Genetic Analysis of the Hereditary Spastic Paraplegias

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ABSTRACT

The Hereditary Spastic Paraplegias (HSP) comprise a group of neurodegenerative diseases characterized by progressive lower limb spasticity. This disease, with a prevalence ranging from 1 to 20 in 100,000 individuals, is currently untreatable. The neuropathological hallmark is axonal degeneration of motor neurons in the corticospinal tract. However, the mechanisms of pathogenesis underlying this neurodegeneration remain poorly understood. Over the last decade, genetic studies of HSP have identified 33 loci including 14 genes. The main objective of this dissertation was to identify and characterize genes in a large North American HSP cohort. Mutation analysis of the two most common genes implicated in HSP, *SPG3* and *SPG4*, led to the detection of nine novel mutations, including an ancestral *SPG4* mutation in five French Canadian families. This screen also allowed for the molecular characterization of the p.del436N mutation in *SPG3*, which suggests a previously unidentified dominant-negative mechanism. Furthermore, a novel deletion in the VPS9 domain of the *ALS2* gene was identified in a family with severe infantile onset HSP. In addition, linkage analysis and whole genome scan efforts resulted in the successful mapping of two novel HSP loci, *SPG27* and *SAX1*. *SAX1* represents the first locus for autosomal dominant spastic ataxia, a complicated form of HSP, with a common ancestor in Newfoundland. Finally, a positional candidate gene strategy at the *SPG8* locus identified three missense mutations in a novel gene encoding strumpellin. Two mutations failed to rescue an axonal phenotype induced by morpholino knock-down of the *SPG8* gene in zebrafish. Our efforts to identify and characterize HSP genes determined the underlying genetic cause in 36% of our cohort. These genetic causes include two novel loci and a novel

gene. The findings are a major contribution to the characterization of the pathophysiology of HSP and significantly broaden the knowledge in the field of motor neuron disease. Analysis of the 15 known HSP genes suggests a common disease mechanism involving disrupted axonal membrane protein trafficking. Unraveling this mechanism will elucidate the functional maintenance of neurons in the corticospinal tract and will facilitate the development of therapies for HSP and related diseases.

RÉSUMÉ

La paraplégie spastique héréditaire (PSH) représente un groupe de maladies neuro-dégénératives caractérisées par une spasticité progressive des membres inférieurs. La prévalence de cette maladie, pour laquelle il n'existe aucun traitement, varie de 1 à 20 individus par 100 000 personnes. Bien que la principale caractéristique de la PSH soit une dégénérescence des neurones moteurs de la voie pyramidale, les mécanismes responsables de cette dégénérescence demeurent largement incompris. Au cours de la dernière décennie, l'étude génétique de la PSH a permis d'identifier 33 *loci* incluant 14 gènes.

L'objectif principal de ce projet est d'identifier et de caractériser des gènes responsables de la PSH dans une large cohorte de patients nord-américains. Ainsi, l'analyse de *SPG3* et *SPG4*, les deux gènes les plus souvent impliqués dans la PSH, a permis la détection de neuf nouvelles mutations, incluant une mutation ancestrale du gène *SPG4* dans cinq familles canadiennes-françaises. Ce criblage a aussi mené à la caractérisation moléculaire de la mutation p.del436N du gène *SPG3* qui suggère, pour la première fois, un mécanisme dominant négatif. Par ailleurs, une nouvelle mutation dans le domaine VPS9 du gène *ALS2* a été identifiée chez une famille souffrant de PSH infantile sévère. Des analyses de liaison génétique et le criblage du génome ont mené à l'identification de deux nouveaux *loci* : *SPG27* et *SAX1*. Ce dernier *locus*, ayant une origine ancestrale à Terre-Neuve, est le premier à être associé à une forme autosomique dominante d'ataxie spastique, une forme complexe de PSH. Finalement, une approche positionnelle de type « gène candidat » au niveau du *locus SPG8* a rendu possible l'identification de trois mutations faux-sens au niveau du gène codant pour la

strumpelline. Deux de ces mutations n'ont pas pu secourir le déficit axonal induit par l'inhibition du gène *SPG8* dans le poisson zébré.

Nos efforts visant l'identification et la caractérisation des gènes responsables de la PSH ont été fructueux, nous permettant de démontrer les causes génétiques de la maladie dans 36% des familles étudiées et d'identifier deux nouveaux *loci* ainsi qu'un nouveau gène causal. Ces résultats représentent une contribution majeure dans la compréhension de la patho-physiologie de la PSH et ont pour effet d'accroître significativement les connaissances dans le domaine des maladies affectant les neurones moteurs. L'analyse des 15 gènes responsables de la PSH suggère un mécanisme pathologique commun, soit une perturbation du trafic protéique membranaire au niveau de l'axone. L'étude de ce mécanisme devrait permettre d'élucider les fonctions de maintien des neurones de la voie pyramidale et de faciliter le développement de traitements pour la PSH et d'autres maladies des neurones moteurs.

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Chapter 2.2:

Inge. A. Meijer, Nicolas Dupré, Bernard Brais, Patrick Cossette, St Onge J, Marie-France Rioux, Melanie Benard, and Guy A. Rouleau. Evidence for a *SPG4* founder effect in French Canada. Submitted to *Can J Neur Sci*, July 2006.

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Chapter 7:

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Reynolds and Drapeau: Zebrafish study design and morpholino injections.

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CLAIMS FOR ORIGINALITY

1. In the *SPG4* mutation screen, 8 novel mutations were identified contributing to the spectrum of spastin mutations. Furthermore, the frequency of spastin mutations in our cohort was determined. This frequency was also determined in the FC population and led to the identification of a common ancestral mutation.

2. The identification of an *ALS2* mutation in a Pakistani family with HSP confirmed the fact that the underlying phenotype at this locus is actually associated with severe upper motor neuron degeneration. Furthermore, the truncation mutation demonstrated the importance of the VPS9 domain in alsin function.

3. A novel HSP locus, *SPG27*, was identified in a FC family with pure recessive HSP. This study highlighted the possibility that HSP loci can be in close proximity to each other.

4. The identification of the novel locus, *SAX1*, for Hereditary Spastic Ataxia (HSA) showed that a clinically variable spectrum can be caused by mutations in the same locus. The clinical characterization of the disorder redefined this subgroup of HSP or autosomal dominant ataxia. Fine-mapping markedly narrowed down the disease gene carrying region and further candidate gene analysis will undoubtedly lead to the identification of the first dominant HSA gene.

5. Mutation analysis of *SPG3A* determined the low frequency of atlastin mutations in our cohort. Furthermore, this project led to the identification of a novel in-frame deletion mutation. Analysis of patients' lymphoblasts suggests that the mutation leads to the degradation of atlastin protein in a dominant-negative fashion. It was previously believed that the disease was caused by loss of GTPase function.

6. The *SPG8* project contributed to the field by adding a novel gene to group of genes mutated in HSP. Although the function of this gene remains largely unknown, we showed the functional effect of the mutations in a morpholino knock-down zebrafish model. This is the first zebrafish model for HSP.

7. This thesis included a first attempt to characterize the genetic basis of HSP in Canada. Two common ancestors were identified, in Newfoundland and Quebec. Furthermore, it was established that atlastin mutations are not a common cause of HSP in our North American cohort and that mutations in strumpellin are relatively frequent in Anglo-Canadians.

ABBREVIATIONS

AA	Amino Acid
AAA	ATPase with various cellular activities
ACC	Agenesis of the Corpus Callosum
ACP33	Acidic Cluster Protein 33
ADHSP	Autosomal Dominant Hereditary Spastic Paraplegia
ALS	Amyotrophic Lateral Sclerosis
AP2	Adaptor Protein Complex 2
ARHSP	Autosomal Recessive Hereditary Spastic Paraplegia
ATP	Adenosine Triphosphate
BBS	Bardet-Biedl Syndrome
BSCL	Berardinelli-Seip congenital lipodystrophy
CLSTN3	Calsyntenin 3
CMT	Charcot–Marie–Tooth
CRASH	Corpus callosum, mental Retardation, Adducted thumbs, Shuffling gait and Hydrocephalus
dHMN	distal Hereditary Motor Neuropathy
DRPLA	Dentatorubral pallidoluysian atrophy
EAAT2	Excitatory Amino Acid Transporter
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmatic Reticulum
ESCRT	Endosomal Sorting Complex Required for Transport
FC	French-Canadian
FSP	Familial Spastic Paraplegia
GAPD	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
HAM/TSP	HTLV1 Associated Myelopathy/ Tropical Spastic Paraparesis
HSA	Hereditary Spastic Ataxia
HSAS	Hydrocephalus due to Stenosis of the Aqueduct of Sylvius
HSP	Hereditary Spastic Paraplegia
HSP60	Heat-Shock Protein 60 also known as HSPD1
HSPB1	Heat-Shock Protein B1
HTLV	Human T Lymphotropic Virus
KCC3	potassium and chloride co-transporter
KIF	Kinesin Family member
L1CAM	L1 Cell Adhesion Molecule

MIT	Microtubule Interaction and Trafficking domain
LMN	Lower Motor Neuron
MND	Motor Neuron Disease
MOI	Mode of Inheritance
MR	Mental Retardation
MRI	Magnetic Resonance Imaging
MT	Microtubule
NMJ	Neuromuscular Junction
NT	Nucleotide
PCR	Polymerase Chain Reaction
PLP	Proteolipid Protein
PLS	Primary Lateral Sclerosis
PMD	Pelizaeus-Merzbacher Disease
Rec/RC	Recombinant
REEP1	Receptor Expression Enhancing Protein 1
RNAi	Ribonucleic Acid interference
RTN	Reticulon
SAX1	Spastic Ataxia 1
SBDS	Shwachman-Bodian-Diamond Syndrome
SCA	Spino-cerebellar Ataxia
SCI	Spinal Cord Injury
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron
SOD1	Super Oxide Dismutase
SPOAN	Spastic Paraplegia, Optic Atrophy, and Neuropathy
SPG	Spastic Paraplegia Gait
TCC	Thin Corpus Callosum
TGN	Trans Golgi Network
UMN	Upper Motor Neuron
USP5	Ubiquitin Specific Peptidase 5
VAMP1	Vesicle-Associated Membrane Protein 1
VAPB	Vesicle-Associated Membrane Protein B
VEGF	Vascular Endothelial Growth Factor
WGS	Whole Genome Scan

CHAPTER 1: GENERAL INTRODUCTION

1.1 PATHWAYS OF THE SPINAL CORD

Human walking is a voluntary rhythmic activity that results from intricate coordination of different muscles controlled by the spinal cord and brainstem. The spinal cord is a small tube of less than two centimeters in diameter that contains neuronal tracts protected by the vertebral column (Sheerin 2005). These neuronal pathways can be divided into the descending and ascending tracts (Lin 2003).

The motor system, which controls our voluntary movement, is part of the descending pathway. It commences in the cerebral cortex, which consists of the primary motor area, the premotor cortex, the supplementary and the postcentral areas. The neurons in the cerebral cortex control contractions of muscles (Gilman and Winans Newman 2003). Traditionally, the motor system can be divided into the pyramidal and the extra-pyramidal system (Figure 1). The pyramidal tract consists largely of the corticospinal tract and the corticobulbar tract, whereas the extra-pyramidal tract includes all other descending pathways. Certain fibers in the corticospinal tract cross over in the lower medulla and are called the lateral corticospinal tract. The fibers that don't cross over form the ventral corticospinal tract (Gilman and Winans Newman 2003).

The major motor neurons that connect the brain with the muscles to generate movement have their cell bodies in the brainstem and spinal cord. These neurons can be divided into the upper motor neurons (UMN) and the lower motor neurons (LMN) (Figure 1). The UMNs originate in the brain and innervate the LMNs directly or through

interneurons. The LMNs then project their axons on the motorplate of muscles (Lin 2003). The proper function of this pathway depends on myelination, which begins near birth and is completed by age two (Lin 2003). The neurotransmitters involved in sending information through these tracts are glutamate (excitatory) from the cortical neurons and glycine in the inhibitory interneurons.

The other important pathway is the corticobulbar pathway, which starts in the sensorimotor cortex and descends to innervate the face muscles (Gilman and Winans Newman 2003). The ascending pathway consists of many tracts, including the spinocerebellar tract. This tract is important for transmitting proprioceptive information from receptors in muscles, tendons, and joints to the cerebellum (Lin 2003). In spinal cord injuries the damage to the spinocerebellar tract can be masked by the effects of lateral corticospinal tract damage (Lin 2003).

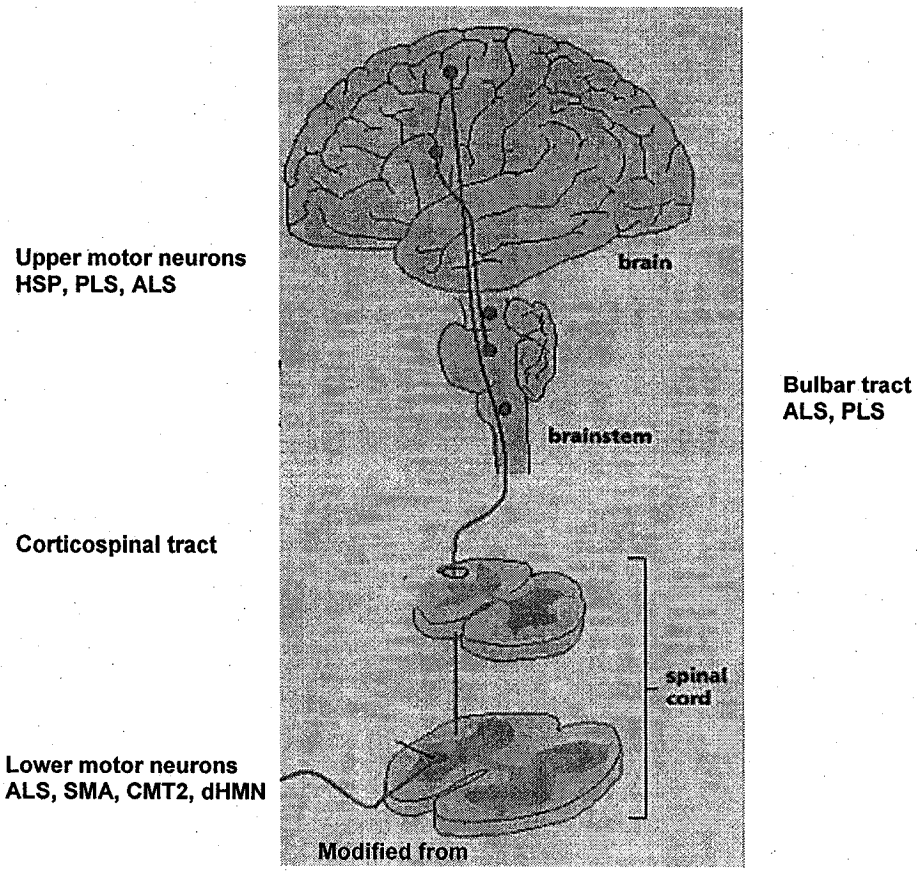


Figure 1: A schematic of the human motor

Figure 1: A schematic of the human motor system.

The human motor system starts with neurons in the motor cortex which connect to the spinal cord and finally synapse on the muscle. The MN diseases are depicted at the neurons they affect.

1.2 INJURIES TO THE SPINAL CORD

The incidence of traumatic spinal cord injury (SCI) is 20 new cases per million a year worldwide. SCIs are physically and mentally disabling and represent a heavy burden on the health care system (Sheerin 2005). Most SCIs result from trauma in motor vehicle accidents and falls (Sheerin 2005). A patient loses all sensation and voluntary movement below the injury in a spinal cord transection injury, whereas hemisections affect one side of the cord and have a higher rate of recovery. After a transection, the patient undergoes spinal shock where all reflexes are absent and spasticity only reappears after weeks. The current treatment includes corticosteroids, which should be administered soon after injury (Gilman and Winans Newman 2003).

The spinal cord can also be damaged by tumors, thrombosis, infection, disc herniation and arthritic changes. Tumors occur outside the spinal cord and can compress nerves, leading to severe pain. The symptoms can indicate where the tumor lies. The thrombotic spinal cord injury occurs when the anterior spinal artery, supplying blood to the corticospinal tracts and the anterior horn cells, blocks. This leads to atrophy, paralysis, spasticity and sensory loss (Gilman and Winans Newman 2003). Syringomyelia is also a SCI characterized by cystic cavities inside the spinal cord, usually at the cervical level. The incidence is approximately 8 new cases/year/100,000 people. The cause of this SCI is inbalance of cerebrospinal fluid (CSF) flow dynamics and can be relieved by immediate surgical treatment (Di Lorenzo and Cacciola 2005).

Damage to the specific nerves can be divided into: peripheral nerve lesions, dorsal root lesions, and motor neuron lesions. Injury of peripheral nerves leads to paralysis and

atrophy of enervated muscle as well as loss of sensation. Widespread peripheral nerve death is called polyneuropathy (Gilman and Winans Newman 2003). When a peripheral axon is severed, the distal part dies off. This process is called Wallerian degeneration (Gilman and Winans Newman 2003). Damage to the dorsal root causes pain, numbness and compromises reflexes.

A lesion of the LMN will cause severe weakness, atrophy and fasciculations, whereas UMN damage presents as minimal weakness, minimal atrophy, no fasciculations, increased tone, increased reflexes and decreased coordination (Wiederholt 2000). In the next section, diseases affecting the motor neurons per se will be discussed.

1.3 MOTOR NEURON DISEASES

Motor neurons are thought to be particularly vulnerable to damage, because they have some of the longest axons in the body (up to 1m long), they do not divide, and they have high energy requirements for transport and axonal support. There are several neurodegenerative diseases that affect these neurons specifically. Apart from the fact that motor neurons in these diseases undergo the same fate, there are other commonalities. Most motor neuron diseases (MNDs) have familial and sporadic forms. The familial forms present all 3 modes of inheritance and most causative genes associated with MNDs are ubiquitously expressed.

MNDs can affect both LMNs and UMNs. The main diseases affecting the former are: Spinal Muscular Atrophy (SMA), distal Hereditary Motor Neuropathy (dHMN) and Charcot–Marie–Tooth (CMT) syndrome (Van Den Berg-Vos et al. 2003).

SMA presents as muscle wasting due to spinal motor neuron death (Talbot and Davies 2001). The majority of patients lack the *survival motor neuron 1* gene on chromosome 5q11.2-13.3. The study of *SMN1* is complicated by the fact that it is situated in an inverted duplication, close to an almost identical gene, *SMN2*. *SMN1* is involved in snRNP biogenesis and pre-mRNA splicing (Pellizzoni et al., 1998; Meister et al., 2000; Hannus et al., 2000).

The dHMNs are characterized by distal upper and lower limb muscle weakness and wasting. The disease slowly progresses thereby affecting gait and other fine motor tasks. Mutations in the small heat-shock protein B1 (HSPB1, previously known as HSP27) are the cause of one form of dHMN, but also of axonal CMT. These mutations disrupt neurofilament assembly and axonal transport (Ackerley et al. 2006).

Lastly, there is the heterogeneous group of CMT diseases. CMT presents as progressive distal muscle weakness and atrophy in the lower limbs, followed by upper limb, gait and foot abnormalities, distal sensory loss and decreased or absent tendon reflexes (Harding and Thomas, 1980). CMT1 mainly affects myelin, whereas CMT2 damages the axons. The most common cause of CMT2 is mutations in the *mitofusin 2 (MFN2)* gene. This gene is a dynamin-like GTPase important for mitochondrial metabolism. Mutations in kinesin family member 1 (KIF1B) also cause CMT2 and result in axonal transport defects (Zuchner and Vance 2005; Verhoeven et al. 2006). Non-genetic diseases affecting the

LMN are monomelic amyotrophy, post-poliomyelitis muscular atrophy and multifocal motor neuropathy (Talbot and Davies 2001).

Amyotrophic lateral sclerosis (ALS) is one of the most common adult onset neurodegenerative disorders and it affects both the UMNs and LMNs. The clinical picture is that of progressive muscle weakness, wasting and, ultimately, paralysis and death typically within 3 to 5 years after disease onset. The majority of cases are sporadic, but genetic studies of a small percentage of familial cases have led to the identification of causative mutations in the *super oxide dismutase (SOD1)* gene (Hand and Rouleau 2002). *SOD1* mutations have been found in 15% of familial cases and also in rare sporadic cases. SOD1 rids the body of free radicals. Many pathogenic mechanisms have been proposed, including oxidative stress, glutamate excitotoxicity, neurofilament disorganization, copper toxicity and apoptotic cell death, but none have been proven (Hand and Rouleau 2002; Gros-Louis et al. 2006). The *ALS4* gene encodes senataxin which causes ALS in the dominant state and leads to Ataxia-oculomotor apraxia in the recessive state. The gene function is unknown but it has a DNA/RNA helicase domain. One private missense mutation was found in the *vesicle-associated membrane protein (VAPB)* gene and seven missense mutations were identified in angiogenin in an Irish and Scottish cohort (Greenway et al. 2006). Many susceptibility genes, such as the genes encoding vascular endothelial growth factor (VEGF), neurofilament and peripherin, have also been reported (Gros-Louis et al. 2006).

The *ALS2* locus was mapped in families with mainly UMN involvement and early disease onset. The disease slowly progresses to quadriparesis with bulbar symptoms. The

UMN degeneration combined with slow progression suggests that this locus actually causes Primary Lateral Sclerosis (PLS). The *ALS2* gene encodes alsin, a Rho guanine exchange factor (GEF). The protein contains three GEF domains: regulator of chromatin condensation (RCC1), pleckstrin-DB1 and a vacuolar protein sorting 9 (VPS9) domain. In addition, alsin has a membrane occupation and recognition nexus (MORN) motif. These alsin domains and motifs suggest a role in signal transduction, protein sorting and membrane localization (Kanekura et al. 2005). The majority of mutations result in protein truncation, but there is no correlation between the size of the truncation and the phenotype. Knock-out alsin mouse models are only slightly affected; they show mild axonal degeneration and reveal a defect in endosomal trafficking (Devon et al. 2006).

The most common genetic UMN disease is Hereditary Spastic Paraplegia (HSP), which will be discussed in the next section.

1.4 CLINICAL DESCRIPTION OF HSP

Hereditary Spastic Paraplegia (HSP), also known as Familial Spastic Paraplegia (FSP) or Strümpell-Lorrain syndrome, comprises a heterogeneous group of disorders characterized mainly by UMN degeneration. This degeneration leads to lower limb spasticity and weakness. HSP was first described by Strümpell in 1880 in a family with two affected brothers and apparent autosomal dominant inheritance. Subsequently, Lorrain reported additional cases (History of HSP reviewed by McDermott) (McDermott et al. 2000). The first attempt to clinically classify HSP was made by Anita Harding (Harding 1981) from

the analysis of 22 families. Harding's classification that holds today is that of pure versus complicated (complex) HSP. The pure form is solely characterized by leg spasticity, sometimes associated with bladder disturbance. The complicated form includes additional features such as optic neuropathy, dementia, ataxia, deafness, mental retardation, skin depigmentation, cataracts and extra-pyramidal disturbance (Harding 1981; Harding 1983; Harding 1993b). The second subclasssification is based on age of onset: HSP type 1 has an onset before 35 years of age and type 2 has an onset after 35 years of age. However, this classification is no longer used because there is no clear phenotypic correlation with age of onset, as it varies greatly, even within families, from early childhood to the mid-eighties. Anecdotal evidence, however, suggests that younger onset progresses slower than later onset HSP. Nonetheless, the severity of symptoms is variable within families and is not a dependable measure for prognosis (Dube et al. 1997; Fink and Hedera 1999).

The clinical exam

The patients generally present with broad based gait at the first neurological consultation. During motor examination, hyperreflexia and occasionally clonus of the lower limbs are observed (Gilman and Winans Newman 2003). Mild hyperreflexia can also be present in the upper limbs. The Babinski sign, also known as the extensor plantar reflex, is usually positive, and a positive Hoffman sign in the hands is rarely observed. The patients also occasionally present with pes cavus (high arched feet). The sensory exam includes a pin prick, vibration and position sense. These tests are usually negative, except for mild decreased sensation in distal lower limbs. The bulbar system is normal and therefore

HSP patients have normal speech and no difficulty swallowing. The cerebrospinal fluid (CSF) shows no abnormalities, and nerve conduction as well as electromyography (EMG) measures are normal (Gilman and Winans Newman 2003). Magnetic Resonance Imaging (MRI) of the brain and spine are usually normal, although a recent report suggests that HSP patients have thinner corpus callosum (CC) and have smaller spinal cord diameters at the thoracic level (Krabbe et al. 1997). The diagnosis of HSP and especially complicated HSP is reached after exclusion of other diseases. These diseases include leukodystrophies, structural spinal abnormalities, metabolic disorders, viral infections and other degenerative diseases, such as multiple sclerosis, ALS and spinocerebellar ataxia (SCA). The majority can be ruled out by abnormalities on MRI. One of the most difficult diseases to distinguish from complex HSP is SCA, which can only be confirmed reliably by genetic tests. ALS, a closely related disorder, can be distinguished by prominent LMN involvement (Harding 1993b; McDermott et al. 2000; Fink 2002).

Course of the disease

The predominant symptom at onset is usually bilateral lower limb spasticity in the form of gait disturbance. Young children affected by HSP usually present with delayed walking. HSP patients wear out shoes at the toes because of their broad based gait. They experience stiffness or cramps in the legs, which can progress and render the patient wheelchair bound. Bladder disturbance is seen in approximately 50% of the cases, whereas fecal incontinence and erectile dysfunction are quite rare (Cartlidge and Bone 1973; Harding 1993b). Patients with pure HSP sometimes also present with decreased

vibration sense in feet, mild upper limb hyperreflexia and mild lower limb muscle wasting later in disease. The disease does not influence lifespan, which in part explains the few available HSP neuropathological reports (Fink and Hedera 1999; McDermott et al. 2000).

Neuropathology

The main neuropathological feature is axonal degeneration at the distal ends of the longest ascending and descending tracts. The axons show typical "dying back" pathology. Dorsal root ganglia and peripheral nerves are usually normal (Behan and Maia 1974; Bruyn 1992; McDermott et al. 2000). A more recent, quantitative study showed a reduction in axonal density and in axonal number in HSP spinal cords. This was accompanied by mild myelin loss. The sensory system was only affected in the upper parts of the spinal cord (Deluca et al. 2004). In three HSP cases with a spastin gene mutation (*SPG4*), novel hyaline inclusions in anterior horn cells were detected. There was also evidence of altered cytoskeleton and mitochondria, together with tau pathology outside the motor system (Wharton et al. 2003).

Few large epidemiological studies have been performed for HSP. The prevalence of HSP is 0.9/100,000 in Denmark (Hazan et al. 1999), 2/100,000 in Portugal (Silva et al. 1997) and 9.6/100,000 in Cantabria, Spain. Moreover, for autosomal dominant HSP (ADHSP) the prevalence is 1.27/ 100,000 in Ireland and 14/100,000 in western Norway (Polo et al. 1991; Hazan et al. 1999; McMonagle et al. 2002). The prevalence of autosomal dominant HSP can therefore be estimated between 0.7-12/100,000. When considering all

forms of HSP combined the prevalence of the disease can be as high as 18.4/100,000 (McMonagle et al. 2002). The prevalence of HSP in Quebec is not known (Dupre et al. 2006).

Complicated HSP

The symptoms associated with complicated HSP vary from additional neurological abnormalities to skin depigmentation. In recent years, many additional types of HSP have been described. These include HSP with frontal lobe dysfunction (Yanase et al. 2004), HSP with peripheral neuropathy (Mostacciuolo et al. 2000), HSP with epilepsy (Al-Yahyaee et al. 2006) and Hereditary Spastic Ataxia (Bouchard et al. 2000).

Hereditary Spastic Ataxia (HSA) consists of a heterogeneous group of progressive neurodegenerative disorders characterized by lower limb spasticity and generalized ataxia with involuntary head jerk, dysarthria, dysphagia, impaired ocular movements, and gait disturbance (Mahloudji 1963; Bouchard et al. 2000). HSA presents as dominant (MIM 108600) or recessive types (MIM 270500). One well studied recessive form in Quebec is autosomal recessive spastic ataxia of Charlevoix- Saguenay (ARSACS, MIM 270550) (De Braekeleer et al. 1993). Mutations have been identified in sacsin, a protein thought to be involved in chaperone-mediated protein folding (Engert et al. 2000). The available neuropathological data also indicates degeneration of the corticospinal tracts and posterior columns.

Treatment for HSP

There is no cure for HSP, nor is there any medication to retard or prevent the disease onset and progression. However, the symptoms of spasticity can be reduced by antispasmodic medication such as baclofen, dantrolene and botox. For bladder spasticity, patients are prescribed oxybutynin. Furthermore, treating physicians always recommend physical therapy to reduce muscle spasticity and maintain general fitness (Fink 2002). A recent clinical trial showed that methylphenidate (eg. Ritalin), an amphetamine that influences neurotransmitters, did not improve HSP symptoms (Klebe et al. 2006b).

1.5 GENETICS OF HSP

1.5.1 Linkage studies

HSP displays genetic heterogeneity in addition to the clinical variability previously described. The genetic heterogeneity in HSP is demonstrated by the number of loci (*SPG1* through *SPG33*) that have been mapped through linkage analysis. These include 4 X-linked, 13 autosomal dominant and 15 autosomal recessive loci (Table 1). There are no genotype-phenotype correlations for the pure forms of HSP, but the clinical diagnosis can aid to determine the underlying locus for the complicated forms. In table 1 all associated phenotypic peculiarities are described as well as the number of families linked to each locus and the ethnic origin of the families. This information can be useful in determining which loci to analyze in a particular family.

Table 1: HSP loci

SPG	Chr.	MOI	Type	Phenotype	Fam*	Origin	Protein	Reference
1	Xq28	X	C	TCC, retardation, hydrocephaly, adducted thumbs, (MASA)	>10	W	L1CAM	(Jouet et al. 1994)
2	Xq22	X	both	Quadriparesis, MR, seizures, (Pelizaeus – Merzbacher)	>10	W	PLP	(Keppen et al. 1987)
3	14q11.2-q24.3	D	P	Childhood onset	>20	W	Atlastin	(Hazan et al. 1993; Zhao et al. 2001)
4	2p21-p24	D	both	Cognitive impairment, epilepsy	>100	W	Spastin	(Hazan et al. 1994; Hazan et al. 1999)
5	8q12-13	R	P	Bladder disturbance, impaired vibration sense	>10	W		(Hentati et al. 1994)
6	15q11.1	D	P	Bladder disturbance	2	W	NIPA1	(Fink et al. 1995; Rainier et al. 2003)
7	16q24.3	R	both	Optic atrophy, bulbar symptoms, cerebelar atrophy	8	Italian,	Paraplegin	(Casari et al. 1998; De Michele et al. 1998)
8	8q23-q24	D	P		2	Caucasian		(Hedera et al. 1999)
9	10q23.3-24.2	D	C	Cataract, esophageal reflux, short stature, amyotrophy	2	Italian,British		(Seri et al. 1999)
10	12q13	D	both	Distal muscle wasting	4	Caucasian	KIF5A	(Reid et al. 1999; Reid et al. 2002)
11	15q13-q15	R	both	MR, TCC, motor-sensory neuropathy	>20	W		(Martinez Murillo et al. 1999)
12	19q13.1	D	P		3	W		(Reid et al. 2000)
13	2q24-q34	D	P	Bladder disturbance, no pes cavus	1	French	HSP60	(Fontaine et al. 2000; Hansen et al. 2002)}
14	3q27-q28	R	C	Distal motor neuropathy, mild MR	1	Italian		(Vazza et al. 2000)
15	14q	R	C	Maculopathy, MR, amyotrophy, dysarthria (Kjellin syn.)	2	Irish		(Hughes et al. 2001)
16	Xq11.2	X	both	Motor aphasia, decreased vision, mild MR, incontinence	2	Japanese.		(Steinmuller et al. 1997; Tamagaki et al. 2000)
17	11q12-q14	D	C	Amyotrophy of hands, sometimes feet (Silver syndrome)	>10	W	Seipin	(Patel et al. 2001)
19	9q33-q34	D	P		1	Italian		(Valente et al. 2002)
20	13q12.3	R	C	Distal muscle wasting, short stature (Troyer syndrome)	2	Amish	Spartin	(Patel et al. 2002)
21	15q22.31	R	C	Spastic dysarthria, pseudo-bulbar signs (MAST syndrome)	1	Amish	Maspardin	(Simpson et al. 2003)
22	Xq13.2		C	Severe MR, dysarthria, ataxia, (Allan-Herndon-Dudley syn.)	>10	W	MCT8	(Claes et al. 2000; Bohan and Azizi 2004;
23	1q24-q32	R	C	Skin depigmentation, cognitive impairment, microcephaly	1	Arab-Israeli		(Blumen et al. 2003)
24	13q14	R	P		1	Saudi		(Hodgkinson et al. 2002)
25	6q23-q24.1	R	C	Multiple disc herniations	1	Italian		(Zortea et al. 2002)
26	12p11.1-q14	R	C	Dysarthria, distal amyotrophy	1	Kuwaiti		(Wilkinson et al. 2005)
27	10q22.1-q24.1	R	P	Spastic bladder	2	French		(Meijer et al. 2004)
28	14q21.3-q22.3	R	P	Childhood onset	1	Morrocan		(Bouslam et al. 2005)

29	1p31.1-p21.1	D	C	Hearing impairment, hiatal hernia	1	Scottish		(Orlacchio et al. 2005)
30	2q37.3	R	C	Mild ataxia and sensory neuropathy	1	Algerian		(Klebe et al. 2006)
31	2p12	D	P		6	? Caucasian	REEP1	(Zuchner et al. 2006a , Zuchner et al. 2006b)
32	8p12-p11.21	R	C	TCC and epilepsy	2	Omani		(Al-Yahyaee et al. 2006)
33	10q24.2	D	P		1	?	ZFVE27	(Mannan et al. 2006)

D: Dominant, R: Recessive, P: Pure, C: Complicated, MR: Mental retardation, TCC: Thin corpus callosum, W: worldwide, MOI: Mode of Inheritance

Modified from Blackstone et al. 2006 (Soderblom and Blackstone 2006) and Tallaksen et al. 2001(Tallaksen et al. 2001)

* Number of families linked to this locus according to OMIM

Apart from the "official" *SPG* loci, other loci have been published with spastic paraplegia as part of a syndrome. Of interest is the addition of the Allan-Herndon-Dudley syndrome as *SPG22* to table 1. The locus name, *SPG22,* has not officially been assigned to this syndrome, but some colleagues are of the opinion that it is appropriate because the patients have leg spasticity as part of a very complex phenotype including hypotonia, dysarthria and MR (Bohan and Azizi 2004; Schwartz et al. 2005). A novel locus for HSP with thin corpus callosum (TCC) and epilepsy was mapped to chromosome 8 and has unofficially been named *SPG32* in table 1 (Al-Yahyaee et al. 2006). Interestingly, only one-third of the patients with HSP-TCC show linkage to the original HSP-TCC locus, *SPG11.* The *potassium chloride cotransporter (KCC3)* gene, that causes hereditary motor and sensory neuropathy associated with agenesis of the corpus callosum (HMSN/ACC), has been excluded in many of these families (Winner et al. 2005). The novel *SPG32* locus might account for some of these HSP-TCC families. Another locus for HSP with optic atrophy and neuropathy on 11q13 was reported as a new syndrome, spastic paraplegia, optic atrophy, and neuropathy (SPOAN) but it overlaps with the *SPG17* locus (Macedo-Souza et al. 2005). A compound phenotype due to disc herniation and spastic paraplegia was mapped to chromosome 6q23.3-q24.1, where three separate blocks of homozygosity were found. Only one block gave a LODscore greater than 3, but the others could not be excluded. Therefore the authors refer to this as a susceptibility locus (Zortea et al. 2002).

The X chromosome already harbors three official HSP loci and there are reports that two more might exist. Two groups reported families with pure or complicated HSP that link to the *SPG2* locus without mutations in the *proteolipoprotein* gene, suggesting that there is another X-linked locus nearby (Steinmuller et al. 1997; Starling et al. 2002). Moreover, an independent linkage study found two additional HSP candidate loci: Xp21.1–Xq21.3 and Xq23–Xq27.1 (Claes et al. 2000).

Finally, it is important to note that even though HSP is known as a hereditary disease, many cases present as sporadic. In these cases HSP may be recessive or acquired. Apart from the linkage studies and mutation screens for candidate genes of HSP, no association studies have been reported.

1.5.2 Gene function

To date, 14 mutated genes have been implicated in HSP pathogenesis (Table 1). The causative proteins have traditionally been grouped into 4 categories: proteins involved in axonal outgrowth and maintenance, mitochondrial function, endosomal trafficking/axonal transport, and various other functions. The majority of HSP genes fall in the trafficking category, suggesting that perturbation of anterograde or retrograde transport in the axons may represent the main cause of HSP (Reid 2003). This hypothesis is supported by the fact that the longest axons require high energy and show "dying back" pathology in HSP. A cartoon drawing displays the intracellular localization of all HSP proteins (Figure 2).

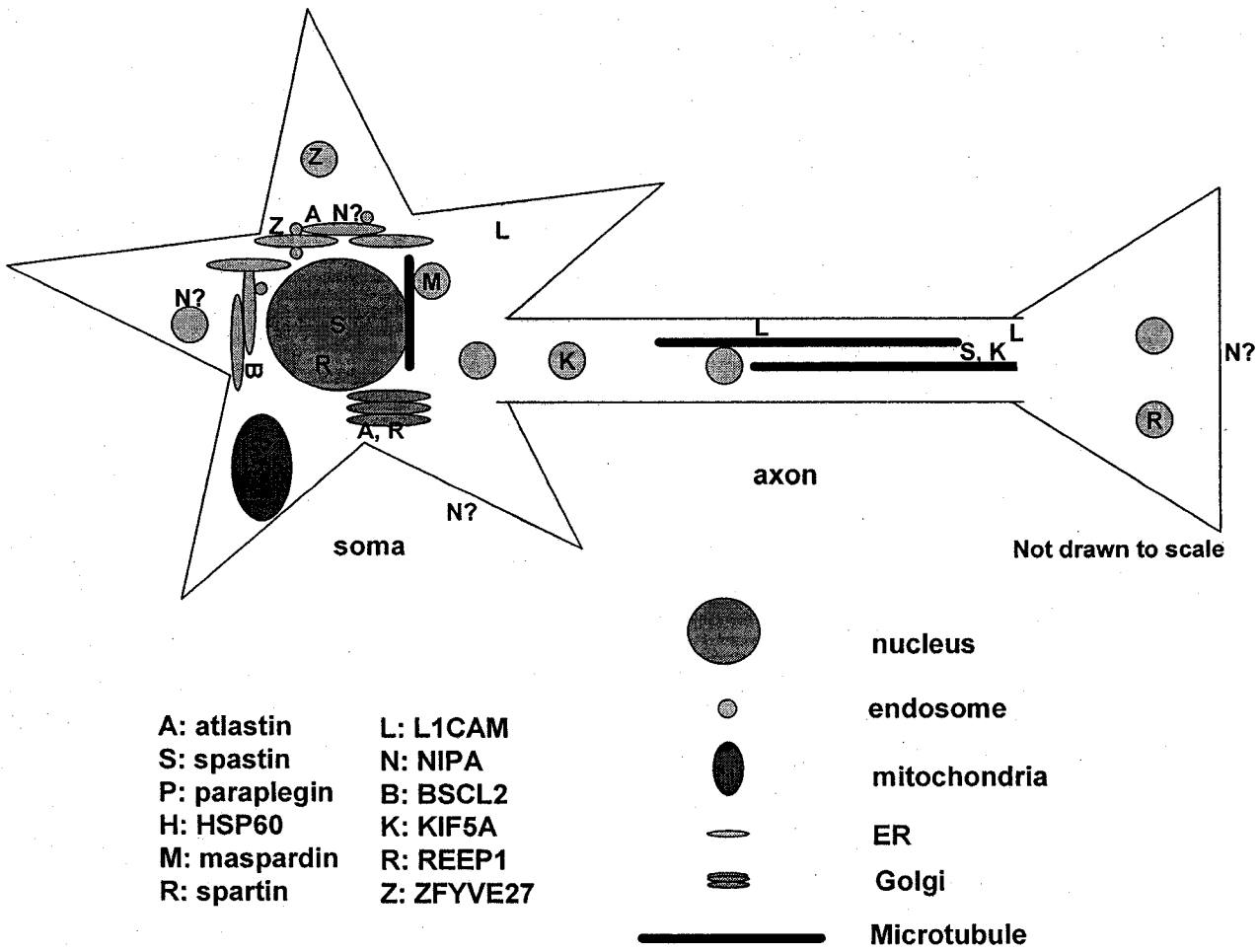


Figure 2: The localization of known HSP proteins

Many studies have focused on determining the localization of HSP proteins in the hope to improve our understanding of their function. This cartoon depicts where these HSP proteins most likely reside in a neuron. PLP1 is expressed in oligodendrocytes.

