Amyotrophic Lateral Sclerosis (ALS) Associated with Superoxide Dismutase 1 (SOD1) Mutations in British Columbia, Canada: Clinical, Neurophysiological and Neuropathological Features

By

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Umeå University 2005
Umeå, Sweden
Front Cover Artwork- “Waterfall at Capilano Canyon”, in oils- by Peggy Smith of West Vancouver, British Columbia, Canada. Peggy was many things- an artist, world traveler, wife, mother, grandmother and great-grandmother. She was diagnosed with ALS in 1999, and though she eventually lost her battle with the disease in 2002, she never lost sight of the beauty around her and continued to paint until shortly before her death. Peggy’s family graciously allowed me to use this beautiful image, from my home in Vancouver, for the cover of my thesis.

Back cover photograph- “Dragonfly”- by Professor Andrew Eisen of Vancouver, British Columbia, Canada. Andrew Eisen (Andy) was Professor of Neurology at the University of British Columbia, and Director of the ALS Clinic at Vancouver General Hospital until his retirement December 2004. He spent the majority of his professional career caring for patients with ALS, while trying to understand disease pathogenesis. He was a pioneer in the neurophysiologic study of ALS and other neuromuscular diseases. As a physician, teacher and mentor, he’s touched and shaped thousands of lives for the better, including my own. I’m blessed to know him.

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Climbing as we fall
We dare to hold on to our fate
And steal away our destiny
To catch ourselves with quiet grace

“The Stairs”
M. Hutchence/A. Farriss
Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by loss of motor neurons and their supporting cells in the brain, brainstem and spinal cord, resulting in muscle paresis and paralysis including the bulbar (speech, chewing, swallowing) and respiratory muscles. The average age at onset is 55 years, and death due to respiratory failure occurs 2-5 years after symptom onset in ~85% of cases. Five to 10% of ALS is familial, and about 20% of familial cases are associated with mutations in the superoxide dismutase 1 (SOD1) gene. To date, 118 SOD1 mutations have been reported worldwide (www.alsod.org). All are dominantly inherited, except for the D90A mutation, which is typically recessively inherited. D90A homozygous ALS is associated with long (~14 years) survival, and some atypical symptoms and signs. The reason for this is not known. In contrast, most other SOD1 mutations are associated with average survival, while some are associated with aggressive disease having lower motor neuron predominance and survival less than 12 months. The A4V mutation, which is the most frequently occurring SOD1 mutation in the United States, is an example of the latter. Understanding the pathogenic mechanisms of SOD1 mutants causing widely different disease forms like D90A and A4V is of paramount importance. Overwhelming scientific evidence indicates that mutations in the SOD1 gene are cytotoxic by a “gain of noxious” function, which although not fully understood results in protein aggregation and loss of cell function.

This thesis explores different ALS-SOD1 gene mutations in British Columbia (BC), Canada. Two hundred and fifty-three ALS patients were screened for SOD1 mutations, and 12 (4.7%) unrelated patients were found to carry one of 5 different SOD1 mutations: A4V (n=2); G72C (n=1); D76Y (n=1); D90A (n=2); and 113T (n=6). Incomplete penetrance was observed in 3/12 families. Bulbar onset disease was not observed in the SOD1 mutation carriers in this study, but gender distribution was similar to previously reported studies. Age at symptom onset for all patients enrolled, with or without SOD1 mutations, was older than reported in previous studies. On average, patients with SOD1 mutations experience a longer diagnostic delay (22.6 months) compared to patients without mutations (12 months). Two SOD1 patients were originally mis-diagnosed including the G72C patient who’s presenting features resembled a proximal myopathy. Neuropathological examination of this patient failed to reveal upper motor neuron disease. The I113T mutation was associated with variable age of onset and survival time, and was found in 2 apparently sporadic cases. The D76Y mutation was also found in an apparently sporadic case. I113T and D76Y are likely influenced by other genetic or environmental factors in some individuals. Two patients were homozygous for the D90A mutation, with clinical features comparable to patients originally described in Scandinavia. Clinical and electrophysiological motor neuron abnormalities were observed in heterozygous relatives of one D90A homozygous patient. The A4V patients were similar to those described in previous studies, although one had significant upper motor neuron disease both clinically and neuropathologically.

Clinical neurophysiology is essential in the diagnosis of ALS, and helpful in monitoring disease progression. A number of transcranial magnetic stimulation (TMS) studies may detect early dysfunction of upper motor neurons when imaging techniques lack sensitivity. Peristimulus time histograms (PSTHs), which assess corticospinal function via recording of voluntarily activated single motor units during low intensity TMS of the motor cortex, were used to study 19 ALS patients having 5 different SOD1 mutations (including 8 of the 12 patients identified with SOD1 mutations from BC). Results were compared with idiopathic ALS cases, patients with multiple sclerosis (MS), and healthy controls. Significant differences were found in corticospinal pathophysiology between ALS patients with SOD1 mutations, idiopathic ALS, and MS patients. In addition, different SOD1 mutants were associated with significantly different neurophysiologic abnormalities. D90A homozygous patients show preserved if not exaggerated cortical inhibition and slow central conduction, which may reflect the more benign disease course associated with this mutant. In contrast, A4V patients show cortical hyper-excitability and only slightly delayed central conduction. I113T patients display a spectrum of abnormalities. This suggests mutant specific SOD1 pathology(s) of the corticospinal pathways in ALS.

Keywords: ALS, SOD1, complete penetrance, incomplete penetrance, mis-diagnosis, upper motor neuron, clinical neurophysiology, transcranial magnetic stimulation, peristimulus time histogram, corticospinal pathway, cortical inhibition, cortical hyper-excitability
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Preface

It is fair to say that our current understanding of the disease amyotrophic lateral sclerosis (ALS) is a “good news”--“bad news” situation. The “good news” is the last decade, perhaps the last half century, has advanced our understanding of this complex disorder through a flurry of scientific research. Paving the way have been advances in molecular and protein analyses, neuroimaging, neuropathological and neurophysiological techniques. The “bad news” is that despite this surge in knowledge about ALS in recent decades, we still have no good therapies that substantially alter the disease course in human beings. This undoubtedly will change in due course (and hopefully a “short” one). In addition, as science finds ways for humans to outlive more common diseases, society and physicians will be faced with ever increasing numbers of individuals with ALS and/or other neurodegenerative disorders. Pre-requisite for effective treatment of ALS is (and will continue to be) a clinician’s ability to recognize the different clinical manifestations of ALS, as well as the earliest signs of disease. Our efforts to treat ALS- the disease biology, symptoms and signs- must be founded in a solid understanding of the phenomenology of the disease and of its biological characteristics.

I. Stewart HG, Eisen A, Fabros M, Mackenzie I, Mezei M, Andersen PM. SOD1 gene mutations in ALS patients from British Columbia, Canada: Clinical features, pathology and ethical issues in management. Manuscript


Relevant publications on which the candidate is a co-author, but are not included in this thesis:


Amyotrophic Lateral Sclerosis- Clinical Features

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that primarily affects the upper motor neurons (UMN) of the motor cortex and brainstem, and the lower motor neurons (LMN) of the brainstem and spinal cord. The lower motor neurons in the spinal cord are also referred to as anterior horn cells (AHC). Skeletal muscles innervated by degenerating upper and lower motor neurons become weak and wasted, including muscles controlling speech, chewing, swallowing and breathing. Degeneration of UMN in the brain and brainstem results in symptoms such as incoordination (loss of dexterity), stiffness, and fatigue in the extremities and trunk, and if the brainstem is affected, difficulty with emotional control (referred to as emotional “lability”). Signs of UMN degeneration include overly active deep tendon reflexes, clonus, increased muscle tone (spasticity), and other pathological or primitive reflexes (Babinski or Hoffman’s sign). Degeneration of lower motor neurons of the brainstem and spinal cord results in symptoms of weakness, muscle fasciculations (twitching) and cramps. Signs of LMN degeneration include muscle atrophy, reduced strength, and decreased muscle tone (flaccidity). Some common lower and upper motor neuron signs observed in affected central nervous system (CNS) regions are summarized in Table 1. The symptoms and signs listed here are those most frequently observed in ALS. The deficits caused by this combination of UMN and LMN degeneration in ALS are progressive, and unfortunately the typical outcome is death from respiratory failure 3-5 years after onset of first symptoms. It is understandable why ALS is considered to be one of the most dreaded of neurological disorders.

Table 1: Lower motor neuron and upper motor neuron signs in four CNS regions

<table>
<thead>
<tr>
<th>Lower motor neuron signs</th>
<th>Brainstem</th>
<th>Cervical</th>
<th>Thoracic</th>
<th>Lumbosacral</th>
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<tbody>
<tr>
<td>weakness</td>
<td>face</td>
<td>neck</td>
<td>back</td>
<td>back</td>
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<td>atrophy</td>
<td>jaw</td>
<td>arm</td>
<td>abdomen</td>
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<tr>
<td>fasciculations</td>
<td>palate</td>
<td>hand</td>
<td>including the chest wall</td>
<td>leg</td>
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<td></td>
<td>tongue</td>
<td>diaphragm</td>
<td></td>
<td>foot</td>
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<td></td>
<td>larynx</td>
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<table>
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<tr>
<th>Upper motor neuron signs</th>
<th>Brainstem</th>
<th>Cervical</th>
<th>Thoracic</th>
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<tr>
<td>hyper-reflexia</td>
<td>clonic jaw</td>
<td>hyper-reflexia</td>
<td>loss of superficial abdominal reflexes</td>
<td>hyper-reflexia</td>
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<td>spread of tendon reflexes</td>
<td>gag reflex</td>
<td>clonus</td>
<td>spasticity</td>
<td>clonus</td>
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<td>exaggerated pseudobulbar features</td>
<td>spasticity</td>
<td>Hoffman sign</td>
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<td>spasticity</td>
<td>preserved tendon reflex in weak, wasted limb</td>
<td>preserved tendon reflex in weak, wasted limb</td>
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Epidemiological Considerations for ALS

Where ascertainment of ALS cases is complete, the incidence of sporadic ALS (SALS) is fairly constant, ranging from 0.86 to 2.5 per 100,000 person-years [1-4]. The more recent studies give an approximate crude incidence rate of 2 per 100,000 person-years. Average survival in ALS is estimated at 3 years, and with prevalence rates being about 3 times incidence rates, the associated crude prevalence of ALS can be estimated at about 6 per 100,000 population on any given date[5]. These incidence and prevalence estimates exclude foci found in the Western Pacific [6-9], namely Guam, the Kii Peninsula in Japan, and western New Guinea. In these areas, variants of ALS (for example ALS combined with Parkinsonism and/or dementia) have been noted in high numbers in the past, and in some villages the incidence was 100 times greater than that observed in Europe or North America. There is general consensus that environment played a significant role in the induction of cases in the Western Pacific foci, which are since decreasing in size, a phenomenon associated with lifestyle modifications including changes in diet [10, 11].

