CNS, but also for preventing disease. Although there are many remaining questions for these forms of HSP, based on the current findings, it would appear that long motor neurons are a tissue that is most susceptible to misregulation of ER structures. It has been suggested that perhaps tubular ER may be necessary at the synapses of these long neurons, and when mutant spastin, atlastin-1, REEP1 or reticulon-2 prevent the proper distribution of the ER, axonal dying back is the outcome. These subsets of HSP have identified an inherent weakness in long projection neurons, and in the future, as the mechanism of other forms of HSP are realized, further weaknesses may become evident. In addition, three autosomal recessive HSP subtypes associated with juvenile parkinsonism, SPG11, SPG15, and SPG48, were discussed. Although there are little data about the function of the proteins involved in these subtypes, evidence appears to link them to a role in the endomembrane system and autophagy. Although HSP is a rare disorder, studying the mechanisms of this disease may provide invaluable insight into the progression of more common disorders that result in axonal degeneration, including amyotrophic lateral sclerosis and multiple sclerosis.

1.1.10 Disease-modelling with induced pluripotent stem cells

Embryonic stem cells (ESC) are pluripotent cells that have two important characteristics. First, they have the ability to proliferate indefinitely, and they can generate any cell type in the adult body, from all three germ layers. In 1981, the first mouse embryonic stem cells were isolated from the inner cell mass of preimplantation blastocysts [98, 99]. These cells could be cultured indefinitely, formed multiple cell types in vitro, and could generate teratocarcinomas when injected into mice. Mouse ESCs have proved to be an invaluable tool for studying mammalian development, and also led to the generation of the field of gene targeting [100]. In 1998, the first human embryonic stem cells were generated by Dr. James Thompson [90]. This reignited interest in stem cell biology with major implications for regenerative medicine. However, ethical issues concerning the destruction of embryos led to the search for additional
sources of pluripotent cells. It was known that cells could be reprogrammed back to an earlier developmental state since John Gurdon transferred the nucleus of a fully differentiated intestinal epithelial cell from Xenopus into oocytes in 1962 [101, 102]. This was the beginning of somatic cell nuclear transfer (SCNT), which was successful in mammals as well, when the sheep Dolly was generated [103] and later in mice [104].

As more was understood about the mechanism of pluripotency in mammalian cells, Dr. Shinya Yamanaka’s group sought to reprogram mouse somatic cells using defined factors [105]. In a groundbreaking report, researchers chose to force somatic cells to express various combinations of 24 transcription factors that are upregulated in ESCs and found that just OCT3/4, KLF4, SOX2 and c-MYC could reprogram cells back to an ESC-like state. The generated cells, termed induced pluripotent stem cells (iPSCs), were shown to display many similar properties of ESC, including self-renewal and the ability to form cells of all three germ layers. A year later, this feat was accomplished in human cells by two independent labs, with Yamanaka’s group using the same four factors from the mouse study (Yamanaka’s factors) [106], and the Thompson lab showing similar results with OCT3/4, SOX2, LIN28 and NANOG (Thompson factors) [107]. Shortly after these reports, some potential benefits became clear to the scientific community, including the ability to generate iPSC lines from patients with genetic disorders to model diseases, and the reduced risk of immune rejection following transplantation of tissues derived from iPSCs that came from the same patient.

In the past eight years since the first generation of iPSCs, enormous progress has been made to increase the efficiency in generating these cells using much safer reprogramming methods. An important focus of many groups has been to design reprogramming methodologies that are integration free, as numerous reports have shown that integrated transgenes can result in increased tumorigenicity and decreased differentiation potential [108].

Over the past several decades, a large amount of effort has been directed towards developing reproducible directed differentiation protocols that allow the generation of clinically
relevant cell populations from pluripotent stem cells [109]. This work has provided researchers for the first time with access to large quantities of human neurons, including spinal motor neurons [110], midbrain dopaminergic neurons [111, 112], telencephalic glutamatergic neurons [113, 114], and medium spiny neurons [115]. The ability to generate defined populations of neuronal subtypes fueled the excitement over the ability to replace affected cells in various neurodegenerative diseases [116]. While progress towards this ambitious goal has progressed slowly, work towards another application for stem cell-derived neurons, “in vitro disease modelling”, has rapidly taken off. The ability to generate patient specific neurons allows researchers to examine processes that are not easily observed in postmortem tissues. The combination of directed neuronal differentiation and iPSCs has allowed the examination of patient-derived neurons that harbor pathogenic mutations. This strategy has successfully recapitulated phenotypes from ALS [117, 118], spinal muscular atrophy [119], Huntington’s disease [120], Parkinson’s disease [121], and Alzheimer’s disease [122]. In addition, the examination of ALS iPSC-derived neurons was able to show that a novel therapeutic compound was more beneficial than two drugs that recently failed clinical trials [123]. Prior to our work, no iPSC-based models of HSP have been reported.

Here, we have utilized human iPSCs to generate models of four HSP subtypes, SPG4, SPG3A, SPG15, and SPG48. Prior to this work, studies on SPG4 have focused on knocking down or overexpressing spastin in cell types not affected in patients, or animal models that do not fully recapitulate phenotypes observed in patients. To date, there have been no published animal models of SPG3A, SPG15, or SPG48, but valuable information on the role of these HSP proteins has been uncovered using engineered cell lines. Because there has been no prior work on these four HSP subtypes using human neurons, we used iPSCs to generate models and perform pilot drug screening studies. This work is presented here, first with a chapter detailing some of the important methods that were used in these studies.
2. Chapter 2
Modeling axonal phenotypes with human pluripotent stem cells

Volume Title: Patient-specific Induced Pluripotent Stem Cell Models Generation and Characterization

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Author Contribution Summary

K.R.D conception and design, data analysis and interpretation, and manuscript writing; C.C.X: manuscript writing support; X.J.L: conception and design, financial support, manuscript writing, data analysis and interpretation, and final approval of manuscript.

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2.1 Summary

Impaired axonal development and degeneration are implicated in many debilitating disorders, such as hereditary spastic paraplegia (HSP), amyotrophic lateral sclerosis (ALS), and peripheral neuropathy. Human pluripotent stem cells (hPSCs) have provided researchers with an excellent resource for modeling human neuropathologic processes including axonal defects \textit{in vitro}. There are a number of steps that are crucial when developing an hPSC-based model of a human disease, including generating induced pluripotent stem cells (iPSCs), differentiating
those cells to affected cell-types, and identifying disease-relevant phenotypes. Here, we describe these steps in detail, focusing on the neurodegenerative disorder HSP.

**Key Words:** hESCs, iPSCs, axon, mitochondria, degeneration

### 2.2 Introduction

It became evident shortly after the first successful derivation of human embryonic stem cells (hESCs) [90] and iPSCs [106] that these cells would be invaluable for researchers looking to study human disorders, particularly diseases affecting the nervous system, where access to tissue is limited. Because these cells retain mutations present in patients, they can recapitulate many of the phenotypes in neurodegenerative disorders while maintaining the correct human genetic background. In addition, developmental biology has progressed at a rate that has allowed the directed differentiation of human pluripotent stem cells (hPSCs) to numerous distinct cellular populations, including various neuronal subtypes [110, 112, 124-131]. These properties of hPSCs make them an ideal system to study neurodegenerative disorders in human cells.

In order to generate an accurate model of a neurodegenerative disorder, there are several important steps that must occur. The first step is to generate hPSCs that will be used to model a disease of interest. This can be accomplished in multiple ways, including generating iPSCs from affected patients and controls, or if a disease occurs through a haploinsufficiency mechanism, knockdown hESCs can be generated. The latter is useful when access to patient tissue is limited, or when confirming findings observed in patient-derived iPSCs. Next, the hPSCs are differentiated to the cell-type that is affected in patients. In our case, forebrain glutamatergic neurons are the main cell-type which are affected in hereditary spastic paraplegia. Confirming that a sufficient percentage of the differentiated cells are the correct cell-type and that there is
not a dramatic difference between lines is crucial, and must be determined prior to examining disease-specific phenotypes. Lastly, once the appropriate neurons are generated, you can examine the phenotype of the mutant cells compared to controls. Here, we will focus on two common defects observed in axonal degenerative diseases: neurite outgrowth deficits and alterations to fast axonal transport of mitochondria.

Impaired axonal functions underlie many neurodegenerative diseases, especially those which affect projection neurons. One process that is often affected is axonal transport. Projection neurons rely heavily on fast axonal transport to deliver membrane proteins, synaptic vesicles, axolemmal components and mitochondria to the distal portions of the cell from the soma [132]. A number of additional adult onset neurodegenerative diseases display defects in axonal transport, including amyotrophic lateral sclerosis [133], spinal muscular atrophy [134], Huntington’s disease [135-137] and Alzheimer’s disease [138-141]. Mutations affecting the plus-end directed MT-based motor kinesin heavy chain (kinesin family, member 5A), result in a form of HSP (SPG10), confirming that perturbations to fast axonal transport can result in axonal degeneration [142]. The fact that the majority of protein is generated within the soma, and must be transported throughout the axon via anterograde transport makes efficient transport particularly critical in neurons [143]. Transport of organelles, including mitochondria, is also essential for neurons [144, 145]. Mitochondria move in a saltatory fashion; where some are rapidly transported long distances within axons, others appear stationary [146-150]. Kinesin proteins mediate transport in the anterograde direction towards the plus-end of microtubules [149, 151, 152], while cytoplasmic dyneins mediate transport in the retrograde direction towards the minus end of microtubules [149, 153]. The precise regulation of mitochondrial localization is necessary for regulating energy demand and preventing cell death [154]. Here we will describe how to image and analyze mitochondrial transport in hPSC-derived neurons.
2.3 Materials

2.3.1 Stock Solutions

1. DMEM (1X): Life Technologies, cat. no. 11965-092.
2. DMEM/F12 (1X): Life Technologies, cat. no. 11330-032.
3. Fetal Bovine Serum (FBS): Life Technologies, cat. no. 16000-044.
5. Neurobasal Medium (1X): Life Technologies, cat. no. 21103-049.
8. Glutamax (100X): Life Technologies, cat. no. 35050-061.
10. Trypsin-EDTA (0.05%): Life Technologies, cat. no. 25300-054.
13. Trypsin Inhibitor (1 mg/mL): Dissolve 50 mg in 50 mL of DMEM F/12, then pass through a 0.22 µm Steriflip filter.
14. FGF2 (100 µg/mL): Dissolve 100 µg into 1 mL of sterile PBS with 0.1% BSA (PeproTech, cat. no. 100-18B).
15. Ascorbic Acid (200 µg/mL): Dissolve 2 mg ascorbic acid in 10 mL PBS. Store at -80°C.
16. Cyclic AMP (1 mM): Dissolve 4.914 mg cyclic AMP in 10 mL sterilized water. Store at -80°C.
17. BDNF, GDNF, IGF1 (100 µg/mL): Dissolve 100 µg of growth factor in 1 mL of sterile water.
18. Mouse Laminin (1 mg/mL): Invitrogen cat. no. 23017-015, aliquot and store at -80°C until use. After thawing, store at 4°C.
19. MitoTracker CMXRos (50 nM): Dilute 0.5 µL 1 mM stock into 10 mL of NDM medium (Life Technologies, cat. no. M-7512).

**2.3.2 Media**

1. **MEF Media (1 L):** Filter 890 mL DMEM, 100 mL FBS, and 10 mL NEAA through a 0.22 µm filter flask.
2. **hESC Media (1 L):** Filter 785 mL DMEM/F12, 200 mL KOSR, 10 mL NEAA, 5 mL Glutamax, and 7 µL β-ME through a 0.22 µm filter flask.
3. **Neural Induction Media (NIM, 500 mL):** Add 5 mL N2, 5 mL NEAA, and 1 mL of 1 mg/mL Heparin to 489 mL of DMEM/F12. Add 1:200 B27, 1:1,000 cAMP, 1:10,000 IGF1 for neural differentiation.
4. **Neural Differentiation Media (NDM, 50 mL):** Add 0.5 mL N2, 0.5 mL B27, 0.5 mL NEAA, to 48.5 mL of neurobasal medium. Prior to use, add 1:1,000 cAMP, 1:1,000 ascorbic acid, 1:1,000 laminin, 1:10,000 IGF1, 1:10,000 BDNF, 1:10,000 GDNF.

**2.3.3 Transfection Reagents**

1. **pCXLE-hOCT3/4-shp53-F:** Addgene plasmid 27077.
2. **pCXLE-hSK:** Addgene plasmid 27078.
3. **pCXLE-hUL:** Addgene plasmid 27080.
4. **pCXLE-EGFP:** Addgene plasmid 27082.
5. **NHDF Nucleofector Kit:** Lonza, cat. no. VPD-1001.

**2.3.4 Tissue Culture Supplies**

1. 10 mL glass pipettes
2. 6-well tissue culture treated dishes
3. T75 non-treated flask
4. Glass bottom 35 cm dish: MatTek, cat. no. PG35G-1.5-14-C

**2.3.5 Antibodies**
1. IgM anti-Tra-1-60: Santa Cruz, 1:50, cat. no. sc-21705.
4. Rabbit IgG anti-Tau: Sigma-Aldrich, 1:100, cat. no. T6402.
5. Rabbit polyclonal anti-Tbr1: Proteintech, 1:1000, cat. no. 20932-1-AP.
6. Mouse monoclonal IgG βIII-tubulin (TuJ-1): Abcam, 1:2,000, cat. no. ab18207.

2.3.6 Software
1. ImageJ [155]
2. ImageJ Plugin Straighten [95]
3. MetaMorph (Molecular Devices)

2.4 Methods
2.4.1 Generating iPSCs
The method for generating iPSC lines from human fibroblasts using episomal vectors is on the basis of the previous study from Dr. Yamanaka’s group [156].

1. Combine 1 µg of each of the four plasmids (pCXLE-hOCT3/4-shp53, pCXLE-hSK, pCXLE-hUL, pCXLE-EGFP) with 82 µL Human Dermal Fibroblast Nucleofector Solution, and 18µL supplement (from the Lonza NHDF Nucleofector Kit) for each line.
2. When fibroblasts are roughly 90% confluent, trypsinize the cells, centrifuge 0.6 x 10^6 cells and remove medium, and resuspend in the Nucleofector solution from step 1.
3. Transfect the cells using a Nucleofector-2b with the program P-022.
4. Allow cells to recover for 10 minutes, and then plate them onto a 6-well TC treated plate.
5. Replace MEF medium every 2 days

6. On day 7, split cells onto a 10cm dish with a mouse embryonic fibroblast (MEFs) feeder layer. Change medium to hESC medium + 4 ng/mL bFGF.

7. Around day 21, iPSC colonies should be large enough to be split. Carefully remove individual colonies using a sterile syringe and a P200 pipette tip, and plate each in its own well of a 12-well dish on MEF feeders. If possible, try to break up the colony a little bit before plating to increase the number of colonies. Try to collect at least 10 clones per line, most of which can be stored as backups. In general, 2-3 clones per line are enough to use for analysis, so the others can be stored in liquid nitrogen.

8. Expand the separate clones and split to a 6-well dish when they colonies become large enough.

2.4.2 Validating iPSC lines

It is important to confirm that the generated iPSC lines are fully reprogrammed. A number of different methods can be employed to show this, however, we generally rely on staining for pluripotency markers such as Tra-1-60, SSEA4, and Nanog, and the teratoma assay [92].

2.4.3 Differentiation to affected neuronal subtypes

One of the main benefits of hPSCs is the ability to differentiate these cells to cell-types which are affected in disease of interest, in our case forebrain glutamatergic neurons. The steps below are summarized in Figure 2-1A.

2.4.3.1 Days 0-4: Collecting hPSCs in suspension

1. To generate telencephalic neurons from iPSCs, stem cells were cultured on a feeder layer of irradiated MEFs in 6-well tissue culture treated plates for around 6
days, with the hESC media (+10 ng/ml fibroblast growth factor [FGF]-2) changed daily.

2. When nearly confluent, each well was washed once with 1 mL of PBS, then 1 mL of Dispase was added. Cells were incubated for around 3 minutes at 37°C, or until the edges of colonies begin to round up.

3. The Dispase solution is removed and the wells are again washed with PBS, then hESC media is added.

4. Next, scrape of the cells off of the well using a 10 mL glass pipette.

5. Transfer the cells to a non-adherent T75 flask with roughly 50 mL of hESC medium (adjust depending on amount of cells). Replace hESC medium every day.

2.4.3.2 Days 5-8: Neural Specification

1. Remove hESC medium and suspend cells in roughly 30 mL of NIM. Replace half of the media every other day.

2.4.3.3 Days 9-16: Formation of neuroepithelial (NE) cells

2. Next, the cells are plated onto 6-well dishes. Remove NIM and resuspend iPSC aggregates in enough NIM + 5% FBS so that 1.5 mL can be used for each well of a 6-well plate. Allow the aggregates to attach overnight, and in the next morning remove the media and replace with standard NIM. Replace media every other day until day 17. During this time, NE cells will develop, and form characteristic “rosette” structures, which resemble the neural tube. (Fig. 2-1B).

3. It is also recommended to test the neural induction efficiency around day 10. To do this, staining for Pax6+ cells is recommended.

2.4.3.4 Day 17-27: Specification of telencephalic (forebrain) neurons
4. On day 17, remove the NE cells by gently blowing media onto the colonies using a P1000 pipette. Collect the isolated NE cells and culture in suspension with NIM supplemented with 1:200 B27, 1:1,000 cAMP, and 1:10,000 IGF1.

5. Replace half of the media every other day until day 27. Once a week, the neurospheres can be fragmented into smaller clusters by passing them through a P200 pipette several times.
Figure 2-1: Forebrain glutamatergic neuron differentiation. (A) Schematic of the neural differentiation protocol used in this study. Cell markers are shown above, and can be checked during the course of differentiation through immunocytochemistry. EBs = embryoid bodies, NE = neuroepithelium, NP = neuronal progenitors. (B) Representative images of cells at various stages of differentiation. Scale bar: 100 µm. (C) Efficient generation of Tbr1+ forebrain glutamatergic neurons, stained at 6 weeks. Images in B and C have been adapted from our previous publication [24]. Scale bar: 50 µm.
2.4.3.5 Day 28: Plating neurons

6. Wash neurospheres with PBS one time, incubate in Accutase for 2 minutes at 37°C.

7. Add an equal volume of Trypsin Inhibitor solution, then centrifuge the cells for 2 minutes at 400 x g.

8. Remove the media and resuspend the neurospheres in 50 µL neural differentiation media (NDM). Pipette up and down several times with a P200 tip to dissociate the cells. Add additional NDM medium so there is a sufficient volume to reduce the density of cells.

9. Add the cells to poly-ornithine/laminin coated coverslips and incubate cells for at least 2 hours at 37°C so that they have time to attach.

10. Once attached, slowly add 500 µL of NDM to prevent cells from detaching. Replace 250 µL of NDM medium every other day until the cells are ready for analysis.

