CK2-mediated Dysregulation of Fast Axonal Transport in Hereditary Spastic Paraplegia

BY

MATTHEW ROBERT BURNS
B.A., Oberlin College, 2001

THESIS

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomy and Cell Biology in the Graduate College of the University of Illinois at Chicago, 2014

Chicago, Illinois

Defense Committee:

Jonathan Art, Chair, Anatomy and Cell Biology
Karen Colley, Biochemistry and Molecular Genetics
Scott Brady, Anatomy and Cell Biology
Ernesto Bongarzone, Anatomy and Cell Biology
Matthew Merigiolli, Neurology
This work is dedicated to my parents Bob and Mary, my wife Katie, and our son Morgan. There is no form or scale, category or condition, no unit of measure for everything you are and all you have given me. I am so grateful. I love you. Thank you.
ACKNOWLEDGMENTS

I would like to thank my committee. Their patience, guidance, and support allowed me to realize this goal. In particular, I would like to thank Dr. Jonathan Art and Dr. Karen Colley. When I initially reached out to them to be members of my committee, I could not have realized what a tremendous blessing they would be. Their mentorship and support has been unwavering and my thesis defense and dissertation would not have been possible without them. These mentors are the examples of scientific, professional, and personal decency and integrity to which I will aspire for the rest of my life.

I would also like to thank the friends, students, staff, and lab members from whom I learned so much and relied on for so much. In particular, I would like to thank Janai Carr and Yalda Afshar. It would have been so lonely without you and I wouldn’t have even known it; and it was so much richer (and funnier and ridiculous...er) with you, and I am forever grateful. Members of the Brady and Morfini lab – Erin, Agnieszka, Bin, Gus, Sarah P., Sarah LB, Yuka – thank you for all your advice and support. I will always regret not expressing my gratitude and admiration more than I did.

I would like to thank the staff of the office of the Anatomy and Cell Biology department: Rory, Mike, Sydelle, Maria, Lee, and Charles. For every time I was
frustrated, impatient, or over my head, you were calm, patient, and a constant live-saver. Thank you.

Finally, to Ina and Sun Kyong: we made it!!! Thank you.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. BACKGROUND AND SIGNIFICANCE</strong></td>
<td></td>
</tr>
<tr>
<td>A. Hereditary Spastic Paraplegias</td>
<td>1</td>
</tr>
<tr>
<td>1. Patient presentation</td>
<td>1</td>
</tr>
<tr>
<td>2. Genetic features</td>
<td>2</td>
</tr>
<tr>
<td>3. Pathophysiologic features</td>
<td>2</td>
</tr>
<tr>
<td>4. SPG4 form of HSP</td>
<td>4</td>
</tr>
<tr>
<td>B. Fast Axonal Transport</td>
<td>5</td>
</tr>
<tr>
<td>1. Cytoskeletal components and molecular motors</td>
<td>5</td>
</tr>
<tr>
<td>2. Axonal transport deficits, motor neuron disease, and length dependence</td>
<td>8</td>
</tr>
<tr>
<td>3. A new paradigm in axonal transport-dependent motor neuron disease</td>
<td>12</td>
</tr>
<tr>
<td>4. Axonal transport regulation and dysregulation in disease</td>
<td>14</td>
</tr>
<tr>
<td>C. Casein Kinase II (CK2)</td>
<td>17</td>
</tr>
<tr>
<td>1. Structure and function</td>
<td>17</td>
</tr>
</tbody>
</table>

| II. MATERIALS AND METHODS | |
| A. Chemicals | 19 |
| B. Materials | 19 |
| C. Cell lines and cultivation | 19 |
| 1. Bacterial strain | 19 |
| 2. Bacterial media | 19 |
| 3. Eukaryotic cell lines | 20 |
| 4. Cultivation media of cell lines | 20 |
| 5. Freezing media | 20 |
| 6. General culture | 21 |
| 7. Differentiation | 21 |
| D. Lentiviral production, concentration, and titration | 21 |
| E. Stable cell line production | 23 |
| F. Antibodies | 24 |
| G. Immunoblotting | 24 |
| H. Oligonucleotides | 25 |
| I. Plasmids | 25 |
| J. Cloning of constructs | 26 |
| K. DNA methods | 27 |
| 1. RNA isolation and cDNA synthesis | 27 |
| 2. cDNA RT-PCR analysis | 28 |
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. Vesicle Motility assay</td>
<td>28</td>
</tr>
<tr>
<td>M. Kinase assay</td>
<td>29</td>
</tr>
</tbody>
</table>

## III. RESULTS

| A. Isoform-specific Effects of Mutant Spastins of FAT in Squid Axoplasm | 30 |
| B. CK2-mediated Effects of Mutant Spastin on FAT | 33 |
| C. Generation of Mutant Spastin Isoform-Expressing Lentiviral Vectors | 37 |
| D. Generation and Characterization of a Stable Human Neuronal Cell Line Expressing Mutant Spastin Isoforms | 40 |
| E. Characterization of CK2 Activity in Mutant Spastin Isoform-expressing Human Neuronal Cell Line | 48 |

## IV. DISCUSSION

| A. A Potential Mechanism | 52 |
| B. Conflicts With This Model | 54 |
| C. The Value of a Gain of Function Mechanism | 55 |
| D. Perspectives from Cancer Biology | 56 |
| E. Mechanistic Connections to Other Cellular Compartments | 57 |

CITED LITERATURE | 59 |

VITA | 67 |
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. SPASTIN MUTATION-EXPRESSING LENVIVIRAL TITERS</td>
<td>38</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>DESCRIPTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Spastin structure and isoforms</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Overview of protein kinases involved in the regulation of fast axonal transport, and their relationship to neuropathogenic polypeptides</td>
<td>16</td>
</tr>
<tr>
<td>3.</td>
<td>Lentiviral production</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>Spastin-IRES-eGFP lentiviral construct</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>Spastin mutations induce deficits in fast axonal transport</td>
<td>31</td>
</tr>
<tr>
<td>6.</td>
<td>Disease-causing mutations in the M1, but not M87 spastin isoform inhibits fast axonal transport</td>
<td>35</td>
</tr>
<tr>
<td>7.</td>
<td>CK2 inhibitors block axonal transport deficits induced by mutant M1 spastin</td>
<td>36</td>
</tr>
<tr>
<td>8.</td>
<td>Lentiviral transgene expression in SH-SY5Y and primary cortical cell culture</td>
<td>39</td>
</tr>
<tr>
<td>9.</td>
<td>Approach for generating stable spastin mutation-expressing human neuronal cell line</td>
<td>41</td>
</tr>
<tr>
<td>10.</td>
<td>Representative phase contrast and fluorescent images of M1 E442Q, M1 STOP and M87 STOP cell lines</td>
<td>42</td>
</tr>
<tr>
<td>11.</td>
<td>Relative spastin gene expression levels in stably expressing M1 STOP and M87 STOP cells</td>
<td>43</td>
</tr>
<tr>
<td>12.</td>
<td>Protein expression of spastin, kinesin, and CK2 alpha/alpha’ in SH-SY5Y cell lines.</td>
<td>44</td>
</tr>
<tr>
<td>13.</td>
<td>Similar GFP transgene protein expression despite apparent increased M1 STOP protein</td>
<td>45</td>
</tr>
<tr>
<td>14.</td>
<td>M1 STOP protein shows increased aggregation relative to M87 STOP despite comparable transgene expression</td>
<td>46</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>15. Relative CK2 alpha and alpha prime gene expression in stably expressing M1 STOP and M87 STOP cells</td>
<td>47</td>
</tr>
<tr>
<td>16. Relative CK2 beta gene expression in stably expressing M2 STOP and M87 STOP cells</td>
<td>48</td>
</tr>
<tr>
<td>17. CK2 activity is significantly higher in M1 STOP cell lysate compared to M87 STOP cell lysate</td>
<td>49</td>
</tr>
<tr>
<td>18. A pharmacological inhibitor of CK2 blocks M1 STOP-induced increases in CK2 activity</td>
<td>50</td>
</tr>
<tr>
<td>19. Potential mechanism for M1 isoform-specific effects of mutations on CK2 activity and FAT</td>
<td>53</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>ATPases Associated with Diverse Cellular Activities</td>
</tr>
<tr>
<td>AD</td>
<td>Autosomal Dominant</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal Recessive</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CDyn</td>
<td>Cytoplasmic Dynein</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein Kinase 2</td>
</tr>
<tr>
<td>DHC</td>
<td>Dynein Heavy Chain</td>
</tr>
<tr>
<td>DIC</td>
<td>Dynein Intermediate Chain</td>
</tr>
<tr>
<td>FAT</td>
<td>Fast Axonal Transport</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>hBDNF</td>
<td>Human Brain-derived Growth Factor</td>
</tr>
<tr>
<td>HSP</td>
<td>Hereditary Spastic Paraplegia</td>
</tr>
<tr>
<td>Htt</td>
<td>Huntingin Protein</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosomal Entry Site</td>
</tr>
<tr>
<td>KHC</td>
<td>Kinesin Heavy Chain</td>
</tr>
<tr>
<td>KLC</td>
<td>Kinesin Light Chain</td>
</tr>
<tr>
<td>LC</td>
<td>Dynein Light Chain</td>
</tr>
<tr>
<td>LIC</td>
<td>Dynein Light Intermediate Chain</td>
</tr>
<tr>
<td>M1</td>
<td>Long Spastin Isoform</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS** (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M87</td>
<td>Short Spastin Isoform</td>
</tr>
<tr>
<td>MBOs</td>
<td>Membrane Bound Organelles</td>
</tr>
<tr>
<td>MIT</td>
<td>Microtubule Interacting and Endosomal Trafficking Domain</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubules</td>
</tr>
<tr>
<td>MTBD</td>
<td>Microtubule Binding Domain</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>PS1</td>
<td>Presinilin 1</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide Dismutase 1</td>
</tr>
<tr>
<td>SPG4</td>
<td>Spastin Gene</td>
</tr>
<tr>
<td>TBCA</td>
<td>(E)-3-(2,3,4,5-Tetrabromophenyl)acrylic acid</td>
</tr>
<tr>
<td>VSVg</td>
<td>Vesicular Stomatitis Indiana Virus Protein G</td>
</tr>
<tr>
<td>α-Syn</td>
<td>Alpha Synuclein</td>
</tr>
</tbody>
</table>
SUMMARY