Increasing incidence of SALS has been reported in various studies from different geographical locations [12, 13], particularly in Southern European regions and amongst females and individuals 75 years of age or older. However, a recent analysis of ALS associated death rates and trends in the United States [14] during the period 1979-2001 showed the average annual age-adjusted ALS death rate was 1.84 per 100,000 persons for 1979 through 1998. A small overall increase in the death rate was observed primarily between 1979 and 1983, with a subsequent plateau. This slight increase reflected increases in the rates among those persons 65 years of age or older, particularly women, and persons in the 20- to 49-year-old age group. There were more ALS deaths in the northern states.

Increasing age is the principal risk factor for the occurrence of SALS [5]. The overall incidence and age-specific incidence rates of SALS are higher in men than women (1.61:1 to 1.3:1 overall), suggesting perhaps hormonal or X-linked susceptibility not yet identified. Some studies suggest that the male weighted gender ratio is decreasing [3, 15], which may reflect greater ascertainment of female ALS cases, improved overall healthcare for women (decreasing mortality in females from other causes), or recent equalization of exposures to exogenous risk factors between males and females. In earlier [16] but also more recent studies [14], it’s been noted that ALS is more common among Caucasian individuals. This may be of true biological significance, however the possibility of case under-ascertainment among non-white individuals, particularly among non-white females over the age of 60, must also be considered.
Historical Perspective: ALS and Jean-Martin Charcot

Jean-Martin Charcot was the first to use the term amyotrophic lateral sclerosis ("sclérose latérale amyotrophique" in French) in 1874 to brand a degenerative condition of the neuromuscular system that resulted in skeletal muscle weakness, wasting and fasciculations (muscle twitching) as well as deformities (contractures) of the joints due to spasticity [17, 18]. However, others had already described similar cases in the medical literature of the day [19, 20]. The British anatomist and surgeon, Charles Bell, had reported by 1830 the case of a middle aged woman with progressive paralysis of the limbs and tongue, preservation of sensation, and pathological evidence of degeneration of the anterior portion of the spinal cord. This confirmed Bell’s hypothesis that the anterior nerve roots of the spinal cord were associated with motor functions, the posterior roots with sensory functions. By 1850, F.A. Aran reported familial cases of what he called “atrophie musculaire progressive” (progressive muscular atrophy, PMA), which he thought to be a primary muscle disease but wisely did not exclude a spinal origin [21]. A few years later, Cruveilhier noted post-mortem atrophy of the anterior roots in one of Aran’s familial PMA cases [22], shifting suspicion from the muscles to the anterior horn cells as the site of the primary lesion in these patients. Finally in 1969, Charcot and Joffroy reported “several patients with the following conditions: paralysis with spasms of the arms and principally the legs, together with progressive amyotrophy, which was confined mostly to the upper limbs and trunk” [23]. They noted that sensation, bladder and bowel functions, and intellect were spared. Post-mortem examinations showed lesions of the gray matter and anterolateral columns of the spinal cord. In some cases, the cranial nerve roots were also atrophic. This is ALS as we know it today, but it would be another 5 years before Charcot formally used the term sclérose latérale amyotrophique to name this disorder [17, 18].

Charcot retains a special historical role in our current understanding of ALS. He was born in Paris, France in 1825, received his medical degree in 1853, and became a staff physician at the Salpêtrière Hospital in 1862. He rose to the ranks of Professor of Clinical Diseases of the Nervous System at the University of Paris, a position specially created for him. An eminent scientist, Charcot was recognized as one of the world’s most prominent professors of neurology, often referred to as the Father of Clinical Neurology. He developed the anatomo-clinical method in which the clinician first defines a condition based on scrupulous examination of large numbers of patients with similar neurological deficits and signs. In doing so, the archetype or classical form of a disease becomes apparent, as do its clinical variants [24]. Charcot was known to make incredibly detailed clinical notes about his patients, even sketching by hand their “deformities” and postures to fully illustrate his patient’s condition. He would later set up a photography studio in the Salpêtrière for this
purpose. The second step of the anatomo-clinical method employed detailed post-mortem studies to correlate anatomic lesions with the clinical signs previously documented. This second step is one that modern-day ALS clinicians have great difficulty making for various logistical and ethical reasons. Charcot identified two primary clinical-pathological correlations of weakness. The first linked muscle wasting and fasciculations with loss of nerve cells in the anterior horn of the spinal cord. The second linked weakness associated with spasticity and contractures of the joints to sclerotic lesions of the lateral spinal cord. Charcot eventually used the term amyotrophic lateral sclerosis (*sclérose latérale amyotrophique*) when he observed both types of weakness and anatomical lesions in the same individual. Now more than a century and later, ALS is still defined and diagnosed by clinical criteria (anatomo-clinical method step 1), and confirmed by post-mortem examination (anatomo-clinical method step 2) [25].

*Charcot’s acute observational skills, his patience for detailed neuro-anatomical post-mortem examinations, and his interest in the corticospinal tract, make him a particularly relevant historical figure to review in this thesis.*

Since ALS is rare (see previous section *Epidemiological considerations*) relative to other neurodegenerative diseases like Parkinson’s disease, the list of “famous” or well known people that have had the disease is short. They include the British theoretical physicist, Stephen Hawking; the British actor, David Niven; the Russian composer, Dimitri Shostakovich; Chinese revolutionary leader, Mao Tse Tung; and Swedish national television broadcaster, Ulla-Carin Lindquist.

In the United States and Canada, ALS is most often associated with baseball player Henry Louis (“Lou”) Gehrig. Gehrig was affectionately nicknamed “The Iron Horse” for his strength and endurance on the field, playing for the infamous New York Yankees. He played 2,130 consecutive games before retiring from baseball due to ALS in 1939. That record was not surpassed until 1995 by Cal Ripken Jr. of the Baltimore Orioles. Before more than 60,000 fans in Yankee Stadium on July 4th, 1939, Gehrig declared himself “the luckiest man on the face of the earth” despite knowing that he would likely die from ALS before his fortieth birthday. Gehrig was praised for his courage, gentlemanly conduct, and warm personality both on and off the field, qualities that have made him a legend and qualities that are so apparent in many ALS patients we see in our clinics today. The personality profile of ALS patients has never been formally studied however.

Finally, it was fitting that Ripken Jr. broke Gehrig’s consecutive game streak. Ripken was also renowned for his dependability and congeniality on the baseball field, and for his community service record off the field. The Cal Ripken Jr.-Lou Gehrig ALS Research Fund at Johns Hopkins School of Medicine was established in September 1995 in commemoration
of Ripken Jr’s record-breaking feat. He has also been instrumental in helping to raise funds for ALS Association chapters throughout the United States and affiliated chapters in Canada.

The Diagnosis of ALS

When reviewing the diagnostic process in ALS, one should attempt to clarify the nomenclature. The term motor neuron disease (MND) is sometimes used interchangeably with ALS, though this can be confusing. The term MND is best used as a broader “umbrella” term that includes ALS among a group of disorders characterized by motor neuron degeneration, among them progressive muscular atrophy (PMA), progressive bulbar palsy (PBP), and primary lateral sclerosis (PLS). The term ALS should be used only to describe the syndrome first described by Charcot [26] (see above). In this context, ALS accounts for about 85 percent of all MND cases. In the United Kingdom in particular, MND is still used more frequently to name the Charcot variant disorder. The diagnosis of ALS requires [27]:

The presence of:

- clinical, electrophysiological or neuropathological evidence of lower motor neuron (LMN) degeneration
- clinical evidence of upper motor neuron (UMN) degeneration and
- progressive spread of symptoms or signs within a CNS region or to other regions, as determined by history or examination, and;

The absence of:

- electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and
- neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

When ALS is in the advanced stages, it is fairly easy for clinicians, even general practitioners, to recognize. Early ALS however remains a challenge for most clinicians to recognize, even neurologists and neurosurgeons. The earliest symptoms of ALS can be vague and difficult for the patient to describe. For example, it is not uncommon for a patient to remark that their affected extremity feels “numb”, when in fact what they’re experiencing is early weakness and/or dexterity problems. The earliest signs of the disease can be equally non-specific, and clinicians, including specialist consultants, will understandably seek out more common and less grave diagnoses first. On average, the diagnosis of ALS is not made until about 12-14 months after the patient was aware of their first symptoms. Diagnostic
delay in ALS is of great importance, due to the fact that the success or failure of candidate new therapies for ALS will depend on when in the disease course treatment commences. To date, the legacy of most human clinical trials of potential new therapies for ALS has been disappointment. Clinical trials in transgenic animal (mice) models of ALS, regardless of the compound being tested, very often delay disease onset and/or slow progression, but subsequently fail to show significant efficacy when administered to human patients. The mice have the advantage of being treated either well before they become symptomatic or show signs of disease, or they’re treated immediately when symptoms/signs begin. All but one of these transgenic clinical trials has translated successfully in human studies (riluzole), probably because disease is too far advanced in human patients when treatment is initiated.

The El Escorial ALS diagnostic criteria

The variability in clinical findings early in the course of ALS, and the lack of any biological diagnostic marker make *absolute* diagnosis difficult and compromise diagnostic certainty in clinical practice, therapeutic trials and other research purposes. Criteria for the diagnosis of ALS were defined in 1994 [28] by the World Federation of Neurology Research Committee on Motor Neuron Diseases at a meeting in El Escorial, Spain. The El Escorial criteria for the diagnosis of ALS seek to classify patients into 3 main categories- *definite*, *probable* and *possible* ALS. Clinical findings of both lower and upper motor neuron disease must both be present in at least one CNS region for the clinical diagnosis to be even possible. Pure lower or upper motor neuron disorders can only be suspected as ALS using these criteria. The criteria were formulated primarily for use in clinical trials, but have nevertheless been used widely in clinical practise, albeit with some difficulties, particularly when the disease is in the early stages. It was felt that the criteria should be revised in order to increase their sensitivity and ease of use, and these revisions were made in 1998 by the same group [27] and are summarized schematically in Figure 1.
Figure 1: Revised El Escorial criteria for ALS. EMG= electromyography; NCS= nerve conduction studies; LMN= lower motor neuron; UMN= upper motor neuron; SOD1= superoxide dismutase one.
The Corticomotoneuronal System and ALS

The corticospinal tract (CST) provides the most direct pathway over which the cerebral cortex controls movement. In rodents and marsupials, this influence is exerted largely upon interneurons in the dorsal spinal gray matter. Ascending the phylogenetic scale through carnivores and primates, the number of corticospinal axons grows, and corticospinal terminations shift progressively toward the interneurons of the intermediate and ventral horn, ultimately forming increasing numbers of synaptic terminations directly on the motor neurons themselves. Degeneration of upper motor neurons (UMNs) is an integral component of ALS, and understanding their particular pathophysiology is important in the global understanding of ALS.