2.4.4 Confirming the generation of forebrain glutamatergic neurons

We perform immunostaining of the generated neurons around 6 weeks after differentiation began to determine the percentage of forebrain glutamatergic neurons that were generated using the markers that are listed in Fig. 1A. In Fig. 2-1C, Tbr1⁺ and βIII-tubulin⁺ cells are shown at 6 weeks. This step confirms that there is not a dramatic difference between control and mutant groups at generating these cells.

2.4.5 Identifying disease-related phenotypes

Once you are confident that the appropriate neuronal subtype has been generated, you can next begin to identify disease-related phenotypes in your mutant cells. We chose to look for axonal defects since HSP is associated with distal axonal degeneration [1]. The two methods
that we will discuss in detail are measuring neurite outgrowth and fast axonal transport of mitochondria.

2.4.5.1 Measuring neurite outgrowth

In order to look at initial neurite outgrowth, cells are analyzed just 48 hrs following plating onto coverslips, representative images are shown in Fig. 2-2A. This time point was also chosen because the coverslips will become too dense with neurites after longer periods to time, making it difficult to trace the morphology of individual cells.

1. Plate dissociated neurons at a low density, and allow neurites to extend for 48 hrs.
2. Fix cells with 4% paraformaldehyde and stain cells for Tau and MAP2 to stain axons or dendrites respectively.
3. Image the cells using a 20X objective and quantify neurite outgrowth using the NeuronJ plugin [96] for ImageJ.
4. For each neuron, trace primary and secondary neurites. Axons can be identified as the longest neurite which also stains the strongest for Tau.

2.4.5.2 Analyzing mitochondrial transport

1. Plate dissociated neurons onto 35 cm glass-bottom dishes (Matek) following the steps as above.
2. When the cells have matured sufficiently, stain the live cells with 50nM MitoTracker CMXRos for 3 minutes at 37°C.
3. Wash the cells two times with NDM.
4. Image cells using a 63X objected with a microscope that has a heated stage (37°C).
Figure 2-2: Neurons 48 hrs post plating stained for Tau. (A) These cells were plated at a good density, making it easy to trace neurites extending from each cell. Scale bar: 20 µm. (B) These cells were not adequately dissociated, and were cultured for 2 weeks after plating, causing the neurites to become too dense. Accurate tracing of neurites would be difficult with this image. Scale bar: 100 µm.
5. Visually identify axons based on morphological characteristics (constant thin diameter, long neurites, no branching and direct emergence from the cell body [157]).

6. After identifying a suitable region, set up a time series by taking an image every 5 sec for at least 5 min.

7. Save the images as TIFs, and open all images within one series in ImageJ. Combine all images into a TIF stack (Image → Stacks → Images to Stack)

8. Next, use the ImageJ plugin “Straighten” to straighten imaged axons (Fig. 2-3A) [95]. Be sure to start with the proximal portion of the axon, as this will appear on the left side of the generated image series, making it easier to calculate directional transport parameters. Adjust filament/wide line value so that the width of the neurite is completely covered.

9. Open the straightened TIF series in MetaMorph and calibrate the scaling for the images.

10. Open the Track Points app (Apps → Track Points) and first set the origin (Set Origin → Click on the far left side, towards the cell body → OK). Also set the interval to 5 sec (Set Interval → User defined → 5 seconds → OK) and configure set the program to record all parameters (Config Log → Enable All).

11. Manually track the location of one mitochondrion within the axon for every frame in the series, then “Add Track” for the next mitochondrion. Repeat until the location of every mitochondrion within the axon has been tracked.
**Figure 2-3: Analyzing mitochondrial transport.** (A) Mitochondria within an axon imaged after staining with MitoTracker CMXRos. The cell soma was located to the left of the field. Bottom image shows results of the Straighten plugin in ImageJ. (B) Representative distance versus time kymograph generated in Metamorph. Scale bar: 10 µm.
12. Open Log
13. Dynamic Data Exchange (opens Excel) → OK (keep default options)
14. F9: Log Data
15. Enters all of the Track Point data into Excel, grouped by each Track # (mitochondrion).
16. Decide what parameters you are interested in: mean velocity, anterograde/retrograde velocity, motile events, anterograde/retrograde events, average run velocity, percentage motile mitochondria per cell, pause/run lengths.
17. Manually calculate velocity with the equation below, since MetaMorph does not differentiate between anterograde and retrograde transport.

\[
\text{Velocity} = \frac{(\text{Distance from origin at current time} + \text{distance at previous time point})}{5 \text{ seconds}}
\]

a. Positive values = anterograde transport
b. Negative value = retrograde transport
18. Determining motility events using a ≥±300 nm/sec threshold, which excludes events that may be mediated by actin based transport and should only include microtubule-based transport [157].
19. If there are 3 consecutive motile events in one mitochondrion’s time series, than that mitochondrion is considered motile.
20. Organize group data and compare control to mutant lines.
21. A distance versus time kymograph can be generated in MetaMorph for graphical representation of mitochondrial transport (Fig. 2-3B).

2.4.5.3 Measuring Mitochondrial Morphology
1. The same straightened images generated for measuring mitochondrial transport can be used to analyze mitochondrial morphology. We generally only analyze the morphology of the mitochondria using the first image of each time series.
2. Within ImageJ, set the scaling of the image to match the objective used.

3. Threshold the image so that all of the mitochondria are highlighted (Image → Adjust → Threshold) (Fig. 2-4)

4. Remove mitochondria outside of cell of interest and delete any overlapping or aggregated mitochondria. A protocol to segment mitochondria can be found in detail here [157].

5. Next, use the Analyze Particles function using the following conditions.
   a. Size = 0.2-Infinity
   b. Circularity = 0-1
   c. Show = Ellipses

6. Important results: area, major axis (length of mitochondrion), AR (aspect ratio)

7. By measuring the length of each imaged axon, and dividing it by the number of mitochondria within that region, you can determine the mitochondrial density (mitochondria/µm axon).

8. You can also calculate the distribution of mitochondria within axons. To do so, use the X,Y coordinates of the centroid of each mitochondrion and calculate the distance between adjacent mitochondria.
Figure 2-4: Analyzing mitochondrial morphology. Mitochondria within an axon before and after thresholding the image. Using the bottom image, the Analyze Particle function can calculate a number of useful mitochondrial morphology parameters. Scale bar: 10 µm.
2.5. Notes

1. Depending on the type of mutation that is present in your iPSCs, it is often useful to generate hESC lines where a gene of interest is knocked down. These knockdown lines can be used to confirm that any phenotypes observed in mutant iPSC-derived cells are caused by haploinsufficiency and are not due to clonal variation. We design shRNAs using http://sirna.wi.mit.edu/ [91].

2. To test the efficiency of neural induction, flow cytometry can be used to compare the percentages of Pax6+ cells between groups [158].

3. It is also recommended to stain and quantify the number of astrocytes (GFAP+) and oligodendrocytes (O4+) that are within neural cultures. This will ensure there are not dramatically different proportions of these cells between control and experimental groups.

4. Depending on your disease of interest, a number of different neuronal subtypes can be generated besides forebrain glutamatergic neurons. These include spinal motor neurons [110] and dopaminergic neurons [159, 160]. It is often useful to compare affected neuronal subtypes to a subtype that is not affected in a particular disease, to show cell-type specific phenotypes in your system.

5. Be sure to not plate neurons too densely for measuring neurite outgrowth, or it will be difficult to trace individual neurites (Fig. 2B).

6. When imaging mitochondrial movement, take care to adjust the exposure time and light intensity to prevent photobleaching.

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3. Chapter 3

Loss of spastin function results in disease-specific axonal defects in human pluripotent stem cell-based models of hereditary spastic paraplegia

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Key Words: iPSCs, hESCs, spastin, hereditary spastic paraplegia, axonal degeneration, RNA interference
3.1 Abstract

Human neuronal models of hereditary spastic paraplegias (HSP) that recapitulate disease-specific axonal pathology hold the key to understanding why certain axons degenerate in patients and to developing therapies. SPG4, the most common form of HSP, is caused by autosomal dominant mutations in the \textit{SPAST} gene, which encodes the microtubule-severing ATPase spastin. Here, we have generated a human neuronal model of SPG4 by establishing induced pluripotent stem cells (iPSCs) from an SPG4 patient and differentiating these cells into telencephalic glutamatergic neurons. The SPG4 neurons displayed a significant increase in axonal swellings, which stained strongly for mitochondria and tau, indicating the accumulation of axonal transport cargoes. In addition, mitochondrial transport was decreased in SPG4 neurons, revealing that these patient iPSC-derived neurons recapitulate disease-specific axonal phenotypes. Interestingly, spastin protein levels were significantly decreased in SPG4 neurons, supporting a haploinsufficiency mechanism. Furthermore, cortical neurons derived from spastin-knockdown human embryonic stem cells (hESCs) exhibited similar axonal swellings, confirming that the axonal defects can be caused by loss of spastin function. These spastin-knockdown hESCs serve as an additional model for studying HSP. Finally, levels of stabilized acetylated-tubulin were significantly increased in SPG4 neurons. Vinblastine, a microtubule-destabilizing drug, rescued this axonal swelling phenotype in neurons derived from both SPG4 iPSCs and spastin-knockdown hESCs. Thus, this study demonstrates the successful establishment of human pluripotent stem cell-based neuronal models of SPG4, which will be valuable for dissecting the pathogenic cellular mechanisms and screening compounds to rescue the axonal degeneration in HSP.
3.2 Introduction

Hereditary spastic paraplegias (HSP) are a heterogeneous group of genetic disorders that result in progressive lower limb spasticity [161]. The symptoms are due to a length-dependent axonopathy, most severely affecting corticospinal motor neurons (CSMN). These neurons display a “dying back” axonopathy, particularly in the spinal cord, resulting in impaired lower motor neuron function which produces prominent lower limb spasticity and typically more mild weakness. Although there are 57 distinct genetic loci associated with HSP, SPG4 is the most common, accounting for nearly 40% of all cases of autosomal dominant HSP [55]. SPG4 is caused by mutations in the \textit{SPAST} gene, which encodes the microtubule-severing ATPase spastin [55, 162-164]. Spastin is a member of the \textit{AAA} ATPase associated with diverse cellular activities (AAA) family that also includes the microtubule-severing protein p60 katanin. The large variety of mutation types present in the \textit{SPAST} gene of SPG4 patients has led to different hypotheses for the pathogenic mechanism of these mutations. The majority are nonsense mutations, deletions, or splice-site mutations. These are believed to reduce the amount of spastin present in a cell, causing disease through a haploinsufficiency mechanism [63]. This seems to be true for the majority of cases; however, there are certain missense mutations in the AAA ATPase domain that appear to act in a dominant-negative, loss-of-function fashion [165], which is possible because spastin functions as a hexamer [67]. Spastin is involved in a variety of functions, including microtubule dynamics [66], membrane remodeling [65], cytokinesis [64, 65], neurite outgrowth [166], and axonal transport [29, 61, 68, 167].

A common observation researchers have made while studying SPG4 is that spastin affects microtubule-based transport. This fits with the role spastin plays in microtubule severing, as microtubule arrays are present through the entire length of axons and both provide structural support and serve as the railways for organelle transport. Axonal transport deficits also neatly match the observation that only the longest projection neurons are affected, since they would put the largest strain on transport systems to deliver cellular contents to the most distal portions.
of the cell. If materials are not properly delivered to the distal regions, it could cause a dying-back degeneration of the axon, as seen in HSP. Some of the best lines of evidence linking spastin and transport come from two different HSP mouse models that possess different spastin mutations \[29, 68\]. These studies showed that cortical neurons cultured \textit{in vitro} could be used to model axonal defects, although the mechanisms underlying the axonal defects in SPG4 remain largely unknown.

To date, the role of spastin has not been investigated in human cortical neurons, but the advent of induced pluripotent stem cell technology \[106, 107\] now provides researchers with a system for studying the specific cell types that are affected by various diseases \textit{in vitro}. This method has been employed for several neurodegenerative disorders including spinal muscular atrophy \[119\], amyotrophic lateral sclerosis \[168\], Parkinson disease \[169\], and Huntington disease \[120\]. Here, we for the first time generated human iPSCs from an SPG4 patient as well as spastin knockdown hESCs to model HSP. The generated human pluripotent stem cell (hPSC) lines serve as a renewable source of cells that can be differentiated into forebrain projection neurons, which include the most severely affected corticospinal motor neurons in HSP. In neurons generated from SPG4 iPSC lines, we observed an increase in the number of axonal swellings and accumulation of mitochondria within these regions, leading us to quantify fast axonal transport. This revealed a significant decrease in the number of motile mitochondria in the SPG4-derived neurons, and decreased motile events in the retrograde direction. It appears as if these defects were due to spastin haploinsufficiency, as the level of spastin protein was significantly decreased in the SPG4-derived neurons. To confirm that loss of spastin function is implicated in the pathogenesis of SPG4, we examined spastin knockdown neurons, and found a similar increase in axonal swellings. Lastly, to show that these cells will be useful as a drug screening platform, we treated iPSC-derived and spastin knockdown neurons with the microtubule-destabilizing drug vinblastine. This treatment led to a significant reduction in axonal swellings as compared to vehicle-treated cells. These findings suggest that
these hPSC-based models of SPG4 will serve as a useful model for HSP, since no therapies exist that can prevent, slow the progression, or cure HSP.

3.3 Materials and Methods

3.3.1 Reprogramming human fibroblasts into iPSC lines. Human iPSC lines were established from human fibroblasts by transfecting them with excisable lentiviruses (kindly provided by Dr. Gustavo Mostoslavsky) [170, 171] or episomal plasmids (Addgene), as reported previously [156]. Briefly, human fibroblasts collected under an IRB-approved clinical protocol (NINDS protocol 00-N-0043) at the NIH Clinical Center were seeded at ~10^5 cells/35-mm dish in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1 mM non-essential amino acids. For lentiviral infection, cells were infected with EF1α-hSTEMCCA-loxP lentiviruses containing pluripotency genes (Oct4, Sox2, c-Myc, Klf4). For episomal transduction, human fibroblasts (~200,000) were dissociated and then infected with episomal plasmids containing pluripotency factors (Oct3/4, Sox2, L-Myc, Klf4, and Lin 28). At around 1 week after lentiviral infection or electroporation transduction, cells were plated onto a 35-mm dish in DMEM supplemented with 10% FBS. After culturing for 7 days, cells were dissociated and seeded onto mouse embryonic fibroblast (MEF) feeder at ~10^5 cells/100-mm dish. Two weeks later, colonies with morphologies similar to hESCs were observed. These colonies were split onto MEF feeder cells to derive iPSC lines. After several passages, homogenous colonies with ESC-like morphology were generated. The lentiviral iPSC lines used in this study were iWT-l1, iSPG4-l4, and iSPG4-l7. The episomal iPSC lines used in this study were iWT-e1, iWT-e3, iSPG4-e6, and iSPG4-e8.

3.3.2 Lentivirus production and transduction of hESCs. To produce high-titer lentivirus, 10 µg of lentiviral transfer vector (pLVTHM), 7.5 µg of lentiviral vector psPAX2, and 5 µg of pMD2.G (VSV-G envelope protein) were cotransfected into HEK293FT cells (Invitrogen) using
the calcium phosphate method. Sixty hours after transfection, cell culture medium containing viral particles was collected and filtered through a 0.45-µm filter (Millipore). Viral particles were further concentrated by ultracentrifugation (SW 28 rotor, Beckman) at 50,000 g for 2 h, and the pellet was resuspended in hESC medium. For transduction of ESCs, hESCs were passaged normally and pelleted by brief centrifugation. Cell pellets were then incubated with 100 µl of concentrated virus (10⁶ transducing units/ml) at 37°C for 30 min. To knock down spastin, pLVTHM lentiviruses contain shRNA targeting spastin or luciferase (as control) were used to infect hESCs, a strategy similar to that we have used for knocking down other genes [158]. RNAi hESCs were expanded and then differentiated as described below.

### 3.3.3 hPSC Neural Differentiation

To generate telencephalic projection neurons from hPSCs, stem cells were cultured on a feeder layer of irradiated MEFs in 6-well tissue culture treated plates for around 6 days, with the hESC media (+10µg/ml FGF-2) changed daily. When almost confluent, cells were detached from the feeder layer to initiate the neural differentiation, as we previously described [128, 172, 173]. The cells were cultured in suspension for 4 days in hESC media, changing the media every day. On day 5, neural induction was started by transferring the generated PSC aggregates to neural induction media (NIM). After 3 additional days in suspension, PSC aggregates were plated onto 6-well tissue culture treated plates in NIM with 10% FBS. After 12 hours, the media was changed with fresh NIM. The media was then changed every other day until day 17, when the generated neuroepithelial (NE) cells were isolated. Mechanically-isolated NE cells were cultured in suspension with NIM (+B27, +cyclic AMP, +insulin-like growth factor 1 [IGF1]) to generate neurospheres for at least an additional 10 days. On about day 28, the neurospheres were dissociated and plated onto polyornithine- and laminin-coated coverslips in neural differentiation media (NDM) containing N2, B27, ascorbic acid, cyclic AMP, laminin, IGF1, brain-derived neurotrophic factor, and glial-derived neurotrophic factor. Half of the media was changed every other day for a total of 6 to 12 weeks, depending
on the analysis to be performed. To treat cells with vinblastine, the media was replaced with standard NDM with 10 nM vinblastine (Sigma-Aldrich) dissolved in water.

3.3.4 Immunocytochemistry. Cells on glass coverslips are fixed with 4% paraformaldehyde for 20 minutes. A 0.2% Triton-X solution was used to permeabilize the cells for 10 minutes followed by several PBS washes. The samples are incubated in a primary antibody solution overnight. On the following day, samples are incubated with the appropriate secondary antibody for 30 minutes [128]. To quantify axonal swellings, at least three blindly-selected fields were imaged for at least three coverslips per group. The number of axonal swellings were counted and divided by the total length of Tau+ axons in each field, which were measured using MetaMorph software.

3.3.5 Antibodies. Mouse IgM anti-Tra-1-60 (Santa Cruz, 1:50), goat IgG anti-Nanog (R&D, 1:500), mouse IgG3 anti-SSEA-4 (DSHB, 1:100), mouse IgG anti-acetylated tubulin (Sigma-Aldrich, 1:10,000), rabbit IgG anti-Tau (Sigma-Aldrich, 1:100), rat IgG2a anti-GFP (Nacalai Tesque, 1:1000), rabbit IgG anti-Tbr1 (Proteintech, 1:1,000), βIII-tubulin, mouse IgG (DSHB, 1:100).

3.3.6 Reverse transcriptase Polymerase Chain Reaction (RT-PCR). RNA was isolated from cells using TRIzol reagent (Invitrogen) following manufacturer's instructions. A total of 2µg of RNA was used to synthesize cDNA using iScriptcDNA Synthesis Kit (Bio-Rad) following manufacturer's parameters: 5 min @ 25°C, 30 min @ 42°C, and 5 min @ 85°C. Semi-quantitative analysis was performed using RT-PCR with GoTaq Green Master Mix (Promega).

