The Cellular and Molecular Pathology of the Motor System in Hereditary Spastic Paraparesis due to Mutation of the Spastin Gene

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Abstract. Hereditary spastic paraparesis (HSP) is a genetically heterogeneous disorder, the most common cause of which is mutation of the spastin gene. Recent evidence suggests a role for spastin in microtubule dynamics, but the distribution of the protein within the CNS is unknown. The core neuropathology of HSP is distal degeneration of the lateral corticospinal tract and of fasciculus gracilis, but there are few neuropathological cases of cases with a defined mutation. We aimed to determine the distribution of spastin expression in the human CNS and to investigate the cellular pathology of the motor system in HSP due to mutation of the spastin gene. Using an antibody to spastin, we have carried out immunohistochemistry on postmortem brain. We have demonstrated that spastin is a neuronal protein. It is widely expressed in the CNS so that the selectivity of the degeneration in HSP is not due to the normal cellular distribution of the protein. We have identified mutation of the spastin gene in 3 autopsy cases of HSP. Distal degeneration of long tracts in the spinal cord, consistent with a dying back axonopathy, was accompanied by a microglial reaction. The presence of novel hyaline inclusions in anterior horn cells and an alteration in immunostaining for cytoskeletal proteins and mitochondria indicates that long tract degeneration is accompanied by cytopathology in the motor system and may support a role for derangement of cytoskeletal function. All 3 cases also demonstrated evidence of tau pathology outside the motor system, suggesting that the neuropathology is not confined to the motor system in spastin-related HSP.

INTRODUCTION

Hereditary spastic paraparesis (HSP), or Strümpell-Lorrain syndrome, is a heterogeneous group of hereditary disorders of the central nervous system in which the primary clinical manifestation is a slowly progressive, bilateral, spastic paraparesis (1). HSP may occur in a clinically pure form, or complicated by additional manifestations such as seizures, dementia, extrapyramidal disturbances, peripheral neuropathy, and amyotrophy (2, 3). Even in pure HSP, sensory findings may be present on clinical examination, particularly in cases with a later disease onset (4). Application of more detailed testing has revealed involvement of different systems within the central nervous system (5), suggesting that even in pure HSP the pathology is not confined to the motor system, blurring the distinction between pure and complex forms.

The detailed neuropathology of HSP has been studied in relatively few cases, perhaps because the disease causes little impairment of life expectancy and patients usually die at home or in nursing care (1). The core feature of the neuropathology is pallor of the lateral corticospinal tracts and, more variably, the anterior corticospinal tracts within the spinal cord. There is loss of both myelin and axons that becomes most marked in the thoracic and lumbar cord. The dorsal columns are also involved, particularly the fasciculus gracilis at cervical levels, with sparing of fasciculus cuneatus. The spinocerebellar tracts are variably involved (6–9). Loss of Betz cells from the motor cortex has occasionally been reported (10) but anterior horn motor neurones are generally reported to be normal. The consistently distal pattern of involvement of the corticospinal tracts and the dorsal column pathway has led to the suggestion that the pathology of HSP represents a dying-back axonopathy.

HSP shows considerable genetic heterogeneity so that a genetic classification of HSP is emerging (11, 12). Nineteen different genetic loci have been identified to date and include autosomal dominant, autosomal recessive, and X-linked patterns of inheritance (13, 14). Genes identified to date include spastin, spartin, kinesin, heat shock protein 60 (hsp60), atlastin, paraplegin, L1 cell adhesion molecule (L1 CAM), and proteolipid protein. Putative functions of these various gene products suggest a number of possible routes to the pathology seen in HSP. Mutations in the L1CAM gene and the proteolipid protein gene have been suggested to cause impaired corticospinal tract development and impaired axo-glial interaction respectively (15). Mutations in the paraplegin gene and in the mitochondrial chaperonin hsp60 suggest a role for impairment of mitochondrial function (16, 17). Mutation in the motor domain of the neuronal kinesin heavy chain, a molecular motor involved in anterograde axonal transport of organelles, suggests a failure of axonal transport as a further putative mechanism (18).

Mutations at the SPG4 locus on chromosome 2p, encoding the spastin protein, is the single most common
cause of HSP, accounting for approximately 45% of autosomal dominant HSP (19). Spastin belongs to the AAA (ATPases associated with diverse cellular activities) family of proteins. The proteins share a common AAA motif and are all magnesium-dependent ATPases. AAA proteins have been described with a wide variety of functions, including metalloproteases, involvement in vesicle and organelle biogenesis, cell cycle regulators, and as components of the 26S proteasome (20). The normal function of spastin currently remains uncertain. The protein has a predicted nuclear localization sequence, suggesting a nuclear function (19). Charvin and colleagues have demonstrated a nuclear localization of spastin with a spastin antibody in HeLa cells and in various mouse tissues (21). These data were contrary to the work of Errico et al who demonstrated a cytoplasmic localization of tagged spastin and suggested a role for spastin in the regulation of microtubule dynamics and interaction with α and β-tubulins (22).

There are few reports of HSP neuropathology in which the gene defect has been characterized so that it is unclear whether the neuropathology varies according to the underlying gene defect. Such data will be required to determine the pathogenesis of corticospinal tract degeneration in different genetic forms of HSP and to allow validation of experimental models of the disease. The aims of this study are to report the molecular neuropathology found within the motor system of 3 cases of HSP due to defined mutations in the spastin gene. Using immunohistochemistry to the spastin protein, we also provide novel data on the distribution of spastin in the normal human nervous system and in HSP due to mutation of SPG4.

MATERIALS AND METHODS

Cases

All HSP cases and control tissues were obtained from the Sheffield Brain Tissue Bank and the Newcastle Brain Tissue Bank. HSP cases were identified from the North of England HSP Database, which contains detailed clinical information and stored DNA on individuals with HSP. Tissue donations were obtained with next-of-kin consent under the most current MRC guidelines for tissue banking. Case details are given in Table 1.

Genotyping

DNA was extracted from either EDTA anticoagulated blood or from fresh-frozen CNS tissue using the Nucleon blood kit or the Nucleon soft tissue kit (Scotlab, Coatridge, UK), respectively, as per manufacturer’s protocol. DNA was screened for mutation in the spastin gene using single stranded conformation polymorphism and mutations confirmed by direct sequencing using methods previously published (23).

Generation and Characterization of Anti-Spastin Antibody

A synthetic peptide corresponding to residues 561–573 of human spastin (Accession No. Q9UBP0) with an amino-terminal cysteine [(C)IRELKEQVKNMS] was synthesized by Dr A. J. Moir, Molecular Biology and Biotechnology, University of Sheffield. The peptide was coupled to SuperCarrier cationized BSA (Pierce, Rockford, IL) and rabbit polyclonal anti-spastin antibodies generated at The Antibody Resource Centre, University of Sheffield in accordance with Home Office guidelines. Antibodies were affinity purified from immune serum using a column containing the synthetic peptide immobilized on SulfoLink gel (Pierce). The specificity of affinity-purified anti-spastin antibodies was characterized by immunoblotting human occipital cortex extracts. Human tissue was homogenized using a tight-fitting glass-Teflon homogenizer in 10 volumes (w/v) of 50 mM Tris-HCl, 0.1 mM Na₂EDTA, 5 mM dithiothreitol, 0.5% Triton X-100, 0.1% Brij 35, 2 mM MnCl₂ (pH 7.5) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Lewes, UK). Protein concentration was assayed by the dye binding method (Pierce) using bovine serum albumin as standard. Protein extracts (45 μg per lane) were resolved on 10% polyacrylamide-SDS gels under reducing conditions, then transferred to Immobilon-P (Millipore, Bedford, MA). Membranes were probed with the anti-spastin antibody at a 1:500 dilution; signal specificity was confirmed by preincubation of the antibody with 100 μg/ml peptide on ice for 1 h.

Histological Evaluation

The following blocks were selected from each case to represent the motor system of the CNS: motor cortex, midbrain,

<table>
<thead>
<tr>
<th>Case number</th>
<th>Tissue</th>
<th>Age at death</th>
<th>Sex</th>
<th>Diagnosis</th>
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<tbody>
<tr>
<td>HSP Case 1</td>
<td>Brain and cord</td>
<td>74</td>
<td>M</td>
<td>HSP</td>
</tr>
<tr>
<td>HSP Case 2</td>
<td>Brain and cord</td>
<td>70</td>
<td>M</td>
<td>HSP</td>
</tr>
<tr>
<td>HSP Case 3</td>
<td>Brain and cord</td>
<td>74</td>
<td>M</td>
<td>HSP</td>
</tr>
<tr>
<td>Control case 1</td>
<td>Brain</td>
<td>82</td>
<td>F</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>Control case 2</td>
<td>Brain</td>
<td>71</td>
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</tr>
<tr>
<td>Control case 3</td>
<td>Brain</td>
<td>63</td>
<td>M</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Control case 4</td>
<td>Spinal cord</td>
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<td>Spinal cord</td>
<td>53</td>
<td>M</td>
<td>Ischemic heart disease</td>
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</table>
medulla, and spinal cord at cervical, thoracic, and lumbar levels. Serial sections were cut and histological investigation performed on all blocks. In addition, for immunohistochemistry to spastin protein, blocks ofpons, hippocampus, basal ganglia, thalamus, association cortex, and cerebellum were selected from controls, and, where available, from disease cases. Tau immunohistochemistry was performed on blocks of association cortex in addition to the motor system blocks of HSP cases, where available (case 1—frontal parasagittal, lateral parietal; case 2—frontal convexity and temporal; case 3—occipital, medial temporal, and hippocampus).

Basic morphology was assessed using hematoxylin and eosin-stained sections and preparations for myelin. Immunohistochemistry was performed on paraffin sections, following dewaxing, using the streptavidin-biotin ABC method and the signal visualized using diaminobenzidine. Primary antibodies used are listed in Table 2. The specificity of immunostaining with the anti-spastin antibody on paraffin-embedded tissues was determined by preincubation of the antibody with spastin peptide at 1.25 μg/ml for 2 h at room temperature. To exclude the possibility of a nonspecific inhibitory effect of the spastin peptide, immunostaining was also carried out on nonphosphorylated neurofilament protein following preincubation with spastin peptide.

Immunocytochemistry was performed using glial fibrillary acidic protein (GFAP) for reactive astrocytes, CD68 for microglia, ubiquitin, abnormally phosphorylated tau using AT8, and α-synuclein (on midbrain and frontal cortex). The cytoskeleton was studied using antibodies to phosphorylated and nonphosphorylated neurofilament protein, and to α, β-tubulin and acetylated tubulin. Acetylated tubulins are usually enriched in a subpopulation of stable microtubules, although acetylation does not seem to cause this stabilization directly (24, 25). Morphological evidence of disturbed axoplasmic flow was sought using antibodies to amyloid precursor protein (APP). The possibility of an alteration in the distribution of cellular organelles was assessed by immunohistochemistry to mitochondria using a commercially available mouse monoclonal antibody. This recognizes a 60-kDa component of human mitochondria (MAB1273, Chemicon International, Temecula, CA) and has been previously used for immunohistochemistry in human material (26).

**RESULTS**

**Mutation Analysis**

The clinical details and the genotypes of cases 1 and 2 have been published previously (23, 27). Briefly, case 1 demonstrated a missense mutation (R424G) within the highly conserved AAA domain. The individual had begun with a progressive spastic paraparesis compatible with pure HSP at the age of 23; however, in the 2 years before his death he suffered a fairly rapid cognitive decline. Another affected family member had a cognitive problem with generalized impairment of verbal learning, nonverbal learning, and memory that were detected as learning problems in childhood. The relevance of these difficulties to her HSP is not known. Otherwise the phenotype in this large multi-generational autosomal dominant pedigree was consistent with pure HSP. The age of onset of symptoms ranged from within the first 2 years of life to 20 years. Case 2 demonstrated a nonsense mutation (S245stop), truncating the spastin protein by almost two thirds. The individual developed a progressive spastic paraparesis at the age of 50. The clinical findings were in keeping with pure HSP as they were in the other members of this large family. No cognitive problems were reported in this family. Case 3 demonstrated a novel nonsense mutation 1292–1293delaa causing a frameshift and premature truncation within the conserved AAA cassette. The phenotype in this individual was of pure HSP. He began with walking difficulties in his early twenties. He had a progressive spastic paraparesis with associated urinary frequency but remained ambulant until his death at the age of 74 years. There was no history of cognitive

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**TABLE 2**

Antibodies and Conditions for Immunohistochemistry*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Antigen retrieval</th>
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<td>O/N 4C</td>
<td>MW 10 min citrate</td>
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<td>Tau-AT8</td>
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<td>MW 10 min citrate</td>
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<td>30 min RT</td>
<td>MW 10 min citrate</td>
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<tr>
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<td>mc</td>
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<tr>
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<td>mc</td>
<td>Sigma</td>
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<td>MW 10 min citrate</td>
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<tr>
<td>Acetylated tubulin</td>
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<td>NFP-NP</td>
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<tr>
<td>Mitochondria</td>
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<td>MW 10 min citrate</td>
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</table>

* Abbreviations: mc, monoclonal; pc, polyclonal; RT, room temperature; O/N, overnight incubation; MW, microwave; GFAP, glial fibrillary acidic protein; NFP-P, neurofilament protein, phosphorylated epitopes; NFP-NP, neurofilament protein, nonphosphorylated epitopes; APP, amyloid precursor protein.
Fig. 1. Specificity of spastin detection. A: Immunoblots of human occipital cortex extracts probed with antibody raised against synthetic peptide corresponding to residues 561–573 of human spastin. Molecular species with apparent molecular masses of approximately 75 and 80 kDa were detected (left panel), and the signals were completely abolished by preincubation of the antibody with the peptide immunogen (right panel). B: Immunohistochemistry to spastin in control spinal cord showing strong reactivity of anterior horn cells (top panel). The staining is abrogated by preincubation with spastin peptide.

Specificity of Anti-Spas tin Antibody

Immunoblotting of human occipital cortex extracts with the anti-spastin antibody detected a major species with an apparent molecular mass of approximately 75 kDa and a minor species with an apparent molecular mass around 80 kDa (Fig. 1A, left panel). Charvin et al (21) also detected 75- and 80-kDa species in multiple human tissues using an antibody raised against residues 129–143 of human spastin, which they attributed to alternative splicing of exon 4. We found that overexposure of immunoblots revealed a short ladder of higher molecular mass species consistent with multiple phosphorylation states or ubiquitin-like modification of spastin (not shown). All signals were abolished by preincubation of the antibody with the synthetic peptide immunogen (Fig. 1A, right panel).

Preabsorption with spastin peptide resulted in complete abrogation of staining with immunohistochemistry using anti-spastin antibody on control spinal cord (Fig. 1B).

Expression of Spastin in the Normal Human CNS

Expression of spastin in control cases was confined to neurons and no staining was observed in glial cells. Expression was greatest within the soma where immunoreactivity was predominantly diffuse. Expression was widespread throughout the brain and spinal cord and not confined to the motor system. In motor cortex, pyramidal neurons, Betz cells, and a proportion of small neurons demonstrated cytoplasmic expression with extension into proximal neurites, including apical dendrites (Fig. 2A). There was diffuse synaptic staining in the neuropil. Nuclear staining was observed in a proportion of cells. This tended to be diffuse, but occasionally a punctate pattern was seen. The hippocampus demonstrated a similar staining pattern with strong expression in pyramidal cells and dentate granule neurons. Basal ganglia demonstrated relatively strong nuclear staining of neurons. Within the cerebellum there was expression in the cytoplasm of dentate nucleus and Purkinje neurons. There was labeling of nuclei in the cerebellar molecular layer and a proportion of...
granule layer neurons (>50%) showed nuclear labeling with a punctate pattern. Within the brainstem, large motor neurons in the nuclei of cranial nerves III, VI, VII, and XII showed a strong, tigroid pattern of staining, similar in distribution to that of their coarse Nissl substance. Staining was also observed in nonmotor neuronal groups such as inferior olive and lateral cuneate nucleus. Within basis pontis, some neurons demonstrated intra-nuclear rod or dot patterns of staining. Strong staining of large neurons was observed within the grey matter of the spinal cord associated with strong staining of neurites (Fig. 2B). Anterior horn motor neurons demonstrated a coarse Nissl-like pattern of cytoplasmic expression with no nuclear expression. A symmetrical diffuse synaptic pattern...
of staining was observed in the posterior horns in the region of the substantia gelatinosa.

No expression of spastin was demonstrated in cerebral white matter. Weak staining, apparently in large axons, was observed in the posterior limb of the internal capsule but not in the anterior limb. Weak axonal expression was demonstrated in pons, medullary pyramids, medial lemniscus within the medulla, and in the white matter of the spinal cord, in dorsal, lateral, and ventral columns (Fig. 2C). Weak axonal expression was also noted in spinal nerve roots.

Expression of Spastin in the CNS of SPG4-Related HSP

Expression of spastin protein in the 3 cases of hereditary spastic paraparesis was similar to the control cases. Case 3 consistently showed stronger immunoreactivity than cases 1 and 2 or controls and the most conspicuous staining of intranuclear rods and dots in pontine and cortical neurons (Fig. 2D). Granulovacuolar degeneration, present in the hippocampal pyramidal neurons of case 3 showed strong spastin expression (Fig. 2E). All 3 disease cases showed areas of loss of Nissl substance from the anterior horn motor neurons of the spinal cords (Fig. 2F). These areas were also devoid of spastin staining and occasional motor neurons showed very little spastin expression. As in the controls, disease cases demonstrated weak axonal expression.

Cellular Pathology of SPG4-Related HSP

Pathology of the Neuronal Soma: Motor cortices were unremarkable on conventional stains, with no abnormalities of Betz cells identified. The anterior horns of the spinal cord showed no loss of motor neurons and no evidence of gliosis or microglial reaction in grey matter. However, occasional motor neurons in all 3 cases demonstrated the formation of large, pale, cytoplasmic hyaline inclusions with distinct edges and no halo formation (Fig. 3A). They did not label for ubiquitin, tau, neurofilament protein, or spastin, but occasional inclusions were observed that labeled for β-tubulin (Fig. 3B). Many spinal and hypoglossal motor neurons appeared pale centrally with peripheral displacement of Nissl substance accompanied by a reduction in cell body staining for β-tubulin and for nonphosphorylated neurofilament protein compared to controls and a displacement of these elements towards the periphery of the cells (Fig. 3C, D). There was an increase in the amount of granular PAS-positive material in many of the anterior horn cells, suggesting an increase in lipofuscin. Occasional chromatolytic motor neurons were observed in case 3. In case 1, motor neurons of the oculomotor nerve demonstrated vacuolation.

Immunohistochemistry to mitochondria demonstrated a strong punctate pattern of staining in the cytoplasm of neurons, their neurites, and in the cytoplasm of other cell types, including glia. Similar staining patterns in neuronal cell bodies were observed in disease and control cases in motor cortex, midbrain, and medulla. However many of the motor neurons of the anterior horns of the spinal cords demonstrated a markedly reduced staining, sometimes with staining confined to an outer rim of cytoplasm, similar to the altered distribution of staining for spastin, neurofilament protein, and tubulin (Fig. 3E, F). Although associated with an increase in PAS-positive lipofuscin in some cases, areas of lipofuscin in controls did not exclude mitochondrial staining, and comparison of cells represented on serial sections suggested that lipofuscin did not account for the changed distribution of this organelle.

Many pale, rounded structures were present in the midbrain and spinal cord of case 3. These were present mostly in grey matter and were not prominent in the corticospinal tract. These structures stained strongly with the PAS method and the presence of similar structures in control cases, although to a lesser extent in grey matter, suggested that they represented a form of corpora amylacea. They labeled poorly with antibodies to ubiquitin but stained with antibodies to both phosphorylated and nonphosphorylated epitopes of neurofilament proteins (Fig. 3D)

Tau pathology was present in all 3 cases (Table 3). Tau-positive glial cells were seen in cerebral white matter from case 1 (Fig. 4A). Case 1 demonstrated occasional neurofibrillary tangles and neuropil threads in the motor cortex. Tau positivity in a pattern resembling tufted astrocytes and astrocytic plaques were seen in motor and association cortex of cases 1 and 2 (Fig. 4B). Neurofibrillary tangles and neuropil threads were present in the CA1, 2 and 3 regions of the hippocampus of case 3, but did not extend into medial temporal isocortex. Neurofibrillary tangle formation was not a feature of association cortex in any of the cases and neuritic plaques were not seen. Tau pathology was present in midbrain and medulla of all 3 cases and was maximal in case 1, consisting of neurofibrillary tangles, neuropil threads, and tufted astrocytes (Fig. 4C). Tau pathology was observed in motor neurons of the spinal cord in case 3, ranging from a fine reticular distribution to an occasional globular tangle (Fig. 4D). Only an isolated neuropil thread was seen in the cord from case 1 and no tau pathology was seen in the spinal cord in case 2. Tau pathology was not observed in the white matter of motor tracts. Ubiquitin immunohistochemistry labeled occasional thickened neurites in the midbrain of case 1, but otherwise did not identify any specific cytopathology.

Pathology of the Corticospinal Tract: Myelin stains demonstrated pallor of the lateral corticospinal tract at thoracic and lumbar levels of the spinal cord (Fig. 5A). Mild pallor of fasciculus gracilis at cervical levels was
noted, as was mild pallor in the posterior spinocerebellar tracts. Case 2 also showed pallor of the anterior corticospinal tract. There was little evidence of upregulation of GFAP to indicate an astrogliosis, but there was an increase in CD68 staining in the lateral corticospinal tract consistent with a mild microglial reaction to tract degeneration (Fig. 5B).

Pallor of myelin staining distally was accompanied by pallor of staining for phosphorylated epitopes of neurofilament protein and tubulin, representing loss of axonal profiles, and indicating that myelin loss has occurred in the context of axonal degeneration (Fig. 5C–F). Neurofilament protein otherwise demonstrated a normal distribution of staining, with nonphosphorylated epitopes expressed

Fig. 3. Lower motor neuron cytopathology. A: Hyaline inclusion in anterior horn motor neuron, case 1. B: Inclusion labeled for β-tubulin, motor neuron in lumbar cord of case 3. C: Antibodies to nonphosphorylated neurofilament protein in control case showing strong labeling of neuronal cytoplasm and proximal neurites. D: In HSP there is a patchy central loss of staining for nonphosphorylated neurofilament protein and 1 neuron appears negative; case 3, lumbar cord. E: Immunohistochemistry to mitochondria, demonstrating a granular staining pattern in control case. F: Patchy loss of staining for mitochondria in anterior horn motor neurons, HSP case 1, cervical cord.
### TABLE 3
Distribution of Neurofibrillary Tangles in HSP Cases*

<table>
<thead>
<tr>
<th>Case</th>
<th>Hippocampus</th>
<th>Entorhinal cortex</th>
<th>Association cortex</th>
<th>Motor cortex</th>
<th>Midbrain</th>
<th>Medulla</th>
<th>Spinal cord</th>
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<tr>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>i</td>
<td>+</td>
<td>++</td>
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<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* NA, not available. 0 = none; i = isolated; + = small; ++ = moderate; +++ = large numbers of neurofibrillary tangles (42).

**Fig. 4.** Tau immunohistochemistry. **A:** Tau-positive glial cell in cerebral white matter, case 1. **B:** Glial tau in frontal association cortex of case 2. **C:** Glial (above) and neuronal tau, midbrain tectum of case 1. **D:** Tau-positive motor neuron, spinal cord of case 3.

Predominantly in perikarya and proximal neurites, and phosphorylated epitopes within axons. No intronaxonal accumulations of neurofilament proteins were identified within long tracts. Similarly, immunohistochemistry to APP demonstrated no evidence of upregulation in cell bodies or of spheroid formation. Pallor of staining was also noted in the lateral corticospinal tracts in the thoracolumbar regions in the HSP cases with immunohistochemistry to β-tubulin, associated with a reduction in the number of axonal profiles. While α and β-tubulin were expressed ubiquitously in cell bodies and neurites, immunohistochemistry to acetylated tubulin demonstrated expression in neurites, but variable expression in neuronal perikarya, although no differences were detected between control and disease cases.

Mitochondria in white matter were observed in glial cell bodies in a dispersed punctate pattern, presumably in axons as well as in glia. There was no reduction in staining in the distal parts of the corticospinal tract in the HSP cases.

**DISCUSSION**

Using immunohistochemistry in human tissue, we have shown that spastin is a neuronal protein with no evidence of expression in glial cells. The protein is predominantly expressed in the cytoplasm, and in large motor neurons it adopts a tigroid expression pattern in the distribution of Nissl substance. Some extension of staining into proximal neurites was observed and staining was noted in axons of long tracts. Cell transfection studies

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Fig. 5. White matter pathology of the spinal cord. A: Low-power view of a myelin stain of the thoracic spinal cord of case 2. There is pallor of the lateral corticospinal tract (CST) and adjacent posterior spinocerebellar tract (1), fasciculus gracilis (2) and the anterior CST (3). B: Antibodies to CD68 demonstrate an upregulation of microglia in the CST in HSP, lumbar cord, case 3. C: Neurofilament protein (phosphorylated) expression in control CST, lumbar cord. D: Reduction in neurofilament protein expression in the CST in HSP, lumbar cord, case 3. E: β-tubulin expression in control CST. F) Reduction in β-tubulin expression in the CST in HSP, case 2.

support a cytoplasmic localization and suggest an association with microtubules (22). However, a proportion of neurons showed a nuclear staining pattern. A nuclear localization signal has been identified in the protein from sequence analysis (19), suggesting that the protein may have a nuclear function in some cells. Charvin et al (21) have also identified a nuclear localization using a spastin antibody in HeLa cells and in neurons in murine spinal cord with no cytoplasmic expression, in contrast to our findings. It is possible that the subcellular distribution of the protein varies between neuronal cell groups, and perhaps species. Differences in distribution may also be related to the detection of different isoforms of the protein with different antibodies. Nuclear staining in our cases
was generally diffuse, but sometimes a punctate pattern was observed. In some neurons, particularly in the pons, intranuclear rod-like structures or dot-like staining was seen. These structures are reminiscent of rod-like neuronal intranuclear inclusions, containing β-tubulin, which may be altered in neurological diseases such as Alzheimer disease (28, 29).

The expression of spastin was not confined to the motor system. The pattern of selective vulnerability in HSP is therefore not determined simply by the normal distribution of the protein. In this regard, HSP is similar to other degenerative diseases of the motor system, such as superoxide dismutase 1-related motor neuron disease, spinal muscular atrophy, and X-linked spinobulbar muscular atrophy where the relevant proteins, superoxide dismutase 1, the survival motor neuron protein and the androgen receptor, respectively, are widely expressed (30–34).

The mutations in our cases were either missense or truncating in type. A variety of mutation types have been described in the spastin gene, including missense, nonsense, frameshift, and splice-site types (23, 35–38). The mutations predict either a truncated or severely altered protein, suggesting that haploinsufficiency, resulting in reduction of function, is responsible for the disease phenotype. However, transfection of mutant spastin results in an alteration of the microtubule cytoskeleton so that the possibility of acquisition of an abnormal function by mutant spastin remains (22). Overall, the pattern of spastin expression in our study was qualitatively similar in both disease cases and controls. However, many anterior horn motor neurons in the disease cases showed areas devoid of both Nissl substance and spastin, and some neurons were virtually spastin-negative, a change that paralleled that of related cytoskeletal proteins (see below).

There are few reports of neuropathological findings in HSP where the underlying mutation has been determined. We have studied the cellular and molecular neuropathology of the motor system in 3 HSP cases with defined mutations in the spastin gene. As previously described (6–8), our cases demonstrated degeneration of the lateral corticospinal tract and fasciculus gracilis, particularly in their most distal parts, as the principal finding with conventional stains. Myelin loss was accompanied by a decrease in neurofilament and tubulin-positive axonal profiles distally, consistent with the concept that the long tract pathology represents a dying back axonopathy. No spheroids or change in cytoskeletal protein distribution was observed and the pathology of this slowly evolving process is subtle. We did, however, demonstrate an increased expression of CD68 in the distal corticospinal tract. Microglial upregulation occurs in corticospinal tract degeneration in other diseases, such as motor neuron disease (39), where it may occur as an early indicator of tract involvement (40). There is also evidence, therefore, of a microglial reaction to the tract degeneration that occurs in HSP.

We have also demonstrated cytopathology in HSP. Although not prominently affected clinically, anterior horn motor neurons of the spinal cord demonstrate the formation of hyaline inclusion bodies in SPG4 patients that are unlike those seen in motor neuron disease (39). Their composition is uncertain, but staining for β-tubulin suggests that microtubular elements may be involved. In addition, anterior horn cells showed variable loss of immunostaining of nonphosphorylated neurofilament protein, β-tubulin, spastin, and mitochondria from their perikarya. This is difficult to evaluate in autopsy studies, but may suggest an altered partitioning of cytoskeletal components and organelles. An increase in lipofuscin staining does not appear to be able to account for all of these changes, which were not observed in relation to the presence of lipofuscin in control cases.

These findings lend support to the hypothesis that cytoskeletal function may be perturbed in HSP. There is evidence that spastin is involved in microtubule dynamics and may have a role in microtubule disassembly, while mutant spastin induces an alteration in microtubule distribution in cell transfection studies (22). Such processes may be particularly critical in motor neurons, which maintain long axons. Thus transport failure is a candidate mechanism for the dying back axonopathy of HSP. The possibility that this may be a general pathogenetic mechanism for some forms of HSP is supported by the recently described mutation in the neuronal kinesin heavy chain gene KIF5A, which codes for a motor protein involved in anterograde axonal transport that is linked to the SPG10 locus (18). Spartin, mutated in Troyer syndrome (SPG20), may also be involved in microtubule function (14).

A primary mitochondrial defect has not been demonstrated in HSP due to spastin mutations (41), in contrast to HSP due to mutation in the gene for the mitochondrial protein paraplegin, in which muscle mitochondria have defects in oxidative phosphorylation (16). It has been suggested, however, that perturbed axoplasmic flow may be important in impaired delivery of organelles, particularly mitochondria, to the distal axon (18), thus resulting in a localized deficit of mitochondrial function.

Our observational data would support the possibility of derangement of the cytoskeleton, with some evidence for altered mitochondrial distribution. We did not demonstrate loss of mitochondria from the distal corticospinal tract, but microglial upregulation may have been responsible for the presence of mitochondrial immunoreactivity in this area, and given the loss of axons at this stage of the disease, it will be difficult to test this hypothesis in
postmortem material. The significance of apparently altered patterns of cellular components determined by immunohistochemistry on autopsy material is difficult to determine and we have not had suitable material for ultrastructural investigation. The observations, however, raise hypotheses that may be investigated further in experimental systems. We have recently shown an alteration in mitochondrial distribution in a cell culture model of HSP, based on transfection of mutant spastin (McDermott et al, manuscript in preparation), suggesting that an altered distribution of cellular components may occur in HSP.

All 3 of our cases also showed tau pathology. The presence of extensive tau pathology has been previously reported for Case 1 (27). The tau pathology consisted of neurofibrillary tangles, neuropil threads, and glial tau pathology, including some white matter tau pathology. Neuritic plaques, however, were not evident. This case showed a late onset dementia in life, but the tau pathology is unlikely to be related to Alzheimer disease (27). Tau pathology was most conspicuous in the brainstem in all cases, especially the midbrain, and was also observed in occasional spinal cord motor neurons. In contrast, neurofibrillary tangle formation was not a feature in association cortex. Thus, these cases did not show associated Alzheimer disease, and, although not formally blocked for scoring, would have only represented approximately Braak stage II (42). In addition to the distribution of the tau pathology, tufted astrocytes and astrocytic plaques are not a feature of Alzheimer disease or brain aging and are reminiscent of those seen in progressive supranuclear palsy and corticobasal degeneration (43). Similar tau pathology was not observed in control cases, which were of a similar age, and with the limited blocks examined would also have achieved a low Braak score (I to II) for tangles. Thus, although not abundant, the distribution and pattern of tau pathology in the HSP cases was unusual and not typical of age-related change or coexistent Alzheimer disease.

Tau is a microtubule-associated protein that is involved in microtubule assembly and stabilization. The mechanisms resulting in its abnormal phosphorylation and its pathogenetic role in various neurodegenerative diseases remain unclear (44). We speculate that the tau pathology in SPG4-related HSP is a further manifestation of cytoskeletal dysfunction in this disorder and may relate to altered microtubule function or stability. The presence of tau pathology in nonmotor systems and, probably, in glial cells implies that the cytoskeletal dysfunction in SPG4-related HSP is not confined to the motor system. Subclinical involvement of nonmotor systems of the CNS is described in cases of pure HSP (5) and cognitive impairment may occur in SPG4-linked HSP (45–47). Prefrontal atrophy, loss of frontal white matter, atrophy of deep grey nuclei, and loss of neurons from atrophic cortex have been described as a correlate of severe mental impairment in HSP (48). We have also previously reported that Case 1 showed α-synuclein-positive Lewy bodies in the substantia nigra (27). We did not demonstrate α-synuclein pathology in the other 2 HSP cases, and it is possible therefore that Lewy body formation in Case 1 is an incidental finding.

In conclusion, to our knowledge this is the first report of the detailed molecular pathology of HSP cases with identified mutations in spastin. In addition to a distal axonopathy of the corticospinal tracts, lower motor neurons demonstrate evidence of a cytopathology. The changes observed lend some support to the concept that the pathogenesis of SPG4-related HSP involves cytoskeletal dysregulation. Experimental studies are required to address the effects of mutant spastin on the cytoskeleton, in particular microtubules, and on the distribution and transport of organelles within neurons. In view of the cytopathology observed in lower motor neurons, it would be also be worth investigating whether there is evidence, clinically and neurophysiologically, that lower motor neuron dysfunction develops later in the course of the disease. The findings in this study also support the concept that the neuropathology of HSP due to spastin mutation is not confined to the motor system and that it may be associated with tau pathology. More detailed studies of the molecular pathology of nonmotor systems within the CNS are warranted to understand the basis of the nonmotor clinical manifestations that may occur in SPG4-related HSP.

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REFERENCES