In 1905, Campbell compiled his histological studies of the cerebral cortex in normal apes and humans, in a number of human amputees, and in two patients with amyotrophic lateral sclerosis (ALS). He noted that the giant Betz cells of the pre-central gyrus occupied the cortical territory where Grunbaum and Sherrington had previously elicited contralateral movement with electrical stimulation in the same individual apes and which degenerated in ALS [29]. Some 50 years earlier J-M Charcot, based on his own anatomical observations in ALS, postulated that “the lesion of the white columns is primary, but always, and necessarily, followed by a lesion of the gray matter. In these cases it is the pyramidal bands which are first attacked and then subsequently the anterior horns”. This “UMN then LMN” hypothesis is now viewed with healthy skepticism, but it remains undetermined whether degeneration of UMNs and the CST is followed by demise of the anterior horn cells (AHC), or if corticospinal degeneration is retrograde and subsequent to death of AHCs. The other hypothesis of course is that upper and lower motor neurons in ALS succumb independently of each other.

Campbell recognized that the cortex containing Betz cells provided the most direct connections from the cerebral cortex to AHCs in the spinal cord (a “cortico-spinal” projection) now commonly referred to as the corticomotoneuronal (CM) projection. Lawrence and Kuypers [30] subsequently described detailed anatomical connections that individual CM axons make with AHCs, showing that they synapse with several or many AHCs belonging to the same motor neuron pool but also those of synergistic muscles. Ascending the phylogenetic scale from rat to cat through monkey and chimpanzee to humans, corticospinal terminations shift progressively more ventrally in the spinal gray, achieving progressively closer interneuronal access to motor output and increasing the number of direct synaptic contacts to the spinal motor neurons themselves [31-33]. Thus as one ascends the phylogenetic scale, there are increased numbers of direct CM connections. Humans probably have more direct CM connections than any other species, and humans therefore depend more on their corticospinal tract for normal movement. The direct CM
system is one of the newest in the development of the nervous system, and biological principles expound that the latest-acquired system is the first to be affected by disease processes. This translates into a greater vulnerability of the latest-expressed genes i.e. those related to the CM and its connections. This large cell is incapable of mitosis, rendering it most prone to environmental insult. The incapacity for cell division, which could be reparative, means it more readily succumbs to such insults.

Although the giant Betz cells often are assumed to be the only neurons from which the corticospinal projection originates, many other large and moderate sized pyramid-shaped cells in layer V of the cerebral cortex contribute axons to the corticospinal tract. This can be illustrated by comparing the number of Betz cells in the human primary motor cortex, 34,000, to the number of axons in the human pyramid, 1,100,000. Only 3% of the CST arises from the giant Betz cells, while the remaining 97% arises from smaller neurons. Nevertheless, the bulk of the lateral corticospinal tract is crossed, and descends in a position close to the dorsolateral aspect of the ventral horn, where the motor nuclei of distal limb musculature are located [33, 34]. In contrast, the bulk of the anterior corticospinal tract is uncrossed, and descends in a position close to the anteromedial aspect of the ventral horn, where the motor nuclei of proximal limb musculature are located. In general the lateral corticospinal tract exerts greater control over distal limb than axial musculature, whereas the anterior corticospinal tract exerts more control over axial and proximal limb than distal limb musculature.

It is now well recognized that CM connections in humans are involved in much more than just movement via direct and indirect excitation of motor neurons. They also [35]:

- control afferent inputs, including nociception;
- are involved in selection, gating and gain of control of spinal reflexes;
- effect autonomic control;
- play an important role in short and long-term plasticity of spinal cord circuits; and
- are involved in trophic function during development, and probably in repair after injury.

The multiple, important functions of the CM must surely have widespread implications in ALS.
Initial studies of the CST and CMs in ALS employed electrical stimulation of the motor cortex [36, 37]. This technique is quite painful for the recipient, and seldom is used anymore to investigate ALS. In many patients, there was marked attenuation or absence of the motor evoked potential (MEP). Less frequently the response had delayed onset, and was accompanied by a modest prolongation of central motor conduction time (CMCT). The abnormalities were more frequent when MEPs were recorded from lower limb muscles. Using transcranial magnetic stimulation (TMS), it was subsequently shown that marked a reduction or absence of the MEP usually accompanied bulbar palsy and was frequently associated with UMN signs [38, 39]. More recent studies have confirmed that central conduction is generally either normal or modestly prolonged in ALS [40, 41]. One interesting exception is the markedly slow central conduction observed in patients homozygous for the D90A SOD1 gene mutation.

The amplitudes of MEPs from TMS are usually much smaller than those of motor responses evoked by conventional supramaximal stimulation of a peripheral nerve. Even in normal subjects, there is considerable variation with sequential cortical stimuli. This variability in MEP amplitude is due to varying desynchronization of the descending volleys, and variable degrees of phase cancellation. This greatly limits the sensitivity of MEP amplitude measurements to detect central motor conduction failure. A recently developed triple stimulation technique (TST) enables resynchronization of the MEP, and allows quantitative information on central conduction failure since it more accurately measures the proportion of motor units activated by the transcranial stimulus [42, 43]. The TST has been shown to be a sensitive method to detect UMN dysfunction in ALS, and abnormalities appear to correlate well with the extent of functional deficit [44].

It is now accepted that cortical excitability is increased in ALS. This is the result of both “increased excitability” and “decreased inhibition” due to dysfunction or loss of local circuit cortical inhibitory interneurons. The finding is not specific to ALS and has been observed in other neurodegenerative diseases. Several different physiological approaches can be used to assess the excitability of the motor cortex in ALS [45]. These include measurement of the intensity required to stimulate the motor cortex (cortical motor threshold), the duration of the cortical silent period and the response to paired magnetic stimulation [46-49].

A magnetic stimulus applied to the motor cortex during voluntary contraction of a target muscle induces a pause in EMG activity—the cortical silent period (CSP). The duration of this cortical silent period, recorded from a hand muscle, is normally about
120 milliseconds (ms). It is dependent on the stimulus intensity. The applied stimulus should be >120–150% of motor threshold [48, 49]. The mechanisms underlying the CSP are complex, multifactorial and incompletely understood [50]. Most of its duration is due to cortical inhibitory mechanisms mediated through local circuit intracortical interneurons. In ALS the duration of the CSP is reduced, but interpretation can be difficult when the cortical motor threshold is high and a sufficiently strong stimulus is not possible. This occurs in advanced ALS and in PLS [51].

Loss of cortical inhibition can also be demonstrated using paired stimulation (conditioning-test stimulus paradigm). In normal subjects there is marked attenuation of the test response following a sub-threshold conditioning stimulus using a very short (1–4 ms) conditioning-test interval [52-54]. This conditioning effect is due to both excitatory and local circuit cortical inhibitory mechanisms. The same type of attenuation does not occur after electrical brain stimulation, which stimulates post-synaptic structures. In ALS the inhibition induced by the conditioning stimulus is markedly reduced or absent [55], revealing a defect in pre-synaptic cortical inhibition. Riluzole, a glutamate antagonist and the only treatment approved (in many countries) for ALS, has been shown to correct this abnormality [56].

Over the last decade peristimulus time histograms (PSTH) have been used to assess the function of the UMN in ALS. This method is advantageous in that it assesses the behavior of individual spinal AHCs and their synaptic inputs. Each AHC receives inputs from a select group of CMs. Information about the function of this group of CMs can be derived by recording single AHC motor unit potentials using the PSTH method. Alterations in the firing of the AHC (motor unit) induced by a series of threshold magnetic stimuli are recorded [38, 57-61]. Corticospinal volleys evoked by the magnetic stimulus perturb the regular firing pattern of a voluntarily activated motor unit, resulting in a large ‘primary’ peak (PP) in the PSTH [48, 62]. In normal subjects and recording motor units in forearm muscles, the PP occurs approximately 20ms after the stimulus, indicating conduction through a fast-conducting, monosynaptic (CM) projection.

The primary peak in normal subjects is well synchronized, but sub-components can be recognized [63]. Sub-components reflect the sequential arrival of indirect descending volleys, or I-waves, typically initiated by a single cortical stimulus [48, 49]. Measurements of the primary peak allow inferences regarding conduction velocity in the CM fibers, the extent of temporal dispersion/desynchronization of descending volleys in the corticospinal tract, the timing of excitatory or inhibitory effects on the spinal motor neuron and the strength of the synaptic input [48, 49]. In sporadic ALS, several abnormalities in the primary peak have been reported. The most consistent is desynchronization and increased duration [60, 64, 65].

The notion that the PSTH abnormalities in ALS result from supraspinal rather than AHC disease is supported by independent observations. For example, comparison of the
PSTH derived from peripheral nerve stimulation of Ia afferent fibers with that derived from cortical magnetic stimulation whilst recording from the same motor unit, has shown that only the cortical PSTH is abnormal [66]. If anterior horn cell disease was responsible for the PSTH abnormalities in ALS, the peripheral PSTH should be equally abnormal since both Ia afferents and descending CM fibers synapse on the same cell membrane. Both inputs are glutamatergic. More direct evidence of the supraspinal origin of the PSTH abnormalities in ALS comes from the study of patients with Kennedy's disease, an exclusively LMN disorder. The PSTH in Kennedy’s disease is normal, thus supporting a supraspinal origin for the primary peak abnormalities in ALS [67].

The mechanisms underlying the PSTH abnormalities in ALS are complex and differ at different stages of the disease [65, 68]. Early in ALS CMs are hyper-excitabile and there is marked repetitive CM firing in response to the cortical stimulus, resulting in desynchronization and an increase in the duration of the PP in the PSTH. The initial volley is not delayed so that the primary peak has a normal onset latency. There is a proportionally greater compound excitatory post-synaptic potential (EPSP) generated at the AHC leading to an increased amplitude of the primary peak. As the pyramidal tracts begin to degenerate, descending CM volleys are less able to raise the AHC membrane to threshold, and to do so requires increased temporal summation. This induces greater desynchronization and a further increase in the duration of the primary peak. Early I-waves may be ineffectual in exciting anterior horn cells so that the primary peak onset becomes modestly delayed. With further CM degeneration cortical threshold rises. These PSTH abnormalities may not be specific to ALS [69, 70] but the spectrum of changes that occur as the disease progresses are possibly highly specific.

The future of human clinical CM physiology using TMS in ALS has to be considered in its own right, and in light of an ever increasing number of cortical imaging techniques. These include magnetic resonance imaging (MRI), functional MRI (fMRI), diffusion tensor MR, magnetization transfer imaging (MTI), positron emission tomography (PET), and magnetic resonance spectroscopy (H^1MRS). There would seem little doubt that some or all of these technologies hold great promise for the future in terms of assessing UMN dysfunction in ALS. However at present, most have some restrictions. These include: cost, which tends to prohibit widespread use and repeated examinations needed for longitudinal studies; complexity of analysis resulting in uncertain specificity of results; and other technical difficulties related to the disease itself, such as inability for many ALS patients to remain prone for lengthy periods. Newer applications of TMS, especially those involving slow and fast repetitive stimulation to assess intracortical excitability have considerable appeal [71]. They can be readily applied in longitudinal studies and have potential application in measuring therapeutic responsiveness.
The Neuropathology of ALS

Charcot provided the first clear neuropathological descriptions of ALS [23], describing not only the shrinkage and loss of anterior horn cells in the spinal cord and bulbar motor nuclei, but also pallor in the lateral columns of the spinal cord. Loss of motor neurons in the cervical and lumbar regions of the spinal cord, and in some patients the bulbar motor nuclei and the motor neurons in the precentral gyrus, are the most prominent microscopic abnormalities [72]. There is relative sparing of the nucleus of Onufrowicz as well as motor nuclei innervating the third, fourth and sixth cranial nerves. Although more difficult to appreciate, there is loss of giant Betz cells in layer 5 of the motor cortex as well as large motor neurons from layers 4 and 6. Easier to appreciate than cortical cell loss is myelin pallor in the corticospinal tract.