3.3.7 Western Blotting. We used a general protocol that was described previously [174]. A mouse monoclonal anti-spastin antibody (Sigma-Aldrich, 1:1000) was used to detect expression
of spastin isoforms, and a mouse monoclonal anti-actin antibody (Sigma-Aldrich, 1:1000) was used as a loading control. The quantification of Western blotting was quantified using ImageJ [155] normalized against actin as a loading control.

### 3.3.8 Live Cell Imaging with MitoTracker

Neurospheres are plated onto polyornithine and laminin coated 35mm dishes (MatTek). At 8 weeks of total differentiation, the cells are stained with 50 nM MitoTracker Red CMXRos (Invitrogen) for 3 minutes to allow visualization of mitochondria and then the media is replaced with fresh NDM. Live-cell imaging was performed using a Carl Zeiss Axiovert 200M microscope equipped with an incubation chamber. The cells are kept at 37°C with 5% CO₂ while imaging. Axons identified according to morphological criteria (constant thin diameter, long neurites, no branching and direct emergence from the cell body) were imaged every 5 seconds for 5 minutes, yielding 60 frames. Quantifications were performed using the same protocol as described previously [133]. In short, the location of each mitochondrion was manually selected using the Track Points function in MetaMorph, and parameters such as distance from cell body and velocity were recorded. A velocity threshold of 300 nm/s was used to select microtubule based transport events [157]. To determine the percentage of motile mitochondria, the total number of mitochondria that were present along the imaged neurite was counted, and those that changed position (velocity >300 nm/s) in at least 3 consecutive frames were considered motile.

### 3.3.9 Statistical analysis

The statistical significance in mean values among multiple sample groups was analyzed with Turkey’s studentized range test after ANOVA. Two-sided t-test was used to examine the statistical significance between two sample groups. The trend between knockdown efficiency and increased swellings was tested using linear regression analysis. The significance level was defined as $p < 0.05$, and all significance tests were conducted using SAS 9.1 (SAS Institute).
3.4 Results

3.4.1 Characterization and differentiation of control and SPG4 iPSC lines

SPG4 iPSCs were generated from dermal fibroblasts of an SPG4 patient with a heterozygous G>T substitution located in intron 4 of the \textit{SPAST} gene that alters the splice acceptor site (c.683-1G>T). The fibroblasts were reprogrammed to iPSCs using both a lentiviral [170, 175] and an episomal method [156], which permitted us to determine if the reprogramming method has any effect on the mutant phenotypes that we observe in the SPG4 neurons. Human iPSC lines derived from normal individuals were also generated and utilized as controls. All of the clones that we analyzed, from SPG4 and controls (wild-type [WT]), displayed characteristic colony morphology and stained positive for the pluripotency markers Nanog, Tra-1-60, and SSEA4 (Fig. 3-1A). The expression of pluripotency genes was analyzed by RT-PCR (Fig. 3-1B); only in the iPSCs was expression of Sox2, Nanog, or Oct4 detected. Teratomas were generated in mice using the iPSC lines to confirm that they were pluripotent. Both the lentiviral and episomal iPSC lines were able to spontaneously differentiate into tissues of each of the three germ layers, confirming pluripotency (Fig. 3-1C). The region surrounding the splice acceptor site of intron 4 was sequenced to confirm that the SPG4-derived iPSC lines maintained the mutation in the \textit{SPAST} gene after reprogramming (Fig. 3-1D). Because it is known that iPSCs are susceptible to chromosomal abnormalities after passaging, karyotype analysis was performed, which did not reveal any defects (Fig. 3-1E).

Next, the iPSC lines were differentiated to the neural lineage to generate forebrain glutamatergic neurons using a protocol we established previously, which leads to the efficient generation of telencephalic neurons [128, 172, 173]. Both control and SPG4 iPSCs efficiently differentiated into neurons with long processes, regardless of the reprogramming method (Fig. 3-1F). These neurons were Tbr1+, confirming that they are glutamatergic (Fig. 3-1F). There were no significant differences in the percentages of Tbr1+ cells between control and SPG4 cells (Fig. 3-1G). Since the spastin mutation in this SPG4 patient would affect RNA splicing, we
performed western blot analysis to examine the levels of spastin present in neurons generated from the iPSCs. Our data showed a reduction in spastin protein levels in the neurons derived from the SPG4 patient lines as compared to controls (~47% of control; Fig. 3-1H). Since SPG4 is caused by autosomal dominant mutations, SPG4 patients likely have ~50% of spastin activity if one allele is non-functional. Thus, the SPG4 iPSC-derived neurons track level of spastin expected in patients, providing a unique model to study the pathological changes caused by reduced spastin levels.

3.4.2 Neurons derived from SPG4 iPSCs exhibit increased axonal swellings
A common pathologic hallmark of SPG4 is the enlargement of axons that accumulate organelles, termed axonal swellings [68]. To determine if the iPSC-derived neurons displayed axonal phenotypes similar to that observed in post mortem spinal cord sections, we performed acetylated tubulin staining to examine the formation of axonal swellings (Fig. 3-2A). Our analysis showed that there was a substantial increase in the number of axonal swellings in the SPG4-derived neurons as compared to controls (Fig. 3-2B). Furthermore, the axons with swellings in SPG4 neurons were significantly longer than those without (SPG4 axons without swellings = 53.16±2.88 µm vs. those with swellings = 93.04±5.87 µm; p<0.01), suggesting that longer axons were more susceptible to these changes. Similar swellings were present in the neurons derived from the episomal SPG4 iPSC lines, indicating that the reprogramming method did not affect this phenotype (Fig. 3-2C). It is plausible that reduced levels of a microtubule-severing enzyme, like spastin, would lead to increased microtubule stability. To test this, we performed Western analysis for stabilized microtubules in extracts from week 6 iPSC-derived neurons, using an acetylated tubulin antibody. This revealed a dramatic increase in acetylated tubulin levels in the SPG4 patient-derived neurons as compared to controls (Fig. 3-2D).
Figure 3-1

Characterization and differentiation of SPG4 iPSCs. (A) Control (iWT-I1, lentiviral) and SPG4 iPSC lines expressed the hESC markers Tra-1-60, Nanog and SSEA-4. Scale bars: 100 µm. (B) Analysis of pluripotency genes in generated iPSC lines (iWT-e3, iWT-e6, iSPG4-e3, iSPG4-e6, episomal) by RT-PCR. (C) Hematoxylin and eosin staining of teratoma sections that were derived from iPSCs. Tissues from each germ layer were formed. Scale bars: 200µm. (D) Sequencing results confirmed the presence of the intron 4 splice acceptor mutation (c.683-1G>T), which was not found in control cells. (E) The lentiviral SPG4 lines maintained a normal 46,XX female karyotype after 10 passages as shown by G-banded analysis. (F) The iPSCs were able to efficiently generate telencephalic glutamatergic neurons, as shown by staining for Tbr1. Scale bars: 50µm. (G) Percentages of cells immunostained positive for Tbr1 in control (iWT-I1) and SPG4 lines (iSPG4-I4 and iSPG4-I7). Data presented as mean ±SD. (H) Western blot analysis revealed a significant decrease in spastin protein in post-mitotic neurons derived from SPG4 iPSCs compared to control neurons. Nuclei are labeled with Hoechst (blue) in A and F.
3.4.3 Fast axonal transport of mitochondria is disrupted in SPG4 neurons

Axonal swellings have been associated with the accumulation of transported cargos [29, 68], indicating that axonal transport may be impaired. To assess axonal transport in SPG4 iPSC-derived cortical neurons, we firstly performed immunostaining for mitochondria and acetylated tubulin. This revealed the accumulation of mitochondrial staining within axonal swellings (Fig. 3-3A), suggesting impaired transport of these organelles. To further analyze mitochondrial fast axonal transport, live cell imaging was performed on week 8 control and SPG4-derived neurons. Images of axons were taken every 5 seconds for 5 minutes. As shown in representative kymographs of mitochondria within proximal axons (Fig. 3-3B), a significant decrease in the mitochondrial movement (indicated along the X-axis) was observed in neurons derived from SPG4 iPSCs. Further analysis of individual trajectories of each mitochondria revealed a significant reduction in the percentage of motile mitochondria; in control iPSC-derived neurons, 10.5% of the mitochondria were motile, compared to just 3.4% in the SPG4-derived neurons (Fig. 3-3C).

Transport vesicles and membranous organelles move along a polarized array of cytoplasmic microtubules down the axon (anterograde transport) and back to the cell body (retrograde transport). It remains unclear whether anterograde or retrograde transport is affected in SPG4. Therefore, using these patient iPSC-derived neurons, we further analyzed the velocity of motile mitochondria and frequency of motile movements in both the anterograde and retrograde directions (Table 3-1). The mitochondrial transport velocity in both anterograde and retrograde direction did not differ between control and SPG4 neurons, suggesting that the ATPase activity of the molecular motors was unaffected. Next, the frequency of fast axonal transport events was calculated; there was a 68% reduction in the frequency of motile events in the SPG4-derived neurons as compared to controls. Subdivision into anterograde and
SPG4 patient-derived neurons display axonal defects. (A) Acetylated tubulin staining revealed the presence of swellings along Tau\(^+\) axons of 6 week-old neurons. Boxed areas are enlarged in insets. (B) Quantification revealed a significant increase in axonal swellings in patient-derived neurons compared to control neurons. Data presented as mean ±SD. **\(P < 0.01\). (C) Episomal SPG4 neurons also possessed axonal swellings. (D) Western blots show that acetylated tubulin levels were dramatically increased in the week 6 SPG4-derived neurons compared to controls. Scale bars: 20 µm.
Figure 3-3

Disrupted fast axonal transport in SPG4-derived neurons. (A) Staining for mitochondria, using MitoTracker Red CMXRos, was enriched within axonal swellings (arrowheads). Scale bars: 20 µm. (B) Representative distance versus time kymographs over a 5 minute recording. Scale bar: 10 µm. (C) Quantification of motile mitochondria in week 8 forebrain neurons. Data presented as mean ±SD. **P < 0.01.
retrograde events revealed a directional selectivity in the effect of reduced spastin activity on fast axonal transport. While the number of anterograde events above the velocity threshold was not significantly different between control and SPG4 cells (though there was a trend toward reduction), there was a prominent 80% reduction in the number of retrograde events in the SPG4 cells, implicating retrograde axonal transport in SPG4 pathogenesis.

3.4.4 Knocking down spastin leads to axonal defects similar to those in SPG4-derived neurons

Considering that SPG4 iPSC-derived neurons recapitulate the disease-specific phenotypes and that the spastin protein is significantly reduced in these neurons, the axonal defects in SPG4 may result from loss of spastin function. To test this, we established spastin-knockdown hESC lines using lentiviral infection of shRNA constructs (Fig. 3-4A). To minimize off-target effects, two shRNAs targeted against different regions of spastin were cloned into the pLVTHM lentiviral vector that allows for the simultaneous expression of GFP and shRNA. After infecting H9 hESCs with the corresponding lentivirus, GFP+ cells were selected to obtain a pure population of knockdown cells (Fig. 3-4B). The spastin knockdown hESCs, as well as the control luciferase RNAi hESCs, were differentiated to the neural lineage and glutamatergic neurons as we described for SPG4 iPSCs. Expression of the shRNA was maintained during neural differentiation, as GFP was robustly expressed (Fig. 3-4B). To confirm the knockdown efficiency of the shRNA constructs, Western analysis was performed on cells at the hESC stage and in differentiated neurons (Fig. 3-4C). In day 40 neurons, there was around 40% and 77% knockdown of spastin protein level in the spastin RNAi lines 1 and 2, respectively. Notably, when the morphology of the neurons was examined, axonal swellings were prominent in neurons from the two spastin knockdown lines (Fig. 3-4D). Quantification revealed a significant increase in the number of swellings in the cells with reduced spastin as compared to controls (Fig. 3-4E). Interestingly, there was a trend toward increased swellings, with the knockdown
Table 3-1: SPG4-derived neurons have decreased frequency of retrograde fast axonal transport motile events.

<table>
<thead>
<tr>
<th></th>
<th>Anterograde Motile Velocity (µm/s)</th>
<th>Retrograde Motile Velocity (µm/s)</th>
<th>Events (&gt;300 nm/s)</th>
<th>Anterograde Events (&gt;300 nm/s)</th>
<th>Retrograde Events (&gt;300 nm/s)</th>
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<tbody>
<tr>
<td>iGM-e3</td>
<td>0.48±0.21</td>
<td>0.50±0.19</td>
<td>0.43±0.09</td>
<td>0.15±0.04</td>
<td>0.29±0.07</td>
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<tr>
<td>iSPG4-e6</td>
<td>0.45±0.12</td>
<td>0.54±0.17</td>
<td>0.14±0.04</td>
<td>0.08±0.02</td>
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<td>(P)-value</td>
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The velocity and frequency of mitochondrial motility were quantified in week 8 control and SPG4-derived neurons. No velocity differences were found in either the anterograde or retrograde direction. The frequency of total motile events was significantly decreased in SPG4 neurons. Though there was a non-significant trend toward reduction of anterograde motile events in SPG4 neurons, retrograde events were significantly reduced. Data are presented as means ±SEM. Statistical significance was determined using a two-sided \(t\)-test.
efficiency ($p<0.01$ by linear regression analysis). These data confirm that loss of spastin function results in the disease-specific axonal pathological changes in hPSC-derived neurons.

### 3.4.5 Microtubule-destabilizing drug rescues the axonal swelling phenotype

In order to show that the cells generated in this study have utility in screening for potential therapeutic compounds, we treated SPG4 iPSC-derived neurons and spastin knockdown hESC-derived neurons with the microtubule-destabilizing drug vinblastine. Vinblastine has been shown to be efficacious at reducing axonal swellings in $SPG4^{Δ/Δ}$ primary mouse neurons [167], increasing the cell body size of olfactory neural progenitors from SPG4 patients [176], and ameliorating the neurodegenerative phenotypes in $Drosophila$ [177]. When week 8 iSPG4-l7 neurons were treated with 10 nM vinblastine for just 24 hours, there appeared to be a reduction in the number of axonal swellings (Fig. 3-5A), and quantification confirmed that vinblastine significantly reduced the number of axonal swellings in these cells (Fig. 3-5B). To confirm the protective effect of vinblastine against axonal defects in human neurons, we treated week 10 spastin knockdown neurons with vinblastine (Fig. 3-5C). Similar to the iPSC-derived neurons, vinblastine treatment significantly reduced the number of axonal swellings (Fig. 3-5D). Taken together, our data revealed that a nanomolar concentration of the microtubule-destabilizing drug vinblastine can ameliorate the axonal defects observed in this human neuronal model of SPG4. This implicates disorganization of microtubules in the axonal phenotypes of SPG4.

### 3.5 Discussion

Human pluripotent stem cells, including hESCs and iPSCs, can differentiate into any cell type in the body, including neurons. Based on developmental principles, different neuronal subtypes have been generated using in vitro differentiation protocols. Using these systems, PSCs have been used to model several neurogenetic disorders [119, 120, 178-186], via
Spastin knockdown in hESC-derived neurons recapitulates SPG4 patient phenotype. (A) Schematic map showing the pLVTHM vector with the shRNA sequence. H9 hESCs were transfected with the indicated lentivirus, and GFP* cells were selected. (B) hESC colony after lentiviral transfection shows efficient generation of knockdown lines. Expression of shRNA continued during differentiation. Scale bars: 100 µm. (C) Western blot analysis confirming knockdown of spastin in both hESCs and neurons after 40 days of differentiation. (D) Immunostaining for acetylated tubulin revealed the development of axonal swellings in two spastin RNAi hESC-derived neurons. Hoechst stains the nuclei. Scale bars: 20µm. (E) Quantification of axonal swellings shows a significant increase in spastin RNAi-derived neurons compared to control cells. Data presented as mean ±SD. *P < 0.05, **P < 0.01. Luc, luciferase.
genetically modifying hESCs or using patient-specific iPSCs and then differentiating these stem cells into the neurons affected by a particular disease. Here, we have differentiated SPG4 iPSCs and spastin-knockdown hESCs to forebrain projection neurons in order to model HSP. We generated iPSCs from an SPG4 patient who possesses a heterozygous mutation (c.683-1G>T) that affects mRNA splicing, resulting in a reduction in spastin levels. Neurons from these lines had a significant increase in axonal swellings which appeared to accumulate mitochondria, suggesting altered axonal transport in these regions. Analysis of fast axonal transport of mitochondria revealed a significant decrease in the percentage of motile mitochondria and a decrease in the frequency of motile events, particularly in the retrograde direction.

To confirm that spastin loss-of-function is responsible for these findings, we generated spastin knockdown lines. Similarly, a significant increase in axonal swellings was found in the knockdown cells compared to a control knockdown line. Moreover, it appeared that cells with the greater reduction in spastin levels exhibited that largest increase in swellings. In addition, the protein expression of stable acetylated tubulin was increased in SPG4 neurons. Treatment with the microtubule-stabilizing drug vinblastine was able to rescue the axonal swelling phenotype in SPG4 and spastin-knockdown neurons. To our knowledge, this is the first study to recapitulate the axonal defects of HSP in human neurons. Our study also implicates the loss of spastin function and subsequent disruption in microtubule organization in the axonal defects in SPG4 neurons.

Our results support the hypothesis that spastin loss-of-function is responsible for mediating disease in SPG4 patients. There was a dramatic decrease in spastin levels in the neurons generated from the SPG4 iPSC lines as compared to lines from control individuals. In addition, SPG4-derived neurons with reduced spastin displayed a significant increase in axonal swellings. Interestingly, the axons that possessed swellings were significantly longer than those
Figure 3-5

Vinblastine treatment ameliorates the SPG4 axonal swelling phenotype. (A) Week 8 iSPG4-l7 neurons treated with 10 nM vinblastine for 24 hours appeared to have a reduction in axonal swellings compared to control cells, while no deleterious effects were observed. Scale bars: 20µm. (B) Quantification revealed that 10 nM vinblastine significantly reduced the number of axonal swellings. (C) Treatment with 10 nM vinblastine for 24 hours also reduced axonal swellings in spastin RNAi-1 neurons. Scale bars: 20µm. (D) Quantification of axonal swellings in cells treated with vehicle or 10 nM vinblastine. Data presented as mean ±SD. **P < 0.01.
without, suggesting that the longer axons may be more susceptible to defects caused by reduced spastin levels. This is concordant with the length-dependent degeneration of the longest CSMN axons in SPG4. Notably, we were able to observe a similar increase in swellings in neurons from spastin knockdown lines, suggesting that this phenotype was dependent on spastin dosage. The presence of axonal swellings in our system, at as early as 6 weeks of differentiation, is very interesting. Neurons at this stage are comparable to immature fetal cells [187], suggesting that defects in patient cells may be present prior to the onset of symptoms. An alternative explanation for this may be the inherent differences in the in vitro environment compared to in vivo environment, which may affect the onset and severity of the phenotype.