Mutations in the microtubule severing protein spastin are the most common cause of Hereditary Spastic Paraplegia. The spastin gene codes for two protein isoforms called M1 and M87. These two isoforms differ by 86 amino acids on the N-terminus of the proteins. M1 includes these 86 amino acids and M87 does not. The role of spastin isoform mutations in the dysregulation of fast axonal transport (FAT) was studied. Specifically, we studied the differing effects of mutations in the context of the two isoforms of spastin. To better understand these isoform-specific effects, several models were used. We used the well-established squid axoplasm model to quantify the effect of several spastin mutations in each isoform on anterograde and retrograde FAT rates. We then generated a human neuronal cell line using lentiviral vectors that expressed these same spastin mutations in each isoform. We then measured the relative kinase activity of Casein Kinase 2 (CK2) among lysates of these cell lines using a standard in-vitro kinase assay.

Rates of anterograde and retrograde FAT were decreased when squid axoplasm was perfused with spastin mutations expressed only in the M1 isoform, but not the shorter M87 isoform. These decreases in transport rates were seen in multiple mutations, but only when the mutations were expressed in the longer M1 isoform. When mutations were expressed in the context of the M87 isoform, no effect was seen on FAT rates. In addition, When M1 mutations were perfused into
SUMMARY (continued)

axoplasm in combination with either a pharmacological or peptide inhibitor of CK2, the decreases in transport rates were blocked. Significantly, the peptide inhibitor is a phosphorylation target of CK2, suggesting that the effect of M1 mutations on FAT is mediated by phosphorylation of a component of FAT by CK2.

CK2 activity was increased in cell lysate of a human neuronal cell line expressing M1 mutations, but not in cell lysates expressing M87 mutations. In addition, a pharmacological inhibitor of CK2 blocked increases in CK2 activity seen in an M1 mutation in these same lysates.
I. BACKGROUND AND SIGNIFICANCE

A. **Hereditary Spastic Paraplegias**

1. **Patient Presentation**

   The hereditary spastic paraplegias (HSPs), also known as familial spastic paraparesis or Strümpell-Lorrain disease, are a genetically diverse group of motor neuron disorders with a common clinical presentation. Patients show progressive lower extremity weakness and spastic paralysis with hyper-reflexia due to degeneration of corticospinal and dorsal column synapses and axons of upper motor neurons. HSP prevalence rates range from 2.0-9.6 : 100,000 depending on the diagnostic criteria, epidemiological methodology, and geographical factors. Onset is highly variable among different affected genes as well as among patients with different mutations in the same affected gene. HSP can have a childhood, adolescent, or adult onset. Rare cases have also been reported describing infantile onset of either motor or associated symptoms.

   Patient presentation is generally classified as pure or complex. Pure forms of HSP tend to be limited to lower limb motor symptoms regardless of age of onset, while complex forms involve other neurological symptoms including ataxia, decreased vibratory sense, amyotrophy, optic atrophy, pigmentary retinopathy, mental retardation, visual disturbances, epilepsy, and extrapyramidal signs. Non-neurological symptoms can be involved as well, including skeletal abnormalities, disc herniation, foot deformities, and hiatal hernias. The most common
complicating symptoms are urinary, most frequently presenting as urinary urgency or incontinence. Despite a diversity of complicating symptoms however, degeneration of the corticospinal and dorsal column synapses and axons remain the defining feature of all forms of HSP.

2. **Genetic features**

Over 45 HSP loci and 20 causative genes have currently been identified, with autosomal dominant (AD), autosomal recessive (AR), and X-linked inheritance patterns all represented among the loci. All genetically defined HSP genes are given the symbol SPG (spastic gait) followed by a number. The AD inheritance pattern represents 70-80% of diagnoses and is most often a pure form. AR forms tend to be complex presentations.

3. **Pathophysiological features**

Despite the diversity of involved genes, inheritance patterns, and complicating symptoms, the degeneration of corticospinal and dorsal column fibers is common to all HSP forms. This common pathological feature has motivated attempts to derive a common pathophysiological mechanism. Any unifying pathophysiological mechanism must address several fundamental observations in HSP. First, while cell death of selected motor neurons represents the final outcome of HSP, it is now well established that these cells first degenerate following a "dying-back" pattern. Specifically, degeneration of motor neurons in HSP begins with synaptic degeneration of the terminal portions of the neurons, followed by an
Axonal pathology that far precedes neuronal cell body degeneration and death.

Pathological observations show a length-dependent dying back axonopathy of the longest axons in corticospinal tracts of HSP patients, suggesting that axonal degeneration represents an early pathogenic event in HSP. Further histological studies are characterized by axonal swellings and varicosities in association with abnormal accumulation of membrane-bound organelles (MBOs) and disorganization of cytoskeletal elements, including neurofilaments. These studies support the idea that early degeneration of synapses and axons constitute an early pathogenic event in HSP. However, little is known about the molecular mechanisms underlying axonal degeneration in HSP, including the neuronal compartments where the primary lesion takes place.

Second, despite wide expression of affected HSP genes among many different neuronal cell types, upper motor neurons are predominantly affected. Among the roughly 20 HSP-causing genes whose functions are known, no common mechanism has been identified and the cellular mechanisms associated with those HSP-causing genes do not seem to explain the susceptibility of motor neurons. Any unifying disease mechanism would have to address the cell specificity of degeneration in HSP.

Finally, the vast majority of HSP mutations results in either an adult onset disease or, in the childhood and adolescent form, seem not to effect the development of the central nervous system grossly or functionally. Any unifying
mechanism would have to account for the apparent adult onset or at least the relative lack of developmental effects.

Attempts to identity a common underlying mechanism is complicated by genetic heterogeneity of HSP. As a result, the field has focused on several representative HSP genes with the hopes that mechanistic insights could be extended to the disease as a whole.

4. **SPG4 form of HSP**

Autosomal dominant mutations in the SPG4 locus cause the most common cause of HSP, accounting about 40% of all HSP cases, and represent a perfect example of HSP’s pathogenic patterns and mechanistic mysteries. The human spastin protein contains several domains (Fig. 1). The SPG4 gene codes for two isoforms of the microtubule-severing protein spastin. The microtubule-binding (MTBD) and AAA (for ATPases Associated with Diverse Cellular Activities) domains are essential for the microtubule binding and severing activity, respectively. A microtubule-interacting and endosomal trafficking (MIT) domain, two nuclear localization signals (NLS), and two nuclear export signals (NES) have also been identified, but their functional significance is not understood.

The mammalian spastin gene includes two start codons, resulting in the translation a 616 amino acid isoform called M1, and shorter isoform missing the first 87 amino acids, called M87 in humans (corresponding to M1 and M85 in
rodents)\textsuperscript{11}. The second in-frame start codon is predominantly expressed in most cell types, consistent with its stronger Kozak sequence\textsuperscript{12,13}.

Spastin monomers assemble into a hexamer, and microtubule severing is achieved through the interaction of the hexamer central pore loop with the c-terminus of tubulin subunits\textsuperscript{14}. The relative roles of the two spastin isoforms and exon 4 deletion in this assembly and microtubule severing process is not currently understood.

Figure 1. Spastin structure and isoforms. NLS-NES: nuclear localization and export signals. MIT: microtubule interacting and endosomal trafficking domain. Ex4: Exon 4 domain. MTBD: microtubule binding domain. AAA: ATPase domain.

B. Fast Axonal Transport

1. Cytoskeletal Components and Molecular Motors

What pathogenic mechanism might explain the common theme of motor neuron degeneration among the variety of forms of HSP? Neurons are some
of the most polar cells in humans. The length of a motor neuron axon can be four orders of magnitude longer than the width of the cell body. As a result, much of the total cell volume is the axonal compartment\textsuperscript{11,15}. Despite neurons being 99\% axon, most of the protein production is confined to the cell body. As a result, transport and delivery of membrane proteins and lipid components along axons, a process collectively referred to as fast axonal transport (FAT)\textsuperscript{11,15}, is essential for cell viability. Almost all axonal and synaptic components are anterogradely transported by microtubule-based molecular motors from the cell body to their distant destination\textsuperscript{11,15,16}. Neuronal survival and maintenance also requires signaling complexes and MBOs containing degradation products to be transported in the retrograde direction from synaptic terminals and axons to the neuronal cell body\textsuperscript{11,15,17}.