Neuropathological features of ALS include an array of protein aggregates and inclusion pathology, including Bunina bodies, ubiquitinated inclusions, and neurofilament-rich hyaline conglomerate inclusions. Protein aggregates in non-neuronal cells may also be seen [73].

ALS Genetics

About the same time that Charcot was first describing ALS, Gregor Mendel was reporting new ideas about genetics in his paper “Experiments in plant hybridization” in 1865. Although the majority of ALS cases are sporadic (SALS) with unknown cause, 5-10% of cases are familial (FALS). Intuitively, FALS refers to the manifestation of ALS in 2 or more blood relatives. However identifying FALS cases in clinical practice is not always straightforward. When ALS is inherited in an autosomal dominant fashion with high penetrance, the situation is clear. Penetrance refers to the frequency or probability of the expression of an allele (manifesting as a phenotype, for example ALS), which can be further modified by interaction with other genes or via external or environmental factors. When disease penetrance is reduced or when inheritance is autosomal recessive, identifying FALS cases can be difficult. For example, a patient may not be aware of any first degree relatives affected by the disease when they’re initially investigated or diagnosed with ALS. Months or years later, it may be learned that “distant” blood relatives whom the patient was not aware of previously, have also been affected by ALS. Such families can be challenging for genealogic investigation, and can be problematic for genetic counselors.

Dominantly inherited FALS is clinically and pathologically similar to SALS, perhaps with variations in age and anatomical site of onset, or disease progression. Recessively inherited FALS frequently has juvenile onset and slow progression. Dominant and recessively inherited forms of ALS have been linked to a number of gene loci (Table 2), and
for some the gene has been identified. The one ALS gene most relevant to this thesis is SOD1, and is discussed in detail below. The reader is directed to the references provide in Table 2 for further details on other ALS associated genes and gene loci.

### Table 2: Genes associated with ALS and other motor neuron disorders

<table>
<thead>
<tr>
<th>Disease</th>
<th>Locus</th>
<th>Gene</th>
<th>Inheritance</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS1</td>
<td>21q22.1</td>
<td>SOD1</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Except D90A</em></td>
<td>Variable phenotypes</td>
<td></td>
</tr>
<tr>
<td>ALS2</td>
<td>2q33</td>
<td>ALS2/alsin</td>
<td>Recessive</td>
<td>Juvenile onset</td>
<td>[76, 77]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Slow progression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Some PLS-like</td>
<td></td>
</tr>
<tr>
<td>ALS3</td>
<td>18q21</td>
<td>Not known</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[78]</td>
</tr>
<tr>
<td>ALS4</td>
<td>9q34</td>
<td>senataxin</td>
<td>Dominant</td>
<td>Juvenile onset</td>
<td>[79-81]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Slow progression</td>
<td></td>
</tr>
<tr>
<td>ALS5</td>
<td>15q15.1-q21.1</td>
<td>Not known</td>
<td>Recessive</td>
<td>Juvenile onset</td>
<td>[82]</td>
</tr>
<tr>
<td>ALS6</td>
<td>16q12.1-2</td>
<td>Not known</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[83-85]</td>
</tr>
<tr>
<td>ALS7</td>
<td>20ptel</td>
<td>Not known</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[85]</td>
</tr>
<tr>
<td>ALS8</td>
<td>20q13.33</td>
<td>VAPB</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Variable phenotypes</td>
<td></td>
</tr>
<tr>
<td>ALS-FTD</td>
<td>9q21-q22</td>
<td>Not known</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[87]</td>
</tr>
<tr>
<td>ALS-PD-D</td>
<td>17q21</td>
<td>tau</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[88, 89]</td>
</tr>
<tr>
<td>X-linked ALS</td>
<td>Xp11-q12</td>
<td>Not known</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[90]</td>
</tr>
<tr>
<td>Progressive</td>
<td>2p13</td>
<td>Dynactin p150</td>
<td>Dominant</td>
<td>Adult onset</td>
<td>[91, 92]</td>
</tr>
<tr>
<td>LMN disease</td>
<td></td>
<td></td>
<td></td>
<td>Slow progression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LMN predominant</td>
<td></td>
</tr>
</tbody>
</table>

SOD1= superoxide dismutase 1; VAPB= vesicle-associated membrane protein B; ALS-FTD= ALS with frontal dementia; ALS-PD-D= ALS with Parkinsonism and dementia; LMN= lower motor neuron

**ALS1: superoxide dismutase 1 (SOD1)**

It was a major breakthrough in ALS research in 1993 when it was reported that about 20% of individuals with familial ALS have mutations in the SOD1 gene on chromosome 21 [74]. The SOD1 gene is small with only 5 exons. The SOD1 protein itself is 153 amino acids long, is ubiquitously expressed, and catalyzes the dismutation of superoxide radicals (O2•−) to hydrogen peroxide (H2O2) and oxygen (O2). SOD1 gene mutations confirmed in patients with ALS now total 118 (www.alsod.org). Mutations occur rarely in exon 3, which encodes the active site of the enzyme. Gain of a novel cytotoxic function, rather than loss of normal
dismutase activity, is implicated in causing motor neuron degeneration. The line of evidence for this includes the following:

- mice with no active SOD1 do not develop an ALS-like disorder but only a subtle motor deficit [75];
- mice transgenic for mutant human SOD1 develop a motor neuron disease phenotype, and disease is conferred independently of SOD1 activity [93-95];
- there is no relationship between superoxide dismutase activity and clinical features in human disease [96, 97]; and
- monosomy for chromosome 21, causing reduced levels of SOD1, is not associated with ALS [98].

Though expressed in all body tissues, and in highest levels in organs such as liver and kidney, motor neurons are predominantly affected. This biological paradox is not well understood. Why all mutations in the SOD1 gene, having widely varying effects on protein structure and stability, all cause ALS is not known.

Various ideas have been put forward as to what the novel mutant associated function might be, including enhancement of protein nitrosylation, enhanced peroxidase activity, exposure of toxic copper at the active site, accumulation or aggregation of abnormal protein, and mutant SOD1-induced mitochondrial damage. This is discussed in more detail under the heading Mutant SOD1 pathophysiology.

**Genetic risk factors for ALS**

A number of genetic risk factors have been implicated in modifying the course or clinical presentation of ALS, including age and site of disease onset, as well as survival. These genes are listed in Table 3 and discussed in more detail below. To date, studies of these risk factors have been unable to show that any of them are major determinants of disease risk or clinical phenotype.

**Neurofilaments**

Two important lines of evidence suggest neurofilaments play a role in ALS pathogenesis. First, accumulation of aggregated neurofilaments is often seen in ALS pathology. Second, over-expression of either the neurofilament light- or heavy-subunit in transgenic mice leads to motor neuron degeneration.

Neurofilaments are present in neuronal cells, and play a central role in intracellular transport and in determining axonal caliber [99]. They are composed of light, medium and heavy subunits, each with a central rod domain plus globular head (N-terminal) and tail (C-terminal) regions. In human beings, a sequence of three amino acids- lysine, serine and proline (KSP)- is repeated 44 or 45 times in the tail end of the heavy neurofilament subunit (NF-H). In ALS, alterations in this domain of the NF-H gene have been found in a number of
studies, though with variable results. An early study showed codon deletions in five SALS patients [100], corresponding to 1.4% of cases. The deletions were not found in healthy controls, but were found in unaffected relatives of the mutation carrying patients. Subsequent studies showed either deletion [101] or insertion [102] of complete KSP repeat motifs in a small number (~1%) of cases, only one case being FALS. Although results have varied from study to study, collectively they suggest that NF-H mutations are a minor risk factor for sporadic ALS.

Table 3: Genetic risk factors for ALS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Observations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurofilament heavy subunit, NEFH</td>
<td>Insertions or deletions of KSP repeats</td>
<td>Found in ~1% of SALS</td>
<td>[103]</td>
</tr>
<tr>
<td>Apolipoprotein E, APOE</td>
<td>ε4 allele</td>
<td>• Shorter survival</td>
<td>[104, 105]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Bulbar onset at earlier age</td>
<td></td>
</tr>
<tr>
<td>Survival motor neuron 1, SMN1</td>
<td>1 or 3 gene copies</td>
<td>Increased risk for ALS</td>
<td>[106]</td>
</tr>
<tr>
<td>Survival motor neuron 2, SMN2</td>
<td>Homozygous deletions</td>
<td>Shorter survival time</td>
<td>[107]</td>
</tr>
<tr>
<td>Vascular endothelial growth factor, VEGF</td>
<td>2 polymorphisms in promotor region</td>
<td>Increased risk of ALS</td>
<td>[108]</td>
</tr>
<tr>
<td>Ciliary neurotrophic factor, CNTF</td>
<td>Homozygous null mutation</td>
<td>Younger age at onset in FALS with SOD1 mutations and SALS</td>
<td>[109]</td>
</tr>
<tr>
<td>GluR2</td>
<td>Altered RNA editing</td>
<td>RNA editing efficiency altered in ALS spinal motor neurons vs. DRPLA and normal controls</td>
<td>[110]</td>
</tr>
</tbody>
</table>

KSP= lysine, serine, proline repeats; SALS= sporadic ALS; FALS= familial ALS; GluR2= subunit of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) glutamate receptor; RNA= ribonucleic acid; DRPLA= dentatorubropallidoluysian atrophy

**Apolipoprotein E**

The ε4 allele of apolipoprotein E (ApoE ε4) is a risk factor in various neurodegenerative diseases, including Alzheimer’s disease, where it is associated with earlier onset. Varied and conflicting results have emerged from a number of studies exploring the role the ε4 allele as a risk factor in ALS. Mui and colleagues did not find an increased frequency of the ε4 allele, nor any association between disease parameters such as bulbar versus spinal onset, age at onset or survival [111]. Siddique and colleagues came to a similar conclusion in their study of ApoE genotypes and ALS [112]. Two other studies [104, 105] also showed
no significant difference in the frequency of the ε4 allele in ALS and control populations; however, Al-Chalabi and colleagues found a significant correlation between ε4 allele carriers and bulbar onset disease, while Drory and colleagues found the ε4 allele correlated with a shortened survival [104]. At best, the collective evidence for ApoE ε4 as a risk factor for ALS suggests a possible role in determining some clinical features (age and location of onset).