Our findings are in agreement with the published studies of two SPG4 mouse models, where a reduction in spastin also led to phenotypes similar to HSP patients and axonal swellings in cortical neurons [29, 68]. Although the knockout mice only had minor motor deficits compared to those of HSP patients, perhaps the prominent differences in absolute size of CSMNs in mice and humans can account for this. One of the difficulties in examining CSMNs using in vitro cultures is the lack of a specific marker, and a directed differentiation method that specifically generates CSMNs is currently unavailable. However, the differentiation method used in this study can produce neurons that express factors enriched in these cells [128], such as FezF2 and Ctip2 [188]. In the future, if conditions for specifying CSMNs are established, it would be interesting to determine whether these neurons are more susceptible to axonal swellings than other neuronal subtypes.

Microtubules are a major component of the neuronal cytoskeleton, and they are needed for the extension of neurites in developing neurons and to serve as railways for cargo transport in mature neurons [189, 190]. They are composed of α- and β-tubulin heterodimers that are selectively arranged in axons so that the plus-end is oriented distal to the soma. All forms of α-tubulin can be acetylated at Lys40, while β-tubulin is not. Long-lived, stable microtubules are
enriched in acetylated tubulin [191], particularly in the proximal portion of axons [192]. Although the exact role of tubulin acetylation has not been fully resolved, studies on Huntington and Parkinson disease models have reported that altered levels of acetylated tubulin result in axonal transport deficits [193, 194]. We found that levels of acetylated tubulin were dramatically increased in SPG4-derived week 6 neurons compared to control neurons. Reduction in the microtubule-severing activity of spastin in the SPG4-derived neurons would be expected to cause such an increase in stabilized microtubules. This result is contrary to what was observed in olfactory neurosphere-derived cells isolated from SPG4 patients [176], where a decrease in acetylated tubulin in SPG4 cells was reported to reflect an increased expression of the microtubule-destabilizing protein stathmin. Differences in the cell types that were analyzed between these two studies may account for the opposing changes in acetylated tubulin levels. This further suggests that differentiating cells to mature neurons that possess axons may be important for recapitulating disease-specific abnormalities.

Deficits in axonal transport are a common observation among different forms of neurodegenerative disorders. This is not surprising, since the axoplasm accounts for >99% of total cellular volume in the longest neurons, which puts enormous strain on the intracellular transport mechanisms needed to traffic proteins, lipids, and other molecules to and from the most distal parts of the cell [132]. Microtubule-severing enzymes are involved in generating the diverse array of microtubules present in axons [195]. As revealed in our study, levels of acetylated tubulin were significantly increased in SPG4 neural cultures. In Sp^{ΔΔ} mice, loss of spastin leads to a decrease in dynamic microtubule ends, and an increase in detyrosinated, stable microtubules along axonal swellings [29]. These changes can lead to alterations in microtubule-associated protein (MAP), kinesin, and dynein binding [189]. The decrease in motile mitochondria we observed in SPG4-derived neurons supports a pathogenic role for disrupted microtubule-based transport in SPG4. Moreover, although the velocity of fast axonal transport
was not altered, the frequency of motile events in the retrograde direction in particular was significantly decreased. Importantly, longer-lived, stable microtubules that are subjected to post-translational modifications and MAP binding may interfere with the binding of molecular motors, altering the frequency of motility events [196]. Retrograde axonal transport is important for bringing distal trophic factors or stress stimuli to the soma so that the neuron can respond properly [197]. Our finding agrees with a recent report on an SPG4 mouse model, SpΔ/Δ, where retrograde transport was also specifically affected in neurons that lack spastin [13]. Moreover, fast axonal transport deficits are implicated in other related neurodegenerative diseases. For example, mutations in subunits of cytoplasmic dynein, a motor protein involved in retrograde transport, can result in motor neuron degeneration, suggesting that retrograde fast axonal deficits are sufficient to cause neurodegeneration [198, 199].

In the future, these SPG4 iPSCs should prove valuable in further unraveling the mechanisms of axonal degeneration in HSP. Understanding the link between microtubule defects found in SPG4 and other forms of HSP will be valuable in understanding the pathways required for axonal maintenance. A better notion of the pathways that spastin regulates will be useful in the development of therapies that may be applicable to multiple forms of HSP. This includes the other forms that are involved in ER morphogenesis: SPG3A, SPG12, and SPG31 [69, 70, 78]. In addition, this hPSC model of HSP will also be a valuable tool in testing future therapeutic strategies. The ability to examine the effects of drugs on the human cell types that are affected by disease is one of the main benefits of using hPSCs. This is particularly important since over 90% of CNS therapies fail during clinical trials, in part because of the lack of accurate models during preclinical stages [187, 200]. The evidence that vinblastine is capable of reducing axonal swellings in human cortical neurons shows that this system will be useful in the future for screening therapeutic agents to rescue axonal degeneration in HSP.
3.6 Conclusion

In this study, we have demonstrated the successful establishment of human neuronal models of SPG4 by generating both SPG4 patient-iPSCs and spastin-knockdown hESCs, and then differentiating these stem cells into telencephalic glutamatergic neurons. These hPSC-derived neurons displayed increased axonal swellings, accumulation of transported cargoes, and impaired mitochondrial transport, recapitulating disease-specific axonal phenotypes. To our knowledge, this is the first evidence of axonal transport deficits in human SPG4 neurons. Moreover, our data support the hypothesis that axonal phenotypes in SPG4 are caused by loss of spastin function, which is buttressed by both the decreased expression level of spastin in SPG4 iPSC-derived neurons and the observation of similar axonal phenotypes in spastin-knockdown neurons. Finally, the expression of stable acetylated-tubulin was increased in SPG4 neurons, and vinblastine, a microtubule-destabilizing drug, rescued the axonal swelling phenotype. These findings suggest that microtubules can be a potential therapeutic target for HSP in human neurons and our hPSC-based models of HSP serve as a unique paradigm to study the pathogenic mechanisms and to screen for therapeutic drugs.

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Chapter 4

Pharmacologic rescue of axon growth defects in a human iPSC model of hereditary spastic paraplegia SPG3A

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Key Words: iPSC, atlastin, endoplasmic reticulum, transport, mitochondria
4.1 Abstract

Hereditary spastic paraplegias (HSPs) are a large, diverse group of neurological disorders (SPG1-71) with the unifying feature of prominent lower extremity spasticity, due to a length-dependent axonopathy of corticospinal motor neurons. The most common early-onset form of pure, autosomal dominant HSP is caused by mutation in the ATL1 gene encoding the atlastin-1 GTPase, which mediates homotypic fusion of ER tubules to form the polygonal ER network. Here we have identified a p.Pro342Ser mutation in a young girl with pure SPG3A. This residue is in a critical hinge region of atlastin-1 between its GTPase and assembly domains, and it is conserved in all known eukaryotic atlastin orthologs. We produced induced pluripotent stem cells (iPSCs) from skin fibroblasts and differentiated these into forebrain neurons to generate a human neuronal model for SPG3A. Axons in these SPG3A neurons showed impaired growth, recapitulating axonal defects in atlastin-1-depleted rat cortical neurons and impaired root hair growth in loss-of-function mutants of the ATL1 ortholog rhd3 in the plant Arabidopsis. Both the microtubule cytoskeleton and tubular ER are important for mitochondrial distribution and function within cells, and SPG3A neurons showed alterations in mitochondrial motility. Even so, it is not clear whether this change is involved in disease pathogenesis. The SPG3A axon growth defects could be rescued with microtubule-binding agents, emphasizing the importance of tubular ER interactions with the microtubule cytoskeleton in HSP pathogenesis. The prominent alterations in axon growth in SPG3A neurons may represent a particularly attractive target for suppression in screens for novel pharmacologic agents.
4.2 Introduction

Hereditary spastic paraplegias are a diverse group of inherited neurological disorders unified by the defining feature of a length-dependent axonopathy of corticospinal motor neurons, resulting in prominent lower extremity spasticity and gait difficulties [54, 201]. These disorders were historically classified as pure or complex based upon the presence (complex) or absence (pure) of associated clinical features such as cognitive impairment, distal amyotrophy, retinopathy, neuropathy, and thin corpus callosum. More recently, a genetic classification scheme has predominated, and HSPs are commonly identified by their affected genes and spastic gait (SPG) loci, SPG1-71, assigned in order of locus identification [18, 54, 201, 202]. Despite the remarkable genetic heterogeneity of HSPs, a relatively small number of common cellular themes have emerged, including perturbations in mitochondrial function, endoplasmic reticulum shaping/distribution, myelination, lipid/cholesterol metabolism, nucleotide metabolism, protein/membrane trafficking, bone morphogenetic protein (BMP) signaling, and endolysosomal function [1, 18, 202].

Disruption in the formation of the tubular ER network in cells has emerged as a key pathogenic theme underlying the HSPs, since over half of patients with autosomal dominant, pure HSP have SPG3A, SPG4, SPG12, or SPG31, each of which is caused by mutations in genes encoding proteins that bind one another and function in the organization of the tubular ER network [1, 54, 203]. Thus, the development of animal and cellular models for these disorders of ER network formation is increasingly important.

The SPG3A protein atlastin-1 along with its human paralogs atlastin-2 and atlastin-3, yeast ortholog Sey1p, and plant ortholog RHD3 are large, oligomeric GTPases that harbor two very closely-spaced transmembrane domains required for proper ER localization; these GTPases mediate homotypic fusion of ER tubules to form the polygonal ER network [71, 72, 204-208]. The SPG31 and SPG12 proteins REEP1 and reticulon-2, respectively, are members of the reticulon/REEP/Yop1p superfamily of proteins that harbor partially-membrane spanning,
hydrophobic hairpin domains and shape the high-curvature ER tubules [209, 210]. Last, the SPG4 protein spastin is an ATPase associated with a variety of activities (AAA), and the larger M1 spastin isoform harbors a hydrophobic hairpin and localizes to ER tubules. These hydrophobic domains are important for self-interactions among these proteins as well as for interactions with one another [69].

To date no mouse model has been reported for SPG3A, the second most common cause of HSP and the most common early-onset form; there have been a few cellular as well as fly and zebrafish models described. For instance, depletion of atlastin-1 in rat cortical neurons in primary culture using shRNA causes prominent axon growth and branching defects [211]. Disruption of the single atlastin ortholog in Drosophila causes synapse and muscle defects that can be rescued with the microtubule-destabilizing drug vinblastine [22], indicating the importance of interactions among ER shaping and microtubule-interacting proteins [22]. In zebrafish, knockdown of the atlastin gene atl1 causes a decrease in larval mobility that is preceded by abnormal architecture of spinal motor axons [20].

Here, we have identified a novel de novo SPG3A mutation (c.1024C>T; p.Pro342Ser) in a 2-year old girl with early-onset, pure HSP. This Pro residue is at a critical position for atlastin-1 within a small linker region between the GTPase domain and three-helical bundle (3HB) assembly domain. In fact, this Pro residue is conserved in all eukaryotic atlastin orthologs [212]. We used skin fibroblasts from this patient to generate induced pluripotent stem cells (iPSCs), which were then differentiated into forebrain neurons to generate the first human neuronal model of SPG3A. These neurons have prominent axonal growth defects that can be partially rescued with microtubule-binding agents, emphasizing the importance of interactions of tubular ER with the microtubule cytoskeleton in HSP pathogenesis [69].
4.3 Results

4.3.1 Cellular effects of SPG3A ATL1 mutation

A 2 year-old girl with a clinical presentation of early-onset, pure HSP had a *de novo*, heterozygous mutation in *ATL1*, c.1024C>T (p.Pro342Ser), identified by commercial DNA sequencing (Athena Diagnostics). Though this mutation has not been previously reported, it occurs at a residue mutated in other patients with SPG3A [213], and is conserved among all eukaryotes in the linker region of atlastin GTPases (Fig. 4-1A); this linker plays a key role in a conformational switch of atlastin important for the ER tubule fusion process (Fig. 4-1B; [212, 214]). This mutation also results in a modest reduction in GTPase activity (Fig. 4-1C-D).

Skin fibroblasts were cultured to assess the effects of this mutation on atlastin-1 protein levels. Immunoblots of SPG3A and control fibroblast cultures indicate that levels of the atlastin-1 protein are not diminished in the mutant cells, suggesting that the mutant protein is present in cells. Levels of a number of other ER proteins, including the atlastin-1 paralogs atlastin-2 and atlastin-3, which are abundant in these cells [207], are also not significantly affected (Fig. 4-2A). The atlastin-1 P342S protein is also present in puncta throughout the peripheral ER, which is mostly tubular ER in contrast to the more perinuclear ER sheets labeled with CLIMP-63 (Fig. 4-2B). The distributions of endogenous atlastin-1 positive puncta appeared similar in control and atlastin-1 P342S fibroblasts (Fig. 4-2C). Since atlastin-2 and -3 are likely the predominant atlastin proteins in skin fibroblasts and may thus provide adequate protein for ER fusion even in the presence of atlastin-1 dysfunction, we examined effects of atlastin-1 P342S overexpression in these cells. Heterologous expression of Myc-tagged, wild-type atlastin in COS7 cells did not result in changes in ER morphology, but expression of the P342S mutant markedly altered ER morphology in all cells examined (Fig. 4-2D).
Figure 4-1. Atlastin-1 P342S SPG3A mutation affects a critical residue for conformational shifts, and alters GTPase activity. (A) Schematic diagram of atlastin-1 domain organization (top) and sequence comparisons among different species in the atlastin-1 linker region (bottom). Asterisks (*) denote conserved residues, and the conserved Pro342 residue is shown in red. Abbreviations: 3HB, three helical bundle; TM, transmembrane domains. (B) Left, superposition of the cytosolic domain of atlastin-1 in two different crystal forms, denoted 1 and 2, with the respective GTPase domains as the reference. Right, Major differences in conformation that comprise a GTPase domain-internal helix and position of the 3HB domain accompanied by restructuring of the linker region with the different positions of Pro342 shown. This image with labels modified slightly is reproduced with permission from Sondermann and Byrnes [212]. (C and D) Myc-tagged wild-type atlastin-1 or the P342 missense mutant were immunopurified from COS7 cells, and GTP hydrolysis was performed in vitro and plotted as a function of time (C). Representative thin-layer chromatography plates show conversion of GTP to GDP (D). Means ± SD are graphed.
4.3.2 Characterization and neural differentiation of control and SPG3A iPSCs

SPG3A iPSCs were generated from skin fibroblasts cultured from the patient with this P342S mutation in atlastin-1. The fibroblasts were reprogrammed using an integration-free episomal method [156], and multiple iPSC clones were generated from both the SPG3A fibroblasts and wild-type controls. These clones had a typical ESC colony morphology, with no obvious differences between SPG3A and control lines, and expressed the characteristic human PSC markers Nanog, SSEA4 and Tra1-60 (Fig. 4-3A). To confirm that the SPG3A iPSCs did not contain any chromosomal abnormalities after passaging, karyotype analysis was performed; this did not reveal any defects (Fig. 4-3B). To confirm the presence of the heterozygous p.Pro342Ser mutation in the ATL1 gene, this region was sequenced in both the iPSCs and the neural cells after differentiation (Fig. 4-3C).

We differentiated the iPSCs to forebrain glutamatergic neurons using a well-established differentiation protocol [128, 172, 173]. Representative images are shown for cells at different stages during the differentiation (Fig. 4-4A). No apparent alterations were observed in the abilities of control and SPG3A cells to generate neurons. Although atlastins are expressed in all eukaryotic cells, atlastin-1 is by far the most abundant member in the central nervous system [71, 207, 211]. To examine how atlastin-1 levels in human neurons change during the course of in vitro differentiation, we performed immunoblot analysis (Fig. 4-4B). Very low levels of atlastin-1 protein were present in iPSCs, but by the neurosphere stage at day 28 of differentiation there was a dramatic increase. Atlastin-1 levels remained high in early generated neurons (week 6) and in more mature neurons (week 15). Conversely, levels of atlastin-2 and atlastin-3 were high at the iPSC stage, and decreased dramatically following neural differentiation (Fig. 4-4C). Levels of atlastin-1 were comparable between controls and SPG3A lines in week 8 neurons (Fig. 4-4D). Thus, similar to what was observed in patient fibroblasts (Fig. 4-2A), the
Figure 4-2. Atlastin P342S localizes to the tubular ER and disrupts ER morphology.  (A) Aliquots of total membranes (10 μg protein per lane) from control or SPG3A P342S human fibroblasts were immunblotted for the indicated ER proteins. Sizes of molecular weight standards (in kDa) are at the left.  (B) Fibroblasts were co-immunostained for endogenous atlastin-1 (green) and CLIMP-63 (red).  (C) Control or SPG3A P342S human fibroblasts were co-immunostained for atlastin-1 (green) and calreticulin (red).  (D) COS7 cells were transfected with Myc-tagged wild-type or P342S mutant atlastin-1 and immunostained for Myc-epitope (green) and β-tubulin (red).  Scale bars: 10 μm in panel B and 20 μm in panels C and D.
Figure 4-3. Generation of SPG3A iPSC lines. (A) Control and SPG3A iPSC lines expressed the human ESC markers Tra-1-60 (red), Nanog (red) and SSEA-4 (green), as visualized by immunofluorescence microscopy. Hoechst nuclear staining is blue. Scale bars: 100 µm. (B) SPG3A iPSC lines maintained a normal 46,XX female karyotype after 10 passages as shown by G-banded analysis. (C) DNA sequencing electropherograms confirm the presence of a heterozygous c.1024C>T, p.Pro342Ser mutation before and after neural differentiation.
p.Pro342Ser mutation did not affect total atlastin-1 protein levels. In addition, levels of the microtubule-severing ATPase spastin, a direct binding partner of atlastin-1 [215, 216] mutated in the SPG4 subtype of HSP [55], were not dramatically different between control and SPG3A neurons (Fig. 4-4E). To confirm that forebrain glutamatergic neurons were generated, immunostaining was performed for Tbr1 and the neuron-specific tubulin isoform βIII-tubulin (Fig. 4-4F). Quantification of Tbr1+ cells did not reveal significant differences between control and SPG3A iPSC lines (Fig. 4-4G).

4.3.3 Axonal outgrowth of neurons derived from control and SPG3A iPSCs

In a zebrafish model of SPG3A, knocking down atl1 resulted in abnormal axonal outgrowth of spinal motor neurons [20]. To investigate axonal morphology in SPG3A iPSC-derived forebrain neurons, neurospheres were plated onto coverslips and axons were allowed to extend for 48 h, then fixed and stained for the axon marker tau. The tau staining extended to the end of the axons, as shown by co-localization with F-actin, which is enriched in growth cones (Supplementary Material, Fig. 4-S2). In addition, the long neurites that extended from the neurospheres were established to be axons by the co-localization of tau with the presynaptic marker bassoon (Supplementary Material, Fig. 4-S3). This revealed an apparent reduction in the length of axons in the SPG3A axons compared to control neurons (Fig. 4-5A). Quantification of neurite outgrowth revealed a significant reduction in the average length of axons (Fig. 4-5B). Analysis of dissociated, singlized neurons revealed a similar reduction in axonal outgrowth in SPG3A neurons derived from two different iPSC clones (Fig. 4-6A-B). Together these data suggest that SPG3A neurons, have impaired axon outgrowth with reduced axon length.
Figure 4-4. Neural differentiation of SPG3A iPSCs. (A) Representative differential interference contrast images of control and SPG3A cells during neural differentiation at various stages. EB, embryoid body; NE, neuroepithelia. Scale bars: 100 µm. (B) Cell aliquots were immunoblotted for atlastin-1 during different stages (weeks 0, 4, 6, and 15) of neural differentiation. Actin levels were monitored as a control for protein loading. (C) Atlastin-2 and -3 levels decrease following neural differentiation. Cell aliquots were immunoblotted during different stages (weeks 0, 4, 6, and 15) of neural differentiation as shown. Actin levels were monitored as a control for protein loading. (D) Immunoblot analysis of atlastin-1 protein levels in extracts from week 15 neurons derived from wild-type (iWT-1 & iWT-3) and SPG3A iPSCs (iSPG3A-6 & iSPG3A-8). Actin levels were monitored as a control for protein loading. (E) Spastin levels are not dramatically altered in SPG3A neurons. Protein samples from week 15 neurons were immunoblotted for spastin. (F) Both control and SPG3A neurons efficiently generated Tbr1+ (red) glutamatergic neurons. Nuclei are identified with Hoechst staining (blue), and βIII-tubulin and Tbr1 were visualized by confocal immunofluorescence microscopy. Scale bars: 50 µm. (G) Quantification of Tbr1+ neurons did not reveal any significant differences between the indicated iPSC lines. Data are presented as means ± SD, n=3. In panels B-E, migrations of molecular mass standards in kDa are indicated to the left.
**Supplementary Figure 1.** Tau stains the entire length of axons. (A) Tau staining extends to the F-actin enriched growth cones, shown by Rhodamine-Phalloidin staining. (B) Higher magnification images of the boxed region in A. Scale bars = 20 µm.
Supplementary Figure 2. Axons grow out of neural clusters. (A) Week 8 iWT-1 neural aggregates stained for axon markers tau and bassoon. Higher magnification image of the boxed region is shown on the right. (B) Week 8 iSPG3A-6 neural clusters also stained for axon markers tau and bassoon. Scale bars = 50 µm.
Figure 4-5. SPG3A neurons display reduced axonal outgrowth. (A) Fluorescence images of Tau^+ axons from iPSC-derived neurons two days after plating. Scale bars: 200 µm. (B) Quantification of Tau^+ axon length shows a significant reduction in SPG3A derived neurons. Data are presented as means ± SD, N=3-6 coverslips, with at least 1100 cells analyzed per group. **P < 0.01 versus control (iWT-1).
Figure 4-6. Reduced axonal outgrowth in singlized SPG3A neurons. (A) Control and SPG3A iPSC-derived neurons were plated and cultured for 48 hrs prior to staining for Tau. (B) Quantification of mean Tau+ process length for each group. N=3 coverslips, with at least 50 cells analyzed per group. Data presented as mean ± SD. *P < 0.05 versus controls.
4.3.4 Fast axonal transport defects in SPG3A neurons

There is abundant evidence that links other forms of dominant, pure HSP to defects in fast axonal transport [29, 30, 61, 68, 217]. To investigate whether transport is disrupted in SPG3A neurons, we analyzed mitochondrial fast axonal transport in week 12 iPSC-derived neurons with live-cell imaging, using the dye MitoTracker CMXRos. After cells were stained, fluorescent images of proximal axons were taken every 5 s for 5 min, and individual trajectories for each mitochondrion within the field were acquired, allowing detailed analysis of multiple transport parameters. Representative position versus time kymographs are shown in Fig. 4-7A, revealing the nature of fast axonal transport in the two groups. Comparison of mitochondrial transport velocities in both anterograde (Fig. 4-7B) and retrograde (Fig. 4-7C) directions revealed no significant differences between control and SPG3A neurons. The frequency of motile events was calculated by counting the number of times each mitochondrion moved with a velocity greater than 300 nm/s. This velocity threshold was used to exclude transport events mediated by actin, which fall well below this threshold [133]. Calculation of the percentage of motile mitochondria for each cell revealed a significant reduction in SPG3A neurons compared to controls (Fig. 4-7D), and the frequency of motile events was lower in the SPG3A neurons as compared to controls (Fig. 4-7E). When these events were separated based on direction of transport, while there was only a non-significant trend towards reduction of events in the retrograde direction (Fig. 4-7F), a significant reduction in anterograde motile events was observed in SPG3A cells (Fig. 4-7G).
Figure 4-7. Decreased mitochondrial transport in SPG3A neurons. (A) Representative distance versus time kymographs show decreased motile mitochondria. Scale bar: 5 μm. (B and C) The velocity of movement events in anterograde (B) and retrograde (C) directions were unaffected in SPG3A neurons. (D) The percentage of motile mitochondria was significantly decreased in SPG3A neurons. (E) The number of motile movement events (velocity >300 nm/s) was also decreased in SPG3A neurons. (F and G) There was a non-significant trend toward a decreased frequency of motile movement events (per mitochondrion over 5 min) in the retrograde direction (F), while the frequency of events in the anterograde direction (G) was significantly reduced in SPG3A neurons. Data are presented as means ± SEM, N = 10-20 cells. *P < 0.05 versus control group (iWT-1); ns, not significant.
4.3.5 Treatment with microtubule-targeting drugs rescues the axon outgrowth phenotype

In a *Drosophila* model of SPG3A, where the single atlastin ortholog was disrupted, neuromuscular junction defects were linked to abnormal accumulation of stable microtubules [22]. It was suggested that this effect of atlastin is mediated through atlastin’s interactions with the SPG4 protein spastin, a microtubule-severing AAA ATPase. Importantly, treatment with the microtubule-destabilizing drug vinblastine partially rescued neuromuscular junction defects and improved the survival of mutant flies [22]. To test whether treatment with microtubule-targeting drugs is similarly effective on human SPG3A neurons, and whether this iPSC-based model will have the potential for drug screening, we treated week 8 SPG3A neurons with several compounds. Briefly, both control and SPG3A neural progenitors were plated onto coverslips and allowed to attach for 24 h. The next day, neurons were treated with 10 nM vinblastine for 48 h, followed by analysis of tau+ axon growth. While vinblastine treatment did not have a significant effect on axon outgrowth in control neurons, it significantly increased outgrowth in the SPG3A cells (Fig. 4-8A-B), suggesting that alterations in microtubule dynamics may be involved in this phenotype.

A previous report investigating a mouse model of SPG4 found that treatment with nanomolar concentrations of both vinblastine and taxol, which are thought to alter only microtubule dynamic instability at these concentrations, significantly reversed the presence of axonal swellings in cortical neurons [167]. Treatment of wild-type iPSC-derived neurons with taxol or vinblastine did not alter axon outgrowth significantly. However, treatment with either taxol or vinblastine significantly increased axon outgrowth in the SPG3A cells (Fig. 4-8A-B). Together, these results reveal that microtubule-targeting drugs are able to rescue axon outgrowth defects in SPG3A patient-iPSC derived neurons.
4.4 Discussion

We have used skin fibroblasts from an SPG3A patient with a novel p.Pro342Ser mutation in the \textit{ATL1} gene to generate iPSC lines and model this common, early-onset, pure form of HSP in human neurons. The SPG3A iPSCs were able to differentiate into forebrain glutamatergic neurons with an efficiency similar to that of control neurons, and levels of atlastin-1 protein were not decreased by the p.Pro342Ser mutation. Since overexpression of this mutant alters ER morphology in a manner similar to atlastin depletion, a dominant-negative, loss-of-function disease mechanism appears most likely, with mutant proteins binding and impairing the function of the wild-type protein in atlastin oligomers. Previously, lymphoblasts from a patient with a p.N436del in-frame deletion in \textit{ATL1} were shown to have markedly decreased atlastin-1 levels, which were postulated to result from increased susceptibility of wild-type/p.del436N heteromers to degrade in a dominant-negative manner [218]. Very recently, multiple affected members of a consanguineous Pakistani family were reported with a homozygous p.Arg118Gln mutation in \textit{ATL1}; heterozygotes had minimal or no clinical signs, consistent with recessive inheritance, though the functional effects of this mutation are unknown [219]. In these cases, a loss-of-function pathogenesis seems most plausible, while also illustrating that loss of atlastin function can arise in a number of ways.

Consistent with a dominant-negative, loss-of-function disease mechanism, SPG3A p.Pro342Ser neurons displayed markedly reduced axonal outgrowth, similar to results from shRNA knockdown of rat cortical neurons [211]. This phenotype is also highly similar to that seen in loss-of-function mutations in the atlastin-1 ortholog RHD3 of the flowering plant \textit{Arabidopsis}, where there are prominent defects in root hair growth [220]. These root hairs are long protrusions from root cells that are reminiscent of axons [54, 221]. Thus, these defects in polarized cell expansion are a broadly conserved function of atlastin proteins across phylogeny.
**Figure 8.** Vinblastine treatment partially rescued the axonal outgrowth phenotype in SPG3A-derived neurons. (A) Immunofluorescence images of control and SPG3A plated neural clusters treated with vehicle, 10 nM vinblastine, or 10 nM taxol for 48 h. Scale bars: 100 µm. (B) Quantification of axon length showed that vinblastine and taxol treatment significantly increased the length of axons in SPG3A-derived neurons. Data are presented as means ± SD, with a minimum of 1100 cells analyzed per group. *P < 0.05 versus controls (iWT-1); #P < 0.05 versus vehicle-treated SPG3A group.
and consequently may represent an important phenotype for rescue in pharmacologic screens. In fact, these axon growth defects in human SPG3A neurons could be rescued by treatment with both vinblastine and taxol at nanomolar concentrations, indicating an important role for ER interactions with dynamic microtubules.

In addition to its interactions with microtubules, the ER has extensive interactions with other organelles, which could be also impacted by changes in ER shape [222]. We investigated fast axonal transport of mitochondria because such defects have already been observed in autosomal dominant SPG4 [29, 68, 167], which is caused by mutations in the spastin AAA ATPase that binds atlastin-1. There were no differences in the transport velocities of mitochondria in either anterograde or retrograde directions, suggesting that activities of the molecular motors were unaffected. However, there were decreases in the frequency of total mitochondrial motile events and events in the anterograde direction, while there was a non-significant trend towards reduced retrograde events in the SPG3A neurons. Since the axoplasm accounts for >99% of total cellular volume in the longest neurons, they are particularly susceptible to defects in intracellular transport [1]. In fact, many neurodegenerative disorders have been linked to deficits in axonal transport, including amyotrophic lateral sclerosis (ALS), Parkinson disease, Alzheimer disease, and Huntington disease [132]. The proper distribution of mitochondria and likely other organelles throughout the axon thus appears critical for maintaining neuronal health.

Defects in axonal transport of mitochondria, other organelles, or proteins may underlie the axon outgrowth defect that is observed in these SPG3A cells. A majority of proteins in the axon and at synapses are generated in the cell body and must be transported down axons via anterograde transport [143]. Mitochondrial localization, which is dependent on fast axonal transport, is also important for regulating the variability of presynaptic strength as well as for axon formation and outgrowth [144, 145]. In fact, mitochondria accumulate in the growth cones of axons during outgrowth [223]. In the SGP3A neurons, reduction in the number of
anterograde motile events could conceivably reduce the net flux of mitochondria towards the distal portions of the axons, negatively affecting axon outgrowth, presynaptic regulation, or both. However, selectively increasing mitochondrial axonal mobility does not slow ALS-like impairment in the SOD1(G93A) mutant mouse model for ALS [224]. Alternatively, other consequences of atlastin dysfunction such as aberrant ER distribution (which could secondarily affect mitochondrial motility) or BMP signaling [1] may play key roles in pathogenesis.

Studies in *Drosophila* have previously shown that there are increased stabilized microtubules in atl mutant flies and that vinblastine treatment can rescue several neuromuscular defects [22]. Loss of atlastin function was hypothesized to result in altered microtubules by disrupting the interaction between atlastin and the microtubule-severing, SPG4 ATPase spastin. We and others have previously shown that treatment with microtubule-targeting drugs can rescue defects in SPG4 cells *in situ*, suggesting that altered microtubules may be a common finding among different forms of HSP [30, 167, 176]. One role of atlastin-1 may be to distribute spastin’s microtubule-severing activity within neurons, since spastin is a direct binding partner [69, 215, 216]. Reduced atlastin-1 within the distal portions of axons may affect the function of spastin in these regions, resulting in increased stabilized microtubules. Treatment with sub-stoichiometric concentrations of either vinblastine or taxol increased axonal outgrowth to levels comparable to wild-type neurons. It will be interesting to examine further whether axon growth and transport deficits can be rescued by these drugs in longer-term cultures.

On initial consideration, it seems hard to reconcile a primary defect in axon growth as a mediator or the pathology in HSPs, since most HSPs are progressive disorders characterized by distal, dying-back axonal degeneration [1, 18, 54, 201, 202, 225]. However, SPG3A in particular is notable for not only being the most common early-onset, pure form of HSP, but also for having a large number of affected individuals presenting with apparently non-progressive forms [6]. Thus, abnormal axon development may play a key role in SPG3A pathogenesis. Our findings suggest that iPSC-based models of SPG3A will be useful for drug screening studies. In
future studies, it will be particularly interesting to assess how rescue of axon growth defects due to SPG3A mutations correlates with rescue of known intracellular functions of atlastins, such as BMP signaling, ER morphology, organelle distribution, and lipid droplet formation [19, 20, 226].

4.6 Materials and Methods

4.6.1 Clinical studies

All study procedures were performed under an Institutional Review Board-approved clinical research protocol (NINDS protocol 00-N-0043) at the National Institutes of Health Clinical Center. The parents of the SPG3A patient provided informed consent.

4.6.2 Cell culture, transfection, and GTPase activity assays

Heterologous expression studies in COS7 cells and in vitro GTPase assays of Myc-atlastin-1 proteins were performed as described previously [71, 227].

4.6.3 Reprogramming fibroblasts into iPSC lines

Human fibroblast cell lines were established from skin punch biopsies and maintained using standard procedures. To generate iPSC lines using episomal transduction, ~200,000 cells were dissociated and transfected with episomal plasmids (Addgene) containing pluripotency factors (Oct3/4, Sox2, L-Myc, Klf4, and Lin 28), as reported previously [156]. At around one week after electroporation transduction, cells were plated onto a 35-mm dish in DMEM supplemented with 10% fetal bovine serum. After culturing for 7 days, cells were dissociated and seeded onto a mouse embryonic fibroblast (MEF) feeder at ~10^5 cells/100-mm dish. Two weeks later, colonies with morphologies similar to human embryonic stem cells (ESCs) were observed. These colonies were split onto MEF feeder cells to derive iPSC lines. Following several passages, homogenous colonies with ESC-like morphology were generated. The iPSC lines used in this study were iWT-1, iWT-3, iSPG3A-6, and iSPG3A-8.
4.6.4 Human iPSC neural differentiation

To generate telencephalic neurons from iPSCs, stem cells were cultured on a feeder layer of irradiated MEFs in 6-well tissue culture treated plates for around 6 days, with the human ESC media (+10 ng/ml fibroblast growth factor [FGF]-2) changed daily. When nearly confluent, cells were detached from the feeder layer to initiate neural differentiation, as previously described [128, 172, 173]. Briefly, iPSC aggregates were cultured in suspension for 4 days in human ESC media and were then transferred to neural induction media (NIM). After 3 additional days in suspension, iPSC aggregates were plated onto 6-well tissue culture treated plates in NIM with 10% fetal bovine serum. After 12 h, the media was replaced with fresh NIM. Media was then changed every other day until day 17, when the generated neuroepithelial (NE) cells were isolated. Mechanically-isolated NE cells were cultured in suspension with NIM (+B27, +cyclic AMP, +insulin-like growth factor 1 [IGF-1]) to generate neurospheres for at least 10 additional days. On about day 28, neurospheres were dissociated and plated onto polyornithine- and laminin-coated coverslips in neural differentiation media containing N2, B27, ascorbic acid, cyclic AMP, laminin, IGF-1, brain-derived neurotrophic factor, and glial-derived neurotrophic factor. Half of the media was changed every other day for 6-12 weeks, depending on the analysis to be performed. For treatment of cells with microtubule-targeting drugs, the media was replaced with standard neural differentiation media with either 10 nM vinblastine (Sigma-Aldrich) or 10 nM taxol dissolved in water.

4.6.5 Immunoblotting and immunocytochemistry

Confocal immunofluorescence microscopy and immunoblotting were performed as described previously [71, 128, 158]. Quantification of immunoblots was performed using ImageJ [155], normalized against actin as a loading control. The percentage of Tbr1+ cells among total cells (Hoechst) was determined by taking 3 randomly selected fields per coverslip and blindly counting cells using MetaMorph software [128]. Three coverslips were analyzed for
Antibodies used in this study included: mouse monoclonal IgM anti-Tra-1-60 (Santa Cruz, 1:50); goat polyclonal IgG anti-Nanog (R&D Systems, 1:500); mouse monoclonal IgG3 anti-SSEA-4 (Developmental Studies Hybridoma Bank, 1:100); mouse IgG2b monoclonal anti-acetylated tubulin (Sigma-Aldrich, 1:10,000); rabbit IgG anti-tau (Sigma-Aldrich, 1:100); rabbit polyclonal anti-Tbr1 (Proteintech, 1:1000); mouse monoclonal IgG βIII-tubulin (TuJ-1; Developmental Studies Hybridoma Bank, 1:100); rabbit polyclonal anti-REEP5 (Proteintech, 1:2000), rabbit polyclonal anti-atlastin-1, -2, and -3 [207], rabbit polyclonal anti-calreticulin (Abcam, 1:1000), mouse monoclonal Myc-epitope (Santa Cruz Biotechnology, 1:1000), mouse monoclonal anti-β-tubulin (Sigma-Aldrich, 1:2000), rabbit polyclonal anti-spastin (Sigma-Aldrich, 1:1,000). Rhodamine-phalloidin (100 nM) was from Cytoskeleton.

### 4.6.6 Axon length measurements

Axon outgrowth properties of neurons plated in small clusters were quantified with MetaMorph software using the Neurite Outgrowth application as described by the manufacturer. This program identifies cell bodies and their neurites and determines the length of each neurite. Three-to-six coverslips for each group, comprising at least 1100 cells per group, were analyzed for neurite outgrowth. Axonal outgrowth of singlized neurons was quantified using the NeuronJ plugin for ImageJ, and the length of the longest process which also had the greatest tau intensity was measured. At least 50 cells were quantified per cell line, for a total of 123 control and 175 SPG3A cells from at least 3 coverslips.

### 4.6.7 Live-cell imaging

Neurospheres were plated onto polyornithine- and laminin-coated 35 mm dishes (MatTek). At 8 weeks of total differentiation, the cells were stained with 50 nM MitoTracker Red CMXRos (Invitrogen) for 3 min to allow visualization of mitochondria, after which the media was
replaced with fresh neural differentiation media. Live-cell imaging was performed using a Zeiss Axiovert 200M microscope equipped with an incubation chamber. The cells were kept at 37°C with 5% CO₂ while imaging. Axons identified according to morphological criteria (constant thin diameter, long neurites, no branching and direct emergence from the cell body) were imaged every 5 s for 5 min, yielding 60 frames. Quantifications were performed using a protocol described previously [133]. In short, the location of each mitochondrion was manually selected using the Track Points function in MetaMorph, and parameters such as distance from cell body and velocity were recorded. A velocity threshold of 300 nm/s was used to select microtubule based transport events [157]. To determine the percentage of motile mitochondria, the total number of mitochondria that were present along the imaged neurite was counted, and those that changed position (velocity >300 nm/s) in at least 3 consecutive frames were considered motile.

4.6.8 Statistical analysis

The statistical significance in mean values among multiple sample groups was analyzed with Turkey’s studentized range test after ANOVA. Two-sided t-tests were used to examine the statistical significance between two sample groups. The significance level was defined as \( P<0.05 \), and significance tests were conducted using SAS 9.1 (SAS Institute).

4.7 Acknowledgements

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5. Chapter 5

Restoring mitochondrial morphology rescues axonal defects in HSP iPSC-derived neurons.

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Kyle Denton: Conception and design, data analysis and interpretation, manuscript writing  
Chong-Chong Xu: Collection and assembly of data  
Craig Blackstone: Provision of study material or patients, data analysis and interpretation  
Xue-Jun Li: Conception and design, financial support, manuscript writing, data analysis and interpretation, final approval of manuscript

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5.1 Abstract

Hereditary spastic paraplegias (HSPs) comprise a large and diverse group of inherited neurodegenerative disorders (SPG1-71). The hallmark of all HSP subtypes is a length-dependent distal axonopathy, resulting in prominent lower limb spasticity, which is primarily caused by the degeneration of cortical-spinal motor neurons. In addition, with many forms of HSP, patients have additional symptoms due the involvement of additional cell-types. Two autosomal recessive forms of HSP, SPG15 and SPG48, are associated with thinning of the corpus callosum, cognitive impairment, ataxia, and juvenile parkinsonism. Interestingly, these patients respond to L-Dopa treatment, suggesting dopaminergic neuron dysfunction. The causative gene for SPG15 is ZFYVE26, which encodes the ubiquitously expressed protein spastizin, with particularly high levels in neurons. In SPG48, the adaptor protein complex 5 zeta 1 (AP5Z1) protein, which was found to bind to spastizin, is lost. These two proteins have been shown to be involved in autophagy, cytokinesis, and potentially in axonal transport. However, the mechanisms by which the loss of these two proteins results in axonal defects are unknown.

In this study, we have generated and validated induced pluripotent stem cell (iPSC) lines from SPG15 and SPG48 patients along with unaffected siblings and other controls individuals. We then differentiated these iPSC lines to telencephalic glutamatergic and midbrain dopaminergic (mDA) neurons, both of which are expected to display disease-specific phenotypes. As a control, cells were also differentiated into spinal neurons, which are unaffected in patients. Control, SPG15, and SPG48 iPSC lines were successfully differentiated into both neuronal subtypes using well-established directed differentiation protocols. Interestingly, neurite number, length, and branching were significantly reduced in SPG15 and SPG48 telencephalic glutamatergic neurons and mDA neurons, suggesting that spastizin and AP5Z1 play a role in neurite development. Next, the morphology of mitochondria was analyzed, as spastizin has been shown to partially localize to the mitochondrial membrane. This revealed a significant reduction in the length and density of mitochondria within neurites of SPG15 and SPG48.
telencephalic glutamatergic neurons and mDA neurons. Mitochondrial membrane potential was reduced in SPG15 neurons, and there was an increase in apoptosis in both SPG15 and SPG48 cells. Treatment with the Drp1 inhibitor, mdivi-1, rescued mitochondrial morphology, neurite outgrowth, and levels of apoptosis in SPG15 neurons. These results link mitochondrial fission/fusion alterations to SPG15 and SPG48, and identify mitochondria as a potential target for therapeutics in the future.

5.2 Introduction

Hereditary spastic paraplegias (HSP) are a heterogeneous group of 71 genetic disorders that result in progressive lower limb spasticity due to a length-dependent degeneration of the corticospinal motor neuron (CSMN) axons [1, 18]. SPG11, SPG15, SPG48 comprise a subgroup of 3 complex recessive forms that have predominantly overlapping phenotypes, including thinning of the corpus callosum, cognitive impairments, and occasionally parkinsonism [228]. The onset of symptoms is mainly during the first two decades of a patient’s life [36]. Interestingly, the gene products affected in these three HSP subtypes can directly interact with each other [83]. SPG11 is the most common, and SPG15 is the second most common recessive HSP subtype associated with thin corpus callosum [229]. The causative gene for SPG15 is \textit{ZFYVE26}, which encodes the ubiquitously expressed ~280 kD protein spastizin, with particularly high levels in neurons [80, 230, 231]. Spastizin harbors a zinc-finger/FYVE domain that is thought to allow it to interact with phosphoinositide-3 phosphate (PI3P) [232]. Spastizin localizes to a number of regions within cells, including the cytoplasm, endoplasmic reticulum, microtubules and mitochondria [230]. The precise role of spastizin within cells, particularly neurons, remains unclear. Spastizin has been suggested to be involved in axonal outgrowth and targeting in zebrafish [91], autophagy [92], cytokinesis [95, 96], and potentially in axonal transport [81]. SPG48 is the result of mutations to the \textit{AP5Z1} gene, which encodes the adaptor for therapeutics in the future.
protein complex ζ subunit (AP5Z1) [84]. AP5Z1 is a subunit of the common heterotetrameric adaptor protein complex 5 (AP-5), an evolutionarily conserved complex thought to be involved in endosomal dynamics, although its precise localization and function are still unclear [85]. Adaptor proteins (APs) are a family of 5 related protein complexes, with AP-5 being the most recently discovered [86]. AP-1 and AP-2 are the most well-characterized, and have been shown to promote clathrin assembly in vitro by providing a bride between clathrin and membranes [87]. APs are comprised of 5 subunits, β1-5, one of either α, γ, δ, ε, or ζ, and μ1-5, and σ1-5 [85]. In addition to AP5Z1, mutations to the AP-4 subunit AP4E1 were shown in 2011 to result in an autosomal recessive form of HSP, SPG51 [88].

SPG15 and the related SPG11 can also present with juvenile onset parkinsonism, which shows significant improvement following dopaminergic therapy [62, 90]. This suggests that in addition to CSMNs, mDA neurons are sensitive to spastizin levels. To date, examination of mDA neurons lacking spastizin or AP5Z1 has not been reported.

Mitochondria are highly dynamic organelles that serve as the main source for ATP. Mitochondrial dysfunction has been implicated in a wide variety of developmental and degenerative neurological disorders. Neurons are highly dependent on mitochondria because of their limited glycolytic capacity [233]. While there are only two HSP genes that encode mitochondrial proteins, paraplegin and HSP60, there are several HSP subtypes associated with mitochondrial trafficking disturbances. In SPG4 and SPG3A, decreased fast axonal transport of mitochondria is observed [24, 30, 62, 68].

Mitochondrial shape and size are determined by a delicate balance between fusion and fission forces. Fusion proteins include mitofusin-1, mitofusin-2, and optic atrophy protein 1 (Opa1), while dynamin-related protein 1 (Drp1) is the main fission protein. The proper
maintenance of mitochondrial morphology is very important for neuronal health, as a variety of neurodegenerative diseases are caused by mutations in genes encoding fission/fusion proteins [234]. Although previously, spastizin has been shown to partially colocalize with mitochondria [82], the contribution of spastizin and AP5Z1 to mitochondrial health has not been examined.

Here, we sought to examine SPG15 and SPG48 using human pluripotent stem cells (hPSCs), including patient-specific induced pluripotent stem cells (iPSCs) and knockdown human embryonic stem cells (hESCs). These cells were differentiated into several neuronal subtypes so that effect of spastizin and AP5Z1 loss could be examined in human neurons with the patient’s genetic background. This revealed cell-type specific defects in neurite projection, apoptosis, mitochondrial morphology and membrane potential. Lastly, these cells were used to test therapeutic agents. The mitochondrial fission inhibitor mdivi-1 was effective at rescuing neurite outgrowth and apoptosis in telencephalic glutamatergic SPG15 neurons, suggesting mitochondrial dynamics may be a potential therapeutic target in the future.

5.3 Materials and methods

5.3.1 Clinical studies

All study procedures were performed under an Institutional Review Board-approved clinical research protocol (NINDS protocol 00-N-0043) at the National Institutes of Health Clinical Center. The SPG15 patient was a 26 year old woman who developed spastic paraparesis around the age of 20. In addition she has developed dysarthria, cognitive decline, fine action tremor, and she is no longer able to work. Magnetic resonance imaging (MRI) identified significant thinning of the corpus callosum.
5.3.2 Reprogramming fibroblasts into iPSC lines

Human fibroblast cell lines were established from skin punch biopsies and maintained using standard procedures. To generate iPSC lines using episomal transduction, ~200,000 cells were dissociated and transfected with episomal plasmids (Addgene) containing pluripotency factors (Oct3/4, Sox2, L-Myc, Klf4, and Lin 28), as reported previously (22). At around one week after electroporation transduction, cells were plated onto a 35-mm dish in DMEM supplemented with 10% fetal bovine serum. After culturing for 7 days, cells were dissociated and seeded onto a mouse embryonic fibroblast (MEF) feeder at ~10^5 cells/100-mm dish. Two weeks later, colonies with morphologies similar to human embryonic stem cells (ESCs) were observed. These colonies were split onto MEF feeder cells to derive iPSC lines. After several passages, homogenous colonies with ESC-like morphology were generated. The iPSC lines used in this study were iWT-1, iWT-2, iWT-Sib, iSPG15-1, iSPG15-2, iSPG48-1, and iSPG48-2.

5.3.3 Lentivirus production and transduction of hESCs.

To knock down spastizin and AP5Z1, shRNAs were cloned into pLVTHM. To produce high-titer lentivirus, 10 µg of pLVTHM-shRNA lentiviral transfer vector, 7.5 µg of lentiviral vector psPAX2, and 5 µg of pMD2.G (VSV-G envelope protein) were cotransfected to HEK293FT cells (Invitrogen) using the calcium phosphate method. Sixty hours after transfection, cell culture medium containing viral particles were collected and filtered through a 0.45-µm filter (Millipore). The viral particles were further concentrated by ultracentrifugation (SW 28 rotor, Beckman) at 50,000 g for 2 h. The pellet was resuspended in the hESC medium. For transduction of ESCs, hESCs were normally passaged and pelleted by brief centrifugation. Cell pellets were then incubated with 100 µl of concentrated virus (106 transducing units/ml) at 37°C for 30 min. The virus and cell mixture was then transferred to a MEF feeder layer overnight before changing
medium on the next day. G418 was used to select transfected cells. The knockdown hESC lines were expanded and then differentiated into telencephalic progenitors as described below.

5.3.4 Human iPSC forebrain neuron differentiation

To generate telencephalic neurons from iPSCs, stem cells were cultured on a feeder layer of irradiated MEFs in 6-well tissue culture-treated plates for around 6 days, with the human ESC media (+10 ng/ml fibroblast growth factor [FGF]-2) changed daily. When nearly confluent, cells were detached from the feeder layer to initiate neural differentiation, as previously described (23-25). Briefly, iPSC aggregates were cultured in suspension for 4 days in human ESC media and were then transferred to neural induction media (NIM). After 3 additional days in suspension, iPSC aggregates were plated onto 6-well tissue culture treated plates in NIM with 10% fetal bovine serum. After 12 h, the media was replaced with fresh NIM. Media was then changed every other day until day 17, when the generated neuroepithelial (NE) cells were isolated. Mechanically-isolated NE cells were cultured in suspension with NIM (+B27, +cyclic AMP, +insulin-like growth factor 1 [IGF-1]) to generate neurospheres for at least 10 additional days. On about day 28, neurospheres were dissociated and plated onto polyornithine- and laminin-coated coverslips in neural differentiation media containing N2, B27, ascorbic acid, cyclic AMP, laminin, IGF1, brain-derived neurotrophic factor, and glial-derived neurotrophic factor. Half of the media was changed every other day for 6 to 12 weeks, depending on the analysis to be performed. For treatment of cells with mdivi-1, the media was replaced with standard neural differentiation media with 10 µM mdivi-1 (Sigma-Aldrich) dissolved in DMSO.

5.3.5 Spinal neuron differentiation:

The procedure for generating neuroepithelial cells and spinal motor neurons from hESCs was essentially the same as described [110, 113]. Briefly, hESCs were induced to neural
lineage by forming ESC aggregations and then culturing in neural medium. The early NE cells were formed around 8-10 days after differentiation from hESCs, which exhibited columnar morphology and started to organize into a rosette-like structure. Human ESC-derived NE cells at day 10 were then treated with RA (0.1 μM) for caudalization in a neural medium, which consisted of DMEM/F-12 medium, N2 supplement, and heparin. One week later (day 17), the posteriorized NE cells were isolated. For specifying spinal motor neurons, these NE clusters were suspended in the same neural medium supplemented with B27, RA and purmorphamine. To generate forebrain neurons, the neuroepithelial cells were cultured in basic neural medium without any caudalizing factors (RA, basic fibroblast growth factor) [128]. These rostral NE cells were also isolated at day 17 and suspended in the same neural medium with B27. For terminal differentiation, the neural progenitor-enriched clusters were plated onto ornithine/laminin-coated coverslips in Neurobasal medium (Invitrogen) supplemented with N2 and B27.

5.3.6 mDA neuron differentiation:
mDA neurons were generated following a previously described protocol [160]. In short, hPSCs were treated using a similar protocol as the telencephalic glutamatergic neuron differentiation, with the addition of several morphogens. From days 10-21, 3μM CHIR99021 was included in the NIM. From days 12-23, 2μM purmorphamine was added to the NIM. From days 21-35, 200ng/ml FGF8 was included in the NIM and NDM. Lastly, starting on day 23 10ng/ml sonic hedgehog (SHH) was included in the NIM and NDM.

5.3.7 Immunoblotting and immunocytochemistry
Confocal immunofluorescence microscopy and immunoblotting were performed as described previously [71, 128, 158]. Quantification of immunoblots was performed using ImageJ [155], normalized against actin as a loading control. The percentage of TBR1+ cells among total cells (Hoechst) was determined by taking 3 randomly selected fields per coverslip.
and blindly counted using MetaMorph software as we described before [128]. Three coverslips were analyzed for each group.

Antibodies used in this study included: mouse monoclonal IgM anti-Tra-1-60 (Santa Cruz, 1:50); goat polyclonal IgG anti-Nanog (R&D Systems, 1:500); mouse monoclonal IgG3 anti-SSEA-4 (Developmental Studies Hybridoma Bank, 1:100); mouse IgG2b monoclonal anti-acetylated tubulin (Sigma-Aldrich, 1:10,000); rabbit IgG anti-tau (Sigma-Aldrich, 1:100); rabbit polyclonal anti-Tbr1 (Proteintech, 1:1000); mouse monoclonal IgG βIII-tubulin (TuJ-1; Developmental Studies Hybridoma Bank, 1:100); mouse monoclonal anti-β-tubulin (Sigma-Aldrich, 1:2000) rabbit monoclonal anti-tyrosine hydroxylase (Pel-Freez, 1:400), rabbit monoclonal anti-Hoxb4 (Abcam, 1:400).

5.3.8 Neurite outgrowth measurements

Axonal outgrowth was quantified using the NeuronJ plugin for ImageJ. Here the length of the longest process which also had the greatest tau intensity was measured. A minimum of 50 cells were quantified per line, from at least 3 coverslips.

5.3.9 Live-cell imaging

Neurospheres were plated onto polyornithine and laminin coated 35 mm dishes (MatTek). At 8 weeks of total differentiation, the cells were stained with 50 nM MitoTracker Red CMXRos (Invitrogen) for 3 min to allow visualization of mitochondria, after which the media was replaced with fresh neural differentiation media. Live-cell imaging was performed using a Zeiss Axiovert 200M microscope equipped with an incubation chamber. The cells were kept at 37°C with 5% CO₂ while imaging. Axons identified according to morphological criteria (constant thin diameter, long neurites, no branching and direct emergence from the cell body) were imaged every 5 s for 5 min, yielding 60 frames. Quantifications were performed using a protocol described previously (28). In short, the location of each mitochondrion was manually selected.
using the Track Points function in MetaMorph, and parameters such as distance from cell body and velocity were recorded. A velocity threshold of 300 nm/s was used to select microtubule based transport events (29). To determine the percentage of motile mitochondria, the total number of mitochondria that were present along the imaged neurite was counted, and those that changed position (velocity >300 nm/s) in at least 3 consecutive frames were considered motile.

5.3.10 Mitochondrial morphology

Neuronal cultures on glass-bottom 35mm dishes were stained with 25nM MitoTracker CMXRos in neural differentiation media for 2 minutes at 37°C. The cells were washed twice with fresh NDM, and 2 ml NDM were added to the dish. Live-cell imaging was performed using a Zeiss Axiovert 200M microscope equipped with an incubation chamber. The cells were kept at 37°C with 5% CO₂ while imaging. Axons identified according to morphological criteria (constant thin diameter, long neurites, no branching and direct emergence from the cell body) were imaged using a Plan-Apochromat 63x/1.40 Oil DIC objective, with identical microscope settings for each group. The 16-bit greyscale images were thresholded in ImageJ and the particle analysis function was used to measure morphological characteristics.

5.3.11 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured based on a previous protocol [235]. Neurons were plated on 35mm glass-bottomed dishes. The fluorescent dye tetramethylrhodamine methyl ester (TMRM, Invitrogen) was used because it accumulates in mitochondria based on Δψₘ. Cells were washed three times with 5mM K⁺, 2mM Ca²⁺ Tyrodes solution, then incubated with 10nM TMRM in 2 ml Tyrodes solution for 45 min at room temperature in the dark. Live-cell imaging was performed using a Zeiss Axiovert 200M microscope equipped with an incubation chamber, using an EC Plan-Neofluar 40x/1.30 Oil DIC
objective. The cells were kept at 37°C with 5% CO₂ while imaging. Microscope settings were optimized using control cells, and these settings were used for all other groups. Randomly selected fields were imaged every 20 s for a total of 600 s. The mitochondrial uncoupler FCCP was added to the media after 300 s so that the final concentration was 1µM. The time-series were analyzed in MetaMorph and at least 20 regions of interest (ROIs) were traced around mitochondrial structures for each cell, along with adjacent background regions. The pixel intensity for each region was determined followed by background subtraction. Change in fluorescence was calculated with the formula ΔF=F-F₀/F₀x100, where F is the fluorescence intensity at any time point, and F₀ is the initial fluorescence.

5.3.12 Caspase 3/7 activity

For measurements of the activities of caspases 3 and 7, the Caspase-Glo 3/7 Assay (Promega) was carried out according to the manufacturer's instructions. Briefly, motor neuron progenitor-enriched cultures were dissociated with Accutase (Invitrogen) and seeded into 96-well plates at 5 000 cells/well in 50 µl of caspase-3/7 reagent. After incubation for 1 h at room temperature, luminescence from each well was then measured using Wallac Victor2 1420 MultiLabel Counter.

5.3.13 Statistical analysis

The statistical significance in mean values among multiple sample groups was analyzed with Turkey’s studentized range test after ANOVA. Two-sided 𝑡-tests were used to examine the statistical significance between two sample groups. The significance level was defined as P<0.05, and significance tests were conducted using SAS 9.1 (SAS Institute).
5.4 Results

5.4.1 Generation and characterization of SPG15 and SPG48 iPSC lines

Dermal fibroblasts (Supp. Fig. 5-1A) were transfected with episomal vectors containing Oct3/4, Klf4, c-Myc, Lin28, and Nanog [156]. The emergent iPSC colonies were expanded for each group and the best clones were used for further analysis. The presence of mutations in the fyve-cent gene was confirmed by Sanger sequences in the iSPG15-1 lines (Supp. Fig. 5-2B). These mutations were not present in an unaffected sibling of the SPG15 patient, providing a control line that shares some genetic background. A homozygous premature stop codon in exon 15 of the AP5Z1 gene was confirmed in the iSPG48-1 iPSC line (Supp. Fig. 5-1C). The generated SPG15 and SPG48 iPSC lines had typical hESC-like colony morphology (Supp. Fig. 5-1D). In addition, the SPG15 and SPG48 iPSCs expressed typical pluripotent stem cell markers, including Nanog, SSEA-4, and Tra-1-60 (Supp. Fig. 5-1E). The fibroblasts from the SPG15 patient and unaffected sibling were previously examined, and were found to lack spastizin expression at the mRNA and protein level [94]. A significant reduction in fyve-cent and AP5Z1 mRNA transcript was observed in SPG15 and SPG48 iPSC-derived neurons (Supp. Fig. 5-2A&B).

5.4.2 Neurite outgrowth defects in SPG15 and SPG48 iPSC-derived neurons

In order to study the effects of lost spastizin and AP5Z1 expression in cell types that are clinically relevant, the iPSC lines were differentiated to telencephalic glutamatergic neurons [128, 172, 173], spinal neurons [110, 127], and midbrain dopaminergic (mDA) neurons [160] using well-established protocols. The differentiation efficiency was determined by examining the percentage of cells expressing markers for telencephalic glutamatergic neurons (Tbr1^+), spinal neurons (Hoxb4^+), and mDA neurons (tyrosine hydroxylase, TH^+). No significant difference between groups in generating these three neuronal subtypes (Fig. 5-1A-D). This data suggests that the absence of spastizin and AP5Z1 does not affect neuronal specification. The ability of
Figure 5-1: SPG15 and SPG48 iPSCs exhibit normal neuronal differentiation. (A)

Immunofluorescence images of week 6 telencephalic, spinal, and mDA neurons. Cells were stained for acetylated tubulin to label neurites, along with the telencephalic glutamatergic marker Tbr1, spinal neuron marker Hoxb4, or the dopaminergic marker tyrosine hydroxylase (TH). Scale bar: 20µm. (B) Quantification of the percentage of Tbr1+ neurons. (C) Quantification of Hoxb4+ spinal neurons. (D) Quantification of TH+ dopaminergic neurons. Data presented as mean ± SEM.
Figure 5-2: SPG15 and SPG48 neurons display altered neurite outgrowth. (A)

Quantification of week 6 neurite outgrowth parameters, including average axon length (longest neurite), average length of all primary neurites, and average number of primary neurites in Tbr1⁺ neurons 48 hr after plating. (B) Hoxb4⁺ neurite outgrowth quantification. (C) TH⁺ neurite outgrowth quantification. All data presented as mean ± SEM. *P < 0.05 compared to iWT-1. #P < 0.05 compared to iWT-sibling cells.
these neurons to project neurites was then examined and compared between neuronal subtype (Fig. 5-2A-C). Interestingly, the length of the longest neurite, which generally develops into the axon, was significantly reduced in the SPG15 and SPG48 Tbr1+ and TH+ neurons, but not the Hoxb4+ neurons. The average length of primary neurites was also reduced in Tbr1+ SPG15 and SPG48 neurons, and in TH+ SPG48 neurons. Lastly, the number of primary neurites was reduced in SPG15 and SPG48 Tbr1+ neurons. Analysis of neurite outgrowth was repeated on additional clones, and again, SPG15 and SPG48 neurons had reduced outgrowth in telencephalic glutamatergic neuron cultures (Supp. Fig. 5-3). This data suggests that neurite outgrowth in telencephalic neurons and mDA neurons is sensitive to the levels of spastizin and AP5Z1, while spinal neurons are not.

A hallmark pathological change found in various HSP subtypes is the presence of enlarged, swollen axons [30, 68, 236]. To examine if swellings are present in week 6 SPG15 and SPG48 iPSC-derived telencephalic glutamatergic neurons were stained for acetylated tubulin. While there were almost no swellings in control lines, there was a significant increase in the SPG15 and SPG48 neurons (Fig. 5-3A&B).

5.4.3 Loss of Spastizin and AP5Z1 alters mitochondrial morphology and function

Previously it has been shown that spastizin partially localizes to mitochondria [82], therefore we examined mitochondrial morphology in the two affected neuronal subtypes. Telencephalic glutamatergic and mDA neuron cultures were stained with MitoTracker CMXRos, and live-cell images were taken of primary neurites. This revealed a significant reduction in mitochondrial length in SPG15 and SPG48 telencephalic glutamatergic neurons (Fig. 5-4A&B). There was also a reduction in aspect ratio (length/width) in the SPG15 neurons (Fig. 5-4C). In addition to the size change, there was a significant reduction in the number of mitochondria per 1µm neurite for the SPG48 neurons (Fig. 5-4D), and a reduction in linear density for both groups (Fig. 5-4E). Neurons in the mDA cultures also showed changes to mitochondrial
Figure 5-3: Increased neurite swellings within SPG15 and SPG48 telencephalic neurons.

(A) Representative images of telencephalic neurons with axonal swellings labeled with arrowheads. Scale bar: 20µm. (B) Quantification revealed a significant increase in axonal swellings in patient-derived neurons compared to control neurons. *P< 0.05 compared to iWT-1. #P< 0.05 compared to iWT-sibling cells.
Figure 5-4: SPG15 and SPG48 neurons have altered mitochondrial morphology. (A)

Immunofluorescence images of mitochondria within neurites taken in live, week 10 telencephalic glutamatergic neurons that were stained with MitoTracker CMXRos. Scale bar: 20µm. (B)

Average mitochondrial length, calculated by measuring the longest axis of each mitochondrion. (C) Average aspect ratio, calculated by dividing the major axis by the minor axis for each mitochondrion. (D) Average number of mitochondria per 1µm neurite. (E) Average linear mitochondrial density, calculated by dividing the total mitochondrial area by the neurite length. (F) Mitochondrial staining in week 10 mDA neuron cultures. (G) Mitochondrial length, (H) mitochondrial aspect ratio, (I) mitochondria per 1µm neurite, and (J) linear mitochondrial density. *P<0.05, **P<0.01, ***P<0.001 versus iWT-1.
morphology. While the mitochondrial length was only altered in the SPG15 neurons (Fig. 5-4G), the number of number and density of mitochondria along neurites was significantly reduced in both SPG15 and SPG48 (Fig. 5-4I&J). There was also an increase in mitochondrial size within the cell bodies of SPG15 and SPG48 mDA neurons (Supp. Fig. 5). This resembles findings in PINK1−/− mouse fibroblasts, cortical neurons, and ES cells were found to have enlarged mitochondrial size via EM analysis [237]. These results suggest that spastizin and AP5Z1 play a role in mitochondrial shape and distribution in neurons.

Next, we sought to examine whether mitochondrial health was affected in SPG15 neurons. The cells were incubated with the fluorescent dye TMRM, which binds to mitochondria based on the membrane potential (Δψm) [238]. This revealed a significant reduction in TMRM fluorescence in SPG15 telencephalic glutamatergic neurons (Fig. 5-5A&B). The TMRM signal was dependent on Δψm, as the uncoupler FCCP reduced TMRM fluorescence, and SPG15 neurons had a significantly greater decrease (Fig. 5-5C). This data reveals that the loss of spastizin has a negative effect on mitochondrial membrane potential.

Considering that alterations in mitochondrial membrane potential can result in the release of cytochrome C and apoptosis [239], we then examined apoptosis in telencephalic and mDA neurons using caspase 3/7 activity as a readout. Week 10 SPG48 telencephalic neurons and SPG15 mDA neurons had significantly increased caspase 3/7 activity (Fig. 5-6A&B).

5.4.4 Inhibition of mitochondrial fission partially rescues SPG15 telencephalic neuron defects.

The reduced size of mitochondria and increased apoptosis in SPG15 and SPG48 neurons suggest that these neurons may have increased mitochondrial fission. To confirm that increased mitochondrial fission is involved in the pathogenesis of SPG15 and SPG48, we tested
Figure 5-5: Reduced mitochondrial membrane potential in SPG15 neurons. (A) Representative images of control and SPG15 neurons stained with TMRM. (B) Quantification of average pixel fluorescence intensity of TMRM before and after FCCP treatment. (C) Change in TMRM fluorescence intensity shown by ΔF which represents the levels of mitochondrial membrane potential. FCCP was added at 300 sec to decrease mitochondrial membrane potential. Data represents mean ± SEM from at least 50 cells from 3 independent experiments. **P<0.01.
Figure 5-6: SPG15 and SPG48 neurons have increased caspase 3/7 activity. (A) Caspase 3/7 activity in week 10 telencephalic neuron cultures. (B) Caspase 3/7 activity in week 10 mDA neuron cultures. Data were presented as mean ± SEM, n=6. **P<0.01.
the effect of mdivi-1 (mitochondrial division inhibitor 1) in SPG15 and SPG48 neurons. This drug is an inhibitor of the large GTPase dynamin-related protein 1 (Drp1), which mediates mitochondrial fission [240]. The concentration of 10µM mdivi-1 was chosen as it was previously reported to be effective in rescuing mitochondrial defects in PINK1 mutant dopaminergic neurons without inducing donut-shaped mitochondria [241]. Telencephalic glutamatergic neurons were plated onto coverslips and 24 hr later, were treated with 10µM mdivi-1 or vehicle for 48 hr. Next, the mitochondrial morphology and neurite outgrowth was examined. Treatment with mdivi-1 significantly increased the number of mitochondria per 1µm in neurites for both SPG15 and SPG48 neurons, while there was no effect on control neurons (Fig. 5-7A&B). While mdivi-1 reduced the linear density of mitochondria in control neurons, it significantly increased linear density in SPG15 and SPG48 neurons (Fig. 5-7C). The average mitochondrial length was also rescued in SPG15 neurons, with a trend of improvement in SPG48 neurons (Fig. 5-7D). Lastly, the aspect ratio was also increased in SPG15 neurons, and there was a non-significant trend towards an increase in SPG48 neurons (Fig. 5-7E). These results suggest that mdivi-1 may improve the health of mitochondria, increasing their transport into neurites, since transport is reduced when mitochondria become damaged [146].

When neurite outgrowth was analyzed following 48 hr mdivi-1 treatment, there was a significant increase in axon and primary neurite length in SPG15 neurons (Fig. 5-8A-C). In contrast, control neurons had a significant reduction in axon length and a non-significant trend towards reduced primary neurite length. The total length of neurites per cell was unchanged in control neurons, but was significantly increased in the SPG15 telencephalic glutamatergic neurons (Fig. 5-8D). Lastly, when we looked at the effect of mdivi-1 on caspase 3/7 activity, there was no effect on control neurons, but a significant reduction in SPG15 neurons (Fig. 5-8E). These findings suggest that restoring mitochondrial normal morphology can rescue the disease-specific phenotypes in SPG15 and SPG48 neurons.
Figure 5-7: Inhibition of mitochondrial fission rescues mitochondrial morphology. (A)
Representative image of axonal mitochondria stained with MitoTracker CMXRos treated with vehicle or 10µM Mdivi-1 for 48 hr. (B) Quantification of the number of mitochondria per 1µm axon. (C) Linear mitochondrial density. (C) Average mitochondrial length along axons. (D) Average mitochondrial aspect ratio. Data represented as mean ± SEM from at least 20 axon segments from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001 versus iWT-Sib DMSO treated. #P<0.05, ##P<0.01, ###P<0.001 versus iWT-Sib Mdivi-1 treated.
Figure 5-8: Inhibition of mitochondrial fusion improves neurite outgrowth in SPG15 forebrain cells. (A) Representative images of telencephalic neurons following 48 hr treatment with vehicle or 10µM Mdivi-1. Scale bar: 20µM. (B) Average length of the longest neurite. (C) Average length of primary neurites per cell. (D) Total length of all neurites per cell. Data presented as mean ± SEM from at least 30 cells from 3 independent experiments. (E) Caspase 3/7 activity following 72 hr treatment with vehicle or Mdivi-1. *P<0.05, ***P<0.001 versus iWT-1 DMSO treated. ##P<0.01 versus iWT-Sib Mdivi-1 treated.
5.5 Discussion

Here, we successfully established human neuronal models for SPG15 and SPG48 by generating patient-specific iPSCs and differentiated these stem cells into different neuronal subtypes. Our results showed that the lack of spastizin or AP5Z1 results in cell-type specific defects in telencephalic and mDA neurons, but not spinal neurons. Our data also implicates dysfunctional mitochondria in SPG15 and SPG48. There was a significant reduction in mitochondrial length and density within neurites, and SPG15 neurons had reduced $\Delta \psi_m$. In addition, there was increased caspase 3/7 activity in SPG15 and SPG48 neurons in long-term cultures. Treatment with the Drp1 inhibitor mdivi-1 rescued mitochondrial morphology defects, and partially rescued neurite outgrowth defects in SPG15 cells. Mdivi-1 also reduced caspase 3/7 activity, suggesting that targeting mitochondria may be beneficial in the future.

5.5.1 Proper neurite outgrowth requires spastizin and AP5Z1:

Both SPG15 and SPG48 telencephalic neurons had a dramatic reduction in axon length, primary neurite length, and number of primary neurites, while there was a more dramatic reduction in neurite outgrowth parameters for SPG48 mDA neurons. This matches the clinical findings from the SPG48 patient, who has parkinsonism, while the SPG15 patient does not. Early neurite outgrowth defects appears to be a common finding among several HSP subtypes, including SPG3A [24], SPG4 [62], and SPG11 [38]. The previous finding that both patient-specific SPG11 iPSC-derived cortical neurons, and spatacsin knockdown neurons have reduced axonal outgrowth in vitro, is interesting, as this HSP subtype is nearly clinically identical to SPG15 and SPG48. The thin corpus callosum that is often associated with SPG15 and SPG48 is thought to be a developmental defect [14], which may arise from the reduced ability of corticofugal axons to reach their target, as shown in our in vitro results.
5.5.2 Abnormal mitochondrial dynamics in SPG15 and SPG48 neurons:

Spastizin was previously shown to partially colocalize with mitochondria [82]. We found that SPG15 and SPG48 cells had smaller, fragmented mitochondria with lower density along neurites. This suggests that spastizin levels may affect mitochondrial fission/fusion balance. While it is possible that spastizin does so directly, it is most likely a result of the reduced autophagic flux that occurs when spastizin levels are reduced [93, 97, 242]. It was recently shown that spastizin is important for autophagic lysosome reformation, a process that generates new free lysosomes [93]. Perhaps a deficiency in free lysosomes that occurs when spastizin is reduced, results in the accumulation of autophagic material, including damaged mitochondria. We observed a decrease in mitochondrial $\Delta \psi_{m}$ and increased mitochondrial fragmentation, which resembles findings from familial forms of Parkinson’s disease, where PTEN-induced kinase 1 (PINK1) or parkin are mutated [241]. The two Parkinson’s disease associated proteins, PINK1 and parkin are directly involved in mitophagy. Parkin is targeted to damaged mitochondria, such as those with low membrane potential, in a PINK-1 dependent manner [243]. Parkin then ubiquitinates mitochondrial proteins, allowing adaptor proteins, such as p62 to bind and trigger phagophore formation, leading to degradation [244]. The fragmented mitochondria observed in SPG15 and SPG48 neurons may be awaiting degradation via mitophagy, as mitochondrial fission has been shown to precede mitophagy [245, 246]. In the future, it will be interesting to further investigate the connection between spastizin, PINK1, and parkin.

5.5.3 Mdivi-1 rescues disease-related phenotypes in SPG15 neurons:

The finding that the Drp1 inhibitor, mdivi-1, can partially rescue SPG15 telencephalic neurons suggests that mitochondrial fission is involved in the pathogenesis of SPG15. Our results are similar to a study where mitochondrial defects in mutant PINK1 N27 neuronal cells could be rescued by the application of 10µM mdivi-1 [241]. Mdivi-1 does not affect mitochondrial fusion, therefore the inhibition of fission would favor increased mitochondrial fusion, which we
can detect by the presence of longer mitochondria along neurites. Regulation of mitochondrial fission and fusion is important during apoptosis [247]. Previous work has shown that mitochondrial fusion is anti-apoptotic [248-250], while fission can be pro-apoptotic [240]. During apoptosis, Drp1 accumulates on mitochondria and increases the rate of division and fragmentation [240]. Inhibition of Drp1 with mdivi-1 can reduce cytochrome-c release following apoptosis stimulation. Our results showed that mdivi-1 can rescue neurite outgrowth deficits and reduce subsequent caspase-3/7 activity in SPG15 telencephalic neurons. In the future it will be interesting to determine the detailed mechanisms by which mdivi-1 improves neurite outgrowth and rescues the axonal degeneration.

In summary, we have developed a novel model of SPG15 and SPG48 using hPSCs. This model was successful in identifying disease-specific phenotypes that may prove useful for drug-screening studies in the future. In addition, abnormalities to mitochondria are a common observation in SPG15 and SPG48. These phenotypes were partially rescued through the inhibition of Drp1, suggesting that targeting mitochondrial dynamics may be a potential therapeutic avenue in the future.
5.6 Supplemental Figures

Supplemental Figure 5-1: Generation and characterization of SPG15 and SPG48 iPSC lines. (A) Representative phase contrast images of SPG15 and unaffected sibling (iWT-1) iPSC lines. Scale bar: 200µm. (B) Sanger sequencing identified compound heterozygous mutations in the *fyve-cent* gene in the SPG15 patient line. One mutation is located at the splice acceptor site of intron 14 (c.2554-1 G>A) resulting in the skipping of exon 15, and the other is a nonsense mutation in exon 19 (c.3417_3418insTA; p. Lys1140X) which results in the truncation of the protein. (C) Sequencing of the SPG48 iPSCs revealed a homozygous C>T nucleotide substitution (c.1732 C>T) resulting in a premature stop codon in exon 15 of the *AP5Z1* gene. (D) SPG15 and SPG48-derived iPSC colonies display typical ESC-like morphology. Scale bar: 100µm. (E) All examined lines stained positive for the pluripotency markers Nanog, SSEA-4, and Tra-1-60. Scale bar: 100µm.
Supplemental Figure 5-2: Reduced spastizin and AP5Z1 expression in SPG15 and SPG48 forebrain neurons respectively. (A) qRT-PCR analysis of spastizin expression in week 15 telencephalic glutamatergic neuron cultures. (B) AP5Z1 expression in control and iSPG15 week 15 telencephalic glutamatergic neuron cultures. Data were presented as mean ± SD, n=6.

*P<0.05
Supplementary Figure 5-3: Reduced telencephalic glutamatergic neuron neurite outgrowth from additional clones. (A) Immunofluorescence images of day 33 forebrain neurons taken 72 hr after plating. (B) Quantification of the longest neurite. (C) Mean length of primary neurites per cell. (D) Number of primary neurite per cell. (D) Number of branches per cell. Data presented as mean ± SEM. *P<0.05, **P<0.01.
Supplemental Figure 5-4: Increased mitochondrial size within cell bodies from mDA enriched cultures. (A) Representative TEM images of neurons within mDA cultures. (B) Quantification of the mitochondrial area within mDA cultures. Data represented as mean ± SEM from \( n \geq 20 \) cells. *\( P<0.05 \), **\( P<0.01 \).
Here, we have shown that multiple forms of HSP can be studied using patient iPSC-derived neurons. Many phenotypes observed in adult patients were recapitulated in iPSC-derived neurons, suggesting that cellular abnormalities may be present prior to clinical diagnosis. We observed length-dependent axonal swellings in SPG4 neurons, and neurite outgrowth abnormalities in SPG3A neurons (Fig. 6-1). These phenotypes were rescued following treatment with the microtubule-targeting drug vinblastine, linking alterations to microtubule dynamics in these forms of autosomal dominant HSP. In addition, the role of spastin in axonal transport was confirmed in human neurons, matching previous findings in mice. A novel role of atlastin-1 in fast axonal transport of mitochondria was identified, suggesting a common deficit in SPG3A and SPG4.

We next investigated SPG15 and SPG48, two autosomal recessive forms of HSP associated with juvenile parkinsonism. This revealed cell-type specific deficits in telencephalic glutamatergic and mDA neurons, two neuronal subtypes affected in patients (Fig. 6-2). Importantly, spinal neurons, which are unaffected in patients, showed no abnormalities. Our analysis also revealed a previously unrecognized role of mitochondria in SPG15 and SPG48. Cells lacking spastizin or AP5Z1 had reduced mitochondrial size and decreased density within neurites. In addition, mitochondria were less polarized, showing reduced Δψm. Treatment with the Drp1 inhibitor, mdivi-1, rescued mitochondrial morphology abnormalities in SPG15 neurons. Interestingly, mdivi-1 partially rescued neurite outgrowth defects in SPG15 cells, suggesting that an imbalance of mitochondrial fission/fusion may result in pathogenic changes.
Figure 6-1: Summary of major phenotypes observed in SPG4 and SPG3A iPSC-derived neurons. The phenotypes that were rescued by vinblastine are also shown.
Figure 6-2: Summary of major phenotypes observed in SPG4 and SPG3A iPSC-derived neurons. The phenotypes rescued by inhibition of mitochondrial fission are also shown.
6.1 Establishing models of HSP

One of the factors that have limited HSP research is the generation of animal models that accurately recapitulate disease phenotypes. To date, out of the 71 HSP-linked loci, only 13 have been studied in zebrafish or *drosophila*, while 12 have been examined in mice. This is due to both fact that the speed with which new HSP gene are identified has outpaced researchers ability to generate animal models and differences in expression of HSP genes between human and animal models [18]. The ability to examine the role of HSP genes in human neurons with stem cell technology allows researchers to overcome the lack of animal models. The availability of stem cell models for every HSP subtype is available should be a reality in the near future. This would greatly enhance the understanding of the pathways necessary for maintenance of long axons. One of the roadblocks to this is the limited availability of HSP patient fibroblasts, making large cohorts of cell lines difficult to employ. The best way to get around this limitation would be to use newer gene targeting techniques, such as the TALEN [251] or CRISPR/Cas systems [252], to introduce mutations identified in patients to every HSP gene in a control cell line, such as H9 hESCs. If all of the lines were generated from the same original control line, they would be in effect isogenic, dramatically reducing the potential variability between control and mutant lines that occurs when comparing lines from individuals with different genetic backgrounds. Stem cell based models also allow the identification of events that precede disease onset, since early born neurons are examined [253]. This is particularly valuable when attempting to identify therapeutic compounds that can prevent disease progression. If a pathogenic event can be detected prior to neurodegeneration, studies can be designed to test for the inhibition of that event. Therapies that prevent disease onset and progression will be more and more valuable as genetic sequencing becomes cheaper and more widespread. Children who are at risk of inheriting HSP can be sequenced at an early age, allowing preventative measures to be taken.
It would also be informative to transplant HSP iPSC-derived neurons into animals to test whether or not the phenotypes observed in vitro are seen in vivo. The conditions that cells are exposed to in vitro are much harsher than those in vivo, resulting in increased oxidative stress, potentially leading to misleading phenotypes [254]. Transplantation of cells would also allow testing therapeutic agents effect in a more physiologically relevant environment. This would also allow neurons to be examined after longer periods of time in vivo than would be possible in vitro. With current culturing techniques stem cell-derived neurons do not survive in vitro indefinitely, but transplanted neurons have been shown to survive at least 2 years in vivo [255].

6.2 Overcoming cellular heterogeneity

One of the difficulties when working with stem cell-derived neural cultures is the heterogeneity of cells generated from directed differentiation protocols. Following neural differentiation, many studies will use immunohistochemistry or transcriptional profiling to determine whether the cell-type of interest is present in the culture, and to what extent. However, often studies fail to fully characterize the other cell-types that are present in the culture, providing a potential significant source of variation, as a number of neurodegenerative diseases result from non-cell autonomous mechanisms. If a culture only contains a small percentage of neurons of interest, this could make it difficult to observe subtle phenotypes, particularly if the other cells in the culture are unaffected in the disease, or perhaps the unaffected cells may support the affected cells by providing metabolic or intracellular signals. One way to get around this is to use a purification technique, such as fluorescence-activated cell sorting (FACS), to obtain pure populations of the neuronal subtype of interest. This however requires a specific surface marker for the particular neuronal subtype, which is not currently available for all clinically relevant cell populations, such as CSMNs. This strategy has worked well for studies examining diseases affecting spinal motor neurons [256] or mDA neurons [257], as specific markers are available to purify these cells. In the case of HSP, using generic
telencephalic glutamatergic neurons is appropriate for some autosomal recessive subtypes, such as SPG15 and SPG48, where widespread abnormalities are seen in neurons whose axons pass the corpus callosum. For autosomal pure HSP subtypes, such as SPG3A and SPG4, the ability to purify neurons with transcriptional or proteomic profiles that match deep layer neurons, particularly CSMNs, will enhance our ability to identify why these neurons are specifically susceptible to degeneration.

6.3 Examining the physiological role of HSP proteins

To date, almost no work has been done to examine the physiological importance of HSP proteins in neurons. The majority of studies have looked at morphological defects of cells when levels of HSP proteins are perturbed. This lack of information needs to be resolved, as a number of HSP proteins are involved in pathways that have direct effect on synaptic transmission and electrophysiology.

In addition, mitochondria play a crucial role to presynaptic function, as these regions rely on high levels of ATP to maintain ionic gradients and membrane potential, and to reload synaptic vesicles [258, 259]. Mitochondria within presynaptic terminals also serve to sequester cytosolic Ca$^{2+}$ during neurotransmission [260]. The involvement of mitochondrial health and transport in multiple forms of HSP suggest it would be interesting to examine whether electrophysiological properties are altered in these HSP neurons.

6.4 Identifying mechanism for cell-type specific susceptibility in HSP.

As mentioned earlier, understanding why particular subpopulations of neuron degenerate when ubiquitously expressed proteins are lost, such as spastin or atlastin-1, is very important. We have attempted to use our iPSC-based SPG4 and SPG3A models to shed light on this question. We previously observed that SPG4 and SPG3A telencephalic glutamatergic
neuron enriched cultures, but not spinal neuron cultures, had increased axonal swellings or decreased neurite outgrowth (Fig. 6-3). We chose to use RNA-sequencing analysis to identify genes and pathways that were specifically altered in forebrain HSP neurons. RNA was collected from week 7 wild-type, SPG4, and SPG3A forebrain and spinal neuron cultures, and libraries were prepared using Illumina’s specifications for polyA-plus stranded reactions. Samples were analyzed using an Illumina HiSeq 2000 sequencer. With the assistance of Dr. Michael Duff, the reads were mapped to the human hg19 genome using TopHat2 [261] and Bowtie2 [262].

Expression quantification in fragments per kilobase gene model per million base pairs (FPKM) was performed using Cuffdiff [263]. A threshold of genes with a minimum FPKM>1 and a maximum FPKM>10 and absolute value of log-fold change is > 1 (so gene fpkm either increased or reduced by a factor of at least 2) were chosen for further analysis. The number of genes that differed between control and SPG4 or SPG3A are shown in Fig. 6-4. In order to exclude changes that also occurred in spinal neurons, those genes were removed from the list. This resulted in 956 genes that were reduced specifically in SPG3A forebrain cells, and 227 genes that were increased. For the SPG4 cells, 1643 genes were specifically reduced, and 475 genes were increased. Because SPG3A and SPG4 are related, we chose to examine the commonly altered genes between these two groups. There were 695 genes that were decreased in both SPG3A and SPG4 forebrain cells compared to controls, and only 73 that were increased.

Next, the list of commonly altered genes was examined using gene ontology (GO) analysis using DAVID (http://david.abcc.ncifcrf.gov/). This revealed a number of GO terms that were enriched in these lists (Table 6-1). A number of the GO terms that were enriched in the gene lists make sense based on the roles of spastin and atlastin-1. The GO term that was most enriched in the genes that were downregulated in SPG3A and SPG4 telencephalic glutamatergic neurons was “regulation of cell morphogenesis.” In the SPG3A list of genes, there
were enriched GO terms related to cellular projections and endocytosis. The genes that were decreased in SPG4 were enriched for GO terms related to cytoskeleton and lipid regulation. Our next step is to validate expression changes using qPCR for a subset of the clinically relevant genes that were altered in SPG3A and SPG4. We have chosen to focus on genes involved in ER stress, as it has been found to be an early pathogenic change in several neurodegenerative disorders [256, 264].

6.5 Implications for other neurodegenerative disorders

One of the aspects that makes studying HSP relevant to neurodegenerative disorders as a whole is the extensive genetic characterization of the different HSP subtypes (Table 1-1). The fact that mutations to 71 genes and counting all lead to the common phenotype of degeneration of the longest axons can provide insight into the pathways that are needed for axonal maintenance in general and in other neurodegenerative disorders. An understanding of the genes and pathways that result in HSP is also beneficial to understanding the mechanisms of other neurodegenerative disorders, as it seems likely that there are pathways affected in multiple diseases, such as axonal transport (Parkinson’s, Alzheimer’s, ALS, Huntington’s, HSP [265]), ER stress (Huntington’s disease [266], ALS [267], SBMA [264]), RNA processing (SMA [268], ALS [269]), mitochondrial quality control (Parkinson’s [270], Alzheimer’s [271], ALS [272], HSP [1]). A recent study performed exome sequencing on 55 families with autosomal recessive HSP, but lacked a genetic diagnosis [18]. They identified 18 unknown HSP genes, and also performing pathway analysis using protein interaction databases to show that HSP genes are more highly connected within the network than expected by chance. There was also a significant overlap between HSP genes and those implicated in Alzheimer’s disease, ALS, and Parkinson’s disease. This finding suggests that common pathways are disrupted in different neurodegenerative disorders, yet different cell types are affected in each. This question of why particular neuronal subtypes are affected in different neurodegenerative diseases has interested
researchers for decades. Perhaps in the future, instead of studying each disease separately, combining analysis through bioinformatic approaches of multiple disorders at once may yield new insights.
**Figure 6-3: Examination of spastin isoform levels between forebrain and spinal neuron cultures.**

(A) Western blot examining the levels of different spastin isoforms between week 11 forebrain and spinal motor neuron cultures. (B) Quantification of the larger M1 spastin isoform, normalized to actin levels. (C) Quantification of the smaller, cytoplasmic M87 spastin isoform. Data represented as mean ± SD. *P*<0.05. (D) Schematic diagram of approach used in part F. (E) Schematic diagram of the approach used in part G. (F) Quantification of axonal swellings in week 8 control and SPG4 neurons. (G) Quantification of week 8 neurite outgrowth in forebrain and spinal neuron cultures 48 hr after plating. Data presented as mean ± SEM. *P*<0.05.
Figure 6-4: RNA-seq analysis of SPG3A and SPG4 iPSC-derived neurons. Venn diagrams depicting the number of genes differentially expressed between groups, either two-fold decreased or increased. Genes that were differentially expressed in spinal MN and forebrain cells were excluded from the forebrain list, to yield forebrain-specific differentially expressed genes. On the right, forebrain-specific differentially expressed genes from SPG3A and SPG4 cells were compared, to identify genes commonly altered genes.
Table 6-1: Gene ontology terms enriched in genes altered in SPG3A and SPG4. Lists of the top GO terms from the lists of genes with decreased expression in SPG3A and SPG4 forebrain neurons. Data was obtained using DAVID (http://david.abcc.ncifcrf.gov/home.jsp)
6.6 Developing therapeutics for HSP

Here, we used iPSC-models of four HSP subtypes to test the effects of several therapeutic compounds. We were able to show that low doses of the microtubule-targeting drug vinblastine rescues the axonal swelling phenotype in SPG4 cells, and the neurite outgrowth phenotype in SPG3A. The finding that targeting microtubule dynamics is beneficial to human SPG4 neurons matches results from primary neurons of a SPAST/− mouse [167]. In that study, only mice completely lacking spastin expression showed any phenotype, while SPG4 patients had roughly 50% spastin expression. This suggests that mice may have a compensatory mechanism that protects them from axonal abnormalities when spastin levels are reduced by 50%. Several potential mechanisms could explain this. First, there is a dramatic difference in the length of axons between humans and mice. Our results would suggest that differences in axon length is responsible, at least in vitro, as our cultured SPG4 cells showed length-dependent axonal swellings in cells that were roughly the same size as the primary cultured mouse cells, yet the heterozygous mouse neurons did not have swellings. Secondly, expression differences between spastin isoforms may partially explain the species differences in the effect of reduced spastin expression. Previous work in mice found that the smaller M87 spastin is the major isoform present in all nervous system tissue, and that the M1 isoform is only detected in the spinal cord [61]. We however detect significant amounts M1-ΔEx4 spastin in both telencephalic and spinal neuron cultures (Fig. 6-3). We also see a small amount of full-length M1 spastin in both types of neurons, with higher levels in the telencephalic glutamatergic cultures. This result suggests that there are differences in spastin isoform abundance between human and mouse neurons, and perhaps human neurons are more sensitive to a decrease in M1 spastin levels, so when one copy of the SPAST gene is disrupted, axon degeneration occurs. A third possibility that may explain the species difference in spastin dosage tolerance may be that other microtubule-severing enzymes, such as katanin or fidgetin, can compensate for the 50% loss of spastin in mice, but not in human neurons. The differences in katanin or fidgetin expression
between human and rodent brain regions have not been compared, yet this may explain why mice are unaffected when spastin expression is reduced by 50%, but this same reduction leads to axon degeneration in humans.

The beneficial effect of targeting microtubule dynamics in SPG4 was confirmed in olfactory neurosphere-derived cells from SPG4 patients [273]. Here, researchers found that very low doses of four microtubule-targeting drugs, vinblastine, taxol, epothilone, and noscapine, could rescue peroxisome trafficking deficit in SPG4 cells. Because the concentration of drugs used was roughly 100-1000 fold lower than the concentrations used to block mitosis and trigger apoptosis in cancer cells [274], the authors suggest that these doses might well-tolerated by patients. All four of these drugs have already passed Phase I clinical trials, suggesting they could be candidates for repurposing. Vinblastine would not likely be helpful to patients, as it does not cross the blood-brain barrier, but epothilone-D does [275], making it a potential candidate for testing the side effects of chronic doses at low concentrations.

We also found that treatment with mdivi-1, an inhibitor of mitochondrial fission, rescued mitochondrial morphology, neurite outgrowth, and caspase activation in SPG15 neurons. This compound has been found to prevent neurotoxicity and increase dopamine release in two mouse models of Parkinson’s disease, \textit{PINK1}^{-/-} mice and 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) mice [276]. They showed that mdivi-1 could penetrate the blood-brain barrier, and intraperitoneal injection once a day for 5 days prevented nigrostriatal dopaminergic neuron loss that occurs following MPTP injection. More work is needed to evaluate whether targeting mitochondrial dynamics is a viable strategy for treating neurodegenerative disorders. Although it has been previously established that altering mitochondrial dynamics in “healthy” cells is detrimental, as Drp1 knockout (loss of mitochondrial fission) or \textit{OPA1} knockout (reduction in mitochondrial fusion) are embryonic lethal [277-280],
more work on the side effects of mdivi-1 treatment are needed. In two studies in mice, there were no adverse effects to short term mdivi-1 treatment for up to a week [276, 281].

While the studies presented here took advantage of stem cell-derived neurons to test for therapeutic compounds, it was on a very small scale. In the future, these systems can be used to screen many more compounds to identify those with the greatest effect and lowest toxicity. To accomplish this, high content screening systems can be employed, such as the PerkinElmer Opera automated confocal microscope. For SPG3A, SPG15, and SPG48, the measurement of neurite outgrowth parameters can be automatically quantified using existing software [282]. The automated identification of axonal swellings is a little more difficult because no software is currently available to do so. Our group is collaborating with researchers at the Sanford-Burnham Medical Research Institute to develop methods for quantifying axonal swellings from high-content images. This would allow large-scale screening of chemical libraries for compounds that can reduce the number of swellings, while not affecting neuron survival or neurite outgrowth.

6.7 Conclusions

Here, we have generated novel models of four HSP subtypes using stem cell technology. This has allowed the identification of novel phenotypes, including altered axonal transport in SPG4 and SPG3A, and impaired mitochondrial health in SPG15 and SPG48. These iPSC-based models were used to show the beneficial effects of vinblastine and mdivi-1 at rescuing disease phenotypes. This confirmed that targeting microtubule dynamics in SPG3A and SPG4 deserves further investigation in HSP patients, as currently no therapies are available for this neurodegenerative disease. The conclusion that inhibiting mitochondrial division rescues neurite outgrowth defects in SGP15 neurons suggests a novel therapeutic approach for this severe form of HSP. There remain many questions that need to be answered before the mechanisms that lead to axonal degeneration in SPG3A, SPG4, SPG15, and SPG48
are fully understood, however the tools and findings reported here should help answer some of those questions in the future.
7. References