Conventional kinesin is the main molecular motor that executes anterograde transport of MBOs, including mitochondria, synaptic vesicles, and axolemmal precursors, among others\textsuperscript{18-22}. The execution of retrograde FAT is carried out by the minus end-directed microtubule-based molecular motor cytoplasmic dynein (CDyn)\textsuperscript{23-25}. Conventional kinesin is a heterotetrameric protein complex composed of a long, coiled-coil rod that incorporates two heavy chains (kinesin-1, KHCs, KIFs\textsuperscript{26}) on one end, and two kinesin light chains (KLCs) forming a fan-like structure at the opposite end. Kinesin-1s are responsible for the mechanoc hemical properties of the conventional kinesin holoenzyme, containing both microtubule-binding and ATPase domains\textsuperscript{15,27}. KLCs play a role in the binding
and targeting of conventional kinesin to selected MBOs. Three kinesin-1 (kinesin-1a, b and c) and two KLC (KLC1 and KLC2) genes exist in the mammalian nervous tissue, giving rise to biochemically heterogeneous forms of conventional kinesin. CDyn exists as a large, multisubunit protein complex formed by at least two heavy chains (DHCs), two intermediate chains (DICs), four light intermediate chains (LICs), and several light chains (LCs). Combinatorial arrangements of CDyn subunits play a role in the regulation and functionality of CDyn, including its binding to selected types of MBOs.

As a result of the unusual demands placed on motor neurons by their polarity, length, and the complex axonal transport machinery, one might expect alterations in axonal transport to contribute to motor neuron disease in particular. In fact, many motor neuron diseases, including HSP, display the distinctive hallmarks of axonal transport deficits. Motor neurons show axonal swellings and accumulations of cytoskeletal components and transported organelles, as well as the degeneration and retraction of synaptic connectivity. This synaptic and axonal pathology begins and progresses well before cell body degeneration and death. As mentioned early, HSP displays such a pattern. In addition, several loss-of-function mutations in key molecular motors of axonal transport have recently been shown to cause this distinctive dying back degeneration of motor neurons in human patients and mouse models. As a result, a link has been made between dying back motor neuron diseases and deficits in axonal transport, with the assumption...
often made that axonal transport deficits are a problem predominantly for long axons.

A growing body of work, however, has called into question this simple correlation. Not only have deficits in axonal transport been found in a broader range of neurodegenerative disease affecting projection neurons of varying length, but these deficits have implications for the differential degeneration that is a hallmark of almost all neurodegenerative disease. As a result, a more complex role for the dysregulation of axonal transport in differential neurodegeneration has begun to be elucidated.

2. **Axonal transport deficits, motor neuron disease, and length dependence**

The field of axonal transport is relatively new territory in neuroscience. After preliminary work showing the transport of subcellular components in axons in the 1940’s and 1960’s, it was not until 1985 that kinesin was discovered. In that same year, research had already begun to focus on the role of axonal transport in disease. Early studies documented and characterized alterations in axonal transport among several motor neuron diseases. Several characteristic pathological patterns emerged. Axonal swellings were common among affected neuronal cell types. These swellings contained disorganized cytoskeletal components including neurofilaments, and membrane-bound organelles including mitochondria. Additionally, synaptic viability and connectivity
were compromised. Intriguingly, these axonal swellings and loss of synaptic connectivity preceded cell death, but correlated well with onset and progression of symptoms. As a result, a “dying back” pattern of degeneration was established, where degeneration of synaptic connectivity and axonal viability and integrity, both of which fundamentally dependent on axonal transport, precede the cell body degeneration seen in post-mortem studies\(^43\).

Direct evidence for the role of axonal transport in motor neuron disease has come more recently from one of the HSP genes. SPG10 codes for one of the three isoforms of the anterograde molecular motor kinesin (Kif5A), where autosomal dominant missense mutations in the motor domain of Kif5A result in HSP\(^35\). The discovery of the SPG10 locus gave direct evidence that reduction (but not complete ablation) of anterograde axonal transport is sufficient to produce adult onset motor neuron degeneration. SPG10 is not the only such example however. Recently, an autosomal recessive mutation in another isoform of kinesin, Kif1A, has been associated with HSP in a Palestinian family and the causative mutation of HSP30\(^44\). Dying back neuropathies have been associated with alterations in retrograde transport as well. The major retrograde molecular motor dynein binds to cargo through a multiprotein complex dynactin\(^45\). Recently, disruption of dynein/dynactin complex interactions was shown to result in adult onset motor neuron degeneration in animal models\(^36,46,47\). In addition, missense mutations in dynein were shown to produce motor neuron degeneration in mice. These animal models suggested that deficits in retrograde transport might be sufficient to
produce dying back degeneration in humans. Subsequent to this work, a mutation in the p150 subunit of dynactin was found in a family with adult onset lower motor neuron disease. Inheritance displayed an autosomal dominant pattern, with patients developing lower motor neuron degeneration with onset in their 30’s.

As a result of the variety of mutations among molecular motors, and their seeming convergence on motor neurons or neurons with long axons, deficits in axonal transport have become associated with motor neuron degeneration\textsuperscript{33,48}. Significantly, these molecular motor mutations have wide expression among neuronal cell types including types of neurons seemingly unaffected by these same mutations. As a result of this seeming conflict, particular features of motor neurons have been thought to make them particularly vulnerable to the effects of transport deficits. Several hypotheses have been advanced. One such explanation is that the large size of motor neurons results in a sheer number of accumulated and uncleared “traffic accidents” insurmountable for the viability of these cells. Another is that the high metabolic demands of long neurons make them particularly sensitive to deficits axonal transport. For example, the sheer number of ATP-dependent ion channels and enzymatic processes occurring in a motor neuron axon at a given time would make a reduction in the delivery of those components particularly damaging for such a cell. Another related hypothesis is that a high demand for cytoskeletal maintenance of long axons makes these cells particularly vulnerable. Both the cytoskeletal and lipid turnover among large cells would again make these cells particularly sensitive to reductions in transport.
A variety of data complicate these explanations, however. For example, although the Kif5A mutation results in degeneration of upper motor neurons, equally long lower motor neurons are not affected\textsuperscript{4,35,49}. These lower motor neurons seem to be spared in SPG10 HSP despite comparable metabolic and cytoskeletal maintenance requirements. Mutations in the p150 subunit of the dynactin complex also produces a predominantly lower motor neuron neuropathy, despite both upper and lower motor neurons of equal length requiring comparable retrograde transport\textsuperscript{37,46,47,50}. Compellingly, although initial genetic studies reported motor neuron degeneration in the cytoplasmic dynein heavy chain mutants \textit{Legs at odd angles} (Loa), \textit{Cramping 1} (Cra), and \textit{Sprawling} (Sw)\textsuperscript{8,51}, more recent studies of these animal models showed proprioceptive sensory neuron loss \textit{without} loss of motor neurons, and some may even selectively affect striatal neurons\textsuperscript{52,53}. Equally confounding is the fact that for all the molecular motor mutations, the resulting disease presents as an adult-onset disease. The metabolic and cytoskeletal demands placed on axons throughout development are significant, but none of the molecular motor mutations produce overt developmental deficits.
3. **A new paradigm in axonal transport-dependent motor neuron disease**

In light of data that complicate a simple length-dependent role for axonal transport in dying back neuropathies, the mutations associated with axonal transport require a more nuanced examination. What conclusions can be drawn from the currently available data on these mutations? First, these mutations all produce dying back neuropathies, in which degeneration and loss of synaptic and axonal connectivity, the accumulation of axonal swellings and varicosities, and the abnormal accumulation of transported organelles (MBOs) and disorganization of cytoskeletal elements all far precede neuronal cell death. These early pathological events correlate well, however, with the onset of the early symptoms of disease. Second, the SPG10 mutations, the dynein mutations, and the dynactin mutations are autosomal dominant. As a result, one functioning allele remains in each case. In the case of SPG10, only one allele of the three isoforms of kinesin 1 heavy chain is lost. This inheritance pattern suggests that a reduction in transport, as opposed to a complete loss of function, is sufficient to produce these dying back neuropathies. Third, mutations in these molecular motors result in an adult onset of disease. This is an intriguing aspect of these diseases given the heavy dependence on fast axonal transport during the development of the nervous system. Proper cell migration, axonal growth, and the establishment and maintenance of axonal and synaptic connectivity rely heavily on sufficient availability and function of molecular motors and motor-associated proteins. Despite this reliance, development seems largely unaffected. Finally, despite broad expression of kinesin, dynein, and dynactin...
mutations, only particular populations of cells are affected while other populations seem to be spared. To be sure, some of these neuropathies affect motor neurons and some affect some populations of neurons with long axons. However, motor neurons are not always affected, nor are all long axons. In the case of one of the dynein mutations, the longest axons seem to be spared while striatal neurons are preferentially affected, while in the SPG10 mutation even the longest lower motor neurons are spared. In all cases however, it is certain populations of projection neurons that preferentially suffer dying back degeneration, while interneurons are generally spared.

In addition, several conclusions can be drawn from the particular ways these mutations affect the functional activities of the molecular motors involved and the cytopathology that results. First, further work on SPG10 has shown that the mutation affects Kif1A’s ability to binding microtubules, suggesting that alterations in kinesin microtubule binding and/or processivity may play a central role in SPG10 pathogenesis. Second, given that the dynactin complex central role in SPG10 pathogenesis. Second, given that the dynactin complex is essential for dynein’s ability to bind and transport cargo, this example suggests alterations to cargo transport may also represent significant pathogenic events in these dying back neuropathies. Taken together, this evidence suggests that alterations in microtubule binding, processivity, and cargo binding of kinesin and dynein may contribute to the pathogenesis of these diseases. Interestingly, mutations in the dynein subunit produce Lewy body like inclusions, and the SGP10
mutation causes gliosis in humans. These particular cytopathologic hallmarks may not immediately be associated with deficits in transport, but suggest that such deficits can have wide-ranging pathological effects.