**Survival motor neuron genes**

Survival motor neuron genes 1 and 2 (SMN1 and SMN2) are highly conserved paralogous genes present on chromosome 5, both of which are often present with more than one copy. Typically, there are 2 copies of SMN1 and 1-2 copies of SMN2. Deletion of SMN1 copies causes recessive spinal muscular atrophy, but the disease phenotype is determined by the copy number of SMN2, with a milder phenotype correlating to more copies [113]. Various studies have investigated whether differences in SMN copy number are also associated with ALS. One study showed that SMN2 gene deletions were approximately four times more common in SALS patients than in controls, and that survival times were shorter in patients with homozygous SMN2 deletions compared to patients without any deletions [107]. Conversely, a subsequent study found no differences in SMN2 copy number between ALS and control groups, whereas the proportion of ALS patients with either 1 or 3 copies of SMN1 was significantly increased compared to controls [106]. Further work is needed to clarify the roles of SMN1 and SMN2 as genetic modifying factors in ALS.

**VEGF**

Vascular endothelial growth factor (VEGF) was first described and named for its role in angiogenesis, but has subsequently been shown to act as a neurotrophic factor [114]. Expression of VEGF increases during hypoxia, due to binding of hypoxia-inducible factors to a hypoxia-response element (HRE) in the VEGF promoter. Deletion of the VEGF allele in mice is embryonic lethal, but deletion of the HRE in the promoter leads to adult onset motor neuron degeneration reminiscent of ALS [115]. Screening of the HRE revealed no sequence alterations in ALS cases from 4 European populations, but single nucleotide polymorphisms in the 5’ untranslated region previously reported to correlate with reduced VEGF expression were found to be over-represented in ALS cases and conferred an increased risk of ALS of 1.8 fold [108]. Both disease and control individuals carrying the “at risk” polymorphisms were found to have lower VEGF levels in serum compared to those without the polymorphisms. However, one of the four populations studied and another independent report [116] failed to show this association between the “at risk” polymorphisms and ALS, suggesting greater complexity of the effect of VEGF as a risk factor. Injection of a lentiviral vector expressing VEGF into various muscles with retrograde transport to spinal motor neurons has recently been reported to significantly delay onset and slow progression of disease in G93A-SOD1
mice, even when the injection is given after onset of symptoms [117]. Intracerebroventricular delivery of recombinant VEGF protein into G93A-SOD1 rats has also recently been shown to significantly extend survival when given either before or at the time of onset of symptoms [118].

**CNTF**

A homozygous mutation in a splice site of ciliary neurotrophic factor (CNTF), present among ~2% of European and Japanese populations, results in production of a biologically inactive protein that causes a 15-20% reduction in motor neuron number, but does not itself cause disease. One individual in a family carrying the V148G-SOD1 mutation developed disease at an unusually early age and subsequent analysis showed this patient to be the only family member to carry the V148G-SOD1 mutation and have the homozygous null mutation in CNTF [109]. 

Analysis of G93A-SOD1/CNTF−/− mice also showed significantly earlier onset of disease compared to G93A-SOD1 CNTF+/+ mice, suggesting that the homozygous null CNTF mutation may be a risk factor for early onset of disease.

**Glutamate transport and receptors**

Glutamate toxicity has long been suspected in ALS pathogenesis. Reduced glutamate transport has been observed in motor cortex and spinal cords from ALS patients [119, 120]. Decreased expression of the glial-specific glutamate transporter EAAT2 (GLT-1) was reported by Rothstein et al. a decade ago [121], but to date the mechanism is not entirely clear. Splice variants in EAAT2 mRNA transcripts leading to loss of expression were not different between ALS patients and controls in 2 different studies [122, 123].

Metabotropic glutamate receptors (mGluRs) are also implicated in glutamate mediated motor neuron death. Using in situ hybridization and immunohistochemistry, Anneser and colleagues [124] found group I and II mGluR mRNAs and protein was significantly upregulated in ALS spinal cord compared to controls (mGluR5 > mGluR1 > mGluR2/3). In vitro, the mGluR group I agonist 3,5-dihydroxyphenylglycine induced proliferation in chick spinal cord astroglial cultures. Moreover, addition of cerebrospinal fluid (CSF) from ALS patients resulted in significantly higher proliferation rates than control CSF. In both cases, the effect could be blocked by addition of the mGluR group I antagonist 1-aminoindan-1,5-dicarboxylic acid. Their data suggested that stimulation of glial mGluRs through mediators present in the CSF may contribute to glial proliferation and astrogliosis in ALS. A subpopulation of motor neurons in ALS patients has recently been shown to have reduced GluR2 subunit mRNA editing efficiency compared to Purkinje cells or motor neurons in controls, a change that would be predicted to result in AMPA receptors with increased calcium permeability [110].
Mutant SOD1 pathophysiology

The mechanism(s) by which mutant SOD1 damages and destroys motor neurons (predominantly) is not well understood, but a number of molecular mechanisms may converge to produce cytotoxic cell death. Evidence from experimental models supports the view that cell–cell interactions are crucial determinants for the induction of motor neuron death in ALS. Although motor neurons are induced to die by the expression of mutant SOD1, other cell types actively participate in the generation of the pathological phenotype. Mutant SOD1 might act at different levels as shown in Figure 2.

Figure 2: Converging molecular mechanisms in motor neuron death induced by mutant superoxide dismutase 1 (SOD1). The beneficial dismutase reaction catalyzed by SOD1 is shown by the reaction going left to right in black text. Reactions indicated by dotted-line arrows are potentially deleterious to the cell. In the figure, (a) - (e) are intra- and extracellular processes whereby mutant SOD1 may act adversely on motor neurons, for example by:
(a) exacerbating the production of oxygen radicals and nitrotyrosine [(1) and (2) in Fig. 2 respectively] with consequent alterations of intracellular proteins and lipids (oxidative stress). In addition to it’s normal dismutase activity, SOD1 can also function as a peroxidase, where it can either catalyze the reverse of the normal dismutase reaction, or it can use the H$_2$O$_2$ produced in the dismutation reaction as a substrate to generate hydroxyl radicals (OH’), both reactions labeled (1) in Fig. 2. Mutant SOD1 has a greater peroxidase activity than wild-type SOD1;

(b) inducing mitochondrial dysfunction and further production of reactive oxygen species (ROS), by activating caspases, and depleting energy (ATP);

(c) impairing the ubiquitin–proteasome pathway (UPP), which is necessary for the degradation and elimination of aberrant or misfolded cellular proteins. This will have consequences for misfolded mutant SOD1 protein and for other cytoskeletal proteins that have been phosphorylated, and require degradation. UPP problems in handling these species could cause ubiquitinated aggregates to accumulate;

(d) inhibiting the activity of the glial glutamate transporter EAAT2. An increase in extracellular glutamate might cause an over-activation of glutamate receptors with consequent increase in calcium influx. An excess of intracellular calcium might cause mitochondrial toxicity and alter a series of calcium-dependent enzymatic pathways leading to the dysfunction of the cell machinery;

(e) activating microglia and astrocytes to produce and release pro-inflammatory cytokines and other potential toxic factors (nitric oxide, NO). These factors might activate different intracellular enzymes, such as the mitogen activated kinase p38, that phosphorylate neurofilaments (NF) with consequent alterations of the cytoskeleton and formation of ubiquitinated (Ub)-inclusions (purple). COX2, cyclooxygenase 2.

Therapeutic Strategies in ALS: Future, Past and Present

Even though the precise mechanism(s) by which mutant SOD1 protein causes motor neuron degeneration and death are not fully understood, being able to selectively shut off the expression of mutant SOD1 before cellular damage occurs would seem the ideal form of therapy.

The duplex of 21-nt RNA, known as small interfering RNA (siRNA), has recently emerged as a powerful gene-silencing tool. Several methods to down-regulate SOD1 protein expression utilizing siRNAs have been developed. For instance, siRNAs have been designed that recognize a single nucleotide alteration in the SOD1 gene. These siRNAs can selectively suppress G93A mutant SOD1 expression in transfected mammalian cells, leaving wild-type SOD1 intact [125]. Earlier this year, Saito el al. [126] reported that crossing
transgenic anti-SOD1 siRNA mice with transgenic G93A mutant SOD1 mice prevented the development of disease in offspring by inhibiting mutant G93A SOD1 production in the central nervous system. They concluded that siRNA-mediated gene silencing can stop the development of familial ALS associated with SOD1 mutations. Also earlier this year, Raoul and colleagues [127] reported that in G93A transgenic mice, intra-spinal injection of a lentiviral vector that produces RNAi-mediated silencing of SOD1 via short hairpin RNAs (shRNAs) significantly delayed onset and slowed progression of disease. Furthermore, SOD1 shRNA can be delivered to motor neurons through intramuscular administration of a viral vector such as the rabies-G-pseudotyped equine infectious anemia viral vector [128], or an adenovirus-associated viral vector [129]. The potential for intramuscular rather than intra-spinal injection in human treatment is attractive.

Most of the present treatment for ALS is directed toward targeting presumed pathogenic pathways, including those of oxidative stress, apoptosis, glutamate excitotoxicity, and neuro-inflammation. All of these approaches have been tested in mutant SOD1 transgenic mice, the average benefit being approximately 20% longer survival. As mentioned previously, the most successful of clinical trials of therapeutics in transgenic mice have proven to be failures in subsequent human trials, except for the anti-glutamate agent riluzole.

The potential for stem cell therapy in ALS is great, but there are numerous difficulties to overcome. Diffuse disease requires that stem cells, or their effects, reach most of the brain and spinal cord. Varying degrees of degeneration, in different regions of the nervous system, further complicates the potential for stem cell therapy. The goal of stem cell therapy, in addition to replacing dead neurons and supporting non-neuronal cells (microglia and astrocytes), should be the delivery of gene products and perhaps growth factors. Stem cells that are successful in differentiating into normal neurons or neuronal like cells might provide a sufficiently normal environment for the rescue and subsequent protection of the host motor neurons.

Despite the present lack of substantial therapy in ALS for the disease pathogenesis, overall care of ALS patients has improved substantially. A holistic-multidisciplinary approach administered through specialized ALS centers may extend life in ALS patients attending them [130], compared to patients attending general neurology clinics. However more study is need in this area. The American Academy of Neurology (AAN) Practice Parameter on ALS published in 1999 [131, 132] was subsequently shown to be associated with improvement in the standard of care of ALS patients in the United States and Canada, although results were biased by data received from specialized ALS centers [133]. Percutaneous endoscopic gastrostomy (PEG) and non-invasive positive pressure ventilation (NIPPV) can have a positive impact on quality of life, palliative care, and survival if administered appropriately,
and support and education given through ALS societies and other patient support groups is highly beneficial.
Materials and Methods

The following information supplements the materials and methods described in the thesis papers.

Subjects

ALS patients

Adults (19 years of age or older) diagnosed with amyotrophic lateral sclerosis at The ALS Clinic at Vancouver General Hospital in Vancouver, British Columbia (BC), Canada were the primary focus of all studies in this thesis (papers I-VI). Some affected and unaffected blood relatives of these ALS patients were also studied (papers I and III).

ALS patients with confirmed SOD1 mutations from Sweden (n=7) and the United States (n=4) were also studied (papers IV-VI).