1.5.3 Axonal outgrowth and maintenance

L1CAM (*SPG1 locus*)

Mutations in the gene encoding the L1 cell adhesion molecule (L1CAM) cause a number of different syndromes: (1) agenesis of the Corpus callosum, mental Retardation, Adducted thumbs, Shuffling gait and Hydrocephalus (CRASH), (2) Hydrocephalus due to Stenosis of the Aqueduct of Sylvius (HSAS), (3) Mental Retardation Aphasia Shuffling gait Adducted thumbs (MASA), (4) *SPG1* and (5) Agenesis of the Corpus Callosum (ACC) and Mental Retardation – Clasped Thumbs (MR-CT). The phenotype associated with these mutations is very variable and this clinical spectrum was named CRASH after mutations were found in the same gene for all the above-mentioned diseases including spastic paraplegia. The common symptom is always mental retardation, a feature seen in other HSP's (Fransen et al. 1997). The HSP phenotype is the milder one in this spectrum of disorders and does not lead to early death. L1CAM is a cell surface glycoprotein involved in cell adhesion between neurons and between neurons and Schwann cells. The protein has a role in axonal outgrowth, guidance and growth cone morphology. The majority of mutations identified lie within the cell adhesion and neurite outgrowth domains (Jouet et al. 1994). The L1CAM protein interacts with itself or with other CAMs and transmits information to the intracellular environment through signal transduction (Fransen et al. 1997; Castellani et al. 2000). L1CAM also plays a role in remodeling after contusive SCI (Jakeman et al. 2006). Studies of mutations in the cytoplasmic domain of L1 have shown that they destabilize L1-ankyrin interactions which may have an effect on L1 endocytosis which is important

for MAPK signaling and neurite outgrowth (Needham et al. 2001). A L1CAM knock-out mouse demonstrated that L1 is important for corticospinal tract development and corpus callosum formation and affects axon pathfinding (Cohen et al. 1998).

PLP (*SPG2 locus)*

The *SPG2* locus for pure HSP and the locus for Pelizaeus-Merzbacher disease (PMD) are allelic. PMD patients present with a more severe phenotype than HSP including mental retardation and early death and the pathology is of reduced white matter (Saugier-Veber et al. 1994; Yool et al. 2000). Mutations in the proteolipid protein (PLP) are the cause of these two diseases. This protein is a membrane protein important for myelin maintenance (expressed in oligodendrocytes) and has two isoforms, PLP and DM20. Missense mutations in this gene have also been identified in the rumpshaker mouse and the paralytic tremor rabbit. These animal models show a mild phenotype, with slight hypomyelination representative of HSP. In the rumpshaker mouse the majority of mutant protein is still targeted to the myelin sheath, where it inserts more slowly than in wild type. The disease mechanism might be dominant-negative, where PLP negatively affects another protein, possibly myelin binding protein (MBP) (McLaughlin et al. 2006). PLP knock-out mice do not show demyelination and have no clinical phenotype but detailed investigation showed that these mice have axonal swelling - indicating that the axons need proper support from oligodendrocytes (Griffiths et al. 1998; Yool et al. 2000). Defects in fast anterograde and retrograde axonal transport underlie the axonal swelling phenotype (Edgar et al. 2004a; Edgar et al. 2004b).

1.5.4 Mitochondrial proteins

Paraplegin (*SPG7* locus)

The detection of a 9.5 kb deletion in a HSP family used in a genome scan led to the identification of the *SPG7* locus. The deletion included the gene encoding the cell adhesion molecule regulator (CAMR), which was later confirmed to be part of the 3'UTR of the paraplegin encoding gene. Paraplegin is an inner-membrane mitochondrial metalloprotease, which complexes with AFG3L2 to form a mitochondrial ATPase with various cellular activities (mAAA) protease. Patients with mutations in paraplegin show ragged red fibers in the muscle, indicative of an oxidative phosphorylation (OXPHOS) impairment. There is a deficiency in respiratory complex 1 and increased sensitivity to oxidative stress (Casari et al. 1998; Atorino et al. 2003). The mitochondrial ribosomal protein MrpL32 needs to be cleaved by the mAAA protease to form the ribosomal complex. The lack of this protease activity leads to a defect in translation and therefore a defect in mitochondrial function (Nolden et al. 2005). A knock-out mouse model of paraplegin presents with distal axonopathy in spinal and peripheral axons. The axonal degeneration is accompanied by swelling and mitochondrial abnormalities in synaptic terminals. It remains unclear whether the degeneration results from a lack of energy or from clogging of the axons. Adeno-associated virus-mediated (AAV-mediated) delivery of paraplegin to the muscles of knock-out mice stopped the progression of neuropathological changes and rescued mitochondrial morphology in the peripheral neurons (Ferreirinha et al. 2004; Pirozzi et al. 2006).

HSP60 (*SPG13* locus)

A missense mutation in the gene encoding HSP60 is thought to be the cause of HSP at the *SPG13* locus. However, the genetic support for the association of a V271I change in heat shock protein 60 (HSP60) is weak. The mutation was identified in only one French family and not in other linked families. Interestingly, the mild V72I change is conserved in *Escherichia coli* which permitted functional testing of the change. The bacterial HSP60 homologue, GroEL, is part of a chaperonin operon essential for bacterial growth. The normal human gene rescues the null bacteria but the mutant HSP60 transformed bacteria failed to grow. Moreover, the disease mechanism is not understood, but the protein is a mitochondrial chaperonin involved in protein folding (Hansen et al. 2002). Interestingly, HSP60 and its neighbour HSP10 are controlled by a bi-directional promoter (Hansen et al. 2003).

REEP1 (*SPG31* locus)

The *SPG31* locus was recently identified and is thought to be the third most common HSP locus (Table 1). Mutations in the receptor expression enhancing protein 1 (REEP1) were identified as the cause for HSP at this locus. This protein was an unlikely candidate gene as it is thought to play a role in G protein coupled trafficking of odorant receptors and other molecules through the endoplasmic reticulum (ER) and Golgi apparatus (Saito et al. 2004). The expression of REEP1 is ubiquitous and it localizes to the mitochondria via a transmembrane domain. The yeast *(Saccharomyces cerevisiae)* ortholog of REEP1, Yop1P, plays a role in Rab-mediated vesicle transport (Zuchner et al. 2006a; Zuchner et al. 2006b).

1.5.5 Vesicle trafficking and axonal transport

Spastin (*SPG4* locus)

The *SPG4* locus was mapped in 1994 to chromosome 2p in several families, prompting the classification of HSPs by their locus instead of clinical features (Hazan et al. 1994; Hentati et al. 1994b). After physical mapping of the region, five mutations were identified in the *SPG4* gene encoding spastin (Hazan et al. 1999). This gene is mutated in 40% of dominant HSP cases and represents the most common cause for HSP. Over 130 mutations have been reported in this 17 exon gene (McDermott et al. 2006). The mutation spectrum includes a majority of missense, nonsense, frameshift and splicing mutations mostly affecting the conserved AAA cassette (Figure 3) (McDermott et al. 2006). Several studies have also shown atypical mutations not likely detected by standard screening methods. These include a large 2307 bp deletion of the 5'UTR and intron 1 (Iwanaga et al. 2005), intronic deletions (Higgins et al. 2001b) and missense changes leading to aberrant splicing (p.406I>V leads to an in frame deletion). It has also been shown that spastin splice mutations are leaky, suggesting that even small amounts of mutant spastin result in neurodegeneration (Svenson et al. 2001b). Two studies have shown that 12% of seemingly sporadic cases have mutations in spastin and this represents the first description of the identification of mutations in sporadic cases (Brugman et al. 2005; Depienne et al. 2006).

Clinical spectrum

Spastin mutations have been associated with a variety of clinical features in addition to lower limb paraparesis. These include subtle cognitive impairment (Tallaksen et al. 2003), juvenile ALS (upper limb and bulbar amyotrophy) (Meyer et al. 2005), epilepsy (Mead et al. 2001), cerebellar ataxia (Nielsen et al. 2004a), thin corpus callosum (TCC) (Alber et al. 2005), dementia, epilepsy (Heinzlef et al. 1998), arachnoid cyst, (Orlacchio et al. 2004a) and LMN involvement (McDermott et al. 2006). Several studies have tried to identify genotype-phenotype correlations but even the simplest correlation with age of onset seems to be absent (Yip et al. 2003).

Function of spastin

Spastin is a member of the ATPase with various cellular activities (AAA) family, and has microtubule (MT) severing functions. The AAA's all posses the characteristic 230-250 amino acid domain containing Walker motifs and confer ATPase activity (Figure 3) (Patel and Latterich 1998). At the N-terminus, spastin has a nuclear localization signal (NLS) and a microtubule interaction and trafficking domain (MIT). Other proteins containing a MIT domain include spartin, skd1, vps4, and snx15, suggesting a function in interaction with membranes (Ciccarelli et al. 2003). The ATPase domain is affected in several mutants and it rearranges the microtubule network into a filamentous network (Errico et al. 2002; McDermott et al. 2003). Further characterization of spastin mutations has shown that most missense mutants bind irreversibly to MT (e.g. p.388K>R), others no longer sever MT and therefore rearrange the MT network (Errico et al. 2002).

Nonsense or frameshift mutations lead to the absence of mutant protein (Charvin et al. 2003; Schickel et al. 2006). Another study also proposes that spastin has a MT bundling role in addition to the ATPase severing activity (Salinas et al. 2005). Spastin has four isoforms resulting from the differential use of two start codons and the alternative splicing of exon 4 (Figure 3) (Salinas et al. 2005). The long form is exported to the cytoplasm and the short form localizes to both the cytoplasm and the nucleus (Claudiani et al. 2005). In neurons, spastin localizes to distal axons and branching regions of NSC34 cells (Errico et al. 2004). The perinuclear staining seen in disrupted mutant spastin cells is reminiscent of kinesin motor defects (McDermott et al. 2003).

The implication of spastin in various cellular processes has been demonstrated by the identification of several interacting partners (Table 2, Figure 3). NA14 was identified as an interacting partner using a human fetal cDNA library. NA14 is a member of the mammalian centrosome, which is a dynamic matrix that holds the proteins necessary for microtubule nucleation. The interaction with a centrosomal protein suggests a role for spastin in cell division and cytokineses, although the human phenotype appears only in postmitotic neurons. It is possible that this MT binding during cytokinesis resembles that of axonal trafficking (Errico et al. 2004). Another interacting partner, CHMP1B, was identified in a yeast two-hybrid screen of an erythroleukemia cDNA library. It is suggested that the MIT domain of spastin is necessary for this interaction. CHMP1B associates with the endosomal sorting complex required for transport (ESCRT)-III, which is responsible for sorting and concentrating of cargo to vesicles. Interestingly, spastin was also localized to punctate staining along the MT tracks reminiscent of vesicle trafficking (Reid et al. 2005). Reticulon 1 (RTN1) was found to interact with spastin in a

yeast two-hybrid study using a human brain cDNA library. Another member of the same protein family, RTN3, also weakly interacts with spastin. These proteins are related to Nogo (RTN4), a protein involved in inhibition of axonal regeneration, but spastin does not seem to directly interact with NOGO. RTN1-C, an RTN1 isoform, interacts with the SNARE complex suggesting a role in intracellular membrane fusion. RTN1 is a positional candidate for *SPG15* but no mutations were found in the linked families (Mannan et al. 2006a). The same group also identified ZFYVE27 as a spastin interacting partner. A ZFYVE27 mutation was discovered in a HSP family and therefore this locus was named *SPG33* (Mannan et al. 2006b).

Most interesting of all the interacting spastin partners is atlastin, the protein mutated at the *SPG3* locus (see next section). This interaction is the first description of two major HSP genes being involved in the same pathway. Sanderson first reported this interaction on the N terminal of atlastin near the second trans-membrane (TM) domain, but it was recently shown that this interaction actually occurs at atlastin's C-terminus. The data suggests that atlastin controls the localization of spastin by recruiting it either to the Golgi or to Golgi derived vesicles (Evans et al. 2006; Sanderson et al. 2006).

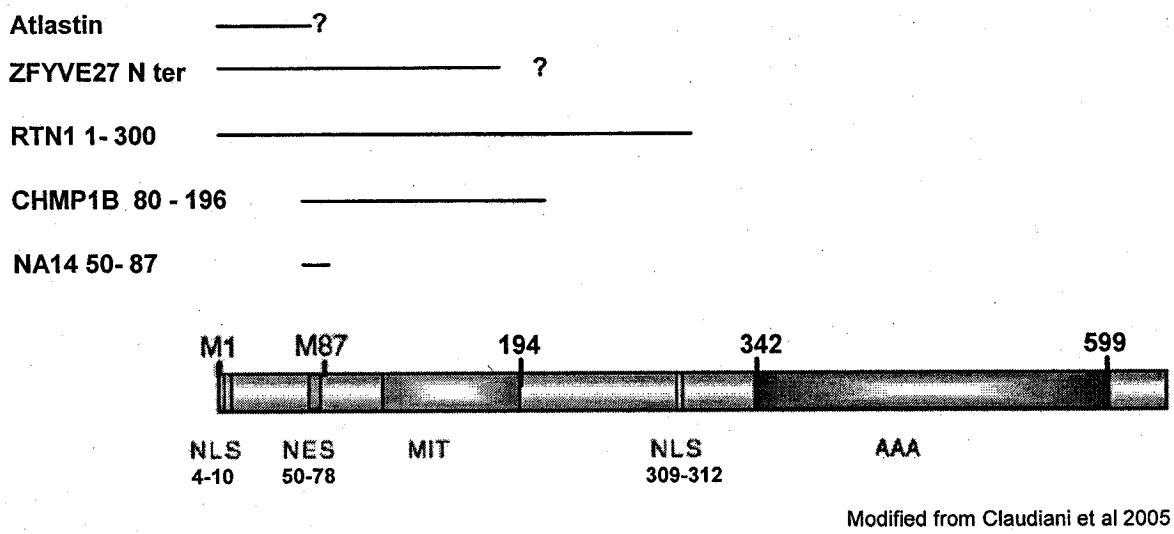


Figure 3: The structure of the spastin.

Spastin contains two NLS, one NES, one MIT and one AAA domain. The location or predicted location of interaction with binding partners is also indicated.

Drosophila spastin models

RNAi experiments in *Drosophila melanogaster* show that D-spastin knock-down in neurons leads to locomotor defects. Neuronal loss of function also leads to reduction of synaptic area and enhancement of neurotransmission. In contrast, overexpression of D-spastin leads to lethality and reduction of neurotransmission. Antibodies against D-spastin label spastin in the cytoplasm of neurons and muscles, with high expression at the neuromuscular junctions (NMJ) of the body wall. The expression of D-spastin green fluorescent protein (GFP) also shows a novel localization for spastin in membrane vesicles (Roll-Mecak and Vale 2005).

Destabilizing the MTs with nocadozole results in reduction of the synaptic transmission phenotype (Trotta et al. 2004). However, *in vivo* use of nocadozole did not improve the phenotype. Vinblastine, another MT destabilizing agent, did lead to attenuation of phenotype *in vivo*, more specifically at the NMJ. In the same study, it was shown that expression of the mutant K467R results in the same phenotype as a D-spastin knock-down suggesting a dominant-negative mechanism (Orso et al. 2005).

Atlastin (*SPG3 locus*)

The second most common cause for dominant HSP are mutations in the *SPG3A* gene, which codes for atlastin (Rainier et al. 2001; Zhao et al. 2001). This gene is mutated in approximately 10% of dominant HSP families and is mostly associated with childhood onset HSP. All reported mutations are missense changes except for one frameshift mutation that leads to a minimally truncated protein (Abel et al. 2004; D'Amico et al.

2004; Durr et al. 2004; Hedera et al. 2004; Sauter et al. 2004; Scarano et al. 2005; Namekawa et al. 2006b). Atlastin is a 64 kDa dynamin family member with GTPase activity. The protein possesses three characteristic GTPase active sites: P Loop, DxxG, and RD domain.

Furthermore, the protein forms a homotetramer and is thought to be an integral membrane protein that localizes to the Golgi and ER and is possibly involved in vesicle trafficking (Zhu et al. 2003; Sanderson et al. 2006; Zhu et al. 2006). Atlastin is expressed ubiquitously but is enriched in many regions of the brain, including the cerebral cortex (Zhao et al. 2001; Zhu et al. 2003). Atlastin was also shown to localize to the growth cone of axons and RNAi studies suggest that atlastin is necessary for axonal growth in cortical neuron cultures (Zhu et al. 2006). The analysis of certain mutants by yeast two-hybrid and gel exclusion assays demonstrates that the mutants can still oligomerize but their GTPase activity is reduced (Zhu et al. 2006). Other localization studies have shown that atlastin colocalizes with spastin (see spastin section above), ER markers, Golgi markers and the Adaptor protein complex 2 (AP2), which interacts with clathrin and other vesicle trafficking proteins (Zhu et al. 2003; Zhu et al. 2006).

KIF5A (*SPG10 locus*)

In a large candidate gene sequencing effort at the *SPG10* locus, Reid and colleagues identified a N256S mutation in the gene encoding kinesin family member 5A (KIF5A). No mutations were found in the second linked family suggesting the presence of another HSP locus nearby (Reid et al. 2002). Since then, two other KIF5A missense mutations (Y276C, R280C) in the kinesin motor domain and A361V in a coiled coil domain were

identified (Fichera et al. 2004; Blair et al. 2006; Lo Giudice et al. 2006). Mouse studies have shown that KIF5A is only expressed in neurons and null mutants die soon after birth. When the gene is knocked out post-natally, the phenotype consists of seizures and sensory neurodegeneration. The histopathology shows loss of large sensory axons and accumulation of neurofilaments (NF) in the dorsal root ganglion but not in brain. KIF5A is a fast motor that is involved in slow axonal transport. It is thought that slow axonal transport is the result of fast transport followed by long pauses (Xia et al. 2003).

Spartin (*SPG20* locus)

Troyer syndrome is a complicated form of HSP associated with dysarthria, distal amyotrophy, mild developmental delay and short stature. The causative gene for Troyer syndrome was mapped using homozygosity mapping in the Old Order Amish. This gene, *SPG20,* has 9 exons, and encodes the 666 amino acid protein spartin. One frameshift mutation was identified (1110delA) that truncates the protein by 268 amino acids. Spartin, like spastin, has a MIT domain and is thought to be involved in endosomal trafficking or microtubule dynamics (Patel et al. 2002; Ciccarelli et al. 2003). A recent study identified a spartin interacting partner, epidermal growth factor receptor pathway substrate 15 (EPS15), an important protein in endocytosis and cell proliferation. Spartin localizes mainly to the cytosol but it is also present in the membrane fraction (Figure 2) (Bakowska et al. 2005).

Maspardin (*SPG21* locus)

Mast syndrome is another complicated form of HSP characterized by spastic paraparesis and dementia or other CNS abnormalities with a high frequency in the Old Order Amish. The gene for Mast syndrome was mapped to chromosome 15q22 and homozygosity mapping narrowed the region to three genes, including the *acidic cluster protein 33 (ACP33)* gene encoding the protein maspardin. A frameshift insertion was identified in the *ACP33* gene (Simpson et al. 2003). Little is known about maspardin function. One report shows that maspardin associates with CD4 and colocalizes with transferrin, a marker for early endosomal recycling pathway (Zeitlmann et al. 2001).

ZFYVE27 (*SPG33* locus)

The protein encoded by the *SPG33* locus was identified through yeast two-hybrid studies using spastin as bait. The protein involved, ZFYVE27, belongs to the FYVE-finger family of proteins. Many members of this family are involved in endosomal trafficking. One missense mutation, G105V was identified in a small ADHSP family and resulted in relocalisation of mutant protein from vesicles to the ER in *in vitro* cell studies (Mannan et al. 2006b). This mutant also disrupts the interaction with spastin.

1.5.6 Various functions

NIPA1 (*SPG6* locus)

The HSP gene at the *SPG6* locus encodes the Not Imprinted in Prader Willi/Angelman (NIPA1) protein. Two NIPA1 missense mutations, T45R and G106R, have been identified in 16 families of different ethnic origin (Rainier et al. 2003; Chen et al. 2005;

Reed et al. 2005). The function of this protein has not been well studied, and it does not share homology to other HSP genes. Prediction programs suggest that NIPA1 functions as a transporter or a receptor through its transmembrane domain. NIPA1 is enriched in the brain but is also ubiquitously expressed at lower levels (Rainier et al. 2003).

BSCL2 (*SPG17* locus)

The *BSCL2* gene was originally identified as the defective gene in a recessive disorder named Berardinelli-Seip congenital lipodystrophy (BSCL) until it was recently implicated in a type of HSP, Silver syndrome (*SPG17).* Silver syndrome is a complex form of HSP characterized by hand amyotrophy in addition to lower limb spasticity. Null mutations in the *BSCL2* gene cause BSCL, whereas missense mutations lead to Silver syndrome and dHMN-V (Windpassinger et al. 2004). The *BSCL2* gene encodes the protein seipin, which localizes to the membrane of the ER. The identified missense mutations, N88S and S90L, are thought to cause HSP by disrupting N-glycosylation of the protein. One seipin isoform is brain specific, but a longer form is ubiquitously expressed. Mutant seipin forms aggresomes (Windpassinger et al. 2004). The function of seipin is also largely unknown, but is thought to be part of large RNA transport complex. The current hypothesis is that the two different diseases result from loss or accumulation of lipids in the motor neurons or adipocytes (Agarwal and Garg 2004).

MCT8 (*SPG22* locus)

Another gene that has been implicated in HSP encodes the mono-carboxylate transporter 8 (MCT8), mutated in Allan-Herndon-Dudley syndrome. The MCT8 protein is a thyroid

hormone transporter and has an important role in transport of triodothyronine into neurons (Schwartz et al. 2005). In keeping with the clinical phenotype, which varies greatly from other HSPs, the gene function also does not fall into any of the HSP functional categories.

Modifier genes

The *Excitatory Amino Acid Transporter 2* gene is a good modifier gene candidate for motor neuron degeneration because of its function as a glutamate transporter (accumulation of glutamate is toxic for neurons) (Meyer et al. 1998). Many groups have searched for mutations in this gene as a cause for ALS and HSP. A heterozygous A79G variant was found in 2 HSP patients of the same family, but the variant did not segregate with the disease. This variant should be considered when looking for a modifier gene of HSP (Meyer et al. 1998).

Interestingly, yeast two-hybrid studies have been performed for many the HSP genes and an increasing number of interacting partners are being identified. The study of HSP interacting partners in addition to HSP genes should give a broader view of HSP pathogenesis. Table 2 lists all the known HSP protein interacting partners reported to date.

Table 2: Interacting partners of HSP genes

HSP protein	Interacting partner	Locus*	Reference
Paraplegin	PLSCR1	3q24	(Rual et al. 2005)
Paraplegin	KRTAP4-12	17q21.2	(Rual et al. 2005)
Paraplegin	MDFI	6p21.1	(Rual et al. 2005)
Paraplegin	RALY	20q11.22	(Lim et al. 2006)
Maspardin	GGA2	16p12.1	(Rual et al. 2005)
Maspardin	C6orf166	6q15	(Rual et al. 2005)
Maspardin	TRAF2	9q34.3 (*SPG19*)	(Rual et al. 2005)
Maspardin	RABAC1	19q13.2 (*SPG12*)	(Rual et al. 2005)
Maspardin	CTPS2	Xp22.2	(Rual et al. 2005)
Maspardin	MTMR9	8p23.1	(Rual et al. 2005)
Maspardin	ZNF263	16p13.3	(Rual et al. 2005)
Maspardin	PRPS1	Xq22.3	(Rual et al. 2005)
Maspardin	CUTC	10q24.2 (*SPG27*)	(Rual et al. 2005)
Maspardin	RTN4/NOGO	2p16.1	(Rual et al. 2005)
Maspardin	CD4	12p13.31 (*SAX1*)	(Rual et al. 2005)
Spartin	EPS15	1p32.3	(Bakowska et al. 2005)
Spartin	KIAA0174	16q22.3	(Lim et al. 2006)
Spastin	KIAA0174	16q22.3	(Lim et al. 2006)
Spastin	Atlastin	14q	(Sanderson et al. 2006)
Spastin	Reticulon 1	14q23.1 (SPG15)	(Mannan et al. 2006b)
Spastin	Reticulon 3	11q13.1	(Mannan et al. 2006b)
Spastin	CRELD1	3p25.3	(Mannan et al. 2006b)

Spastin	COPS5	8q13.2 (*SPG5)*	(Mannan et al. 2006b)
Spastin	CHMP1B	18p11.21	(Reid et al. 2005)
Spastin	NA14	9q34.3 (*SPG19)*	(Errico et al. 2004)
Spastin	ZFYVE27	10q24.2 (*SPG27)*	(Mannan et al. 2006b)
Seipin	TMEM19	12q21.1	(Rual et al. 2005)
Seipin	NID67	5q33.1	(Rual et al. 2005)
Seipin	MDS032	19p13.11	(Rual et al. 2005)
KIF5A	TP53BP2	1q41	(Lim et al. 2006)
L1CAM	PRNP	20p13	(Lim et al. 2006)
PLP1	MBP	18q23	(Lim et al. 2006)

* This HSP interacting partner is a positional candidate for the HSP locus in brackets.

The table was prepared in collaboration with Dr. F. Blondeau.

Most of these interacting partners have been identified recently and the nature of the interactions has not been well characterized. Therefore, future functional studies are necessary to elucidate the importance of these interactions in HSP patho-physiology and neuronal maintenance.

1.6 HTLV INFECTIONS, A NON GENETIC CAUSE OF HSP

Human T Lymphotropic Virus type 1 (HTLV1) infections are mainly associated with two clinical phenotypes: Adult T cell Leukemia (ATL) and progressive neurological disease. However, the majority of carriers remain asymptomatic (Ferreira et al. 1997; Araujo and Hall 2004; Proietti et al. 2005). The HTLV1 related neurological disorder was first described as Tropical Spastic Paraparesis (TSP) in 1897 by Henry Strachan and Henry Scott in Jamaican patients. TSP is also known as multiple neuritis (Zunt 2001). The virus was identified in 1980 and the link between TSP and HTLV1 was made five years later (Gessain et al. 1985).

Clinical description

TSP was renamed HTLV1 Associated Myelopathy/Tropical Spastic Paraparesis by the (HAM/TSP) World Health Organization. HAM/TSP is characterized by weakness in legs, hyperreflexia of lower limbs, loss of vibration sense, absence of upper limb involvement and spastic bladder (Montgomery 1989). The symptoms normally appear between the ages of 20 and 50 years and consist of lower back or leg pains. Within 10 years many patients are wheelchairbound. In some cases the upper limbs are affected and loss of vibration sense has also been noted. HTLV1 infections have also been associated with sporadic ALS (Silva et al. 2005). Neuropathological studies have shown inflammation of the spinal cord with demyelination and loss of axons (Montgomery 1989). Using b-amyloid precursor protein as a marker for impaired axonal trafficking,

Umehara and colleagues suggest that there is axonal damage and impairment of trafficking in the areas of active inflammation (Umehara et al. 2000). HTLVI specific immune responses play a critical role in the pathogenesis of HAM/TSP (Proietti et al. 2005).

Transmission

The virus is transmitted through breastfeeding, sexual contact, blood transfusion, and needle sharing. Approximately 5% of HTLVI infected individuals develop disease, while the vast majority of them remain asymptomatic (Proietti et al. 2005). More women are infected with HTLV1, as is the case in many other sexually transmitted disorders. The virus can be diagnosed through enzyme linked immuno sorbent assay (ELISA), Western blot analysis or polymerase chain reaction (PCR) (Ferreira et al. 1997).

Epidemiology

TSP has been described in Jamaica, Martinique, Seychelles, Colombia, Japan, India, Peru, and West/Central Africa (Oomman and Madhusoodanan 2003; Gotuzzo et al. 2004; Proietti et al. 2005). An estimated 15–20 million people currently live with HTLV1 infections worldwide, but studies are biased because they focus on blood donors (Orland et al. 2003).

HTLV2 is considered a more benign virus compared to HTLV1. There are approximately 200,000 HTLV2 infected individuals in the U.S. alone. Infections occur in similar fashion to HTLV1 except HTLV2 is more prevalent amongst intravenous drug

users and Amerindian tribes (Roucoux and Murphy 2004). Even though the literature is somewhat divided on the clinical outcome of HTLV2 infections (Araujo and Hall 2004), studies have shown that it can also lead to a milder form of HAM/TSP. The confounding factor in the clinical diagnosis of HTLV2 induced HAM/TSP is the fact that these patients are also frequently chronic drug users. Some reports suggest that HTLV2 infections can lead to other neurological syndromes such as HAM with ataxia, neuropathy and spinocerebellar ataxia. Other clinical symptoms associated with HTLV2 infections are increased risk of pulmonary, skin and soft tissue infections, and increased lymphnode activity. Interestingly, HTLV2 and HIV often occur together and HTLV2 is suggested to hasten HIV progression (Araujo and Hall 2004; Roucoux and Murphy 2004).

Treatment for HAM/TSP

Treatment of HAM/TSP includes corticosteroids, other immunosuppressive medications, high dose vitamin C and interferons, and supportive care with antispasmodics and physical therapy (Proietti et al. 2005).

1.7 THE STUDY OF MENDELIAN TRAITS

Stemming from Mendel's revolutionary observations in peas, modern clinicians and geneticists came to the realization that certain human traits are determined by transmission of single genes, which are Mendelian traits. Mendelian traits are usually rare, highly penetrant traits, with a clear mode of inheritance (MOI). The study of Mendelian diseases has been successful for many years and remains important, for it allows relatively easy gene identification and characterization. There are currently 1945 phenotypes with known molecular basis in the Online Mendelian Inheritance in Man catalog (OMIM). In addition, there are 1534 entries of phenotypes without a known molecular basis. Elucidating a particular pathway through the study of a gene function also leads to a better understanding of more frequent complex diseases with lower penetrance (Antonarakis and Beckmann 2006). Interestingly, five out of the ten top selling drugs in 2004 in the US had Mendelian phenotypic correlates, illustrating the importance of gene finding in the development of treatment (Brinkman et al. 2006). The identification of disease genes has also lead to the development of disease specific treatments. There are, for example, 95 gene transfer trials currently underway. Other types of gene-based therapies are antisense or RNAi technology for dominant disease, and embryonic stem (ES) cell therapy (O'Connor and Crystal 2006).

Positional cloning

The general procedure involved in gene identification is positional cloning. Positional cloning starts with the characterization of the phenotype and determination of criteria for a clear diagnosis. Phenotypic characterization is followed by the collection of large families with a segregating phenotype. The families need to be sufficiently large to have power for reaching statistical significance. This can be determined by the statistical program SIMLINK. At a first stage, a karyotype should be performed to rule out large chromosomal aberrations. If a normal karyotype is obtained, the project can proceed to linkage analysis. Linkage analysis calculates the cosegregation of a trait with polymorphic marker alleles in a family. When a marker allele segregates with the disease, it means that the marker is close to the disease gene. The statistical measure for segregation is the LODscore (log of odds), which is calculated by comparing the likelihood of two loci being linked over the likelihood of two loci being unlinked (theta $\geq$ 0.5). This can be calculated using various programs such as the LINKAGE software package. A LODscore ≥ 3 indicates that the two loci are linked and a LODscore of ≤ -2 suggests that the loci are unlinked. Multiple markers can be analyzed in multipoint linkage analysis using programs such as LINKAGE or GENEHUNTER (Pericak-Vance 1996).

Linkage analysis of markers at known loci should first be investigated. If no linkage is found and the locus can be excluded, the positional cloning effort can proceed to a whole genome scan (WGS). In a WGS, markers at set densities are genotyped over the entire genome and analyzed for linkage. Loci with LODscores over a certain cut-off (usually +1.5 or +2) are further investigated. The analysis of additional markers at a locus of

interest and the collection of additional families are part of the fine-mapping process. The determination of phase of marker alleles (identity by descent), allows for the identification of recombination events (haplotyping). These recombination events delimit the borders of the candidate region. In large populations, in particular those descending from a common founder, linkage disequilibrium (LD) mapping can be applied to narrow down the candidate region (Morton 2003). In general, the mapping of recessive genes in consanguineous families is easier because one can search for homozygosity and one can be more certain about cross-over events (Botstein and Risch 2003).

Once the critical candidate interval is established, the project proceeds to the candidate gene screening phase. At this point, bioinformatics tools become crucial. A gene list is made of all candidates in the region, containing relevant information such as functional data, expression data and structural data. This list serves to prioritize genes in the gene screening process. Primer design, PCR amplification and sequencing follow. The multiple variants identified should follow rigorous criteria in order to be validated as a mutation or eliminated as a polymorphism. The criteria for a mutation are the absence of the variant in a set of control samples and the cosegregation of the variant with the disease. Other analyses are sequence comparison for conservation, homology modeling and functional tests. According to the human mutation database, the most common mutations are coding changes (Table 3), which is why the focus should first be on exons and flanking intronic sequences (http://www.hgmd.cf.ac.uk/ac/).

Table 3: Number of entries in the HGMD by mutation type as of August, 2006.

Mutation type	Number of entries
Micro-lesions -	
Missense/nonsense	30563
Splicing	5050
Regulatory	670
Small deletions	8737
Small insertions	3478
Small indels	769
Gross lesions -	
Repeat variations	140
Gross insertions/duplications	459
Complex rearrangements	378
Gross deletions	2964
Total	*53208*

The identification of Mendelian genes through positional cloning has led to the realization that Mendelian traits can be complex. The variable expressivity, incomplete penetrance and extreme genetic heterogeneity of loci complicate genotype-phenotype correlations. Furthermore, the mapping of genes for Mendelian traits has revealed new mechanisms of mutations (Botstein and Risch 2003). An example is mutations in the *Shwachman-Bodian-Diamond Syndrome* (*SBDS*) gene due to gene conversion with its pseudogene, which results in protein truncation (Boocock et al. 2003). Epistasis was shown to occur in Bardet-Biedl Syndrome (BBS), a disorder of basal bodies and cilia, after the identification of individual genes (*BBS1-BBS11*). Epistatic interactions in BBS were demonstrated in families and in zebrafish, where mutations at different loci modify the age of onset and severity of the disease (Badano et al. 2006; Stoetzel et al. 2006). Lessons from phenylketonuria taught us that environmental factors such as diet can greatly impact the phenotypic preservation of a trait (Scriver and Waters 1999).

The genetics of complex or multifactorial traits is currently the new wave of human genetics. The genetic analysis of these traits consists largely of case-control association studies and transmission disequilibrium tests. Many positive associations have been reported; however few have been replicated or validated. Amongst the replicated studies, most are due to genes with major effects and often the variants in those studies are not functionally validated (Brinkman et al. 2006). The association of a 5' regulatory SNP in the *XPNPEP2* gene with APP activity was well established but no functional data has been reported (Duan et al. 2005). The strong association of leprosy with an 80 kb region between *PARK2* and *PACRG* is also yet to be supported by functional studies (Mira et al.

2004). An alternative approach to gene-finding for complex traits would be to search for endo-phenotypes in the hope to identify more genes of major effect by positional cloning. In addition, positional cloning and gene characterization have been revolutionized, mostly because of the invention of PCR and the large-scale sequencing projects. We now have access to fast genotyping, denser genetic maps, almost complete physical maps, model organisms, and gene annotation databases (Morton 2003).

Bioinformatics

The human genome project would not have been of much use if the sequence was not properly annotated and made available to the public. The tremendous amount of data generated can now easily be accessed with the help of excellent annotated databases such as the ucsc, ncbi and ensembl genome browsers (http://genome.ucsc.edu/, http://www.ncbi.nlm.nih.gov/, http://www.ensembl.org/index.html). Every year a list of all current tools is reported and provides an up-to-date overview of what is available. The tools most valuable to human gene finding projects include gene prediction programs (structure), expression databases, 3D modeling predictors (validation step), and sequence comparison programs (Fox et al. 2006). The tool that constitutes the basis of bioinformatics is the Basic Local Alignment Search Tool (BLAST - http://www.ncbi.nlm.nih.gov/BLAST/) and its derivative BLAT, which allow for fast sequence comparisons. Recent advances in development of tools focus on 3D predictions and functional RNA analysis (Fox et al. 2006). The geneticist is mostly on the user end of bioinformatics and works in close collaboration with the programmers to develop tools. The web interfaces integrating gene information of all databases have become the

gold standard for gene hunters. Three of these tools are Moby (http://www.biomoby.org/), Bioinformatic Harvester (http://harvester.embl.de/) and Suspects (http://www.genetics.med.ed.ac.uk/suspects/). These types of tools render the gene cloning process much more efficient.

1.8 ZEBRAFISH AS A MODEL FOR HUMAN DISEASE

The use of model organisms in HSP has been limited to the mouse and the fruit fly (see previous section on HSP genes). A relatively new addition to the model organism list for genetic manipulations is the zebrafish, *Danio rerio*. The zebrafish is an excellent model organism, given its high fecundity, easy care, transparent embryos, easy injection of water soluble substances and significant morphological and physiological similarities to mammals (http://zfin.org/) (Westerfield 1995).

The zebrafish genome sequencing project started in 2001 at the Sanger Institute and has led to the development of new tools such as morpholino knock-down, transgenic fish lines and ENU mutagenesis projects. Large scale ENU mutagenesis screens have already resulted in the identification of many genes. One such screen was featured in the 123rd issue (1996) of Development. These advances, combined with the well established electrophysiology techniques for fish, enable us to study human disease genes in the zebrafish (Penberthy et al. 2002).

Morpholino knock-down technique

Morpholinos are modified oligonucleotides with resistance to degradation (Gene Tools, LLC). Injection of a gene specific morpholino can reduce protein expression by 90%. One type of morpholino binds to the ATG of mRNA and blocks translation. Another type of morpholino binds to intron-exon boundaries and disrupts splicing. The knock-down with the correct dose of morpholino is usually specific and lasts at least up to two

days post fertilization (Nasevicius and Ekker 2000). Furthermore, the availability of GFP-expressing transgenic lines facilitates immuno-histochemical analysis of morphants. One such line, isl1, expresses GFP in motor neurons and enables the visualization of the cell bodies and main axons (Higashijima et al. 2000).

This morpholino system can be used to study inherited developmental defects and loss of function of genes involved in late-onset diseases. Several disorders have been modeled in the zebrafish, ranging from cardiac defects to neurological disorders (Penberthy et al. 2002). Recently, a knock-down model for Joubert syndrome, a cystic kidney disease, was reported. The knock-down of the *cep290* gene in zebrafish led to a phenotype in retinal, cerebellar and otic cavity development and resulted in the hallmark cysts in the kidney tubules (Sayer et al. 2006). An elegant study by McWhorter and colleagues used morpholino technology to model SMA, a LMN disorder. The zebrafish knock-down model of SMA offered the opportunity to study the motor neuron degeneration *in vivo*. Their study showed a defect in motor neuron axon outgrowth and excessive branching (McWhorter et al. 2003). Taken together, it can be concluded from these studies that zebrafish model system is a useful tool in the field of genetics and can be applied as a model for neurodegenerative diseases such as HSP.

1.9 OBJECTIVES

In summary, the Hereditary Spastic Paraplegias encompass a group of neurodegenerative diseases characterized by severe progressive leg spasticity and paraparesis. This disease affects a significant number of individuals worldwide (20,000 people in the U.S.) and there is no treatment available. Several mutated genes have been identified in HSP and the study of these genes will elucidate the mechanism of UMN death in the corticospinal tracts seen in HSP. HSP gene characterization should also improve our understanding of how the nervous system functions normally, in particular with regards to the maintenance of healthy neurons over time. These studies could ultimately lead to the development of therapies for people with spinal cord injuries and neurodegenerative illnesses in general.

The main objective of this dissertation is to identify and characterize additional genes and their gene products involved in the neurodegenerative disease, HSP. Furthermore, we would like to genetically stratify our collection of HSP patients of mostly North American, English Canadian and French Canadian descent. Our laboratory has a large collection of families consisting of over 80 HSP families and three large HSA families with mostly autosomal dominant and recessive modes of inheritance. The large families with sufficient statistical power will allow us to map disease genes using family-based linkage analysis. These positional cloning projects aim to identify novel genes in an attempt to pinpoint a possible common pathway that results in specific neuronal cell death in the corticospinal tracts of HSP patients. The smaller families in our collection are less suitable for linkage analysis, and can be included in large-scale mutation screens

of known HSP genes to broaden the mutation spectrum and characterize the nature of these novel mutations. The confirmation of known mutations and the identification of novel mutations in known genes contribute to genotype-phenotype correlations, determines the genetic make up of our HSP cohort and excludes families from future linkage studies.

This thesis focused on mutation analysis, linkage mapping and positional cloning at different HSP loci. In the first chapter a general overview of the literature was provided. In the next three chapters (chapters two, three and four) the mutation analysis of known HSP genes in our cohort is described. Linkage mapping and positional cloning efforts follow in chapters five, six, and seven. Finally, chapter seven includes gene identification and mutation validation.

CHAPTER 2: MUTATIONS IN THE *SPG4* GENE

Reference:

Inge A. Meijer, Collette K. Hand, Denise A. Figlewicz and Guy A. Rouleau. Spectrum of *SPG4* mutations in a large collection of North American families with Hereditary Spastic Paraplegia. *Arch Neurol.* 2002 Feb;59(2):281-6

and

Submitted to the *Canadian Journal of Neurological Sciences* (CJNS) in July 2006.

Reference:

Inge. A. Meijer, Nicolas Dupré, Bernard Brais, Patrick Cossette, St Onge J, Marie-France Rioux, Melanie Benard, Guy A. Rouleau. Evidence for a *SPG4* founder effect in French Canada. 2006

2.1 RATIONALE

At the start of this project, 11 HSP loci had been mapped, including 4 genes that had been identified (McDermott et al. 2000). Our collection of HSP families consisted of 49 dominant, 11 recessive, and 1 X-linked family. In addition, there were 10 families with uncertain pattern of inheritance and 6 seemingly sporadic cases. Previous linkage studies had been attempted, but lack of human genomic data made positional cloning difficult. This project started the year the human genomic data was published, which greatly facilitated this kind of research.

The *SPG4* gene encoding spastin was cloned in 1999 and mutations in this gene were shown to be the most common cause of ADHSP (Hazan et al. 1999). We decided to screen this gene in our cohort to identify novel mutations and to exclude families from further analysis. The cohort selected for *SPG4* screening consisted of dominant, recessive and sporadic cases (non-ADHSP samples were included to investigate whether spastin mutations could also cause other forms of HSP).

Section 2.2 describes the initial screening of *SPG4* in our cohort (Meijer et al. 2002a) and the following section (2.3) focuses on one mutation in exon 15 with a possible common ancestor in the French Canadian population.

2.2 SPECTRUM OF *SPG4* MUTATIONS IN A LARGE COLLECTION OF NORTH AMERICAN FAMILIES WITH HEREDITARY SPASTIC PARAPLEGIA

2.2.1 Abstract

Background: Hereditary Spastic Paraplegia (HSP) is a neurodegenerative disease characterized by progressive spasticity and weakness of the lower limbs. The most common form of HSP is caused by mutations in the *SPG4* gene, which codes for spastin, an adenosine triphosphatase with various cellular activities (AAA) protein family member.

Objective: To investigate a large collection of predominantly North American patients with HSP for mutations in the spastin encoding gene, *SPG4*.

Methods: DNA from 76 unrelated affected individuals was studied for mutations by single stranded conformational polymorphism analysis and direct sequencing. Each new variant identified was then analyzed in 80 control subjects to determine whether the variant is a common polymorphism or a rare mutation. All DNA samples were amplified by Polymerase Chain Reaction, followed by electrophoresis and autoradiography.

Results: We identified 8 novel and 5 previously reported mutations in 15 affected individuals. The novel mutations are 4 missense, 1 nonsense, 1 frameshift, and 2 splice mutations. Two polymorphisms (one in an affected individual) were also identified.

Conclusion: Our collection of families with HSP is different on a genetic level from

those previously described. The percentage of our families with a SPG4 mutation is 10% lower than the 40% estimate of families with autosomal dominant HSP noted to be linked to this locus, and splice mutations are not predominant in our collection. Interestingly, we also identified two recurring mutations in specific populations (R562Q and G559D), which may facilitate the development of future spastin diagnostic testing in these populations.

2.2.2 Introduction

Hereditary Spastic Paraplegia (HSP) also known as Familial Spastic Paraplegia (FSP) or Strümpell-Lorrain syndrome comprises a heterogeneous group of neurodegenerative disorders characterized by progressive lower limb spasticity and weakness associated with bladder disturbance in approximately 50% of the cases (Harding 1981; Harding 1993b). Clinically, there are two types of HSP: the pure form which is characterized exclusively by leg spasticity often with bladder disturbance and the complicated form which includes additional neurological abnormalities such as optic neuropathy, dementia, ataxia, deafness, mental retardation, and extra-pyramidal disturbance (Harding 1981; Harding 1993b). The main neuropathological feature is axonal degeneration of the distal ends of the longest ascending and descending tracts. This neurodegeneration results in spasticity of the lower limbs which causes difficulty walking and in severe cases the patients become wheelchair bound (Dube and Rouleau 2000; McDermott et al. 2000). The age of onset varies greatly, even within families, from early childhood to mid-eighties. The genetic heterogeneity in HSP is demonstrated by the number of loci (*SPG1* through *SPG17*) that have been mapped. These include three X-linked (Keppen et al. 1987; Jouet et al. 1994; Tamagaki et al. 2000), 9 autosomal dominant (Hazan et al. 1993; Hazan et al. 1994; Fink et al. 1995; Hedera et al. 1999; Reid et al. 1999a; Seri et al. 1999; Fontaine et al. 2000; Reid et al. 2000; Patel et al. 2001) and five autosomal recessive loci (Hentati et al. 1994a; De Michele et al. 1998; Martinez Murillo et al. 1999; Vazza et al. 2000; Hughes et al. 2001). Of all described families 40% are linked to the autosomal dominant locus known as *SPG4* (OMIM182601) (Hazan et al. 1999). The defective gene for *SPG4* was cloned recently and encodes a 616 amino acid protein named spastin. The

protein is an ATPase associated with various cellular activities (AAA) with a characteristic AAA cassette in the C-terminus from amino acid 342 to amino acid 599. According to computer predictions the protein possesses a nuclear localisation signal, Walker motif A and B, AAA minimal consensus sequence, Leucine Zipper motifs and Helix Loop Helix domain. Furthermore, the protein shows strong homology to two yeast proteins (Yta6p and Sap1) and the mouse Skd1 protein. Recent evidence suggests that spastin might interact with tubulin (Casari and Rugarli 2001), but its main function remains unknown despite its homology to other proteins and functional domains. To date, more than 50 mutations have been reported in the spastin encoding gene, including splice site, nonsense, missense and frameshift mutations (insertion and deletion) affecting either directly or indirectly the conserved AAA cassette (Burger et al. 2000; Fonknechten et al. 2000; Hentati et al. 2000; Lindsey et al. 2000; Santorelli et al. 2000; De Bantel et al. 2001; Higgins et al. 2001a; Namekawa et al. 2001; Svenson et al. 2001a), suggesting the functional importance of the AAA cassette. No clear genotype phenotype correlation has been reported, but most articles suggest that *SPG4* is caused by haploinsufficiency of spastin, which implies a critical spastin threshold (Casari and Rugarli 2001).

Herein we report 8 novel mutations and 2 novel polymorphisms identified in the spastin encoding gene and the recurrence of two mutations in two populations, which could be important for the development of large scale *SPG4* mutation detection efforts.

2.2.2 Patients and Methods

PATIENTS

The participants included five controls and seventy-six unrelated patients from families with HSP available in our laboratory. The families FSP4 (Zmax = 1.09 - unpublished data), 7 (Zmax = 2.7), and 28 (Zmax = 0.77) (Dube et al. 1997) had previously shown suggestive linkage to the *SPG4* locus. The families were diagnosed as having autosomal dominant HSP (ADHSP; 49 families) or autosomal recessive HSP (11 families), and the other 16 families are small families in which the pattern of inheritance is unclear. All probands were seen by a neurologist and the diagnostic criteria were lower limb spasticity in absence of any evidence for a structural lesion or demyelination. Our collection of patients consists of mostly French Canadian and other Caucasian (Canada, U.S.) families with the exception of two Middle Eastern families. Patients as well as controls in this study have given informed and written consent. Genomic DNA from these individuals was prepared from peripheral blood leukocytes by standard extraction methods.

MUTATION SCREENING

Single stranded conformational polymorphism (SSCP) analysis

Polymerase Chain Reaction (PCR) primer pairs were designed from the genomic DNA sequence of the human *SPG4* gene (Genbank accession number : AL121655, AL121658) as shown in table 1.

The 17 coding exons were amplified by PCR using approximately 20ng genomic DNA and radiolabelled dATP in a total volume of 13 µl for SSCP analysis. The reactions were

performed in thermocyclers (Perkin Elmer Inc, Forest City, Calif), starting with an initial denaturation of 5 minutes at 94°C followed by 30 cycles of 30 seconds denaturation, 30 seconds annealing at primer specific temperature and 45 seconds extension at 72°C. Electrophoresis of PCR products was performed on 9.5% polyacrylamide (5% glycerol) and on 50% Mutation Detection Enhancement gels (Biowhittaker Molecular Applications, Rockland, ME) followed by autoradiography. Samples showing altered migration patterns were selected for further analysis.

Sequencing

Genomic DNA from selected samples was amplified in 50 ul nonradiolabelled PCR mix. The PCR products were purified with a gel extraction kit (QIAEX11, QIAGEN, Mississauga, Ontario), after which the purified fragments were sequenced using a thermosequenase P^{33} cycle sequencing kit (United States Biochemical Corp, Cleveland, OH) and run on 6 % polyacrylamide denaturing gels.

Variations identified by sequencing were analysed for cosegregation in family members when available. All newly identified disease associated variations were examined for presence in 80 white controls (160 chromosomes) by SSCP analysis.

2.2.3 Results

One affected member from each of the 76 kindreds was screened for *SPG4* mutations. Most families were too small to test for linkage and 3 families had shown suggestive linkage to the *SPG4* locus in a previous study. We identified 13 mutations in 16 families and 2 polymorphisms (Table 2, 4). Our results show 8 novel mutations in the *SPG4* gene: one frameshift (687del1), two splicing mutations (A1538-2G, G1854-1C), four missense mutations (C1321T, C1591T, G1810A, G1801A), and one nonsense mutation (G1425T) (Table 3). Five previously reported mutations were also found in our collection. The transmission of these mutations correlates with the clinical status of the families except for some carrier individuals who are asymptomatic, which could be due to age dependent, incomplete penetrance or simply subtle symptoms. Interestingly, mutations were identified in only 2 out of 3 families previously linked to *SPG4* (family FSP4 and FSP7). Seven of the novel mutations are in the AAA cassette spanning exon 7 to 16 (Hazan et al. 1999). The last novel mutation is a 1 basepair deletion in exon 3, which results in a frameshift leading to a premature stop codon, truncating the protein before the AAA cassette. This mutation was found in individual R11540 and is one of the rare mutations occurring outside of the AAA cassette of spastin (Higgins et al. 2001b), and further suggests the importance of the AAA cassette in spastin function. DNA for the rest of the family was unavailable for testing of cosegregation. The other nonsense mutation found in individual R12477 creates a stop codon in the third conserved domain of the protein, which results in a protein lacking the predicted helix loop helix domain as well as a leucine zipper motif.

This study identified 4 novel missense mutations, all occurring in the AAA cassette. The mutation, C1321T in exon 9, changes a serine (polar and hydrophilic) into a leucine, a hydrophobic amino acid. A mutation in exon 12 leads to a C1591T change, where proline with a cyclic ring is substituted by a hydrophobic linear amino acid, which occurs close to the conserved helix loop helix domain. The mutation G1801A (exon 15) gives rise to an amino acid change from an uncharged glycine to aspartic acid in the conserved 6226 domain. This mutation was found in two French Canadian families with HSP. Lastly, a missense mutation G1810A in exon 15 was found in two white US families and changes a basic amino acid arginine, to an uncharged glutamine. The novel missense mutations all occur in a domain or near a domain of the AAA cassette, suggesting the importance of conserved amino acid structure in this region. None of the variants were found in 160 control chromosomes by SSCP analysis. There is only one report of a coding polymorphism, and it is not the same as any of the above mentioned mutations (Svenson et al. 2001a).

We found two new splice site mutations (A1538-2G and G1854-1C) in the acceptor splice site of exon 11 and 15, respectively (Strachan and Read 1996). For both mutations the predicted truncated protein lacks a part of the AAA cassette, which probably renders the protein nonfunctional and causes disease. Another possibility is that the splicing machinery uses an alternative cryptic splice acceptor, which results in a defective protein.

We also detected two possible polymorphisms. One polymorphism leads to an amino acid change (R431Q) in the third conserved domain of spastin of a spouse of an affected included in the screening as a control. This individual was seen by a neurologist

and was not affected with HSP. The proband of this family is individual R12320, who has a missense mutation. The other polymorphism is an intronic substitution 16 nucleotides away from the exon in an affected individual and does not show cosegregation in the family.

2.2.4 Comments

Thirteen mutations, 8 that are novel, were identified in the *SPG4* gene of patients from a collection of 76 predominantly North American with HSP families. Interestingly, mutations in the spastin gene were found in only 15 (31%) of the 49 known families with ADHSP who were analyzed, which suggests that our collection of families represents a subpopulation that does not exactly conform to the 40% estimate of families with ADHSP linked to *SPG4*. A previous report (Dube et al. 1997) of linkage analysis from our laboratory showed that only 14% of the families in that study linked to *SPG4*, which correlates with the lower percentage of mutations found in the screen. Some of the 16 small families with an unclear pattern of inheritance could also be families with ADHSP, which would decrease the percentage of our families linked to *SPG4*. Furthermore, 11 recessive families were included in the screen because Lindsey and colleagues (Lindsey et al. 2000) reported a homozygous mutation in spastin, S44L in a kindred with autosomal recessive HSP. There were no mutations detected in any of our families with recessive disease. These data suggest that *SPG4* mutations may be responsible for a smaller fraction of ADHSP than previously reported, and that spastin screening is only worthwhile in kindreds showing clear autosomal dominant inheritance. However, it is possible that our screen underestimated the fraction of spastin mutation-positive families because SSCP analysis is only 80%-100% effective in detecting mutations (Dracopoli et

al. 1994), and mutations occurring in the noncoding regions, such as the promotor and other regulatory sequences, would not be detected by our screen which analyzed the exons and the intron–exon junctions. Nevertheless, our methods are similar to those used in other spastin mutation screens.

While most reports of *SPG4* mutations show a predominance of splice mutations, missense mutations were the most frequent ones found in our study. Only 3 splice mutations were found out of 13 mutations identified in affected individuals. This could be related to the fact that our families are North American, and to our knowledge no large scale study has ever been performed on this population. We do not think it is likely that variants in the splice junction were not detected because the primers used in this study included approximately 30 base pairs of the flanking intronic regions. The missense mutations found in affected individuals were not found in 160 control chromosomes and showed cosegregation with the disease phenotype in the respective families. All affected individuals in a *SPG4* positive family had the identified mutation. Because HSP has incomplete and age-dependent penetrance some seemingly unaffected individuals (Fam33, R7050, some family members tested for cosegregation) in our screen carried a mutation in spastin. In addition, it is estimated that 25 % of individuals are asymptomatic or unaware of their symptoms (Durr and Brice 2000). These data strongly support a causative role for these mutations in HSP.

The spastin gene is highly conserved through species, which suggests that amino acid changes, especially when present in the AAA cassette, are likely to have a consequence on protein function. There are no reports of a polymorphism in the coding region in this gene except for one variant, G1004A (Svenson et al. 2001a), which was

first detected in a proband of a family with HSP, but not in 80 control individuals. Our study identified the same variant in a second affected individual. Unfortunately, no other affected individuals are available in this kindred for testing, but the variant was not present in 80 control individuals. Because this variant has not been detected in a total of 320 control chromosomes and has now twice been identified in affected individuals, it is likely that the variant is a mutation involved in disease etiology. The variant, which does not change the amino acid sequence, could possibly disrupt an exonic splicing enhancer or silencer, thereby affecting the patterns or efficiency of mRNA splicing (Liu et al. 2001). Future mRNA work is required to further investigate the effects of this mutation. Two additional polymorphisms, one of which leads to an amino acid substitution, were also identified in this study. The characterization of variants as polymorphisms should be confirmed by mRNA RT PCR analysis and studies of larger control populations.

Five mutations were identified in individuals with complicated HSP showing atypical symptoms such as restless legs, atypical seizures and dysarthria. One interesting case has a nonsense mutation in exon 10 (age 45), with severe manifestation of disease showing spasticity, footdrop and signs of ataxia, dysarthria and nystagmus. The severe phenotype could be classified as a complicated HSP or spastic ataxia and suggests that this gene might also be involved in other clinical entities. Contrary to the nonsense mutation mentioned above, a conservative mutation in exon 10 for patient R5913 (age 66) caused early onset progressive spasticity rendering the patient bedridden, with rather uncommon fecal incontinence (Table 2). Last, there are 2 patients R7050 and Fam33 who have no mobility problems and were unaware that they are affected. They both have a previously reported splice mutation (Svenson et al. 2001a) and a nonsense mutation respectively.

One would expect that the individual with the nonsense mutation in exon 15 and, therefore, lacking the C terminal end of the AAA cassette, might have a relatively severe phenotype, but remarkably that is not the case. The fact that a splice alteration in acceptor exon 15 causes disease suggests that the C terminal part of the AAA cassette is important for function and having even a part of cassette is not sufficient for normal function. More in-depth phenotype-genotype correlation studies would be necessary to further support that there is no actual correlation.

In addition, patients R9259 and R12320 have the same mutation but different phenotypes, particularly age of onset. The clinical variability that occurs in individuals with the same or similar mutations might be due to modifier genes; this hypothesis is supported by a report of mutations found in the *EAAT2* gene (already involved in Amyotrophic Lateral Sclerosis) in some patients with HSP (Meyer et al. 1998). The modifier gene theory can also partly explain why there are reports of complicated HSP as well as pure HSP at the SPG 4 locus. The hypothetical modifier might be related to the conserved AAA cassette. Spastin bound to ATP could bind a modifier or activate a secondary gene, which allows for greater expressivity of the phenotype. Unfortunately, the function of spastin can only be hypothesized.

To our knowledge, this study is the first to identify 2 recurring mutations. The G1801A mutation was identified in two seemingly unrelated French Canadian families, and the G1810A in two seemingly unrelated Caucasian American families. This suggests that these two mutations show a higher frequency in the specific populations, which could be helpful for future *SPG4* diagnostic testing in these populations. It is important to identify frequent mutations in specific populations because the spectrum for spastin

mutations is so great, making efficient genetic testing only feasible in subpopulations. There were 24 other French Canadian families included in the screen that did not show any significant variation in the spastin gene. These families are not all from the same region in Quebec, but are assumed to belong to the general French Canadian population. The results suggest that there is at least one more defective gene responsible for HSP in the French Canadian population.

In conclusion, this report presents 8 novel mutations in the *SPG4* gene, contributing to the large spectrum of mutations found in this gene. Continuing to identify new mutations is important particularly for developing diagnostic testing programs based on the frequency of mutations in specific populations. Further phenotype-genotype correlations studies are required to unveil the reason behind the great variable expressivity for the *SPG4* form. A better understanding of the function of spastin is vital to discovering a treatment for HSP, and future studies phenotype-genotype studies and localization studies for the protein will be necessary.

2.2.5 *Tables*

Table 1: Primers designed for amplification of all 17 exons.

Exon	Forward Primer	Reverse Primer	Size, bp
1A	CCACCGACTGCAGGAGGAGA	GCGCCGCCGGAGCCTTTCTTCTTC	260
1B	TTATGGCGGCGGCGGCAGTGAGAG	CCATGAGGGCGCGGGAGAAGC	292
1C	CACCTGGGGCTCCTCTTC	GACCCCACCGCCTTCTT	263
2*	TTTTTATGTATTACCTCTCAA	AAAAATAAATAAATAAATAAATAG	266
3	CTCCCCATGAAAGTAGTT	ATGTTAAAAAGCCTGGAC	290
4	TATCATGTAACAATCTGGTA	TTATAAAATCAAATCAACAT	307
5	TTTTCTAATCACAATGGT	TATGATCAACTTAAGCAGGAAT	309
6	ATGTTAGGTTGTATTTTCA	GTATTTATTATCTATTTCACTCCT	269
7	TGTCATAGGGCTTAGGCTTCA	ATGGATTCAGTAACAGATGGTATT	226
8	CTGTTTGGGAAGATGCT	GTAAATAATAGACTCAAGGACAAG	273
9*	GCATGAGCCACCACACCTG	AGATAAGCTCCTAGAAAAATA	316
10	GTGCTAGATTTTCAACATA	GCCCTTCTTTAAAACTTCTTCC	270
11	ACTCACATAGCTTGGTCTT	AGGAAAATATACTAAAATG	196
12	ATGGCCAAGGTTAAAAATACAA	CTGGAAGAAAATAGTGAAT	281
13	CTTTTCCTGTCATTTGCTGTTTC	TTAATATTGTCAAGATGGTAGTTC	186
14	TCGGGAGGCTGAGATGG	AATAAATAAAAGCTGTAAGATAAA	300
15	AAAAAGCGGGAGGGGAAATA	TGGGCAACAGACTGAGACC	248
16*	ATTGTACTTGGTTTTGCCCTTCA	GAGCCGATATCATGCCAGACT	259
17	ACCACCATATACCTGTTGAT	CTGTTTCTGTAGCCGATGAC	263

* These exons have also been amplified using primers by Lindsey et al, 2000 (Lindsey et al. 2000).

Table 2 : Clinical information of probands[§]

Identifier	Age of onset	Gait	upper limb hyper-reflexia	bladder disturbance	Babinski	Additional comments
Fam33	...	no mobility problems	+	-	+	...
R3428	childhood	unable to walk	-	-	-	foot drop
R4650	50's	problems running	-	+	+	fecal incontinence, nocturmal myoclonus, restless leg
R4888	40's	unable to walk	-	-	-	...
R5913	childhood	unable to stand	-	+	...	fecal incontinence
R7050	...	no mobility problems	+	-	+	patient unaware of symptoms
R9109	40's	problems walking	+	-	+	dysphagia
R9259	25	able to run	-	+	+	...
R11540	37	problems running	+	-	+	arthritis
R11872	childhood	uses walker	-	-	...	late onset atypical seizures and memory loss
R12320	70	problems walking	-	+	+	...
R12416	60	unable to walk	+	-	-	...
R12447	40	trips often, can still walk	+	+	+	assymetrical spasticity, depression, dysarthria, nystagmus, footdrop.
R13912	childhood	problems walking	-	-	...	dysarthria

*Age of onset as observed by patient

§ Relevant clinical data for individual 78 are unavailable. All probands experienced lower limb hyperreflexia. + indicates present; -, absent; and ellipses, data not available

Table 3: Summary of mutations identified in probands with HSP.

Sample	Location	Mutation*	Amino acid change	consequence	Origin
R11540	Exon 3	687Del1	PTC + 7a.a.	frameshift	French Canadian
R4888	Exon 6	G1004 A (Svenson et al. 2001b)	P293P	Possible mutation of splice enhancer/silencer?	White (U.S.)
R11872	Intron 8	G1298 +1 A (Lindsey et al. 2000)	…	splicing mutation	White (U.S.)
R7050	Exon 9	A1367G (Svenson et al. 2001a)	K414K	splicing mutation	French Canadian
R12416	Exon 9	C1321T	S399L	missense	French Canadian
R5913	Exon 10	C1401G (Fonknechten et al. 2000)	L426V	missense	White (U.S.)
R12447	Exon 10	G1425T	Q434STOP	nonsense	French Canadian
R13912	Intron 11	A1538 - 2 G	…	splicing mutation	French Canadian
R9109	Exon 12	C1591T	P489L	missense	French Canadian
R9259	Exon 15	G 1801A	G559D	missense	French Canadian
R12320	Exon 15	G1801A	G559D	missense	French Canadian
Fam33	Exon 15	C1809T (Fonknechten et al. 2000)	R562STOP	nonsense	White (U.S.)
78	Exon 15	G1810A	R562Q	missense	White (U.S.)
R4650	Exon 15	G1810A	R562Q	missense	White (U.S.)
R 3428	Intron 16	G1854 -1C	…	splicing mutation	White (U.S.)

The nucleotide location was assigned using SPG4 cDNA available from www.genoscope.cns.fr.

PTC: protein truncation

…..: functional effect unknown

2.2.6 Acknowledgements

This work was supported by the Muscular Dystrophy Association, Tucson, Ariz; and the Canadian Institute of Health Research, Ottawa, Ontario (Dr Rouleau). We thank all the families with HSP (and their referring physicians), who participated in this study, and André Toulouse, M.Sc./Ph.D., for carefully reading the manuscript.

2.3 EVIDENCE FOR A *SPG4* FOUNDER EFFECT IN FRENCH CANADA

2.3.1 Abstract

Background: The most common cause of autosomal dominant Hereditary Spastic Paraplegia (HSP) is mutations in the *SPG4* gene. We have previously identified novel *SPG4* mutations in a collection of North American families including the c.G1801A mutation present in 2 families from Quebec. The aim of this study is to estimate the frequency of the c.G1801A mutation in the French Canadian (FC) population and to determine whether this mutation originates from a common ancestor.

Methods: We collected and sequenced exon 15 in probands of 37 families. Genotypes of markers flanking the *SPG4* gene were used to construct haplotypes in 5 families. Clinical information was reviewed by a neurologist (ND) with expertise in HSP.

Results: We have identified 3 additional unrelated families with the c.G1801A mutation and haplotype analysis revealed that all 5 families share a common ancestor. The mutation is present in 7% of all our FC families and explains half of our spastin linked FC families. The phenotype associated with the c.G1801A genotype is pure HSP with bladder involvement.

Conclusion: In this study we have determined that the relative frequency of the c.G1801A mutation in our FC collection is 7%, and approximately 50 % in the spastin

positive FC group. This mutation is the most common HSP mutation identified in this population to date and is suggestive of a founder effect in Quebec.

2.3.2 Introduction

The Hereditary Spastic Paraplegias are a clinically and genetically heterogeneous group of neurodegenerative disorders characterized by progressive lower limb spasticity and weakness (Harding 1993b). HSP is clinically grouped as pure or complicated HSP. The complicated form of HSP includes patients with lower limb spasticity and additional neurological features such as optic neuropathy, dementia, ataxia, deafness, mental retardation, or extra-pyramidal disturbance (Harding 1993b; Reid 2003). The genetic heterogeneity in HSP is demonstrated by the large number of loci mapped for the disease (*SPG1* through *SPG31*) (Hodgkinson et al. 2002; Fink 2003a; Simpson et al. 2003; Klebe et al. 2006a; Zuchner et al. 2006a). Identification of eleven HSP genes has revealed that several pathophysiological pathways are involved in this disease including impaired axonal transport, a common link with other neurodegenerative diseases (Crosby and Proukakis 2002; Fink 2003a; Rainier et al. 2003).

The most common cause of HSP is mutations in the *SPG4* gene, explaining 40% of all autosomal dominant HSP cases (Durr et al. 1996; Soderblom and Blackstone 2006). Recently, Depienne and colleagues also reported *SPG4* mutations in 12% of apparently sporadic HSP cases (Depienne et al. 2006). The *SPG4* gene encodes spastin, which has been implicated in various processes such as endosomal trafficking and cytoskeletal rearrangement, but the most studied function is microtubule severing (Errico et al. 2002) through its MIT (microtubule-interacting and trafficking molecules) domain (Roll-Mecak

and Vale 2005) and the AAA (ATPase associated with various cellular activities) domain. Since the cloning of the *SPG4* gene (Hazan et al. 1999), many groups have reported mutations across the gene (Burger et al. 2000; Fonknechten et al. 2000; Hentati et al. 2000). The majority of missense mutations occur in the AAA cassette including the c.G1801A mutation we reported in two FC families (Meijer et al. 2002a; Yip et al. 2003). In this study we determine the relative frequency of the c.G1801A mutation in our French Canadian collection, we provide evidence that the subjects carrying this mutation originate from a common ancestor, and we describe the associated phenotypic features.

2.3.3 Methods

Genotyping

Polymorphic markers were amplified by PCR incorporating radiolabeled S^{35} dATP into the product. The products were separated on 6% denaturing polyacrylamide gels and visualized on autoradiographic film. The following markers were genotyped in and around the SPG4 gene: D2S2201, D2S1383, D2S352, D2S2347, D2S367, D2S1325, and D2S2328. Haplotype construction assuming minimal recombination was performed manually and ordered according to the goldenpath physical map (http://www.genome.ucsc.edu/).

Mutation Detection

Exon 15 was amplified using flanking primers previously reported by Meijer et al. 2001 (Meijer et al. 2002a) and PCR products were sequenced at the McGill University and Genome Quebec Centre for Innovation.

Subjects

Subjects gave written informed consent to participate in our study, which was approved by the local ethics review board (Centre Hospitalier de l'Université de Montréal). Peripheral blood samples were obtained from 37 probands and DNA was extracted by standard methods.

2.3.4 Results

We have previously identified spastin mutations in 15 North American families including 8 novel mutations and 2 recurring mutations in Caucasian families (Meijer et al. 2002a). The c.G1801A mutation was detected in 2 out of 32 FC probands, which was suggestive of a common FC mutation. In the present study we have collected and sequenced another 37 families for this mutation in exon 15. We identified 3 additional families with the common c.G1801A mutation (Fig.1). The c.G1801A mutation therefore has a 7% frequency (5/69) in our FC sample. This also represents approximately half of the spastin mutations found in the FC cohort. To determine whether the families share the mutation because of a genetic link or because of a mutational hotspot, we performed haplotype analysis around the *SPG4* locus (Table 1). Haplotype analysis indicates that all 5 families share a segment of at least 4 Mb and thus originate from a common ancestor.

The phenotype associated with this mutation is that of pure HSP with or without bladder involvement (Table 2). The age of onset varies greatly between families and mild sensory symptoms are only seen in two probands. The detailed clinical information of sample R19935 was not available, but the family history is also that of pure adult onset HSP.

2.3.5 Discussion

We have identified the first common spastin mutation in Quebec, which is suggestive of a founder effect. This c.G1801A mutation has not been reported by other groups and seems to be specific to the FC population. Our laboratory has the largest collection of FC HSP cases and the c.G1801A mutation is the only recurring HSP mutation in this population, with a frequency of 7%. We have also identified other genetic causes for HSP in Quebec namely: PLP, atlastin, spastin and *SPG27,* but they occur at a much lower frequency (Meijer et al. 2002a; Meijer et al. 2004) (personal communication).

Founder mutations have previously been studied in French Canada because of historical roots of the populations and many genes have been identified for these mostly recessive diseases such as Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay (ARSACS) and Hereditary Motor and Sensory neuropathy with Agenesis of the Corpus Callosum (HMSN/ACC) (Laberge et al. 2005; Dupre et al. 2006). The c.G1801A mutation is one of the few examples of a common ancestor in Quebec for a dominant disease. The only other reported founder mutation in spastin was reported by Orlacchio et al. This p.N386S change was detected in 9/15 Scottish families (Orlacchio et al. 2004b). Recently, Namekawa and colleagues also showed evidence of a common ancestor for the R495W atlastin mutation in the French population (Namekawa et al. 2006a).

The phenotype associated with *SPG4* mutations is very variable and mutation carriers can present with pure HSP, ataxia (Nielsen et al. 2004a), Primary Lateral Sclerosis (PLS), early onset Amyotrophic Lateral Sclerosis (ALS) (Meyer et al. 2005), or

dyplastic corpus callosum (Alber et al. 2005). The probands with the c.G1801A mutation present with a pure form of HSP and the disease is uniform in all five families.

Even though the FC HSP population is genetically diverse, it is advisable to screen for the c.G1801A mutation as a priority in patients with dominant or sporadic adult onset HSP to improve efficiency of genetic testing. Interestingly, successful prenatal diagnosis for spastin mutations has been reported and offers an option for families with severe early onset HSP (Nielsen et al. 2004b).

A recent report showed that in a *Drosophila* model of spastin with adult onset neurodegeneration, the phenotype was partially alleviated by treatment with vinblastine, a modulator of microtubule stability (Orso et al. 2005). These findings offer hope for the development of a treatment for spastin related HSP and further underlines the importance of testing for spastin mutations.

2.3.6 Acknowledgements

The authors would like to thank the families for their participation in this study as well as Melanie Benard and Isabelle Thibault for patient recruitment. GAR, PC and ND are supported by the Canadian Institutes for Health Research (CIHR). BB is a scholar of the Fonds de la Recherche en Sante du Quebec (FRSQ)

2.3.7 Tables

Table 1: Haplotype analysis around the *SPG4* locus

Marker	Location Mb	X2754	R9259	R12045	R13395	R19537	CEPH freq
D2S2201	21	2	2	4	2	2	
D2S2383	29	11	2	11	11	11	
D2S352	31	5	5	5	5	5	0.125
SPG4x15	32	A	A	A	A	A	n/a
D2S2347	33	4	4	4	4	4	0.054
D2S367	34	8	8	8	8	8	0.250
D2S1325	35	2	2	2	2	2	0.310
D2S2328	40	10	5	5	5	12	

Table 2: Clinical features of probands with the c.G1801A mutation

Subjects	R12045	R9259	X2754	R13395
Family History	+	+	+	+
Age of onset	70	25	12	35
Age at evaluation	77	40	25	38
Hyperreflexia in the arms	-	-	+	+
Hyperreflexia in the legs	+	+	+	+
Leg spasticity	+	+	-	+
Urinary symptoms	+	+	-	-
Vibration at the toes	decreased	normal	normal	decreased
Plantar responses	extensor	extensor	indifferent	extensor
MRI results	not available	Total spine normal	Total spine normal	Total spine and brain normal

2.3.8 Figures

Figure 1

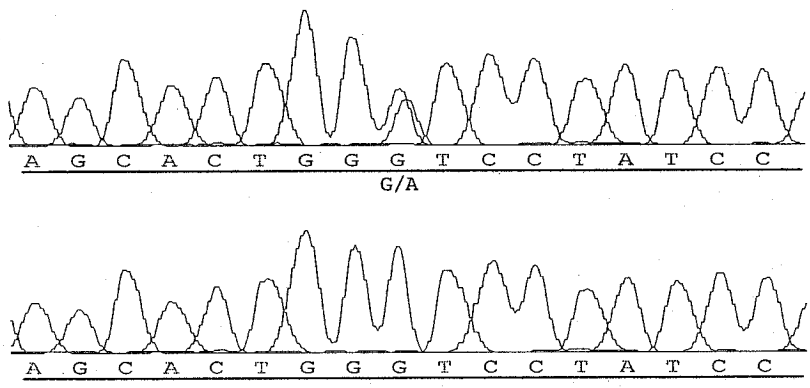


Figure 1: Sequence analysis

The chromatogram of patient DNA carrying the c.G1801A heterozygous mutation compared to a control sample. This mutation leads to a p.G559D missense change in the AAA cassette.

CHAPTER 3: NOVEL MUTATION IN THE *SPG3A* GENE

Submitted to *Annals of Neurology*, August 2006.

Reference:

Inge A. Meijer B.Sc., Patrick Dion Ph.D., Sandra Laurent, Nicolas Dupré M.D./M.Sc., Bernard Brais M.D./Ph.D., Annie Levert, Jacques Puymirat M.D., Marie France Rioux M.D., Michel Sylvain M.D., Cynthia Soderblom B.Sc., Craig Blackstone M.D./Ph.D., and Guy A. Rouleau M.D./Ph.D. A novel deletion in the *SPG3A* gene suggests a dominant-negative mechanism for Hereditary Spastic Paraplegia. 2006.

3.1 RATIONALE

Mutation analysis of the *SPG4* gene described in the previous chapter yielded 8 novel mutations and identified the cause for HSP in 18 families in our HSP collection (Meijer et al. 2002a). When the second most common ADHSP gene, *SPG3A*, was identified in 2001 (Zhao et al. 2001), we decided to also screen this gene in our cohort without a priori linkage data. Probands from families previously shown to be unlinked to spastin were selected and newly recruited samples were added to the cohort, representing a total of 70 samples. The *SPG3A* gene encodes for atlastin and mutations in this gene have been predominantly associated with young onset HSP (Zhao et al. 2001). However, our cohort was not preselected for childhood onset cases, because incomplete penetrance has been reported for *SPG3A* mutations, suggesting that defects in this gene might also cause later development of the disease. Our samples included mainly North American and French Canadian HSP cases, including 14 childhood onset cases. Chapter 3 describes the screening of the *SPG3A* gene in this cohort and the characterization of a novel mutation.

3.2 ABSTRACT

Objective

The Hereditary Spastic Paraplegias (HSPs) are a clinically and genetically heterogeneous group of neurodegenerative disorders characterized principally by progressive lower limb spasticity and weakness. Mutations in the *SPG3A* gene, which encodes the large oligomeric GTPase atlastin, are the second most common cause of dominant HSP and the most common cause of young onset HSP. The objective of this study was to determine the frequency and nature of *SPG3A* mutations in a collection of 70 North American HSP cases.

Methods and Results

Mutation detection of the 14 exons of *SPG3A* was performed using the dHPLC WAVE technique combined with automated sequencing. We identified one novel segregating in-frame deletion of p.del436N in a large French Canadian kindred that was not present in controls. This represents the first report of an in-frame *SPG3A* deletion mutation. Immunoblot analyses of lymphoblasts demonstrated that atlastin protein levels were much lower in patients as compared to controls. Interestingly, we also show that the p.del436N mutant protein can still form oligomers, but that GTPase activity is reduced.

Interpretation

Thus, patients with this *SPG3A* deletion likely have loss of atlastin function through dominant-negative interactions of mutant atlastin with wild-type atlastin, resulting in both increased degradation and loss of GTPase activity.

3.3 INTRODUCTION

The Hereditary Spastic Paraplegias are a group of neurodegenerative disorders characterized by progressive lower limb spasticity and weakness (Harding 1993b). Classically, the HSPs have been grouped as either pure or complicated. Pure forms of HSP typically present as lower limb spastic weakness alone, though they are sometimes associated with mild sensory changes and bladder dysfunction. Complicated forms of HSP exhibit additional neurological features such as optic neuropathy, dementia, ataxia, deafness, mental retardation or extra-pyramidal disturbances (Harding 1993b; Reid 2003). The existence of large HSP families has enabled the mapping of over 30 loci for autosomal dominant, recessive and X-linked forms of HSP (*SPG1* through *SPG 31*) (Hodgkinson et al. 2002; Fink 2003a; Simpson et al. 2003; Klebe et al. 2006a; Soderblom and Blackstone 2006; Zuchner et al. 2006a). The genetic heterogeneity is complicated by the fact that the disease presentation of pure HSP is relatively uniform, rendering genotype – phenotype correlation efforts difficult. Eleven genes have been identified and their encoded proteins have a variety of functions such as mitochondrial functions, myelin maintenance, axonal growth and endosomal trafficking (Crosby and Proukakis 2002; Fink 2003a; Rainier et al. 2003; Soderblom and Blackstone 2006).

The *SPG3A* gene is mutated in approximately 10% of dominant HSP families which makes it the second most common cause for dominant HSP (Rainier et al. 2001; Zhao et al. 2001) and the most common cause of young onset HSP (Namekawa et al. 2006b). Over 20 mutations have been identified, all of which are missense changes except for a single nucleotide insertion mutation that leads to a small protein truncation at the C-terminus (Tessa et al. 2002; Abel et al. 2004; D'Amico et al. 2004; Durr et al.

2004; Hedera et al. 2004; Sauter et al. 2004; Scarano et al. 2005; Namekawa et al. 2006b). The *SPG3A* gene consists of 14 exons giving rise to a 2.2 kb mRNA transcript and a 558 amino acid (a.a.) protein (Zhao et al. 2001). The protein, atlastin, is a member of the dynamin superfamily of large GTPases (Praefcke and McMahon 2004), with a tripartite GTP binding motif consisting of a P Loop, DxxG, and RD domain in exons 2, 4 and 7 respectively. Furthermore, atlastin is an integral membrane protein with two transmembrane domains spanning residues 448 to 496 (Zhu et al. 2003). Atlastin localizes to the Golgi apparatus as well as the endoplasmic reticulum and is possibly involved in vesicle trafficking (Zhu et al. 2003; Sanderson et al. 2006; Zhu et al. 2006). In the rat brain atlastin has been localized to the cerebral cortex and the pyramidal neurons of the hippocampus (Zhu et al. 2003). Localization and knock down studies in cortical neuron cultures have demonstrated that atlastin is necessary for axonal growth (Zhu et al. 2006). Interestingly, Sanderson and colleagues have recently shown that atlastin interacts with spastin, the most commonly mutated protein in HSP (Sanderson et al. 2006). The interaction between these two proteins suggests involvement in the same pathophysiological pathway. Our aim in this study is to determine the frequency of atlastin mutations in our large North American cohort and to characterize the nature of these mutations.

3.4 METHODS

Subjects

Probands and their families gave written informed consent to participate in our study approved by the local ethics review board (Centre Hospitalier de l'Université de Montréal). Peripheral blood samples were obtained from 70 subjects and their families, and DNA extraction was performed using standard salt extraction methods (Miller et al. 1988). Immortalized lymphoblastoid cell lines were established for at least one individual per family (Anderson and Gusella 1984).

Mutation Detection

Primers were designed to amplify the 14 exons and at least 50 basepairs of the flanking sequence. The primer sequences can be made available upon request. The PCR products were separated on agarose gel to confirm amplification and then analyzed for heteroduplexes by dHPLC-WAVE (Transgenomics). All variants were sequenced and tested for segregation in the family when available. To determine whether the variants unknown to dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) are polymorphisms, Caucasian controls were analyzed for these variants by direct sequencing, Allele Specific Oligomerization (ASO) (Labuda et al. 1999) or radiolabeled polyacrylamide Gel Electrophoresis (PAGE) for the 3 bp deletion.

Co-immunoprecipitation and immunoblot analyses

Total protein extracts were prepared by homogenizing lymphoblasts in Laemmli buffer. After boiling the samples for 10 minutes, 50 μg of protein was electrophoresed on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. Immunoblotting was performed with rabbit polyclonal anti-atlastin (#5409) (Zhu et al. 2003; Zhu et al. 2006) (1:1000) and mouse monoclonal anti-actin (1:20,000; Chemicon) antibodies. After addition of secondary antibodies, immunoreactive bands were visualized using enhanced chemiluminescence (Perkin Elmer). Co-immunoprecipitation of HA- and myc-tagged wild type and p.del436N atlastin proteins expressed in COS-7 cells was performed as described previously (Zhu et al. 2006). Briefly, HA-atlastin was co-expressed with either wild-type myc-atlastin or else myc-atlastin Δ436 in COS-7 cells. After immunoprecipitation with anti-HA antibodies, precipitates were immunoblotted with anti-myc antibodies (Zhu et al. 2006). In control experiments, the myc-tagged atlastin proteins were expressed alone, and they were not precipitated by anti-HA antibodies.

Yeast two-hybrid assays

Yeast two-hybrid assays were performed between the wild-type and Δ436 mutant atlastin cDNAs cloned into bait (pBHA) and prey (pGAD10) constructs as described previously (Zhu et al. 2003; Zhu et al. 2006). Strength of interaction was assayed by *HIS3* and *β-gal* induction as described by Zhu et al. (Zhu et al. 2003; Zhu et al. 2006). In all cases *HIS3* and *β-gal* induction correlated. Site-directed mutagenesis was performed using the QuickChange method (Stratagene).

GTPase activity assay

Assays for GTP hydrolysis of calmodulin-binding protein (CBP) fusion proteins of wild-type (WT) and Δ436 mutant atlastin proteins were performed as described in Zhu et al. (2003). GTPase activity was expressed as a ratio of GDP to total guanine nucleotides (GDP+GTP) at each time point, and expressed as a percentage of maximal GTPase activity for wild-type atlastin.

3.5 RESULTS

Mutation Detection

The amplicon for exon 13 contains two SNPs (rs3080579 and rs3080581), which complicates analysis. However, since the first publication by Zhao et al (Zhao et al. 2001) a presumed splice variant was added to the database NM_181598 that does not contain this exon and thus exon 13 might not be part of the mRNA.

Many variants were detected by dHPLC-Wave, which were further analyzed by sequencing. Table 1 summarizes all identified variants unknown to dbSNP and their frequency in controls tested in this study. The 3'UTR and the IVS6 +74 G>A changes were not pursued because of non segregation and the lack of appropriate controls respectively. The most interesting variant is the deletion of one amino acid, p.del436N, which abolishes a consensus N-glycosylation site. This variant is absent in 380 control chromosomes and segregates in two related families (Fig. 1). The sequence trace of a patient clearly shows the 3 bp deletion in both directions compared to the control. Since the variant is absent in controls and segregates in two families, it can be concluded that the p.del436N is a novel mutation in the *SPG3A* gene. It should also be noted that the frequency of *SPG3A* mutations in our cohort is quite low, 1.4% (1/70) compared to previous studies (Abel et al. 2004; Sauter et al. 2004; Namekawa et al. 2006b).

The clinical features of the individuals with the p.del436N mutation are summarized in Table 2. The majority of patients presented in childhood with delayed walking and/or toe walking. The upper limbs are generally unaffected, and there are no reported urinary symptoms. All patients have normal cognition and bulbar functions, and no one

is wheelchair bound. MRI results of the CNS were available for only one affected individual (Table 2) for whom the results were normal. The sensory system is not affected in family 41, but two individuals in family 17 have decreased vibration sense in the toes. The disease progression is typically rapid in the years following initial symptoms but stabilizes later in life. All carriers present with uniform, early onset pure HSP and foot symptoms. Two patients have required surgeries to alleviate tight Achilles tendons and one patient has marked foot atrophy. One of the mutation carriers has very mild symptoms. He is an obligate carrier of the mutation because he has a severely affected son, and sequencing confirmed he carries the p.del436N mutation. Interestingly, this individual performs intense physical training as part of his profession during which he never noticed any weakness.

Immunoblot analysis of atlastin

To further characterize the defect in our patients we studied the atlastin levels in the *SPG3A* patients' lymphoblasts by immunoblotting, with actin as a loading control (Fig. 2). This showed a marked reduction in atlastin protein level in patients' lymphoblasts as compared to control samples. The patients carry the mutation in the heterozygote state but, contrary to what might be expected, the level of atlastin is significantly less than half (Figure 2). These results suggest that the normal copy of the protein is also affected, perhaps by binding to the mutant form and undergoing more rapid degradation.

Oligomerization and GTPase activity of p.del436N atlastin

The deletion described in the patients above was also introduced by site-directed mutagenesis into an atlastin expression construct and yeast two-hybrid vectors to study

the effects of this mutation on atlastin oligomerization. Yeast two-hybrid assays showed that the wild-type atlastin protein can interact with the p.del436N mutant protein (Fig. 3A). Similarly, co-immunoprecipitation studies of wild-type and p.del436N atlastin proteins overexpressed in COS7 cells confirmed this interaction (Fig. 3B). The atlastin protein functions as a GTPase, and it has previously been shown that mutations in the GTPase domain reduce this activity *in vitro* in CBP-atlastin fusion proteins (Zhu et al. 2003; Zhu et al. 2006). The GTPase activity was therefore also tested in the p.del436N mutant, and it was found to be significantly reduced as well (Figure 3C).

3.6 DISCUSSION

Screening of a large collection of HSP families for atlastin mutations allowed us to identify a novel deletion mutation, p.436delN, in a large family. The collection of samples analyzed for atlastin mutations included mostly spastin-negative samples, but also included untested probands. The sample set was not selected for early age of onset which is frequently associated with atlastin mutations (Namekawa et al. 2006b). These factors may explain the low frequency of mutations in our sample group and suggest that one can increase the efficiency of atlastin screening by careful patient selection. The low frequency identified may also indicate that the French Canadian populations had a lower prevalence of atlastin mutations.

Though atlastin mutations are distributed throughout different ethnic groups, with mutations reported in African American, Italian, French, Portugese, English, and German families (Namekawa et al. 2006b), the p.del436N deletion mutation described here is the first atlastin mutation identified in the French Canadian population. Additional families of French Canadian descent with childhood-onset HSP should therefore be screened for the p.436delN mutation as a priority. The clinical picture associated with the p.436delN mutation is that of an early onset, slowly progressive form of pure HSP. The first sign of the disease is usually delayed onset of walking, and can be confused with cerebral palsy. The upper limbs are relatively unaffected, and the patients do not display any cognitive or cerebellar symptoms; sensory findings, when present, are mild. This description closely compares to the phenotype associated with other *SPG3A* mutations previously published (Wilkinson et al. 2003; Durr et al. 2004; Sauter et al. 2004). Also, the cases in this study do not have bladder disturbance. One

individual had mildly increased lower limb reflexes and was unaware of symptoms. Previous groups have reported incomplete penetrance in individuals presenting with mild pyramidal tract involvement and more strikingly for the R415W mutation where several individuals were clinically normal (Tessa et al. 2002; D'Amico et al. 2004). The favoured explanation is the involvement of modifying genes that can influence expression of atlastin but interestingly our mildly affected case has a profession that requires extreme physical training, which might have slowed the progression of the disease symptoms.

The p.436delN mutation occurs in exon 12, which supports the finding that atlastin mutations tend to cluster in exons 7, 8 and 12 (Namekawa et al. 2006b). There are no clear protein domains encoded by exon 12, but there are predicted phosphorylation and consensus N-glycosylation sites. Zhu and colleagues showed by *in vitro* glycosylation assays that a N436Q mutant does not influence atlastin migration with or without N glycosidase F treatment (Zhu et al. 2003). Our immunoblot results confirm that N436 is not glycosylated *in vivo,* and topologically residue 436 has been shown to be cytoplasmic (Zhu et al., 2003), which strongly argues against any role for changes in glycosylation.

In addition, the immunoblot shows that there is a marked reduction of atlastin protein in lymphoblast extracts from mutation carriers as compared to controls. The p.del436N mutation might lead to an increased susceptibility to degradation of the wild-type and mutant copy of the protein. One previous study has investigated the effect of an atlastin mutation on its level of expression (Tessa et al. 2002). The insertion mutation truncates the protein by 37 a.a. and RNA analysis did not show a change in level of atlastin expression in skeletal muscle. These results support the hypothesis that

the mutations might affect protein degradation rather than expression. We cannot however be certain that the observed decrease in atlastin protein in lymphoblasts is representative of what occurs in the brain where atlastin is most expressed.

In support of the immunoblot analysis which suggests that the normal atlastin copy is also targeted to the degradation pathway, we show that the wild type and mutant copy of atlastin can interact and potentially form oligomers. Furthermore, *in vitro* GTPase activity studies indicate that p.del436N atlastin homomers have reduced activity as compared to wild-type homomers, reminiscent of previous studies showing that mutations in the GTPase domain reduce GTPase activity (Zhu et al. 2006). This assay, however, is performed on purified atlastin bacterial fusion proteins, and therefore does not reflect the heterozygous state of the dominant mutations. Taken together these results suggest that the p.del436N mutation acts in a dominant-negative fashion on protein degradation, and likely also on reducing GTPase activity. In the future, it will be interesting to study the effect of other *SPG3A* mutations on atlastin levels in lymphoblasts to determine whether increased protein degradation is a common theme. Additional studies such as the development of animal model systems are necessary to better understand the role of atlastin in maintenance of healthy corticospinal tract neurons.

3.7 ACKNOWLEDGEMENTS

The authors would like to thank the families for their participation in this study. GAR is supported by the Canadian Institutes for Health Research (CIHR). Special thanks to Adrienne Lei, Rislaine Benkelfat and Julia Stadler for technical assistance. This work was supported in part by the Intramural Research Program of the NIH, NINDS.

3.8 TABLES

Table 1: A list of all variants unknown to dbSNP detected in our cohort

Variants	Cases	Controls	Note
exon 2, c.50A>G	4	4/31	p.P28P
exon 12, c.1306-1308delAAT	9	0/190	p.del436N
3' UTR, c.1916C>T	1	0/183	Not segregating
IVS2-74 G>C	4	1/31	
IVS4 -19G>T	12	1/4	(Durr et al. 2004)
IVS6 - 24 G>C	12	7/95	(Durr et al. 2004)
IVS6 + 74G>A	1	0/160	Ethiopian origin
IVS9 - 53A>G	13	3/4	

Table 2a: A clinical summary of Family 17 with the P.del436N mutation

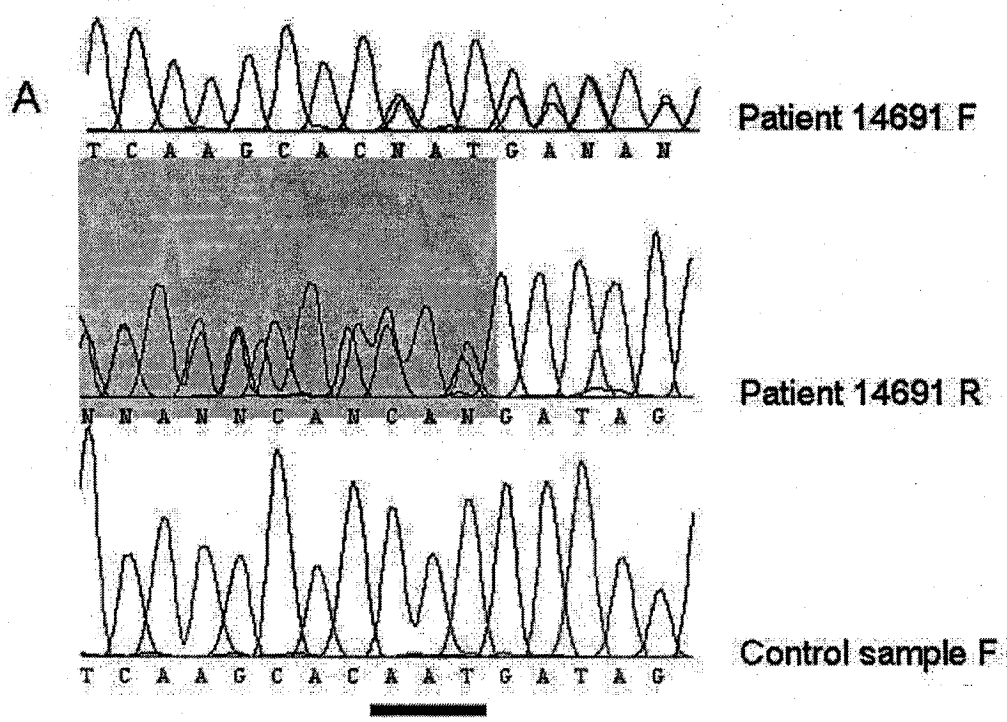
Subjects	R29030	R29029	R29028	R29031	X2751
Age of onset	1-2	1-2	1-2	1-2	1-2
Age at evaluation	43	23	22	18	26
Hyperreflexia in the arms	+	-	-	-	-
Hyperreflexia in the legs	+	+	+	-	+
Leg spasticity	+	+	+ mild	+	+
Urinary symptoms	-	-	-	-	-
Vibration sense at the toes	normal	normal	normal	normal	normal
Plantar responses	Extensor	Extensor	Extensor	Extensor	Extensor
Additional comments					+ve Hoffman

Table 2b: A clinical summary of Family 41 with the P.del436N mutation

Subjects	R14691	R29238	R29239	X5499
Age of onset	18	16 months	?	16
Age at evaluation	57	4	36	63
Hyperreflexia in the arms	-	+	-	-
Hyperreflexia in the legs	++	+++	+	++ (knees) - (ankles)
Leg spasticity	+	+++	-	+
Urinary symptoms	-	-	-	-
Vibration sense at the toes	Abnormal	Normal	Normal	Abnormal
Plantar responses	Extensor	Extensor	Flexor	Extensor
Additional comments			Pes cavus	Sensory-motor axonal polyneuropathy Distal atrophy in the legs

3.9 FIGURES

Figure 1



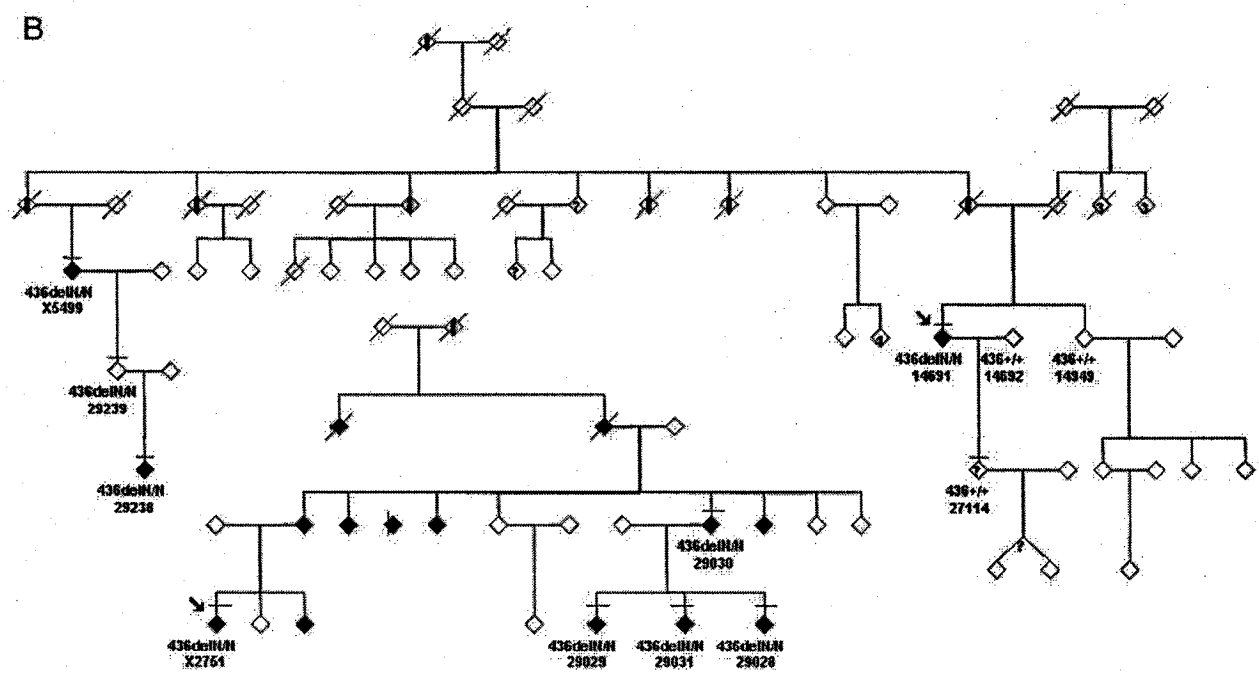

B
436delN/N
X5499
436delN/N
29239
436delN/N
29238
436delN/N
X2751
436delN/N
29030
436delN/N
29029
436delN/N
29031
436delN/N
29028
436delN/N
14691
436+/+
14692
436+/+
14949
436+/+
27114

Figure 1: Sequence analysis and family tree

A, The sequence trace of patient 14691DNA carrying the c. del1306-1308AAT compared to a control sample. This mutation leads to a deletion of a single amino acid, p.del436N. The first two rows represent sequencing across this region in either direction (F - forward and R – reverse sequence).

B, The pedigrees of the two large autosomal dominant French Canadian families in which the p.del436N segregates. Note that individual 29239 is depicted here as an unaffected individual but has very mild symptoms. These two families share a last name and are most likely related. The bar above the symbol indicates that the patient was seen by a neurologist.

Figure 2

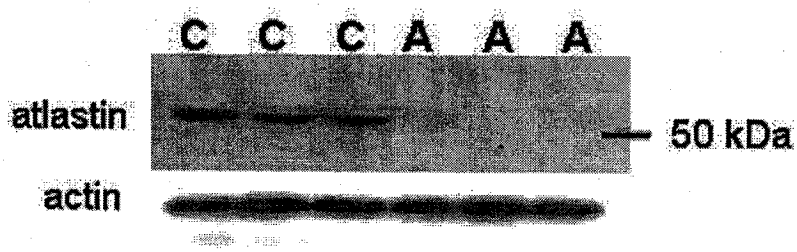
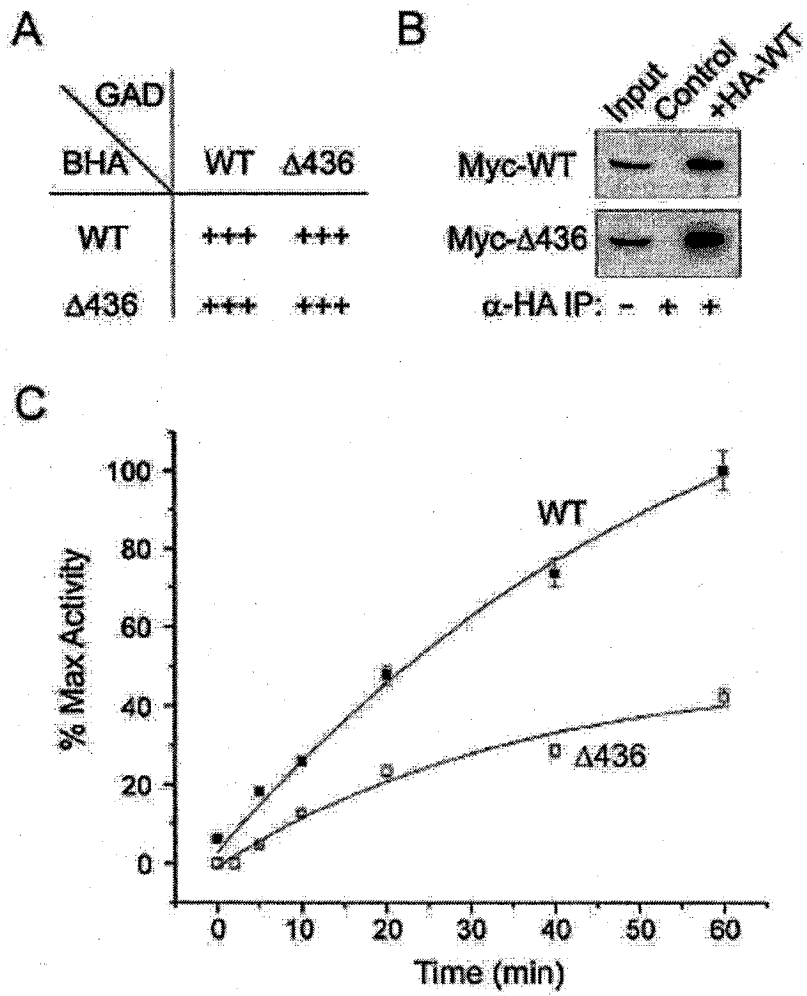


Figure 2: Immunoblot analysis of atlastin in patient cell lines

Equal amounts of protein extracts from lymphoblasts of three affected individuals and three controls were separated on a 10% gel. Followed by the detection of the atlastin protein using the #5409 affinity-purified rat polyclonal antibody (Zhu et al. 2003). There is a marked reduction of atlastin protein in the *SPG3A* patient samples compared to actin, which was used as a protein loading control.


A
GAD
BHA
WT
Δ436
WT
+++
+++
Δ436
+++
+++
B
Input
Control
+HA-WT
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α-HA IP: - + +
C
% Max Activity
100
80
60
40
20
0
WT
Δ436
0
10
20
30
40
50
60
Time (min)

Figure 3: Atlastin Δ436 interacts with wild-type atlastin but has impaired GTPase activity.

A, Matrix of yeast two-hybrid assays showing interactions of wild-type and Δ436 mutant atlastin proteins, with bait (BHA) and prey (GAD10) constructs indicated. Relative strength of interaction was assayed by *HIS3* and *β-gal* induction as described by Zhu et al. (Zhu et al. 2003; Zhu et al. 2006). In all cases *HIS3* and *β-gal* induction correlated.

B, Co-immunoprecipitation of wild-type and Δ436 mutant atlastin proteins. HA-atlastin was co-expressed with either wild-type myc-atlastin or else myc-atlastin Δ436. After immunoprecipitation with anti-HA antibodies, precipitates were immunoblotted with anti-myc antibodies. In control experiments, the myc-tagged atlastin proteins were expressed alone, and they were not precipitated by anti-HA antibodies.

C, Assays for GTP hydrolysis of CBP fusion proteins of wild-type (WT) and Δ436 mutant atlastin proteins were performed as described in Zhu et al. (Zhu et al. 2003) Data are expressed as means ±SD (n=3) of the % maximal GTP hydrolysis for the CBP-atlastin wild-type fusion protein at the indicated time points.

CHAPTER 4: MUTATIONS IN THE *ALS2* GENE CAUSE INFANTILE ONSET HSP

Reference:

Gros-Louis F[¶], Meijer IA[¶], Hand CK, Dube MP, MacGregor DL, Seni MH, Devon RS, Hayden MR, Andermann F, Andermann E, and Rouleau GA. An *ALS2* gene mutation causes hereditary spastic paraplegia in a Pakistani kindred. *Ann Neurol.* 2003 Jan;53(1):144-5.

[¶] Contributed equally to this work, even though the journal did not allow equal contributing authors at the time.

4.1 RATIONALE

Our two mutation screening projects of the most commonly mutated HSP genes resulted in the identification of mutations in 20 dominant families of our cohort. The majority of the large statistically powerful families had now been linked to *SPG4* or *SPG3A*. In an effort to find the genetic cause of HSP in other families of our cohort, we focused on the recessive families with consanguinity, since consanguinity increases the power of the families and therefore provides an enhanced opportunity to identify linkage in smaller families.

The largest of these recessive families is a family collected in Pakistan with the help of Dr Andermann. This family consists of 6 living affected members and 2 other individuals had already succumbed to the disease. The pedigree depicted in Figure A demonstrates the high degree of consanguinity in this kindred. In order to identify the genetic defect that underlies HSP in this family, a WGS analysis was performed for 5 affected individuals. Homozygosity mapping did not result in any positive loci. It can be hypothesized that the negative result was due to sample mix-up and low density of genome scan markers.

At a later time, mutations in the *ALS2* gene were identified as the cause for juvenile onset ALS (Hadano et al. 2001; Yang et al. 2001). This atypical form of ALS leads to quadriplegia, but the neurodegeneration is restricted to the UMN. In addition, the *ALS2* locus lies near an HSP locus, *SPG13* (Hansen et al. 2002) (Figure B). The gene responsible for HSP at *SPG13* is the *HSP60* gene. This gene was mutated in a single family and two other *SPG13* linked families did not show any variation in this gene

(Hansen et al. 2002). The lack of mutations in the *HSP60* gene and the UMN phenotype associated with the *ALS2* mutations led us to the hypothesis that the *ALS2* gene might also be mutated in HSP families. A proband of the Pakistani kindred was analyzed for mutations in the *ALS2* gene and the results are described in the next section.

Figure A

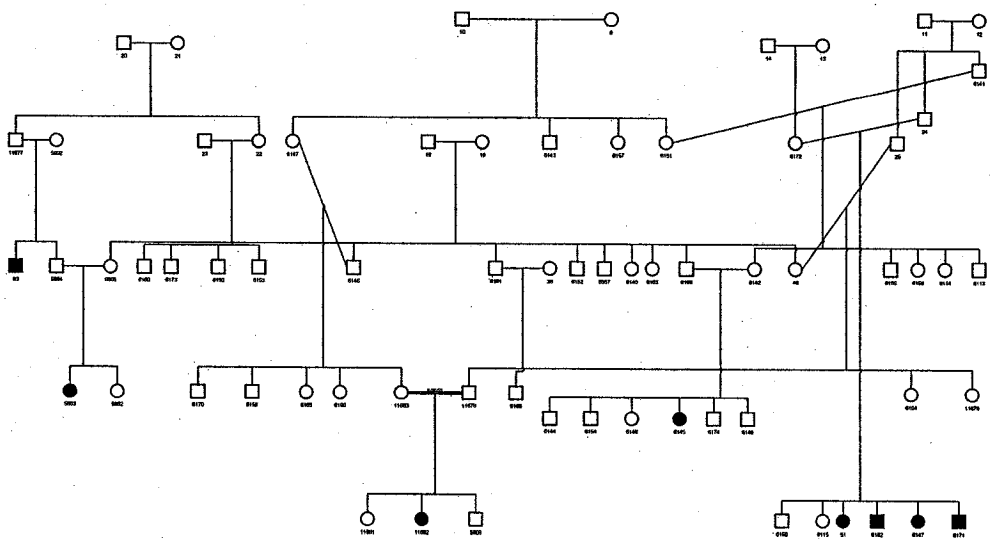


Figure A: The pedigree of a large consanguineous Pakistani kindred with complicated HSP.

Figure B

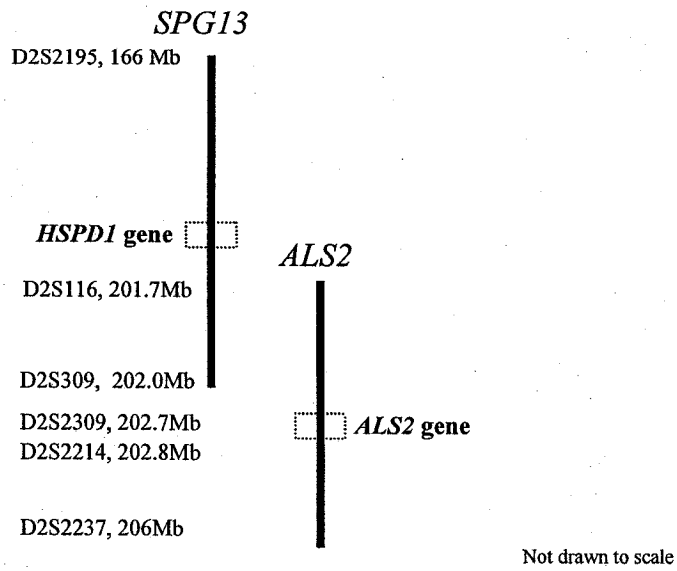


Figure B: The *HSP/ALS* locus. The loci for *SPG13* (Hansen et al. 2002) and *ALS2* (Hadano et al. 2001) were mapped in close proximity to each other, suggesting a common mutated gene for the two forms of motor neuron degeneration.

4.2 LETTER

Hereditary Spastic Paraplegia (HSP) and Amyotrophic Lateral Sclerosis (ALS) are genetically heterogeneous progressive neurodegenerative disorders distinguished by differential motor neuron involvement. In ALS both upper and lower motor neurons are affected, whereas in HSP only upper motor neuron function is affected resulting in a less severe disease. Recently, juvenile forms of ALS (*ALS2*) and Primary Lateral Sclerosis (PLS) were shown to be caused by mutations in the *ALS2* gene, which encodes a putative GTPase regulator (Hadano et al. 2001; Yang et al. 2001).

The *ALS2* gene has 34 exons with at least two splice variants. The long variant (6394 nucleotides) is expressed in various tissues with highest expression in the brain (Hadano et al. 2001). The protein, alsin, shows similarity to three domains (RCC1, Pleckstrin-DB1, VPS9) and to Membrane Occupation and Recognition Nexus (MORN) repeat motifs, which are characteristic of various guanine exchange factors (Ran, Rho and Rab respectively).

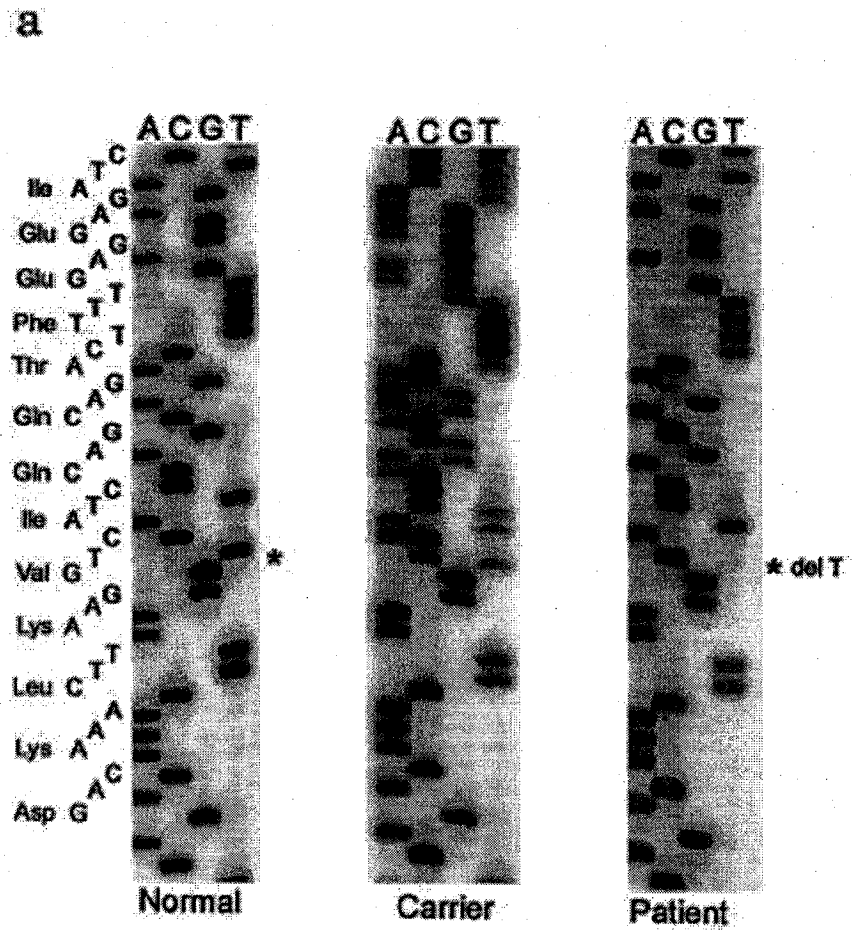
We report the identification of a novel *ALS2* mutation in a large consanguineous Pakistani family with infantile recessive complicated HSP. The proband initially presented with gait disturbance and hyperreflexia at age 18 months. Currently, at age 12 years, she is anarthritic and confined to a wheelchair. Family history indicates that the disease slowly progresses to tetraplegia and death by the fourth decade of life, with relatively preserved intellect. The clinical picture is similar to that recently reported by Eymard-Pierre and colleagues (Eymard-Pierre et al. 2002). Because our family is clinically related to previously reported *ALS2* families (Hadano et al. 2001; Yang et al. 2001) and the fact that an HSP locus (*SPG13*) (Fontaine et al. 2000) initially overlapped

with the *ALS2* locus, the proband was analyzed for mutations in the *ALS2* gene using the DHPLC-WAVE system (Transgenomics, Mountain View, CA). Sequencing of a DHPLC variant in exon 32 showed a 1 bp deletion (4844delT) (Figure 1a). Co-segregation of the mutation and the disease in the family was confirmed and the mutation was absent in 155 control individuals. The deletion occurs at the beginning of the VPS9 domain and adds 43 unique residues to the truncated protein (Figure 1b). Our results show that absence of a functional VPS9 domain of alsin is sufficient to cause neurodegeneration. The yeast VPS9 protein and its mammalian homolog RABEX-5 are guanine nucleotide exchange factors for specific proteins thought to be involved in vacuolar endocytic transport (Esters et al. 2001). Disruption of intracellular trafficking has long been suggested to cause selective degeneration of the long axons of the pyramidal tract in HSP, but identification of additional mutations, functional studies and animal models are necessary to further understand the pathogenesis resulting from *ALS2* mutations.

4.3 ACKNOWLEDGEMENTS

This work was supported by the Canadian Institute of Health Research (GAR, FGL and EA) and IAM is partially funded by a MUHC Research Institute studentship. We would like to express our gratitude to the family for their participation in the study. We also thank D. Rochefort for technical assistance and Dr. A. Toulouse for carefully reading manuscript.

Figures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A C G T
A C G T
Ile A T C
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Glu G A G
Phe T T T
Thr A C T
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Gln C A G
Ile A T C
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Lys A A G
Leu C T T
Lys A A A
Asp G A C
*
* del T
Normal
Carrier
Patient

b

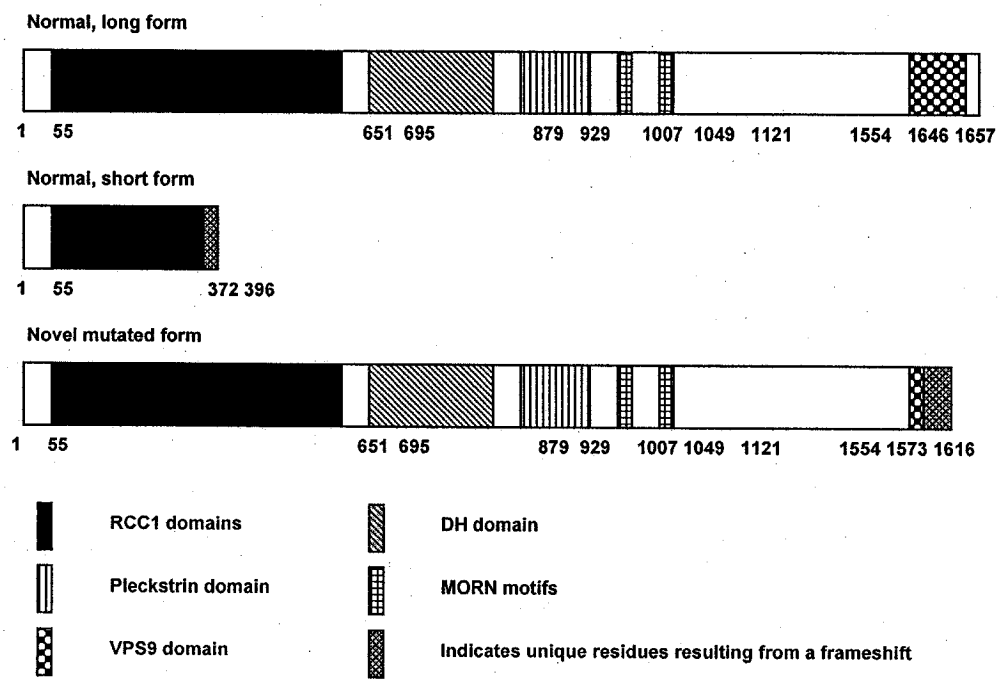
Normal, long form
1 55 651 695 879 929 1007 1049 1121 1554 1646 1657
Normal, short form
1 55 372 396
Novel mutated form
1 55 651 695 879 929 1007 1049 1121 1554 1573 1616
RCC1 domains
DH domain
Pleckstrin domain
MORN motifs
VPS9 domain
Indicates unique residues resulting from a frameshift

Figure 1

(a) Sequencing analysis for exon 32 of the *ALS2* gene in a healthy individual, a carrier and a patient. Sequencing shows a deletion of nucleotide 4844 resulting in a frameshifting event.

(b) Schematic diagram of the alsin protein. The mutation identified in this family truncates the VPS9 domain and results in 43 unique additional residues at the carboxy-terminal of the protein.

CHAPTER 5: A NOVEL LOCUS, *SPG27* FOR RECESSIVE HSP

Reference:

Inge A. Meijer B.Sc., Patrick Cossette M.D./M.Sc., Julie Roussel, Melanie Benard B.A., Sylvie Toupin R.N./B.Sc., Guy A. Rouleau M.D./Ph.D.

A novel locus for pure recessive Hereditary Spastic Paraplegia maps to chromosome 10q22.1-10q24.1 *Ann Neurol.* 2004 Oct;56(4):579-82.

5.1 RATIONALE

The identification of a novel *ALS2* mutation in a clinically complicated HSP family was an exciting finding in the field, but mutations were restricted to families of Middle Eastern descent with severe UMN involvement (Gros-Louis et al. 2003; Hand et al. 2003). Our collection also included a large autosomal recessive family with pure HSP from Abitibi, Quebec. This family (FSP7) was tested for linkage to the known recessive loci: *SPG5, SPG7* and *SPG11* (Hentati et al. 1994a; De Michele et al. 1998; Martinez Murillo et al. 1999). No linkage to these recessive loci was found. In addition, the fact that half of the 14 siblings in this one-generational family had HSP was suggestive of a dominant MOI. We therefore proceeded to test linkage to the known dominant loci. Weak linkage was observed under a dominant model to *SPG9* and more detailed analysis revealed that the patients shared a compound heterozygote haplotype. This chapter describes the mapping of a novel HSP locus, *SPG27*, to chromosome 10q in family 7.

5.2 ABSTRACT

The Hereditary Spastic Paraplegias (HSP) are a group of clinically and genetically heterogeneous disorders characterized by progressive lower limb spasticity. In this study we performed linkage analysis on an autosomal recessive pure HSP family and mapped the disease to chromosome 10q22.1-10q24.1, a locus partially overlapping the existing *SPG9* locus. We have either identified a novel locus for pure recessive HSP (*SPG27*) or we have found the first case of allelic disorders with different mode of inheritance in HSP. If the disorders are indeed allelic, our results have reduced the *SPG9* interval by 3 Mb with D10S536 and D10S1758 as flanking markers.

5.3 INTRODUCTION

The Hereditary Spastic Paraplegias are a clinically and genetically heterogeneous group of neurodegenerative disorders characterized by progressive lower limb spasticity and weakness often associated with bladder disturbance (Harding 1993b). Clinically HSP is classified as either pure or complicated HSP. In the complicated form of HSP the lower limb spasticity does not occur in isolation, but is accompanied by additional neurological features such as optic neuropathy, dementia, ataxia, deafness, mental retardation, and extra-pyramidal disturbance (Harding 1993b; Reid 2003). The genetic heterogeneity in HSP is demonstrated by the large number of loci mapped for the disease (*SPG1* through *SPG 24*) (Hodgkinson et al. 2002; Fink 2003b; Simpson et al. 2003). (Both *SPG18* and *SPG22* have not yet been reported in the literature). There are 3 X-linked, 10 dominant, and 9 recessive loci described for both pure and complicated

forms of HSP. Identification of ten HSP genes has shown that several pathophysiological pathways are involved in this disease including impairment of axonal transport, a common link with other neurodegenerative diseases (Crosby and Proukakis 2002; Fink 2003b; Rainier et al. 2003).

In 1999, Seri and colleagues mapped a locus (*SPG9*) for autosomal dominant complicated HSP to chromosome 10q23.3-q24.2 in a large Italian family. This family presented with lower limb spasticity and bilateral cataracts. Other minor features included persistent vomiting, amyotrophy, peripheral neuropathy and anticipation (Seri et al. 1999). The critical disease interval of 12 cM (~ 9.2 Mb) was slightly refined to ~ 7 Mb when a second family with a motor system disorder linked to the *SPG9* locus (Seri et al. 1999; Lo Nigro et al. 2000). In addition to the motor system feature this British family presented with bilateral cataracts, short stature, learning difficulties, muscle weakness, skeletal abnormalities and anticipation (Slavotinek et al. 1996). It has been suggested that the syndrome described in the two families is genetically homogeneous (Lo Nigro et al. 2000). Furthermore, a family with HSP and epilepsy was excluded for linkage to the *SPG9* locus, which overlapped a partial epilepsy locus (Lo Nigro et al. 2003).

In this study, we present a large single-generation French Canadian family with pure recessive HSP for which we have mapped the disease locus, *SPG27*, to chromosome 10q22.1-10q24.1 by linkage analysis. This recessive locus overlaps with the *SPG9* locus.

5.4 PATIENTS AND METHODS

Clinical picture

One neurologist experienced in the assessment of HSP (P.C.) assessed the family. All individuals gave informed consent for participation in the study. The family consists of two unrelated healthy parents, and 14 offspring of which half are affected with pure HSP (Fig.1). All affected individuals presented with moderate to severe spastic paraparesia of the lower limbs and with spastic bladders. Upon examination, they also showed lower limb hyperreflexia, positive Babinski signs and moderate to severe decrease of vibration sense in the feet. Muscle strength in both upper and lower limbs was normal. In addition, individuals II:2 and II:3 had slower rapid alternating movements in feet and of the tongue with mild dysarthria. Age of onset ranged between 25 and 45 years. One affected individual was wheelchair bound, but the other individuals walked independently or with the help of a cane. Except for one individual with neurosensorial deafness secondary to chronic exposure to noise, there was no evidence for hearing impairment, optic neuropathy, cognitive decline, ataxia, or extra-pyramidal signs in affected individuals from this family. Detailed electrophysiological evaluations have been performed in individuals II:1 and II:3. Nerve conduction studies revealed normal amplitude for both sensory nerve and compound muscle action potentials, as well as normal conduction velocities. Electromyography did not show denervation changes in distal muscles in the lower limbs. In turn, somatosensory evoked potentials were clearly abnormal in these two individuals. For individual II:3, a severe decrease in amplitude in both upper and lower limbs has been observed with relative preservation of latencies. For patient II:1, no significant evoked potential has

been recorded after stimulation of various nerves in the lower limbs. Considering normal nerve conduction studies, these latter results suggest a severe impairment of sensitive pathways within the central nervous system.

Genotyping

Polymorphic markers were amplified by PCR incorporating radiolabeled S^{35} dATP into the product. The products were separated on 6% denaturing polyacrylamide gels and visualized on autoradiographic film. Genotyping was initially performed for the following markers: D8S166, D8S260 (*SPG5*), D16S2621, D16S413 (*SPG7*), ACTC, D15S118 (*SPG11*), D14S747, D14S288 (*SPG3*), D2S1325, D2S352 (*SPG4*), D15S128, D15S822 (*SPG6*), D8S1179, D8S586 (*SPG8*), D10S1755, D10S1680 (*SPG9*), and D12386, D12S83 (*SPG10*). After linkage was established to the SPG9 locus, additional markers were genotyped at that locus and haplotype construction assuming minimal recombination was performed. Markers and their order were obtained from the Marshfield genetic map (Centre for Medical Genetics, Marshfield Medical Research Foundation).

Linkage analysis

Two point parametric linkage analysis was performed with the MLINK program of the FASTLINK (version 5.1) software package (Cottingham et al. 1993b). The following parameters were used: equal allele frequencies, equal male/female recombination, 100% penetrance, disease gene frequency of 1/1000 assuming a recessive mode of inheritance.

Mutation Detection

The Denaturing High Performance liquid chromatography (DHPLC)-WAVE system (Transgenomics, Mountain View, CA) was used to detect heteroduplex formation in samples of affected individuals mixed with equal amounts of control PCR product and carriers. Segregating variants were sequenced. Intronic primers were designed to amplify all 21 coding exons of the *KIF11* gene under standard conditions and primers are available upon request.

5.5 RESULTS

There was no evidence for linkage to the recessive loci (*SPG5, 7, 11*) reported at the start of the study (data not shown). We then proceeded to investigate the known dominant loci (*SPG3, 4, 6, 8, 9, 10*). Linkage analysis under a recessive model with *SPG9* markers identified linkage of our family to this locus with a maximum LOD score of 3.04 for marker D10S1755 (Table 1). Analysis of additional markers in the region revealed a higher LOD score of 4.49 at theta 0 for markers D10S1786 and D10S1765, which lie outside the *SPG9* locus.

Dense haplotype construction revealed two different alleles inherited together by affected individuals with absence of a homozygous shared region (Fig.1). The critical disease interval in our family is determined by markers D10S606 and D10S1758 and spans approximately 26 Mb (Table 2). Interestingly, there is an overlap of approximately 6.1 Mb with the existing *SPG9* locus and this reduces the critical *SPG9* interval by 3 Mb if the two forms of HSP are allelic. The overlapping region contains

40 genes including *KIF11*, a member of the kinesin motor protein family. This gene was screened using DHPLC-WAVE technology followed by sequencing of variants and no mutations were found.

5.6 DISCUSSION

The HSP loci are generally associated with a particular mode of inheritance, with the exception of a homozygous inherited recessive S44L mutated allele in spastin at the dominant locus, *SPG4* (Lindsey et al. 2000). It is also known that families with pure and complicated HSP can be linked to the same loci (eg. *SPG4* and *SPG7*) (De Michele et al. 1998; Heinzlef et al. 1998). In the present study we have identified linkage of a pure HSP family with recessive inheritance to a known dominant complicated locus *SPG9*. The critical intervals for both forms overlap 6.1 Mb. In contrast to the *SPG9* families that show motor neuropathy and several additional features such as cataracts, skeletal abnormalities and gastroesophageal reflux, our family presented with pure CNS involvement restricted to the upper motor neurons. This clinical difference together with the difference in mode of inheritance might suggest that we have mapped our family to a novel recessive HSP locus, *SPG27,* near *SPG9*. The large candidate region (~26Mb) found in our French Canadian family may have contributed to an apparent overlap between the two loci. However we cannot exclude that the two disorders are allelic, although allelic disorders have not previously been reported for HSP. Considering this hypothesis, our haplotype results would reduce the critical *SPG9* interval by 3Mb. The issue of allelic disorders versus novel locus cannot be resolved until the disease causing mutations are identified.

Interestingly, the overlapping critical interval of 6.1 Mb contains a good candidate gene, *KIF11* also known as *hEG5*. The protein is involved in mitotic spindle formation, but recent evidence suggests that rodent EG5 contributes to regulation of microtubules in axons and dendrites of post mitotic neurons (Ferhat et al. 1998). Because of its possible role in axonal trafficking we screened this gene but did not detect any mutation. Other interesting candidates include neuronally expressed genes *SLIT1* and *SORBS1*. Further candidate gene screening is underway.

The affected individuals in our family are compound heterozygotes. We hypothesize that there is a higher frequency of one or both of the haplotypes in the French Canadian population. We are unaware of other large recessive French Canadian HSP families, but there are many seemingly sporadic HSP cases. The possibility that these single cases share a haplotype with our family needs to be explored.

Our work suggests that in future exclusion studies one should investigate both dominant and recessive loci regardless of the mode of inheritance observed in a given family. The identification of the disease causing mutations at the *SPG9* locus and other loci is necessary to further our understanding of disease pathogenesis in HSP and other related diseases.

5.7 ACKNOWLEDGEMENTS

This work was supported by the Canadian Association of Familial Ataxias (IAM) and the Canadian Institutes for Health Research (PC and GAR). We thank the family for their participation in this study. We acknowledge the technical assistance of K. Brisebois.

5.8 TABLES

Table 1. Linkage analysis within and near the *SPG9* locus

	Position	LOD score at θ						
Marker	cM	0	0.01	0.05	0.1	0.2	0.3	0.4
D10S606	93.37	- ∞	-0.99	0.77	1.24	1.26	0.86	0.32
D10S580	96.72	**4.49**	4.40	4.05	3.60	2.66	1.66	0.63
D10S1765	108.79	**4.49**	4.40	4.05	3.60	2.66	1.66	0.63
D10S1755*	114.19	- ∞	2.70	**3.04**	2.88	2.23	1.42	0.54
D10S1680*	117.42	1.78	1.75	1.64	1.49	1.15	0.75	0.30
D10S1758	118.94	- ∞	-1.29	0.50	1.01	1.09	0.75	0.27

* Initial evidence for linkage detected with these markers.

Table 2. Overlap between the recessive haplotype of our family and the *SPG9* locus

Marker	Location (Mb)[+]	Maternal haplotype	Paternal haplotype	*SPG9* (Lo Nigro et al. 2000)
D10S606	72.7	3	4	-
D10S1765	89.3	8	10	4
D10S1753	92.1	1	2	4
D10S536	92.5	-	-	5
D10S1755	94.1	5	3	2
D10S583	94.0	1	3	3
D10S185	94.9	3	4	-
D10S1680	95.3	4	1	1
D10S677	95.6	2	3	-
D10S574	98.0	10	3	4
D10S1736	98.1	-	-	4
D10S1758	98.6	2	5	-
D10S603	101.7	-	-	1

[+] According to the July 2003 Freeze of UCSC web browser

The borders represent the observed recombinations in our recessive HSP family and the box delimits the *SPG9* locus. The dashed line indicates a ~ 3 Mb reduction of the previously published *SPG9* locus if the recessive and dominant forms of HSP are indeed allelic.

5.9 FIGURES

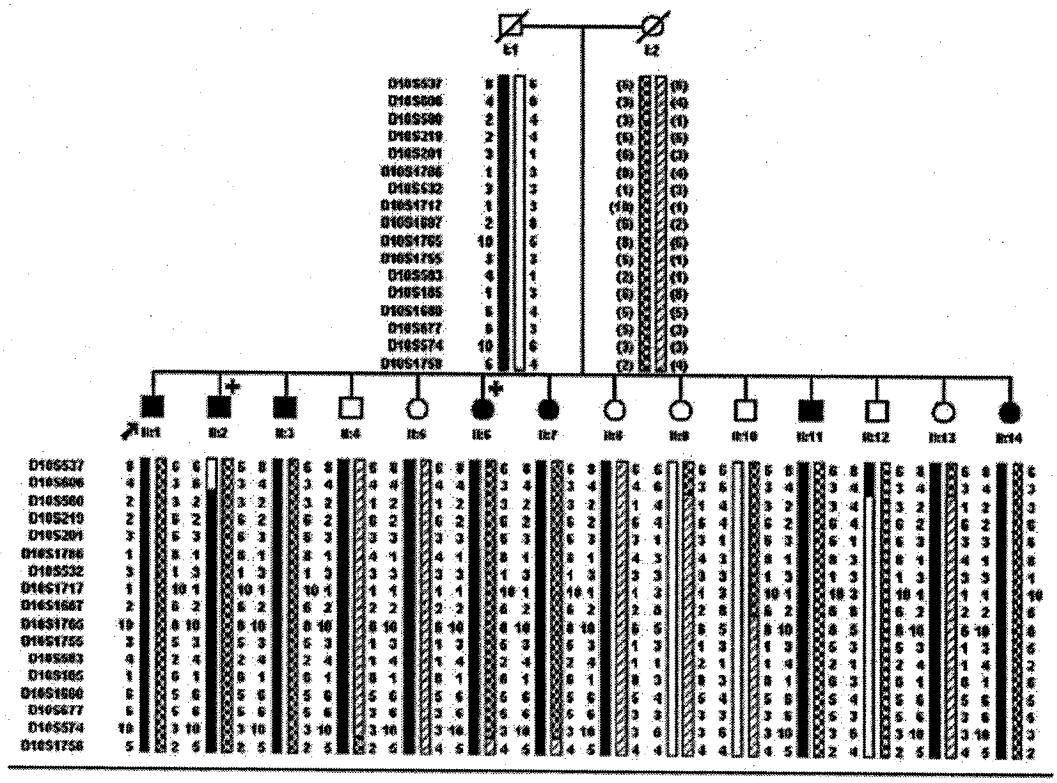

Figure 1. Family tree of the recessive pure HSP family with the haplotypes within and telomeric to the *SPG9* locus. The black and the diamond bars represent the paternal and maternal haplotypes respectively. The key recombinants for each haplotype are indicated by the + symbol.

CHAPTER 6: THE MAPPING OF THE *SAX1* LOCUS

Reference:

Meijer IA, Hand CK, Grewal KK, Stefanelli MG, Ives EJ, and Rouleau GA. A Locus for Autosomal Dominant Hereditary Spastic Ataxia, *SAX1*, Maps to Chromosome 12p13. *Am J Hum Genet.* 2002 Mar;70(3):763-9.

and

Reference:

Grewal KK, Stefanelli MG, Meijer IA, Hand CK, Rouleau GA, and Ives EJ. A founder effect in 3 large families with a novel clinically variable spastic ataxia and supranuclear gaze palsy. *Am J Med Genet A.* 2004 Dec 15;131(3):249-54.

and

Unpublished data

Meijer IA and Rouleau GA. Fine-mapping and candidate gene analysis at the *SAX1* locus.

6.1 RATIONALE

After the success of the mapping endeavours described in previous chapters, where the focus was on large recessive families, we decided to extend our linkage study to the remaining large families in our cohort most consistent with harbouring a dominant locus. Although the majority of the large ADHSP families had been previously linked to the *SPG4* locus, our collection included three large families from Newfoundland with a complicated phenotype. When these families were initially collected, the diagnosis was that of HSP in two families and spinocerebellar ataxia in the other. The absence of cerebellar atrophy and nystagmus, together with a common clinical picture upon closer examination in all three families indicated that these families had complex HSP with severe ataxia. In an attempt to clinically classify this form of HSP, the term Hereditary Spastic Ataxia (HSA) has been established (Mahloudji 1963; Eadie 1991). The genetic characterization of this subgroup should clarify whether it shares a common pathophysiological mechanism with other HSPs. In section 6.2, the mapping of a novel locus, *SAX1,* is described (Meijer et al. 2002b). The mapping study is followed by a detailed clinical study (section 6.3) and fine-mapping results (Grewal et al. 2001). The third section in this chapter contains unpublished data, which includes the candidate gene analysis at the *SAX1* locus. Concurrently with the candidate gene analysis, fine-mapping efforts and the search for additionally linked families were performed and are also described.

6.2 LOCUS FOR AUTOSOMAL DOMINANT HEREDITARY SPASTIC ATAXIA, *SAX1*, MAPS TO CHROMOSOME 12P13

6.2.1 Summary

The Hereditary Spastic Ataxias (HSA) are a group of clinically heterogeneous neurodegenerative disorders characterized by lower limb spasticity and generalized ataxia. Three unrelated autosomal dominant families from Newfoundland presenting mainly with severe leg spasticity, dysarthria, dysphagia, and ocular movement abnormalities were diagnosed with HSA. A genome wide scan was performed on one family and linkage was identified to a novel locus *SAX1*, on chromosome 12p13. Fine-mapping confirmed linkage in the two large families with the third smaller family showing LOD scores suggestive of linkage. Haplotype construction using 13 polymorphic markers revealed that all three families share a disease haplotype, which key recombinants and overlapping haplotypes refine to approximately 5 cM flanked by markers D12S93 and GATA151H05. *SAX1* is the first locus for autosomal dominant HSA.

6.2.2 Introduction

Hereditary Spastic Ataxia (HSA) comprises of a heterogeneous group of progressive neurodegenerative disorders characterized by lower limb spasticity and generalized ataxia with dysarthria, impaired ocular movements, and gait disturbance (Bouchard et al. 2000; Mahloudji 1963). HSA includes dominant (OMIM 108600) as well as recessive types (OMIM 270500), which includes autosomal recessive spastic

ataxia of Charlevoix- Saguenay (ARSACS, OMIM 270550) (De Braekeleer et al. 1993). The disease gene causing ARSACS has been identified on chromosome13q 11 (OMIM 604490) and encodes for sacsin, which is thought to be a protein involved in chaperone-mediated protein folding (Engert et al. 2000). Three large families (13, 27 and 71) with autosomal dominant hereditary spastic ataxia were identified in Newfoundland (Grewal et al. 2001). The affected individuals present with progressive leg spasticity by the age of twenty followed by moderate to severe dysarthria, ocular movement abnormalities including slow saccades and impaired vertical gaze, and involuntary head jerk. This phenotype resembles that observed in ARSACS, however, these three families are from a different population and show a clear pattern of dominant inheritance with a later age of onset than seen in ARSACS (Bouchard et al. 2000).

In order to map the gene causing HSA in these three dominant kindreds a genome wide scan was performed on one of the families, family 71. Strong linkage to chromosome 12p13 was identified and fine-mapping confirmed linkage to this family. Haplotype analysis established a disease haplotype of 10.5 cM. Examination of these critical markers in two additional families identified a shared haplotype within the three families. Overlapping the disease haplotypes defined by key recombinants in each family reveals that all affected individuals inherit a minimal DNA segment of 5.1 cM. This is the first report of a locus for autosomal dominant spastic ataxia.

6.2.3 Subjects and Methods

Clinical information

Three families originating from the province of Newfoundland were independently assessed mostly by a single neurologist (M.S.) using a standard protocol. The majority of affected individuals present initially with progressive leg spasticity of variable degrees followed by ataxia in the form of involuntary head jerk, dysarthria, dysphagia, and ocular movement abnormalities with no signs of amyotrophy. The lower limbs show hyperreflexia and hypertonicity. The ocular movement abnormalities include slow saccades, impaired vertical gaze and in some cases lid retraction. A few patients have additional features such as dystonia, pes cavus, mild ptosis, and decreased vibration sense in lower limbs. The severity of the phenotype varies greatly within and among the families and the age of onset ranges from early childhood to early twenties, although the majority of patients start presenting with symptoms between 10 and 20 years of age. The available neuropathological data indicates degeneration of the corticospinal tracts and posterior columns. The lifespan and cognition of patients is not affected and there is no obvious kinship between these three families (unpublished data; Grewal KK).

Twenty-nine individuals have been collected from family 71 of which 14 are clinically affected and 15 unaffected (Figure 1). The largest family is family 13, of which 50 individuals have been collected: 21 affected and 29 unaffected (Figure 2). Finally, family 27 is a small collection of 7 individuals of which 3 are affected (Figure 3). Blood was collected from consenting family members, 19 years and older, and DNA was extracted using a salting out extraction method (Miller et al. 1988).

Genotyping and Linkage analysis

Although not the largest pedigree, a genome wide scan of the 22 autosomes was performed on family 71, because the individuals in this family are more closely related and fewer individuals were required for significant linkage results. Approximately 400 evenly spaced polymorphic microsatellite markers throughout the genome were used. Two point parametric linkage analysis was performed for each marker using the FASTLINK (version 4.1) software package. The parameters in the linkage analysis are: an estimated disease gene frequency of 1 in 10,000, a penetrance of 90%, equal male and female recombination frequencies, and CEPH marker allele frequencies.

Additional polymorphic markers were genotyped to further investigate the positive linkage results. These markers and their order were obtained from the Marshfield genetic map and the primer sequences from the GDB and the CHLC database. These polymorphic markers were amplified by Polymerase Chain Reaction (PCR), which incorporated a radiolabelled nucleotide (S^{35}- dATP) in the product. The PCR products were separated on 6% denaturing polyacrylamide gels and detected by exposure to autoradiographic film. The alleles were assigned on basis of their size with comparison to a M13mp18 sequence ladder. The MLINK program of the FASTLINK software package was then used to perform two point parametric linkage analysis on the genotype data (Cottingham et al. 1993). Haplotypes of individuals with inferred genotypes were constructed assuming minimal recombinantion.

6.2.4 Results

Linkage analysis from the genome wide scan data identified linkage of family 71 to marker D12S374 on chromosome 12 with a LOD score slightly greater than 3 at theta = 0. No other LOD scores greater than 2 were found. Following the genome scan, seven additional markers over an 11 cM interval were genotyped and the results confirmed linkage to a novel locus for Hereditary Spastic Ataxia on chromosome 12p13 (Table 1). The highest LOD score was obtained for markers D12S374 (4.8 at theta = 0) and marker D12S93 (5.1 at theta = 0). The locus for DRPLA is in the vicinity of marker D12S374, but two individuals from each of the three families tested negative for expansions in that gene (unpublished data; Rouleau GA).

Once linkage of family 71 was confirmed all markers were also tested in families 13 and 27. Family 13 showed highly significant linkage to the new locus with highest LOD score of 7.2 at theta = 0 for both marker D12S93 and D12S374, that are 1.6 cM apart (Table 2). The highest LOD score for family 27 was 0.45 at thetha = 0 for D12S1623. The SLINK program of the LINKAGE software program was used to simulate linkage in family 27 and the highest LOD score attainable was predicted to be 1.18 at theta = 0.01. Interestingly, none of the markers showed a negative LOD score and given the small number of samples, these results indicate suggestive linkage of this family to the new locus (Table 3).

A disease haplotype was established for family 71 and key recombinants (VI:4, VI:14) defined the smallest critical region as 10 cM flanked by markers D12S1685 and GATA151H05. The haplotype was constructed following the order of markers available from the Human Genome Project Working Draft database. Haplotype analysis

was conducted for the same critical markers in family 13 and 27. An identical disease haplotype was identified for both families, suggesting that these two families originate from a common ancestor. It is highly unlikely that two families share the same alleles over an 11 marker interval by chance. For family 13, the key recombinants V:14 and VI:11 define the critical region to a 8 cM interval between marker D12S1725 and D12S397.

When the disease haplotypes of the three families are overlapped the smallest common disease haplotype is 5.1 cM, flanked by markers D12S93 and GATA151H05 (Table 4). All affected individuals in each family inherited this identical segment of DNA. However, there are individuals who are presently unaffected, but share a part of this 5.1 cM region. These individuals might be presymptomatic or asymptomatic, which can be explained by age-dependent or reduced penetrance of HSA. In family 13 two individuals (VII:14, VII:15) share almost the entire haplotype, but are younger and possibly presymptomatic. In family 27 individual III:3 shares the entire haplotype and is unaffected (Figure 3).

According to the Human Genome Working Draft sequence the smallest disease haplotype of 5.1 cM corresponds to approximately 3.7 Mb on the physical map. There are 40 known genes and 52 predicted genes estimated in this interval. The genomic sequence in the region is not yet complete and the order of markers and the size of the region may be altered as sequencing progresses. Additional polymorphic markers will become available as the sequence gaps are resolved. Meanwhile, the collection and haplotype construction of additional affected individuals will continue.

6.2.5 Discussion

In an effort to find the molecular cause of a phenotypically distinct clinical disorder, dominant HSA, a genome wide screen was conducted and identified the first locus for dominant hereditary spastic ataxia on chromosome 12p13. This locus has been assigned the symbol *SAX1* by the HUGO nomenclature committee. At present, three families have been linked to this novel locus and the smallest commonly inherited DNA segment is 5.1 cM. Mapping dominant HAS to a novel locus suggests that there is locus heterogeneity in HSA: *SAX1* and ARSACS.

The shared haplotype in the three Newfoundland families suggests that they originate from a common ancestor in Newfoundland. It is therefore likely that a common mutation will be identified for these three families, which would facilitate a genetic testing program in Newfoundland. However, there is currently no genealogical evidence that these families are related and available family records will be further studied in search of a common ancestor.

The approximately 3.7 Mb locus contains three large unsequenced gaps, which complicates the fine-mapping efforts. However, more polymorphic dinucleotide repeats can be identified using the Human Genome Working Draft database. Furthermore, single nucleotide polymorphisms (SNP's) in the known genes can also be used to further refine the disease haplotype. Finally, the collection of affected family members in particular for family 27 and possibly new families will continue in an effort to identify more recombinations to further narrow down the region. This work will continue until the smallest disease haplotype is established by haplotype analysis. The 3.7 Mb region contains 40 genes and 52 predicted genes and candidate genes will be

screened with priority on expression profiles, and possible molecular function in disease pathogenesis.

HSA is clinically related to the Hereditary Spastic Paraplegias (HSP) and the Spinocerebellar Ataxias (SCA) (Bouchard et al. 2000; Tallaksen et al. 2001). Lower limb spasticity is marked in HSA, but is also the hallmark of HSP. The main pathological feature is axonal degeneration of the terminal ends of the corticospinal tracts and the dorsal column pathway tracts (McDermott et al. 2000). HSP causing mutations have been identified in four genes. L1 cell adhesion molecule (L1CAM) is the defective protein identified for SPG1 (X-linked) and is involved in the development of the nervous system. Proteolipid Protein (PLP) is involved in later stages of myelin sheet compaction and maintenance and is mutated in SPG2 patients (X-Linked). Finally, there are two AAA (ATPase with various cellular activities) family members, paraplegin and spastin implicated in HSP. Paraplegin is a mitochondrial metalloprotease mutated in SPG 7 recessive HSP families and *SPAST*, is responsible for 40% of the dominant HSP cases and encodes a protein, spastin, thought to act as a chaperone (Casari and Rugarli 2001).

Spinocerebellar Ataxias (SCA) are another group of clinically heterogeneous neurodegenerative disorders. This group is characterized by degeneration of the cerebellum, brain stem and spinal cord (Harding 1993). There are 17 loci reported to date of which 7 are caused by trinucleotide repeat expansions (Nakamura et al. 2001; Tan and Ashizawa 2001) and include Dentatorubral pallidoluysian atrophy (DRPLA) and Machado-Joseph disease (MJD). Evidence suggests that trinucleotide repeat expansions are the main pathogenic mechanism for autosomal dominant cerebellar

ataxias (progressive ADCA), although the underlying cause of the cell degeneration observed remains unknown (Klockgether et al. 2001).

Genes homologous to any of the known HSP, SCA or ARSACS genes: AAA family members, L1CAM, PLP and sacsin present in the 3.7 Mb interval will be investigated. Interestingly, *DRPLA* is present in the disease interval, but two patients from each of the three families have tested negative for expanded repeats (unpublished data, Rouleau GA). However, *DRPLA* is not excluded as a candidate gene and will be considered for point mutation screening. Anticipation has not been observed in any of the three families, which suggests that a DNA repeat expansion is less likely responsible for *SAX1*. Other interesting candidates in the region are vesicle associated membrane protein 1 (VAMP1), a synaptobrevin involved in vesicle transport to the synapse (Archer et al. 1990), and gamma enolase (ENO2), a neuronal specific enzyme with increased expression during cell death (Craig et al. 1990; Lafon-Cazal et al. 1992).

The mapping of this first locus for dominant HSA will lead to the identification of a disease causing mutation in these families. Pinpointing the disease gene would facilitate clinical diagnosis, which is often difficult in HSA. In addition, a genetic testing program and improved clinical diagnosis in Newfoundland would be feasible with the evidence of a common ancestor. The characterization of the disease causing gene and defective protein will provide more insight into the disease pathogenesis of this and other related neurodegenerative diseases such as HSP, SCA and amyotrophic lateral sclerosis (ALS).

6.2.6 Acknowledgements

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6.2.7 Tables

Table 1: LOD scores for chromosome 12 in Family 71.

		Recombination fraction θ						
Marker	Position (cM)	0	0.01	0.05	0.1	0.2	0.3	0.4
D12S1725	9.52	0.909	1.007	1.190	1.223	1.031	0.667	0.241
D12S314	11.37	1.439	1.450	1.455	1.401	1.174	0.846	0.420
D12S93	12.60	**5.092**	5.026	4.729	4.294	3.265	2.057	0.766
D12S374	14.23	**4.884**	4.819	4.521	4.085	3.057	1.852	0.575
D12S1623	15.69	-1.248	-1.056	-0.676	-0.450	-0.229	-0.118	-0.051
D12S397	17.72	-4.083	0.903	1.834	2.070	1.820	1.178	0.396
D12S1695	19.68	-1.447	1.704	2.429	2.535	2.119	1.322	0.373
D12S77	20.27	-1.773	2.314	2.920	2.907	2.316	1.397	0.371

Table 2: LOD scores for chromosome 12 in Family 13.

		Recombination fraction θ						
Marker	Position (cM)	0	0.01	0.05	0.1	0.2	0.3	0.4
D12S1725	9.52	2.904	6.058	6.180	5.691	4.241	2.527	0.882
D12S314	11.37	-2.461	5.086	5.997	5.9142	4.948	3.500	1.776
D12S93	12.60	**7.293**	**7.142**	**6.526**	**5.736**	**4.104**	2.447	0.905
D12S374	14.23	**7.223**	**7.324**	**7.325**	**6.9685**	**5.749**	4.106	2.135
D12S1623	15.69	1.107	1.265	1.489	1.545	1.374	0.981	0.458
D12S397	17.72	-4.342	1.562	2.398	2.384	2.261	1.553	0.713
D12S1695	19.68	2.487	6.808	7.204	6.932	5.719	4.028	2.017
D12S77	20.27	0.679	5.291	5.948	5.918	5.071	3.678	1.917

Table 3: LOD scores for chromosome 12 in Family 27.

		Recombination fraction θ						
Marker	Position (cM)	0	0.01	0.05	0.1	0.2	0.3	0.4
D12S1725	9.52	0.226	0.241	0.273	0.276	0.217	0.123	0.037
D12S314	11.37	0.355	0.367	0.391	0.380	0.285	0.154	0.042
D12S93	12.60	0.280	0.271	0.238	0.197	0.122	0.059	0.016
D12S374	14.23	0.354	0.367	0.389	0.378	0.281	0.148	0.039
D12S1623	**15.69**	**0.448**	0.437	0.391	0.333	0.217	0.110	0.030
D12S397	17.72	0.259	0.273	0.303	0.301	0.230	0.126	0.036
D12S1695	19.68	0.333	0.346	0.370	0.359	0.266	0.137	0.035
D12S77	20.27	0.349	0.362	0.386	0.374	0.279	0.148	0.039

Table 4: *SAX1* locus on chromosome 12p13 determined by key recombinants and overlapping of haplotypes.

Markers	Family 71[1]	Family 13[1]	Family 27[1]	Minimal commonly inherited segment[2]
D12S1685	13	6	6	
D12S1725	1	6	6	
D12S314	5	8	8	
D12S93	7	1	1	
D12S374[3]	5	5	5	5 (209 bp)
D12S1623	4	4	4	4 (127 bp)
DRPLA	2	2	2	2 (180 bp)
GATA151H05	5	5	5	
D12S397	3	3	3	
D12S1695	6	6	6	
D12S77	4	4	4	
D12S89[3]	8	8		
D12S391	5	4		

[1]This table shows the disease haplotype of the *SAX1* locus on chromosome 12p13 for each linked family (71, 13, 27). The boxed areas indicate the minimal disease interval determined by key recombinants. Note that there were no key recombinants identified for family 27 in this interval.

[2]The shaded area represents the minimal commonly inherited disease haplotype of 5.1 cM obtained by overlapping the critical disease intervals of each family. To standardize the results the size of the marker alleles in basepairs is provided.

[3]All three families share part of their haplotypes from marker D12S374 to D21S89.

6.2.8 Figures

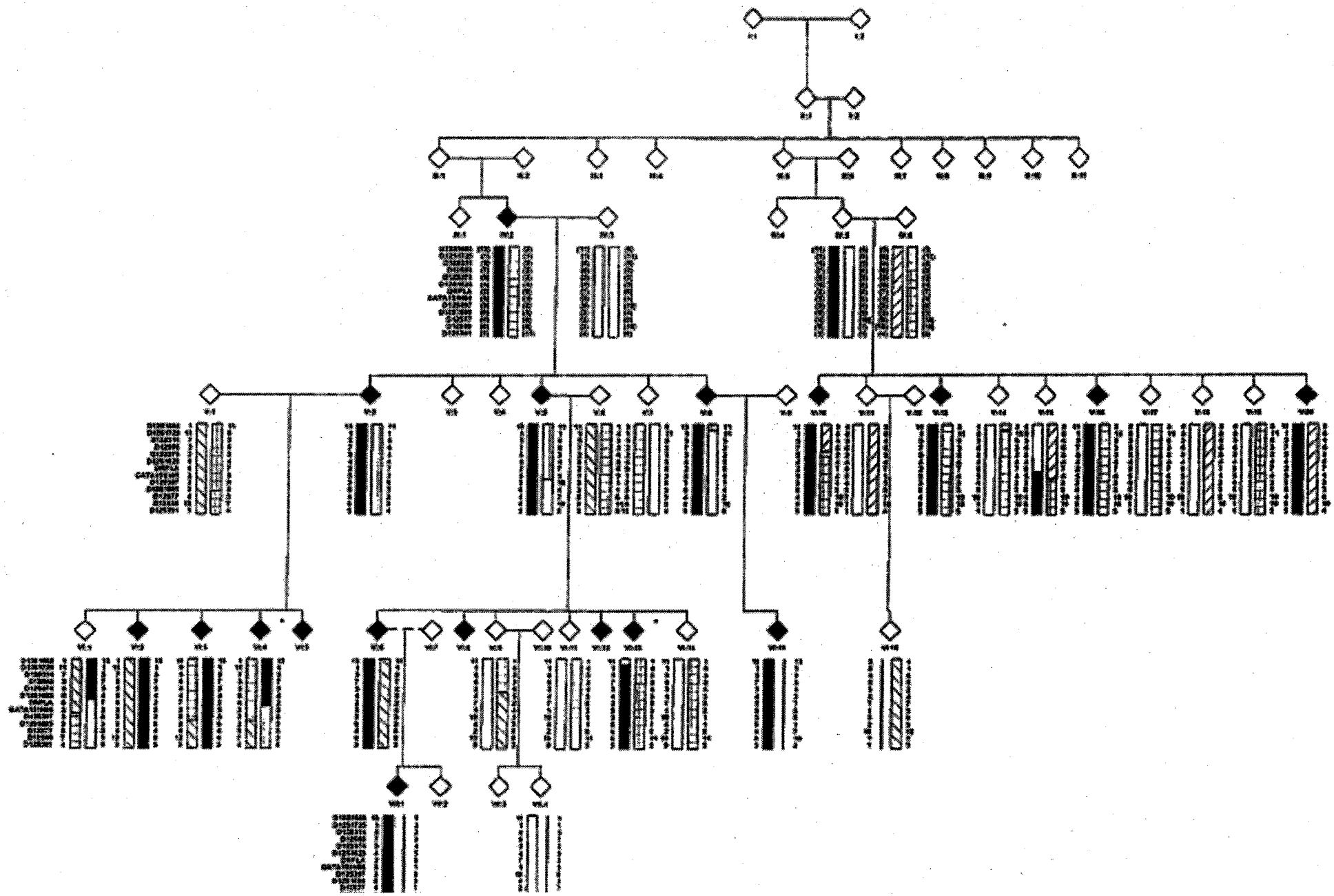


Figure 1

Figure 1: Informative section of family 71 with the chromosome 12p13 haplotype. The disease haplotype is in black, and key recombinants are indicated by an asterix (VI4, and VI14). These recombinants determine a critical disease interval of 10 cM flanked by markers D12S1685 and GATA151H05.

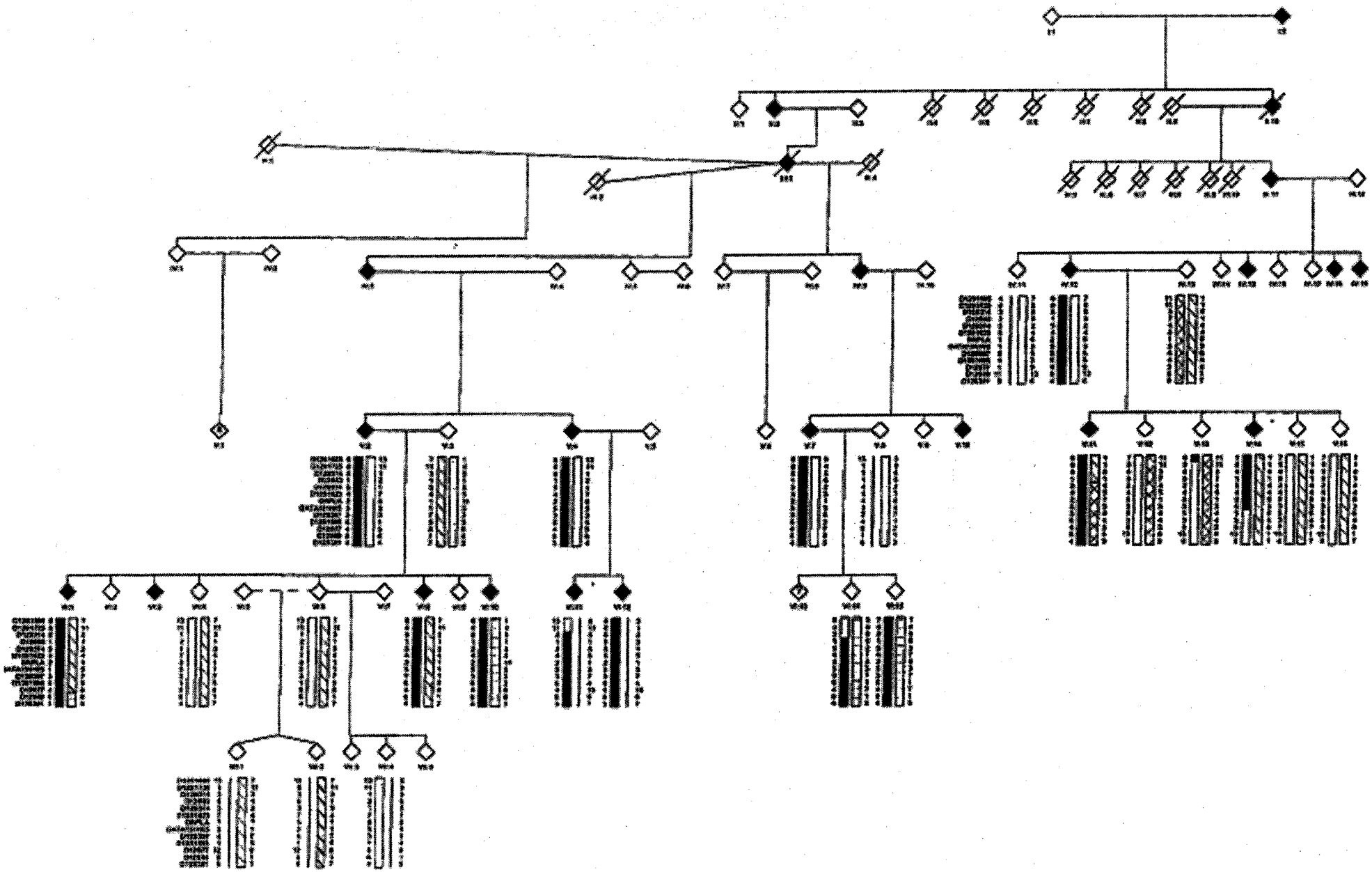


Figure 2

Figure 2: Informative section of family 13 with the chromosome 12p13 haplotype. The disease haplotype is in black, and key recombinants are indicated by an asterix (V:14, VI:11). They define the critical region to an 8 cM interval between marker D12S1725 and D12S397.

Figure 3

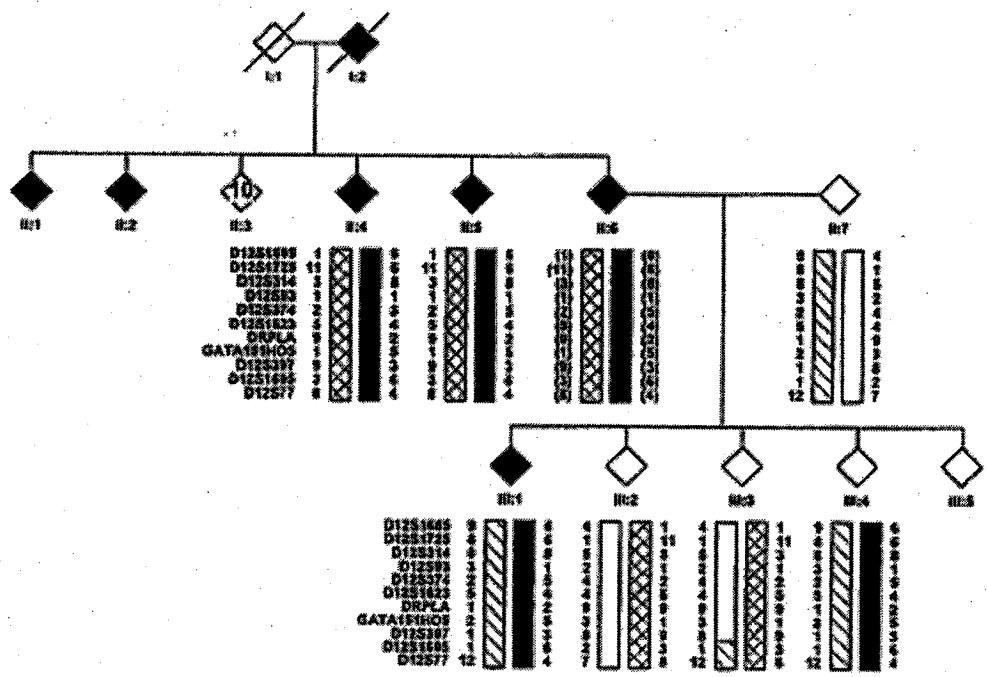


Figure 3: Informative section of family 13 with the chromosome 12p13 haplotype. The disease haplotype is in black. Individual III:3 shares the complete haplotype and is unaffected.

6.3 A FOUNDER EFFECT IN THREE LARGE NEWFOUNDLAND FAMILIES WITH A NOVEL CLINICALLY VARIABLE SPASTIC ATAXIA AND SUPRANUCLEAR GAZE PALSY

6.3.1 Summary

A distinctive slowly progressive neurodegenerative disorder, which falls under a new category of neurological diseases, the hereditary spastic ataxias (HSA), is described in three independently ascertained Newfoundland kindreds. HSA is a heterogeneous group of disorders in which pyramidal tract features overlap cerebellar characteristics. The families are assumed to have the same condition as, although apparently unrelated, all originate in a historically isolated cluster of rural communities and link to the same locus at 12p13, *SAX1*. Clinically the phenotype is very variable but lower limb hypertonicity and hyperreflexia are early and prominent generally preceded by eye movement abnormality, an impaired vertical downward saccade and a typical involuntary head jerk. These are followed by variable levels of ataxia, dysarthria, and dysphagia. Onset occurs in the first two decades and can be detected in most by early adulthood. Significant mobility problems are present by the fourth decade with a broad based ataxic and spastic gait. MRI scans of brain and spinal cord were normal. Neuropathology showed degeneration of corticospinal tracts and posterior columns and midbrain neuronal loss. The phenotype is striking in its diversity among and within families and the variability of expression can be observed within the same sibship. Pedigree analysis shows no evidence of anticipation or any sex differences in severity.

The condition is unusually prevalent in the province of Newfoundland, which is characteristic of a founder effect followed by isolation and large family size. Fine-mapping efforts have reduced the critical interval of the *SAX1* locus to 1.9 Mb. Identification of the *SAX1* gene will help to clarify the pathogenesis of this type of HSA.

6.3.2 Introduction

Hereditary spastic ataxias (HSA) are a group of progressive, clinically heterogeneous neurodegenerative disorders, which encompass both pyramidal tract and cerebellar characteristics (Eadie et al. 1991). The reason for a separate classification of HSA distinct from spinocerebellar ataxia (SCA) and hereditary spastic paraplegia (HSP) occurred due to the level and increased importance of the cerebellar component. Clinical similarities between HSA and both SCA and HSP can be observed {Tallaksen et al 2001; Bouchard et al. 2000). An almost universal finding in both HSA and HSP is lower limb spasticity. Lower limb spasticity resulting in gait disturbance is a prominent finding in HSA and generalized ataxia with dysarthria, dysphagia, and eye movement abnormalities are also frequent features. The HSA group is also genetically heterogeneous and families compatible with both autosomal dominant and recessive inheritance have been reported (Mahloudji 1963; Bouchard et al. 2000; Hedera et al. 2002). A well-characterized early-onset form is the autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) the gene for which has been mapped to chromosome 13q11 and isolated (MIM604490). We describe the full clinical spectrum in three large multigenerational apparently unrelated HSA families (71, 27, and 13) from the province of Newfoundland with a novel autosomal dominant, complex, markedly variable, and

clinically distinct form of the disorder (MIM108600) (Grewal and Stefanelli 2001). An additional small nuclear family from the same geographic area has also been identified. All families map to a locus at 12p13 now called *SAX1* for spastic ataxia locus 1 (Meijer et al. 2002b) and gene isolation is in progress. A shared haplotype strongly supports the idea that they are the result of a founder effect. *SAX1* is the first locus for autosomal dominant HSA.

6.3.3 Methods

Patient Ascertainment and Study

All the study families were separately ascertained through referral from the Neurology service to Genetics. The clinical and genetic study of the families was approved and informed consent obtained as specified by the Human Investigation Committee of Memorial University. Family histories were obtained and the majority of patients were examined by a single physician following a set protocol. Blood was collected for DNA studies. Age-of-onset of symptoms was obtained by interview and where possible corroborated by another family member. Demographic data on the origins of the research families was examined in provincial genealogical resources.

Genotyping and Haplotype Construction

Polymorphic markers were amplified by PCR incorporating radiolabeled ^{S35}dATP into the product. The products were separated on 6% denaturing polyacrylamide gels and visualized on autoradiographic film. In an effort to reduce the critical region of the *SAX1* locus four additional markers were genotyped around the initial flanking markers

(D12S356, D12S808, VWF378/379, REP736AAT). We have developed primers, REP736F-CAG AGG TTG CAG TGA TTG G and REP736RATG TGT CTT TTA TGA GGT TCT TAC, for amplification of a trinucleotide repeat (AATn) in the region. All markers in the *SAX1* region were also amplified for 15 recently collected affected individuals of family 27. These genotypes were used to construct haplotypes assuming minimal recombination. Markers and their order were obtained from the Marshfield genetic map (Centre for Medical Genetics, Marshfield Medical Research Foundation) and UCSC Human Genome Browser. Mutation Detection Total RNA was extracted from cultured lymphoblasts using a Rneasy midi kit (Qiagen Inc., Mississauga, Ontario, Canada). A cDNA library was synthesized by RT-PCR using poly dT and random hexamer oligonucleotides. Exonic primers were designed to amplify overlapping fragments of cDNA and genomic primers were designed to amplify the exons and at least 50 bp of the flanking intronic sequence. The PCR products were sized by gel electrophoresis and sequenced at the Montreal Genome Centre.

6.3.4 Results

Clinical Presentation

Age-of-onset.

Attempts to determine the age-of-onset (Table I) were made by a combination of interview, family observation, and clinical examination. A characteristic rapid head nod or brief involuntary jerk is a prominent early feature present in a significant number in each pedigree and taken as a measure of affection status by family members. Affected children often show subtle signs of general clumsiness and the disorder can be identified

clinically by early adulthood in the majority of cases. Significant progression usually occurs in the late thirties and early forties. Older family members can usually only recall if difficulties began in childhood or the teenage years and a few individuals are unaware that they are affected. Clinical examination is detecting the condition at an earlier age but otherwise, as shown later there are no significant intergenerational differences in age-of-onset.

Neurological features and progress.

The frequency of individual neurological features is shown in Table II and as can be seen there is wide variation among the families in the frequency of the individual characteristics. There is also striking phenotypic diversity within each family. This marked variation ranges from a few very mild symptoms to an extremely severe complex and disabling phenotype regardless of age. Some individuals are noticeably affected at a young age whereas others display only subtle findings at an advanced age and this can be seen even within the same sibship. The disorder in all the families is characterized by pronounced pyramidal signs and mild to moderate ataxia, the latter possibly affected by the individual degree of spasticity. An early feature is the increased tone and reflexes in the lower limbs and to a much lesser degree in the upper limbs. Upgoing plantars and some ankle clonus are frequent. Power is usually preserved until the later stages even when spasticity limits mobility so only older individuals appear weak. Many patients complain of leg stiffness and have an awkward spastic and ataxic gait worse in cold weather. The gait widens significantly over time, tandem gait becomes difficult and there are problems on irregular ground and with balance. Falls are common and by the fifties several individuals use a cane or wheelchair. Limb ataxia is

observed quite frequently although rare in the upper limbs. A fine tremor of the head or mild intention tremor of the limbs is noted occasionally but vibration, position sense, and sensation are generally normal. Dystonia, usually cervical presenting as torticollis, seen in a few individuals is an unusual component of the phenotype. By the third or early fourth decades, speech has often become dysarthric and scanning in nature suggesting both spastic and ataxic components. Ocular motility abnormalities are also an almost universal and early feature but nystagmus is not observed. Initially there are problems with saccadic gaze greater than pursuit for vertical movements. Downward gaze is often more impaired than upward and eventually horizontal gaze also becomes affected. However, lateral eyeball movements are clearly much less affected than vertical. These difficulties are overcome by oculocephalic maneuvers suggesting they are supranuclear in origin. The authors believe that the characteristic head nod or jerk is secondary to early vertical saccadic gaze failure. It is a compensating mechanism for the inability to gaze down and the earliest sign, which allows identification of an affected individual by the previous generation. Finally cognition is not generally affected in this disorder as abnormalities in the mini mental status exam are rarely seen. Of 15 school-aged children examined, only one had a mild learning disability and most affected adults have normal employment. The life span does not appear to be affected to any appreciable degree.

Special Investigations

In the past, SSEP (somatosensory evoked potential) and nerve conduction studies were carried out on several individuals and were non-contributory. MRI of the cerebral and cerebellar hemispheres and brainstem as well as the cervical spinal cord in three severely affected individuals from families 27 and 13 is normal for age. Neuropathology

is available on two affected individuals in family 13 a female and male aged 77 and 63, respectively at death. The former showed severe degeneration of the lateral corticospinal tracts and dorsal columns. There was also neuronal loss and gliosis involving the subthalamic nucleus, tectum and tegmentum of the midbrain which probably correlates with the patient's supranuclear palsy. Despite typical clinical findings the latter individual showed only degeneration in the lateral corticospinal tracts with no significant abnormality in dorsal columns or midbrain. In both, the cerebellum was normal.

Genetics

Members of these three large families have been known to Neurology service for over 20 years but during the current research study were examined by a single neurologist (MS). In family 71, 29 individuals were seen of whom 14 (ages 4–78 years) were clinically affected. In family 27, 28 individuals have now been seen of whom 19 (ages 11–70 years) were clinically affected and in family 13, 52 individuals have been seen with 23 clinically affected (ages 21–71 years) and the majority contributed DNA samples. The fourth small nuclear family has two affected individuals, a father and daughter. The pedigrees of the three families are shown in Figure 1 indicating those individuals affected by history or examined previously and in the present study. In the three large families, there are 53 affected and 44 unaffected males and 52 affected and 53 unaffected females so that neither segregation nor sex ratios differ significantly from 1:1 and are consistent with autosomal dominant inheritance over four generations. There is no evidence for a sex of parent influence on the age-of-onset in their offspring. We examined parent–child pairs for changes in age at onset across the generations in the

three large families. Using the age ranges in Table I, 15 affected male parents had 25 affected offspring 12 with onset in childhood, 12 in teenage, and 1 as an adult while 18 affected female parents had 27 affected offspring 13 with onset in childhood, 10 in teenage, and 4 as an adult. These distributions are not significantly different. Similarly there is no evidence for an earlier age of onset in more recent generations since among these 52 parent–child pairs only 9 offspring had onset earlier than the parent while in 43 it was the same or later. The lack of an associated familial structural chromosome anomaly was verified by a normal karyotype in two typically affected but otherwise phenotypically normal members of family 13. No known relationship could be established between the three large families whose founders however are from the same geographic area (Fig. 2). The ancestors of the small nuclear family could not be traced but its origins are also in the same area. The three large families were not initially recognized as having the same condition due to the marked diversity in clinical presentation. Accordingly two affected individuals from each were tested for known *SCA* and *DRPLA* gene expansion mutations and normal alleles were found in each case. In due course results of a genome scan in family 71 revealed linkage to markers at 12p13 and, subsequently, the other three kindreds were found to share the same marker alleles at this locus, *SAX1*. The original *SAX1* critical region was 5.1 cM or ~3.7 Mb. In this study, genotyping of recently collected affected individuals together with analysis of four additional polymorphic markers (Table III) has led to the identification of a recombination in family 27 that further refines the critical interval. This interval spans approximately 1.9 Mb and is flanked by markers VWF378/379 and GATA151H05 (Table III). One interesting candidate, the *DRPLA* gene, has been analyzed for mutations

in genomic DNA and in mRNA of two patients and no mutation was found. Further fine-mapping efforts and analysis of additional candidate genes are underway.

6.3.5 Discussion

The newly described disorder is sufficiently distinct from SCA and HSP to merit a separate assignment. The older classification for SCA and HSP (Harding 1993b; Harding 1993a) based on the clinical features and neuropathology is no longer applicable. As in the condition described here there can be great variability in the clinical phenotype within one family and correlation of clinical findings with available neuropathology is often poor. A molecular classification has supervened as the mutant genes in different families are mapped and identified. Over 20 varieties of SCA, with the prominent feature of ataxia, and a similar number of varieties of HSP, with predominant lower limb spasticity, are now known. Previously the term HSA was coined (Bouchard et al. 2000) to accommodate the problem of those families in which both spasticity and ataxia figure significantly. It is felt that the present condition fits best into this category and the genetic locus at 12p13 for this autosomal dominant form has been named *SAX1* (Meijer et al. 2002b). This new disorder shows some similarity to other reported families of HSA. In the well-described condition ARSACS (MIM270550) cerebellar signs precede spasticity but progression is much more rapid, the phenotype more consistent and genetic transmission is autosomal recessive. Furthermore the gene for ARSACS maps to 13q11 (MIM604490) and is known to encode for sacsin, a protein believed to be involved in chaperone mediated protein folding (Engert et al. 2000). Hedera et al. (Hedera et al. 2002) have described a family

in which spastic paraplegia and ataxia are associated with mild mental retardation (SPAR). The phenotype is quite variable over three generations and suggestive of anticipation but, other than this variability, differs in many respects from the one described here. In this present condition, the most consistent finding is the lower limb spasticity typical of the HSP group but the variability of all neurological findings does not permit unequivocal clinical differentiation from other SCA and HSP. It is of interest that among the many genetic loci associated with HSP, a number of genes have now been characterized and information on pathological mechanisms affecting the long corticospinal tracts is beginning to emerge (Reid 2003). A common explanation for this aspect and specific unusual components of the phenotype, notably the supranuclear gaze palsy, occasional dystonia and pathologically normal cerebellum, will be required to clarify the pathogenesis of the present disorder. Although the phenotype is variable, there is no consistent trend to an earlier onset of symptoms, as an aspect of increased severity, in successive generations as shown by comparison of a significant number of parent–child pairs. This lack of genetic anticipation is against the possibility that this is a trinucleotide repeat disorder. The sex of the transmitting parent likewise does not appear to influence onset of symptoms in offspring so that genomic imprinting is also unlikely. Factors such as modifier genes or environmental influences may contribute to clinical variability but as yet no supportive evidence is available. Identification of the gene and the protein involved is critical to further understanding of genetic mechanisms in this condition. In the present study, we have reduced the critical interval to half its original size by additional genotyping and haplotyping. This critical interval spans ~1.9 Mb and contains approximately 53 known genes. The *DRPLA* gene did not show any mutations in the genomic sequence or in the mRNA of patients. It seems unlikely that a

mutation in splice enhancers or repressors will cause such a dominant neurodegenerative disease. Therefore, we are continuing our candidate gene screening process, prioritizing genes by their expression in the nervous system and their function. An explanation for the underlying pathogenesis of this disorder, including perhaps the variability, awaits isolation of the *SAX1* gene and knowledge of its function. The clinical features are similar in all four families, which were separately ascertained and there are no apparent relationships amongst the current generations. But if one explores their ancestry they can all be traced back to the counties of Dorset and Devon in southwestern England from which migration of Protestant families from the port of Poole was largely complete by the 1820s. Common surnames still occur in these counties but to date the local neurologists have not reported a similar disorder in their region. In Newfoundland their descendants reside in a string of small nearby coastal communities in a largely rural area and the families have mostly remained in the same general area as their ancestors (Fig. 2). Archival studies examining genealogical records have not identified a single common ancestor to date but all four sets of family surnames go back to earliest settlement of the island and appear as early as 1805 in records of land ownership in adjacent properties and communities (Governor Gower's Plantations book, online@NFGenWeb). Geographic isolation and the tendency towards large family size probably served to perpetuate the disorder to the degree to which it occurs today. This is consistent with the idea of a founder effect, which has been noted in other dominant conditions in Newfoundland (Rahman et al. 2003). At the molecular level all four families share a common disease haplotype with several tightly linked flanking markers over at least a 10 cM region, which also adds weight to the idea that a common founder was responsible. It is highly probable that a common mutation will be

found in all four families. Interestingly, no case has been found who carries the entire disease haplotype and is still unaffected past the oldest age-of-onset recorded. This suggests that the mutation at the *SAX1* locus is highly penetrant. A prevalence figure for this disorder based on the currently identified families alone in the province is 2.16 per 10,000, which exceeds those reported in the literature for similar neurological conditions such as HSP, approximately 0.64–1 per 10,000 (Polo et al. 1991; Silva et al. 1997). This is in accordance with enrichment of the condition and the concept of a founder effect. The disorder thus contributes disproportionately to the burden of adult neurological degenerative disease in the area. Identification of the gene and specific mutation will facilitate appropriate genetic testing and easier clinical diagnosis. The haplotype for the *SAX1* locus can already be used to test any suspect new sporadic cases or families. Genetic counseling is important for information and support although variability in clinical severity and progression makes prognosis difficult to predict. This variability similarly impacts on experience with and attitudes to the disorder including reproductive decisions. While genetic and clinical delineation of the disorder makes it possible to identify those at risk, the question of offering predictive genetic testing to those still clinically unaffected, as with other untreatable neurological conditions, is too complex an issue to address here and will require further study. At risk individuals can be followed for earlier clinical diagnosis than had occurred in the past. This could be helpful in children in relation to physical education or classroom modification or, in adults, for realistic occupational expectations.

6.3.6 Acknowledgements

We thank the families for their participation in the study and Dr. Pryse-Philips for referral to genetics and early work on this disorder.

6.3.7 Tables

Table I. Age of Onset*

Age Range (Years)	Family 71 No (%) n=14	Family 27 No (%) n=19	Family13 No (%) n=23
0 – 12	4 (31)	7 (37)	8 (35)
13 – 19	8 (57)	9 (47)	11 (48)
20 – 39	2 (15)	3 (16)	4 (17)
40+	0	0	0

*As determined by the appearance of head jerk or general clumsiness.

Table II: A Comparison of the Clinical Features of Families 71, 27 and 13

Clinical Features	Family 71 No (%) n = 14	Family 27 No (%) n = 19	Family 13 No (%) n = 23
Hypertonicity	12 (86)	16 (84)	17 (74)
Hyperreflexia	12 (86)	17 (89)	19 (83)
Eye movements:			
Impaired vertical gaze	12 (86)	15 (79)	17 (74)
Slow saccades	10 (71)	13 (68)	7 (30)
Lid retraction	3 (21)	0	6 (26)
Dysarthria	10 (71)	8 (42)	11 (48)
Dysphagia	8 (57)	4 (21)	15 (65)
Spastic gait	8 (57)	9(47)	11 (48)
Limb ataxia	9 (64)	5 (26)	12 (52)
+ve Babinski	5 (36)	5 (26)	6 (26)
Pes cavus	2 (14)	0	6 (26)
Involuntary head jerk	4 (28)	9 (47)	15 (65)
Tremor	1 (7)	4 (21)	10 (43)
Proprioceptive errors	2 (14)	0	4 (17)
Decreased vibration sense	2 (14)	0	4 (17)
Dystonia	3 (21)	0	3 (13)
Mild ptosis	1 (7)	2 (10)	1 (5)

Table III: Refined physical map of the *SAX1* locus.

Marker	Position (Mb)*	Common Haplotype Fam 71	Fam 13	Fam27	Lower RC Fam 71	Upper RC Fam 27	Critical region	Allele size(bp)$
D12S1685	3.7	13	6	6	13	-		
D12S1725	4.2	1	6	6	1	1		
D12S314	4.7	5	8	8	5	-		
D12S93	5.1	7	1	1	7	6		
D12S356	5.6	1	2	2	1	2		
D12S374+	5.7	5	5	5	5	2		
VWF378/379	6.0	3	3	3	3	5		
D12S1623	7.1	4	4	4	4	4	4	209
DRPLA	6.9	2	2	2	2	2	2	127
REP736AAT	7.3	4	4	4	4	4	4	191
D12S808	7.4	3	3	3	3	3	3	257
GATA151H05	7.9	5	5	5	3	5		
D12S397	8.1	3	3	3	8	-		

* Approximate position in megabases according to the July freeze 2003 of the UCSC human genome browser (golden path)
$ CEPH allele numbers were used when available and marker allele size is provided for the critical region
+ All three families share a common haplotype starting from marker D12S374
Markers in bold have been added to the original haplotype (Meijer et al, 2002)
RC: recombinant

6.3.8 Figures

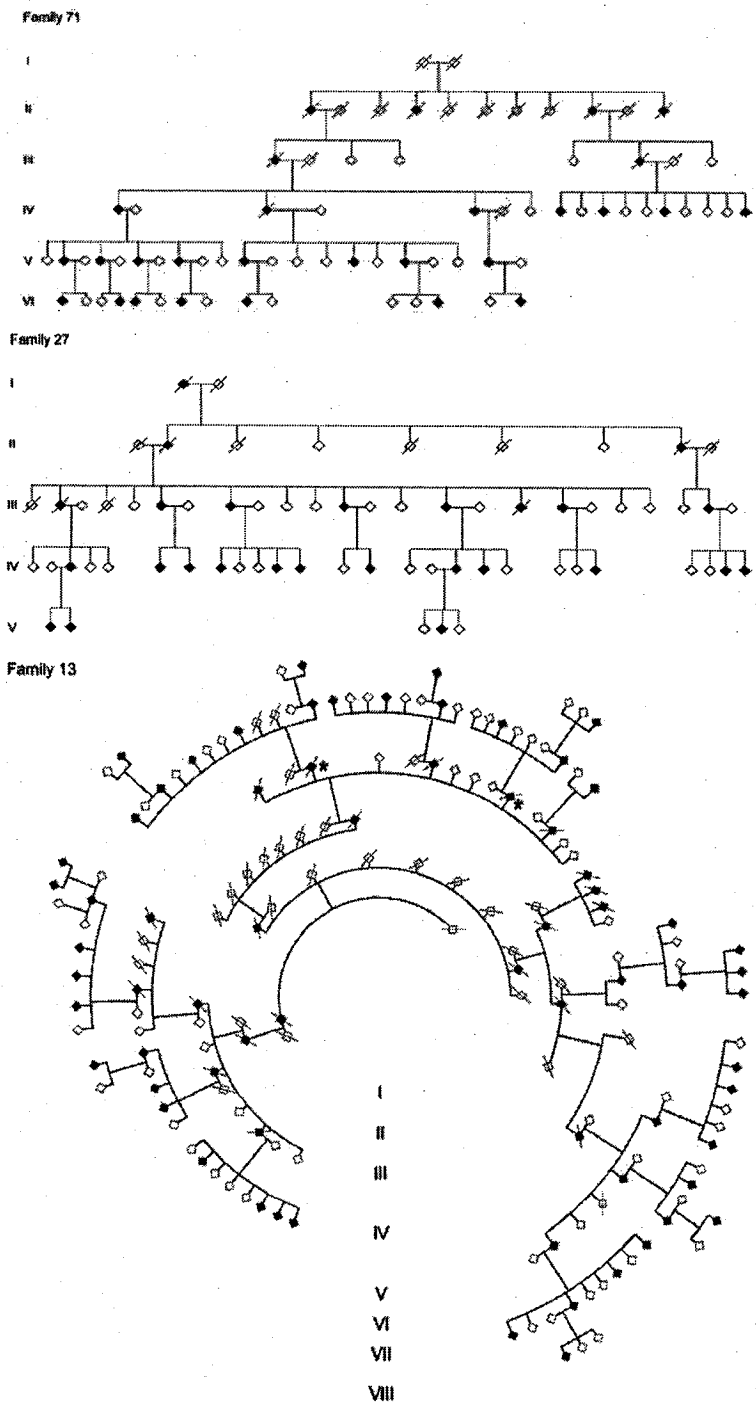


Figure 1

Fig. 1 A-C: Anonymized pedigrees of the three families to preserve confidentiality. Solid symbols, affected members; open symbols, unaffected members (A, Family 71; B, Family 27; C, Family 13).

*Examined prior to this study by another physician.

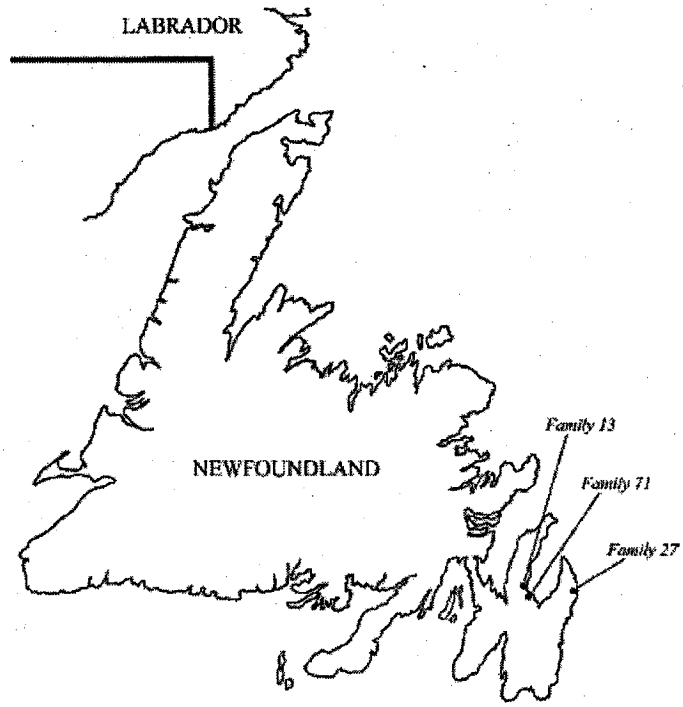


Fig. 2. Map of Newfoundland showing approximate location of the three families on the Avalon peninsula.

6.4 CANDIDATE GENE ANALYSIS AT THE *SAX1* LOCUS

6.4.1 Candidate gene screening

In the previous section, fine-mapping data and clinical characterization of the *SAX1* locus has been presented. The critical candidate gene interval was mapped to 1.9 Mb bordered by markers VWF378/379 and GATA151H05. This region contains 53 known genes. Since the region contained many genes, a two-way approach was applied to maximize the gene finding efficiency. The first approach was fine-mapping to narrow down the candidate interval as well as the number of genes to be screened and the second approach was candidate gene analysis of a single gene at a time. In addition, the variants identified in the candidate gene analysis were incorporated in the haplotype to contribute to the fine-mapping efforts. Fine-mapping with additional markers and samples from family R0027 in the candidate region allowed us to identify two new critical recombinants. These recombinants reduced the candidate region to a 1.6 Mb region flanked by markers LTBR and GATA151H05. This critical region, however, still contains approximately 50 known genes. Table 1 presents the tightly conserved disease haplotype with the flanking markers. The distance between each flanking marker and the next typed marker is small and suggests that maximum density in fine-mapping has been achieved. The flanking regions between the recombinant marker and the next do not contain many genes, therefore further fine-mapping would not significantly reduce the number of candidate genes.

Table 1: Critical candidate gene interval at the *SAX1* locus.

		Fam 71		Fam 13		Fam 27	
Markers	**Position**	**R12615**	**X2259**	**R9934**	**X1414**	**X2682**	**Overlap**
	Mb″	upper RC	lower RC	upper RC	lower RC	upper RC	
D12S1685	**3.8**	11	13	9	x	x	
D12S1725	**4.3**	1	1	4	6	1	
D12S314	**4.8**	5	5	8	8	x	
D12S93	**5.2**	7	7	1	1	6	
NTF3 rs6332	**5.4**	A	A	A	A	A	
D12S356	**5.7**	1	1	2	2	2	
D12S374	**5.8**	5	5	5	5	2	
VWF	**5.9**	3	3	3	3	5	
LTBR	**6.3**	1	1	1	1	2	
VAMP1	**6.4**	G	G	G	G	G	G
GAPD rs3741916	**6.5**	G	G	G	G	G	G
D12S1623	**6.7**	4	4	4	4	4	4
GRCA exon 5	**6.8**	C	C	C	C	C	C
USP5 rs2071064	**6.8**	T	T	T	T	T	T
DRPLA (CAG)	**6.9**	2	2	2	2	2	2
CLSTN3 rs6488452	**7.1**	A	A	A	A	A	A
REP736AAT	**7.3**	4	4	4	4	4	4
D12S808	**7.5**	3	3	3	3	3	3
GATA151H05	**7.8**	5	3	5	5	5	
D12S397	**8**	3	8	3	5	3	

″March 2006 freeze ucsc genomebrowser
RC: recombinant

In parallel, candidate gene analysis was undertaken and priority was given to genes with functional similarities to genes involved in HSP and SCA and to genes highly expressed in the nervous system (Figure 1; Table 2).

Figure 1

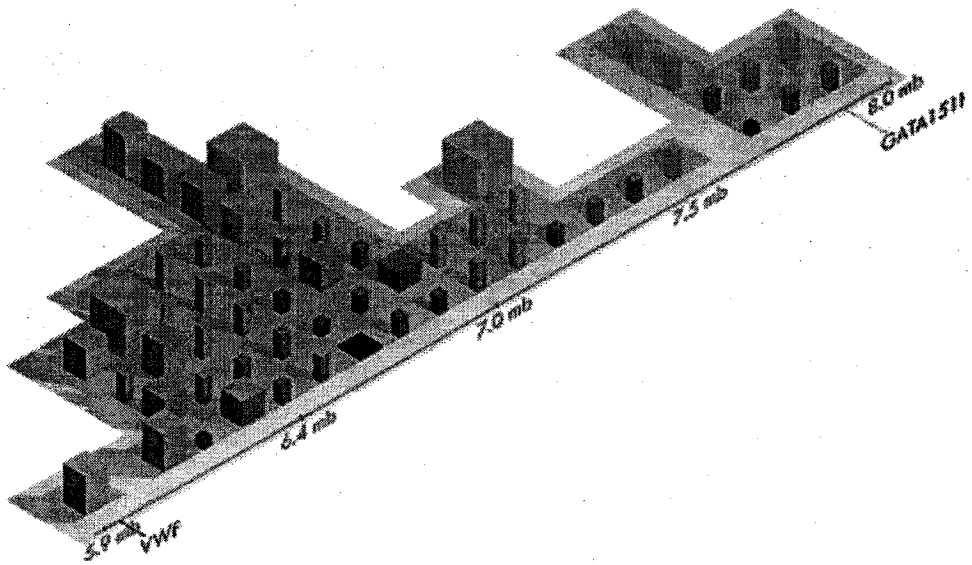


Figure 1: Candidate genes at the *SAX1* locus.

A graphical representation of the *SAX1* candidate region and the genes it contains between markers VWF and GATA151H05. Each cube respresents a gene, and the height and width of each cube indicates the relevance of the candidate based on CNS expression and functional similarities. The picture was generated by the Suspects tool (http://www.genetics.med.ed.ac.uk/suspects/index.shtml).

Table 2: Positional candidates at the *SAX1* locus.

	Gene symbol	Priority*	Function	CNS expression	Method
1	NTF3[¶]	1	controls survival and differentiation of neurons, NTF3 - mice have severe limb movement abnormalities	mature neurons	DNA
2	VWF[¶]	2	VON WILLEBRAND FACTOR- PROCOAGULANT ACTIVITY	high in brain	NT**
3	FLJ10665/PLEKHG6[¶]	1	Rho gef domain, interacts with pleckstrin, probable exchange factor for a small ras-like gtp- binding protein.	low in CNS	RNA
4	TNFRSF1A[¶]	3	mediate apoptosis and function as a regulator of inflammation/ causes Hibernian fever	low in brain and pancreas	NT
5	SCNN1A[¶]	3	AMILORIDE-SENSITIVE sodium non voltage gated channel	not in CNS	NT
6	LTBR	3	NF member, the development and organization of lymphoid tissue and tranformed cells.	ubiquitous, downregulated in CNS	DNA/hybrids
7	TNFRSF7	3	tumor necrosis factor receptor 7, Hodgkins lymphoma, TNF recessive family	brain, lung, spleen	DNA/hybrids
8	TAPBP-R	3	member of variable-constant Ig family linking (MHC) class I molecules to the transporter	low brain expression	DNA
9	VAMP1	1	synaptic vesicle specific	NS specific	DNA and hybrids
10	MRPL51	2	catalyze protein synthesis within the mitochondrion	high in Fetal Pancreas, low in brain	DNA and hybrids
11	KIAA0159/CNAP1	2	chromosome condensation-related SMC-associated protein, leucine zipper	low in brain	RNA
12	GAPD	1	carbohydrate metabolism, binds to normal polyQ tracts, tubulin	ubiquitous	DNA
13	DFKZP58612223	1	This gene is a member of the intermediate filament family.	high in brain and whole blood	RNA
14	NOL1	2	pos role in regulation of cell cycle and increased nucleolar activity associated with proliferation.	low in brain	DNA and hybrids
15	CHD4	2	chromatin organization modifier and SNF2-related helicase/ATPase domains.	in embryo head mainly, ubiquitous	RNA
16	ING4	1	candidate tumor suppressor p33 ING1 homolog, may act in chromatin-mediated transcriptional regulation; contains PHD-finger.	high in brain	RNA/ DNA/hybrids
17	LAG3	2	lymphocyte-activation gene 3, Ig superfamily, common ancestor with CD4	possibly not in brain	DNA/hybrids
18	PTMS	2	parathymosin may mediate immune function by blocking the effect of prothymosin alpha which confers resistance to certain opportunistic infections.	present in brain	DNA/hybrids
19	ACRBP	3	acrosin binding protein, This protein is located in the spermacrosome.	testis only	DNA/hybrids
20	GPR92	1	similar to rat and mediates cellular responses to nucleotides and stimulates intracellular calcium flux; member of the rhodopsin family of G protein-coupled receptors.	low in brain, mostly in leukopheresis	DNA and hybrids
21	MLF2	3	myeloid leukemia factor 2	higher in brain	DNA and hybrids
22	COPS7A	2	strongly similar to murine :ELONGATION FACTOR 1-ALPHA 1(Mus musculus); 98% similarity over 113 a.a, inv in protein synthesis.	in brain	DNA and hybrids

23	ZNF384	1	CAG repeat domain polyglutamine-rich expanded repeat domain, may function as a transcription factor, with a potential role in the regulation of neurodevelopment	in brain	DNA
24	CD4	2	T-cell surface glycoprotein; has role incell-cell interactions and may act in signal transduction , antigen	in brain	DNA/hybrids
25	A/GPR162	1	Function unknown	high in brain	DNA
26	LEPREL2	2	Low similarity over N-terminus to cartilage ssociated proteins; also has low similarity over N-terminus to synaptonemal complex proteins	high in neuroepithelial cells	RNA/DNA/hybrids
27	GNB3	2	component of G-protein complexes; heterotrimer transduces signals from G protein-coupled receptors to intracellular effectors- causes hypertension and obesity.	in CNS	RNA/DNA
28	GRCC8/MGC2577	3	unknown	no CNS, mostly in colonic mucosa	DNA/hybrids
29	USP5	1	ubiquitin-specific cysteine(thiol) protease that disassembles free branched polyubiquitin chain.	expressed in brain and cerebellum	RNA
30	TPI1	1	catalyzes interconversion of glyceraldehyde 3-P and dihydroxyacetone-P during glycolysis,causes Hemolytic anemia	expressed in brain and cerebellum	DNA
31	GRCC9/MCG2519	1	SPRY domain-containing SOCS box protein SSB-2, cytokine signaling domain Domain in SPla and the RYanodine Receptor	in CNS	DNA/hybrids
32	B7	2	Low similarity to the regulatory subunit of protein phosphatases; may mediate protein-protein interactions; contains leucine rich repeats.	high in optic nerve	DNA/hybrids
33	ENO2	1	enzyme in glycolitic pathway found in mature neurons and cells of neuronalorigin.	only in nervous system	RNA
34	DRPLA	1	involved in DRPLA, has a CAG repeat	ubiquitous	DNA/RNA/hybrids
35	C10/LOC113246	2	cytokine	in CNS	RNA
36	PTPN6	2	src and sh2 domains, tyrosine phosphatases. Suspected to cause severe autoimmune and immunodeficiency syndrome.	expressed in CNS	DNA/hybrids
37	REA	3	repressor of estrogen receptor activity	low in nervous system	DNA/hybrids
38	C2F/NEP1	2	new, similar to S plombe region, optic nerve, esophagus, pharynx expression.	low in nervous system	DNA/hybrids
39	C3F	2	putative protein similar to nessy (Drosophila), homeobox-target gene involved in mesoderm formation	low in brain	DNA/hybrids
40	C1S	2	Complement component C1s, a serine protease involved inmultiple autoimmune diseases	present in CNS	DNA/hybrids
41	C1R	2	new also lupus related, in tandem with C1S duplicated	high in SC	RNA/DNA/hybrids
42	LOC51279/C1RL	2	similar to C1R may be a serine protease; member of the trypsin family, contains extracellular CUB domain,	mainly in head	DNA/hybrids
43	RBP5	3	retinol-binding protein 5, cellular putative cellular retinol-binding protein CRBP III, RETINOL-BINDING PROTEIN I, similar to RAT homologue 55%	not necessarily in NS	DNA/hybrids
44	CLSTN3	1	cadherin domains, high similarity to clstn1 which is a postsynaptic membrane protein with a calcium binding domain.	high CNS expression	RNA/DNA/hybrids

45	PXR1	1	directs proteins with PTS1 signals from the cytosol to the peroxisome, an inability to assemble functional peroxisomes, MR etc early death.	high CNS expression	RNADNA/hybrids
46	GDF3	2	a member of the bone morphogenetic protein (BMP) family, function unknown	high in neuroepithelial cells	RNA
47	APOBEC1	3	B mRNA editing protein is involved in the production of apolipoprotein B (animal model:liver dysplasia,hepatocellular carcinomas)	only in blood and intestines	DNA/hybrids
48	CLECSF11	3	C-type (calcium dependent,carbohydrate-recognition domain) lectin, superfamily member 11, blood dendritic cell antigen 2 protein	only in hepatic parenchymal cells	DNA/hybrids
49	FLJ12581	1	Contains homeobox domains.	in CNS	DNA/hybrids
50	STELLA	3	unknown		DNA/hybrids
51	M160/CD163L	3	scavenger receptor cysteine-rich type 1 protein M160 precursor	not in CNS nor leukocytes	DNA/hybrids
52	CD163	3	macrophage-associated antigen	not in CNS nor leukocytes	DNA/hybrids
53	AY151139	3	unknown	unknown	DNA/hybrids
54	AK056500	3	unknown	unknown	DNA/hybrids
55	BC011980	3	unknown	unknown	DNA/hybrids
56	NANOG	3	homeobox transcription factor	not in CNS	DNA and RNA
57	DKFZp547D2210	3	unknown	high in CNS	DNA/hybrids
58	AL833364	3	unknown	low in brain	DNA/hybrids

* Priority at the start of the study, 1 signifies high priority
** NT: not tested
¶ These genes are outside of the smallest candidate interval

The best candidates were screened first and consisted of genes highly expressed in the nervous system and/or with functional relevance to HSP. These included DRPLA, USP5, GAPD, CLSTN3, and VAMP1. The candidate gene list also includes mRNAs of unknown function with incomplete open reading frames. We chose to screen candidate genes by automated sequencing of genomic DNA or cDNA. For the latter, total RNA was extracted from lymphoblasts and cDNA was generated by RT-PCR using random and poly dT primers. To improve our mutation detection efforts, concurrently with the gene screening process we have also sent lymphoblasts from a patient, R12615 (family R0071), to a company (GMP genetics Inc., Waltham) for isolation of chromosome 12 by cell hybrid technology. The isolation of the chromosomes allows us to sequence and compare the normal and mutated alleles of genes separately and more efficiently (Papadopoulos et al. 1995). The fragments for each gene were amplified in one affected member each of families R0013 and R0071, in the hybrids and in one control sample. Sequence analysis was performed with the program Seqman (DNAstar package) and by visual inspection of traces. All identified unknown segregating variants were tested for their presence in control individuals by sequencing analysis. We have a collection of 40 Newfoundland and 200 Caucasian controls in the laboratory for variant validation. Several interesting variants required follow-up in controls (Table 3), but were eliminated as causative mutations after being detected in control samples. As more sequencing data became available, some of these variants were added to the dbSNP database. Unfortunately, after candidate gene analysis of all known genes and mRNAs known to the database in our interval, no mutation was identified.

Table 3: Interesting variants identified at the *SAX1* locus.

Variant	Gene	Location	Controls	Comments
CODING				
AAC>AAT, N150N	CLSTN3	nt 991, exon 3	17	in 2 controls: C/T, only in mRNA of affected hyb 42
CGT>CAT, R119H	C1S	exon 3	19	in controls: 3 A/A and 2 G/A, rs12146727 only in one screened affected sample
CGA>CAA, R84Q	GRCA	nt 4607, exon 4	5	present in 1 control, only in 1 tested affected individual
G286A, G564A	GRCA	nt 5512, exon 5	106	1 htz spouse 40 Newfoundlanders, 96 Caucasians
G/A, V797M	CNAP	nt 3188, CNAP_8	0	in control and 1 patient, rs10849482
G>A, P83P	NTF3	nt 424	19	in controls: 6 htz, 3 hmz A/A
G>A, K248K	ING4	nt 12373, exon 7_8	18	in controls: 2 G/A, synonymous
S145S	ING4	nt 10847 C>T, exon 5	18	in controls: 1 C/T, 1 T/T, only in 1 tested affected individual
INTRONIC				
IVS11-170insT	C1S	C1Sx11a	20	absent in controls, only in 1 tested affected individual
3'UTR	LTBR	nt 7367 G/T	13	in controls 6 G/T, rs12354

htz: heterozygote
hmz: homozygote
nt: nucleotide

There are several explanations for the fact that the causative mutation remains unknown. The most likely explanation is that the detection efficiency of our study was not 100%. All sequence traces should be reviewed where possible by using a different sequence

analysis program, such as mutation surveyor, and should also be assessed by a different person. Errors in the establishment of the critical candidate interval are often the reason for not finding the disease gene. Additional SNPs should be typed to confirm the size and borders of the region, but this is an unlikely explanation. Due to time and financial limitations not all genes were screened by RNA, DNA and hybrid analysis and this could be revisited in the future.

Another limitation to this study was that our candidate gene analysis only included the coding region and not always the UTR sequences. Studies have shown the importance of 5'and 3' UTRs in gene function. miR-134, for example, is a brain-specific micro RNA that inhibits translation of the lim domain containing protein kinase 1 (LIMK1) by interacting with its 3'UTR (Schratt et al. 2006). These UTR regions should therefore be completely screened, alternatively the protein quantity of all good candidates could be studied by Western blot analysis. However, the antibodies to all these proteins are not always readily available.

The mutation can also lie in a region that has not yet been annotated as a functional gene or part of a gene. Many examples of difficulties in gene finding due to this reason have been reported. Progressive myoclonus epilepsy, for instance, is caused by mutations in an atypical single-exon gene of 1180 bp that showed some homology with other vertebrates (Chan et al. 2003).

In one type of Usher syndrome 51 additional exons were identified after Northern Blot analysis indicated the existence of additional splice forms (van Wijk et al. 2004). This

possibility can be pursued by searching the candidate region for EST's and cDNAs of other vertebrate, although the region is already quite gene-dense.

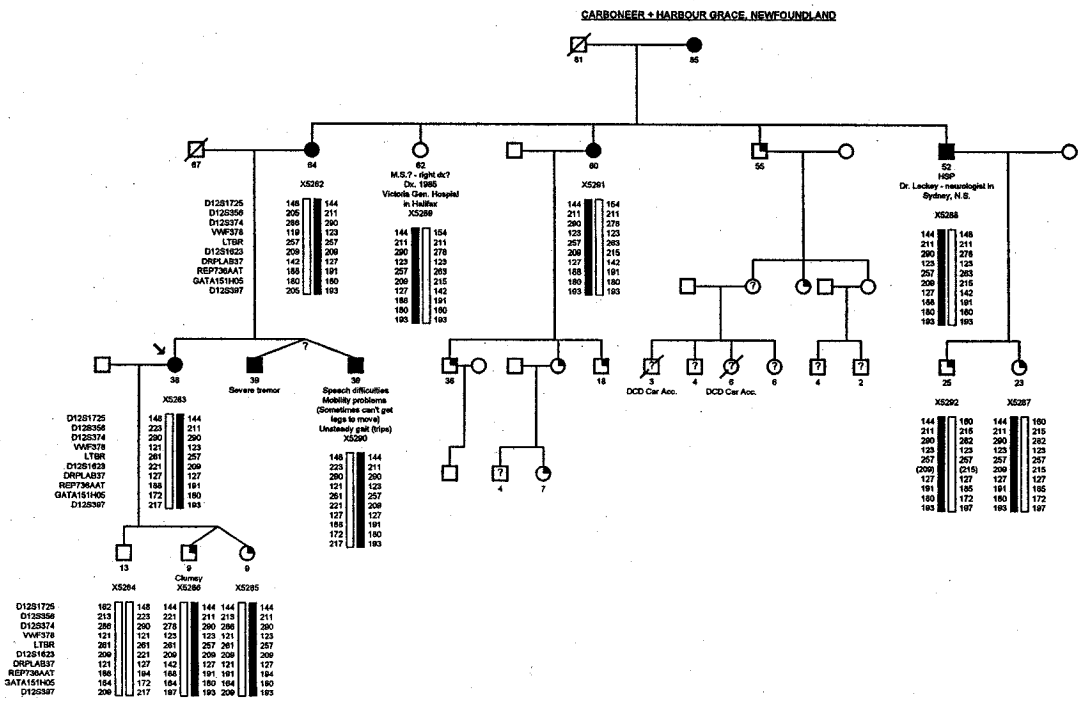
Finally, there is a possibility of a duplication mutation. We have detected a rare sequence gap in the region flanked by the *C1R* gene. The region is also incompletely sequenced in the mouse and chimpanzee. The difficulty in sequencing this region might be due to a repetitive sequence. In support of a duplication event in the region, the candidate region contains a few genes that are very similar and likely arose from gene duplication, *C1R* and *C1RL*, *M160* and *CD163*. Long-range PCR or large fragment electrophoresis could be performed to determine whether there is a difference in size between patients and controls in our candidate region. In addition, long-range PCR could also aid in determining the size of the gap. A more recent technique would be to perform Comparative Genomic Hybridization to detect copy number variants (NimbleGen Systems, Madison, WI).

6.4.2 The search for additional alleles

The search for the *SAX1* gene is complicated by the fact that the candidate region is extremely gene rich and that there is only one disease-causing mutation in our families. In an attempt to find additional alleles, we contacted several neurologists to obtain additional spastic ataxia families. So far, eight additional spastic ataxia families (FSPAT1,2,3,4,5,6,7,8) have been recruited. Five families from English-Canadian (FSPAT1), French Canadian (FSPAT3), Portuguese (FSPAT4), Australian (FSPAT5) and Nova Scotian (FSPAT6) origin were genotyped for several markers in the *SAX1* region. Unfortunately, of the eight additional families collected, FSPAT2 (French Canadian) was too small to be analysed by haplotype construction and analysis for FSPAT7 and 8 has not been completed. This haplotype analysis enabled us to exclude linkage to the *SAX1* locus in families FSPAT 1, 3, 4, and 5. Interestingly, the maternal grandmother in the Nova Scotia family was originally from Carbonear, Newfoundland (Figure 2). It was therefore not surprising when results showed that this family shares a large part of the haplotype seen in the other 3 Newfoundland families. The clinical picture of FSPAT6 is variable as reported in the other Newfoundland families, although dystonia and supranuclear gaze palsy seem to be more prominent in this family. Albeit genealogically interesting that these families share such a large chromosomal region (they are unaware of their relationship), it also prevented us from finding any recombinants.

The exclusion of linkage to the *SAX1* locus in most FSPAT families suggests that there is genetic heterogeneity for dominant HSA. Our results also imply that the *SAX1* locus seems to be specific to the Newfoundland population.

Figure 2: Pedigree of the FSPAT6 family from Carbonear, Newfoundland.



In conclusion, the search for the *SAX1* mutation is still ongoing. Once the gene is identified, functional studies will ensue. Depending on the availability of data about the protein involved, future experiments will be determined. Molecular characterization of this protein should shed light on the variable phenotype and on a link with the other HSP proteins.

CHAPTER 7: IDENTIFICATION OF THE *SPG8* GENE

Submitted to *American Journal of Human Genetics (AJHG)* in August, 2006

Reference:

Paul N. Valdmanis *, Inge A. Meijer *, Annie Reynolds, Adrienne Lei, Patrick MacLeod, David Schlesinger, Mayana Zatz, Evan Reid, Patrick Dion, Pierre Drapeau, Guy A. Rouleau. Mutations in the *KIAA0196* gene at the *SPG8* locus cause Hereditary Spastic Paraplegia. 2006.

* These two authors contributed equally to this work.

7.1 RATIONALE

Previous work in our lab had established linkage of a large U.S. family to the *SPG8* locus (Reid et al. 1999b) (unpublished data). The candidate interval was too large to pursue positional cloning at that time. By applying the same strategy of focusing on large ADHSP families as previously mentioned, we identified a second large Canadian family that linked to the *SPG8* locus. The presence of 2 disease alleles as well as the reduced candidate interval made the study feasible and interesting for candidate gene analysis. Fine-mapping, candidate gene analysis, gene identification and mutation validation at the *SPG8* locus is presented in chapter 7. This chapter also includes haplotype analysis of additional ADHSP families, as well as mutation analysis in probands of spastin- and atlastin-negative samples (genetic validation). In an attempt to biologically validate the mutations identified, a morpholino knock-down zebrafish model was developed.

7.2 ABSTRACT

Hereditary Spastic Paraplegia (HSP) is a progressive upper motor neurodegenerative disease. The eighth HSP locus, *SPG8*, is on chromosome 8p24.13. The three families previously linked to the *SPG8* locus present with relatively severe, pure spastic paraplegia. We have identified three mutations in the *KIAA0196* gene in five families linked to the *SPG8* locus. One mutation, V626F, segregated in three large North American families with European ancestry. An L619F mutation was found in a Brazilian family. The third mutation, N471D, was identified in a smaller family of European origin, and lies in a spectrin domain. None of these mutations were identified in 300-500 control individuals. Both the L619 and V626 residues are strictly conserved across species and likely have a notable effect on the structure of the protein product, strumpellin. Rescue studies with human mRNA injected in zebrafish treated with morpholino oligonucleotides to knockdown the native gene showed that mutations at these two residues impaired the normal function of the *KIAA0196* gene. However, the function of the 1159 amino acid strumpellin protein is relatively unknown. The identification and characterization of the *KIAA0196* gene will enable further insight into the pathogenesis of HSP.

7.3 INTRODUCTION

Hereditary Spastic Paraplegia (HSP) has a worldwide prevalence between 1-18 in 100,000 (Polo et al. 1991; Silva et al. 1997; McMonagle et al. 2002) and is characterized by central motor system deficits leading to lower limb spastic paraperesis (Harding 1983; Harding 1993b; Fink 1997). This is due to a "dying back" phenomenon whereby upper motor neurons degenerate progressively, commencing with the longest axons (Behan and Maia 1974; Deluca et al. 2004). HSP can be classified into pure and complicated forms (Harding 1983). In pure HSP, lower limb spasticity is the only major presenting symptom. Alternatively, in complicated HSP, this spasticity can be accompanied by other neurological or non-neurological symptoms such as ataxia, dementia, mental retardation, deafness, epilepsy, ichthyosis, retinopathy, ocular neuropathy and extra-pyramidal disturbances (Harding 1983; Soderblom and Blackstone 2006). There is clinical heterogeneity within families, where age of onset and severity can differ markedly; between families that map to the same locus; and certainly between families which map to separate loci. This complicates genotype-phenotype correlations for HSP.

HSP is also extremely genetically heterogeneous. Eleven genes have been identified out of over 30 loci mapped (*SPG1-31*). This disease can be transmitted in a dominant (12 loci), a recessive (14 loci) or an X-linked manner (3 loci) (Klebe et al. 2006a; Soderblom and Blackstone 2006; Zuchner et al. 2006a). By far the most common locus for the disease is *SPG4*, with mutations in the microtubule severing protein spastin

accounting for ~40 percent of dominant HSP cases (MIM604277) (Hazan et al. 1999; Meijer et al. 2002c).

SPG8 is considered to be one of the more aggressive subtypes of HSP with disease onset occurring for patients as early as their 20s or 30s. It was first identified in a Caucasian family as a 6.2 cM region between the markers D8S1804 and D8S1774 (Hedera et al. 1999). The family contained 15 patients affected with spasticity, hyperreflexia, extensor plantar reflexes, lower limb weakness, decreased vibration sensation and limited muscle wasting. The candidate region was further reduced to 3.4 cM due to a lower recombinant in a second family, narrowing the interval between markers D8S1804 and D8S1179 (Reid et al. 1999b). This family as well as a third Brazilian family linked to *SPG8* also presented with pure adult onset HSP (Rocco et al. 2000). For two of the families, a muscle biopsy was performed (Hedera et al. 1999; Rocco et al. 2000); however, no gross histological or histochemical abnormalities were observed. Ragged red fibers have been observed in muscle biopsies of HSP patients with paraplegin mutations (Casari et al. 1998).

In this study, we identified four additional families which are linked to the *SPG8* locus. Upon gene screening in an expanded candidate *SPG8* locus defined by our collected families, we identified three point mutations in the *KIAA0196* gene encoding the strumpellin protein product (name pending HUGO approval) in the Brazilian family and our four *SPG8*-linked families.

7.4 MATERIAL AND METHODS

Subjects

Protocols were approved by the ethics committee of the CHUM. Patients gave informed consent after which patient information and blood was collected. DNA was extracted from peripheral blood using standard protocols.

Genotyping and locus exclusion

PCR amplified fragments incorporating α-^{35}S-dATP were resolved on 6% denaturing polyacrylamide gels. Alleles were run alongside an M13mp18 sequence ladder and scored based on allele sizes and frequencies from the Fondation Jean Dausset CEPH database. LOD score calculations and multipoint analysis were performed using the MLINK program of the LINKMAP software package (Cottingham et al. 1993a).

Mutation Screening

The 28 exons of *KIAA0196* were screened by automated sequencing, including at least 50bp of each intronic region. Primers were designed using the PrimerSelect program (Lasergene) and were synthesized by Invitrogen Canada Inc. Primers for exon 11 are 5'-TGCTCCAGGCATTTTTGTCG-3' (forward) and 5'-GAACAGACTGCTGGGTGGGTCATA-3' (reverse). ASO primers for exon 11 are 5'-ACTAGAAAACCTTCAAGCT-3' (normal) and 5'-ACTAGAAGACCTTCAAGCT-3' (mutated). Exon 14 primers are 5'-TTTGCATGTCTAATAAGTTCCCAGGTC-3' (forward) and 5'-TTTGCATGTCTAATAAGTTCCCAGGTC-3' (reverse) with ASO primers of 5'-GGAGAGTTGGTATC-3' (normal) and 5'-GGAGAGTTCGTATC-3' (mutated). For

the exon 15 change, primers used were 5'-TTTGCAGCATTTTTAGAAGGATTAGC-3' (forward), and 5'-TTCCCCTGAGAATACTGAGGCGAACA-3' (reverse), with ASO primers of 5'-CACTGAAGGTTTTG-3' (normal) and 5'-CACTGAAGTTTTTG-3' (mutated). Primer sequences for the remaining 25 exons will be provided upon request.

Variants were first tested in 12 controls by sequencing, followed by allele-specific oligomerization (ASO) (Labuda et al. 1999; Bourgeois and Labuda 2004). Briefly, 4μl of PCR product was hybridized onto Hybond-N+ Nylon membranes (Amersham Biosciences) using a dot blot apparatus. $^{P-32}$-labelled probes specific to the mutation or normal sequence were hybridized then visualized on autoradiographic film after overnight exposure.

Protein sequence alignment

Cluster analysis was performed using the Probcons v. 1.09 program. Proteins from aligned species included *Homo sapiens* (Q12768), *Canis familiaris* (XP_532327), *Pan troglodytes* (XP_519952), *Drosophila melanogaster* (CG12272), *Caenorhabditis elegans* (CE13235), *Xenopus tropicalis* (MGC89323), *Rattus norvegicus* (XP_343250) *Danio rerio* (BC045490), *Gallus gallus* (XP_418441), *Dictyostelium discoideum* (EAL63144), and *Mus musculus* (NP_705776.2).

Homology modeling

The size of the strumpellin protein (1159aa) made it prohibitive to obtain a template for the entire protein. Instead, 200 amino acids around the two mutations were selected (aa 501-725) and inputted in the Phyre program version 2.0. The template with the highest

score was selected, namely 1dn1b from the Neuronal-Sec1 Syntaxin 1a complex. The SwissProt database viewer version 3.7 (Guex and Peitsch 1997) was used to visualize the model concentrating on the alpha helix in which the two mutations lie and on a second alpha helix nearby in 3D space (fig. 5). Peptides incorporating one or the other identified point mutation were visualized in the same manner.

Expression studies

Northern Blot and RT-PCR analysis

A 1 kb probe specific to the c-terminal region of strumpellin was generated by digesting KIAA0196-pBluescript vector with XhoI and NotI. 30 ug of total RNA per sample was loaded. RNA was extracted from various regions of the brain of a control individual. A reverse-transcriptase reaction was performed using MMLV-RT (Invitrogen). Primers in exons 10 (Forward) and 15 (Reverse) of *KIAA0196* were used. *GAPD* cDNA was amplified as a control.

Constructs

The *KIAA0196* cDNA pBluescript clone was kindly provided by the Kazusa DNA Research Institute. Each mutation was introduced into the clone by site-directed mutagenesis using the primers 5'- CTGGAGAGTTCGTATCCTATGTG – 3' for the exon 14 variant and 5' – CCTATGTGAGAAAATTTTTGCAGATC – 3' for the exon 15 variant, along with primers of their complementary sequence. Wildtype and mutant *KIAA0196* cDNAs were cloned into a pCS2 vector and transcribed in vitro using the SP6 mMESSAGE mMachine kit (Ambion) for zebrafish studies.

Zebrafish knockdown studies

Morpholino injections

Wildtype zebrafish were raised and mated as previously described (Westerfield 1995). Antisense morpholinos (AMO) were designed and purchased from Genetools LLC (Philomath, Oregon). The morpholino sequences were designed against the zebrafish strumpellin ortholog, BC045490. The oligonucleotide, CTCTGCCAGAAAATCAC[CAT]GATG (KIAA MO) binds to the ATG of the *KIAA0196* gene and CTCTcCCAcAAAATgAg[CAT]cATG (CTL MO) is a five basepair mismatch control. AMO injections were performed as previously described at a concentration of 0.8 mM (Nasevicius and Ekker 2000). The rescue injections were performed as mentioned above with a morpholino and mRNA concentration of 0.8 mM and 50 ng/ul respectively.

Immunohistochemistry

Standard protocols were used for immunohistochemistry (Westerfield 1995). Briefly, three day old embryos were fixed in 4% paraformaldehyde, washed, and blocked at room temperature. Primary antibodies [anti-acetylated tubulin, 1:50 (Sigma)] were added overnight. After extensive washing, the embryos were incubated with the fluorescently labelled secondary antibody Alexa 568 (Molecular Probes). Imaging was performed on an UltraView LCI confocal microscope (Perkin Elmer) using Methamorph Imaging software (Universal Imaging Corporation). The statistical significance between the different conditions was calculated using a chi square test.

7.5 RESULTS

Clinical information and family details

The *SPG8* family FSP24 is from the province of British Columbia, Canada. It is composed of 49 members, 12 of which are affected with a spastic gait and lower limb stiffness (fig. 1A). Symptoms were first observed in individuals between the ages of 35 and 53. Intrafamilial phenotypic heterogeneity exists as noted by the symptoms presented and the range in disease severity in patients. Deep tendon reflexes were brisk or increased, and decreased vibration sensation was also noted in three patients. Occasional bladder control problems were also observed. Walking aids were required for some individuals while one is confined to a wheelchair. Together, these features are consistent with a pure, uncomplicated HSP similar to that described for other families linked to the *SPG8* locus.

Family FSP29 is of European descent residing in the United States. There are 31 affected individuals in the family, and 10 have been collected (fig. 1B). Age of onset was quite variable with symptom onset ranging in patients from their twenties to their sixties. The family was negative for mutations in the spastin gene. FSP34 is originally from Great Britain, residing in Canada. The family has 9 affected individuals, 3 of whom have been collected (fig. 1C), and again is spastin-negative. Onset of symptoms occurred in the fourth or fifth decade for patients, and they occasionally presented with bladder urgency. Family FSP91 is originally from Europe, collected in the United States and contains 3 affected members with onset in their twenties. The fifth family is from Brazil, of Caucasian ancestry, with 16 affected members affected with pure spastic paraplegia of adult onset (Rocco et al. 2000).

Linkage analysis

For FSP24, seven markers spanning the *SPG8* locus from markers D8S586 to D8S1128 were genotyped in all individuals collected. A disease haplotype segregated with the disease in all affected individuals and no genotyped unaffected individuals contained any portion of the disease haplotype (table 1). A recombination event occurred in individual III:6 between makers D8S586 and D8S1804 defining the upper border of the locus in this family. No lower recombinant was identified nor searched for since the haplotype extended beyond the limits of the *SPG8* locus. The maximum LOD score for this family was 3.43 at $\theta = 0$ using CEPH allele frequencies for the marker D8S1804.

Family 29 included many informative recombination events. The upper recombinant occurred between markers D8S1799 and D8S1832 in three individuals (Fig. 1B, V:10, V:11 and VI:5), and the lower recombinant was between markers D8S1179 and D8S1128. This yielded a candidate interval of 3.15 Mb. The maximum LOD score for this family was 5.62 ($\theta = 0$) for the marker D8S1179 when using CEPH allele frequencies. Multipoint analysis was also conducted for this family in this region yielding a maximum LOD score of 6.73, 0.5 cM centromeric to the D8S1128 marker.

Gene screening

The previously published *SPG8* locus spanned 3.4 cM (1.04 Mb) between markers D8S1804 and D8S1179 on chromosome 8q23-8q24. We screened all 9 annotated genes in this candidate region as annotated in the UCSC human genome browser May 2004 update along with many clustered ESTs and mRNAs that aligned to the locus without detecting a mutation. Therefore, we opted to redefine the candidate region based on the

critical interval determined by an upper recombinant in our FSP29 family at the marker D8S1832 and a lower recombinant at D8S1774 was based on published data (fig. 2A) (Hedera et al. 1999). This increased the size of the region to 5.43 cM (3.15 Mb), which contains 7 additional known genes (fig. 2B). These additional genes were screened and three mutations were identified in the *KIAA0196* gene (fig. 2C).

Mutation analysis

A valine-to-phenylalanine mutation was identified in amino acid 626 for families FSP24 and FSP29 (p.V626F) (fig. 3A). All affected individuals collected from each family (8 and 11 respectively) were screened and were positive for this mutation. This G to T nucleotide change is at position 1956 of the mRNA (NCBI accession NM_014846.2). A total of 500 ethnically matched control individuals were negative for this mutation after screening by a combination of allele-specific oligomerization (ASO) and sequencing. No unaffected members and spouse controls in any family were positive for the mutations.

A second mutation was identified in the Brazilian family in exon 14, a G to C transition at position 1937 of the mRNA (fig. 3B). This leucine-to-phenylalanine change (p.L619F) is only 7 amino acids away from the V626F mutation. It was also not found in 500 controls using ASO.

The *KIAA0196* gene was screened in probands from 24 additional dominant HSP families that are negative for mutations in both spastin and atlastin, resulting in the identification of two more families with missense mutations in the *KIAA0196* gene.

FSP34 has the same p.V626F change in 3 affected members. Haplotype analysis of this family with markers D8S1804, D8S1179, D8S1774 and D8S1128 indicated that there is allele sharing between this family and family FSP29 suggesting an ancestral haplotype (table 1). An additional mutation was found in three affected siblings of another family. This c.A1491G transition results in an asparagine to aspartate amino acid change (p.N471D), and is not present in over 300 controls tested (fig. 3C).

Mutated amino acids at positions 619 and 626 are strictly conserved across all eleven species examined all the way to the social amoeba, *Dictyostelium discoideum* (fig. 3D). Indeed, the entire region surrounding these two mutations appears to be functionally relevant for the protein as 73 consecutive amino acids (aa 576-649) are 100% identical between the human, dog, chicken, mouse, rat and orangutan. Position 471 is conserved across all species save for *Drosophila melanogaster* with a glutamine residue and *Xenopus tropicalis* with a histidine.

The exon 15 mutation is in the very first nucleotide of the exon, which leads to the speculation that the splicing of this exon might be compromised in these families. The *KIAA0196* gene was expressed within all regions of the brain examined by RT-PCR (fig. 3E). There are no alternative splice isoforms detected in control brain samples and the patient whole blood samples by RT-PCR and Northern analysis (fig. 3E, 3F). In addition, both normal and mutant alleles were observed in cDNA analysis of the patients.

KIAA0196 profile

The *KIAA0196* gene spans 59.7 kilobase pairs of genomic DNA, is 28 exons long and codes for a protein of 1159 amino acids that we have named *SPG8*. The gene is expressed ubiquitously, including in the brain (fig. 3E). The EBI institute's InterPROScan program predicts a spectrin-repeat containing domain from amino acids 434 to 518. Thus, the mutation at position 471 may abrogate the binding of the spectrin domain with other spectrin-repeat containing proteins. In examining the secondary structure using PSIPRED (Jones 1999), 74% of the protein is considered to be alpha-helical. The program further predicts an alpha helix in the protein from amino acids 606 to 644, encompassing the two other mutations which have been identified.

Homology modeling

Given the high proportion of *KIAA0196* considered to be alpha-helical, it is not surprising that the optimal homology modeling candidates are similar in secondary structure composition. This is true for 1dn1b, a stat-like t-SNARE protein neuronal-Sec1 Syntaxin 1a complex. This is the most appropriate model for strumpellin according to the Phyre program. The two mutated residues lie within an alpha-helix from amino acids 619-628 which is in close 3D proximity to another alpha-helix from residues 665-670 (fig. 4A). A mutation in either Val-626 or Leu-619 to a phenylalanine residue would appear to have significant structural implications given the change in bulkiness between each of the residues. In addition, Tyr-622 points in the same direction from the alpha-helix residue. To have two amino acids with aromatic rings in such physical proximity could force apart the alignment of the two alpha-helices or induce alterations in the alpha-helix backbone.

Zebrafish rescue experiments

In order to validate the functional phenotype of the *SPG8* mutations *in vivo*, we developed a zebrafish model. Morpholino oligonucleotide knockdown of the *KIAA0196* ortholog in zebrafish (KIAAMO) resulted in an enlarged heart cavity along with a curly tail phenotype which severely impaired the ability of the fish to swim properly. The overall phenotype ranged in severity and was classified in 3 major groups: normal, slightly curly, and severely curly. This phenotype was clearly visible after dechorionating by 1 day post fertilization (dpf). At 3dpf, wildtype zebrafish are ~5 mm long with a straight tail (fig. 5A). Fish injected with a mismatch-control morpholino (CTLMO) were initially used to titer a KIAAMO specific non-toxic injection dose (fig. 5B). Injection of the KIAAMO resulted in 66 of 178 (37%) severely curly fish and 50 of 178 (28%) slightly curly fish (table 2; fig. 5C, D). The KIAAMO fish had a significantly different distribution of phenotypic groups compared to CTLMO injections ($p<0.001$). When wildtype human *KIAA0196* mRNA was co-injected with KIAAMO, the curly tail phenotype was rescued to levels comparable to CTLMO injections ($p= 0.51$) (fig. 5E, F). This suggests that in zebrafish, human *KIAA0196* mRNA can compensate for the loss of endogenous zebrafish mRNA. Conversely, co-injection of human *KIAA0196* mRNA incorporating either the exon 14 or exon 15 mutation failed to significantly rescue the phenotype (fig. 5G, H). Injection of mutant exon 14 or exon 15 mRNA alone (without morpholinos) did not lead to a curly tail phenotype, suggesting that the two mutations do not exert a dominant-negative effect. The exon 11 mutation was not tested for functionality in zebrafish, but has been genetically validated using controls and segregation analysis. Approximately 200 embryos were injected per experimental

condition (table 2). The difference in distribution between KIAAMO injection alone and KIAAMO co-injection with wildtype mRNA was significant ($p<0.001$). Similarly, co-injection of wild type mRNA versus either exon 14 or exon 15 mutant mRNA was significantly different with a p-value <0.001. There was no statistical difference between the co-injection of the exon 14 mutant and the exon 15 mutant ($p=0.10$). Upon histochemical analysis of the embryos using an anti-acetylated tubulin stain for growing axons, we found that the motor neurons in the spinal cord did not develop normally (fig. 6). Motor neuron axons in fish injected with KIAAMO alone or with the mutant mRNAs were shorter and showed abnormal branching. The structure of interneurons in the spinal cord was also different. The absence of the *KIAA0196* gene or mutations in this gene during early development thus seemed to hamper axonal outgrowth.

7.6 DISCUSSION

HSP is one of the most genetically heterogeneous diseases, caused by mutations in at least 31 different genes. This means that >0.1% of genes in the human genome can be mutated resulting in one predominant neurological outcome: the degeneration of upper motor neuron axons. This heterogeneity may in part explain why it was originally difficult to identify the *SPG8* gene leading to an expansion of the candidate interval. It is possible that two spastic paraplegia genes exist on chromosome 8q23-24, and the overlap of linkage results from both loci yielded a region between the two causative genes. This is similar to the *SPG33* gene *ZFVE27* which is in close proximity to the *SPG9* (MIM 601162) and *SPG27* (MIM609041) loci (Mannan et al. 2006b). Alternatively, one originally reported family may have had a false positive linkage result to chromosome 8. The families described in this study display convincing evidence of linkage to the *SPG8* locus.

SPG8 is a pure form of hereditary spastic paraplegia with relatively little interfamilial variability in phenotype. Interestingly, two missense mutations were identified in highly conserved amino acids in a predicted α-helix. The helix consists of a heptameric repeat with hydrophobic residues aligning in inaccessible regions at the center of the helix. The hydrophobic lysine and valine amino acids are seven amino acids apart in the protein sequence; thus it is expected they would be buried in the helix, close in 3D space (fig. 5A). When replaced by a bulky phenylalanine residue at either position, the stability of the α-helix could very well be disrupted.

The one known domain in strumpellin is a spectrin repeat which consists of three α-helices of a characteristic length wrapped in a left-handed coiled coil (Djinovic-Carugo

et al. 2002). The spectrin repeats appear in the spectrin/dystrophin/α-actinin family. The spectrin proteins have multiple copies (15-20) of this repeat which can then form multimers in the cell. Spectrin also associates with the cell membrane via spectrin repeats in the ankyrin protein. Likewise, four spectrin repeats are found in α-actinin beside two N-terminal calponin homology domains which anchor the complex to actin (Ylanne et al. 2001). This effectively connects the cell membrane with the actin cytoskeletal network. The stability and structure of this network also provides appropriate routes for intracellular vesicular transport, a mechanism already linked to other mutated HSP genes. Proteins with three spectrin repeats or fewer can be considered to have transient association with the spectrin network. The single repeat in strumpellin is more likely to be involved in docking with one of the cytoskeletal spectrin repeats, which could help in protein localization or signal transduction. It will be interesting to determine which protein(s) strumpellin interacts with through its spectrin domain and particularly how the mutation identified at the core of this domain influences this potential interaction.

Proteins with a spectrin repeat have been identified in other neurological disorders, most notably dystrophin, mutated in myotonic dystrophy (MIM300377) (Koenig et al. 1988). The repeat also has been found in a form of cerebellar ataxia (MIM117210) (Ishikawa et al. 2005). β-III spectrin itself is found to be mutated in SCA5 (Ikeda et al. 2006). While none of the genes mutated in HSP have a spectrin domain, L1CAM (*SPG1*) has an indirect association (Jouet et al. 1994; Soderblom and Blackstone 2006). L1CAM is a single-pass transmembrane protein with a glycosylated extracellular component which facilitates the outgrowth and migration of neurons in the corticospinal tract. The

intracellular C-terminus however binds to the spectrin-repeat containing protein, ankyrin, linking the cell membrane to intracellular spectrin. Thus, strumpellin with its spectrin domain may also be involved in this process.

The only details known about the human *KIAA0196* gene so far is that it has previously been implicated in prostate cancer (Porkka et al. 2004). An increase in gene copy number was assayed by real-time quantitative PCR and fluorescence *in-situ* hybridization, determining over ten-fold overexpression of the gene in PC-3 prostate cancer lines, and in ~1/3 of advanced prostate cancers examined (Porkka et al. 2004). How this relates to a spastic paraplegia phenotype is not clear.

Analysis of other species has provided some insight into a potential function for *KIAA0196*. A 118 kDa homologue of the strumpellin protein was identified as part of a TATA-binding protein-related factor 2 (TRF2) complex in a *Drosophila* nuclear extract (Hochheimer et al. 2002). Eighteen proteins were pulled down along with TRF2 in this complex including NURF, and SWI, with functions for chromatin remodeling and transcription activation. TRF2 is selective for promoters lacking TATA or CAAT boxes. One protein of the complex is DREF which binds to DRE elements common in controlling genes involved in cell cycle regulation and cell proliferation (Hirose et al. 1993; Shimada et al. 2003).

With little known about the function of *KIAA0196*, we decided to test the functionality of the missense changes with a zebrafish knockdown model. Interestingly, the KIAAMO injected fish showed a severe tail phenotype characterized by abnormal motor

neuron outgrowth in the spinal cord. The knocked-down zebrafish resemble other mutants affecting midline development (Brand et al. 1996). Injection of wildtype human *KIAA0196* mRNA concurrently with zebrafish KIAAMO knockdown rescued the phenotype to values not significantly different from a control morpholino injection. However, co-injecting human *KIAA0196* mRNA containing either the exon 14 or exon 15 mutation yielded a phenotype comparable to injection of KIAAMO alone. These experiments demonstrate the importance of the *KIAA0196* gene in early development of zebrafish and suggest that the two missense mutations impair the normal function of strumpellin. Further characterization of the *KIAA0196* knockdown phenotype is necessary to better understand the role of this gene in early fish development, more precisely in motor neuron outgrowth.

The identification of *KIAA0196* as the gene mutated in *SPG8* adds another component to the various genes already identified for the disease. Two missense mutations in a conserved part of the protein have been identified, including one mutation common to 3 families. Additional work will aid in clarifying the function of the protein and how it relates to other proteins implicated in HSP, and the overall disease pathogenesis. As more of the genes involved in HSP emerge, the responsible pathways and mechanisms of toxicity will be better understood. This will also help in elucidating the pathophysiology of a related disease, amyotrophic lateral sclerosis, in which the reduced lifespan of patients complicates the cloning of genes by linkage analysis.

7.7 ACKNOWLEDGEMENTS

We sincerely appreciate the co-operation of the families involved in this study. We would like to thank Melanie Benard in patient recruitment, and Daniel Rochefort, Pascale Hince, Liliane Karemera, Judith St-Onge, Kara Melmed, and James Lee for expert technical assistance. PNV, PD and GAR are supported by the Canadian Institutes of Health Research.

7.8 TABLES

Table 1. Haplotype comparison between SPG8 linked families

Marker	Position (Mb)	FSP24	FSP29	FSP34
D8S586	121.2	1	11	11
D8S1804	124,8	NT	3	3
D8S1832	125,4	2	2	NT
D8S1179	125,9	3	9	9
KIAA0196	126,1	L619F	L619F	L619F
rs2293890	126,4	G	C	C
D8S1774	127,5	3	5	4
D8S1128	128,5	7	5	1

The candidate interval for each family is boxed. NT = not typed

Table 2. Phenotype profile from zebrafish morpholino oligonucleotide knockdown expressed in percent (total number)

Condition	Normal	Slight curve	Severe curve	Dead	Total
KIAA0196 morpholino	19.1 (34)	28.1 (50)	37.1 (66)	15.7 (28)	178
Control morpholino	56.1 (83)	24.3 (36)	7.4 (11)	12.2 (18)	148
Wildtype rescue	63.2 (127)	19.4 (39)	8.0 (16)	9.5 (9)	201
Mutant x14 rescue	16.0 (32)	37.0 (74)	36.0 (72)	11.0 (22)	200
Mutant x15 rescue	13.2 (29)	37,4 (82)	30.1 (66)	19.2 (42)	219

7.9 FIGURES

Figure 1.

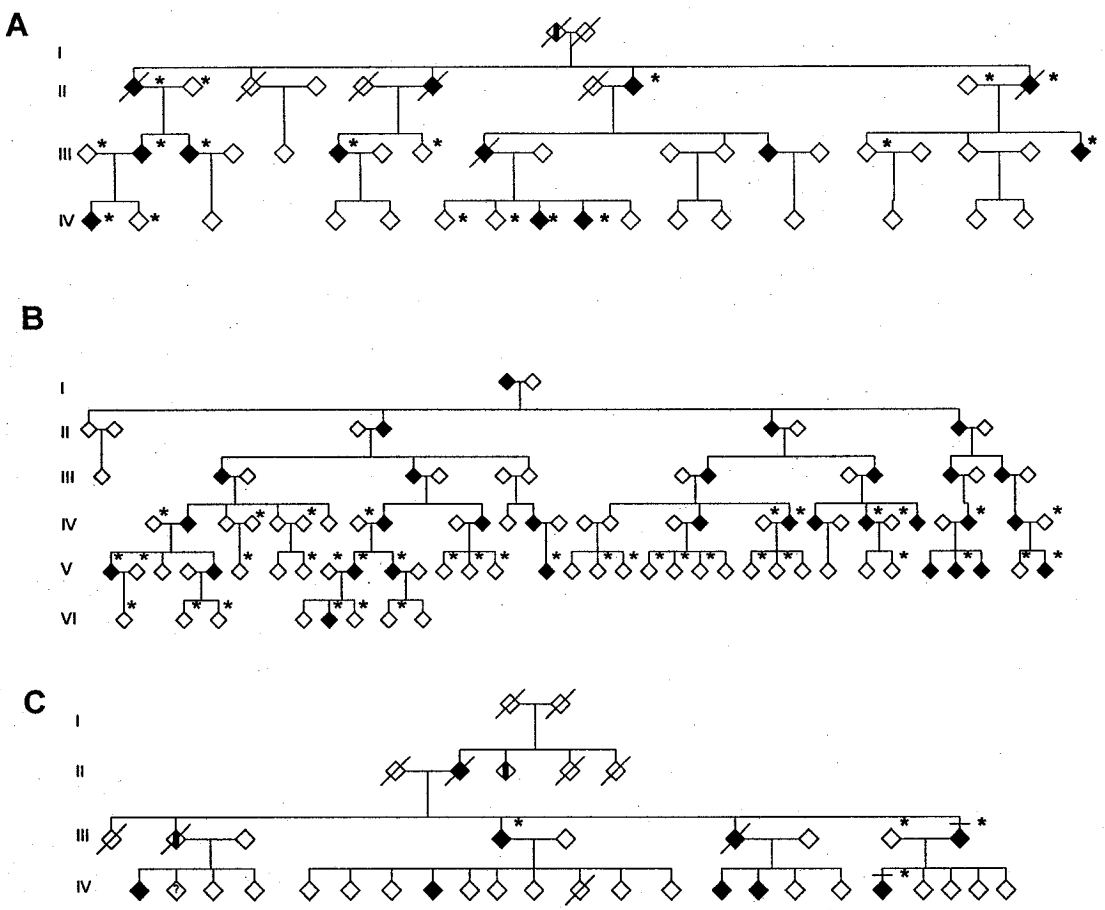


Figure 1

Pedigrees for families with *KIAA0196* mutations. A) Family FSP24, B) Family FSP29, C) Family FSP34. Blackened boxes represent affected individuals, and a diagonal line through the symbol means the individual is deceased. A vertical black bar indicates an individual with an unconfirmed phenotype. Sex of each individual has been masked to preserve confidentiality. A star indicates that DNA and clinical information have been collected for the particular individual. All collected affected patients are heterozygous for the c.1956C->T mutation in *KIAA0196* (NCBI accession # NM_014846.2)

Figure 2.

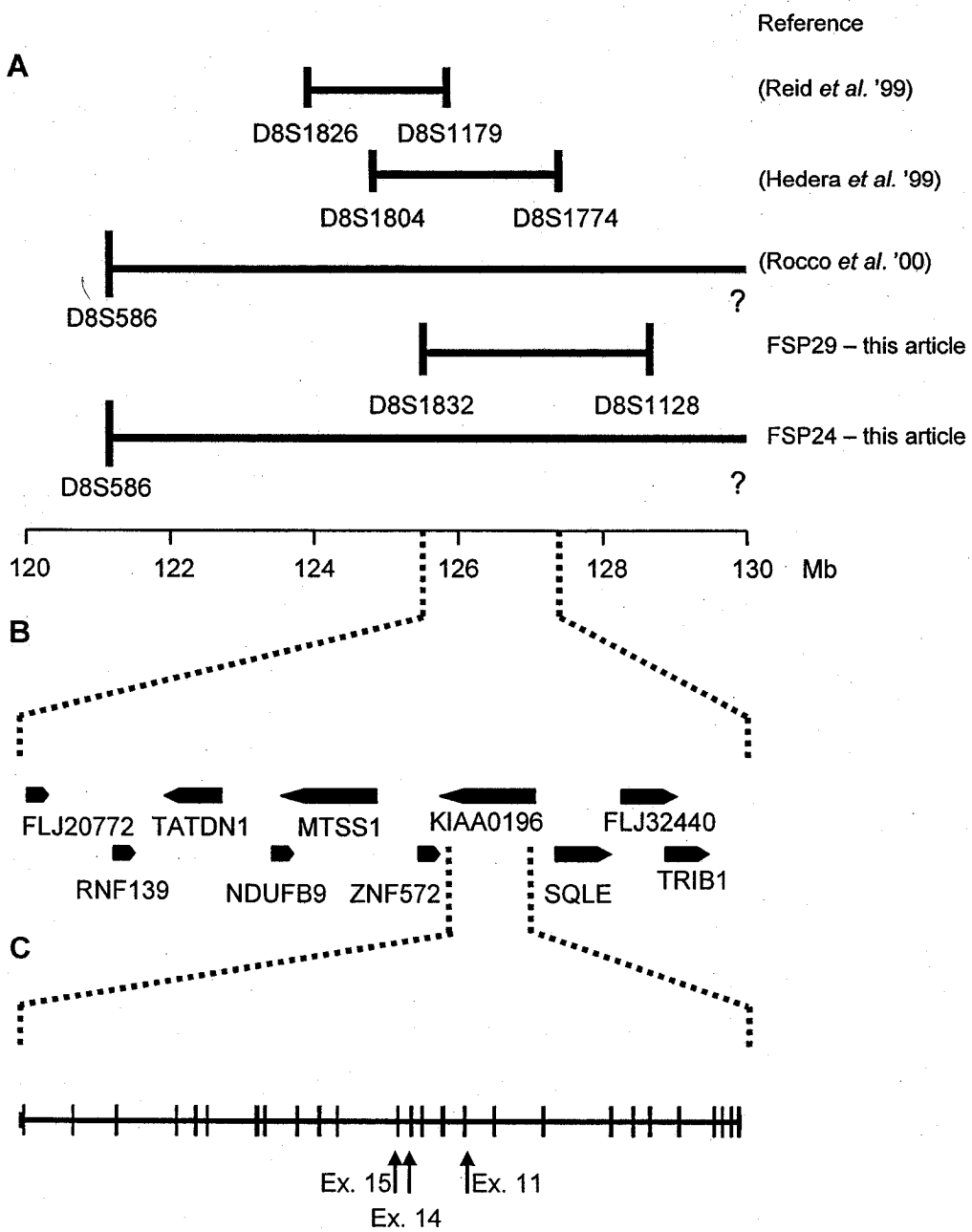

Reference
A
(Reid et al. '99)
D8S1826
D8S1179
(Hedera et al. '99)
D8S1804
D8S1774
(Rocco et al. '00)
D8S586
?
FSP29 – this article
D8S1832
D8S1128
FSP24 – this article
D8S586
?
120
122
124
126
128
130
Mb
B
FLJ20772
TATDN1
MTSS1
KIAA0196
FLJ32440
RNF139
NDUFB9
ZNF572
SQLE
TRIB1
C
Ex. 15
Ex. 14
Ex. 11

Figure 2

Region spanning the *SPG8* locus. A) Markers defining the borders of each described *SPG8* family and their scaled position on chromosome 8q24.13. B) Candidate region used to search for the *SPG8* gene between markers D8S1832 and D8S1774. Genes in the region are shown in their observed orientation. C) The 28-exon *KIAA0196* gene drawn to scale with the location of 3 mutations in exons 11, 14 and 15 highlighted.

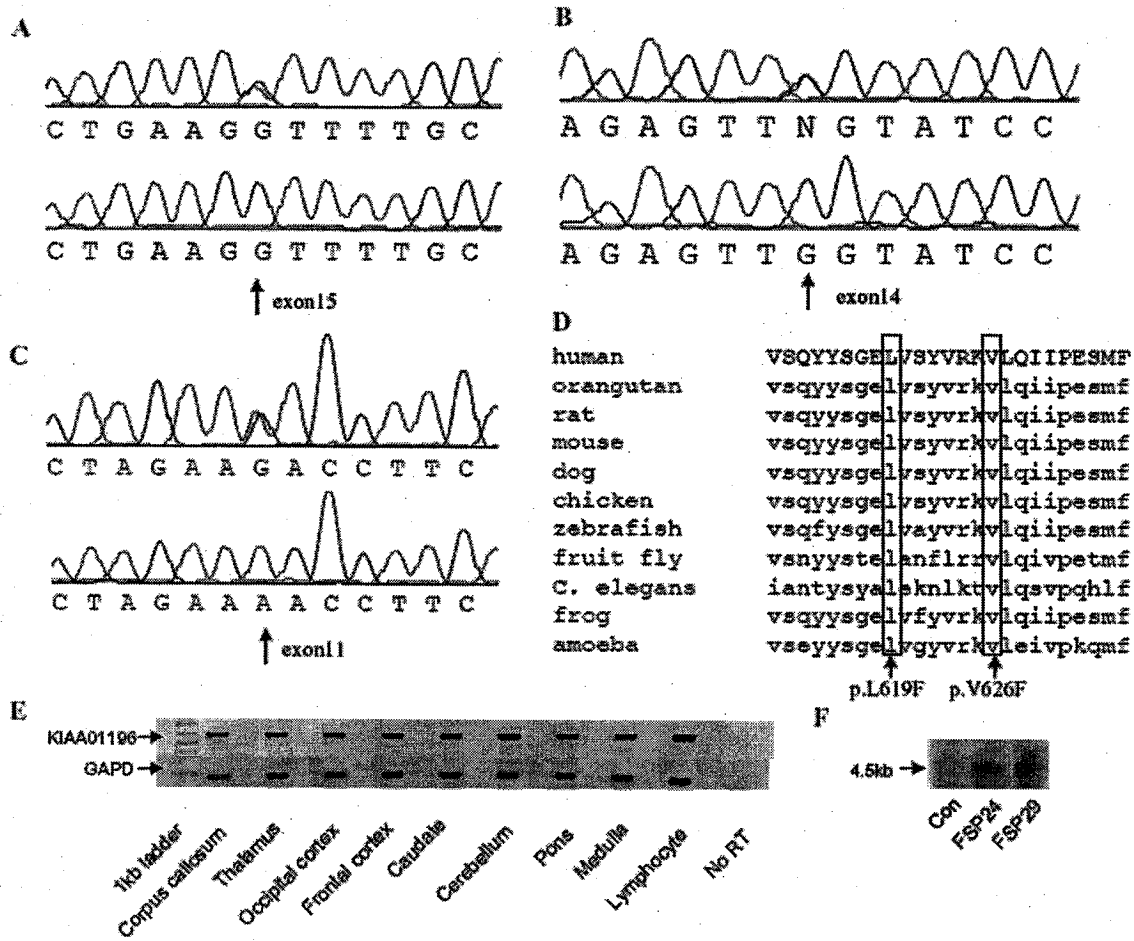

A
C T G A A G G T T T T G C
C T G A A G G T T T T G C
exon15
B
A G A G T T N G T A T C C
A G A G T T G G T A T C C
exon14
C
C T A G A A G A C C T T C
C T A G A A A A C C T T C
exon11
D
human VSQYYSGELVSYVRFVLQIIPESMF
orangutan vsqyysgelvsyvrkvlqiipesmf
rat vsqyysgelvsyvrkvlqiipesmf
mouse vsqyysgelvsyvrkvlqiipesmf
dog vsqyysgelvsyvrkvlqiipesmf
chicken vsqyysgelvsyvrkvlqiipesmf
zebrafish vsqfysgelvayvrkvlqiipesmf
fruit fly vsnyystelanflrrvlqivpetmf
C. elegans iantysyalsknlktvlqsvpqhlf
frog vsqyysgelvfyvrkvlqiipesmf
amoeba vseyysgelvgyvrkvleivpkqmf
p.L619F
p.V626F
E
KIAA01196
GAPD
1kb ladder
Corpus callosum
Thalamus
Occipital cortex
Frontal cortex
Caudate
Cerebellum
Pons
Medulla
Lymphocyte
No RT
F
4.5kb
Con
FSP24
FSP29

Figure 3

Mutation analysis of the *KIAA0196* gene. A - C) Sequence trace of an HSP patient above the sequence trace of a control individual. Exon 15 (A), 14 (B) and 11 (C) heterozygous point mutations are indicated. D) Multiple sequence alignment for strumpellin homologues surrounding the two coding changes (boxed). The Probcons (v.1.09) program was used for cluster analysis. E) RT-PCR of multiple brain regions using a *KIAA0196*-specific probe. F) Northern blot of the *KIAA0196* transcript using 30 ug of total RNA and a 1 kb C-terminal probe.

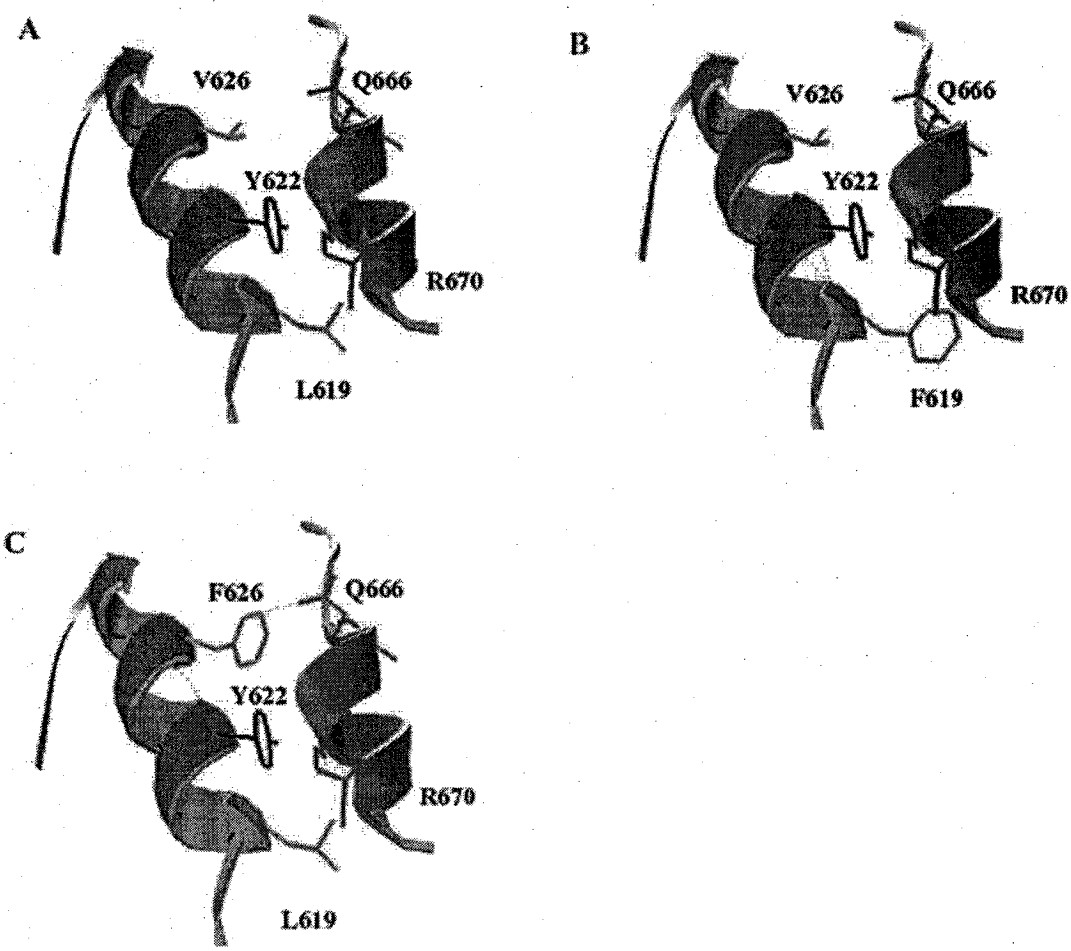
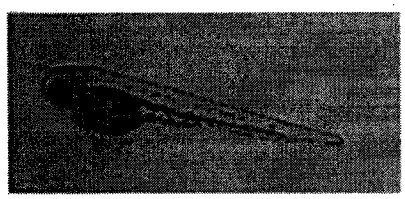

A
V626
Q666
Y622
R670
L619
B
V626
Q666
Y622
R670
F619
C
F626
Q666
Y622
R670
L619

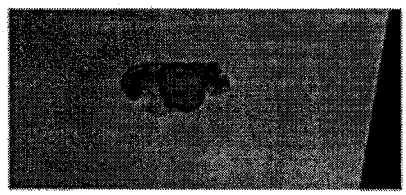
Figure 4

Three-dimensional modeling of strumpellin using d1dn1b as a template using SwissProt database viewer. Two helices from the 1159aa protein are shown including amino acids 614-634 in one alpha helix and amino acids 662-672 from a nearby alpha helix in the antiparallel direction. A) Residues L619 and V626 are in the same orientation in an alpha helix opposite a second helix in an antiparallel direction. Only residue side-chains which are closest in physical space are shown. B) The L619F mutation adds a bulky phenylalanine side-group which likely exceeds the space available between the two alpha helices C) The V626F mutation. The epsilon carbon of the F626 aromatic ring also may force apart the two alpha-helices and impinges on Q666.

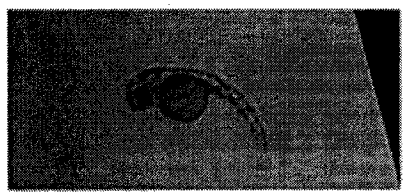
A. WT



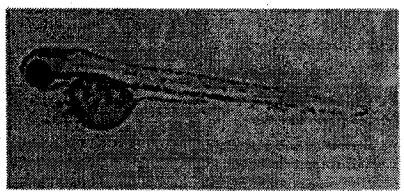
B. Control MO



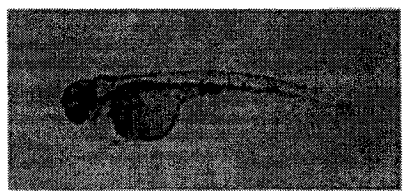
C. KIAA MO



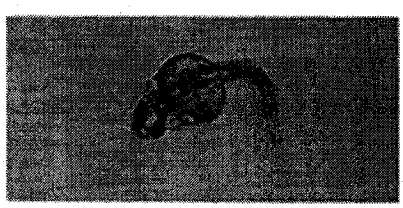
D. KIAA MO



E. KIAA MO + WT mRNA



F. KIAA MO + WT mRNA



G. KIAA MO + x14 mRNA

H. KIAA MO + x15 mRNA

Figure 5

Zebrafish knockdown and rescue of *KIAA0196* function. A) The gross morphological features of normal zebrafish are depicted at 3 dpf. B) Injection of a 5 base-pair mismatch morpholino results in no obvious phenotype. C,D) The KIAAMO injected fish present with a severely curly tail (C) or with a slightly curly tail (D). Their heart cavities are also enlarged, which is commonly seen in injected fish. E, F) When the KIAAMO is injected along with normal human *KIAA0196* mRNA, the fish partially develop the curly tail (F) or not at all (E) depending on the injected quantity. G), H) The phenotype is not alleviated when the KIAAMO is injected with the mutant forms of the human mRNA. These fish resemble the KIAAMO fish.

A. WT B. Control MO C. KIAAMO

D. KIAAMO + WT mRNA E. KIAAMO + x14 mRNA F. KIAAMO + x15 mRNA

Figure 6

Immunohistochemical analysis in zebrafish of the *KIAA0196* knockdown phenotype. A) The motor neurons in the ventral roots of zebrafish are segmented and oriented at 3 dpf. The spinal cord consists of the cell bodies of motor neurons and interneuron bundles. The picture was taken near the gut of the fish. B) The mismatch control has a similar motor neuron distribution compared to the wildtype. C, E, F) Zebrafish injected with KIAAMO and fish co-injected with mutant mRNA have shorter, branching motor neurons which are not oriented. D) Wildtype co-injections partially rescue the motor neuron phenotype; the axons are longer and oriented.

CHAPTER 8: DISCUSSION AND CONCLUSION

This chapter is devoted to a specific discussion of the results generated for this dissertation. Furthermore, this chapter will address the limitations of the project and the recent developments in the field of HSP. More general topics such as the usefulness of tools applied in this study and what impact this work has had on the field of HSP will also be discussed. Finally, the future directions in HSP genetic research and functional studies of nervous system pathways will be highlighted.

8.1 DISCUSSION

8.1.1 General discussion of chapters

SPG4 mutation analysis

SPG4 mutation analysis in a cohort of 76 HSP patients resulted in the identification of eight novel mutations and a 31% mutation frequency. The spastin mutation frequency obtained in our study was 10% lower than previously published frequencies. As previously mentioned, one cause for a lower mutation frequency in our population is 80% mutation detection efficiency. Another possible explanation would be the presence of mutations outside the coding sequence. These possibilities have since been explored by several groups. Iwagana and colleagues identified a large 5' deletion by Southern blotting, which is thought to interfere with translation initiation (Iwanaga et al. 2005). Interestingly, a large deletion spanning exons 8-17 was detected by multiplex ligation-dependent probe amplification in one of our spastin-negative families, FPS28 (or FSP7D), as part of a collaboration (Beetz C, in submission 2006). This family had shown suggestive linkage to the *SPG4* locus. It is estimated that large deletion mutations represent 14% of all spastin mutations (Beetz C, in submission 2006). In addition, it has since been confirmed that the 1004G>A in exon 6 identified in our study is a private polymorphism (Proukakis et al. 2003). Two other polymorphisms, S44L and P45Q, are thought to function as modifiers and decrease the age of onset (Chinnery et al. 2004; Svenson et al. 2004; McDermott et al. 2006). The presence of modifier effects of other genes on spastin function should be further studied.

SPG3A gene analysis and characterization

The mutation screen of the *SPG3A* gene in a cohort of 70 North American families resulted in the identification of a single mutation in two related French Canadian families. The p.del436N mutation decreases GTPase activity and patients with the mutation in heterozygote state show almost no protein, when compared to controls. This proposed dominant-negative effect of the p.436delN mutation was only observed in a single cell type. The analysis of atlastin levels in other tissue types of patients such as fibroblasts would aid in validating this mechanism. The other dominant HSP genes, *BSCL2*, *SPG13* and *SPG4,* suggest a haploinsufficiency mechanism. However, the spastin mutations that lead to constitutive binding to MTs can be considered gain-of-function mutations. Moreover, *NIPA1* mutations have not been well studied, but can be either dominant-negative or gain-of-function mutations, since the heterozygous deletion of the entire NIPA1 encoding gene does not result in an HSP phenotype. Understanding the mechanism by which these mutations affect protein function is an important step in elucidating the pathophysiology of this disease and this type of study should be extended to atlastin. Future studies should include the treatment of atlastin-positive lymphoblasts with protease inhibitors to further investigate whether the mutation acts in a dominant-negative fashion. If the atlastin levels remain comparable to control samples levels after protease inhibitor treatment, this would suggest that proteases degrade the mutant atlastin protein under normal conditions. Furthermore, a zebrafish atlastin knock-down model could be developed and the atlastin protein level could be detected and compared after rescue experiments with wild type or mutant mRNA.

In addition, the interaction between atlastin and spastin should be further analyzed, while focusing on mutation-positive samples. RNAi knock-down of atlastin affects spastin levels and localization, but the reverse experiment does not impact on atlastin expression (Evans et al. 2006). The 1688A insertion mutation in atlastin also disrupts the atlastin-spastin interaction (Evans et al. 2006). It would be interesting to determine whether the p.436delN mutant can interact with spastin. Furthermore, the effect of mutations on the cellular distribution and cell viability should also be analyzed.

ALS2 gene function

A deletion mutation was identified in the *ALS2* gene that causes severe infantile onset HSP. This frameshift mutation remains the most C-terminal mutation identified in *ALS2* and truncates the VPS9 domain. Unfortunately, the expression level of alsin in lymphoblasts has not yet been determined. Interestingly, a missense mutation, p.G540E, in the RCC1 domain was recently identified and it was shown to cause delocalization of alsin from endosomal structures (Panzeri et al. 2006). In addition, alsin associates with the centrosome, as does spastin (Millecamps et al. 2005). The question remains if these two proteins colocalize and whether they are involved in a common pathway.

Furthermore, alsin has been shown to control survival and growth of spinal motor neurons in spinal motor neuron specific RNAi experiments. This knock-down results in smaller endosomes and intracellular accumulation of L1CAM and transferrin (Jacquier et al. 2006). The link of alsin function with L1CAM, an HSP gene, supports the fact that the *ALS2* phenotype is more representative of severe HSP.

Mapping of the *SPG27* locus

In chapter 4 a novel locus for pure recessive HSP, *SPG27*, was mapped to chromosome 10q, and overlaps with a previously mapped dominant HSP locus, *SPG9*.

The *SPG27* locus was recently confirmed by Ribai and colleagues in a recessive Tunisian family with spastic paraparesis and sensorimotor polyneuropathy (Ribai et al. 2006). The candidate gene region was narrowed down from 25 to 19.6 cM, which contains over 200 genes. The disease locus cannot be further reduced in our family. Additional genotyping of other families has revealed that this locus is not a common cause of HSP in our cohort.

In collaboration with Dr. Mannan and colleagues, the FSP7 family was screened for mutations in a positional candidate gene which lies in the *SPG27/SPG9* region, *ZFYVE27*, which is also a spastin-interacting partner and has been shown to cause HSP in a German kindred (Mannan et al. 2006b). No mutations were identified in the FSP7 family, nor in the *SPG9* linked family (Mannan et al. 2006b). The group, who initially reported the *SPG9* locus, is screening candidate genes in the *SPG9/SPG27* overlap and this group is including one of our samples in their screening process. Furthermore, we will continue to monitor the region and investigate whether newly identified HSP interacting partners map to this locus.

Gene finding at the *SAX1* locus

The first locus for autosomal dominant spastic ataxia, *SAX1*, was mapped to chromosome 12p13 in three large Newfoundland families. The distinct phenotypic spectrum of spasticity and ataxia associated with this locus raises the question whether complicated HSPs should be classified as separate disorders. The fact that there can be

diagnostic ascertainment differences between neurologists complicates matters even more. Ideally, the most concrete classification should rest on the underlying genetic cause and should be applied for all genetic neurological disorders.

After extensive fine-mapping, additional sample collection and thorough candidate gene analysis, the causative gene at the *SAX1* locus remains elusive. The candidate gene analysis focused on coding sequences, and even though UTRs are an unlikely candidate for mutations in this case, they should nevertheless be completely screened. The possibility of a large deletion mutation was ruled out by the use of haploid alleles derived from the cell hybrids. However, our mutation analysis did not account for duplications. This avenue of copy number change should be further explored by extensive SNP typing or Comparative Genomic Hybridization (CGH). Despite the fact that the genetic data supporting the candidate locus are overwhelming, the physical map still contains a rare sequence gap flanked by the *C1R* gene. Interestingly, a locus for Bowen-Conradi syndrome, a lethal recessive disorder, maps to the same region as the *SAX1* locus (Lamont et al. 2005) and the group has not been able to identify the causative gene either (personal communication). It is possible that the region is much more gene rich than previously thought, or that there is a large genomic aberration or even that the genes have incomplete structural annotations. The search for the *SAX1* mutation will be continued and the main focus should be the review of all generated data and the analysis of novel coding sequences. Again, the identification of the *SAX1* gene and the characterization of its function should reveal whether this gene also plays a role in the various mechanisms proposed in HSP.

The identification of the *SPG8* gene

The identification of a novel gene responsible for HSP on chromosome 8 (*SPG8*) is an exciting finding in this field of research. Three mutations in five families were found in the *KIAA0196* gene, which encodes strumpellin. It was shown that two of the mutations are not able to rescue a knock-down phenotype in zebrafish, though the function of the gene remains unclear. With no obvious conserved domain, except for the spectrin repeat, the biochemical characterization of the strumpellin protein will be a daunting task. Future experiments will focus on the generation of antibodies, the search for interacting partners, protein localization studies and further characterization of the zebrafish phenotype. Additional neuronal markers should be studied to detail the extent of motor neuron damage in the knock-down model. Another interesting experiment would be to knock-down other HSP genes in zebrafish and determine whether a similar phenotype to the strumpellin knock-down is obtained. Such an experiment would elucidate whether the HSP genes affect the same pathway in zebrafish. Furthermore, the search for strumpellin interacting partners should start with yeast two-hybrid analysis between all known HSP genes. Ultimately, embryonic stem cell knock-outs of the *KIAA0196* gene can be used for the generation of a knock-out mouse model. Furthermore, a transgenic mouse model or zebrafish model can also be developed to study the effect of a dominant mutation. These models would aid in elucidating the function of strumpellin in the nervous system and the transgenic models would allow us to study the effects of the mutations *in vivo*.

8.1.2 Limitations of studies

In this thesis project two HSP loci were mapped, three HSP genes were screened for mutations in a large cohort and a novel HSP gene was identified. However, the project was complicated by several limitations. In general, the number of affected members in HSP families tends to be high. Unfortunately, the cost of collecting the entire family is also high and not all family members are willing to participate, which often leads to partially collected families with lower statistical power. Furthermore, the nature of the disease and our collection procedure only allowed us limited access to tissue from patients: as a standard we collect blood DNA and establish lymphoblastoid cell lines for each participant. The lymphoblasts are a continuous and reliable source of RNA and protein, but gene expression in those cells is not necessarily representative of expression in the nervous system. The lack of nervous tissue samples from patients complicates and limits the characterization of mutations. Alternatively, this characterization can also be performed in an *in vitro* model (cell transfection assays) or in tissue expression studies in the mouse nervous system, for example. Instead of exclusively focusing on generating and refining genetic data, our approach could have included more functional and cell biology studies, such as the development of antibodies against spastin and strumpellin. The study of HSP genes in model organisms should also occur in parallel with the human studies, potentially in collaboration with other specialized laboratories. The lack of bioinformatics tools also decreased the gene analysis efficiency. Useful integrative tools such as Bioharvester and Suspects were only made public later during this thesis project. To facilitate our gene finding efforts, an algorithm could have been

developed for determining gene priority and gene structure by integrating all available data from the public databases.

8.1.3 Efficiency of linkage mapping and positional cloning

The use of linkage mapping as a tool to identify disease loci has been successful in this study, with the identification of two novel loci and one novel gene. The *SPG27* locus has since been confirmed by another group and the *SAX1* locus has been confirmed by our group through the identification of a fourth linked family with Newfoundland roots. Interestingly, almost as many recessive as dominant HSP loci have been mapped, even though recessive HSP is much rarer. This observation supports the idea that mapping recessive loci is easier because of homozygosity mapping.

The success of linkage mapping for Mendelian traits, again demonstrated in this thesis, begs the question whether this approach should also be employed for complex traits. Instead of collecting thousands of samples to reach association statistical power in association studies, one could focus on "Mendelianized forms of complex traits" with severe phenotypes or endophenotypes that segregate in families.

One of the major limitations of linkage mapping in humans is the rarity of critical recombinations. In the case of the *SAX1* locus, we are confronted with a young population where the mutation lies on a well-conserved haplotype. In addition, the success of a positional cloning effort depends largely on the gene density in the candidate interval. Another approach to linkage analysis of Mendelian traits would be to study other species with similar phenotypes. The clinical characterization of neurodegenerative traits has improved in animals such as the dog (Siso et al. 2006) and

the swine (Kratzsch et al. 1999). The genetic studies of these animals are statistically more complex, but have the advantage of more recombinants.

To conclude, the mapping of Mendelian traits mostly focus on "negative" phenotypes. The study and mapping of genes involved in "positive" traits, such as longevity (Perls and Terry 2003), strong enamel, exceptional eyesight and resistance to HIV infection can also contribute tremendously to our understanding of the function and maintenance of wide-ranging systems in the human body and should be explored.

8.1.4 Genetic make up of our HSP cohort

The different approaches of candidate gene analysis, linkage mapping and positional cloning resulted in the identification of the underlying genetic cause in many HSP families (Table 1).

Table 1: A summary of all genetic causes identified in our cohort.

# Fam	Locus	Protein
1	*SPG2*	PLP
2	*SPG3*	atlastin
18	*SPG4*	spastin
4	*SPG8*	strumpelin
2	*SPG27*	?
1	*ALS2*	alsin
4	*SAX1*	?

The study allowed us to identify the underlying locus is 36% (32/90) of all families tested. In the French Canadian subgroup, we identified four genetic causes for HSP,

namely *SPG2* (personal communication), *SPG3, SPG4* and *SPG27* in a total of 15 families. In concordance with findings by other groups, the most commonly mutated gene for HSP in our cohort is *SPG4.* It is important to address the fact that the yield of mutations in this cohort was not high, most likely due to the extreme genetic heterogeneity observed in HSP, which complicates the cost-efficient screening of HSP patients on a population level. Nevertheless, patients in Canada can now take advantage of HSP gene screening tests. The HSP patients in Quebec should first be screened for the common *SPG4* mutation and the less frequent *SPG3* mutation. In addition, Anglo-Canadians should preferentially be tested for *SPG4* and *SPG8* mutations.

In an academic laboratory, the cost of mutation screening of known genes is quite high and the cheaper genotyping prices will eventually warrant direct WGS approaches in large families, instead of first performing exclusion analysis by linkage analysis or mutation screening. Furthermore, our candidate gene analysis focused on coding sequence and splice junctions, where mutations in non-coding and/or potentially regulatory sequences might have been overlooked.

8.1.5 Genetic complexity behind a simple Mendelian trait

The genetic complexity observed in HSP demonstrates the fact that genes would not be identified if it was not for the existence of large HSP families. If for a very penetrant neurodegenerative disease, mutations in 30 different genes can result in disease, how many genes of small effect contribute to so-called complex traits? And will we ever be able to detect such weak signals? A more positive hypothesis would be the common variant-common disease theory, which should be identified more readily. In such a case a common variant would be detected when over-transmitted in cases versus controls,

especially in founder populations with increased genetic homogeneity. The absence of genotype-phenotype correlations complicates the genetic studies even further. With a relatively easy to diagnose disease such as HSP, the phenotypes associated with spastin mutations are wide ranging: neuropathy, dementia, and ALS. This phenotypic variation must have an even greater impact on genetic studies for diseases that are more difficult to diagnose such as neuropsychiatric diseases.

Another interesting phenomenon that emerged from the mapping of HSP loci is that several of the loci initially overlapped: *SPG26/SPG10*; *SPG9/SPG2/SPG33*; *SPG3/SPG28* and *SPG13/ALS2*. This suggests that many more HSP loci are yet to be discovered, or that the diseases are actually allelic or that there are common cis-acting factors specific to the nervous system at these loci.

Finally, the 33 HSP loci and genes mapped to date represent 0.1% of all genes in the genome (~30,000), which demonstrates that the study of Mendelian traits can contribute to the characterization of the human phenome. This forward genetics approach of gene finding can be complemented by reverse genetics and systems biology. An elegant yeast two-hybrid study which included all known SCA genes in yeast revealed a network of genes and physical interacting partners involved in transcriptional regulation, RNA splicing, ubiquitination, cell cycle control and other functions (Lim et al. 2006). In addition, this network confirmed previously identified genetic modifiers in *Drosophila*. Such a high throughput search for interacting partners has yet to be undertaken for HSP genes. Moreover, several partners have been identified for HSP genes in a whole genome interacting partner study (see table in General Introduction) (Rual et al. 2005).

The systems biology approach can also be applied in *C. elegans* with RNAi knock-down genes. In this approach a certain gene is knocked-down and the resulting phenotype is matched in a knock-down library of genes. These genetic interactions can then be analyzed by yeast two-hybrid analysis to determine if they are physical interactions. Large-scale studies in *C. elegans* have shown that most genes interact with only a few genes (specific modulators) and few genes interact with many (modifier) genes (Lehner et al. 2006). In addition, this approach of systems biology could serve as an alternative tool for gene finding in complex traits (Lim et al. 2006).

8.1.6 Genetic similarities between HSP and other diseases

Genetic research in the field of neurodegeneration affecting the motor system has advanced tremendously in recent years. The number of loci identified for HSP is now up to 33, compared to 29 loci mapped for spinocerebellar ataxia (Duenas et al. 2006). The late onset and normal reproductive fitness in these similar diseases result in the existence of large families, which facilitates linkage analysis. In addition, several HSP genes were identified in a yeast two-hybrid screen for SCA gene interacting partners (Lim et al. 2006).

The identification of mutations for HSP in the *ALS2* gene highlighted the fact that HSP and ALS are closely related disorders. Both disorders display genetic heterogeneity, have sporadic forms of the disease and have ubiquitously expressed disease genes. These similarities greatly complicate clinical diagnosis and underline the importance of genetic results in the clinic. Furthermore, the complicated HSPs also show how mutations in the same gene can lead to different motorneuron diseases. Mutations in the *BSCL2* gene cause Silver syndrome and dHMN. Moreover, HSA families linked to the

SAX1 locus, were initially diagnosed as SCA or HSP. Interestingly, a zebrafish knock-down model of the *SMN1* gene (SMA disease) resulted in axonal outgrowth and branching defects similar to those observed in the *SPG8* knock-down model. Together, these findings support the hypothesis that there are a limited number of mechanisms that result in motor neuron death. What are these mechanisms?

8.1.7 Molecular mechanisms underlying HSP

The mechanisms of HSP patho-physiology proposed in the field are described in the general introduction (Chapter 1). They consist of axon outgrowth dysfunction, impaired myelin maintenance, defects in axonal trafficking and mitochondrial dysfunction.

The HSP proteins that have been identified are mostly ubiquitously expressed, but yet defects in these proteins lead to degeneration of motor neurons specifically. What makes motor neurons so vulnerable? These long neurons have high energy requirements for transport and synaptic transmission and they require a large intact infrastructure for transport. In addition, motor neurons are extremely polar and do not divide. In the following sections the different proposed mechanisms will be discussed. Furthermore, the recently identified genes will be incorporated in these mechanisms.

Mitochondrial dysfunction

Mitochondria are the source for energy in our body. They also act as the main calcium buffers and control reactive oxygen species. In addition, mitochondria can be found in high density at the synapse (Kwong et al. 2006).

Two HSP proteins, paraplegin and HSP60 are directly involved in mitochondrial function. The recent identification of REEP1 as an HSP protein that also localizes to the mitochondrial membrane contributes to this category. Interestingly, five other genes involved in axonopathies have a function in mitochondria (Zuchner and Vance 2005). Mitochondrial dysfunction has been linked to many diseases such as MND, Alzheimer disease, Huntington, and ataxias. There is no doubt that proper mitochondrial function is essential to the maintenance of healthy motor neurons.

Vesicle trafficking and/or axonal transport

The mechanism favored by most researchers to explain axonal degeneration with a "dying back" pattern is a defect in axonal transport. The majority of the HSP genes have been grouped in this category although some have been merely associated with membrane trafficking (see following section). Interestingly, four out of seven axonopathy proteins involved in endosomal trafficking are HSP proteins and one out of five has a role in axonal transport (Zuchner and Vance 2005). Molecular motors have important roles in neurons, where they are necessary for migration, pathfinding, synapse stabilization, and active transport (Holzbaur 2004). A defect in this type of protein can clearly impact the maintenance or development of a motor neuron. The HSP proteins that are directly implicated in axonal transport are spastin (the microtubule severing protein) and KIF5A (a fast anterograde motor). Many questions regarding this hypothesis remain unanswered: is anterograde or retrograde transport affected? It has not yet been determined whether there is a problem in transporting cargo to the synapse or whether recycling of cargo from the synapse does not reach the soma. Secondly, the question remains as to what the mysterious cargo is? Many groups have been trying to

characterize the contents of the axonal swelling observed in animal models of PLP, paraplegin and spastin. One of the organelles accumulating in these swollen structures is actually mitochondria (McDermott et al. 2003). Another possibility is that the traffic disrupted in HSP is in fact the transport of proteins from the ER to the Golgi.

Endosomal trafficking

Membrane proteins undergo maturation in the ER and Golgi after which they get transported to the plasma membrane from the trans-Golgi network (TGN) (Winckler 2004). Many of the HSP proteins and interacting partners (atlastin, spastin, spartin, maspardin, ZFYVE27, NIPA1, BSCL2, EPS15, REEP1, CHMP1B and alsin) localize to the Golgi, ER or other membrane structures and are thought to have a function in membrane trafficking. NIPA1 and seipin, in the various functions category of chapter 1, could also play a role in endosomal trafficking since they both have a transmembrane domain and BSCL2 localizes to the ER.

The newly identified strumpellin contains a spectrin domain and a spectrin-actin network surrounds the cytoplasmic surface of Golgi and TGN. Motor proteins such as KIFs, dynactin and $P150^{glued}$ involved in other MNDs also play an important role in targeting ER-Golgi vesicles to MTs for transport (Vaughan 2005). Winckler proposes an interesting mechanism involving selectivity filters for polarized transport in neurons, which incorporates the suggested function of most HSP proteins (Winckler 2004). In this mechanism, proteins destined to the plasma membrane of the axon first get packaged in specialized vesicles tagged for the axon or dendrite. These vesicles are then transported via microtubules by axonal-specific motors, one of which has been shown to be KIF5A. Once arrived at the membrane in the axon or synapse, selective membrane

fusion takes place. If the protein manages to evade these three filters, misincorporated proteins can be taken up by endocytosis and recycled back to the soma. The process of retrograde endosomal transport is poorly understood, but is a necessary pathway for sending signaling cues from the periphery to the cell body (Deinhardt and Schiavo 2005; Nixon 2005). Even though retrograde transport has not been well studied, it serves as an attractive candidate for the "dying back" hypothesis. A defect in this process can lead to accumulation of unwanted proteins in the axon and synapse, where they may become toxic to the cell.

The endosomal trafficking theory does not exclude the axonal transport hypothesis; instead axonal transport is an integral part of this proposed mechanism. The filter hypothesis can also explain the late onset of HSP, where the many checkpoints are redundant and actual accumulation of mislocalized proteins or lack of proteins in the axon occurs over time. Interestingly, this hypothesis also includes the HSP proteins involved in axon outgrowth (L1CAM, strumpellin) since membranes and membrane-associated proteins are probably incorporated in a similar fashion into the growing axon. HSP (and other MNDs) are probably the result of the dysfunction of not only one of the mechanisms proposed above, but rather due to a combination of two or more disrupted pathways. It seems that each HSP presentation has a "private" way of "going wrong", where the mutations cause a major phenotype of lower limb spasticity and specific mechanisms lead to local subtle dysfunctions. These milder defects in possible other pathways give rise to the multitude of phenotypes associated with this type of disease. This scenario is different from, for example, the dominant ataxias, where there is a common mutational mechanism but where the specificity of neuronal death seems to be determined by both the specific protein which carries the mutation and/or modifier

proteins. In HSP the proteins and mutations are different, but a shared pathway leads to specific upper motor neuron death.

Elucidating the molecular mechanisms underlying HSP is key to developing treatments. The identification of individual HSP genes can lead to the development of gene-specific treatments such as an intramuscular viral delivery of paraplegin to paraplegin-deficient mice (Pirozzi et al. 2006) and treatment with vinblastine of *Drosophila* spastin mutants (Orso et al. 2005). Another potential treatment for HSP caused by spartin mutations involves Akt regulators in HSP (Duenas et al. 2006). Spartin interacts with eps15, which in ubiquitinated state controls Akt signaling. Akt signaling regulates anti-apoptotic proteins and promotes neuronal survival (Fallon et al. 2006). Interestingly, alsin also mediates neuroprotection through the Akt pathway (Kanekura et al. 2005). Other single gene based therapies include RNAi treatment, which is not applicable to haploinsuffiency mutations.

The treatments that apply to motor neuron degeneration in general can also be tested as a treatment for HSP. Minocycline is a tetracycline that has neuroprotective properties because it inhibits microglial activation and inhibits apoptosis. Furthermore it has been shown to slow disease progression in mouse models of ALS and is therefore a good candidate for HSP (Kriz et al. 2002; Festoff et al. 2006).

Other therapies involve treatment after the damage has occurred. For example, ES cell derived MNs have been shown to reestablish functional motor units in paralyzed rats (Deshpande et al. 2006). With the constant improvement of our understanding of the mechanisms involved in neurodegeneration, we are getting closer to developing better therapies.

8.2 CONCLUSION

Over the course of this PhD project we have screened a North American cohort for mutations in two key HSP genes, spastin and atlastin. The mutation spectrum for these genes has been determined in this cohort and the unlinked families can now be used in future linkage studies. We also characterized a novel atlastin mutation and revealed a possible dominant-negative mechanism. Furthermore, we have identified the cause of a very severe form of HSP to be a mutation in an ALS gene, *ALS2*. This finding broadened the spectrum of clinical disorders previously linked to the *ALS2* locus. In addition two novel HSP loci were mapped, *SPG27* and *SAX1*. We are now one step closer to cloning the disease genes at these two loci. Finally, we have identified the gene at the *SPG8* locus.

Taken together, this PhD project contributed to the field of HSP, HSA, ALS and motor neuron dysfunction in general. Many interesting avenues have been brought to light and our laboratory will continue the efforts to identify and characterize additional genes. Indeed we have come a long way from the initial classification of the HSP by Dr. Anita Harding. In 1983 she wrote: "Ideally, this scheme will be modified when specific metabolic markers for some conditions are identified or when mutant genes can be linked to polymorphisms on specific chromosomes" (Harding 1983). The identification of 15 HSP genes suggests a common mechanism of disruption of axonal membrane protein trafficking, which can also apply to other neurodegenerative disorders. Understanding the patho-physiology of HSP will facilitate the development

of therapies for MNDs and SCIs and will help unravel the mechanisms that control voluntary movement in the corticospinal tract.

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UCSC Human Genome Project Working Draft (Golden Path), http://genome.ucsc.edu/

APPENDIX

Ethical approval

Radioactivity training certificate

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