In sum, the available genetic data suggests that reductions in either anterograde or retrograde fast axonal transport are sufficient to produce adult onset dying back degeneration of particular neuronal populations of projection neurons through alterations to the microtubule binding, processivity, and cargo binding of kinesin and dynein. Additionally, these specific transport deficits can result in wide ranging pathology, including aggregate formation and gliosis.

4. **Axonal transport regulation and dysregulation in disease**

Interestingly, growing evidence suggests that kinesin and dynein-independent axonal transport is regulated by a variety of kinases and phosphatases, and that this kinase and phosphatase-dependent transport regulation is altered in dying back neuropathies. In recent years, our group and others provided experimental evidence demonstrating that mutations in proteins associated with a variety of neurodegenerative disease can dysregulate FAT through activation of protein kinases, resulting in phosphorylation of molecular motors

For example, familial Alzheimer's disease-related mutations in presenilin-1 (PS1) inhibit anterograde FAT by a mechanism involving activation of GSK3, phosphorylation of KLCs, and detachment of conventional kinesin from
cargoes\textsuperscript{18,43,60,62}. Pathogenic forms of Androgen Receptor (AR, the protein mutated in Spinal Bulbar Muscular Atrophy) and Huntingtin (Htt, responsible for Huntington’s disease) were also found to inhibit FAT through a mechanism involving activation of JNK, phosphorylation of kinesin-1, and inhibition of conventional kinesin binding to microtubules. Selective effects on anterograde or retrograde FAT were found in association with pathogenic forms of tau\textsuperscript{63}, SOD-1 and a-synuclein (Morfini and Brady, \textit{unpublished}), and the Parkinsonian metabolite MPP+\textsuperscript{56,57}. Finally, oligomeric, but not soluble β-amyloid species were found to inhibit FAT by activating CK2\textsuperscript{64}. Thus, a variety of unrelated pathogenic proteins can similarly alter FAT by different molecular mechanisms\textsuperscript{16,55}. 
Figure 2. Overview of protein kinases involved in the regulation of fast axonal transport, and their relationship to neuropathogenic polypeptides. GSK-3: glycogen synthase kinase 3, JNK: c-Jun N-terminal kinase, CK2: Casein Kinase 2, P-38: p38 mitogen-activated protein kinase, PKC: Protein Kinase C. T-shaped lines represent inhibition; arrowheads represent activation. Figure adapted from Berth, Leopold and Morfini

These observations provide a possible explanation for the homogeneous clinical manifestation of HSP patients resulting from mutations in structurally and functionally unrelated genes. Consistent with this view, FAT alterations have been
documented in association with HSPs including SPG2 (PLP mutations\textsuperscript{66}), SPG7 (paraplegin mutations\textsuperscript{67}), SPG11 (spatacsin\textsuperscript{69}), SPG10 (kinesin-5A mutations\textsuperscript{35}), and SPG30 (Kif1A mutations\textsuperscript{44}). The complex regulation of FAT may provide a common pathogenic mechanism linking different types of HSP. Experimental precedents above suggest the possibility that pathogenic spastin might induce unique alterations in one or more regulatory pathway(s) for FAT.

C. **Casein Kinase II (CK2)**

1. **Structure and function**

   One such kinase implicated in fast axonal transport deficits is Casein Kinase 2 (CK2). In cells, CK2 exists primarily as a tetrameric protein comprised of two catalytic subunits, $\alpha$ and/or $\alpha'$ (derived from different genes), and two regulatory $\beta$ subunits, which interact to form a heterotetramer\textsuperscript{69,70}. CK2 $\alpha$ and $\alpha'$ subunits bear the domains responsible for the catalytic activity of CK2, whereas the regulatory $\beta$ subunit modulates both the substrate selectivity and the catalytic activity of the CK2 holoenzyme\textsuperscript{71}. Until recently, CK2 was considered a constitutively active protein kinase\textsuperscript{72}. However, recent experimental evidence identified several mechanisms that allow for its regulation \textit{in vivo}, including targeting to specific substrates by interacting proteins, alterations in the interactions of $\alpha$ and $\beta$ subunits, binding to small metabolites and phosphorylation by other protein kinases\textsuperscript{71,73}. Experimental evidence indicates that protein–protein interactions play a major role in the regulation of CK2 through a variety of distinct mechanisms, including \textit{direct} effects on the catalytic activity of CK2, or \textit{indirect}
effects that specify the phosphorylation of specific cellular substrates by targeting CK2 to particular locations and/or controlling the access of CK2 to these substrates. For example, an interaction of CK2 with the FACT multiprotein complex facilitates selective phosphorylation of the protein p53. Interestingly, CK2 α' subunits have been shown to associate with kinesin-1C, suggesting the possibility that an association of mutant spastin with CK2 might target its kinase activity to conventional kinesin. CK2 interacting protein-1 (CKIP-1) also interacts with CK2 α subunits, recruiting CK2 α to the plasma membrane. The region of CKIP-1 responsible for its interaction with CK2 contains various protein interaction domains including a PH domain, a leucine zipper domain, and five PXXP motifs, which are known to interact with WW and SH3 domains of various proteins. Sequence analysis revealed the presence of several PXXP motifs within the unique amino terminus of M1 spastin, suggesting that pathogenic M1 spastin could interact with CK2 in a manner analogous to that of CKIP-1.
II. Materials and Methods

A. **Chemicals**

Acidic acid (Invitrogen)  
Ampicillin (Merck)  
b-Mercaptoethanol (Sigmaaldrich)  
BSA (Roth)  
Calcium chloride (Merck)  
DMSO (Sigmaaldrich)  
EDTA (Merck)  
Ethanol (Merck)  
Ethidium bromide (Sigmaaldrich)  
Glycerol (Roth)  
HEPES (Roth)  
L-glutamine (Sigmaaldrich)  
Methanol (Merck)  
Milk (Roth)  
MOPS (Sigmaaldrich)  
PBS, Mg2+ and Ca2+ free (Invitrogen)  
Penicillin (Sigmaaldrich)  
Polybrene (Millipore)  
Ponceau S (Serva)  
SDS (sodium dodecyl sulfate) (Sigmaaldrich)  
Sodium pyruvate (cellculture tested) (Sigmaaldrich)  
Streptomycin (cellculture tested) (Sigmaaldrich)  
Sucrose (Merck)  
Tris, Tris base (Sigmaaldrich)

B. **Materials**

Cultivation dishes/flasks/well plates for cell lines (BD Falcon)  
DMEM (Gibco)  
F12 (Invitrogen)  
FBS (Invitrogen)  
Light-sensitive films for ECL (Amersham)  
Maxi/mini plasmid prep kits (Qiagen)  
MEM (Invitrogen)  
Nitrocellulose  
NuPage 4-12% Bis-Tris gells  
PCR reaction kit (Promega, Bio-Rad)  
Radioactivity (P-32)  
Trypsin (Invitrogen)
C. **Cell lines and cultivation**

1. **Bacterial strains**
   
   E.coli DH5α (Invitrogen)

2. **Bacterial media**
   
   **L-broth medium (LB)**
   
   0.5% (w/v) NaCL
   
   1% (w/v) trypton
   
   0.5% (w/v) yeast extract
   
   20 mM Tris/pH 7.5

3. **Eukaryotic cell lines**
   
   293T/17 (ATCC number: CRL-11268)
   
   SH-SY5Y (ATCC number: CRL-2266)

4. **Cultivation media of cell lines**
   
   **293T**
   
   MEM
   
   10% fetal bovine serum
   
   2 mM L-glutamine
   
   0.1 mM MEM non-essential amino acids
   
   1% penicillin/streptomycin

   **SH-SY5Y**
   
   50% MEM
   
   50% Nutrient Mixture F-12, HAM
   
   1% MEM non-essential amino acids
   
   1% L-glutamine
   
   1% penicillin/streptomycin
   
   5% fetal bovine serum

5. **Freezing media**
   
   Normal cultivation media plus 10% DMSO
6. **General culture**

SH-SY5Y cells were ordered from ACCC and cultured as described before\(^7\). Briefly, cells are plated at \(\sim 20\)-30% density on standard tissue culture dishes in Gibco 1:1 F12/DMEM media supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep).

7. **Differentiation**

SH-SY5Y cells are differentiates as described with some modification\(^7\). On day zero, cells are plated at appropriate densities in F12 media with 5% FBS and 1% Pen/Strep onto dishes coated with 0.3mg/ml poly-L-lysine. One day after plating, media is replaced with fresh F12 media with 2% FBS and 10 uM all-trans retinoic acid (R.A). On day three, one half of the media is refreshed with 1% FBS + 10 uM R.A. On day five, R.A. media is removed and cells are gently washed in serum free F12 media. After washing, serum free media with 12ng/ml hBDNF (brain-derived growth factor) is added to the cells. Cells are fully differentiated 2-3 days after the addition of hBDNF.