Disease controls

Ten individuals diagnosed with primary progressive or relapsing-remitting variants of multiple sclerosis (MS) at The MS Clinic at the University of British Columbia in Vancouver, British Columbia, Canada, were studied as controls in paper V.

Healthy controls

Thirty-two individuals without neurological or other relevant disease, including conditions contraindicated by study related procedures, were studied as controls in papers IV, V (each n=11) and paper VI (n=21).

Ethical Considerations

The studies described in this thesis were performed according to guidelines set forth in the Declaration of Helsinki [134]. All studies were approved by the clinical research ethics boards at The University of British Columbia (Vancouver, British Columbia, Canada) and Umeå University. All subjects gave informed consent prior to their voluntary participation.

Ethical considerations for genetic research

Because genetic material is by its nature shared by both close and distant biological relatives, identifying a genetic causative agent in a primary study subject has implications and possible risks for individuals beyond the primary subject. Genetic research studies are unique in that they have the potential for indirectly gathering knowledge about biological relatives or groups by studying only a few individuals. In addition, a primary subject may indirectly give informed consent on behalf of their family members or community when they provide information about their family members or social structures to investigators as part of a genetic research study protocol.
Institutional ethics review boards (ERBs) must take matters like these into consideration when reviewing genetic research projects. Unfortunately there are no well defined guidelines at this time for ERBs to follow regarding this issue. Extra precautions must be taken by study investigators to ensure the privacy of all members of a family or group who may be at increased risk for disease when a few members are found to be carriers of disease associated genes.

**Human genetic research in Canada**

It’s important to stress that most ALS patients participating in this study were informed of their genetic test results. In Canada, under Article 8.1 of the Tri-Council Policy Statement on the Ethical Conduct for Research Involving Humans [135], an individual participating in a genetic research study has the right to know their test results. The researcher shall “…report results to that individual if the individual so desires.” This was required to be clearly stated in the informed consent document. Participants were instructed that at any time, they had the right to request their test result. Only the patient or an individual they designated in writing on their informed consent document (usually next of kin) could request the test result. Only when the patient had received sufficient information and counseling to understand the benefits and risks of having this information were the tests results given to them. This legal requirement in Canada blurs the boundaries between genetic testing for research and clinical purposes. However, because the study was conducted at a world renowned ALS clinical center, we could take all necessary precautions to protect the autonomy, privacy and well-being of each individual and their family members. Subjects were encouraged to exercise discretion in discussing their involvement in this study with others, to help ensure that sensitive information about them remains private.

**Subject Recruitment**

**SOD1 gene analysis (Paper I)**

**Patients:** ALS patients seen at the Vancouver ALS Clinic were informed about the study, and if interested, asked to read a detailed consent form outlining the study rationale and objectives, inclusion/exclusion criteria, study related procedures, potential risks, etc. They were given the opportunity ask questions of the study investigators, and encouraged to discuss the matter further with their family members and general practitioner before making a decision. ALS patients found to carry SOD1 mutations and who requested their results were counseled accordingly.

**Limitations:** Though the Vancouver ALS Clinic is the primary referral center for ALS in the province of BC, not all ALS patients diagnosed and managed there could be screened for SOD1 mutations during the study period. Hence our data does not fulfill criteria for a true epidemiologic study. Rather, the intent of the study was to describe genotypic and
phenotypic features of a select group of BC ALS patients with SOD1 mutations, a study that has been done only rarely in other geographical areas, but not previously in Canada. See the Summary of Paper 1 for further discussion about this study’s limitations.

Patient’s Relatives: It was left to the patient’s discretion whether or not to discuss the results with adult (19 years of age or older) family members, particularly those who were unaffected but most at risk of being gene carriers. They were offered family counseling should it be required in future. In most cases patients elected to inform their adult family members who would then contact us for further information regarding the study. The investigators never made unsolicited contact with a patient’s family members. Whenever possible, face to face interviews were conducted with interested family members. Those choosing to participate in the study did so with the knowledge that although the results would be used for research purposes, they retained the right to know their genetic testing results, as stated in the study informed consent document. Consequently, most unaffected, at-risk individuals wishing to participate in the study did so under guidelines similar to those used for pre-symptomatic testing in Huntington’s disease [136] and those proposed for use in ALS [137].

Neuropathological analysis (Paper II)

In some cases, subjects gave written informed consent prior to death for an autopsy to be performed, and for their tissues to be used for research purposes. Consent forms were co-signed by next of kin, usually the patient’s spouse, and consent was re-confirmed with the next of kin upon the patient’s death.

Sample Collection

Blood

To maintain confidentiality, subjects were assigned a unique code upon enrollment in the study. The code consisted of a number and the subject’s initials, but in scrambled order. The code was linked to personal identifying information at the study site in Vancouver. Blood samples were labeled only with this code number.

Blood (on average 30mls) was drawn into ethylenediamine tetraacetic acid (EDTA) containing vacuum tubes. Samples were packed according to international standards for shipping non-infectious human blood samples, and couriered within 24 hours from Vancouver to Umeå. Total transit time averaged 2 days. In Umeå, samples are assigned a second unique 4 digit code number for laboratory specific cataloguing and tracking. Samples were centrifuged to separate blood into plasma, erythrocytes, and leukocytes (buffy coat) then stored at -80°C until further analyzed.
Tissue

Autopsies (Papers I and II) were performed 24-48 hours after death at Vancouver General Hospital. The whole brain and spinal cord were removed. In patients with SOD1 mutations and some SALS patients, sections of the fresh brain, spinal cord, and various non-CNS tissues were frozen at -80° C for later biochemical analyses. For histological and immunohistochemical studies, tissues were immersion-fixed in phosphate buffered 10% formalin (3.7% formaldehyde, pH 7), and paraffin embedded blocks were made.

SOD1 Analysis

SOD1 enzymatic activity

The direct spectrophotometric method was used to measure SOD1 activity. Most assays of the superoxide anion radical (O2•-) are indirect because of the very rapid, spontaneous disproportionation of O2•- in aqueous solutions at neutral pH. The direct spectrophotometric method uses alkaline conditions under which the O2•- radical is more stable, and which allows for measurement of O2•- decay using an ordinary spectrophotometer at 250 nm.

Before assay, packed erythrocytes are lysed by addition of 17 volumes 5mM Na HEPES buffer, pH 7.4. The assay is performed directly on the hemolysates without prior precipitation of the hemoglobin. An excess of O2•- is produced by KO2 at pH 9.5. Addition of hemolysates containing SODs catalyzes the decay of O2•- to hydrogen peroxide and oxygen in a first degree order. By measuring the speed of the decay, and after correction for the very slow second degree spontaneous disproportionation of O2•-, the SOD activity can be calculated. One unit is defined at the SOD1 enzymatic activity that brings about a decay of O2•- at a rate of 0.1 sec⁻¹ in 3ml buffer. One unit in the assay corresponds to 4.25ng wild-type human SOD1. Results are expressed as units/mg of hemoglobin. Hemoglobin is determined with a standard cyanomethemoglobin assay.

For the differentiation between SOD2 and the CuZn SODs, 3 mM of cyanide is added to the buffer which almost completely inactivates CuZn SODs. Currently there is no such possibility in discriminating between SOD1 and SOD3 activity in this assay. The assay is very sensitive for CuZn SOD activities but for the detection of SOD2 the assay is much less sensitive and only about 10% of that of CuZn SODs.

SOD1 genotyping

All SOD1 genetic analyses reported in Papers I-VI were performed at Umeå University between January 1st, 1998 and December 31st, 2004. Genomic DNA was isolated from leukocytes according to standard methods using the Nucleon BAAC2 kit (Amersham). Exons 1 to 5 of the SOD1 gene were screened for mutations using the polymerase chain reaction single-stranded conformational polymorphism (PCR-SSCP) analysis described by
Andersen et al [138]. The primers and PCR conditions used are shown in Table 4. PCR-SSCP is based on the principles that: a. specific regions of genomic sequences can be efficiently labeled and amplified simultaneously by using labeled substrates in the polymerase chain reaction, and; b. in non-denaturing polyacrylamide gels, the electrophoretic mobility of single-stranded nucleic acid depends not only on its size, but also on its sequence. Hence, a change in the expected nucleotide sequence will cause an altered banding pattern. Samples that showed altered SSCP banding patterns were subsequently sequenced using an Applied Biosystems 3730xL automated fluorescent DNA sequencer.

**Table 4: Primers and PCR conditions used for genotyping**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Direction</th>
<th>Oligonucleotide Sequence</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward</td>
<td>5'-TTC CGT TGC AGT CCT CGG AA-3'</td>
<td>158 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGG CCT CGC AAC ACA AGC CT-3'</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Forward</td>
<td>5'-TTC AGA AAC TCT CTC CAA TT-3'</td>
<td>208 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGT TTA GGG GCT ACT CTA CTG T-3'</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Forward</td>
<td>5'-CAT ATT GCT TTT TCT TCT T-3'</td>
<td>138 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCA TTG ATT ACA AGA GTT AAG G-3'</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Forward</td>
<td>5'-CAT CAG CCC TAA TCC ATC TGA-3'</td>
<td>236 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGC GAC TAA CAA TCA AAG TGA-3'</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward</td>
<td>5'-GTT ATC TTC TTA AAA TTT TTT ACA G-3'</td>
<td>133 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTC AGA CTG TTA AAA TTT TTT ACA G-3'</td>
<td></td>
</tr>
</tbody>
</table>

PCR conditions were:
Exon 1: 95° C for 15' then 35 cycles of 94° C for 1', 65° C for 1', 72° C for 1', ending with 72° C for 10'  
Exons 2-5: 95° C for 15' then 35 cycles of 94° C for 1', 55° C for 1', 72° C for 1', ending with 72° C for 10'

The D90A (Asp90Ala) mutation in exon 4, in which adenine is replaced by cytosine at base pair 1078, causes the introduction of a new site for the restriction enzyme ItaI (Fnu4HI) as shown:

\[
\begin{align*}
3'...G C N G C... & \rightarrow \\
5'...G C N G C... & \leftarrow \\
\end{align*}
\]

This restriction enzyme test provides rapid and specific screening for the D90A allele, for example, in untested ALS patients with clinical characteristics resembling D90A homozygous ALS, or in relatives of ALS patients confirmed to have D90A.
**SOD1 antibodies**

For western immunoblots and immunohistochemistry studies performed on human tissues in Paper II, we used antibodies raised against amino acids 57-72 (amino acids CTSAGPHFNPLSRKHG) and 131-153 (amino acids CNEESTKTGNAGSRLACGVIGIAQ) of the human SOD1 sequence.

The SOD1 primary antibodies were polyclonal rabbit antibodies raised against keyhole limpet hemocyanin (KLH) conjugated peptides. All peptides have a cystein on the N or C-terminal end of the peptide to be able to link the peptide to KLH or to a sulfolink coupling gel which was used for purifications of the antibodies. The purification process was made in two steps. First the rabbit antisera were purified on a protein A sepharose (Amersham) which was followed by purification on the sulfolink coupling gel (Pierce) with the corresponding peptides coupled.

**Neuropathological Studies**

For Paper II, histological and immunohistochemical studies were performed on both CNS and non-CNS tissues. In the brain, the following areas were examined: primary motor cortex, pre-motor and pre-frontal cortices, hippocampus, basal ganglia, thalamus, cerebellum, midbrain, and lower brainstem. The spinal cord was examined at the cervical, thoracic and lumbar levels. The liver, kidney and quadriceps muscle were also examined.

**Staining procedures (Vancouver)**

Formalin fixed, paraffin-embedded tissue sections were stained using hematoxylin and eosin (HE), Luxol fast Blue (LFB), Gallyas and modified Bielschowsky silver methods. Immunohistochemistry was performed using the Ventana ES automated staining system. The primary antibodies employed recognized:

- ubiquitin (DAKO anti-ubiquitin; 1:500, following microwave antigen retrieval);
- nonphosphorylated neurofilament (NF) (DAKO anti-neurofilament protein; 1:2,000, following protease digestion);
- phosphorylated neurofilament (pNF) (Sternberger SMI 31; 1:8,000, following protease digestion);
- hyperphosphorylated tau (Innogenetics AT-8, pS202/pS205; 1:2,000 following heat pretreatment and Sigma TAU-2; 1:1,000 with 3 hours initial incubation at room temperature);
- α-synuclein (Zymed anti-α-synuclein; 1:10,000, with heat pretreatment);
- glial fibrillary acidic protein (DAKO anti-glial fibrillary acidic protein; 1:4,000);
- CD68 (DAKO KPI, 1:800 with heat pretreatment) and MHC class II (DAKO CR3/43; 1:500, with heat pretreatment).
Staining procedures (Umeå)

SOD1 immunohistochemistry was performed in Umeå using a Ventana AEC immunohistochemistry system, following the manufacturer’s protocol with pre-treatment by microwave irradiation of the sections in citric acid buffer for 3x5 minutes. The antibodies used for SOD1 staining were previously described.

Western Blots

Homogenization of samples

In Paper II, human tissues were homogenized in 25 volumes of PBS (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.0, in 137 mM NaCl, 2.7mM KCl) with EDTA-free Complete (Roche Diagnostics) antiproteolytic cocktail added, using an Ultraturrax (IKA) for two minutes. This was followed by sonication of the homogenate using a Sonifier Cell Disruptor (Branson) for 1 minute.

Blotting procedures

Samples were solubilized 1:1 in 2X SDS-PAGE (SDS-polyacrylamide gel electrophoresis) sample buffer (100 mM Tris-HCl, pH 6.8, 10% b-mercaptoethanol, 20% glycerol, 4% SDS, and bromphenol blue) and heated for 10 min at 95° C, and then separated on SDS-PAGE gels (Bio-Rad). The gels were then electroblotted onto polyvinylidine difluoride (PVDF) membranes (Amersham) Blots and were probed with the SOD1 antibodies described above. Biotinylated anti-rabbit IgG antibody and horseradish peroxidase-conjugated streptavidin (Amersham) were used for detection of bands, and chemiluminescence was generated using substrate from Pierce or Amersham. Bands were visualized on film and by using a Fluor-S Multiimager or Chemidoc imager and Quantity One software (Bio-Rad).

Statistical Analysis

Statistical calculations were performed using UNISTAT version 4.0 for Windows (Papers IV and V), NCSS Statistical System for Windows[139] (Paper VI) and Microsoft Excel (descriptive statistics only, Paper I).

In papers IV and V, differences between groups were analyzed by one-way analysis of variance (ANOVA). When significant differences were found with ANOVA, analysis continued with pairwise comparisons (F test, t test) to determine differences between groups. If the F test revealed significantly different variances between groups, the separate variance t test was applied, otherwise the pooled variance t test was used.

More conservative statistical approach was used in paper VI. Groups were compared initially using the non-parametric Kruskal-Wallis one-way analysis of variance by ranks. Post-hoc pairwise comparisons were made using the Kruskal-Wallis multiple comparison Z test.
When tests for equal variances failed, pairwise comparisons were performed directly using the Aspin-Welch unequal variance $t$ test, or alternatively the Kolmogorov-Smirnov test for different distributions. Fisher’s exact test was used to compare proportions.
Objectives of this Thesis

The overall aim of this thesis was to investigate the role(s) that mutations in the SOD1 gene play in patients with ALS in the western Canadian province of British Columbia (BC). The thesis has 4 main routes of investigation: molecular genetic analysis, genealogical investigation, neuropathological examinations, and neurophysiological studies. No similar studies have been conducted in Canada previously, and it is hoped this thesis will enhance the current global knowledge of ALS associated with SOD1 gene mutations.

The specific objectives of each study were:

- **Paper I**- to estimate the frequency of SOD1 gene mutations in patients with ALS in BC, to conduct thorough clinical and genealogical investigations of the mutation carrying patients and their families, and to compare the clinical and demographic features of mutation carrying patients to patients without SOD1 mutations.

- **Paper II**- to describe the clinical and neuropathological phenotype of an ALS patient with atypical presenting features which resulted in a significant diagnostic delay, and who was found to have a novel mutation in exon 3 of the SOD1 gene.

- **Paper III**- to describe the atypical clinical and neurophysiological findings in D90A heterozygous relatives of a homozygous D90A ALS patient.

- **Paper IV**- to examine corticospinal function in ALS patients homozygous for the D90A SOD1 mutation and ALS patients without SOD1 mutations using peristimulus time histograms (PSTHs).

- **Paper V**- to examine corticospinal function in patients with multiple sclerosis using PSTHs, and to compare the findings to those previously recorded in ALS patients homozygous for the D90A SOD1 mutation.

- **Paper VI**- to examine corticospinal function in patients with different SOD1 mutations using PSTHs, and to compare the findings amongst patients with these mutations and with findings previously recorded in D90A homozygous patients.
Results and Discussions of Papers

Paper I - SOD1 gene mutations in ALS patients from British Columbia, Canada:
clinical features and ethical issues in management

- 253 ALS patients were screened for SOD1 mutations, all of whom resided in British Columbia, but none of whom were related to each other;
- 41% of patients were female (n=104), giving a male:female ratio in this cohort of 1.43:1;
- of 205 patients from the study cohort on which familial history was available, 22% (46) were familial cases, indicating a selection bias for FALS cases;
- 4.7% of all screened patients (12/253) carried SOD1 gene mutations in exons 1, 3 or 4; 25% (3/12) of SOD1 mutation carriers were apparently sporadic cases;
- stated alternatively, 19.6% (9/46) of known FALS and 1.8% (3/159) of known SALS in this study carried SOD1 mutations;
- 5 different SOD1 mutations were identified in the study cohort: A4V (n=2), D76Y (n=1), G72C (n=1), D90A (n=2) and I113T (n=6); the D90A cases were homozygous;
- median age at onset of first symptoms in mutation carrying patients was slightly younger than patients without SOD1 mutations (54.9 and 60.3 years respectively);
- as a group, patients with SOD1 mutations experienced a diagnostic delay (time from onset of first symptoms to diagnosis) almost twice as long as patients without SOD1 mutations (22.6 and 12 months respectively);
- as a group, patients with SOD1 mutations survived on 15 months longer than patients without mutations (38 and 23.2 months respectively);
- age at onset of first symptoms among the SOD1 mutation carrying patients shows a spectrum, with patients carrying the I113T mutation (and the single G72C carrier) forming the older end of the spectrum (48 years or older at onset), and A4V, D76Y and D90A patients at the opposite (younger) end (Fig. 3);
- all patients with SOD1 mutations had spinal onset disease, 75% with first symptoms in the lower extremities; in contrast, 26% of patients without SOD1 mutations have bulbar onset disease, while 49% of patients without SOD1 mutations and with spinal onset disease have first symptoms in the lower extremities (Table 5);
- patients with SOD1 mutations were twice as likely to have either fast or slowly progressing disease than patients without SOD1 mutations (Table 5);
- unusual clinical symptoms and signs in the patient carrying the G72C mutation, and unexpected genealogical information regarding the relatives of one of the D90A homozygous cases, prompted more detailed studies of these cases and their families (reported in Papers II and III respectively).
Table 5: Clinical features of ALS patients at study enrollment (n= 205*)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Bulbar</th>
<th>Spinal</th>
<th>Lower Limb</th>
<th>Upper Limb</th>
<th>Diffuse</th>
<th>Truncal or Respiratory</th>
<th>Definite or Probable</th>
<th>Probable, Lab Support</th>
<th>Possible or Suspected</th>
<th>Progression Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>ALL SOD1 cases</td>
<td>12</td>
<td>0</td>
<td>100 (12)</td>
<td>75 (9)</td>
<td>23 (3)</td>
<td>0</td>
<td>0</td>
<td>50 (6)</td>
<td>33 (4)</td>
<td>17 (2)</td>
<td>33.3 (4)</td>
</tr>
<tr>
<td>ALL nonSOD1 cases</td>
<td>193</td>
<td>26 (44)</td>
<td>74 (127)</td>
<td>49 (62)</td>
<td>42 (53)</td>
<td>3 (4)</td>
<td>6 (7)</td>
<td>59 (101)</td>
<td>19 (33)</td>
<td>21 (36)</td>
<td>49 (86)</td>
</tr>
<tr>
<td>nonSOD1 Female</td>
<td>80</td>
<td>41 (29)</td>
<td>59 (42)</td>
<td>60 (25)</td>
<td>33 (14)</td>
<td>5 (2)</td>
<td>2 (1)</td>
<td>60 (44)</td>
<td>18 (13)</td>
<td>22 (16)</td>
<td>54 (38)</td>
</tr>
<tr>
<td>nonSOD1 Male</td>
<td>113</td>
<td>15 (15)</td>
<td>85 (85)</td>
<td>44 (37)</td>
<td>47 (39)</td>
<td>2 (2)</td>
<td>7 (6)</td>
<td>58.7 (57)</td>
<td>20.6 (20)</td>
<td>20.6 (20)</td>
<td>46 (48)</td>
</tr>
<tr>
<td>nonSOD1 Sporadic</td>
<td>160</td>
<td>27 (39)</td>
<td>73 (106)</td>
<td>51 (54)</td>
<td>42 (46)</td>
<td>2 (2)</td>
<td>5 (5)</td>
<td>60 (86)</td>
<td>19 (27)</td>
<td>21 (30)</td>
<td>53 (78)</td>
</tr>
<tr>
<td>nonSOD1 Familial</td>
<td>33</td>
<td>19 (5)</td>
<td>81 (21)</td>
<td>43 (9)</td>
<td>38 (8)</td>
<td>9.5 (2)</td>
<td>9.5 (2)</td>
<td>56 (15)</td>
<td>22 (8)</td>
<td>22 (6)</td>
<td>30 (8)</td>
</tr>
</tbody>
</table>

In the table, results are reported as percent (n). *Although 253 patients were enrolled in the study and screened for SOD1 mutations, data was missing on 48 patients at the time of press, and these patients were not included in the descriptive statistics summarized in Table 5.

Figure 3: Age at onset of first symptoms in 12 ALS patients found to carry mutations in the SOD1 gene. Patients are shown in ascending order (left to right) of age at onset. The key for identifying mutations is shown at the bottom of the chart. Patients with the I113T mutation (dark grey bars), and the single G72C patient (stippled bar), were the oldest at onset of first symptoms.
In Paper I, one patient of English ancestry was found to carry a novel mutation, G72C, in exon 3 of the SOD1 gene;

- a family history of ALS was reported in the propositus’ sister and father (Fig. 4A and B); all 3 cases were spinal onset, but with widely varying age at onset (sister-49, father-55 and propositus-72 years);
- survival was also variable within this family, the sister of the propositus surviving only 12 months, the father nearly 20 years, and the propositus 4.5 years; neither of the affected relatives of the propositus could be genotyped for SOD1;
- the paternal family history has been difficult to trace as the propositus’ father and 3 siblings were orphaned as small children; none of the siblings died of ALS or other similar neurological disorder;
- the propositus had disease onset in the proximal limb girdle muscles (symmetrically), and never manifest UMN signs or fasciculations, contributing to increased diagnostic delay;
- an autopsy was performed, revealing marked reduction of lower motor neurons in the dorsal horns of the spinal cord, and SOD1 immunoreactive inclusions in remaining motor neurons; there was no significant pathology in the upper motor neurons or the corticospinal tract;
- SOD1 levels were reduced in CNS tissues compared to control values, and were comparable to another patient from the same study cohort (Paper I) found to carry the A4V mutation;
- G72 is in a loop of the SOD1 molecule adjacent to H71 which ligands the stabilizing zinc ion; substituting a larger cysteine residue for glycine is predicted to result in misfolding of the protein and instability, corresponding with the more than 50 percent decrease in SOD1 enzyme activity and protein levels measured;
- though not discussed in the paper, this patient was not prescribed the ALS approved medication, riluzole, due to her multiple other health issues and in part due to the advanced state of her ALS.
Figure 4: A shows the large pedigree of a patient with the G72C SOD1 mutation, with the propositus’ immediate family framed, and enlarged in B. The propositus is indicated by an arrow to the bottom right of the symbol.
in Paper I, one of 2 patients found to be homozygous for the D90A SOD1 mutation had an unusual family history in that her father reportedly had a thinning leg over many years; the family was of Finnish ancestry;

- weakness of the right leg was noticed by the propositus’ father in the late 1950’s when he became unable to rise up on his right toes, then subsequently unable to run;

- subsequent SOD1 genotyping showed the propositus’ father and mother were heterozygous carriers of the D90A mutation; the mother was healthy;

- examination revealed wasting of both the right leg and thigh compared to the left side (Fig. A and C), and weakness of ankle flexion, extension, inversion, eversion and toe flexion on the right side;

- EMG examination revealed diffuse chronic denervation, with active denervation in the right anterior compartment muscles; NCS and MEPs were unrevealing;

- imaging of the spine was normal;

- CT scan of the right leg and thigh at the junction of the pelvis and upper thigh showed fatty replacement of the anterolateral thigh muscles; a more severe pattern of muscle atrophy and fatty replacement was noted in the right posterior compartment muscles (not shown in figure), with milder involvement of the anterior compartment muscles, the left leg was normal;

- no new weakness or wasting has been noted 7 years since the original examination and this individual has remained otherwise healthy;

- an older brother of the propositus, also heterozygous for D90A, has had longstanding back pain, cramping and fasciculation in the posterior compartment muscles, but denies weakness;

- he had asymmetrical reflexes with spread in the legs, otherwise his examination was normal;

- EMG showed some evidence of chronic denervation.
Figure 5: Anterior (A, B) and posterior (C, D) views of the lower limbs of III:2, father of index D90A homozygous ALS patient, Case 5 in Paper I. III:2 is heterozygous for D90A. These images show modest but noticeable asymmetry in muscle bulk between the right and left sides. Muscle wasting was noted in both the right leg and thigh. A and C were photographed in December 1997. Leg circumference at that time, measured 12cm below the inferior border of the patella, was 5cm smaller on the right side. The right thigh was 2.5cm smaller than the left, measured 20cm above the superior border of the patella. B and D were photographed in October 2005, and little change in muscle bulk or leg asymmetry is noted. However, measurements of the legs and thighs were not made at the same time the recent photos were taken.

Figure 6: Computed tomography (CT) scan at the junction of the pelvis and upper thigh in III:2 showing fatty replacement (white arrowhead) of the anterolateral thigh muscles on the right side. A more severe pattern of muscle atrophy and fatty replacement was noted in the right posterior compartment muscles (not shown), with milder involvement of the anterior compartment muscles. The left leg was normal.
Papers IV - Preserved slow conducting corticomotoneuronal projections in amyotrophic lateral sclerosis with autosomal recessive D90A CuZn-superoxide dismutase mutation

- 8 ALS patients homozygous for the D90A SOD1 gene mutation, 12 ALS patients without SOD1 mutations, and 11 healthy control subjects underwent studies of corticospinal (corticomotoneuronal) function using peristimulus time histograms (PSTH);
- 6 of the 8 D90A homozygous patients participating in this study were from Sweden and traveled to Vancouver, Canada to be examined; one D90A was from the United States, and the last was from Vancouver; all other patients and controls were from Vancouver;
- all D90A patients participating in this study were of Scandinavian heritage, either of Swedish or Finnish extraction; all displayed the stereotypical phenotype associated with D90A homozygosity previously well described [138, 140], including the Canadian and American patients;
- PSTHs were constructed from 3-7 voluntarily recruited single motor unit potentials recorded with a monopolar needle electrode in the extensor digitorum muscle;
- primary peaks (PP) in the PSTHs were observed in all but one experiment (one motor unit) in a patient without a SOD1 mutation;
- a number of PSTH primary peak parameters were measured and compared amongst study groups: onset latency, number of excess bins, duration, synchrony and amplitude;
- all PSTH parameters differed significantly between healthy control subjects and D90A patients;
- PPs were delayed in D90A patients relative to healthy controls and SALS without SOD1 mutations; in addition, delayed primary peaks in D90A were usually preceded by an interval of motor unit inactivity or suppression;
- stimulus threshold and PP amplitude were not significantly different between SALS without SOD1 mutations and healthy controls, but all other PP parameters were abnormal in SALS;
- stimulus threshold and PP synchrony were not significantly different between D90A and SALS patients.

Paper V - The physiological basis of conduction slowing in ALS patients homozygous for the D90A CuZn-SOD mutation

- 10 patients with multiple sclerosis (MS) were studied subsequently to Paper IV, using identical methodology, and results compared to previously reported data for D90A and ALS patients without SOD1 mutations;
- the intent of the study was to identify more subtle differences in the slow corticospinal conduction observed in both D90A and MS patients using conventional MEP studies;
- PP onset latencies in MS patients were somewhat delayed relative to normal, and similar to those observed in ALS patients without SOD1 mutations, but were significantly shorter than those recorded in D90A patients;
stimulus threshold and PP amplitude were also abnormal in MS patients relative to healthy controls;

PPs were significantly more delayed and dispersed in D90A relative to MS, and cortical thresholds were significantly higher.

**Paper VI- Corticomotoneuronal dysfunction in ALS patients with different SOD1 mutations**

- patients with 4 other SOD1 mutations (A4V, I113T, G41D and G114A), as well as 21 new healthy controls subjects were examined using PSTHs;
- the intent of the study was to compare corticospinal function in patients with markedly different clinical disease phenotypes caused by different SOD1 mutants, including the D90A patients studied previously;
- the cortical threshold was significantly reduced in 3 patients with the A4V mutation relative to patients with other SOD1 mutations, SALS patients and D90A patients studied previously, but also relative to healthy controls;
- PP amplitudes, also referred to as EPSP amplitudes in Paper VI, were larger in A4V patients relative to all other groups apart from D90A homozygous patients studied previously;
- PPs were well synchronized in A4V patients relative to all other patient groups, and similar to healthy controls;
- patients with I113T mutations had significantly more dispersed PPs than SALS patients, but otherwise, clinical features and PSTH parameters were similar between I113T and SALS without SOD1 mutations.

**Conclusions**

**Paper I-** Although analysis of data for this manuscript is preliminary at the time this book went to press, the estimated frequency of SOD1 mutations amongst ALS patients in the western Canadian province of British Columbia (4.7%) was similar to what has been reported from previous studies worldwide. The patients with SOD1 mutations are slightly older on average than SOD1 mutation carrying patients in previously reported North American studies, and none of the SOD1 patients in this study had bulbar onset disease. The reason for this is unclear, but is not likely a factor of age as the median age of onset of the SOD1 mutation carrying patients was similar to that of patients without SOD1 mutations. Furthermore, 26% of patients without SOD1 mutations had bulbar onset disease, including 19% of familial non SOD1 cases. One quarter (3/12) of SOD1 mutation carrying patients identified were apparently sporadic cases, making further investigation and genetic counseling of these families extremely challenging.

**Paper II-** The unusual myopathic-like, lower motor neuron exclusive phenotype of the patient with the novel G72C SOD1 mutation contributed to a diagnostic delay of 33 months from onset of first symptoms, about 11 months longer than the average diagnostic delay for patients without SOD1 mutations. We conclude that SOD1 gene testing earlier in the diagnostic workup of this
patient would have dramatically shortened the diagnostic delay. When more meaningful therapies for ALS are available in the (near) future, an excessive diagnostic delay such as this could have serious negative consequences for therapeutic response. The absence of upper motor neuron disease in this case was proven neuropathologically. We conclude also that the G72C SOD1 mutation found in this patient was disease causative, despite the impossibility of genotyping the previously affected relatives, and despite the mutation not being reported (to date) in any other ALS patients worldwide.

**Paper III:**
The heterozygous D90A father and brother of a homozygous D90A patient in our study cohort each showed symptoms and signs of motor system involvement, including abnormal neurophysiological testing. This was particularly evident in the father, who had clinical and EMG findings compatible with a focal motor neuron disorder, monomelic amyotrophy (of the leg). Other diagnoses that may have explained his clinical presentation had been excluded. Though similar results from other D90A recessive families had not been reported before (or since) we published this article, our results suggest that a phenotype can develop in heterozygous carriers of the D90A mutation, even in families where true ALS is inherited recessively.

**Papers IV, V and VI:**
PSTHs indicate that slow conducting or/polysynaptic corticomotoneuronal connections are preserved and functional in D90A homozygous cases, a phenomenon that might not be detected using conventional MEP studies. In D90A homozygous ALS, cortical inhibition is preserved if not exaggerated. These features may reflect the slow disease progression associated with this unusual SOD1 mutant. The same slow pathway and exaggerated cortical inhibition is not apparent in patients with idiopathic disease and typical survival. Individuals with MS show different PSTH abnormalities to D90A, with the same degree of PP dispersion, but significantly shorter onset latencies and near normal cortical thresholds supporting the hypothesis that corticospinal desynchronization in D90A and MS patients is caused by different pathologies. Patients with A4V and I113T SOD1 mutations have distinctive corticomotoneuronal changes that are different from those in D90A and patients without SOD1 mutations.
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References