D. **Lentiviral production, concentration, and titration.**

Low pass 293T cells (<p10) were plated at 50% confluency into 15 cm\(^2\) dishes (180 cm\(^2\) surface area per well) with DMEM high glucose with 5% FBS and 1% P/S one day prior to transfection. We plate 6x15cm\(^2\) dishes per transgene. 2 hours prior to transfection, we removed the old media and add 20 ml of new media
to each dish. 6 ml of 0.1 T.E was mixed with 3 ml of sterile water in a 50 ml falcon tube. To this mixture, transgene, VSVg and DeltaR8.9 helper plasmid DNA was added in a molar ratio of 2:1:1 for a total of 186 ug/plate. 900 ul CaCl$_2$ was then added and the T.E.-DNA solution was incubated at room temperature for 5 minutes. Finally, 9 ml of 2x HBS was added dropwise while vortexing over a one-minute period. Add complete solution evenly into cell media, 3 ml per dish. Rock plates gently to mix (Fig. 3).

After 14-16 hours, media was changed to 3% FBS F12 media, and viral supernatant was harvested 48 hours after media change. The viral supernatant was filtered using a 0.45 um low-protein binding vacuum filter connected to a 500 ml receiver container. 30 ml aliquots of viral supernatant were ultracentrifuged over a 4 ml 20% sucrose cushion at 20,000g for 4 hours. The media and cushion was then poured off and viral pellet was re-suspended in 400 ul Mg$^{2+}$ and Ca$^{2+}$ free sterile Ph7.4 PBS. 10 ul aliquots of concentrated virus were then stored at -80°C until use.

To establish the concentration of concentrated viral aliquots, HEK293T cells were plated at a density of ~50% into 6 well plates. 10 ul of concentrated virus was then diluted into 1 ml of DMEM media + polybrene. Polybrene aids in reducing the charge-charge repulsion between the viral pseudocoat and the cell surface, improving viral binding to the cell surface. A serial dilution is then made from that volume of 10$^{-2}$ viral dilution to a 10$^{-7}$ dilution. Each dilution is added to the corresponding well of HEK293T cells, and the plate is incubated for 48-72 hours.
After incubation, cells are examined by fluorescent microscope and 4-5 random fields are chosen and florescent cells are counted, with each fluorescing cell considered to represent one active viral particle. The concentration of viral particles per 10 ul aliquot is then calculated from the number of fluorescing cells, the surface area of each field, and the total surface area of one well of a 6-well plate.

E. **Stable cell line production**

Undifferentiated SH-SY5Y cells were infected with the spastin lentiviral constructs in 96 well plates depicted in figure 10. Limited dilutions of these polyclonal cultures were then performed and spastin-expressing monoclones selected for expansion. Monoclonal cell stock was then frozen and stored at -80°C. Expression of exogenous spastin and eGFP transgene was then confirmed by RT-PCR and western.
Figure 3. Standard calcium phosphate transfection techniques are used to transiently triple-transfect low pass 293T cells with lentiviral envelope (VSVg), packaging (Delta R8.9), and transgene constructs. Viral media is then harvested from 293T cell culture and viral particles are concentrated through ultracentrifugation. Viral pellets are then re-suspended in sterile PBS and stored at -80°C until use.

F. **Antibodies**
H2 (antigen: KHC; species: mouse; developed by Brady lab)
CK2 alpha/alpha’ (species: mouse/human; abcam)
CK2 beta (species: mouse/human; abcam)
Spastin (species: mouse/human; Santa Cruz)
DM1A (alpha-tubulin; species: mouse/human; Sigma Aldrich)
GFP (species: mouse; Santa Cruz)

G. **Immunoblotting**
Protein samples loaded onto a 4-12% bis/tris gradient gel (Invitrogen) and separated using 35 mAmps/gel for 2 hours. Proteins from the gels were transferred (at 4°C) to Immobilon-P transfer membranes (PVDF, Millipore) at 0.4 Amps for 2 hours in 1X Tobin buffer. After transfer, the membranes were stained with Ponceau
red to visualize proteins, then cut as needed. Membranes were blocked at RT for 60 minutes with 5% milk in PBS. Primary antibodies in 1% BSA were added overnight at 4°C with gentle rocking. Primary antibodies were washed 3 x 10 minutes with PBST (0.25% Tween), and secondary antibodies were added for 1 hour at 4°C with gentle rocking. Membranes were again washed 3 x 10 minutes and visualized with ECL (Amersham) and exposed on film (Kodak) for HRP secondary.

H. **Oligonucleotides**

*Spastin STOP fwd*
AAT TTG GTT ATG GCCAAG GAC CGC

*Spastin STOP rev*
CCA TTG CGG CAT GCC AAG TTA GTA

*hCyclophilin fwd*
GCA GAC AAG GTC CCA AAG ACA G

*hCyclophilin rev*
CAC CCT GAC ACA TAA ACC CTG G

I. **Plasmids**

pcDNA3.1
VSVg
DeltaR8.9
FCbAGW
J. **Cloning of constructs**

**Figure 4. Spastin-IRES-eGFP lentiviral construct.** SD: splice donor site. RRE: Rev responsive element. cPPT: central polypurine tract. SA: splice acceptor site. CAG prom.: CMV early enhancer beta-Actin rabbit beta Globin gene promoter. IRES: Internal Ribosome Entry Site. wPRE: woodchuck hepatitis virus posttranslational regulatory element

cDNA constructs coding for human spastins were generated (Fig. 4). A c-Myc tag at the C-terminus of all spastin constructs was added to help distinguish these from endogenous spastins using immunochemical methods. All spastin constructs were cloned in mammalian expression vectors (pcDNA3.1 plasmids) for the purposes of *in vitro* translation and perfusion of resulting polypeptides in isolated squid axopasm. In addition, some of these constructs were subcloned in the lentiviral plasmid vector FCbAGW-IRES-GFP. Designed to accommodate expression cassettes up to 7 kb. An IRES sequence was introduced in the original FCbAGW-GFP vector78, which allows for the simultaneous and independent expression of cMyc-tagged spastins and Green Fluorescent Protein (GFP).
K. **DNA methods**

1. **RNA isolation and cDNA synthesis**

   Semi-quantitative PCR was used to evaluate relative expression levels of exogenous M1 and M87 spastin mutations as well as CK2 catalytic subunits alpha, alpha prime, and regulatory subunit beta. Three SH-SY5Y clones expressing M1 STOP (named D2, D3, and H4) and three clones expressing M87 STOP (named D5, D6, and F8) were plated in one well each of a 6-well plate and differentiated using standard protocols using retinoic acid and human BDNF as described above. Once differentiated, cells were homogenized using 1 ml Trizol Reagent on ice and aliquoted into 1.5 ml eppendorf tubes. 0.2 ml chloroform was then added and the mixture was vortexed for 15 seconds and centrifuged at 12,000g max for 15 minutes at 4°C. The samples separate into a red phenol chloroform phase and a colorless upper aqueous phase. The aqueous phase as transferred to a fresh 1.5 ml tube, mixed with 0.5 ml isopropanol, and incubated at room temperature for 10 minutes. The samples were then centrifuged at 12,000g max for 10 minutes at 4°C. The RNA pellet forms a white pellet at the bottom of the tube. The RNA pellet was then washed once with 1 ml of 75% ethanol and then centrifuged at 7,500g max for 5 minutes at 4°C. The pellet was then air dried and re-suspended in 30 ul of RNase free water. 5 ul aliquots were made and stored at -80°C. The RNA was then treated with DNase following standard Promega protocols and cDNA was generated using Bio-Rad iScript Synthesis kit protocol.
2. cDNA RT-PCR analysis

RT-PCR primers were designed and purchased from Integrated DNA Technologies (IDT) using their PrimerQuest primer design software. Two primer sets were ordered for each gene of interest and optimized in triplicate by evaluating optimum running temperature and melting curve profile using the Bio-Rad iCycle system. In addition, several standard housekeeping gene primer sets were optimized. Human cyclophilin was identified as superior to GAPDH for these samples, consistent with previous findings in our lab. Once the optimal primer set and running temperature was identified, samples were run in triplicate over 2-5 trials along with a serial dilution of pooled samples of all six clones for each primer set. These serial dilutions were then used to optimize the regression curve for each primer set.

L. Vesicle motility assay

Buffer X
20 mM HEPES, pH 7.2
350 mM potassium aspartate
130 mM taurine
70 mM betaine
50 mM glycine
13 mM MgCl₂
10 mM EGTA
3 mM CaCl₂
1 mM glucose
supplemented with 10mM ATP

A Zeiss Axiomat microscope with video image enhancement and quantitation of vesicle velocities is used. The squid giant axon is dissected and extruded onto a
glass coverslip. The X/2 buffer, which contains ATP and closely resembles the intracellular environment of the squid axon, is then perfused with spastin proteins and inhibitors of interest. The rate of bulk anterograde and retrograde flow of the organelles is measured by matching the speed of calibrated cursors to that of moving organelles. Anterograde and retrograde velocities are measured for 50 minutes and plotted in graphs as illustrated in preliminary results.

M. **Kinase assay**

**10x CK2 buffer**
250mM HEPES, pH 7.5  
120mM MgCl₂  
1 M NaCl

**Inhibitors**
500 nM Okadiac Acid  
2 uM Microcystine  
1/100 Protease inhibitor cocktail

Kinase assays were performed according to well-characterized kinase assays⁷⁹,⁸⁰. Spastin cell lysates were incubated in triplicate with recombinant CK2 (containing alpha and beta subunits, commercially available from New Englad Biolabs) and a highly specific CK2 peptide substrate (RRREEETEEE, Sigma Genosys). Reactions were started by addition of radioactive ATP and halted by spotting onto P81 phosphocellulose paper in triplicate, as described elsewhere⁸⁰. A scintillation counter was then used to quantify and record p32 emissions.
III. Results

A. Isoform-specific effects of mutant spastins on FAT in squid axoplasm.

Wild type and SPG4-HSP-related mutant spastin constructs were generated (see Fig. 5). We first evaluated the effects of recombinant wildtype, or full length (FL) spastins, and Truncated spastin constructs, comprising a.a. 1-273 truncation of wild-type spastin that resemble truncated spastin polypeptides predicted to result from multiple nonsense mutations causative of SPG4-HSP81. The full length and truncation constructs used for this experiment express both isoforms. Additionally, the truncation mutation results in proteins that lack both microtubule severing and microtubule binding functions due to their lack of AAA and MTBD domains, respectively.

Spastin polypeptides were produced using in vitro translation techniques. Reactions without cDNA did not result in detectable protein synthesis, but two functional start codons in the full-length wild type spastin construct (M1/M87) resulted in the translation of both M1 (a.a. 1-614), and M87 (a.a. 85-614) polypeptides, as previously described11,82. Translation of the truncated spastin construct M1/M85 STOP yielded truncated versions (a.a. 1-273) of both M1 and M85 lacking both the MTBD and the AAA domains.

Perfusion of full-length spastin had no effect on average anterograde or retrograde fast axonal transport rates (Fig 5). However, perfusion of the truncation polypeptides resulted in a significant decrease in both anterograde and retrograde
transport rates. Data derived from our collaborative work with Dr. Peter Baas suggested a novel pathogenic mechanism in SPG4-HSP based on the distinct distribution, developmental expression pattern, and toxic properties of spastin isoforms\textsuperscript{11}. Specifically, M1 spastin is almost absent from developing neurons and from most adult neurons. However, M1 comprises 20-25\% of the total spastin in the adult spinal cord, the precise location of axons degenerating in HSP\textsuperscript{11}.

\textbf{Figure 5. Spastin mutations induce deficits in fast axonal transport.} Vesicle motility assays in isolated squid axoplasm. Individual velocity measurements (arrowheads) are plotted as a function of time. Anterograde (blue right-pointing arrowheads and line), and retrograde (green left-pointing arrowheads and line) FAT rates are shown. \textbf{Right panel} Perfusion of full-length wild type spastin (resulting in the expression of both M1 and M85 isoforms) has no effect on FAT. \textbf{Left panel} Perfusion of mutant spastin dramatically decreases anterograde and retrograde FAT. \textbf{n: number of independent experiments}
The selective expression of M1 in the adult spinal cord also matched with the adult onset characteristic of HSP. Moreover, spastin was absent in the adult sciatic nerve, consistent with the fact that HSP does not involve degeneration of axons of the peripheral nervous system\textsuperscript{11}. These observations suggested that HSP-related spastin mutations might lead to axonal degeneration through an isoform-specific axonal transport-mediated mechanism. To evaluate isoform-specific effect of pathogenic spastin on FAT, we prepared separate pools of M1-STOP and M85-STOP using \textit{in vitro} translation methods and individually perfused these polypeptides in isolated squid axoplasm\textsuperscript{11}(Fig. 5). Results from these experiments showed that M1-STOP inhibited FAT, whereas M85-STOP had no effect (Fig. 5). Additionally, we generated an E440Q missense mutant spastin constructs in the context of both M1 and M85 isoforms. The missense mutants showed the same isoform specific pattern of axonal transport deficit as the STOP mutants, with M85 E440Q showing no effect on anterograde or retrograde transport, while the M1 E440Q mutant induced dramatic anterograde and retrograde fast axonal transport deficits. A silent mutation was introduced in all M1 constructs, which prevents translation from methionine 85\textsuperscript{11} (see Fig. 6).

Although perfusion of mouse spastin M1 and M85 mutants showed an M1 isoform-specific effect on transport, we continued to further test the effects of \textit{human} mutant spastins on FAT, additional constructs were generated representing
the human form of previously perfused mouse STOP and E440Q missense mutation (Fig 6). In addition, two human disease-causing missense mutations, E112K, L195V, were generated for perfusion as well. Perfusion of either M1 STOP, representing a truncation at amino acid 173, or M1 E442Q, resulted in dramatic reductions in anterograde and retrograde fast axonal transport when perfused in squid axoplasm, consistent with data obtained with mouse constructs. Perfusion of M87 STOP or M87 E442Q however, had no effect on either direction of transport (Fig. 6). In addition, two disease-causing E112K and L195V mutations were perfused, showing a similar pattern of M1 isoform-specific fast axonal transport reduction. These studies indicate that mutant spastin inhibit FAT through an M1 isoform specific mechanism.

B. **CK2-mediated Effects of Mutant Spastin on FAT**

Based on prior work from our lab suggesting a role of several kinases in FAT dysregulation, we co-perfused mutant M1 spastin with several specific kinase inhibitors to see if we could block decreases seen in figures 5 and 6. Both a highly specific pharmacological inhibitor of CK2 or a specific peptide substrate for the kinase CK2 blocked the axonal transport deficits induced by missense and truncated M1 spastin (Fig. 7). Specific inhibitors of other kinases, however, could not prevent the effect thus supporting the specificity of the effect (data not shown). This data suggests CK2 mediates the inhibitory effect of mutant M1 spastin on fast axonal transport. In addition, given that co-perfusion of M1 mutants with a CK2 specific phosphorylation substrate was able to prevent anterograde and retrograde
transport, this data further suggests that M1 isoform-specific effects are mediated by CK2 phosphorylation of specific targets of fast axonal transport components. Finally, the inhibitory effect of mutant M1 spastins on FAT is independent of transcription and translation, because extruded axoplasms are separated from cell bodies. More significantly however, the lack of effect of the M87 mutant on axonal transport strongly argues against a mechanism involving microtubule dynamics or a nonspecific effect. The lack of a functional ATPase domain in EQ442 and truncated mutant spastins further supports this idea. Taken together, these studies indicate that mutant spastin polypeptides inhibit FAT through an isoform specific mechanism independent of microtubule dynamics or transcriptional effects.
Figure 6. Disease-causing mutations in the M1, but not M87 spastin isoform inhibit fast axonal transport. Vesicle motility assays in isolated squid axoplasm. A) Perfusion of common disease-causing spastin M1 missense polypeptides in axoplasm inhibits both anterograde and retrograde FAT rates. Perfusion of spastin M87 missense or truncation did not affect FAT rates. A different disease-causing M1 missense mutation inhibits FAT, while M87 mutation shows mild anterograde deficit only. n: number of independent experiments.
Figure 7. CK2 inhibitors block axonal transport deficits induced by mutant M1 spastin. A) Co-perfusion of M1E440Q (mouse) and CK-specific pharmacological inhibitor DMAT blocked deficits in FAT. Co-perfusion of M1 spastin missense mutation with CK2-specific phosphorylation substrate also blocks deficits in FAT. Similar blockade is seen for M1 truncation mutation using either CK2 specific inhibitor (partial blockade) or phosphorylation substrate (complete blockade).
C. **Generation of Mutant Spastin Isoform-Expressing Lentiviral Vectors**

Having established an M1 spastin isoform specific CK2 phosphorylation-mediated effect on anterograde and retrograde transport in squid axoplasm, we were interested to corroborate this result. To do so, we pursued a human neuronal cell model and further explore the biochemical mechanism by which mutant spastin, CK2, and components of axonal transport interact. Specifically, we identified the human neuroblastoma cell line SH-SY5Y. This cell line is a tractable model with a well-established differentiation protocol inducing robust neurite outgrowth and the expression of cortical neuronal markers consistent with the cell types affected in HSP. Unfortunately, no SH-SY5Y cell lines exist that stably express spastin isoforms and mutants. As a result, we turned to a lentiviral gene delivery system. This system allows for high-efficiency neuronal infectivity and genomic transgene incorporation for stable expression of genes of interest. We received the two lentiviral packaging plasmids (VSVg and ΔR8.9) as a generous gift from our collaborator Dr. Ernesto Bongarzone. The spastin isoforms and mutants used for the squid axoplasm results discussed above were cloned into a bicystronic lentiviral vector as described in the methods section by a talented lab tech Donyang Huang. These vectors were triple-transfected using a standard calcium phosphate transfection protocol into low-pass 293T HEK cells, and viral media was harvested 24-36 hours later. Viral media was then ultracentrifuged over a sucrose cushion and concentrated viral pellets re-suspended in Magnesium and Calcium-free sterile PBS. Viral titers were then calculated by extrapolating number of infective viral
particles from fluorescing 293T HEK cells of a ten-fold limited dilution across five
orders of magnitude from $10^{-2}$ to $10^{-7}$ in a 6-well plate.

<table>
<thead>
<tr>
<th>Lentiviral construct</th>
<th>Titer (T.U. = viral particles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 full length-IRES-GFP</td>
<td>$5.95 \times 10^8$ T.U.</td>
</tr>
<tr>
<td>M1 E442Q -IRES-GFP</td>
<td>$5.1 \times 10^9$ T.U.</td>
</tr>
<tr>
<td>M1 STOP -IRES-GFP</td>
<td>$6.54 \times 10^{11}$ T.U.</td>
</tr>
<tr>
<td>M87 full length-IRES-GFP</td>
<td>$4.33 \times 10^9$ T.U.</td>
</tr>
<tr>
<td>M87 E442Q-IRES-GFP</td>
<td>$3.4 \times 10^{10}$ T.U.</td>
</tr>
<tr>
<td>M87 STOP - IRES-GFP</td>
<td>$6.0 \times 10^9$ T.U.</td>
</tr>
</tbody>
</table>

**Table 1: Spastin mutation-expressing lentiviral titers.** All transgene constructs produced high-titer virus, ranging from $\sim 10^8$ to $\sim 10^{10}$ T.U.

To test the efficiency of infectivity of concentrated viral stock, 300,000 SH-SY5Y cells at several stages of differentiation were infected with $1\times 10^8$ viral
particles at a Multiplicity of Infection (M.O.I) of 1000 viral particles per cell.

Infection of differentiating cells after 5 days retinoic acid and after full
differentiation (5 days retinoic acid + 2 days of human brain-derived neurotrophic
factor) showed high efficiency and robust eGFP expression and fluorescence (Fig. 8).

Additionally, similar efficiency and fluorescence were seen in undifferentiated SH-SY5Y cells (data not shown). No obvious toxicity was seen with viral infection as
judged by gross morphologic changes or large-scale cell death. Undifferentiated
cells infected, grown, and repeatedly passed in culture showed no morphological
changes or alterations in long-term viability.
To evaluate the breadth of tropism of these viral vectors, primary rat cortical culture was also infected with concentrated viral vectors at DIV4 (Fig. 8). Primary rat cortical culture also showed strong efficiency and eGFP expression.

Figure 8: Lentiviral transgene expression in SH-SY5Y and primary cortical cell culture. Lentiviral vectors show robust expression and normal morphology at various stages of differentiation of SH-SY5Y cells (top 4 panels) and rat primary cortical culture (DIV4).
D. **Generation and Characterization of a Stable Human Neuronal Cell Line Expressing Mutant Spastin Isoforms**

Despite robust efficiency of infection and expression in these cell types, limited access to primary culture and the time-intensive nature of regular viral productions made acute infection for large-scale biochemical assays let attractive. The capacity of lentiviral vectors to produce genomic incorporation of the transgene, provided a tractable approach to producing stable spastin mutation-expressing cell lines. As a result, naïve SH-SY5Y cells were infected with high-titer lentiviral vectors. These "polyclonal" cell populations were then expanded, and limited dilutions of these cells were performed, with single clones selected based on fluorescence. These single clones were then expanded and frozen stock stored at -80°C.
Figure 9: Approach for generating stable spastin mutation-expressing human neuronal cell line. SH-SY5Y cells were plated and transfected with spastin-IRES-GFP virus. Cells were then plated into 96 well plates at varying densities and wells monitored for single fluorescing colonies. These colonies were then expanded and stored at -80°C.

Three clones were identified each for M1 STOP (D2, D3, and H4), M87 STOP (D5, D6, and F8), two for M1 E442Q (F6 and G5), and two for GFP (1F6 and 2G4). Unfortunately, repeated attempts to produce an M87 E442Q cell clone failed, likely due to lower titers in the concentrated viral stock and resultant low efficiency expression of the source polyclones.
Brightfield and fluorescent images of M1 STOP, M87 STOP, and M1 E442Q clones showed strong fluorescence, normal morphology, and no gross toxicity over multiple passages (Fig. 10).

Figure 10. Representative phase contrast and fluorescent images of M1 E442Q, M1 STOP, and M87 STOP SH-SY5Y stable cell lines. Representative 20x images of undifferentiated SH-SY5Y stable cell line. Cells display 100% efficiency, robust GFP expression, normal cell growth, and no gross cell toxicity. Spastin transgene expression was confirmed by western (data not shown). Several different clones were produced, expanded, and stored in liquid nitrogen for each mutation. M1 STOP clones labeled D2, D3, and H4. M87 STOP clones labeled D5, D6, and F8.
Given the non-targeted nature of genomic lentiviral transgene incorporation and the potential for multiple transgene incorporations, gene expression levels of total spastin were evaluated using semi-quantitative Reverse Transcriptase Polymerase Chain Reaction assays. CDNA was harvested from M1 STOP and M87 STOP fully differentiated SH-SY5Y clones, spastin primers were optimized and spastin transgene expression was evaluated in triplicate relative to expression levels of the housekeeping gene cyclophilin. Although relative gene expression varied among the six clones evaluated, equivalent total spastin gene expression was seen in M1 STOP D2 and H4, and M87 STOP D5 (Fig. 11).

**Figure 11.** Relative spastin gene expression levels in stably expressing M1 STOP and M87 STOP SH-SY5Y cells. M1 STOP clones are labeled D2, D3, and H4. M87 STOP clones are labeled D5, D6, and F8. Gene expression is normalized to cyclophilin expression. Note that D2, H4, and D5 all have equivalent relative expression.
In addition, expression levels of CK2 catalytic subunits alpha and alpha prime, as well as regulatory subunit CK2 beta were evaluated to ensure that spastin transgene incorporation and expression did not alter CK2 subunit expression levels. Primers for CK2 alpha, alpha prime, and beta subunits were optimized and relative gene expression was evaluated normalized to cyclophilin. Although gene expression varied across the six spastin clones tested, equivalent CK2 alpha, alpha prime, and beta levels were seen in the M1 STOP D2 clone as compared to the M87 STOP D5 clone (Figures 12 and 13).

**Figure 12:** Relative CK2 alpha and alpha’ gene expression levels in stably expressing M1 STOP and M87 STOP SH-SY5Y cells. M1 STOP clones are labeled D2, D3, and H4. M87 STOP clones are labeled D5, D6, and F8. Gene expression is normalized to cyclophilin expression. Note that D2 and D5 all have equivalent relative expression.
Figure 13: Relative CK2 beta gene expression levels in stably expressing M1 STOP and M87 STOP SH-SY5Y cells. M1 STOP clones are labeled D2, D3, and H4. M87 STOP clones are labeled D5, D6, and F8. Gene expression is normalized to cyclophilin expression. Note again that D2 and D5 all have equivalent relative expression.

Based on the above RT-PCR evaluation of the spastin SH-SY5Y stable cell lines, the M1 STOP D2 and M87 STOP D5 clone show equivalent expression of total spastin and CK2 subunit expression levels normalized to cyclophilin expression.

To further characterize protein expression levels of spastin, CK2, kinesin, and GFP levels, spastin SH-SY5Y clones were differentiated and cell lysate was harvested for
evaluation by SDS-PAGE electrophoresis. Equivalent expression of kinesin heavy chain (probed with H2 antibody) was seen in all clones with the exception of M1 STOP D3 (Fig. 14). Additionally, CK2 alpha and alpha prime expression was evaluated in M1 STOP D2 and M87 STOP D5 and was seen to be equivalent.

Figure 14: Protein expression of spastin, kinesin, and CK2 alpha/alpha’ in SH-SY5Y cell lines. Although kinesin and CK2 alpha/alpha’ protein expression was similar when comparing M1 STOP and M87 STOP clones, apparent M1 STOP protein expression was increased with notable degradation products.
GFP expression was also evaluated in all clones and was largely the same, with a slight increase in expression in the M1 STOP D2 clone (Fig. 15 and 16).

Figure 15: Similar GFP transgene protein expression despite apparent increased M1 STOP protein. Similar GFP transgene expression suggests that differences in one M1 STOP and M87 STOP clone are due to mutant protein aggregation and not expression.

Interestingly, despite largely similar expression of kinesin heavy chain, CK2 alpha/alpha prime, and GFP, and equivalent expression of total spastin and CK2 as seen with RT-PCR, total protein amounts of M1 STOP were much higher as compared to M87 STOP for all three M1 STOP clones. Additionally, westerns show
what appear to be either degradation or aggregation products, suggesting M1 mutant isoform-specific protein processing (Fig. 16).

**Figure 16:** M1 STOP protein shows increased aggregation relative to M87 STOP despite comparable transgene expression. Across all clones generated, M1 STOP protein aggregated at high levels with increased degradation products relative to M87 STOP despite comparable GFP expression (refer to green and red arrows as example).

**E. Characterization of CK2 Activity in Mutant Spastin Isoform-expressing Human Neuronal Cell Line**

Having established equivalent spastin and CK2 subunit expression in the M1 STOP D2 and M87 STOP D5 clones, the isoform-specific effect of spastin mutations on CK2 activity was evaluated in differentiated SH-SY5Y cell lysate.
Figure 14: CK2 activity in the differentiated M1 STOP D2 clone is significantly higher at all time points measured compared to the differentiated M87 STOP D5 clone. Not that M87 STOP CK2 activity is equivalent to lysate alone, suggesting an isoform-specific mechanism as opposed to a dose-dependent, non-specific effect of transgene overexpression.

CK2 activity was found to be significantly high in cell lysates expressing the M1 STOP mutation. Cell lysates expressing the M87 STOP mutation had equivalent CK2 activity to control cells. When spermine, a CK2 activator was added to M87 STOP, CK2 activity increased significantly, illustrating a lack of potential CK2 inhibitor activity on the part of the M87 STOP mutation.
We also evaluated the ability of a highly specific pharmacological inhibitor of CK2 to block the M1 isoform-specific mutation-induced activation of CK2 in differentiated SH-SY5Y cell lysate. Figure X shows that the CK2 inhibitor TBCA inhibits the increase in CK2 activity seen in cell lysate expressing the M1 STOP mutation.

**Figure 15:** Addition of a highly specific pharmacological inhibitor of CK2 to M1 STOP lysate inhibited the M1 isoform-specific CK2 activation seen in figure X.
Taken together, data from squid axoplasm and a human cell line show that mutations in the context of the long spastin isoform (M1) decrease rates of anterograde and retrograde transport and increase CK2 phosphorylation activity. This decrease in transport rates is mediated by the phosphorylation of CK2 of as yet unidentified substrates of FAT. Additionally, preliminary western blot data from stable cell line lysate suggest differential processing and/or aggregation of M1 isoform mutations relative to M87 mutations.
IV. Discussion

A. **Potential Mechanism**

The data shown here provide a link between mutations in the M1 isoform of human spastin, the activation of CK2, and the dysregulation of anterograde and retrograde transport. In addition, these data suggest that mutant M1 isoform-specific activation of CK2 results in the phosphorylation of fast axonal transport components including kinesin heavy chain. These results are consistent with a growing body of evidence implicating mutations in neurodegenerative disease-linked proteins to the activation of axonal kinases and resultant deficits in anterograde transport, retrograde transport, or both. The generation of stable isoform-specific spastin mutation-expressing human cell lines described here have allowed for the corroboration of data from an ex vivo squid axoplasm model of fast axonal transport. This human cell line should continue to provide insights into the relationship between mutations in spastin, deficits in fast axonal transport, and Hereditary Spastic Paraplegia.
Figure 16. Potential mechanism for M1 isoform-specific effects of mutations on CK2 activity and FAT

Based on the data currently available, a potential pathogenic mechanism can be proposed. Mutations in the context of the M1 spastin isoform may expose an otherwise cryptic N-terminal poly-cationic epitope. This exposed poly-cationic epitope may then interfere with the charged interaction between the acidic and basic domains of the CK2 catalytic alpha subunits and the regulatory beta subunits. Once released from their regulatory subunits, CK2 alpha has the capacity to phosphorylate kinesin light chains, resulting in release of transported cargo. In addition, CK2 alpha may phosphorylate kinesin heavy chain, releasing the heavy chain from microtubules. This CK2 phosphorylation of kinesin light and heavy chains results in the dysregulation and inhibition of anterograde transport and the axonal accumulation of transported cargo and cytoskeletal components that are an established pathological feature of HSP.
B. **Conflicts With This Model**

This model is a departure from the standard loss-of-function models associated with SGP4 HSP. Traditionally, the assumption has been that if the physiological function of spastin is microtubule severing, mutations in the spastin protein must result in HSP through a loss of microtubule severing. Several groups have suggested that the loss of severing capacity results in microtubules too long to transport to required regions of the axon. Alternatively, dominant negative mechanisms have been proposed, suggesting that incorporation of the mutant protein into the spastin hexamer reduces or prevents microtubule severing. Although loss-of-function or dominant negative mechanisms are reasonable starting points, several observations complicate these mechanisms. First, SGP4 HSP is an adult-onset disease, with symptoms beginning anywhere from the second to the eighth decade of life. Patients do not experience noticeable developmental delays or defects. Additionally, there is no appreciable phenotype at birth. Given the significant demands for microtubule severing throughout development, it is reasonable to assume that any loss of severing capacity would have serious effects on axonal growth and path-finding, but this seems not to be the case. Second, expression studies suggest that total expression levels of spastin decrease throughout development and reach relatively low levels in the adult. As a result, any loss of microtubule severing would result in a much larger total decrease during development than in the adult, but again, SGP4 HSP is reliably adult-onset. Finally, work done more recently by Dr. Peter Baas has show no significant decrease in
microtubule severing with the expression of the L195V mutant relative to controls in an in vitro assay of microtubule severing. This data argues against a dominant negative mechanism.

C. **The Value of a Gain of Function Mechanism**

The gain of function mechanism proposed here should not be viewed as eccentric, but is consistent with a growing body of work from our lab and others implicating mutation-induced or protein conformation-dependent kinase activation. In addition, work done by Dr. Piers Nash has identified a number of modular protein-protein interaction domains and has shown the significance of the evolution of these domains in the development of protein functions and signaling. Taken together, this suggests that proteins may have multiple protein-protein interacting domains that are constrained to varying extents by conformation, localization, or both. Although we often think of evolutionary pressure in terms of cells or organisms, these same pressures exist at the level of the protein. As a result, many proteins may contain the evolutionary vestiges of cryptic signal transduction domains. In the context of the results of the genome project, which continues to revise drastically downward its estimate of the total number of human genes to a number similar to that of a worm, it is not unreasonable to question the “one gene, one protein, one function” supposition. It may in fact be the case that proteins may have, even in a single cellular or subcellular context, multiple functions defined by the a set of epitopes whose exposure to potential protein interactions is dependent
not only on adaptor, scaffolding, or chaperone proteins, but on a set of steady-state conformational possibilities. The appropriate analogy may not be that proteins are keys as much as proteins are key chains bearing multiple keys.

D. **Perspectives from Cancer Biology**

Although the concept of pathogenic mutation-induced signal transduction epitope exposure is discussed here in the context of axonal transport, the relationship between pathogenic kinase activation and neurodegenerative disease should be viewed as a more central theme, with axonal transport as one prominent “read-out” allowing for the elucidation of what may not be a transport dependent neurodegenerative pattern so much as a kinase dependent pattern of neurodegeneration. Alternatively, the role of kinase-induced alterations in the functions of molecular motors may have import for synaptic vesicle budding, Golgi-ER function, and critical steps in cytokinesis. In fact, the relationship between kinase activation or dysregulation and disease has long been a focus of cancer research, in which aberrant and pathogenic signal transduction has been identified in many cancers and the targets of many well-established and developing therapeutics. This long-help focus of cancer biology may be of benefit to the field of neuroscience, as an increasing number of neurodegenerative diseases have revealed alterations in signal transduction as a central pathogenic feature. For example, the pharmaceuticals company Cyclene is currently in clinical trials with CX-4945, a small peptide inhibitor of CK2 with the goal of using this oral chemotherapeutic as adjunct therapy for several solid-state tumors.
In actuality, the divide between the pathogenic mechanisms of tumorigenesis and neurodegenerative disease may be closing. For example, loss-of-function mutations in the gene coding for Ataxia Telangiectasia Mutated (ATM) results not only in degeneration of cerebellar neurons but also an increased risk of breast cancer and hematologic malignancy\textsuperscript{83}. The increased gene expression of beta amyloid in Down syndrome is another example. Patients with Down syndrome are at increased risk for early-onset Alzheimer’s-type dementia as well as acute leukemia and transient myeloproliferative disorders. Yet another example of this confluence is found in mutation of the gene that codes for Parkin, which results in half of the early onset inherited Parkinson’s cases. Parkin has also been identified as a tumor suppressor gene with its deletion or downregulation a key feature of several tumor types.

E. **Mechanistic Connections to Other Cellular Compartments**

By the same token, the distinction between axonal and synaptic pathologies may have more significance for study groups than for pathogenic mechanisms. As discussed earlier, the loss-of-function Kif5A mutation results in a full dying back degeneration, which involves both early axonal and synaptic degeneration. Other examples emphasize an axonosynaptic pathogenesis. Mutations in the Drosophila Kinesin-3, *immaculate connections (imac)*, result in failure of synapses to form despite normal motoneuron extension and guidance. Despite normal membrane addition and growth cone guidance, there is a decrease in active zone proteins and synaptic vesicles are absent\textsuperscript{84}. Another example involves syntabulin. Traditionally
syntabulin was considered an important active zone protein until work suggested that it may function to link Kif5B to the synaptic protein bassoon. RNAi against syntabulin results in failure of bassoon anterograde transport, further linking synaptic and axonal physiologies. Work from our lab has identified an isoform of the MAP kinase JNK in alterations in fast axonal transport in the context of Huntington’s disease, but this same kinase has also been associated with modifications of the Drosophila neuromuscular junction as well as the number of synapses formed. P-38, another kinase linked to neurodegenerative alterations in transport, has been shown to be required for presynaptic development.

What may, in a wide range of neurodegenerative diseases, draw the pathogenic mechanisms in axonal and synaptic compartments together is kinase dysregulation. This work has shown such a link, and is consistent with a large and growing body of literature making similar strides. Growing emphasis on kinase dysregulation may also provide a better understanding of the differential degeneration of certain cell populations that are a hallmark of almost all neurodegenerative disease. With a growing insight into the kinaseome of vulnerable neuronal population, not only can more light be shed on pathogenic mechanisms, but the effective therapeutics to address them.


CITED LITERATURE (continued)


CITED LITERATURE (continued)


CITED LITERATURE (continued)


CITED LITERATURE (continued)


NAME: Matthew Robert Burns

EDUCATION: B.A., Physics, Religion, Oberlin College, Oberlin, Ohio, 2001

M.D., University of Illinois at Chicago College of Medicine, Chicago, Illinois, 2014

Ph.D., Anatomy and Cell Biology, University of Illinois at Chicago College of Medicine, Chicago, Illinois, 2014

TEACHING: Professor of Neuroscience, Adjunct Faculty, Liberal Arts Department, School of the Art Institute of Chicago, Chicago, Illinois, 2011-2012

Teaching Assistant, Laboratory section, Human Neuroanatomy Course, University of Illinois at Chicago College of Medicine, Chicago, Illinois, 2008

Course Instructor, MCAT and GRE preparation, Kaplan Test Preparation, Chicago, Illinois, 2004-2005


HONORS: Medical School Class Secretary, University of Illinois at Chicago College of Medicine, Chicago, Illinois, 2006-2007

Medical School Student Summer Research Fellowship, American Academy of Neurology, Chicago, Illinois, 2006

General Assembly Scholarship, Chicago, Illinois, 2005-2006

PROFESSIONAL MEMBERSHIP: Society for Neuroscience

American Society for Neurochemistry

VITA (continued)


PUBLICATIONS:


CHAPTERS: