The pathophysiology of amyotrophic lateral sclerosis

Author:
Vucic, Ostoja Steve

Publication Date:
2007

DOI:
https://doi.org/10.26190/unsworks/17578

License:
https://creativecommons.org/licenses/by-nc-nd/3.0/au/
Link to license to see what you are allowed to do with this resource.

Downloaded from http://hdl.handle.net/1959.4/40729 in https://unsworks.unsw.edu.au on 2023-08-05
The pathophysiology of amyotrophic lateral sclerosis

Ostoja Steve Vucic

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Medicine, University of New South Wales, November 2007
ACKNOWLEDGMENTS

I am forever indebted to my supervisor, Associate Professor Matthew Kiernan, for the immense energy, effort and time he has invested in my career over the last 3 years. The sense of fulfilment that I have obtained from research and the achievements derived thereof, are in no small part attributed to his sharp research acumen, wisdom and his dedication to fostering a supportive research environment that inspires medical scientists to develop an “eye for detail”. I would like to thank my co-supervisor, Professor Garth Nicholson, for his constant support and attention to the genetic aspects of these studies. I would also like to express immense gratitude to Dr Con Yiannikas, who inspired me to enter the world of neurophysiology and who has supported my career from the registrar days to present. I would also like to acknowledge James Howells and Louise Trevillion for their immense help with the development of the cortical excitability technique used in the present thesis. I am also grateful to Professor Hugh Bostock for designing the axonal excitability technique used in these studies. I am grateful to my colleagues at the Prince of Wales Medical Research Institute, Department of Neurology Westmead Hospital, Institute of Neurological Sciences, Prince of Wales Hospital and Department of Neurology, Neuromuscular Unit, Massachusetts General Hospital, Boston. Grant support was provided by the Motor Neuron Disease Research Institute of Australia and the National Health and Medical Research Council of Australia. Finally, I am grateful to my family, especially my wife Radmila for her endless support, patience and words of wisdom along with my children, Katarina and Jovan, for putting things into perspective.
This thesis examines the pathophysiology of motor neurone dysfunction, along with site of disease onset, in amyotrophic lateral sclerosis (ALS). The rationale for this thesis is the “dying forward” hypothesis, which suggests that corticomotoneurons cause anterograde excitotoxic degeneration of motor neurons in ALS.

Initially, axonal excitability studies were applied to ALS patients and revealed widespread axonal ion channel dysfunction, with increases in persistent Na$^+$ conductances and reduction in K$^+$ currents. Such changes result in axonal hyperexcitability, thereby resulting in generation of fasciculations and cramps. Subsequently, axonal excitability studies were applied to Kennedy’s disease (KD) patients, a pathological control group, revealing similar changes to ALS and suggesting that upregulation of persistent Na$^+$ conductances was responsible for generation of fasciculations.

To better understand the mechanisms underlying fatigability and to assess whether Na$^+/K^+$ pump dysfunction contributes to neurodegeneration in ALS, activity-dependent changes in axonal excitability were measured after a maximal voluntary contraction. The increase in threshold was more pronounced in ALS patients with predominantly lower motor neuron involvement, suggesting that peripheral factors were responsible for fatigue in ALS and that Na$^+/K^+$ pump function was preserved.
Having documented abnormalities of axonal excitability, a novel threshold tracking transcranial magnetic stimulation (TMS) technique was developed for assessment of cortical excitability. This technique overcomes the marked variability in the motor evoked potential with consecutive stimuli, a major limitation of the previous “constant stimulus” technique. After establishing normative data, threshold tracking TMS established that cortical hyperexcitability was an early and prominent feature in ALS. Similar changes were found in flail-arm variant ALS, a pure lower motor neuron form of ALS. In KD patients, cortical excitability was normal, thereby suggesting that cortical hyperexcitability is a primary event in ALS rather than a down-regulation of inhibitory control over the motor cortex in order to compensate for anterior horn cell loss.

In order to determine whether cortical hyperexcitability underlies motor neurodegeneration, longitudinal studies were undertaken in familial ALS subjects with the copper/zinc superoxide-dismutase-1 gene mutation. These studies established that cortical hyperexcitability precedes the development of clinical ALS, thereby suggesting that cortical hyperexcitability underlies the basis of motor neurodegeneration in familial ALS.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>PREFACE</td>
<td>vi</td>
</tr>
<tr>
<td>PUBLICATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>AWARDS</td>
<td>viii</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>2</td>
</tr>
<tr>
<td>Genetics of ALS</td>
<td>6</td>
</tr>
<tr>
<td>Pathophysiological mechanisms in ALS</td>
<td>11</td>
</tr>
<tr>
<td>Where does ALS begin</td>
<td>21</td>
</tr>
<tr>
<td>Axonal excitability testing</td>
<td>23</td>
</tr>
<tr>
<td>Activity-dependent changes in axonal excitability</td>
<td>36</td>
</tr>
<tr>
<td>Assessment of cortical excitability</td>
<td>38</td>
</tr>
<tr>
<td>Kennedy’s disease</td>
<td>49</td>
</tr>
<tr>
<td>METHODOLOGY</td>
<td>54</td>
</tr>
<tr>
<td>Chapter 1 Axonal excitability properties in amyotrophic lateral sclerosis</td>
<td>75</td>
</tr>
<tr>
<td>Chapter 2 Pathophysiological insights into motor axonal function in Kennedy’s disease</td>
<td>96</td>
</tr>
<tr>
<td>Chapter 3 Fatigue and axonal excitability changes in ALS</td>
<td>114</td>
</tr>
<tr>
<td>Chapter 4 Assessment of cortical excitability using threshold tracking techniques</td>
<td>135</td>
</tr>
<tr>
<td>Chapter 5 Novel threshold tracking techniques suggest that cortical hyperexcitability is an early feature in ALS</td>
<td>150</td>
</tr>
<tr>
<td>Chapter 6 Abnormalities in cortical and peripheral excitability in flail-arm variant ALS</td>
<td>173</td>
</tr>
<tr>
<td>Chapter 7 Cortical excitability in Kennedy’s disease</td>
<td>194</td>
</tr>
<tr>
<td>Chapter 8 Cortical hyperexcitability precedes the development of familial ALS</td>
<td>211</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>232</td>
</tr>
<tr>
<td>GLOSSARY OF ABBREVIATIONS</td>
<td>240</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>243</td>
</tr>
</tbody>
</table>
In 1869, Jean Martin Charcot (1825-1893) described a woman who suffered with extreme muscle weakness. Although her symptoms were initially attributed to hysteria, a mental disturbance that Charcot attributed to physiological causes, subsequent post-mortem examination of the woman revealed "sclerosis" in areas of the spinal column along with loss of anterior horn cells (spinal motor neurons). In 1874, Charcot provided a definitive name for the disorder, *amyotrophic lateral sclerosis*, based on his observation of 20 patients and five autopsy studies. Although Charcot’s clinical and pathological descriptions remain accurate, forming the basis for diagnosing amyotrophic lateral sclerosis (ALS), the site of disease onset remains elusive. In 1992, a “dying forward” hypothesis was proposed, suggesting that ALS was primarily a disorder of corticomotoneurons, with anterior horn cell degeneration mediated via an anterograde excitotoxic process (Eisen et al., 1992). In contrast, others have suggested that ALS begins in the muscle and that upper motor neurons are affected in a retrograde process (Appel, 1981) or that upper and spinal motor neurons degenerate independently (Kiernan and Hudson, 1991; Pamphlett et al., 1995). Resolving this issue would not only enhance the understanding of the pathophysiological processes in ALS, but will serve diagnostic and therapeutic purposes. This thesis will attempt to clarify the site of ALS onset by applying novel threshold tracking excitability techniques to both the central and peripheral nervous system.
PUBLICATIONS

Chapters 1-8 represent separate studies, which have been published in peer-reviewed journals as follows:

**Literature review**


**Chapter 1:**


**Chapter 2:**


**Chapter 3:**


**Chapter 4:**


**Chapter 5:**

Vucic S, Kiernan MC. Novel threshold tracking techniques suggest that cortical hyperexcitability is an early feature of MND. *Brain* 2006; 129: 2436-46.

**Chapter 6:**


**Chapter 7:**

Vucic S, Kiernan MC. Cortical excitability distinguishes Kennedy’s disease from ALS. *Clinical Neurophysiology* (Submitted, 25/10/07)

**Chapter 8:**

AWARDS

These studies have been awarded the following prizes.

1. TOW prize, Coast Medical Association, Open Junior Division, Prince of Wales Hospital, 2006 (Chapter 5).

2. Golseth Young Investigator Award, American Association of Neuromuscular and Electrodiagnostic Medicine (AANEM), 2007 (Chapter 7).


4. Nina Buscombe Travel Award, Motor Neurone Disease Association of Victoria, 2007 (Chapter 8).
LITERATURE REVIEW
INTRODUCTION

Motor neuron disease (MND) is a progressive neurodegenerative disorder of motor neurons in the spinal cord, brainstem, and motor cortex first described by Charcot (Charcot and Joffroy, 1869). The varied clinical presentations of MND include; (1) amyotrophic lateral sclerosis (ALS), which presents with a combination upper and lower motor neuron signs; (2) progressive muscle atrophy (PMA), with only lower motor neuron signs; (3) primary lateral sclerosis (PLS), with predominantly upper motor neuron features; (4) progressive bulbar palsy, with degeneration of bulbar nuclei and preservation of anterior horn cell and upper motor neuron function (Ross, 1997; Desai and Swash, 2002); and (5) frontotemporal dementia with ALS, whereby the features of ALS are preceded or develop in conjunction with a dementia characterized by socially inappropriate, impulsive behaviour and general deterioration in ability to perform routine daily tasks (Pinsky et al., 1975; Hosler et al., 2000).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is the most frequent form of MND with an incidence of 0.86-2.4/ 100 000 (McGuire et al., 1996; Nelson and McGuire, 2006; Sorenson et al., 2002) and prevalence of 4-6/100,000 (Chancellor and Warlow, 1992; Traynor et al., 1999; Seljeseth et al., 2000; Kiernan, 2003; Mandrioli et al., 2003; Nelson and McGuire, 2006). Age of onset is between 45-60 years, with a lifetime risk of 1 in 2000 (Boillee et al., 2006; Gros-Louis et al., 2006). Men are more frequently affected than women, with median survival being 2-3 years (Nelson and McGuire, 2006). Other MND phenotypes are less frequent, including PMA, which accounts for 10%, PLS 1-3 % and progressive
bulbar palsy 1-2% of all MND cases (Dumitru and Amato, 2002). Ten percent of MND cases are familial, in which two or more family members are clinically affected, with ~20% of cases attributed to mutations in the copper/zinc superoxide-dismutase-1 gene (SOD-1) on chromosome 21q22.1 (Rosen et al., 1993). For the purposes of this thesis, MND is synonymous with ALS.

**Clinical features and diagnosis**

ALS is characterized by the presence of upper and lower motor neuron features in accordance with the “El Escorial criteria” (Brooks et al., 2000b). Lower motor neuron (LMN) features include fasciculations, muscle wasting and weakness, while upper motor neuron (UMN) features include weakness and atrophy, slowness of movement, increased tone, hyper-reflexia, and extensor plantar responses. The majority of ALS patients (75-80%) present with asymmetrical weakness and wasting of the distal upper limb muscles, typically spreading along the neuraxis to affect contiguous motor neurons (Rowland, 1998; Francis et al., 1999; Talbot, 2002; Rocha et al., 2005). Fasciculations are often taken as the cardinal sign of ALS but are unusual as the initial presenting symptom (Gubbay et al., 1985). Bulbar-onset disease occurs in 20% of ALS cases and presents with progressive dysphagia and dysarthria. (Gubbay et al., 1985). Respiratory symptoms develop in a majority of ALS patients with advanced disease (Lechtzin et al., 2002; Houseman and Kelley, 2005; Rocha et al., 2005), and rarely is the presenting feature (Scelsa et al., 2002; Czaplinski et al., 2003). In 10% of cases, a slowly progressive form of ALS occurs, termed flail-arm variant, characterized by neurogenic weakness initially confined to shoulder girdle muscles with absence of UMN signs (Vulpian, 1886; Gowers,
In addition to motor symptoms, mild frontal lobe-type cognitive abnormalities are evident in 30-50% (Hanagasi et al., 2002; Abrahams et al., 2004; Abrahams et al., 2005; Ringholz et al., 2005) and dementia in 3.5% of ALS patients (Hanagasi et al., 2002). Extraocular and sphincter muscles, innervated by motor neurons not receiving direct projections from the motor cortex, are characteristically spared in ALS (Eisen et al., 1992; Kiernan, 2003).

**Neurophysiological features**

Motor nerve conduction studies (NCS) are normal in early stages of ALS, but in advanced disease the compound muscle action potential (CMAP) amplitude becomes reduced, reflecting denervation (de Carvalho and Swash, 2000). The CMAP changes may be accompanied by a mild reduction in motor conduction velocity (not less than 70% of the lower limit of normal) and mild prolongation of distal motor and F-wave latencies (nerve more than 30% of upper limit of normal) due to loss of large diameter fast-conducting fibres (Iijima et al., 1991; Daube, 2000; de Carvalho and Swash, 2000). Sensory NCSs are normal and overt abnormalities of sensory NCSs should raise suspicion of an alternative diagnosis (Daube, 2000; Eisen and Swash, 2001).

Electromyography (EMG) is essential in identifying LMN loss (Daube, 2000). The most frequently recognized abnormalities on EMG are fasciculations and spontaneous
“denervation” discharges [fibrillation potentials and positive sharp waves (PSWs)], indicative of ongoing motor neuron loss (Daube, 2000; Eisen and Swash, 2001). Fibrillation potentials and PSWs may not develop until one third of the motor neurons have deteriorated (Daube, 2000). Importantly, fibrillation potentials and PSWs may be evident in clinically normal muscles and hence EMG may aid in an early diagnosis (McComas, 1987; Daube, 2000; Eisen and Swash, 2001). Collateral sprouting of surviving motor axons results in large-amplitude, long-duration motor unit potentials [MUPs] (Daube, 2000; Eisen and Swash, 2001; Dumitru and Amato, 2002; Dumitru and Zwarts, 2002).

Surviving motor units may fire spontaneously, as fasciculations. Fasciculations are a classical feature of ALS and usually become widespread, but is rarely the presenting symptom (Gubbay et al., 1985). When detected in the tongue, fasciculations are highly specific for ALS (Li et al., 1986). Alterations in axonal membrane conductances (see section on axonal excitability), particularly increased persistent sodium (Na⁺) and reduced potassium (K⁺) conductances, contribute to the generation of fasciculations (Bostock et al., 1995; Horn et al., 1996; Mogyoros et al., 1998a; Kanai et al., 2006). Fasciculations are generated at the nerve terminals, though some arise at more proximal regions including at the level of the motor neuron (Wettstein, 1979; Roth, 1982; Roth, 1984; Layzer, 1994; Miller and Layzer, 2005). As ALS progresses, fasciculations develop a complex morphology with increased duration, amplitude and degree of polyphasia (Daube, 2000).
Within the gene, the coding sequences are called exons, while the non-coding regions are called introns. Each gene is composed of a polymer called deoxyribonucleic acid (DNA). DNA forms a double-stranded helix structure consisting of alternating sugar and phosphate residues to which a nitrogenous base is attached. Four types of bases are present in the human DNA; adenine (A), cytosine (C), guanine (G) and thymine (T). Adenine is paired to thymidine, and guanine with cytosine. In the exons, the DNA bases are arranged into codons, a triplet of bases that specify a particular amino acid, the building blocks of proteins.

An alternative form of the same gene is called an allele, and there are usually two alleles for the same gene, one transmitted for the mother and one from the father. When two alleles are the same they are referred to as being homozygous, while two different alleles are said to be heterozygous. The physical expression of alleles is called a phenotype. The patterns by which alleles are transmitted from one generation to the next include autosomal dominant, autosomal recessive and X-linked (dominant or recessive). Autosomal dominant mode of inheritance occurs when the phenotype is expressed even if the gene, which is located on an autosome, is heterozygous. In contrast, with autosomal recessive inheritance, the gene needs to be homozygous. X-linked inheritance refers to the allele being located on one of the sex determining chromosomes. In FALS, autosomal dominant and recessive modes of inheritance have been reported (Dewil et al., 2004; Andersen, 2006a; Gros-Louis et al., 2006).
Clinical evidence of UMN dysfunction may be elusive in ALS and obscured by motor neuron loss. Transcranial magnetic stimulation (TMS) may used in the clinical setting to assess for UMN dysfunction. The TMS parameters routinely measured in ALS patients include; corticomotor threshold, MEP amplitude, CMCT and CSP duration. Abnormality of these parameters, although inconsistently reported in ALS, establish UMN dysfunction, and may aid in the early diagnosis of ALS (Eisen et al., 1990; Caramia et al., 1991; Eisen et al., 1993; Desiato and Caramia, 1997; Ziemann et al., 1997c; Magistris et al., 1998; Eisen and Swash, 2001; Zanette et al., 2002b; Komissarow et al., 2004).

**Genetics of ALS**

Familial ALS (FALS) constitutes 0.8-13.5% of all ALS cases (Murros and Fogelholm, 1983; Li et al., 1988; Haverkamp et al., 1995; Fong et al., 1996; Andersen, 2006a). To date 59 genetic loci and seven genes have been identified, with one gene remaining uncharacterised (Gros-Louis et al., 2006; Dunckley et al., 2007). Mutations in one of these genes, SOD-1 gene, results in classical ALS, called ALS1, and will be discussed in this thesis (Dewil et al., 2004; Andersen, 2006a; Gros-Louis et al., 2006; Pasinelli and Brown, 2006).

Genes are functional units that encode specific products that exert activity within or outside the cell and are located on chromosomes. All adult human cells, including neurons, contain two homologous sets of 23 chromosomes, 22 non-sex determining chromosomes (autosomes) and a pair of sex chromosomes (X and/or Y). Females have two X chromosomes (XX), whereas males have one X and one Y chromosome (XY).
ALS1

The gene for ALS1 was first mapped to the long arm (q) of chromosome 21 [21 q22.1] (Siddique et al., 1991). Subsequently, 11 different mutations were identified in the SOD-1 gene confirming it as the causative gene (Rosen et al., 1993). Mutations in the SOD 1 gene underlie 14-23% FALS and 1-7 % of sporadic ALS (SALS) cases (Cudkowicz et al., 1997; Orrell et al., 1997; Shaw et al., 1998; Andersen, 2006a).

The SOD-1 gene spans 11 kilobases of genomic DNA, comprises five exons and four introns (Levanon et al., 1985). The SOD-1 gene encodes a highly conserved 153-amino acid long protein, which together with a catalytic copper (Cu) ion and a stabilizing zinc (Zn) ion form a subunit (Levanon et al., 1985; Andersen, 2006a). A disulfide bridge stabilizes each subunit, and the two identical subunits combine through non-covalent bonds to form the Cu-Zn SOD-1 enzyme. The main function of the SOD-1 enzyme is in free radical scavenging whereby the enzyme catalyses the conversion of the superoxide anion to molecular oxygen and hydrogen peroxide, which in turn is reduced to water by glutathione peroxidase and catalase (Fridovich, 1986; Shaw and Kuncl, 2002; Andersen, 2006a). The Cu-Zn SOD enzyme constitutes 0.5-1% of soluble protein in the brain and spinal cord, and is located within in the cytosol, nucleus and between the two mitochondrial membranes (Bowling et al., 1995; Pardo et al., 1995; Andersen, 2006a). To date over 122 different mutations have been reported in the SOD-1 gene (Gros-Louis et al., 2006). The majority are missense mutations causing a change of one amino acid for another but preserving the SOD-1 protein length. The remaining mutations are either nonsense or deletion mutations that either introduce novel nucleotides or remove existing
nucleotides resulting in alteration of polypeptide length (Dewil et al., 2004; Andersen, 2006a; Gros-Louis et al., 2006). Autosomal dominant pattern of inheritance is evident with most mutations, except for the D90A mutation, which may be transmitted in an autosomal recessive manner. Compound heterozygosity has also been reported with SOD1 FALS, where two siblings with a slowly progressive ALS phenotype were found to be carriers of both the D90A and D96N mutations (Hand et al., 2001).

The SOD-1 mutations are widely distributed throughout the gene with preponderance for exon 4 and 5 (Radunovic and Leigh, 1996; Cudkowicz et al., 1997; Andersen et al., 2003; Andersen, 2006b). Globally, the most frequent mutation is the substitution of aspartate for alanine (D90A), followed by alanine to valine (A4V) and isoleucine for threonine (I113T) (Dewil et al., 2004; Andersen, 2006a). In Australia, the following mutations have been reported: alanine to threonine (A4T); leucine to valine (L38V); histidine to arginine (H43R); asparate to alanine (D90A); glutamic acid to glycine (E100G); isoleucine to threonine (I113T); asparate to valine (D124V); and valine to glycine (V148G) (Aggarwal and Nicholson, 2005; Andersen, 2006a). SOD-1 FALS is clinically and pathologically indistinguishable from SALS (Cudkowicz et al., 1997; Orrell et al., 1997; Dewil et al., 2004; Gros-Louis et al., 2006). For most mutations there is intra- and interfamilial variation in penetrance (i.e when the disease becomes clinically manifest), age and site of disease onset, rate of disease progression and survival (Andersen, 2006a). Penetrance in FALS is age dependent, with ~50% of patients expressing the disease by age 43 and more than 90% by 70 years (Cudkowicz et al., 1997; Robberecht, 2002). Only 17 mutations are associated with this high degree of penetrance (Andersen, 2006a).
Some mutations, such as the I113T may be transmitted asymptomatically from grandparents to grandchildren (Suthers et al., 1994; Jones et al., 1995).

The mean age of disease onset of SOD-1 FALS is 47 years, but varies from 6 to 94 years (Andersen, 2006a). Some mutations are associated with an earlier age of onset, such as the L38V, I104F and G37R mutations (Ikeda et al., 1995; Kawamata et al., 1997; Robberecht, 2002), while others (I113T and D90A) have a later age of onset (Andersen et al., 1996; Cudkowicz et al., 1997). The site of disease onset can also vary depending on the mutation (Aoki et al., 1994; Andersen et al., 1996; Andersen et al., 1997; Cudkowicz et al., 1997; Ohi et al., 2002; Andersen, 2006a). A clinical LMN pattern of involvement is the rule for SOD1 patients, with predominantly UMN disease phenotypes, such as PLS, never reported (Ikeda et al., 1995; Abe et al., 1996; Radunovic and Leigh, 1996; Cudkowicz et al., 1997; Orrell et al., 1997; Andersen et al., 2003).

There is a great deal of variation in survival among patients with different mutations. Some mutations are associated with rapid disease progression and shorter survival (~ 1.4 years for the A4V mutation), while other mutations (D90A) are associated with long survival (Aoki et al., 1993; Aoki et al., 1994; Cudkowicz et al., 1997; Juneja et al., 1997; Robberecht, 2002). Co-inheritance of protective or disease-modifying genes may provide an explanation for this variability in survival (Andersen et al., 1997; Al-Chalabi et al., 1998; Simpson and Al-Chalabi, 2006).
**Pathophysiological mechanism in ALS**

The mechanisms underlying neurodegeneration in both familial and sporadic ALS remain elusive and on the information currently available are likely to be multifactorial. Evidence is emerging of a complex interaction between genetic factors, oxidative stress, glutamate overactivity and mitochondrial dysfunction, which may result in damage of critical target proteins and organelles within the motor neuron.

1. **Oxidative stress and “toxic gain of function” of the SOD-1 enzyme**

Current understanding is that SOD-1 gene mutations result in acquisition of new cytotoxic activity by the SOD-1 enzyme, termed a “toxic gain of function” (Robberecht, 2002; Dewil et al., 2004; Andersen, 2006a). Support for this toxic gain of function is provided by several lines of evidence. Firstly, some SOD-1 mutations (D90A and G37R) do not affect the activity of the SOD-1 enzyme but yet still result in ALS (Andersen et al., 1995; Bowling et al., 1995; Sjaland, 1995). Transgenic mice overexpressing the mutated SOD-1 gene develop an ALS-like syndrome despite normal or elevated SOD-1 enzyme activity (Gurney et al., 1994; Ripps et al., 1995; Wong et al., 1995; Bruijn et al., 1997a). Mice in which the SOD1 gene has been “knocked out” fail to develop ALS (Reaume et al., 1996).

What the acquired activity is and how it underlies neurodegeneration of motor neurons remains debatable (Pasinelli and Brown, 2006). Studies have suggested that the SOD-1 enzyme develops aberrant biochemical activity (Beckman et al., 1993; Bruijn et al., 1997a; Andrus et al., 1998; Bruijn et al., 1998; Bruijn et al., 2004). In addition, SOD-1
mutations induce structural changes in the SOD-1 protein such that substrates other than the superoxide anion gain access to the active centre, resulting in increased production of hydroxy and free radicals (Bogdanov et al., 1998; Liu et al., 1998). Others have suggested that the mutated SOD-1 enzyme may also accept peroxynitrate as a substrate, resulting in nitration of tyrosine residues on proteins (Beckman et al., 1993; Beckman and Koppenol, 1996; Crow et al., 1997) and ultimately cell injury and cell death (Beckman et al., 1993). Upregulation of protein-tyrosine nitration has been reported in anterior horn cells in ALS (Chou et al., 1996; Abe et al., 1997) and elevation of 3-nitortyrosine has been reported in the spinal cord of ALS patients and transgenic SOD-1 mice (Beal et al., 1997; Ferrante et al., 1997).

In addition to altering the SOD-1 enzyme activity, mutations in the SOD-1 gene may result in improper binding of zinc to the mutated SOD-1 peptide, thereby allowing reduction of SOD-1 bound copper, which in turn results in formation of superoxide anion and cell injury (Estevez et al., 1999). Furthermore, diminished metal ion binding by the mutated SOD-1 peptide may result in release of zinc and copper ions, thereby triggering neurotoxicity and degeneration (Pasinelli and Brown, 2006).

Oxidative stress, imparted by aberrant activity of the SOD-1 enzyme, may also cause neurodegeneration via inflammation by upregulating proinflammatory cytokines (Hensley et al., 2006). Increased expression of proinflammatory mediators such as nitric oxide, interleukin (IL)-1, IL-6, and IL-12 were reported in the transgenic SOD-1 mouse model and these inflammatory mediators were demonstrated to be toxic to motor neurons
in spinal cord preparations (Kim et al., 2006). Furthermore, antagonism of these agents with neutralizing antibodies resulted in increased motor neuron survival.

An alternative hypothesis is that SOD-1 mutations result in conformational instability and misfolding of the SOD-1 peptide, resulting in formation of intracellular aggregates. In the transgenic SOD-1 mouse model and in some human ALS cases, immunoreactive SOD-1 aggregates have been reported in motor neurons and glial cells (Bruijn et al., 1998; Jonsson et al., 2006; Zetterstrom et al., 2007). Whether the intracellular aggregates are toxic to motor neurons remains unknown, although a number of possible cytotoxic mechanisms have been proposed, including: (i) co-aggregating with vital cellular constituents, (ii) inhibiting normal proteosomic function, and (iii) exerting mechanical or biochemical effects on the cell, such as disruption of axonal transport systems (Bruijn et al., 1997a; Bruijn et al., 1998; Williamson and Cleveland, 1999; Pasinelli and Brown, 2006).

2. Glutamate excitotoxicity

In addition to oxidative stress, there is emerging evidence that glutamate-mediated excitotoxicity is an important pathophysiological mechanism underlying neurodegeneration in ALS (Boillee et al., 2006). Glutamate is the major excitatory neurotransmitter in the CNS (Watkins and Evans, 1981; Heath and Shaw, 2002), synthesized from either reductive deamination of alpha-ketoglutarate or from the action of amino acids of aminotransferases, with ~20% of the total glutamate pool stored in presynaptic nerve terminals (Heath and Shaw, 2002). During normal transmission,
glutamate is released from presynaptic neurons by depolarization, diffuses across the synaptic cleft and activates receptors on postsynaptic neurons. The excitatory signal is terminated upon removal of glutamate from the synaptic cleft by specific glutamate re-uptake transporters located on both neurons and astrocytes (Vandenberg, 1998; Dong et al., 1999). Within the presynaptic astrocyte, glutamate is converted into glutamine by the enzyme glutamine synthetase and subsequently, glutamine is returned to the neuron for resynthesis of glutamate (Laake et al., 1995). In addition to being involved in the re-uptake of neurotransmitters, astrocytes mediate the re-uptake of extracellular K⁺, and provide a supporting network, nutrition and protective covering for neurons (Kandel, 1991).

Glutamate receptors are broadly classified into ionotropic or metabotropic receptors (Heath and Shaw, 2002). Binding of glutamate to ionotropic receptors results in a conformational change that allows passage of sodium and calcium ions through a central pore. Metabotropic glutamate receptors are linked via G proteins to second-messenger enzymes, which in turn, can regulate a host of cellular activities (Simeone et al., 2004). The present thesis will focus on ionotropic receptors, as these have been implicated in the pathogenesis of ALS.

Based on pharmacological studies, glutamate ionotropic receptors are further classified as; (i) N-methyl-D-aspartate (NMDA), (ii) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and (iii) kainite receptors. This pharmacological classification is further supported by subsequent cloning studies that have identified six
different families of glutamate ionotropic receptors that conform to the original agonist studies (Simeone et al., 2004). The following discussion will focus on NMDA and AMPA ionotropic receptors which have been implicated in the pathogenesis of ALS.

**N-methyl-D-aspartate** receptors are permeable to influx of Na\(^+\) and Ca\(^{2+}\) and efflux of K\(^+\) (Simeone et al., 2004). An essential feature of NMDA receptors is voltage-dependent blockade by magnesium (Mg\(^{2+}\)) binding within the channel pore, which can be alleviated by depolarization (MacDermott et al., 1986). NMDA receptors are involved in excitatory neurotransmission, which is characterized by a slow rise time and decay. As such, the NMDA receptors are involved in complex physiological processes, such as generation of rhythmic motor activity (Traven et al., 1993), regulation of neuronal migration during embryogenesis (Komuro and Rakic, 1993) and in memory formation (Bliss and Collingridge, 1993).

The NMDA receptor complex is composed of different subunits derived from 6 genes; NMDAR1 (eight splice variants described), NMDAR2 (A-D) and NMDAR3 (A,B) (Heath and Shaw, 2002; Simeone et al., 2004). While the NMDAR1 subunit forms the basic structure of the receptor (Heath and Shaw, 2002), the NMDAR2 subunit determines the ion channel properties and forms ligand-binding sites (Kutsuwada et al., 1992; Meguro et al., 1992; Michaelis, 1998). Functional and pharmacological properties of NMDA receptors are determined by a specific combination of NMDAR1 and NMDAR2 subunits (Kutsuwada et al., 1992; Monyer et al., 1992). In addition, there are regional variations in the expression of NMDA receptor subtypes (Kutsuwada et al., 1992;
AMPA receptors mediate a rapid influx of monovalent ions, such as Na\(^+\), K\(^+\) and chloride (Cl\(^-\)), but unlike NMDA receptors are impermeable to calcium (Ca\(^{2+}\)) (Heath and Shaw, 2002). Four AMPA receptor subtypes have been cloned (GluR1-4) and are composed of three transmembrane domains (M1, M3, M4) and a fourth cytoplasmic hairpin loop (M2), which contributes to the pore-lining region (Dingledine et al., 1999; Simeone et al., 2004). The AMPA receptor exists as a pentameric structure in vivo, which is formed by the arrangement of subunits to create receptor diversity (Heath and Shaw, 2002). The GluR2 subunit influences the Ca\(^{2+}\) permeability of AMPA receptors. Specifically, AMPA receptors lacking the mature GluR2 subunit are more permeable to Ca\(^{2+}\) ions, which upon activation of AMPA receptors, may result in excessive intracellular concentration of Ca\(^{2+}\) and ultimately neurodegeneration through activation of Ca\(^{2+}\)-dependent pathways within the cell (Heath and Shaw, 2002; Simeone et al., 2004). Failure of normal posttranscriptional editing of the GluR2 pre-messenger RNA, usually 90% efficient, whereby glutamine is replaced by a positively charged arginine in the M2 pore forming loop (Donevan and Rogawski, 1995), results in this increased Ca\(^{2+}\) permeability of AMPA receptors.
What is the evidence that glutamate-mediated excitotoxicity underlies neurodegeneration in ALS?

Glutamate excitotoxicity is mediated by excessive activation of postsynaptic glutamate receptors (Heath and Shaw, 2002). Evidence for glutamate excitotoxicity in ALS has been provided by studies that have investigated the expression and function of astrocytic glutamate transporter systems responsible for removal of glutamate from the synaptic cleft (Rothstein et al., 1993; Trotti et al., 1999; Boillée et al., 2006; Ionov, 2007). A 70% reduction in expression of excitatory amino acid transporter-type 2 (EAAT2), the main astrocytic glutamate transporter (Trotti et al., 1999), was reported in the motor cortex and spinal cord in ALS patients (Rothstein et al., 1995), with oxidative reactions inactivating EAAT2. Reduced expression of glutamate transortper type 1 [GLT-1] (equivalent to human EAAT2) and function of the Na⁺-dependent uptake of glutamate, have been reported in the transgenic SOD-1 mouse model (Bruijn et al., 1997b; Canton et al., 1998). Further, increased expression of NMDA receptors along with expression of AMPA receptors with an unedited GluR2 subunit, rendering them more permeable to Ca²⁺, have been reported in ALS (Takuma et al., 1999; Van Damme et al., 2002; Kawahara et al., 2004; Kwak and Kawahara, 2005; Van Damme et al., 2005). The editing defect appears to be specific for sporadic ALS, and it has been suggested that the sensitivity of motor neurons to excitotoxicity relies on the presence of AMPA receptors lacking the functional GluR2 subunit (Heath and Shaw, 2002; Cox et al., 2007).

Further support for glutamate excitotoxicity in ALS has been provided by the effectiveness of the anti-glutamate agent, riluzole (Bensimon et al., 1994; Gurney et al.,
Riluzole inhibits glutamate release and blocks persistent Na$^+$ current (Louvel et al., 1997; Ludolph, 2000; Urbani and Belluzzi, 2000). In addition, riluzole exerts neuroprotective effects on motor neurons by binding with high affinity to the inactivated form of the Na$^+$ channel and stabilizing the channel in an inactivated state (Hebert et al., 1994; Turner et al., 2005a).

Neurophysiological and radiological studies have also provided support for glutamate excitotoxicity. Specifically neurophysiological studies in the form cortical transcranial magnetic stimulation (TMS) have demonstrated increased cortical excitability in ALS (Caramia et al., 1991; Prout and Eisen, 1994; Kohara et al., 1996a; Yokota et al., 1996; Mills and Nithi, 1997; Ziemann et al., 1997c; Sommer et al., 1999; Weber and Eisen, 2000; Desiato et al., 2002; Zanette et al., 2002b; Mills, 2003; Komissarov et al., 2004). Further, positron emission tomography studies have revealed widespread activation of the cortex contralateral to limb movement in sporadic and FALS cases (Kew et al., 1994; Kew et al., 1993).

For the glutamate hypothesis to be plausible as a mechanism of motor neuron degeneration, it must explain how motor neurons are selectively damaged by overactivity of the glutaminergic system and provide a mechanism by which degeneration occurs. A number of cell-specific molecular features of motor neurons render them vulnerable to glutamate toxicity. Firstly, motor neurons preferentially express an AMPA receptor lacking the functional GluR2 subunit, thereby rendering the motor neurons more permeable to Ca$^{2+}$ (Kawahara et al., 2004; Kwak and Kawahara, 2005; Van Damme et
al., 2005; Van Damme et al., 2002). Secondly, motor neurons vulnerable to degeneration lack the intracellular expression of Ca\(^{2+}\) binding proteins parvalbumin and calbindin D28k, required to buffer intracellular Ca\(^{2+}\) (Alexianu et al., 1994; Ince et al., 1993). Recently, increased expression of the inositol 1,4,5-triphosphate receptor 2 (ITPR2) gene was reported in ALS (van Es et al., 2007). The ITPR2 is involved in glutamate-mediated neurotransmission, whereby stimulation of glutamate receptors results in binding of inositol 1,4,5-triphosphate to ITPR2, which subsequently increases intracellular calcium (Choe and Ehrlich, 2006; van Es et al., 2007). Aberrant activity of ITPR2 results in higher intracellular concentration of Ca\(^{2+}\) and ultimately apoptosis (Gutstein and Marks, 1997).

Although details of the molecular mechanisms by which glutamate exerts neurotoxicity are still to be clearly elucidated, several pathways have been defined. Initially, there is an influx of Na\(^{+}\), Cl\(^{-}\) and water resulting in acute neuronal swelling that is reversible with removal of agonist (Choi, 1987; Stys, 1998; Shaw and Kuncl, 2002). Subsequently, an influx of Ca\(^{2+}\) ions through NMDA receptors, Ca\(^{2+}\) permeable AMPA receptors, or indirectly through voltage-gated Ca\(^{2+}\) channels occurs (Choi, 1987; Miller et al., 1989), resulting in increased intracellular Ca\(^{2+}\) concentration and activation of Ca\(^{2+}\)-dependent enzymatic pathways that mediate neuronal death (Meldrum and Garthwaite, 1990; Regan et al., 1995; Shaw and Kuncl, 2002; Cox et al., 2007). Further, glutamate excitotoxicity results in production of free radicals that can further damage the intracellular organelles and cause cell death (Bondy and Lee, 1993; Lees, 1993; Maher and Davis, 1996).
3. Mitochondrial dysfunction

Numerous studies have focussed on the role of mitochondrion dysfunction in neurodegeneration evident in ALS (Boillee et al., 2006; Pasinelli and Brown, 2006). There is now compelling evidence that mitochondrial dysfunction, in conjunction with oxidative stress and glutamate excitotoxicity, may exert an important role in neurodegeneration evident in ALS (Boillee et al., 2006). Mitochondria are intracellular organelles whose main function is to generate energy for the cell in the form of ATP. Under conditions of Ca\(^{2+}\) load, as occurs with activation of glutamate receptors (Dugan and Choi, 1994; Dykens, 1994), mitochondrial production of free radicals increases. The oxidative stress may ultimately result in injury of proteins and DNA within neurons, thereby resulting in neurodegeneration. In addition, mitochondria are sensitive to free radical damage at both the protein and DNA level, resulting in further mitochondrial dysfunction (Bowling and Beal, 1995). Mitochondrial damage may also enhance glutamate excitotoxicity by disrupting the normal resting membrane potential, thereby resulting in a loss of the normal voltage-dependent Mg\(^{2+}\) mediated block of NMDA receptor channels (Heath and Shaw, 2002; Shaw and Kuncl, 2002a).

Mitochondrial degeneration has been reported in both ALS patients and in the transgenic SOD-1 mouse model (Higgins et al., 2003; Kong and Xu, 1998; Wong et al., 1995; Xu et al., 2004). Ultrastructural abnormalities of muscle mitochondria, paracrystalline inclusions, and abnormal cristae have been reported in ALS (Comi et al., 1998; Chung and Suh, 2002; Lederer et al., 2007). Dysfunction of mitochondrial enzymes involved in energy generation, such as cytochrome c oxidase and respiratory chain complexes I and
IV, as well as down-regulation of nuclear genes encoding mitochondrial components within the motor cortex have been reported in ALS (Comi et al., 1998; Fujita et al., 1996; Jung et al., 2002; Kirkinezos et al., 2005; Lederer et al., 2007). This dysfunction in energy metabolism would result in reduced intracellular ATP levels, thereby increasing the susceptibility of neurons to damage by oxidative and excitotoxic stress (Lederer et al., 2007). Further impetus for mitochondrial dysfunction was provided by experiments showing that coenzyme Q$_{10}$ (a cofactor in the mitochondrial electron transport chain) prolonged the lifespan in the transgenic FALS mouse model (Matthews et al., 1998). Studies are currently underway to assess the efficacy of coenzyme Q$_{10}$ in humans (Ferrante et al., 2005; Traynor et al., 2006).

**Where does ALS begin?**

Despite Charcot’s initial observation of concomitant upper and lower neuron pathology in ALS, and research attempting to determine the underlying mechanisms of neurodegeneration in ALS, the issue of where ALS begins has not been resolved. Resolution of this question may not only enhance the understanding of the pathophysiology of ALS, but may also have diagnostic and therapeutic implications.

In 1992, Eisen and colleagues proposed that ALS was primarily a disorder of upper motor neurons, or corticomotoneurons, which connect monosynaptically with anterior horn cells (Eisen et al., 1992). Dysfunction of these corticomotoneurons was postulated to result in alteration of glutamate metabolism and thereby anterior horn cell degeneration via an anterograde or “dying forward” process (Eisen et al., 1992). This dying forward
hypothesis was based on a number of poignant observations, including: (i) motor neurons located within the oculomotor, abducens, and Onuf’s nuclei, which lack a monosynaptic connection with corticomotoneurons, are typically spared in ALS; (ii) absence of an animal model of ALS was ascribed to a paucity of corticomotoneuronal-anterior horn cell connections (Armand, 1982; Lemon and Griffiths, 2005); (iii) pure lower motor neuron forms of ALS are relatively rare, while subclinical upper motor neuron involvement is invariably detected with TMS studies (Eisen and Shtybel, 1990); (iv) the asymmetric and variable motor deficits evident in ALS may be anticipated by the anatomy of corticomotoneurons, whereby a single corticomotoneuron synapses with several anterior horn cells (Porter, 1985; Porter, 1987; Lemon and Griffiths, 2005).

In contrast, the “dying back” hypothesis of ALS proposes that there is a deficiency of a motor neurotrophic hormone, which is normally released by postsynaptic cells and retrogradely transported up the presynaptic axon to the cell body where it exerts its effects (Appel, 1981). This hypothesis implies that ALS begins within the muscle cells or neuromuscular junction. The dying back hypothesis is supported by studies reporting that synaptic denervation precedes motor neuron degeneration (Gould et al., 2006; Pagani et al., 2006; Pun et al., 2006). This synaptic denervation, which is reportedly an early feature, is possibly mediated by dysfunction of perisynaptic Schwann cells that accumulate mutant SOD-1 proteins (Gould et al., 2006; Neusch et al., 2007). In addition to the “dying back” hypothesis, some have suggested that upper and lower motor neurons degenerate independently (Kiernan and Hudson, 1991; Pamphlett et al., 1995; Ravits et al., 2007).
In an attempt to resolve the issue of where ALS begins, and thereby enhance the understanding of the mechanisms underlying neurodegeneration in ALS, the present thesis will assess sporadic ALS and clinically affected familial ALS (FALS) patients, along with longitudinally monitoring asymptomatic SOD1 mutation carriers, through a combination of novel peripheral axonal excitability testing and threshold tracking methods adapted for cortical excitability testing.

**Axonal excitability testing**

Indirect information regarding resting membrane potential and axonal ion channel function may be gained through axonal excitability studies by using a technique called threshold tracking (Bostock et al., 1998; Burke et al., 2001). Threshold in this context refers to the stimulus current required to produce a specific potential. With threshold tracking, changes in the test stimulus current intensity required to generate a fixed amplitude can be adjusted on-line to keep the target amplitude constant (see Methodology). Assessment of axonal ion channel function may provide insight into mechanisms responsible for generation of fasciculations and cramps, as well as mechanisms responsible for motor neuron loss in ALS. Using a recently developed protocol, the following parameters of axonal excitability will be measured: threshold; strength-duration time constant; rheobase; threshold electrotonus; current/threshold relationship; and recovery cycle (Kiernan et al., 2000a).
1. Threshold

The stimulus current required to activate an axon and thereby produce a compound potential of specific amplitude is referred to as threshold (Bostock et al., 1998; Burke et al., 2001). Threshold may be used as a surrogate marker of membrane potential, with membrane depolarization reducing and hyperpolarization increasing threshold. However, threshold may not be an accurate indicator of membrane potential in all circumstances, for example with hyperventilation and nerve ischemia, and in such cases the ambiguity may be resolved by measuring other indices of axonal excitability (Baker and Bostock, 1989; Bostock et al., 1994; Mogyoros et al., 1997; Grosskreutz et al., 1999; Grosskreutz et al., 2000).

2. Strength-duration time constant and rheobase

As the duration of test stimulus current increases, the strength of current required to activate a specified fraction of a compound muscle action potential decreases (Bostock et al., 1998). Strength-duration time constant and rheobase are measures of the relationship between the intensity of a threshold stimulus to its duration (Bostock, 1983; Mogyoros et al., 1996a; Bostock et al., 1998; Burke et al., 2001).

Strength-duration time constant ($\tau_{SD}$) provides a measure of the rate at which the threshold current decreases as its duration increases (Weiss, 1901; Bostock, 1983; Mogyoros et al., 1996a; Mogyoros et al., 1999). In human peripheral nerve, $\tau_{SD}$ can be calculated by using the ratio between stimulus-response curves for two different stimulus durations according to Weiss' formula (Weiss, 1901). Rheobase is defined as the
threshold current (mA) for stimulus of infinitely long duration (Bostock et al., 1998).

Both the rheobase and $\tau_{SD}$ are properties of the nodal membrane, being dependent on passive membrane properties and persistent Na$^+$ channel conductances ($I_{NaP}$) (Bostock and Rothwell, 1997).

Persistent Na$^+$ currents are conducted through voltage-gated Na$^+$ channels that consist of alpha ($\alpha$) and four beta ($\beta$1-4) subunits (Catterall, 2000; Isom, 2001; Catterall et al., 2005). The $\alpha$ subunits are organized in four homologous domains (I-IV), each of which contains six transmembrane $\alpha$ helices (S1-S6) and an associated pore loop located between the S5 and S6 segments, that acts as a selectivity filter. The pore loops line the outer, narrow entry to the pore, whereas the S5 and S6 segments line the inner wider exit from the pore. The S4 segments in each domain functions as a voltage sensor.

Inactivation of Na$^+$ channels is mediated by a short intracellular loop connecting homologous domains III and IV, which fold into the channel structure and block the pore from the inside during sustained membrane depolarization. With persistent Na$^+$ channels, which constitute 1-2% of the total Na$^+$ current (French et al., 1990; Crill, 1996; Bostock and Rothwell, 1997), inactivation is either very slow or incomplete (Llinas, 1988; Stys et al., 1993; Baker and Bostock, 1998). Ten distinct Na$^+$ channel isoforms have been identified, Na$_v$ 1.1 to Na$_v$ 1.9 and Na$_x$ (Catterall, 2000; Catterall et al., 2005), with $I_{NaP}$ conducted by the Na$_v$1.6 isoform, an isoform abundantly expressed at the nodes of Ranvier (Catterall, 2000; Tzoumaka et al., 2000; Goldin, 2001).
Although the exact mechanisms underlying INaP are yet to be determined, it has been suggested that a uniform population of Na\(^+\) channels may generate both transient and persistent Na\(^+\) currents by switching between different gating modes (Alzheimer et al., 1993; Brown et al., 1994). Phosphorylation of amino-acid residues on the alpha subunit of voltage-gated Na\(^+\) channels, mediated by protein kinase C and cAMP-dependent protein kinases, may underlie these gating changes (Carr et al., 2003; Chen et al., 2006).

Changes in the resting membrane potential will affect rheobase and \(\tau_{SD}\), and thereby persistent Na\(^+\) conductances, with depolarization reducing the rheobase and prolonging \(\tau_{SD}\), while hyperpolarization exerts the opposite effects. The \(\tau_{SD}\) may also be affected by changes in nerve geometry, such as axonal loss, peripheral nerve demyelination and discrete changes in nodal Na\(^+\) conductances (Brismar, 1981; Mogyoros et al., 1996a; Kiernan et al., 2005b; Kuo et al., 2005). Of clinical relevance, prolongation of the \(\tau_{SD}\) has been reported in ALS (Mogyoros et al., 1998a; Kanai et al., 2003; Kanai et al., 2006), suggesting that upregulation of INaP may underlie the generation of excessive ectopic axonal activity, and thereby fasciculations (Kanai et al., 2003; Kuo et al., 2005).

Furthermore, upregulation of INaP may underlie neurodegeneration by increasing the intracellular concentration of Ca\(^{2+}\) (Stys et al., 1991; Stys et al., 1993; Stys, 1998).

3. Threshold electrotonus

Threshold electrotonus (TE) is the only technique that provides insight into both nodal and internodal membrane conductances. This technique measures changes in threshold current produced by a long-duration polarizing current (Bostock and Baker, 1988;
Bostock et al., 1998). Conditioning currents are subthreshold and do not trigger an action potential, but rather result in local changes in the membrane potential (Bostock et al., 1998; Kiernan and Burke, 2004a). TE is usually measured with 1 ms current pulses, a duration which is long compared to the time constant of the nodes of Ranvier but short compared with the time constants of the internodal membrane and slowly activating ion channels (Kiernan and Burke, 2004a). In a protocol developed for clinical use (Kiernan et al., 2000; Kiernan et al., 2001c), changes in threshold current in response to subthreshold conditioning currents are measured at varying conditioning-time intervals. Conventionally TE is plotted such that an increase in excitability (or threshold reduction) produces an upward deflection, while reduction in excitability (or threshold increase) produces a downward deflection (Fig. 1).

The initial fast response ("F" phase, Fig. 1) reflects rapid changes in threshold at the node of Ranvier, resulting from the application of depolarizing or hyperpolarizing subthreshold currents. This is followed by slower changes in threshold over tens of milliseconds in both depolarizing and hyperpolarizing directions, called the "S1" phase, and reflects the spread of current to the internodal membrane (Fig. 1).

During depolarization, the S1 phase peaks at 20 ms after the onset of the current pulse, after which threshold begins to return to baseline, the S2 phase. This S2 phase occurs due to activation of nodal and internodal slow K⁺ channels (Baker et al., 1987; Bostock and Baker, 1988; Bostock et al., 1998) that are activated by long duration subthreshold currents (Kiernan and Burke, 2004a).
In hyperpolarization, the S1 phase peaks at 100-150 ms after current pulse onset and begins to return to baseline. This accommodative phase, the S3 phase, is due to activation of the hyperpolarizing-activated inward rectifying currents ($I_h$, see section on current-threshold relationship) (Pape, 1996). On termination of the subthreshold currents there is an overshoot of threshold with both depolarization and hyperpolarization. With

**Figure 1**: Threshold changes to polarising currents of 100 ms duration set to ± 40% of resting threshold. Changes are plotted with threshold reductions, with depolarisation represented as an upward deflection and hyperpolarization in a downward direction.
depolarization, slow K⁺ channels mediate the overshoot, while in hyperpolarization the overshoot is mediated by I₉.

Voltage-gated K⁺ channels are members of the voltage-gated ion channel protein superfamily (Yu et al., 2005), and are composed of four alpha pore-forming subunits and accessory β subunits (Judge and Bever, 2006). In human myelinated axons, K⁺ currents are activated by depolarization and at least three types of currents have been identified (Dubois, 1981), including two fast components (I₉₁ and I₉₂, see section on refractoriness) and one slow current (I₉₃) conducted via slow (S) channels. These S channels, which underlie the S2 phase of TE, exhibit slower deactivating kinetics, are blocked by TEA and barium (Baker et al., 1987; Gordon et al., 1988), and are present at both the node and internode (Roper and Schwarz, 1989; Safronov et al., 1993). Since S channels require prolonged depolarization for activation, as occurs with high-frequency activity, these channels are responsible for the production of a hyperpolarizing afterpotential that serves to limit inappropriate repetitive firing of axons (Baker et al., 1987). In addition, the internodal S channels may be important in the maintenance of the internodal resting membrane potential, thereby contributing to the nodal resting potential and increasing the safety factor of impulse conduction (Chiu and Ritchie, 1984).

Alterations in membrane potential result in changes in threshold electrotonus. Specifically, with membrane depolarization there is a reduction in the resistance of the internodal membrane, due to activation of paranodal and internodal K⁺ channels, resulting in reduction of the S1 phase in both depolarizing and hyperpolarizing directions,
thereby causing a “fanning in” appearance of TE (Kiernan and Bostock, 2000; Burke et al., 2001; Kaji, 2003; Kiernan and Burke, 2004a). Conversely, hyperpolarization closes the paranodal and internodal K⁺ channels, thereby increasing the internodal membrane resistance and the S1 phase in both depolarizing and hyperpolarizing directions producing a fanning out appearance (Kiernan and Bostock, 2000; Burke et al., 2001; Kaji, 2003; Kiernan and Burke, 2004a).

Of clinical relevance, alterations in TE were first described in ALS where two distinct responses were described; (i) TE recordings with greater threshold reductions during depolarization (Type 1 response) and (ii) TE recordings with an unexpectedly rapid increase in threshold during depolarisation (Type 2 response) (Bostock et al., 1995). Reduction in internodal slow K⁺ channel conduction was reported as the underlying mechanism. Abnormalities of TE have also been reported in multifocal motor neuropathy with conduction block (Kiernan et al., 2002a) and metabolic neuropathies including diabetes (Horn et al., 1996; Krishnan and Kiernan, 2005) and uremia (Kiernan, 2002; Krishnan et al., 2005b; Krishnan et al., 2006b).

4. Current-threshold relationship

Changes in threshold can also be plotted in response to long duration subthreshold currents (200ms duration) applied in a ramp like fashion, referred to as the current-threshold relationship (I/V plot) (Kiernan et al., 2000; Kiernan et al., 2001c). Conventionally, threshold increases (hyperpolarization) are plotted to the left and threshold decreases (depolarization) to the right [Fig. 2] (Baker et al., 1987; Kiernan et
al., 2000; Kiernan et al., 2001c). The I/V relationship estimates rectifying properties of both nodal and internodal segments of the axon (Baker et al., 1987; Bostock et al., 1998). The I/V gradient induced by depolarizing sub-threshold currents reflects conduction through outward rectifying K⁺ channels, while the I/V gradient during hyperpolarizing sub-threshold currents reflects inwardly rectifying conductances (Pape, 1996; Bostock et al., 1998).

**Figure 2:** Current-threshold relationship: normalised threshold changes at the end of 200 ms duration currents are plotted, with depolarization represented to the right and hyperpolarization to the left. The conditioning current is varied from +50 % to –100 % of control threshold.
Two basic types of current underlie inward rectification (Mayer and Westbrook, 1983). The first type, or classical inward rectifier, is a pure K\(^+\) conducting channel, which is activated at membrane potentials negative to the K\(^+\) equilibrium potential (Rudy, 1988; Pape, 1996; Jan and Jan, 1997). As such, an increase in extracellular K\(^+\) concentrations increases conductance of this inward rectifier K\(^+\) channel. The second type of inward rectification is mediated by a channel that exhibits conductance for both Na\(^+\) and K\(^+\) (Baker et al., 1987). This current begins to activate at between -45 mV and -60 mV and peaks at -110 mV (Angstadt and Calabrese, 1989; Banks et al., 1993; Bayliss et al., 1994; Pape, 1996). This second \(I_H\) current activates and deactivates slowly, and the magnitude of the \(I_H\) current is also dependent on extracellular K\(^+\) concentration (Bader and Bertrand, 1984).

A major function of internodal \(I_H\) is to limit electrogenic hyperpolarization and the consequent reduction of axonal excitability in response to high-frequency activity (Bergmans, 1970; Bostock and Grafe, 1985; Baker et al., 1987). Given that high-frequency activity can induce failure of impulse conduction in axons with a reduced safety factor of transmission (Vagg et al., 1998), the \(I_H\) current may be critical in preventing such conduction failure. Motor nerves may have a predilection to develop activity-dependent conduction failure when affected by disease, particularly primary demyelinating neuropathies (Cappelen-Smith et al., 2000; Kaji et al., 2000), in contrast to sensory nerves, due to the reduced expression of \(I_H\) in motor nerves (Bostock et al., 1994; Lin et al., 2002; Kiernan et al., 2004).
5. The recovery cycle of axonal excitability

Following conduction of an action potential, axons undergo a series of stereotyped excitability changes known as the recovery cycle (Fig. 3). Initially, there is a period of total inexcitability that lasts for 0.5-1 ms, during which the axon cannot generate an action potential, called the **absolute refractory period**. Subsequently, the axon enters a **relative refractory period (RRP)**, during which an action potential may be generated by stronger than normal stimulus currents. The RRP lasts up to 4 ms and may be measured as an increase in current required to generate a potential (termed refractoriness), or as the duration of the RRP, the point where the recovery cycle curve crosses the x-axis (Fig.3).

**Figure 3:** Recovery cycle of excitability is assessed by tracking the changes in threshold that occur following a supramaximal conditioning stimulus of 1-ms duration. RRP refers to the relative refractory period.
The absolute refractory period results from inactivation of nodal voltage-gated transient \( \text{Na}^{+} \) channels, while the RRP results from gradual recovery of these channels from inactivation (Hodgkin and Huxley, 1952). Like persistent \( \text{Na}^{+} \) channels, these transient channels are of the \( \text{Na}_{1.6} \) isoform, and consist of alpha and beta (\( \beta \)) subunits (Catterall, 2000; Catterall et al., 2005). However, the kinetics of transient \( \text{Na}^{+} \) channels are markedly different, such that these channels exhibit ‘fast’ activation and inactivation kinetics, i.e. the channels open rapidly with depolarization and inactivate rapidly (Scholz et al., 1993). Transient \( \text{Na}^{+} \) currents are first detected at membrane potentials of -55 mV, peaking at ~ -30 mV (Brown et al., 1994), and underlie the rapid phase of depolarization of the action potential (Burke et al., 2001).

Refractoriness is followed by a period of increased axonal excitability, called superexcitability. Superexcitability lasts for ~ 15 ms and is mediated by re-excitation of the nodal membrane by the discharge of current stored on the internodal membrane following an action potential, also known as the depolarizing long-lasting afterdepolarization (DAP) (Barrett and Barrett, 1982; Burke et al., 2001). The amplitude and time course of DAP, and thereby superexcitability, is limited by the activation of paranodal and juxtaparanodal \( \text{K}^{+} \) channels, which shunt current outward and reduce charging of the internodal membrane (David et al., 1995; McIntyre et al., 2002). Paranodal \( \text{K}^{+} \) channels are kinetically classified into fast (F) channels and intermediate (I) channels (Jonas et al., 1989; Safronov et al., 1993). Although both F and I channels are blocked by 4-aminopyridine (4-AP), F channels activate at potentials between -40 and +40 mV and deactivate very rapidly at potentials between -120 mV and -65 mV (Roper...
and Schwarz, 1989; Wang et al., 1993; Vabnick and Shrager, 1998; Reid et al., 1999). In contrast, I channels are first activated by depolarization to around -70 mV, become fully activated at around -40 mV and deactivate slowly (Reid et al., 1999).

Superexcitability varies with changes in membrane potential, such that membrane depolarization reduces superexcitability by limiting Na\(^+\) influx and increasing K\(^+\) efflux through paranodal and juxtaparanodal fast K\(^+\) channels (Barrett and Barrett, 1982; David et al., 1995; Burke et al., 2001). Conversely, membrane hyperpolarization increases DAP and therefore superexcitability. As such, superexcitability may be used as an indicator of membrane potential (Kiernan and Bostock, 2000).

The late phase of decreased axonal excitability, termed late subexcitability, is mediated by activation of nodal slow K\(^+\) channels (Baker et al., 1987; Burke et al., 2001) and lasts for ~100 ms after passage of action potential. Late subexcitability is also dependent on differences between the membrane potential and the K\(^+\) equilibrium potential. As such, current induced membrane depolarization, without changes the electrochemical gradient for K\(^+\), increases late subexcitability. Conversely, membrane depolarization secondary to increases in extracellular K\(^+\) concentration, as occurs with ischemia or renal failure, results in reduction of late subexcitability (Kiernan and Bostock, 2000; Krishnan et al., 2005b).
Activity-dependent changes in axonal excitability

Although fatigue is a common symptom in ALS, the underlying mechanisms appear complex, with dysfunction of central and peripheral nervous systems independently reported as contributing factors. To investigate the mechanisms of fatigue in ALS, changes in axonal excitability following a maximal voluntary contraction were undertaken in the present thesis.

During a maximal voluntary contraction (MVC), trains of impulses are conducted by motor axons, resulting in membrane hyperpolarization (Burke et al., 2001; Lin et al., 2006). Two types of activity-dependent hyperpolarization have been described. The first type, known as the H₁ period, develops after 7-10 impulses and represents the accumulation of late subexcitability due to activation of slow K⁺ channels (Bergmans, 1970; Baker et al., 1987; Miller et al., 1996; Kiernan et al., 1997b; Lin et al., 2000; Burke et al., 2001). The H₁ period increases the threshold of cutaneous afferents by up to 40% and decays over ~ 100 ms.

The second type of post-tetanic hyperpolarization, referred to as the H₂ period is produced by long trains of impulses and is attributed to activation of the Na⁺/K⁺ pump secondary to intra-axonal accumulation of Na⁺ (Bergmans, 1970; Bostock and Bergmans, 1994; Kiernan et al., 1997a; Kiernan et al., 1997b; Burke et al., 2001). The Na⁺/K⁺ pump is a 270 kDa transmembrane spanning protein composed of two polypeptides: a transmembrane catalytic alpha subunit and a glycoprotein regulatory beta subunit. The alpha subunit has a binding site for Na⁺ and ATP on the intracellular side and binding
sites for K\(^+\) and ouabain on the extracellular surface (Koester, 1991). Because the pump extrudes three Na\(^+\) ions out of the cell for every two K\(^+\) ions into the cell, thereby resulting in a net efflux of positive charge, it is known as electrogenic. One of the main functions of the Na\(^+/K^+\) pump is in regulating the resting membrane potential (Thomas, 1972; Grafe et al., 1994), and paralysis of the Na\(^+/K^+\) pump results in membrane depolarization (Ritchie and Straub, 1957; Kaji and Sumner, 1989; Felts et al., 1995).

In addition to regulating the resting membrane potential, the Na\(^+/K^+\) pump exerts a significant role in the restoration of the Na\(^+\) and K\(^+\) gradients after high-frequency impulse activity. Specifically, after a period of tetanization, the accumulated intracellular Na\(^+\) ions increase Na\(^+/K^+\) pump activity resulting in membrane hyperpolarization, called **activity-dependent hyperpolarisation (ADH)**, and a reduction in axonal excitability (Bergmans, 1970; Bostock and Grafe, 1985; Gordon et al., 1990; Bostock and Bergmans, 1994). This activity-dependent hyperpolarization, and therefore Na\(^+/K^+\) pump function, can be indirectly assessed in-vivo by measuring changes in threshold after a maximal voluntary contraction (Burke et al., 1998; Vagg et al., 1998). Maximal voluntary contraction for 15 seconds can reduce excitability for as long as 10 minutes, with longer contraction (60 seconds) inducing changes for up to 20 minutes (Burke et al., 1998).

Assessment of ADH in ALS patients may not only clarify the mechanisms of fatigue, but also determine whether Na\(^+/K^+\) pump dysfunction underlies neurodegeneration in ALS, as has been recently reported in the SOD-1 mouse model (Ellis et al., 2003).
Assessment of cortical excitability testing

To assess the entire neurological axis (central and peripheral nerves), and thereby determine the site of disease onset in ALS, new techniques for the assessment of cortical excitability were developed during this thesis. Cortical excitability was assessed using transcranial magnetic stimulation (TMS), a relatively painless neurophysiological technique first described by Barker and colleagues (Barker et al., 1985).

Principles of magnetic stimulation

Magnetic stimulators consist of a capacitor, a device for storing charge, which when discharged, initiates a flow of current through a coil and generates a magnetic field. The magnetic field induces an electric field in a nearby conductor (cortical neurons), thereby resulting in current flow and neural stimulation (Rossini et al., 1994; Mills, 2004). The position at which the nerve is excited by magnetic stimulation depends on the voltage gradient parallel to the nerve fibre. Given that neural anatomy in the brain is complex, the point of excitation occurs at bends, branch points or at the transition from cell body to axon (Abdeen and Stuchly, 1994). As such, the orientation of neurons, relative to the induced electric field, is critical in determining which neurons are activated.

Physical properties of the coil will also influence neural excitation. Circular coils, used in this thesis (see Methodology) induce maximum current at the coil circumference. As such, coils placed at the vertex, with the edge overlying the hand area, will preferentially stimulate the primary motor area. Use of a more focal “figure-of-eight coil”, formed by two smaller adjacent circular coils, requires specific positioning and orientation over the
motor cortex to ensure adequate activation (Rossini et al., 1994; Mills, 2004). The
direction of current flow will dictate which hemisphere is stimulated. Intracellular
current is most effective at stimulating the motor cortex when flowing from a posterior-
anteor direction (i.e. inion to nasion). For a circular coil positioned at the vertex,
clockwise current in the coil (viewed from above) stimulates the right hemisphere
(Rossini et al., 1994).

Horizontally oriented neurons that are closest to the brain surface are activated by
magnetic stimulation. From animal experiments it has been demonstrated that cortical
stimulation results in generation of complex corticomotoneuronal volleys composed of
direct or D-waves (due to direct stimulation of the corticospinal axon) and multiple
indirect or I-waves arising from transynaptic excitation of pyramidal cells via excitatory
cortical interneurons (Patton and Amassian, 1954). In humans, TMS activates the motor
cortex at a depth of approximately 1.5 to 2.1 cm (Epstein et al., 1990; Rudiak and Marg,
1994) and cervical epidural recordings have confirmed the presence of D and I-waves, at
intervals of 1.5-2.5 ms (Kaneko et al., 1997; Di Lazzaro et al., 1998b). The I-waves are
numerically labelled such that the first I wave is called I1, the second I2, the third I3 and
so on (Di Lazzaro et al., 1998a). I-waves are best elicited by cortical currents directed in
a posterior to anterior direction, whereas D-waves are produced preferentially if the
current runs in a latera to medial direction (Mills et al., 1992; Werhahn et al., 1994;
Kaneko et al., 1996; Sakai et al., 1997; Ziemann and Rothwell, 2000; Di Lazzaro et al.,
2003a; Di Lazzaro et al., 2003b). The production of I-waves may not be sequential, such
that I3 waves may be recruited first if the stimulating conditions are optimal (Day et al.,
Although numerous models have been proposed to attempt to explain how I-waves are produced within the motor cortex, the mechanisms continue to remain elusive (Amassian et al., 1987; Day et al., 1989; Chen et al., 1995; Sakai et al., 1997; Ziemann and Rothwell, 2000).

In the clinical setting, the assessment of cortical excitability and the integrity of corticospinal pathways are best assessed by measuring the following parameters: (i) threshold; (ii) motor evoked potential (MEP) amplitude; (iii) central conduction time; (iv) cortical silent period; and (v) paired-pulse techniques assessing intracortical inhibition and facilitation.

1. Motor threshold

Motor threshold (MT), or the ease with which the corticomotoneurons can be excited, is defined as the minimum intensity necessary to elicit a small motor evoked potential (MEP) in the target muscle in at least half the trials (Rossini et al., 1999). With adaptation of threshold tracking to TMS, MT was re-defined as the minimum intensity required to elicit and maintain a target potential (Fisher et al., 2002). MT reflects the density of corticomotoneuronal projections onto the anterior horn cell, which is greatest for intrinsic hand muscles. As such, MT is lowest for intrinsic hand muscles of the dominant hand and correlates with the ability to perform fine finger tasks (Brouwer and Ashby, 1990; Macdonell et al., 1991; Triggs et al., 1994; Triggs et al., 1997; Chen et al., 1998).
MT is mediated by excitability of cortico-cortical and thalamo-cortical axons via voltage-dependent Na\(^+\) channels (Amassian et al., 1987; Epstein et al., 1990; Rudiak and Marg, 1994). Inhibition of voltage-dependent Na\(^+\) channels with medications such as carbamazepine, phenytoin and lamotrigine, increases MT (Mavroudakis et al., 1994; Ziemann et al., 1996c; Chen et al., 1997a; Boroojerdi et al., 2001). MT may also be influenced by neurotransmission through AMPA receptors, such that agents that enhance transmission through AMPA receptors reduce MT (Di Lazzaro et al., 2003c).

2. Motor evoked potential amplitude and stimulus-response curve

From epidural recordings, it has been established that MEP amplitude reflects summation of D- and I-waves (Amassian et al., 1987; Di Lazzaro et al., 1998c). At threshold, TMS elicits I\(_1\)-waves, which are followed by later I-waves, at intervals of 1.5 ms with increasing stimulus intensity (Di Lazzaro et al., 1998c). During this process, the increase in MEP amplitude that occurs with increasing stimulus intensity, may be used to generate the *stimulus-response (SR) curve*. This SR curve is known to follow a sigmoid function (Devanne et al., 1997).

As with MT, the MEP amplitude and the gradient of the SR curve, reflect the density of corticomotoneuronal projections onto anterior horn cells. The MEP amplitude and steepness of the SR curve are highest for intrinsic hand muscles (Brouwer and Ashby, 1990; Devanne et al., 1997; Chen et al., 1998). MEP amplitude may be expressed as a percentage of the maximum CMAP amplitude recorded following electrical stimulation (Rossini et al., 1999). This may be used to better differentiation between upper and lower
motor neuron lesions. For intrinsic hand muscles, the MEP:CMAP ratio is 0.5-0.6. However, there is a large inter-subject variability (Hess et al., 1987), thereby reducing the sensitivity of this measure in detecting abnormalities of the corticomotoneurons.

Neuromodulation of cortical activity influences the MEP amplitude and gradient of the SR curve. Medications that enhance transmission through the gamma-aminobutyric acid type A (GABA<sub>A</sub>) receptors, such as benzodiazepines, result in suppression of I-waves and ultimately reduction of the MEP amplitude and SR curve gradient (Inghilleri et al., 1996; Boroojerdi et al., 2001; Ziemann, 2004b). Conversely, norepinephrine, AMPA receptor agonists and serotonin reuptake inhibitors, all increase the MEP amplitude and SR curve gradient (Boroojerdi et al., 2001; Ilic et al., 2002a; Di Lazzaro et al., 2003c; Ilic et al., 2003). These changes in MEP amplitude and SR curve occur without changes in MT, thereby suggesting that the physiology underlying the generation of the MEP amplitude and MT is different.

3. Central motor conduction time

Central motor conduction time (CMCT) refers to the time interval between stimulation of the cortex and the onset of the EMG response (Rossini et al., 1999). Factors contributing to CMCT include the time to activate the pyramidal cells, conduction time of the descending volley down the corticospinal tract, synaptic transmission and activation of spinal motor neurons, motor axon conduction and neuromuscular transmission (Mills, 2004).
To calculate the CMCT, the peripheral component clearly needs to be estimated. This may be achieved using two methods, (i) The F-wave method (see Methodology) and (ii) the cervical nerve root stimulation method. With the F-wave method, the peripheral component is calculated according to the following formula (Mills and Murray, 1986; Claus, 1990):

\[
\text{Peripheral conduction} = \frac{(F+M-1)}{2}
\]

Where \( F \) is the minimum F-wave latency from 20 trials, \( M \) is the distal motor latency of the CMAP response and 1 is the estimated turnaround time at the anterior horn cell. F-wave refers to the late excitation of a muscle by an antidromic volley reaching and activating the spinal motor neuron.

Alternatively, CMCT may be calculated by stimulating the nerve roots at their exit foramina and then subtracting the nerve root-muscle latency from the cortex-muscle latency (Mills and Murray, 1986). It should be stressed that these methods provide an estimation of CMCT and there remain limitations with use of either method (Rossini et al., 1994; Mills, 2004).

Central motor conduction time may be influenced by a variety of technical, physiological and pathological factors (Mills, 2004). As such, there is a wide range of normative data and each laboratory needs to establish their own data. In ALS, CMCT may be prolonged in ~ 20% of cases and may be particularly useful when UMN signs are equivocal (Eisen et al., 1990; Mills and Nithi, 1997; Mills, 2003). Sensitivity is improved if recording is undertaken from both upper and lower limb muscles, and from the tongue, particularly in patients with bulbar signs (Urban et al., 1998; Urban et al., 2001; Mills, 2004). Abnormality of initiating I-waves and desynchronization of corticomotoneuronal volleys,
have been proposed as potential mechanisms underlying CMCT prolongation (Eisen et al., 1996; Kohara et al., 1996b; Komissarov et al., 2004).

4. Cortical silent period

Cortical silent period (CSP) is defined as interruption of voluntary EMG activity in the target muscle contralateral to the stimulated motor cortex which is typically preceded by an MEP response (Cantello et al., 1992). The CSP onset is defined from the onset of the MEP response, while the CSP offset is defined as resumption of voluntary EMG activity. CSP duration is measured from MEP onset to resumption of EMG activity (Cantello et al., 1992; Ziemann, 2004a) and correlates with stimulus intensity (Cantello et al., 1992; Inghilleri et al., 1993; Triggs et al., 1993).

The mechanisms underlying CSP generation are complex. The early segment of the CSP is mediated by inhibition at the level of the spinal motoneurons (Cantello et al., 1992; Inghilleri et al., 1993; Chen et al., 1999). The late segment of CSP is mediated by long-lasting inhibitory post-synaptic potential (IPSP), generated via gamma-aminobutyric acid type B (GABA_B) receptors (Connors et al., 1988; Inghilleri et al., 1993; Ziemann et al., 1993; Avoli et al., 1997). Evidence for this relationship is provided by pharmacological studies whereby GABA_B receptors agonists, such as baclofen, and GABA re-uptake inhibitors, such as tiagabine, lengthen the CSP duration (Siebner et al., 1998; Werhahn et al., 1999).
GABA_B receptors are metabotropic receptors that couple to Ca^{2+} and K^+ channels via G proteins and second messenger systems (Kornau, 2006). GABA_B receptors are located at pre- and postsynaptic sites of the nerve terminals. Post-synaptic GABA_B receptors mediate CSP by inducing an increase in K^+ efflux upon activation of a specific G-protein, thereby resulting in hyperpolarization of the postsynaptic membrane (Kofuji et al., 1995; Luscher et al., 1997; Kornau, 2006). Presynaptic GABA_B receptors mediate inhibition of N and P/Q voltage-gated Ca^{2+} channels resulting in inhibition of neurotransmitter release (Wu and Saggau, 1997; Takahashi et al., 1998; Filippov et al., 2000; Poncer et al., 2000). In addition to being modulated by GABA_B receptors, CSP is also influenced by motor attention, the extent of voluntary drive and other neuromodulators, such as dopamine (Priori et al., 1994; Ziemann et al., 1996a; Mathis et al., 1998).

Of clinical relevance, reduction of CSP duration has been reported in disorders affecting the GABA system, such as autoimmune-mediated stiff-person syndrome (Sandbrink et al., 2000) and in patients with infarction of the motor cortex (Schnitzler and Benecke, 1994). Shortening of CSP has also been reported in Parkinson’s disease due to cortical depletion of dopamine (Priori et al., 1994). Studies investigating changes of CSP duration in ALS have revealed conflicting results. While some studies have reported either an absence or reduction in CSP duration, most prominent early in the disease course (Desiato and Caramia, 1997; Siciliano et al., 1999; Zanette et al., 2002b; Mills, 2003), others have failed to demonstrate any abnormalities of CSP (Prout and Eisen, 1994; Ziemann et al., 1997c; Turner et al., 2005b).
5. Paired-pulse techniques

Transcranial magnetic stimulation may assess cortical excitability via a paired-pulse technique in which a conditioning stimulus modulates the effects of a second test stimulus. Seven different paired-pulse paradigms have been described (Ziemann, 2004a). The following discussion will focus on two paradigms measuring short interval intracortical inhibition and intracortical facilitation.

**Short interval intracortical inhibition** is measured via a paired-pulse paradigm, in which a subthreshold conditioning stimulus (set to 70-80% of resting MT) is delivered before a suprathreshold test stimulus (Kujirai et al., 1993; Nakamura et al., 1997; Hanajima et al., 1998b). In this paradigm, the conditioning and test stimuli remain constant and the effects of the conditioning stimulus are measured by recording changes in the amplitude of the test response. If the interstimulus interval (ISI) lasts between 1-5 ms the test response is inhibited, termed **short interval intracortical inhibition (SICI)**, while at ISIs of 7-20 ms the test response is facilitated, referred to as **intracortical facilitation (ICF)**.

A major limitation of the “constant stimulus” paired-pulse technique is the marked variability in the motor evoked potential (MEP) amplitude with consecutive stimuli (Kiers et al., 1993; Hanajima et al., 1998b). This variability results in part from spontaneous fluctuations in the resting threshold of cortical neurons. As a result, multiple stimuli must be delivered at each conditioning-test stimulus interval to ensure validity of the measurement. Recently, a novel method of assessing intracortical inhibition has been
reported using a computer-assisted “threshold tracking” technique (Awiszus et al., 1999; Fisher et al., 2002). In this method, a constant target MEP amplitude is tracked on-line by a test stimulus using different conditioning-test paradigms. The ability of this method to minimize recording variability, and to be adapted for clinical investigation, will be explored by this thesis (Chapter 4).

There is compelling evidence that SICI and ICF originate at the level of the motor cortex (Nakamura et al., 1997; Di Lazzaro et al., 1998c). Epidural recordings have established that SICI is associated with reduction in number and amplitude of late I-waves, mediated by inhibitory cortical interneurons via GABA_A receptors (Nakamura et al., 1997; Hanajima et al., 1998b; Di Lazzaro et al., 2000; Illic et al., 2002b; Ziemann, 2004b; Ziemann, 2004a). Conversely ICF was associated with an increase in the I-wave amplitude and is reduced by GABA_A receptor agonists (Nakamura et al., 1997; Ziemann, 2004a).

GABA_A receptors are ionotropic in that they directly gate Cl⁻ selective ion channels and possess modulatory binding sites for benzodiazepines, barbiturates, neurosteroids and ethanol (Bormann, 1988; Macdonald and Olsen, 1994). GABA_A receptors consist of five protein subunits arranged around a central pore, there are important structural differences (Mohler, 2006). Each subunit consistent of an extracellular N-terminal domain which forms the binding site for agonist/antagonists, followed by three membrane spanning domains (M1-3), an intracellular loop and a fourth membrane spanning domain (M4) (Mohler, 2006). The second membrane-spanning domain (M2) forms the wall of the
There are 16 different subunits comprising the GABA<sub>A</sub> receptor, namely: α1–6, β1–3, γ1–3, δ, ε, π and 0, with the α1β2/3γ2, α2β3γ2, and α3β3γ2 subunit combinations being the most frequent (Barnard et al., 1998; Whiting, 2003; Rudolph and Mohler, 2006). SICI does not appear to be mediated by GABA<sub>A</sub> receptors not bearing the alpha 1 subtype (Di Lazzaro et al., 2007).

Threshold tracking TMS techniques have reported two physiologically distinct phases SICI (Fisher et al., 2002). The first phase occurs at an ISI of ≤1 ms and the second peaks at ISI of 2.5 ms. Although the second phase of SICI is mediated by cortical interneurons, there is debate regarding the first phase of SICI. While some have argued that the first phase of SICI is mediated by relative refractoriness of pyramidal neurons (Fisher et al., 2002), others have suggested that this first phase of SICI is best explained by activation of inhibitory circuits different to that which mediate SICI at ISI of 2.5 ms (Roshan et al., 2003).

SICI and ICF are also modulated by other cortical neurotransmitter systems. Glutamate antagonists such as riluzole (Schwenkreis et al., 2000; Stefan et al., 2001), dopamine (DA) receptor agonists (Ziemann et al., 1996a; Ziemann et al., 1997b) and norepinephrine (NE) antagonists (Korchounov et al., 2003) increase SICI, while DA antagonists (Ziemann et al., 1997b) and NE agonists reduce SICI (Illic et al., 2003). Glutamate antagonists (Liepert et al., 1997), selective serotonin re-uptake inhibitors and NE antagonists (Illic et al., 2002a; Korchounov et al., 2003) decrease ICF, while NE agonists increase ICF (Plewnia et al., 2001; Plewnia et al., 2002).
Abnormalities of SICI and ICF have been reported in a variety of neurological disorders. Reduction in SICI and ICF have been reported in ALS (Yokota et al., 1996; Ziemann et al., 1997c; Hanajima and Ugawa, 1998; Sommer et al., 1999; Stefan et al., 2001; Zanette et al., 2002b), and is believed to result from a loss of parvalbumin-positive inhibitory cortical interneurons along with glutamate mediated down-regulation of SICI (Nihei et al., 1993; Stefan et al., 2001). In addition, reduction of SICI has also been reported in movement disorders such as Huntington’s disease (Abbruzzese et al., 1997), Parkinson’s disease (Ridding et al., 1995a; Strafella et al., 2000; Bares et al., 2003; Lefaucheur, 2005), task specific dystonias (Ridding et al., 1995b; Rosenkranz et al., 2005) and Tourette’s syndrome (Ziemann et al., 1997a), believed to be a product of altered afferent signalling to the motor cortex.

If TMS studies establish the presence of cortical hyperexcitability in ALS, it could be argued that down-regulation of intracortical inhibition in response to anterior horn cell loss, may explain this increase in cortical excitability. In order to investigate for this possibility, cortical excitability studies in this thesis were undertaken on Kennedy’s disease (KD) patients, a pathological control group.

**Kennedy’s disease**

Kennedy’s disease, or spinobulbomuscular atrophy, is a slowly progressive, X-linked recessive inherited neurodegenerative disorder of motor and sensory neurons (Kennedy et al., 1968). Clinical features usually appear between the ages of 30 and 50 (Kennedy et al., 1968; Harding et al., 1982; Greenland and Zajac, 2004) and males are affected, with
females being asymptomatic carriers (Ishihara et al., 2001; Schmidt et al., 2002; Gallo, 2004). Kennedy's disease may be misdiagnosed as ALS in ~2% of cases (Parboosingh et al., 1997). Unlike ALS, the life expectancy in KD is normal (Kouyoumdjian et al., 2005).

The genetic mutation underlying KD was identified as an increased expansion of the polymorphic cytosine-adenine-guanine (CAG) repeat sequence (coding glutamine) in the androgen receptor (AR) on the X chromosome, at Xq11-12 (La Spada et al., 1991). AR receptors are expressed on motor neurons where they function to promote axonal regeneration (Sar and Stumpf, 1977; Yu and McGinnis, 1986; Simerly et al., 1990; Jones, 1994). In normal individuals, the number of CAG repeats in the AR gene is 9-38, while in KD patients this repeat sequence is > 40 (La Spada et al., 1992; Zoghbi and Orr, 2000). Germline instability, whereby the size of the CAG repeats increases with successive generations, is evident in KD, with male to female transmissions being more unstable (La Spada et al., 1992; Spiegel et al., 1996). Further, the size of CAG repeat expansion positively correlates with disease severity (Doyu et al., 1992; La Spada et al., 1992) and age of onset (Zoghbi and Orr, 2000).

The pathophysiological mechanism by which the expanded CAG repeat underlies neurodegeneration has not been determined. An emerging view is that the mutated insoluble AR protein, which forms inclusion bodies within the nucleus and cytoplasm of motor neurons (Ordway et al., 1997; Stenoien et al., 1999), binds to transcription factors, ubiquitin, proteosomal proteins and molecular chaperones, thereby interfering with the
transcriptional machinery and the cells ability to dispose of mutant proteins (Bence et al., 2001; Chai et al., 1999; Jana et al., 2001; La Spada et al., 2001; Nucifora et al., 2001; Okazawa et al., 2002; Shimohata et al., 2005; Shimohata et al., 2000; Zuccato et al., 2003). Interestingly, the development of neurodegeneration in KD requires the presence of circulating androgens (Katsuno et al., 2002; Katsuno et al., 2003; Gallo, 2004; Katsuno et al., 2006).

Clinical features
Kennedy’s disease begins in early adulthood, with generalized fasciculations, muscle cramps, fatigue and postural hand tremor being the earliest features (Kennedy et al., 1968; Harding et al., 1982; Huang et al., 1998; Meriggioli et al., 1999; Gallo, 2004; Greenland et al., 2004; Lee et al., 2005). Subsequently, proximal muscle weakness and wasting develop (Gallo, 2004), often accompanied by bulbar features, such as jaw weakness, dysarthria and dysphagia (Kennedy et al., 1968; Harding et al., 1982; Huang et al., 1998; Meriggioli et al., 1999; Gallo, 2004; Greenland et al., 2004; Lee et al., 2005). Facial fasciculations with pursing of the lips, called contraction-fasciculations, are a specific feature of KD and may be evident in female heterozygote or homozygous mutation carriers (Schmidt et al., 2002; Gallo, 2004).

In addition to neurological features, partial signs of androgen resistance have been reported ~ 80% of KD patients, with gynaecomastia being most prominent (Kennedy et al., 1968; Harding et al., 1982; Huang et al., 1998; Meriggioli et al., 1999; Dejager et al., 2002; Gallo, 2004; Greenland et al., 2004; Lee et al., 2005). Alteration of testicular
exocrine function has also been reported in KD, with elevated testosterone levels documented in 70% of KD patients (Dejager et al., 2002). Testicular atrophy and reduced fertility have been reported less frequently in KD (Greenland and Zajac, 2004). Androgen insensitivity appears later in the disease process, and correlates with the size of the CAG repeat expansion (Dejager et al., 2002).

**Neurophysiological findings**

Although the diagnosis of KD is established by genetic testing, most KD patients are initially referred to ALS clinics where they undergo neurophysiological testing as a diagnostic work-up for ALS. In KD, sensory nerve conduction studies reveal reduced or absent sensory responses in most patients (Harding et al., 1982; Olney et al., 1991; Ferrante and Wilbourn, 1997; Meriggioli et al., 1999), reflecting a loss of dorsal root ganglion neurons (Harding et al., 1982; Sobue, 1995).

Accompanying the sensory findings are chronic neurogenic changes (large amplitude, long duration and polyphasic motor unit potentials) on needle EMG testing (Harding et al., 1982; Olney et al., 1991; Ferrante and Wilbourn, 1997). In addition, generalized and persistent fasciculations are evident in KD (Meriggioli et al., 1999; Hirota et al., 2000). Unlike ALS, the fasciculation potentials in KD patients are simple and their frequency is less (Hirota et al., 2000). In addition, the contraction-fasciculations, that are characteristic of KD, are electrically characterised by either (i) myokymia-like discharges that are induced by facial movement or (ii) 20-40 Hz discharges of individual motor unit action potentials of 0.1 to several second duration (Olney et al., 1991).
Although KD has been regarded as a pure lower motor neuron disorder (Kennedy et al., 1968), subclinical upper motor neuron involvement has been recently reported in KD (Attarian et al., 2006; Pachatz et al., 2007). Such a finding may argue against the hypothesis that ALS is primarily a disorder of corticomotoneurons. Consequently, the current thesis will apply novel axonal and cortical excitability techniques in sporadic and familial ALS patients, and will compare these findings to KD patients, in order to determine the site of disease onset and thereby the pathophysiological mechanisms underlying neurodegeneration in ALS.
METHODOLOGY
Subjects
The healthy subjects used as controls had no clinical evidence of a peripheral nerve
disorder or any history of medical conditions known to affect peripheral nerve function.
Patients with ALS did not have a history of other illnesses known to cause neuropathy,
and all were diagnosed with clinically probable or definite ALS as defined by the revised
El Escorial criteria (Brooks et al., 2000b). Patients with familial ALS (FALS) and
asymptomatic subjects from families with FALS all had genetically confirmed mutations
in the copper/zinc superoxide dismutase-1 gene (SOD-1). In patients with Kennedy’s
disease (KD), the diagnosis was confirmed on genetic testing. All subjects gave written
informed consent to the procedures, which had been approved by the South Eastern
Sydney Area Health Service Human Research Ethics Committee (Eastern Section).

Equipment
The following equipment was used to carry out the studies in this thesis:

Hardware requirements:
1) IBM-compatible personal computer fitted with a 16-bit data acquisition card (National
   Instruments PCI-MIO-16E-4) that sampled signals at 10 kHz.
2) A purpose-built isolated linear bipolar constant stimulator.
3) Conventional non-polarisable 5-mm Ag-AgCl surface EMG electrodes (ConMed,
   Utica, USA) for stimulation and recording.
4) Preamplifier and filter (3 Hz-3 kHz) for recording sensory and motor potentials (GRASS ICP511 A.C. amplifier, Grass-Telefactor, Astro-Med Inc., West Warwick, RI, USA).

5) Two high-power magnetic stimulators which were connected via a BiStim device (Magstim Co., Whitland, South West Wales, UK).

6) 90 mm circular coil for magnetic stimulation.

7) Purpose built thermometer for measuring skin temperature.

**Software requirements:**

Data acquisition and stimulation delivery (both electrical and magnetic) were controlled by a computerised threshold tracking programme, QTRACS software version 0.9.7 (© Professor Hugh Bostock, Institute of Neurology, Queen Square, London, UK).

**Stimulating and recording paradigms**

Peripheral nerve studies described in Chapters 1-8 were undertaken on motor axons of the median nerve (Fig. 4). The median nerve was stimulated electrically at the wrist via 5-mm Ag-AgCl surface electrodes (ConMed, Utica, USA), with the cathode placed at the wrist crease and the anode located in the mid-forearm. The resultant compound muscle action potentials (CMAPs) were recorded via surface electrodes with the active recording electrode positioned over the motor point of the abductor pollicis brevis (APB) and the reference electrode placed 4 cm distally (Kiernan et al., 2000). The peak-to-peak amplitude and onset latency for the CMAP were determined.
Cortical excitability studies, described in Chapters 4-8, were performed by transcranial magnetic stimulation (TMS), whereby the motor cortex was stimulated by means of a 90 mm circular coil oriented to induce a current flow in a posterior-anterior direction in order to activate the motor cortex. The coil, initially centered over the vertex, was moved in the antero-posterior and medial-lateral directions in order to find the optimal position for evoking responses of maximal amplitude from the APB muscle. The currents were generated by two high-power magnetic stimulators which were connected via a BiStim device (Magstim Co., Whitland, South West Wales, UK) so that conditioning and test stimuli could be independently set and delivered through one coil. The magnetic evoked potentials (MEPs) were recorded over the APB using surface electrodes and the peak-to-peak amplitude and onset latency were determined for MEPs.

**Figure 4.** Configuration of the testing paradigm for peripheral nerve stimulation. The median motor is stimulated at the wrist with recording over the abductor pollicis brevis muscle (APB). The magnetic evoked potentials are recorded over the abductor pollicis brevis using the same recording electrode configuration.
Threshold tracking and excitability protocols

Axonal excitability can be investigated using threshold tracking, where ‘threshold’ refers to the stimulus current required to produce a target potential (Bostock et al., 1998; Burke et al., 2001). Axonal excitability studies incorporating threshold tracking provide information about the membrane properties of axons at the site of stimulation. With threshold tracking, resting threshold is measured and nerve excitability is altered by changing the nerve environment, such as with ischemia, or by applying a conditioning polarizing current (Bostock et al., 1998). These techniques provide information about membrane potential and axonal ion channel function.

The threshold tracking software used in this thesis was an automated tracking system whereby the test stimulus intensity was automatically increased or decreased in percentage steps after each response, depending on the difference between the recorded and target responses (Fig. 5). For measurement of multiple excitability parameters, an automated multiple excitability protocol, TRONDXM2 (QTRAC version 0.9.7, Institute of Neurology, Queen Square, London, UK) was used that contained a proportional tracking system, in which the change in test stimulus current intensity was proportional to the difference (or error) between the recorded response and target response. Proportional tracking can be more efficient, especially when excitability changes abruptly.
Multiple excitability measures: sequence of recordings

To commence the protocol, stimulus-response (SR) curves were generated using test current impulses of 0.2- and 1-ms (Fig. 6A, B). The peak amplitude, measured from baseline to negative peak, generated by the current intensity of 1 ms duration was used to set the target response (40% of supramaximal CMAP response). Stimuli were increased in 4% steps, with two responses averaged at each step until three averages were considered maximal. The ratio between the SR curves for two different stimulus durations that produced the same CMAP response were used to calculate the rheobase, defined as the threshold current for a target response when the stimulus is of infinitely long duration (Bostock et al., 1998), and strength-duration time constant (τSD; Fig. 6D) of motor axons of different thresholds using Weiss’ formula (Weiss, 1901; Mogyoros et al., 1996a; Bostock et al., 1998). The threshold changes that occur in response to subthreshold depolarizing and hyperpolarizing pulses, referred to as threshold electrotonus, were measured by altering nerve excitability using prolonged sub-threshold polarizing currents of 100 ms duration, set to + 40% (depolarizing) and - 40%

Figure 5. In threshold tracking, the target response is set to 40% of the supramaximal compound muscle action potential (CMAP) amplitude. When the CMAP amplitude is smaller than the target response (dashed black line), the subsequent test stimulus current intensity is increased. When the CMAP amplitude is larger than the target response (grey line), the subsequent test stimulus current intensity is reduced. If the CMAP amplitude is equal to the target response, the subsequent test stimulus intensity remains unchanged.
(hyperpolarizing) of controlled threshold current (Bostock et al., 1998; Kiernan et al., 2000; Burke et al., 2001). Three stimulus combinations were tested sequentially: test stimulus alone (measured control threshold current); test stimulus + depolarizing current; test stimulus + hyperpolarizing current. Threshold was tested at 26 time points before, during and after the 100 ms polarizing pulse. The stimulus combinations were repeated until three valid estimates were recorded within 15% of target response (Kiernan et al., 2000).

A current-threshold relationship (I/V) was obtained by tracking the changes in threshold of 1 ms test pulses that occurred following sub-threshold polarizing currents of 200-ms duration which were altered in ramp fashion from +50% (depolarizing) to -100% (hyperpolarizing) of controlled threshold in 10% steps. Stimuli with conditioning currents were alternated with test stimuli until three valid threshold estimates were recorded. The I/V relationship estimates rectifying properties of both nodal and internodal segments of the axon (Baker et al., 1987; Bostock et al., 1998). The I/V gradient during depolarizing sub-threshold currents reflects conduction through outward rectifying K+ channels, while the I/V gradient during hyperpolarizing sub-threshold currents reflects inwardly rectifying conductances activated by hyperpolarization (Pape, 1996; Kiernan et al., 2000).

Finally, the recovery of axonal membrane excitability, referred to as the recovery cycle, was assessed by tracking the changes in threshold that occurred following a supramaximal conditioning stimulus of 1 ms duration. Eighteen conditioning-test
stimulus intervals were studied, decreasing from 200 to 2 ms. Three stimulus combinations were recorded: (i) unconditioned test stimulus (1 ms duration); (ii) supramaximal conditioning stimulus alone; (iii) conditioning and test stimuli in combination. The response in (ii) was subtracted on-line from response in (iii) so as to eliminate contamination of the measured CMAP response by the supramaximal conditioning response at short interstimulus intervals. Each stimulus combination was repeated until 4 valid estimates were obtained. Following completion of the recovery cycle, a profile of nerve excitability was generated using a customized plotting program (QTRACP version 0.0.5), consisting of six different plots (Fig. 6).

The 95% confidence limits were calculated as mean ± t_{0.05} SD (where t_{0.05} is the value of Student’s t-test which the probability of a larger value is 0.05) such that 95% of individual observations would fall within the limits if the variables were distributed normally. For data plotted on logarithmic axes, the logarithm of the variable was assumed to be normally distributed, and the mean plotted is the geometric mean. Differences in excitability parameters were analysed using Student’s t-test. A probability (P) value of < 0.05 was considered statistically significant. All results are expressed as mean ± standard error of the mean.
**Figure 6:** Six plots of excitability parameters recorded from abductor pollicis brevis muscle for a single subject. **(A)** Absolute stimulus-response relationship. **(B)** Normalised stimulus-response relationship. **(C)** Current-threshold relationship. **(D)** Strength-duration time constant. **(E)** Threshold electrotonus. The conditioning-test interval corresponding to hyperpolarising threshold electrotonus at 90-100 ms (TEh 90-100 ms) and depolarising threshold electrotonus at the same time interval (Ted 90-100 ms) are depicted. **(F)** Recovery cycle, demonstrating timepoint at which the relative refractory period (RRP) is measured, as well as superexcitability and late subexcitability.
Data analysis

Upon completion of the excitability protocol, values for multiple excitability measures were automatically generated. Normative values for these parameters have been established for the median nerve motor axons (Kiernan et al., 2000). The stimulus intensity (mA) was measured as the current required to elicit a target response set to 40% of maximal CMAP for a stimulus of 1 ms duration. Strength-duration time constant (ms), which reflects nodal persistent Na⁺ conductances (Bostock and Rothwell, 1997), was calculated for nine motor axonal populations, starting from axons contributing to CMAP responses between 5-15% up to the maximum of 85-95%, increasing in 10% batches (Kiernan et al., 2000). Rheobase (mA), defined as the threshold current for a target response when the stimulus is of infinitely long duration, was also calculated. Stimulus-response slope was calculated from the normalized SR curves by subtracting the stimulus that evoked a 25% maximal CMAP response from that which evoked a 75% response and dividing the result by the stimulus evoking a 50% response.

Threshold electrotonus parameters were calculated from data in Fig. 6E. TEd (peak) refers to the peak threshold reduction produced by a subthreshold depolarizing current. The reduction in threshold was measured at three time points relative to onset of the subthreshold depolarizing current, i.e. at 10-20 ms, TEd (10-20 ms), 40-40 ms TEd (40-60 ms), and at 90-100 ms TEd (90-100 ms). Threshold changes were also measured at similar latencies following a subthreshold hyperpolarizing current and are referred to as TEh (10-20 ms) and TEh (90-100 ms). S2 accommodation was calculated as the difference between the peak threshold reduction in the depolarizing direction and the
plateau value, i.e. TEd (peak) – TEd (90-100 ms). The peak threshold increase at the end of depolarizing current and peak threshold reduction at the end of hyperpolarizing current were averaged over 20 ms and are referred to as TEd (undershoot) and TEh (overshoot).

From the I/V graph, the following parameters were recorded; (i) resting I/V slope, calculated from polarizing currents between +10% to -10%, minimal I/V slope, calculated by fitting a straight line to each three adjacent points in turn, and (iii) hyperpolarizing I/V slope, calculated from polarizing current between 0-100%.

For the recovery cycle of axonal excitability the following parameters were measured; (i) relative refractory period (RRP, ms), defined as the first intercept at which the recovery curve crosses the x-axis; (ii) superexcitability, expressed as a percentage reduction in threshold current, was calculated as the minimum mean of three adjacent point at conditioning-test intervals of 5-15 ms and (iii) late subexcitability (%), as the maximum mean of three adjacent points at interstimulus intervals > 15 ms.

**Activity-dependent excitability studies**

For excitability studies assessing activity-dependent changes in Chapter 3, median nerve excitability was tracked before and after maximal voluntary contraction (MVC) of APB for 60 seconds (Vagg et al., 1998). Stimuli were delivered at 1.2 Hz and rotated sequentially through a series of six different conditioning-test combinations (Fig. 7). On channel 1, a fixed supramaximal stimulus of 0.2 ms was delivered to produce a CMAP of maximal amplitude. A stimulus 20% greater than channel 1 was delivered on channel 2.
and commenced following the period of voluntary contraction. This was done in order to ensure that the post-contraction maximal CMAP on channel 1 had remained truly maximal (Cappelen-Smith et al., 2000). On channel 3, a stimulus of 0.1 ms duration was delivered and its intensity fixed for the entire duration of the study to 70% of the pre-contraction maximal CMAP, in order to assess the effects of contraction-induced excitability changes on CMAP amplitude.

**Figure 7:** Configuration of stimulus patterns in Chapter 6. Vertical arrows indicate threshold tracking of test potential (set to 70% of the maximal potential, as measured on stimulus 1). Stimuli were delivered at 1.2 Hz, rotating through the 6 stimulus channels sequentially.
On channels 4-6, proportional tracking was used to produce a target CMAP 70% of maximal (Bostock et al., 1998). On channels 4 and 5, 0.1 ms and 1 ms stimuli were used respectively to achieve the target response and from the data obtained the strength duration time constant ($\tau_{SD}$) was calculated using Weiss’ formula (Weiss, 1901; Mogyoros et al., 1996a). Channel 6 tracked the changes in superexcitability, a period of increased axonal excitability which follows the RRP and is due to passive discharge of the myelin sheath (Barrett and Barrett, 1982; David et al., 1995). To assess superexcitability, a conditioning supramaximal stimulus was delivered 7 ms before the test stimulus. The test response on this channel was measured after online subtraction of the conditioning stimulus obtained in isolation (using the response obtained from channel 1). Superexcitability was measured as the reduction in stimulating current required to produce the target CMAP and expressed as a negative threshold change.

During the period of voluntary contraction, subjects abduced the thumb against resistance and were encouraged to maintain maximal effort for the entire duration of the 60 seconds. Resistance was provided by the same person in all studies. Stimuli were not delivered during the period of contraction and the hand and forearm were stabilized to limit contraction of other muscle groups and to ensure that there was no displacement of electrodes.

For nerve excitability studies post-MVC, values were compared to data obtained from normal controls ($N = 25$, 14 males, mean $45 \pm 3.1$ years, range 26-73). Although the controls were younger than ALS patients, sub-group analysis in healthy controls
suggested that age did not significantly affect the activity-dependent changes in threshold as indicated by the fact that the threshold increase in younger controls (age < 45 years, mean age 33.0 years, N = 14) of 1.20 ± 0.02%, was similar to that for the older group (age >45 years, mean age 60.3 years, N = 11) of 1.22 ± 0.07%, P = 0.3. For comparison of the magnitude of activity-dependent changes in excitability, ALS patients were assigned by two blinded neurologists as having either predominantly lower motor neurone or upper motor neurone involvement as measured by the upper motor neurone (UMN) score (Turner et al., 2004). Specifically, in ALS patients with predominantly lower motor neurone involvement the UMN score was ≤ 7, while an UMN score of 8-16 indicated predominantly UMN involvement. Single comparisons in excitability parameters were analysed using Student’s t-test. Repeated measures analysis of variance (ANOVA) was used for multiple comparisons and correlations were analysed using Pearson’s correlation coefficient. A probability (P) value of < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.

Cortical excitability studies

For studies assessing cortical excitability described in Chapters 4-8, TMS was applied to the motor cortex by means of a 90 mm circular coil oriented to induce a current flow in a posterior-anterior direction in order to activate the motor cortex. The coil, initially centered over the vertex, was moved in the antero-posterior and medial-lateral directions in order to find the optimal position for evoking responses of maximal amplitude from the APB muscle. The currents were generated by two high-power magnetic stimulators which were connected via a BiStim (Magstim Co., Whitland, South West Wales, UK) so
that conditioning and test stimuli could be independently set and delivered through one coil.

**TMS threshold tracking** (Fig. 8): In the conventional paired-pulse technique, the conditioning and test stimuli are kept at constant intensities, and changes in the MEP amplitude are measured. In this thesis, the output (MEP response) was fixed and changes in the test stimulus intensity required to generate a target response, when preceded by either sub- or suprathreshold conditioning stimuli, were measured. This technique is analogous to that used in the assessment of peripheral nerve excitability (Bostock et al., 1998; Burke et al., 2001).

![Figure 8](image.png)

*Figure 8.* Demonstration of threshold tracking transcranial magnetic stimulation experimental design used in the present thesis.

The threshold tracking strategy used for the present study was developed on the basis of preliminary observations that the stimulus-response relationship was exponential
(Devanne et al., 1997). Fisher and colleagues (2002) established that the relationship
between the logarithm of the MEP amplitude and the stimulus was close to linear over a
hundred-fold range of responses, from about 0.02 to 2 mV (Fisher et al., 2002). Based on
these observations a small target response of 0.2 mV (±20%), in the middle of this linear
range, was selected for the present study and subsequently tracked. Resting motor
threshold (RMT) was defined as the stimulus intensity required to produce and maintain
the target MEP response (0.2 mV peak-to-peak).

Initially, the SR curve for cortical stimulation was determined by increasing the intensity
of the magnetic stimulus to the following levels: 60, 80, 90, 100, 110, 120, 130, 140 and
150% RMT. Three stimuli were delivered at each level of stimulus intensity. The
maximum MEP amplitude (mV) and MEP onset latency (ms) were recorded. Central
motor conduction time (CMCT, ms) was calculated according to the F-wave method
(Mills and Murray, 1986; Claus, 1990; Cros et al., 1990).

The cortical silent period (CSP) induced by single-pulse TMS was recorded while
performing a weak voluntary contraction, broadly estimated by the investigators as
representing 10-30 % of maximum voluntary contraction. The magnetic stimulus
intensity was varied as for the SR curve. The duration of the silent period was measured
from the beginning of MEP to the return of EMG activity (Cantello et al., 1992).

A paired-pulse paradigm was developed, adapted from previous studies that investigated
short interval intracortical inhibition (SICI) (Kujirai et al., 1993; Hanajima et al., 1998b).
In this experimental paradigm a subthreshold conditioning stimulus preceded a suprathreshold test stimulus at increasing interstimulus intervals (ISIs) as follows: 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 7, 10, 15, 20, and 30 ms. The subthreshold conditioning stimulus (70% RMT) was such that it did not evoke a response. Stimuli were delivered sequentially as a series of three channels (Fig. 9): channel 1 tracked the stimulus intensity required to produce the unconditioned test response (i.e., RMT; shown in Fig. 10A,B); channel 2 monitored the subthreshold conditioning stimulus so as to ensure that an MEP response was not produced and that the subject remained relaxed; and channel 3 tracked the stimulus required to produce the target MEP when conditioned by a subthreshold stimulus equal in intensity to that on channel 2. Tracking was deemed acceptable when the test stimulus produced two consecutive MEP responses that were within 20% of the target response (0.2 mV) or consistently oscillated about the target. The three channels were applied sequentially. Stimuli were delivered every 5-10 seconds (stimulus delivery was limited by the charging capability of the BiStim system) and the computer advanced to the next ISI only when tracking met the target criteria.

Precision of the tracking method was limited by the fact that the intensity of the magnetic stimulus was restricted to integral values from 1% to 100% of maximum stimulator output.
Figure 9: Experimental paradigm and configuration of stimulus patterns used in Chapters 4-8. Cortical excitability was assessed by measuring changes in stimulus intensity required to generate a target magnetic evoked potential response of 0.2 mV, recording over the abductor pollicis brevis. Channel 1 = unconditioned test stimulus, measuring resting motor threshold (RMT); Channel 2 = conditioning stimulus, which was set to subthreshold (70% RMT) when assessing short interval intracortical inhibition; Channel 3 = conditioned test stimulus at different interstimulus intervals (ISIs). SICI was measured by increasing ISI from 1-30 ms.
Figure 10: (A) Illustration of threshold tracking. The dashed horizontal line represents the target output of 0.2 mV which was “tracked”. The circles (clear and filled) represent the magnitude of the motor evoked potential (MEP) amplitude with each stimulus. (B) Illustration of three MEP responses of different amplitude. The MEP response is initially larger (a), then smaller (b), and again larger (c) than the target output of 0.2 mV in three consecutive stimuli. These are depicted as filled circles in Figure A. The dashed horizontal lines represent the tracking windows, which were set to 0.2 mV (peak-to-peak).
Intracortical inhibition induced by a conditioning stimulus was measured as the increase in the test stimulus intensity required to evoke the target MEP. Inhibition was calculated off-line using the following formula (Fisher et al., 2002):

\[
\text{Inhibition} = \frac{(\text{Conditioned test stimulus intensity} - \text{RMT})}{\text{RMT}} \times 100
\]

Facilitation was measured as the decrease in the conditioned test stimulus intensity required to evoke the target MEP.

Each data point was weighted (QTRACS software version 0.9.7) such that any measures recorded outside the threshold target window (0.2mV, peak-to-peak) contributed least to the data analysis. All results are expressed as mean ± standard error of the mean. Student t-test was used for assessing differences between two groups. Analysis of variance (ANOVA) was used for multiple comparisons to assess the difference between the conditioned test and unconditioned test stimuli at different ISIs. A probability (P) value of < 0.05 was considered statistically significant.

**Staging of Amyotrophic Lateral Sclerosis**

A neurological history and physical examination were undertaken in all ALS and KD patients. All ALS patients were clinically staged using the amyotrophic lateral sclerosis functional rating scale-revised (ALSFRS-R) (Cedarbaum et al., 1999). The ALSFRS-R is a questionnaire-based, 12-item functional scale administered to the patient or, if the patient can not communicate effectively, to an informant such as spouse. The ALSFRS-R incorporates items assessing bulbar function, fine motor function, gross motor function and respiratory function. Each item is rated from 0 (complete dependence for that
function) to 4 (normal function), resulting in a total ALSFRS-R score ranging from 0 to 48 (normal).

In addition to assessing the ALSFRS-R, the hand strength in all patients was assessed using the Medical Research Council (MRC) rating scale (Medical Research Council, 1976), while hand function was assessed with the Trigg’s hand function score (Triggs et al., 1999). The MRC scale is a five point scale ranging from 0 (no movement) to 5 (normal strength). The Trigg’s hand function score is a four point scale and is graded as follows: 0 = normal; 1 = mild to moderate hand weakness without impairment of dexterity; 2 = weak with significant impairment of dexterity (i.e., difficulty with handwriting and buttoning clothes); and 3 = marked weakness-major disability and loss of fine motor control.

Upper motor neuron (UMN) signs were graded using the UMN score (Turner et al., 2004). This UMN score comprised a sum of pathologically brisk reflexes that included assessment of biceps, supinator, triceps, finger, knee and ankle reflexes, with plantar responses, facial and jaw jerks, all bilaterally, for a maximum possible score of 16 (Turner et al., 2004).
Chapter 1

Axonal excitability properties

in amyotrophic lateral sclerosis
SUMMARY

In order to investigate the mechanisms underlying the generation of fasciculations in amyotrophic lateral sclerosis (ALS), axolemmal ion channel function was assessed in ALS using axonal excitability testing. Specifically, the threshold tracking protocol was implemented to measure multiple indices of axonal excitability in 26 ALS patients by stimulating the median motor nerve at the wrist. The excitability indices studied included: stimulus-response curve (SR); strength-duration time constant ($\tau_{SD}$); current/threshold relationship; threshold electrotonus to a 100ms polarizing current; and recovery curves to a supramaximal stimulus. Compound muscle action potential (CMAP) amplitudes were significantly reduced in ALS patients (ALS 2.84±1.17 mV; controls 8.27±1.09 mV, P < 0.0005) and the SR curves for both 0.2 and 1 ms pulse widths were shifted in a hyperpolarized direction. Threshold electrotonus revealed a greater threshold change to both depolarizing and hyperpolarizing conditioning stimuli, similar to the “fanned out” appearance that occurs with membrane hyperpolarization. The $\tau_{SD}$ was significantly increased in ALS patients (ALS 0.50±0.03 ms; controls 0.42±0.02 ms, P < 0.05). The recovery cycle of excitability following a conditioning supramaximal stimulus revealed increased superexcitability in ALS patients (ALS 29.63±1.25 %; controls 25.11±1.01 %, P < 0.01). Threshold tracking studies revealed widespread dysfunction in axonal ion channel conduction, including increased persistent Na⁺ channel conduction, and abnormalities of fast paranodal K⁺ and internodal slow K⁺ channel function, in ALS patients. An increase in persistent Na⁺ conductances coupled with reduction in K⁺ currents would predispose axons of ALS patients to generation of
fasciculations and cramps. Axonal excitability studies may provide insight into mechanisms responsible for motor neuron loss in ALS.
INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder of motor neurons in the spinal cord, brainstem and motor cortex (Desai and Swash, 2002). Fasciculations are an almost inevitable feature of ALS and reflect ectopic activity of motor axons (Wettstein, 1979; Conradi et al., 1982; Roth, 1982; Roth, 1984; Layzer, 1994; Miller, 2002; Miller and Layzer, 2005). Such ectopic activity may arise due to abnormalities in axonal excitability and changes in resting membrane potential (Mogyoros et al., 1996a).

Threshold electrotonus is the only clinically available method that enables insight into nodal and internodal axonal membrane conductances in humans in vivo. With this technique, long-lasting DC current pulses are used to alter the potential difference between nodal and internodal membrane (Bostock et al., 1998; Burke et al., 2001). Such a process may produce a non-propagating change in membrane potential, and thereby change the activity of voltage-dependent conductances with resultant changes in axonal excitability.

The first clinical application of threshold electrotonus involved the investigation of ALS patients (Bostock et al., 1995). This study established that peripheral motor axons in ALS had abnormal membrane properties. Specifically, the majority of patients (63.6 %) demonstrated greater threshold reduction and thereby increased excitability during the depolarization phase of threshold electrotonus (type I response). Other patients (36.4%) demonstrated an exceptionally rapid increase in threshold to depolarizing current (type 2
response). From these studies it was hypothesized that patients with ALS may have a relative deficiency of axonal $K^+$ channels. Other studies, however, have suggested less frequent abnormalities in ALS patients, in addition to a greater variability (Kodama et al., 1995; Horn et al., 1996).

Subsequent to these original studies, a new protocol for testing multiple measures of axonal excitability were developed for clinical investigation that combine threshold electrotonus with other techniques to explore nerve excitability (Kiernan et al., 2000). As some of the difficulties in interpreting the variability of findings with threshold electrotonus in ALS patients may yet be overcome by measuring multiple excitability parameters simultaneously (Kiernan and Burke, 2004a), the present study employed such a protocol in an attempt to clarify whether specific changes in resting membrane potential and axonal conductances underlie excitability abnormalities in peripheral axons in ALS.

**METHODS**

**Patients**

Studies were undertaken in 26 patients with clinically probable or definite ALS (18 men, 8 women: age range 43-71, mean 59.6 years) as defined by the revised El Escorial criteria (Brooks et al., 2000b). All patients were referred from the multidisciplinary ALS clinical service held at Prince of Wales Hospital and were clinically assessed using the ALS-functional rating scale-revised (ALSFRS-R) (Cedarbaum et al., 1999), hand function score (Triggs et al., 1999), and hand strength using the Medical Research Council (MRC) rating scale (Medical Research Council, 1976). The patients were classified according to
the site of disease onset into predominantly limb or bulbar-onset groups. CMAP amplitude was used as a broad marker of disease severity, using CMAP amplitude < 4 mV as a cut-off for more severe disease involvement, based on data obtained from control subjects (see Chapter 4).

**Nerve excitability testing**

Nerve excitability studies were performed according to a previously described protocol that measures multiple parameters of nerve excitability (Kiernan et al., 2000). In all studies, the median nerve was stimulated at the wrist with the resultant CMAP recorded over the APB (see Methodology). Skin temperature was monitored close to the site of stimulation for the duration of each study.

The following excitability parameters were recorded using the previously described TRONDXM2 multiple excitability protocol (see Methodology): stimulus-response (SR) curves using stimuli of 0.2 and 1 ms duration, rheobase and strength-duration time constant (τSD), threshold electrotonus to 100 ms polarizing currents (a marker of internodal axonal membrane function), current-threshold relationship (a marker of inwardly rectifying K⁺ conductance), refractoriness (due to inactivation of transient Na⁺ channels), superexcitability (dependent of paranodal fast K⁺ channels) and late subexcitability (determined by nodal slow K⁺ conductance). The values of measured excitability parameters were compared to previously reported normal control data obtained from 29 subjects (21 men; 8 women aged 24-58 years) (Kiernan et al., 2000). Student’s t-test was used to compare differences in means between ALS patients and
control subjects. A probability (P) value of < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.

RESULTS

The clinical features of all 26 ALS patients are summarized in Table 1.1. A full sequence of excitability measurements was recorded from APB in each patient. There was a trend towards rightward shift of the stimulus-response curves in the ALS group for stimuli of both 0.2 ms and 1 ms duration, suggesting that axons in ALS patients were of higher threshold than controls (Fig. 1.1, P = 0.09), a uniform finding in ALS patients, independent of CMAP amplitude. The CMAP amplitude was significantly reduced in the ALS group when compared to control values (ALS group, 2.84 ± 1.17 mV; controls, 8.27 ± 1.09 mV, P < 0.0005).

Threshold electrotonus

Threshold electrotonus was abnormal in 18 of 26 (69.2%) ALS patients. A “fanned out” response was evident in 10 (38.5%) patients. The type I abnormality, in which there is a greater change in response to a sub-threshold depolarizing pulse (Bostock et al., 1995), is depicted for an illustrative ALS patient (Fig. 1.2 A). Overall, the type I abnormality was evident in 26.9% of ALS patients in the present study. The type II abnormality, in which there is a sudden decrease in membrane excitability marked by an abrupt increase in threshold, was not evident in any of the ALS patients from the present study. In one patient with advanced ALS (patient # 7, Table 1, CMAP = 0.4 mV), a “fanned in” response was evident. Analysis of mean data established greater threshold changes to
both depolarizing and hyperpolarizing sub-threshold conditioning pulses in ALS patients,
similar to the “fanned out” response that occurs with membrane hyperpolarization (Fig. 1.2 B-D).

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Disease onset</th>
<th>Disease Duration (months)</th>
<th>ALSFRS</th>
<th>Bulbar</th>
<th>Fine motor</th>
<th>Gross motor</th>
<th>Respiratory</th>
<th>Triggs hand score</th>
<th>MRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59,M</td>
<td>UL</td>
<td>40</td>
<td>34</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>69,M</td>
<td>UL</td>
<td>9</td>
<td>45</td>
<td>12</td>
<td>5</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>69,M</td>
<td>UL</td>
<td>48</td>
<td>36</td>
<td>6</td>
<td>6</td>
<td>14</td>
<td>10</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>67,F</td>
<td>UL</td>
<td>6</td>
<td>40</td>
<td>12</td>
<td>4</td>
<td>12</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>56,M</td>
<td>UL</td>
<td>19</td>
<td>40</td>
<td>11</td>
<td>6</td>
<td>15</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>51,M</td>
<td>UL</td>
<td>36</td>
<td>42</td>
<td>12</td>
<td>12</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>43,F</td>
<td>UL</td>
<td>18</td>
<td>40</td>
<td>9</td>
<td>5</td>
<td>14</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>44,F</td>
<td>UL</td>
<td>12</td>
<td>46</td>
<td>12</td>
<td>6</td>
<td>16</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>62,M</td>
<td>UL</td>
<td>6</td>
<td>42</td>
<td>12</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>50,M</td>
<td>UL</td>
<td>24</td>
<td>42</td>
<td>12</td>
<td>8</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>71,M</td>
<td>UL</td>
<td>96</td>
<td>42</td>
<td>12</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>70,M</td>
<td>UL</td>
<td>10</td>
<td>42</td>
<td>11</td>
<td>4</td>
<td>16</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>13</td>
<td>54,M</td>
<td>UL</td>
<td>14</td>
<td>46</td>
<td>12</td>
<td>6</td>
<td>16</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>54,M</td>
<td>UL</td>
<td>14</td>
<td>37</td>
<td>12</td>
<td>8</td>
<td>5</td>
<td>12</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>59,M</td>
<td>UL</td>
<td>24</td>
<td>45</td>
<td>12</td>
<td>5</td>
<td>16</td>
<td>12</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>70,M</td>
<td>UL</td>
<td>18</td>
<td>46</td>
<td>12</td>
<td>6</td>
<td>16</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>17</td>
<td>58,F</td>
<td>UL</td>
<td>10</td>
<td>43</td>
<td>12</td>
<td>4</td>
<td>15</td>
<td>12</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>18</td>
<td>47,M</td>
<td>LL</td>
<td>5</td>
<td>42</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>19</td>
<td>64,M</td>
<td>LL</td>
<td>21</td>
<td>36</td>
<td>12</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>69,M</td>
<td>LL</td>
<td>48</td>
<td>36</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>12</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>59,M</td>
<td>Bulbar</td>
<td>18</td>
<td>39</td>
<td>6</td>
<td>6</td>
<td>16</td>
<td>11</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>22</td>
<td>56,F</td>
<td>Bulbar</td>
<td>16</td>
<td>40</td>
<td>8</td>
<td>4</td>
<td>16</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>23</td>
<td>53,F</td>
<td>Bulbar</td>
<td>20</td>
<td>44</td>
<td>8</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>24</td>
<td>66,M</td>
<td>Bulbar</td>
<td>6</td>
<td>38</td>
<td>8</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>25</td>
<td>68,F</td>
<td>Bulbar</td>
<td>21</td>
<td>36</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>26</td>
<td>62,F</td>
<td>Bulbar</td>
<td>10</td>
<td>40</td>
<td>7</td>
<td>6</td>
<td>15</td>
<td>12</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Mean</td>
<td>59.6</td>
<td></td>
<td>21.9</td>
<td>40.7</td>
<td>10.3</td>
<td>6.3</td>
<td>12.3</td>
<td>11.7</td>
<td>1.2</td>
<td>4.1</td>
</tr>
<tr>
<td>SEM</td>
<td>1.7</td>
<td></td>
<td>3.7</td>
<td>0.7</td>
<td>0.5</td>
<td>0.4</td>
<td>0.7</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 1.1. Clinical details for 26 patients with amyotrophic lateral sclerosis (ALS). The site of disease onset was classified as either upper limb (UL), lower limb (LL), or bulbar. Disease duration refers to the period from symptom onset to date of testing. The patients were clinically graded using the amyotrophic lateral sclerosis functional rating scale revised (ALSFRS-R), with a maximum score of 48 when there is no disability. The ALSFRS-R is comprised of 4 sub-scores; bulbar (maximum score 12), fine motor (maximum score 8), gross motor (maximum score 16), and respiratory (maximum score 12). Muscle strength was clinically assessed using the Medical Research Council (MRC) for the abductor pollicis brevis, as this muscle was utilized for excitability testing.
Figure 1.1. Stimulus response (SR) curves obtained using test stimuli of 0.2 and 1 ms duration in controls (dotted lines) and patients with amyotrophic lateral sclerosis (ALS, solid lines). The SR curves are shifted to the right in ALS patients indicating that the median nerve at the wrist is of increased threshold. The maximum compound muscle action potential (CMAP) amplitude was reduced in ALS patients.
Figure 1.2. Threshold electrotonus refers to changes in membrane excitability in response to long duration polarizing currents. Threshold reduction with depolarization is represented in an upward direction and hyperpolarization in a downward direction. (A) The figure illustrates a recording from one patient with amyotrophic lateral sclerosis (ALS) superimposed on mean data ± standard error of mean from 29 controls. This patient exhibited the type I response in which there is a greater threshold reduction to a sub-threshold depolarizing current. Group data revealed a significant increase in threshold to (B) sub-threshold depolarizing currents at 40-60 ms, TEd (40-60 ms), and (C) sub-threshold depolarizing currents at 90-100 ms, TEd (90-100 ms), in ALS patients. (D) The threshold increase to sub-threshold hyperpolarizing currents at 90-100 ms, TEh (90-100 ms) was significantly increased in ALS patients. *P < 0.05; **P < 0.01.
Current/threshold (I/V) relationship

The I/V relationship estimates rectifying properties of both nodal and internodal segments of the axon (Baker et al., 1987; Bostock et al., 1998). The I/V gradient during depolarizing sub-threshold currents reflects conduction through outward rectifying K⁺ channels, while the I/V gradient during hyperpolarizing sub-threshold currents reflects inwardly rectifying conductances activated by hyperpolarization (Pape, 1996; Kiernan et al., 2000). The hyperpolarizing I/V gradient was not significantly different in ALS patients when compared to controls (ALS, 0.38 ± 0.03; controls, 0.37 ± 0.01, P = 0.69), nor was the resting I/V gradient (ALS, 0.55 ± 0.03; controls, 0.62 ± 0.02, P = 0.12, Fig. 1.3).

Strength duration time-constant and rheobase

Strength-duration time constant, which reflects nodal persistent Na⁺ conductances (Bostock and Rothwell, 1997), and the rheobase, defined as the threshold current for a stimulus of infinitely long duration (Bostock et al., 1998) can both be used to define strength-duration properties (Weiss, 1901; Bostock, 1983; Mogyoros et al., 1996a). To estimate τSD, nine axonal populations were studied from the SR curves depicted in Figure 1.4, starting from axons contributing to CMAP responses between 5-15% up to the maximum of 85-95%. As illustrated in Figure 1.4 A, the nine τSD were significantly longer in ALS patients when plotted against their CMAP responses and the mean τSD was significantly longer in the 26 ALS patients compared to controls (Fig. 1.4 B). While there was no significant difference in the rheobase (ALS, 3.30 ± 1.08 mA; controls, 3.05 ± 1.06 mA, P = 0.39), the previously documented negative correlation between the τSD
and rheobase established for controls (Mogyoros et al., 1998a), was maintained in ALS patients from the present study (R = -0.48, P < 0.05).

**Figure 1.3.** The current threshold relationship was not significantly different in amyotrophic lateral sclerosis (ALS, clear circles) when compared to controls (filled circles). Data expressed as mean ± standard error of mean in both groups.
Figure 1.4. The strength-duration time constant (τ_{SD}) reflects nodal persistent Na⁺ conductances. (A) The distribution of τ_{SD} in nine different populations of axons contributing to the compound muscle action potential (CMAP) response, starting from axons that contribute 5-15% of maximal response and increasing in 10% increments up to a maximum of 85-95%. The τ_{SD} was significantly increased in amyotrophic lateral sclerosis patients (ALS, clear circles) compared to controls (filled circles) for each of the populations. (B) Mean data illustrating that the τ_{SD} was significantly increased in ALS patients compared to controls. *P < 0.05
Recovery cycle of excitability

The recovery cycle reflects changes in membrane excitability in response to a supramaximal conditioning stimulus (Eisen et al., 1982; Kiernan et al., 1996b). The initial phase of the recovery cycle is the refractory period due to the recovery from inactivation of transient Na⁺ channels. The duration of the RRP was not significantly different when compared to controls (ALS, 3.22 ± 1.04 ms; controls, 3.15 ± 1.02 ms, P = 0.60). Superexcitability, a period of increased axonal excitability due to a depolarizing afterpotential spreading to the internodal axolemma (Barrett and Barrett, 1982), was significantly increased in ALS patients as a group compared to controls (Fig. 1.5 A, B). The final phase of the recovery cycle, referred to as the late subexcitability period, probably reflects activation of slow K⁺ channels (Waxman and Ritchie, 1993; Kiernan et al., 1996b; Burke et al., 2001), and was not significantly different when compared to controls (ALS, 15.06 ± 0.94%; controls, 14.87 ± 0.74%, P = 0.85).

Correlation with clinical parameters and disease duration

When ALS patients were analyzed as a group, there was correlation between the CMAP amplitude and threshold electrotonus [TEh (20-40 ms, R = 0.55, P < 0.01), and TEh (90-100 ms, R = 0.56, P < 0.01)]. In a sub-group of ALS patients with limb-onset disease the CMAP amplitude again correlated with threshold electrotonus [TEh (20-40 ms, R = 0.46, P < 0.05), TEh (90-100 ms, R = 0.50, P < 0.05)] and furthermore, the ALSFRS-R bulbar sub-score correlated with threshold electrotonus [TEh (20-40 ms, R = 0.54, P < 0.05), TEh (90-100 ms, R = 0.50, P < 0.05), TEd (90-100 ms, R = 0.62, P < 0.01), and TEd (40-60 ms, R = 0.54, P < 0.05)]. Taken together, these findings suggest that the change in
threshold electrotonus, TEh (90-100ms) is an early feature of ALS, occurring when the peripheral disease burden is limited.

**Figure 1.5.** (A) Illustration of the recovery cycle, which depicts changes in axonal excitability following a single supramaximal conditioning stimulus, in a single patient with amyotrophic lateral sclerosis (ALS) superimposed on mean data ± standard error of mean from 29 controls. Relative refractory period (RRP, ms), refers to the first intercept on the $\chi$-axis and reflects the recovery of inactivated transient Na$^+$ channels. Superexcitability (%) is expressed as the percentage change in the threshold current. Late subexcitability (%) refers to the largest increase in threshold following superexcitability. Superexcitability was increased in this representative patient. (B) Group data revealed a significant increase in superexcitability in ALS patients. **P < 0.01
This is supported by the greater change in TEh (90-100 ms) in ALS patients with limited peripheral disease, as determined by the CMAP amplitude (ALS less severe disease, CMAP > 4mV, TEh [90-100 ms] 138 ± 4.2; ALS severe disease, CMAP < 4mV, TEh [90-100 ms], 112.8 ± 7.6 %, P < 0.01).

DISCUSSION

The present study has established abnormalities of axonal excitability in ALS patients. As previously reported, the $\tau_{SD}$ was prolonged in ALS patients (Mogyoros et al., 1998a). Type I abnormalities were evident during threshold electrotonus in 26.9% of patients but no type II abnormalities were observed. Overall however, there was a greater threshold reduction to depolarizing and hyperpolarizing sub-threshold currents in ALS patients, suggestive of a “fanned out” response. These changes observed during threshold electrotonus were accompanied by an increase in superexcitability during the recovery cycle of excitability. Taken in total, it would not be possible to attribute these excitability changes to an alteration in a single axonal membrane conductance. Rather, these combined abnormalities may suggest more widespread dysfunction of axonal membrane ion channel function, the basis of which will be further explored in the discussion.

Threshold electrotonus and ALS

Threshold electrotonus refers to changes in membrane excitability in response to long duration polarizing currents and provides information on internodal membrane conduction (Bostock and Baker, 1988; Bostock et al., 1998). Threshold electrotonus is sensitive to changes in both membrane potential and alterations in internodal slow K$^+$ channels.
channel conduction (Bostock et al., 1998). The previously reported type I abnormality, resulting from reduced internodal slow K+ channel conduction (Bostock et al., 1995) was less frequent in ALS patients from the present study and the type II abnormality, postulated to be due to regenerative depolarization was not observed (Bostock et al., 1995). However, in keeping with a previous study (Horn et al., 1996), a “fanned out” response was noted in ALS patients, similar to that seen with membrane hyperpolarization. The changes in threshold electrotonus correlated with CMAP amplitude and ALSFRS-R bulbar sub-scores, which may suggest that the “fanned out” response occurs early in ALS when disease burden is small. That the “fanned out response” is an early feature of ALS is further supported by the fact the TEh (90-100 ms) is greater in ALS patients with limited peripheral disease, as evidenced by CMAP amplitude > 4 mV. The subsequent “pseudo-normalization” of excitability changes as the disease progresses may indicate that the “sickest” or most abnormally responding axons have already died (Kiernan and Burke, 2004a; Winhammar et al., 2005). This process may also underlie the variability reported from previous studies in ALS patients (Bostock et al., 1995; Kodama et al., 1995; Horn et al., 1996).

While hyperpolarization may partly account for the observed changes in threshold electrotonus, as supported by an increase in superexcitability, the absence of change in other excitability parameters, such as the resting I/V slope and late subexcitability would argue against significant membrane hyperpolarization as the sole explanation for this finding. In the absence of uniform excitability changes to implicate membrane hyperpolarization, an alternative explanation at least for the changes during threshold
electrotonus, is that they reflect axonal K⁺ channel dysfunction (Bostock et al., 1995). Further support for such an explanation may also be derived from recent gene expression studies that documented reduced mRNA expression of K⁺ channel genes, both paranodal fast K⁺ channel genes (KCNA1 and KCNA2) and the nodal K⁺ channel gene (KCNQ2), in spinal motor neurons of ALS patients (Jiang et al., 2005).

Strength-duration time constant and persistent Na⁺ conductances in ALS

Strength-duration time constant is a measure of the rate at which the threshold current for a target potential declines as stimulus duration increases and reflects persistent Na⁺ channel conductances (Bostock, 1983; Bostock and Rothwell, 1997). Mogyoros and colleagues (1998) established that the τSD was longer in ALS patients and the present study has confirmed the original finding.

Prolongation of the τSD may have resulted from changes in nerve geometry due to axonal loss. Although axonal loss is a major pathological feature of ALS, rheobase a parameter sensitive to changes in nerve geometry (Mogyoros et al., 1996a), was unaffected. Furthermore, there was a normal preservation of the negative correlation between τSD and rheobase in ALS patients (Mogyoros et al., 1998a). In addition to axonal loss, nerve demyelination may also prolong the τSD by exposing internodal membrane with a higher time constant (Brismar, 1981). As ALS is not recognized as a demyelinating disorder (Murakami, 1990), this could not explain the prolonged τSD in the present study.
What then underlies this reproducible change in the $\tau_{SD}$?

Strength-duration time constant is voltage-dependent and may be expected to increase with axonal depolarization. Membrane depolarization seems an unlikely explanation for the prolonged $\tau_{SD}$ observed for the ALS patients in the present study, given that other indices of membrane excitability did not suggest such a change. Specifically, superexcitability, the most sensitive parameter of membrane potential (Kiernan and Bostock, 2000) increased, or in other words moved in the opposite direction to that expected for membrane depolarization; and threshold electrotonus if anything fanned out, both changes arguing against membrane depolarization as an explanation for the prolongation in the $\tau_{SD}$.

While an increase in the $\tau_{SD}$ has been previously linked to the process of axonal regeneration and sprouting (Kanai et al., 2003), and metabolic abnormalities in degenerative motor neurons affecting Na$^+$ channel gating properties can not be excluded (Mogyoros et al., 1998a), the increase in the $\tau_{SD}$ in ALS patients may reflect upregulation or increase in persistent Na$^+$ conductances. Such an increase has been recently demonstrated in motor neurons cultured from transgenic SOD-1 mice (Kuo et al., 2005). Of further relevance, riluzole, the only disease modifying treatment for ALS, is known to block persistent Na$^+$ channels (Urbani and Belluzzi, 2000) and also to reduce the excess persistent Na$^+$ currents expressed in mutant SOD-1 motor neurons (Kuo et al., 2005).

Coincidentally, Kanai and colleagues (Kanai et al., 2006) have recently reported on the presence of similar widespread dysfunction of axonal excitability in a group of Japanese
patients with ALS. Specifically, reductions in K\(^+\) channel conductances and increases in persistent Na\(^+\) conductances were documented, much as established in the present study. To help clarify these findings, the authors applied a mathematical model of a human motor axon (Kiernan et al., 2005a) and established that a reduction in fast and slow K\(^+\) channel conductances drives membrane depolarization, partly accounting for an increase in the \(\tau_{SD}\). Of further relevance, the increase in superexcitability, as similarly documented in ALS patients from the present study, resulted from an increase in nodal leak conductance and reduction of internodal fast K\(^+\) conduction. The changes in axonal excitability established for ALS patients in the present study are entirely in keeping with the modeled hypothesis, further validating the findings from two independent patient groups.

**Clinical implications**

In addition to suggesting further therapeutic targets in ALS, the combination of reduction in axonal K\(^+\) currents with an increase in persistent Na\(^+\) conductances, may contribute to the clinical symptoms of ALS, particularly cramps and fasciculations. Reduction in axonal K\(^+\) conductances would decrease hyperpolarizing tendency while an increase in persistent Na\(^+\) conductances would increase depolarizing drive. Together, these changes could underlie the hyperexcitability typical of ALS (Kiernan and Burke, 2004a). Fasciculations, a prominent feature of ALS, reflect ectopic activity in motor axons (Wettstein, 1979; Conradi et al., 1982; Roth, 1982; Roth, 1984; Layzer, 1994; Miller and Layzer, 2005). Bostock and colleagues linked a reduction in K\(^+\) conductances with fasciculations in ALS (Bostock et al., 1995), and the present study provides further
supportive evidence, as do previous studies that documented ectopic activity arising from the neuromuscular junctions following presynaptic block of K⁺ channels (Dodson et al., 2003).

In consort with these changes in axonal K⁺ conductances, an increase in persistent Na⁺ conductances will further contribute to the hyperexcitability of motor axons in ALS, leading to the almost inevitable symptoms of cramps and fasciculations.
Chapter 2

Pathophysiological insights into motor axonal function in Kennedy’s disease
SUMMARY

Having established the presence of widespread axonal ion channel dysfunction in amyotrophic lateral sclerosis (ALS, see Chapter 1) as the pathophysiological mechanisms underlying the generation of fasciculations, in the present chapter, axonal excitability techniques were applied to patients with Kennedy’s disease (KD) in order to assess whether similar mechanisms were mediating the generation of fasciculations in KD, a disorder that mimics ALS. Kennedy’s disease or spinobulbomuscular atrophy, is a slowly progressive inherited neurodegenerative disorder, marked by prominent fasciculations that typically precede the development of other symptoms. Although the genetic basis of KD relates to triplet (CAG) repeat expansion in the androgen receptor (AR) gene on the X chromosome, the mechanisms underlying the clinical presentation in KD, especially fasciculations that are also a prominent features of ALS, have yet to be established. Peripheral nerve excitability studies were undertaken in 7 KD patients with compound muscle action potentials (CMAP) recorded from the right abductor pollicis brevis. Strength-duration time constant (KD 0.54±0.03 ms; controls, 0.41±0.02 ms, P < 0.01) and the hyperpolarizing current/threshold gradient (KD 0.42±0.01; controls, 0.37±0.01, P < 0.05) were significantly increased in KD. Strength-duration time constant correlated with the CMAP amplitude (R = 0.68) and the fasciculation frequency (R = 0.62). Threshold electrotonus revealed greater changes in response to a sub-threshold depolarizing (KD TEd [90-100 ms], 50.75±1.98%; controls, TEd [90-100 ms], 45.67±0.67%, P < 0.01) and hyperpolarizing (KD TEh [90-100 ms], 128.5±6.9%; controls, TEd [90-100 ms], 120.5±2.4%) conditioning pulses. Measurements of refractoriness, superexcitability and late subexcitability, changed appropriately for axonal
hyperpolarization, perhaps reflecting the effects of increased ectopic activity. In total, the increase in the strength-duration time constant may be the primary event, occurring early in course of the disease, contributing to the development axonal hyperexcitability in KD, and thereby to the generation of fasciculations, a characteristic hallmark of the disease.
INTRODUCTION

Kennedy’s disease (KD), or spinobulbomuscular atrophy, is a slowly progressive, X-linked inherited neurodegenerative disorder of motor and sensory neurons (Kennedy et al., 1968). An increase in the triplet repeat (CAG) expansion in the androgen receptor (AR) gene on the X chromosome, at Xq11-12 (La Spada et al., 1991), has been identified as the underlying cause of KD.

The clinical hallmarks of Kennedy’s disease include widespread and prominent fasciculations that may precede the development of other neuromuscular symptoms such as muscle weakness and wasting, postural hand tremor, dysarthria and dysphagia (Kennedy et al., 1968; Harding et al., 1982; Olney et al., 1991; Gallo, 2004). Neurophysiologically, fasciculations in Kennedy’s disease may be complex, consisting of two or more components, and are indistinguishable from fasciculations evident in ALS (Meriggioli et al., 1999; Hirota et al., 2000). Reinnervation through collateral axonal sprouts underlies the generation of these complex fasciculations, as supported by the presence of chronic neurogenic changes on electromyography (EMG) (Sobue et al., 1989; Olney et al., 1991; Trojaborg and Wulff, 1994; Ferrante and Wilbourn, 1997; Meriggioli et al., 1999; Hirota et al., 2000). In addition to spontaneous fasciculations, twitching around the chin and mouth, termed “contraction-fasciculations”, is a widely reported clinical feature in KD, although given that such activity is triggered by voluntary contraction it is clearly distinct from purely spontaneous fasciculations (Olney et al., 1991).
Fasciculations reflect motor nerve hyperexcitability, typically generated at the motor nerve terminal or the motor neuronal level (Wettstein, 1979; Conradi et al., 1982; Roth, 1982; Roth, 1984; Layzer, 1994; Miller and Layzer, 2005). Alterations in axonal membrane conductances and consequently resting membrane potential contributes to the development of fasciculations in ALS (Mogyoros et al., 1998a; Kiernan and Burke, 2004b; Kanai et al., 2006) and acquired auto-immune neuromyotonia (Maddison et al., 1999; Nagado et al., 1999; Kiernan et al., 2001b). Given that fasciculations are an inevitable accompaniment of KD, clinically and physiologically indistinct from fasciculations evident in ALS (Hirota et al., 2000), the present study combined clinical findings with nerve excitability techniques to provide information about nodal and internodal axonal membrane conductances in KD, compared to ALS, and thereby potential insight into pathophysiological mechanisms underlying nerve degeneration in KD.

**METHODS**

**Patients**

Studies were undertaken in 7 patients with KD (aged 35-68, mean 53 years) with the diagnosis confirmed by genetic testing (La Spada et al., 1991). Specifically, the cytosine-adenine-guanosine (CAG) triplet repeat in the androgen receptor gene on X chromosome was expanded in all patients, with the size of the expansions varying from 40-46 (normal 9-36, values considered diagnostic of KD > 38) (Andrew et al., 1997). Patients were clinically graded using the Medical Research Council (MRC) rating scale (Medical Research Council, 1976). Frequency of fasciculations was calculated by dividing the
number of fasciculations recorded during a 2 minute period, using surface electrodes positioned over the abductor pollicis brevis (APB) muscle, to yield a frequency per minute value.

**Nerve excitability testing**

Nerve excitability studies were performed according to a previously described protocol (Kiernan et al., 2000). The median nerve was stimulated at the wrist with the resultant CMAP recorded using surface electrodes positioned over the APB (see Methodology). The following axonal excitability parameters were recorded; stimulus-response curves were recorded separately for stimuli of 0.2 and 1 ms duration, rheobase and strength-duration time constant ($\tau_{SD}$), threshold electrotonus, current-threshold relationship and the recovery cycle of excitability (see Methodology). Skin temperature was monitored close to the site of stimulation for the duration of each study.

**Statistical analysis**

The values of nerve excitability were compared to normal control data obtained from 29 subjects (21 men; 8 women aged 24-58 years; (Kiernan et al., 2000) and corrected for age, gender, and temperature (Kiernan et al., 2000; Kiernan et al., 2001a) and to 50 ALS patients (32 men; 18 women aged 26-78 years). Excitability changes were further compared to a subgroup of age-matched normal male patients (N = 12, aged 39-56 years, mean 49 years). Student’s t-test and analysis of variance (ANOVA) was used to compare differences in excitability data between the groups. The relationship between excitability indices and clinical assessment were analyzed by Spearmen’s rank correlation test. A
probability (P) value of < 0.05 was considered significant. Results are expressed as mean ± standard error of the mean.

RESULTS

The clinical features of the 7 KD patients are summarized in Table 2.1. Sensory symptoms in the form of paraesthesiae, numbness, and burning, were evident in 29% of patients. The CMAP amplitude was smaller in KD and ALS patients (KD 6.8 ± 0.5 mV, ALS 5.8 ± 0.6 mV; controls 10.4 ± 0.7 mV, P < 0.001). There were no differences in the stimulus intensity required to generate SR curves in the KD patients compared to ALS and controls for stimuli of 0.2 ms and 1 ms duration, suggesting that axons in KD patients were of similar threshold (KD 4.4 ± 1.1 mA; ALS 4.8 ± 1.1 mA; controls 4.5 ±1.1 mA).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Triplet (CAG) repeat length</th>
<th>Disease duration (months)</th>
<th>Thenar muscle strength MRC</th>
<th>CMAP amplitude (mV)</th>
<th>Fasciculation Frequency (per minute)</th>
<th>Dysarthria</th>
<th>Dysphagia</th>
<th>Sensory features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>46</td>
<td>168</td>
<td>4</td>
<td>4.1</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>46</td>
<td>240</td>
<td>4</td>
<td>6.8</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>46</td>
<td>84</td>
<td>5</td>
<td>6.1</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>44</td>
<td>180</td>
<td>5</td>
<td>8.0</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>44</td>
<td>60</td>
<td>5</td>
<td>7.6</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>44</td>
<td>72</td>
<td>5</td>
<td>7.4</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>45</td>
<td>48</td>
<td>5</td>
<td>7.8</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mean SEM</td>
<td>56.7</td>
<td>45.0</td>
<td>121.7</td>
<td>4.7</td>
<td>6.8</td>
<td>3.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Clinical details for the 7 Kennedy’s disease (KD) patients. The diagnosis of KD was confirmed on genetic testing in all cases revealing an increase in the triplet repeat (CAG) expansion in the androgen receptor gene on the X chromosome (> 38 repeats abnormal). Hand strength was assessed using the Medical Research Council score (MRC) from the tested muscle, abductor pollicis brevis.
Strength duration time-constant and rheobase

The strength-duration time constant, reflecting persistent nodal Na⁺ conductances (Bostock and Rothwell, 1997), was estimated using nine axonal populations starting from axons contributing to CMAP responses between 5-15% up to the maximum of 85-95%. The nine τSD were significantly longer in KD patients when plotted against their corresponding CMAP responses (Fig. 2.1A) and mean τSD was increased accordingly in the KD patients compared to controls, but was not different when compared to ALS patients (KD 0.53 ± 0.03 ms; ALS 0.52 ± 0.02 ms; controls 0.42 ± 0.01 ms, P < 0.001, Fig. 2.1B).

Rheobase, defined as the threshold current for a stimulus of infinitely long duration (Weiss, 1901; Bostock, 1983; Mogyoros et al., 1996a; Bostock et al., 1998), was similar in KD patients, ALS patients and controls (KD 2.7 ± 1.1 mA, 3.1 ±1.1 mA; controls 3.0 ± 1.1 mA). The previously established negative correlation between the τSD and rheobase in healthy controls (Mogyoros et al., 1998a), was maintained in KD patients (R = -0.8).

Current/threshold (I/V) relationship and threshold electrotonus

The I/V relationship reflects rectifying properties of both nodal and internodal segments of the axon (Baker et al., 1987; Bostock et al., 1998). The I/V gradient during depolarizing sub-threshold currents reflects conduction through outward rectifying K⁺ channels, while the I/V gradient during hyperpolarizing sub-threshold currents reflects inwardly rectifying conductances activated by hyperpolarization (Pape, 1996; Kiernan et al., 2000). The hyperpolarizing I/V gradient demonstrated a significant increase in KD
patients when compared to ALS patients and controls (KD 0.43 ± 0.01; ALS 0.39 ± 0.02; controls 0.37 ± 0.01, P < 0.05, Fig. 2.2 A, B).

**Figure 2.1.** The strength-duration time constant reflects nodal persistent Na⁺ conductances. (A) The distribution of strength-duration time constant in nine different populations of axons contributing to the compound muscle action potential (CMAP) response, starting from axons that contribute 5-15% of maximal response and increasing in 10% increments up to a maximum of 85-95%. The strength-duration time constant was significantly increased in patients with Kennedy’s disease (KD, filled circles) compared to controls (clear circles) for each of the populations. (B) Mean data illustrating that the strength-duration time constant was significantly increased in KD patients compared to controls. **P < 0.01**
Figure 2.2. The current/threshold (I/V) relationship estimates rectifying properties of both nodal and internodal segments of the axon. In the present protocol, threshold was tested with 1-ms pulses following a sub-threshold polarizing current of 200-ms duration. The polarizing current was altered from +50% (depolarizing) to -100% (hyperpolarizing) of the control threshold in 10% steps. (A) The hyperpolarizing I/V slope was significantly increased in patients with Kennedy’s disease (KD) compared to controls. (B) Mean data illustrating a significant increase in the I/V slope of KD patients compared to controls. *P < 0.05
In addition to these changes in the I/V relationship, abnormalities in threshold electrotonus, which refer to changes in membrane excitability in response to long duration polarizing currents (Bostock and Baker, 1988; Bostock et al., 1998), were also evident in both KD and ALS patients. Specifically, group data revealed greater threshold changes in response to a sub-threshold depolarizing conditioning pulse in KD and ALS patients compared to controls (TEd [40-60 ms] KD 59.03 ± 2.65%; ALS 56.8 ±1.1%; controls 53.12 ± 0.79 %, P < 0.01; TEd [90-100 ms] KD 51.35 ± 1.71%; ALS 50.4 ± 1.0%; controls 45.76 ± 0.67 %, P < 0.001, Fig. 2.3 A, B). Hyperpolarizing threshold changes were also increased in KD and ALS patients relative to controls (TEh [90-100 ms] KD 128.5 ± 6.9%, ALS 126.3 ± 3.8%; controls 120.5 ± 2.4 %), although the difference was not significant.

**Recovery cycle of excitability**

The recovery cycle reflects changes in membrane excitability in response to a supramaximal conditioning stimulus (Kiernan et al., 1996b). Three components of the recovery cycle were measured: (i) relative refractory period (RRP), determined by recovery from inactivation of transient Na⁺ channels; (ii) superexcitability, reflecting a period of increased axonal excitability due to spread of a depolarizing afterpotential to the internodal axolemma (Barrett and Barrett, 1982); and (iii) late subexcitability, to a period of reduced axonal excitability that probably results from activation of slow K⁺ channels (Waxman and Ritchie, 1993; Kiernan et al., 1996b; Burke et al., 2001). The duration of the RRP (KD 3.0 ±1.06 ms; ALS 3.1 ± 1.0 ms; controls 3.16 ± 1.02 ms), the extent of superexcitability (KD 28.73 ± 2.44%, ALS 27.4 ± 1.1%; controls 23.44 ± 1.50
%, and late sub-excitability (KD 14.44 ± 1.60 %; ALS 13.5 ± 0.7%; controls 13.88 ± 0.95 %) were not significantly different when compared to ALS patients and controls.

**Figure 2.3.** Threshold electrotonus refers to changes in membrane excitability in response to long duration polarizing currents. Group data revealed a significant increase in threshold to (A) sub-threshold depolarizing currents at 40-60 ms, TEd (40-60 ms), and (B) sub-threshold depolarizing currents at 90-100 ms, TEd (90-100 ms), in patients with Kennedy’s disease (KD). **P < 0.01
Correlations of triplet repeat expansions with indices of axonal excitability and clinical features

It has previously been reported that the size of the triplet (CAG) repeat expansion positively correlates with disease severity in KD, such that the greater the number of CAG repeats the more severe the disease phenotype (Doyu et al., 1992; La Spada et al., 1992) and the earlier the age of onset (Zoghbi and Orr, 2000). In the present series, $\tau_{SD}$ correlated with CMAP amplitude ($R = 0.68$, Fig. 2.4A) and the frequency of fasciculations ($R = 0.62$, Fig. 2.4B), such that the longer $\tau_{SD}$ occurred in patients with relatively preserved CMAP amplitude and a higher frequency of fasciculations. Further, $\tau_{SD}$ correlated with disease duration ($R = -0.41$) and the MRC score ($R = 0.50$). The size of triplet repeat expansion correlated with the CMAP amplitude ($R = -0.42$, Fig. 2.4C). In total, these correlations suggest that upregulation of persistent $Na^+$ conductances, as assessed by the $\tau_{SD}$, may be an early finding in KD, being more prominent in patients with preserved CMAP amplitudes and with normal muscle strength. Further, upregulation of persistent $Na^+$ conductances is associated with an increase in the frequency of fasciculations.

DISCUSSION

The present study has established abnormalities of axonal membrane excitability in patients with KD or progressive spinobulbar muscular atrophy. Specifically, strength-duration time constant was prolonged and the hyperpolarizing current/threshold gradient was increased. These changes were accompanied by changes in threshold electrotonus, revealing greater changes in response to a sub-threshold depolarizing pulse.
Figure 2.4. Correlation studies illustrating (A) that the strength-duration time constant correlated with the compound muscle action potential (CMAP) amplitude in 6 progressive spinobulbar muscular atrophy patients (Kennedy’s disease). (B) The strength-duration time constant correlated with the frequency of fasciculations. (C) The size of the triplet repeat (CAG) expansion correlated with the CMAP amplitude in the same patients.
Prior to interpreting these findings, a potential limitation of axonal excitability studies is that only surviving axons may be assessed. Consequently, in neurodegenerative diseases such as KD, axonal excitability findings may be influenced by a few long surviving axons that may possess different excitability properties compared to short surviving axons (Kiernan and Burke, 2004b). In the present study, formal evaluation of motor unit numbers was not undertaken, although CMAP amplitude was incorporated as a marker of peripheral disease burden, and was within normal limits in most KD patients. This would suggest that axonal excitability studies were not based on a few remaining long surviving motor axons. Furthermore, omission of data from one KD patient, in whom the CMAP amplitude was reduced, did not alter the overall findings. However, CMAP amplitude should be interpreted with caution as such measures are indirect markers of axonal loss and may be influenced by collateral reinnervation (Bromberg and Larson, 1996).

An increase in the $\tau_{SD}$, an indirect measure of persistent Na$^+$ conductances (Bostock, 1983; Bostock and Rothwell, 1997; Kanai et al., 2003), has been reported to contribute to the generation of fasciculations in ALS (Mogyoros et al., 1998a; Kanai et al., 2006) (see Chapter 1) and some of the spontaneous activity evident in acquired neuromyotonia (Maddison et al., 1999). Fasciculations are prominent in KD, often preceding the development of other neuromuscular features and are clinically and physiologically indistinguishable from complex fasciculations evident in ALS (Kennedy et al., 1968; Harding et al., 1982; Sobue et al., 1989; Olney et al., 1991; Trojaborg and Wulff, 1994; Ferrante and Wilbourn, 1997; Meriggioli et al., 1999; Hirota et al., 2000). Although reinnervation through collateral axonal sprouts underlies the generation of fasciculations
in both KD and ALS, as supported by findings of chronic neurogenic changes on needle EMG testing (Sobue et al., 1989; Olney et al., 1991; Trojaborg and Wulff, 1994; Ferrante and Wilbourn, 1997; Meriggioli et al., 1999; Hirota et al., 2000), the present study establishes that upregulation of persistent Na\(^+\) conductances is an early feature of KD, being most prominent in those patients with limited peripheral disease burden. Such a finding is likely to underlie the early development of widespread fasciculations that are prominent in early stages of KD. These findings in KD would also be in keeping with previous studies ALS where upregulation of Na\(^+\) conductances was reported to contribute to the generation of fasciculations (Mogyoros et al., 1998a; Kanai et al., 2006) (see Chapter 1).

As an alternative explanation to upregulation of persistent Na\(^+\) conductances, prolongation of \(\tau_{SD}\) may also result from changes in nerve geometry secondary to axonal loss. Although axonal loss is a pathological feature of KD (Kennedy et al., 1968; Harding et al., 1982; Sobue, 1995), rheobase, a marker of excitability sensitive to changes in nerve geometry (Mogyoros et al., 1996b), was not significantly affected and there was normal preservation of the negative correlation between \(\tau_{SD}\) and rheobase (Mogyoros et al., 1998a). Similarly, nerve demyelination may also prolong the \(\tau_{SD}\) by exposing internodal membrane with a higher time constant (Brismar, 1981). Given that there is no evidence of demyelination in the motor nerves of KD patients (Harding et al., 1982; Sobue, 1995), this would seem an unlikely explanation for the prolonged \(\tau_{SD}\) in the present study.
Strength-duration time constant may also be prolonged with axonal depolarization (Bostock et al., 1998; Burke et al., 2001). Although the increase in the I/V slope may suggest that membrane depolarization contributes to the prolongation in the $\tau_{SD}$, the fact that other indices of membrane excitability were not appropriately altered would argue against significant membrane depolarization as the likely explanation.

The mechanisms underlying neurodegeneration in KD remain unclear (Gallo, 2004). Triplet repeat expansion (CAG) in the AR gene results in dysfunctional proteins with long polyglutamine sequences, that, when translocated to the nucleus result in formation of the characteristic polyglutamine nuclear inclusions and dysregulation of transcription (Gallo, 2004). Expression of ARs is particularly abundant in motor neurons (Sar and Stumpf, 1977; Yu and McGinnis, 1986; Simerly et al., 1990; Menard and Harlan, 1993), where androgens play an important role in motor axonal regeneration.

Fasciculations are a prominent feature of KD (Kennedy et al., 1968; Harding et al., 1982; Olney et al., 1991; Ferrante and Wilbourn, 1997) and persist throughout the entire course of the disease (Kennedy et al., 1968). Previous studies that have investigated activity-dependent effects have established characteristic changes in axonal excitability (Kiernan et al., 1996a; Kiernan et al., 1997b; Kiernan et al., 2004). Specifically, activity-dependent hyperpolarization is associated with a reduction in refractoriness, an increase in superexcitability and late subexcitability. In addition, membrane hyperpolarization results in a “fanned out” appearance of threshold electrotonus (Kiernan and Bostock, 2000; Burke et al., 2001). Although not significant, such a pattern of change in the
recovery cycle of excitability and in threshold electrotonus in KD patients from the present series, suggests the presence of membrane hyperpolarization. So that while, upregulation of persistent Na\(^+\) conductances may have contributed to the generation of fasciculations in KD, increased inward rectifying conductances may occur as a result of this ectopic activity, and thereby contribute to the changes observed in the I/V relationship.

The present study may also provide insight into the mechanisms underlying axonal loss in KD (Kennedy et al., 1968; Harding et al., 1982; Sobue, 1995). Specifically, upregulation of persistent Na\(^+\) conductances and the consequent effects of increased activity would tend to result in accumulation of intra-axonal Na\(^+\). Such an increase in intra-axonal Na\(^+\) may in turn trigger reverse operation of the Na\(^+\)-Ca\(^{2+}\) exchanger, and thereby neurodegeneration (Stys et al., 1991; Stys, 1998) through activation of Ca\(^{2+}\)-dependent intracellular enzymes, such as calpain, phospholipases, and protein kinase C (Stys, 1998).
Chapter 3

Fatigue and activity-dependent changes
in axonal excitability in ALS
SUMMARY

While patients with ALS may complain of fatigue, the underlying mechanisms appear complex, with dysfunction of central and peripheral nervous systems independently reported as contributing factors. In this chapter, the mechanisms underlying increased fatigability in ALS are investigated by measuring activity-dependent changes in axonal excitability following a maximum voluntary contraction (MVC). Nerve excitability changes were recorded before and after a MVC of abductor pollicis brevis in 16 ALS patients and 25 controls. In ALS patients, there was a greater increase in threshold (36.5±5.9%; controls 19.6±3.5%, P < 0.05) as a result of MVC, with reduction in the compound muscle action potential amplitude generated by a submaximal stimulus (ALS 49±7.6%; controls 41.0±5.4%). These changes were associated with an increase in superexcitability (ALS 65.1±25.4%; controls 42.3±5.7%) and reduction in strength-duration time constant (ALS 20±4.9%; controls 10±2.5%, P < 0.01) indicative of axonal hyperpolarization. The increase in threshold was more pronounced in ALS patients with predominantly lower motor neuronal involvement. Higher firing rates of surviving motor axons attempting to compensate for neurogenic weakness are likely to explain the greater activity-dependent changes in ALS. As such, the present study suggests a further peripheral factor underlying the development of fatigue in ALS.
INTRODUCTION

Amyotrophic lateral sclerosis is a progressive neurodegenerative disorder that affects motor neurones in the spinal cord, brainstem and motor cortex (Desai and Swash, 2002; Kiernan, 2003). The consequences of this neurodegeneration are motor deficits in the limbs, bulbar and respiratory muscles (Rowland and Shneider, 2001). Although the mechanisms of neuronal dysfunction, and ultimately the development of symptoms in ALS remain unknown, glutamate excitotoxicity (Rothstein et al., 1993; Almer et al., 1999; Trotti et al., 1999), increased levels of inducible nitric oxide synthase levels (Almer et al., 1999) and, in cases of inherited ALS, oxidative stress secondary to mutations in the superoxide dismutase-1 (SOD-1) gene, have been proposed (Rosen et al., 1993; Beckman and Koppenol, 1996; Crow et al., 1997; Andrus et al., 1998).

Increased fatigability, defined as an inability to sustain a predictable maximal force during voluntary contraction, is a common symptom of ALS (Sharma et al., 1995; Sanjak et al., 2001; Thomas and Zijdewind, 2006). The mechanisms underlying fatigue in ALS are complex, and contributions from both the central and peripheral nervous systems have been reported (Sharma et al., 1995; Sanjak et al., 2001). Central fatigue refers to a reduced excitatory drive to motor neurones, secondary to central nervous system dysfunction, resulting in incomplete motor unit recruitment and sub-maximal motor unit discharge rates. In contrast, peripheral fatigue typically refers to impaired muscle activation, caused by dysfunction at or below the anterior horn cell (Carpentier et al., 2001; Thomas and Zijdewind, 2006). Perhaps somewhat counterintuitively, fatigue in ALS appears to be independent of muscle strength and disease severity (Robbins et al.,...
2001; Feasson et al., 2006). Regardless of the underlying mechanism, fatigue in ALS severely impacts on the patient’s quality of life (Robbins et al., 2001; Feasson et al., 2006).

The ability to sustain a motor output may be assessed by measuring changes in axonal membrane threshold following a voluntary contraction. Specifically, in peripheral nerves, voluntary contraction activates the axonal membrane Na\(^+\)/K\(^+\) pump (Burke et al., 1998), which attempts to return the resting membrane potential to baseline after contraction has ceased (Bostock and Grafe, 1985; Morita et al., 1993; Bostock and Bergmans, 1994; Burke et al., 2001), resulting in activity-dependant hyperpolarization (ADH). The magnitude of ADH is determined by the impulse load (Kiernan et al., 2004) and, in neurological diseases where the safety margin for impulse conduction has been reduced as occurs for instance in demyelinating neuropathy, may be sufficient to induce conduction failure (Cappelen-Smith et al., 2000; Kaji et al., 2000; Nodera et al., 2006).

In an attempt to further delineate the mechanisms underlying fatigability and weakness in ALS, the present study measured activity-dependant changes in axonal excitability induced by voluntary contraction.

**METHODS**

**Patients**

Studies were undertaken in 16 patients with clinically probable or definite ALS as defined by the revised El Escorial criteria (Brooks et al., 2000b). There was no history of other significant co-morbidities, which may have caused neuropathy in these patients.
Specifically, focal compressive mononeuropathies, such as median nerve dysfunction at the level of the wrist, were excluded in all patients. All patients were receiving riluzole at the time of the study, for which fatigue may be a potential side-effect (Lacomblez et al., 1996; Kiernan, 2005; Zoing et al., 2006). Fatigue was assessed using the multidimensional fatigue inventory (MFI) (Smets et al., 1995). The MFI is a 20-item self-report instrument designed to measure five dimensions of fatigue including: general, physical and mental fatigue, reduced activity and reduced motivation. There are four items in each dimension, with the score for each item ranging from 1 (no fatigue) to 5 (severe fatigue), and the score for each dimension ranging from 4 (no fatigue) to 20 (fatigue). The total MFI score may range from 5 (no fatigue) to 100 (severe fatigue).

**Nerve excitability studies**

All ALS patients were clinically staged using the ALS-functional rating scale-revised (ALSFRS-R) (Cedarbaum et al., 1999), Medical Research Council (Medical Research Council, 1976) clinical grading of power in conjunction with Trigg’s hand function score (Triggs et al., 1999) and forced vital capacity (FVC). ALS patients were classified according to site of disease onset as either limb or bulbar-onset, and clinical phenotype as predominantly lower or upper motor neurone. CMAP amplitude was also broadly used as a marker of disease severity, with a CMAP amplitude < 4 mV determining those patients with more severe disease involvement (see Chapter 4 and Chapter 5).

In all studies, the median nerve was stimulated at the wrist and the resultant compound muscle action potential (CMAP) was recorded from the abductor pollicis brevis (APB)
muscle (see Methodology). The procedure lasted for 14 minutes, consisting of 3 minutes baseline recording, 1 minute of MVC and 10 minutes recovery (see Methodology). Skin temperature was maintained at > 32°C during voluntary contraction. Prior to excitability studies, CMAP amplitude and onset latency, F-wave latency and frequency were all measured. The neurophysiological index (NI) was derived according to a previously reported formula (de Carvalho and Swash, 2000) (see Chapter 5).

Median nerve excitability was tracked before and after maximal voluntary contraction (MVC) of APB muscle for 60 seconds. Stimuli were delivered at 0.8 ms intervals and rotated sequentially through a series of six different conditioning-test combinations (see Methodology). On channel 1, a fixed supramaximal stimulus of 0.2 ms was delivered to produce a CMAP of maximal amplitude. A stimulus 20% greater than channel 1 was delivered on channel 2 and commenced following the period of voluntary contraction. This was done in order to ensure that the post-contraction maximal CMAP on channel 1 had remained truly maximal (Cappelen-Smith et al., 2000). On channel 3, a stimulus of 0.1 ms duration was delivered and its intensity fixed for the entire duration of the study to 70% of the pre-contraction maximal CMAP, in order to assess the effects of contraction-induced excitability changes on CMAP amplitude. On channels 4-6, proportional tracking was used to produce a target CMAP 70% of maximal (Bostock et al., 1998). On channels 4 and 5, 0.1 ms and 1 ms stimuli were used respectively to achieve the target response and from the data obtained the strength duration time constant ($\tau_{SD}$) was calculated using Weiss' formula (Weiss, 1901; Mogyoros et al., 1996a). Channel 6 tracked the changes in superexcitability, a period of increased axonal excitability which
follows the relative refractory period (RRP) and is due to passive discharge of the myelin sheath (Barrett and Barrett, 1982; David et al., 1995). To assess superexcitability, a conditioning supramaximal stimulus was delivered 7 ms before the test stimulus. The test response on this channel was measured after online subtraction of the conditioning stimulus obtained in isolation (using the response obtained from channel 1). Superexcitability was measured as the reduction in stimulating current required to produce the target CMAP and expressed as a negative threshold change.

During the period of voluntary contraction, subjects abducted the thumb against resistance and were encouraged to maintain maximal effort for the entire duration of the 60 seconds. Resistance was provided by the same person in all studies. Stimuli were not delivered during the period of contraction and the hand and forearm were stabilized to limit contraction of other muscle groups and to ensure that there was no displacement of electrodes.

**Statistical analysis**

Values were compared to data obtained from normal controls (N = 25, 14 males, mean 45 ± 3.1 years, range 26-73). Although the controls were younger than ALS patients, subgroup analysis in healthy controls suggested that age did not significantly affect the activity-dependent changes in threshold as indicated by the fact that the threshold increase in younger controls (age < 45 years, mean age 33.0 years, N = 14) of 1.20 ± 0.02%, was similar to that for the older group (age > 45 years, mean age 60.3 years, N = 11) of 1.22 ± 0.07%, P = 0.3 (see Methodology). For comparison of the magnitude of
activity-dependent changes in excitability, ALS patients were assigned by two blinded neurologists as having either predominantly lower motor neurone or upper motor neurone involvement as measured by the upper motor neurone (UMN) score (Turner et al., 2004). Specifically, in ALS patients with predominantly lower motor neurone involvement the UMN score was ≤ 7, while an UMN score of 8-16 indicated predominantly UMN involvement. Single comparisons in excitability parameters were analyzed using Student’s t-test. Repeated measures analysis of variance (ANOVA) was used for multiple comparisons and correlations were analyzed using Pearson’s correlation coefficient. A probability (P) value of < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.

RESULTS

The clinical features of 16 ALS patients are summarized in Table 3.1. All patients reported fatigue, with the total MFI score ranging from 27-93.

Neurophysiological findings

The CMAP amplitude (ALS 6.5 ± 1.9 mV; controls 9.5 ± 0.5 mV, P <0.05) and neurophysiological index (ALS 0.6 ± 0.1; controls 2.6 ± 0.2 mV, P <0.00001) were significantly reduced in ALS patients. As previously reported (Mogyoros et al., 1998a), baseline τ_SD, which reflects activity of persistent Na⁺ conductance (Bostock and Rothwell, 1997), was significantly longer in ALS patients (ALS 0.45 ms ± 0.04; controls 0.36 ± 0.02, P < 0.05). Baseline superexcitability was not significantly different between the two groups (ALS -18.3 ± 2.0 %; controls -17.3 ± 1.4%, P = 0.3).
Activity-dependent changes in axonal excitability

Following MVC, there was an increase in peak threshold in ALS patients for stimuli of 0.1 and 1 ms duration as illustrated in a representative ALS patient (Fig. 3.1A, B). Mean data revealed a significantly greater increase in threshold for 0.1 and 1 ms stimuli durations in ALS patients when compared to controls (Fig. 3.1A, B). Repeated measure ANOVA confirmed that threshold remained significantly higher (P < 0.05) in ALS patients of 0.1 ms duration.

Table 3.1. Summary of clinical features in 16 amyotrophic lateral sclerosis (ALS) patients. The site of disease onset was classified as upper limb (UL), lower limb (LL) or bulbar. Disease duration refers to the period from symptom onset to date of testing. Patients were clinically graded using the ALS functional rating scale revised (ALSFRS-R). In four patients, the Multidimensional Fatigue Inventory (MFI) was not quantitated (NQ) at time of testing. Muscle strength was clinically assessed using the Medical Research Council (MRC) score. Forced vital capacity (FVC) was also assessed in each patient.
patients for the first 8 minutes with stimuli of 0.1 ms and for the first 3 minutes for stimuli of 1 ms duration in the immediate post-contraction period (Fig. 3.2A, B).

This increase in threshold was accompanied by a reduction in $\tau_{SD}$ (Fig. 3.1C) and an increase in superexcitability (Fig. 3.1D) as illustrated for a representative ALS patient.

Group data confirmed a significant reduction in $\tau_{SD}$ in ALS patients post-MVC compared to controls (Fig. 3.3A, $P < 0.01$). The reduction in $\tau_{SD}$ was accompanied by an increase in superexcitability in both ALS and normal controls following MVC (Fig. 3.3B). These changes in the $\tau_{SD}$ and superexcitability were consistent with hyperpolarization of the
axonal membrane (Bostock and Grafe, 1985; Burke et al., 1998) and correlated with an increase in threshold for both ALS patients and controls (Fig. 3.4A, B).

**Figure 3.2.** Group data for 16 ALS patients and 25 normal controls revealed a significantly greater increase in normalized threshold (NT) in ALS patients for stimuli of (A) 0.1 ms and (B) 1 ms duration, following a maximum voluntary contraction (MVC) of 60 seconds duration. Threshold changes are expressed as mean ± standard error of the mean. The filled bar represents the period of MVC.
Figure 3.3. Group data in 16 ALS patients and 25 normal controls (A) revealed a significant reduction of the strength-duration time constant in ALS patients when compared to controls after a maximum voluntary contraction (MVC) of 60 seconds duration. (B) Superexcitability, a sensitive parameter of membrane potential, was increased in ALS patients and controls after MVC. The filled bar represents the period of MVC.
Figure 3.4. The increase in threshold for stimuli of 1 ms duration correlated with (A) reduction in the strength-duration time constant (SDTC), (B) an increase in superexcitability, and (C) reduction in submaximal compound muscle action potential (CMAP) amplitude post maximum voluntary contraction, in ALS patients (filled diamonds) and controls (open squares). The changes in these excitability parameters are consistent with membrane hyperpolarization in both ALS patients and controls.
These changes in axonal excitability were accompanied by a reduction in the CMAP amplitude produced by a submaximal stimulus of fixed intensity (Fig. 3.5A). The reduction in submaximal CMAP amplitude correlated with the increase in threshold in ALS patients and controls (Fig. 3.4C). However, there was no reduction of the CMAP amplitude to a maximal stimulus in ALS patients post MVC (Fig. 3.5B).

Figure 3.5. (A) Group data for 16 ALS patients and 25 controls showing a reduction in the compound muscle action potential (CMAP) amplitude generated by a submaximal stimulus. (B) The changes in CMAP amplitude for a fixed supramaximal stimulus revealed a reduction in the CMAP amplitude after MVC.
Correlations between membrane excitability and clinical parameters

To explore the clinical significance of these activity-dependent excitability changes in ALS patients, correlations were undertaken with clinical and neurophysiological findings. In ALS patients with limb-onset disease the activity-dependent changes in threshold correlated with the ALSFRS-R gross motor score (Fig. 3.6A) and with the neurophysiologic index (Fig. 3.6B). This suggested that greater activity-dependent changes in threshold resulted from an increase in firing rates of the surviving motor axons, possibly in an attempt to compensate for the development of neurogenic weakness.

The possibility that greater activity-dependent changes resulted from higher firing rates of motor axons was supported by a greater increase in threshold in ALS patients with predominantly lower motor neurone signs, as indicated by an UMN score of ≤ 7 (lower motor neurone normalized threshold 1ms pulse, 1.44 ± 0.16; upper motor neurone 1.19 ± 0.05; controls 1.19 ± 0.04, P < 0.05, Fig. 3.6C). Furthermore, the changes in threshold were greater in ALS patients with advanced disease as reflected by CMAP amplitude (threshold in ALS patients with CMAP < 4 mV 44.4 ± 8.1%; ALS CMAP > 4mV 30.2 ± 5.9%, P = 0.09).

To explore whether greater changes in threshold following activity in ALS patients reflected alteration in axonal Na⁺/K⁺ pump function, recovery of threshold in the early post-contraction period was compared between ALS patients and controls. When expressed as a percentage of the maximal change in threshold and then normalized, the
slopes of the recovery in threshold were almost identical in the early post-contraction period for the ALS patients and controls, arguing against any significant difference in Na\(^+/K^+\) pump function in the ALS patient group (Fig. 3.7A, B). Furthermore, the fact that slope was similar would explain the longer time required for recovery in ALS patients, given the initial higher threshold increases.

**Figure 3.6.** The increase in normalized threshold correlated with (A) the ALSFRS-R gross motor sub-score and (B) the neurophysiologic index, a marker of peripheral nerve disease burden. (C) The activity-dependent changes in normalized threshold were significantly greater in ALS patients with predominantly lower motor neurone (LMN) compared to upper motor neurone (UMN) signs. *P < 0.05
Figure 3.7. Recovery of threshold for stimuli of (A) 0.1 ms and (B) 1 ms duration for axons from ALS patients (filled diamonds) and controls (open squares) in the early post-contraction period. The ratio of normalized threshold recorded during the post-contraction period compared to the maximal change in threshold immediately following the period of contraction was used as an index of threshold recovery (NT/max). The ratio shows a similar relationship between recovery of threshold for axons from ALS patients and controls, suggesting that Na⁺/K⁺ pump function remains unaffected in ALS patients.
DISCUSSION

The present study investigated changes in axonal excitability after voluntary contraction in ALS patients, to further understand the peripheral factors that may contribute to muscle fatigue and weakness in ALS. As a group, the mean total MFI score was 58 ± 6.4, confirming that fatigue was a common symptom in the present series of ALS patients. Maximum voluntary contraction induced greater activity-dependent changes in threshold in ALS patients, when compared to controls. Changes in threshold were associated with an increase in superexcitability, reduction of $\tau_{SD}$, and sub-maximal CMAP amplitude, all features consistent with membrane hyperpolarization (Burke et al., 1998). While these activity-dependent changes were more pronounced in ALS patients with predominantly lower motor neurone involvement, they were insufficient to induce conduction failure. In total, these findings demonstrate that there is a major contribution of peripheral factors to muscle fatigue, particularly in ALS patients with predominantly lower motor neurone dysfunction.

Mechanism of activity-dependent hyperpolarization

The electrogenic $\text{Na}^+$/K$^+$ pump is integral to the maintenance of normal resting membrane potential and extrudes three Na$^+$ ions for every two K$^+$ ions into the axon, leading to a net deficit of positive charge on the inner aspect of the axonal membrane (Rakowski et al., 1989). Activity alters resting membrane potential through effects mediated by the Na$^+$/K$^+$ pump, with membrane depolarization occurring during MVC resulting from Na$^+$ influx, followed by an increase in Na$^+$/K$^+$ pump activity immediately following contraction that results in membrane hyperpolarization (Bergmans, 1970;
In vitro studies have demonstrated that the post-tetanic increase in axonal threshold (Bergmans, 1970; Kiernan et al., 1997b; Kiernan et al., 2004) may be abolished following application of ouabain (Kaji and Sumner, 1989), a potent inhibitor of Na⁺/K⁺ pump (Bostock and Grafe, 1985; Morita et al., 1993).

In the present study, there was a greater increase in threshold following voluntary contraction in ALS patients compared to controls. These changes were accompanied by changes in other excitability parameters indicating membrane hyperpolarization. Given that threshold changes in ALS patients as a group were significantly greater compared to controls, a simple explanation for this finding may be that the Na⁺/K⁺ pump is overactive. However, since the slopes of threshold recovery were almost identical in ALS patients and controls (Fig. 3.7A, B), Na⁺/K⁺ pump overactivity is clearly not the cause. Alternatively, given that the magnitude of the activity-dependent hyperpolarization depends on the impulse load delivered to the axon (Burke et al., 1998), greater activity-dependent changes in threshold may result from higher firing rates of surviving motor units in the setting of neurogenic weakness. Supporting this hypothesis are findings that the increase in threshold correlated with measures of peripheral disease burden and was most evident in ALS patients with predominantly lower motor neurone involvement. As such, these findings would suggest a major contribution of peripheral factors in increased fatigability in a sub-group of ALS patients with predominantly lower motor neurone involvement.
These findings are in keeping with a previous study reporting impairment in muscle activation as a mechanism of fatigue in ALS (Sharma et al., 1995). Dysfunction of excitation contraction coupling (ECC) was demonstrated to be the mechanism underlying the failure of muscle activation. Given that impaired ECC in the surviving motor units results in faster firing frequencies (Vollestad et al., 1988), and therefore potentially greater activity-dependent hyperpolarization, the findings in the present study would be in keeping with dysfunction of ECC as a mechanism of fatigue in ALS.

Exercise, fatigue and neurodegeneration in ALS

Given that ALS patients suffer from higher fatigability during physical exertion, the role of exercise in ALS remains an open discussion. Specifically, while some have reported an adverse effect of exercise in terms of precipitating the onset of ALS, others have suggested a beneficial role of physical activity in maintaining muscular conditioning and diminishing symptoms of fatigue.

It has also been suggested that Na\(^+/\) K\(^+\) pump dysfunction may be responsible for motor neurone loss in ALS (Ellis et al., 2003). Disruption of resting membrane potential may lead to secondary effects mediated by changes in intracellular Na\(^+\), with resultant reverse activation of the Na\(^+/\)Ca\(^{2+}\) exchanger, leading to intracellular increases in Ca\(^{2+}\) concentration, activation of Ca\(^{2+}\)-dependent enzyme systems and neuronal death (Stys et al., 1991). Of relevance, widespread loss and dysfunction of Na\(^+/\) K\(^+\) pump function has been demonstrated in the SOD-1 ALS mouse model (Ellis et al., 2003). In the present study, the development of activity-dependent hyperpolarization would argue against Na\(^+/\)
K⁺ pump dysfunction at the axonal level, consistent with previous findings (Mogyoros et al., 1998b). However, the present study does not absolutely dismiss the possibility of Na⁺/K⁺ pump dysfunction as a mechanism of neurodegeneration in ALS. Specifically, it could be argued that Na⁺/K⁺ pump dysfunction occurred in the axons that had prematurely died, with resultant reduction in CMAP amplitude, and that the surviving axons, which could be assessed in the present study, had either normal pump function or had a stronger reserve capacity of the Na⁺/K⁺ pump. As such, in-vitro studies assessing axonal pump function would be required to further address this critical issue.
Chapter 4

Assessment of cortical excitability using threshold tracking techniques
SUMMARY

After characterising the nature of axonal ion channel dysfunction in ALS (see chapter 1), cortical excitability was assessed using the paired-pulse transcranial magnetic stimulation (TMS). Although the constant stimulus paired-pulse TMS technique has been extensively used, this technique is limited by the marked variability in the motor evoked potential (MEP) amplitude with consecutive stimuli. In order to overcome this limitation, the threshold tracking technique, used in peripheral axonal excitability studies, was adapted for assessing cortical excitability using the paired-pulse TMS techniques.

Studies were undertaken in 26 healthy controls, tracking the motor evoked potential (MEP) response from the abductor pollicis brevis muscle. Short interval intracortical inhibition (SICI) occurred up to an interstimulus interval (ISI) of 7-10 ms, with two distinct peaks evident, at ISIs of ≤1 and 3 ms, followed by intracortical facilitation to an ISI of 30 ms. In this study, the threshold tracking TMS technique was confirmed as a reliable and reproducible measure of cortical excitability. Simultaneous assessment of upper and lower motor neuronal function with threshold tracking techniques will be applied to patients with amyotrophic lateral sclerosis and related neuromuscular disorders to help to determine the site of disease onset and patterns of disease progression.
INTRODUCTION

Transcranial magnetic stimulation (TMS) has been used to assess cortical excitability via a paired-pulse paradigm, in which a conditioning stimulus is delivered at preset time intervals before a suprathreshold test stimulus (Kujirai et al., 1993; Nakamura et al., 1997). In this paradigm, the conditioning and test stimuli remain constant and the effects of the conditioning stimulus are measured by recording changes in the amplitude of the test response. Typically, if the interstimulus interval (ISI) is between 1-5 ms the test response is inhibited, while at ISIs of 7-20 ms the test response is facilitated (Kujirai et al., 1993; Nakamura et al., 1997; Di Lazzaro et al., 1998c; Hanajima et al., 1998a).

The constant stimulus paired-pulse technique has been used extensively to investigate cortical excitability in healthy controls (Kujirai et al., 1993; Nakamura et al., 1997; Boroojerdi et al., 2001; Ziemann, 2004b) and patient groups (Ridding et al., 1995a; Ridding et al., 1995b; Abbruzzese et al., 1997; Chen et al., 1997b; Ziemann et al., 1997a; Ziemann et al., 1997c; Hanajima et al., 1998a; Zanette et al., 2002b; Rosenkranz et al., 2005). A major limitation of this technique is the marked variability in the motor evoked potential (MEP) amplitude with consecutive stimuli (Kiers et al., 1993). This variability results in part from spontaneous fluctuations in the resting threshold of cortical neurons. As a result, multiple stimuli must be delivered at each conditioning-test stimulus interval to ensure validity of the measurement. Further, the constant test stimulus technique is limited by restrictions in the range of MEP amplitude reduction and by the dependence of the paired-pulse paradigm on factors other than the integrity of the cortical synaptic pathways, such as effects of spinal and peripheral mechanisms on the output signal.
Recently, a novel method of assessing intracortical inhibition has been reported using a computer-assisted “threshold tracking” technique (Awiszus et al., 1999; Fisher et al., 2002). In this method, a constant target MEP amplitude is tracked on-line by a test stimulus using different conditioning-test paradigms. Although threshold tracking has been validated in electrical studies of peripheral axons in healthy controls (Mogyoros et al., 1996a; Kiernan et al., 2000; Kiernan et al., 2001c; Krishnan et al., 2004) and in the investigation of disease pathophysiology in a wide array of neurological disorders (Bostock et al., 1998; Burke et al., 2001; Cappelen-Smith et al., 2001; Kiernan et al., 2001b; Kiernan et al., 2002a; Kiernan et al., 2002b; Kiernan et al., 2005a; Kiernan et al., 2005b; Krishnan et al., 2005a; Krishnan and Kiernan, 2005; Krishnan et al., 2006a), limited data exists for the application of threshold tracking to TMS to study cortical excitability. Consequently, the aim of the present study was to determine the adaptability of threshold tracking to TMS techniques to investigate the processes of intracortical inhibition and facilitation. If this proved possible, a further aim was to establish normative data to enable similar investigations to be undertaken in patients with neurological disease.

METHODS

Studies were undertaken on 26 healthy volunteers (14 men and 12 women, aged range 23-63 years). Prior to assessment of cortical excitability with TMS, the median nerve was stimulated electrically at the wrist and the resultant compound muscle action potential was recorded over the abductor pollicis brevis muscle (see Methodology). The peak-to-peak amplitude and onset latency for the CMAP were determined. Stimulus
response (SR) curves were recorded separately for both stimulus widths. The SR data was used to estimate the strength-duration time constant, by comparing the ratio between the 0.2 ms and 1 ms stimuli required to evoke the same response using Weiss’s formula (Weiss, 1901). Ten F-waves were then recorded in each subject and onset latencies were determined. Skin temperature was monitored close to the site of stimulation for the duration of the study.

Following these brief peripheral studies, TMS was applied to the left motor cortex by means of a 90 mm circular coil oriented to induce a current flow in a posterior-anterior direction in order to activate the motor cortex with the resultant MEP recorded over the APB muscle (see Methodology). The following cortical excitability parameters were recorded using the threshold tracking TMS protocol (see Methodology): resting motor threshold, short interval intracortical inhibition, intracortical facilitation, magnetic SR curve and cortical silent period.

RESULTS

A complete sequence of recordings was obtained from all subjects. All tolerated the study well, no one reported finding the TMS painful, and there were no complications or early terminations of any study.

Stimulus-response curves

Stimulus response curves for electrical test stimuli of 0.2 and 1 ms duration were generated for the group (Fig. 4.1A). Mean maximum peak-to-peak CMAP amplitude for
the APB muscle was 10.0 ± 0.7 mV. SR curves were steep for the APB muscle, as
previously documented (Kiernan et al., 2000), because alpha-motor axons have a
restricted range of thresholds, and there is little contamination with potentials

![Figure 4.1. Mean stimulus-response curves obtained following peripheral (electrical) and central (transcranial magnetic stimulation, TMS) stimulation in 26 normal subjects. (A) Stimulus response curves for test stimuli of 0.2 and 1 ms duration recorded from the abductor pollicis brevis (APB) muscle following electrical stimulation of the median nerve at the wrist. The strength-duration time constant was determined by comparing the ratio between the 0.2- and 1-ms stimuli (see Methodology). (B) Stimulus response curve recorded from APB using TMS. The dashed horizontal line represents the target output of 0.2 mV which was “tracked”.
]
generated by other median-innervated muscles. Strength-duration time constants were calculated from the SR curves, with a mean value of 0.40 ± 0.02 ms, similar to that reported previously (Mogyoros et al., 1996a; Kiernan et al., 2000).

The magnetic SR curve (Fig. 4.1 B) was non-linear as previously reported (Devanne et al., 1997). Mean maximum MEP amplitude was 2.4 ± 0.5 mV, while the mean CMCT was 5.2 ± 0.3 ms, both of which were within the normal range (Claus, 1990; Triggs et al., 1997).

**Short interval intracortical inhibition**

SICI, as reflected by an increase in the conditioned stimulus intensity required to track a constant target MEP of 0.2 mV, is depicted for two illustrative controls in Figure 4.2. The difference between the conditioned and unconditioned stimulus intensities indicated that SICI occurred up to an ISI of 10 ms (Fig. 4.2 C, D). Group data revealed that SICI occurred up to an ISI of between 7-10 ms, with two phases of SICI evident (Fig. 4.3 B): an initial smaller peak at an ISI of ≤1 ms, representing a 6.8 ± 1.3 % increase in conditioned stimulus intensity; and a larger peak occurring at an ISI of 3 ms, representing a 10.8 ± 1.9 % increase in conditioned stimulus intensity. The conditioned stimulus intensity was significantly greater compared to the unconditioned stimulus intensity over the period of testing up to 10 ms (ANOVA P < 0.05, Fig. 4.3 A). The unconditioned stimulus intensity (RMT) remained reasonably stable over the period of testing, from ISI 1-30 ms, indicating that the subjects were adequately relaxed and that the testing environment remained stable during this period of testing.
Intracortical facilitation

Intracortical facilitation (ICF), as reflected by a decrease in the test stimulus intensity required to maintain the target MEP of 0.2 mV, developed at an ISI of between 7-10 ms and was still evident at an ISI of 30 ms (Fig. 4.3 B). Facilitation for the group peaked at an ISI of 15 ms, as represented by a $2.2 \pm 2.1\%$ decrease in conditioned stimulus intensity. In addition, the conditioned stimulus intensity was significantly lower.
compared to the unconditioned stimulus intensity over the period of testing from 10-30 ms (ANOVA P < 0.05, Fig. 4.3 A).

Figure 4.3. Mean data for short interval intracortical inhibition (SICI) and facilitation in 26 healthy subjects. (A) Conditioned test (CT, open circles) and unconditioned test (UCT, filled diamonds) stimuli (mean ± SEM, percent maximum stimulator output, % MSO) are plotted for each interstimulus interval (ISI). Repeated measures ANOVA over time revealed a significant difference between the two testing stimuli at each ISI (P < 0.05). (B) The difference between CT and UCT (mean ± SEM) is normalized as a percentage of threshold (see Methods). SICI developed up to an ISI of between 7-10 ms. Two phases of SICI can be discerned occurring at an ISI of ≤1 and 3 ms. Intracortical facilitation occurred at an ISI of 10-30 ms.

**Cortical silent period**

In a contracting muscle, the MEP is followed by a period of electrical silence that interferes with ongoing EMG activity (Fig. 4.4 A). The early part of this cortical silent
period (CSP) likely reflects spinal inhibition (Cantello et al., 1992; Inghilleri et al., 1993; Ziemann et al., 1993; Chen et al., 1999), while the later part originates from cortical inhibition (Connors et al., 1988; Inghilleri et al., 1993; Ziemann et al., 1993; Avoli et al., 1997). The mean CSP duration, as defined from onset of MEP to resumption of voluntary EMG activity, increased from $22.6 \pm 5.4$ ms to $198.2 \pm 6.5$ ms as the stimulus intensity increased from 60 to 150% RMT (Fig. 4.4 B). This increase in the CSP recruitment curve was non-linear.

Figure 4.4. (A) Cortical silent period (CSP) measured from the onset of the motor evoked potential to the beginning of EMG activity. The CSP duration increased with increasing stimulus intensity. (B) Mean data ($\pm$ SEM) illustrating a non-linear increase in CSP duration with increasing stimulus intensity.
Reproducibility

In order to test the reproducibility of threshold tracking TMS, studies were repeated in 4 subjects on two occasions and in one subject on five occasions. The variability in SICI and ICF in one subject, tested on 5 occasions, is depicted in Figure 4.5A and B. Peak SICI when compared for each subject demonstrated good reproducibility (Fig. 4.5C).

![Graphs showing reproducibility](image)

**Figure 4.5.** (A) Raw traces and (B) mean data ± SEM illustrating intersession variability in short-interval intracortical inhibition (SICI) and facilitation in one subject tested on five separate occasions. (C) The variability in maximum SICI is illustrated in five subjects who underwent repeat testing on two separate occasions.
DISCUSSION

In the present study threshold tracking techniques were successfully applied using paired-pulse TMS paradigms to investigate the processes of intracortical inhibition and facilitation. SICI persisted up to an ISI of 7-10 ms. Two distinct phases of SICI were evident, occurring at ISIs of ≤1 and 3 ms. This was followed by ICF, peaking at an ISI of 15 ms. Threshold tracking paired-pulse TMS proved to be both a feasible and reproducible technique for assessing cortical excitability.

A major limitation of the paired-pulse constant stimulus method is the marked variability in the MEP amplitude and area, from stimulus to stimulus, that may be up to 200% (Kiers et al., 1993). As such, it is difficult to discern whether changes in the MEP produced by a constant test stimulus are secondary to a true change in cortical excitability or alternatively represent fluctuations related to the delivery of the test stimulus. By setting a tracking target of 0.2 mV, located in a steep portion of the non-linear SR curve (Devanne et al., 1997), variability in MEP amplitudes translate to smaller changes in stimulus intensity, potentially overcoming this limitation. This is illustrated in Figure 4.2 A, where the unconditioned stimulus, as recorded on channel 1 (see Fig. 9, Methodology), varied by a mean of 1.2% during the testing period, while the variability in the MEP amplitude was much greater.

It is commonly accepted that intracortical inhibition and facilitation reflect changes in the cortical circuitry. As such, it is assumed that the spinal and peripheral mechanisms reliably convert messages from the cortex to EMG responses (Fisher et al., 2002). As
suggested by Fisher and colleagues (2002), measurement of intracortical inhibition and facilitation using threshold tracking methods limit the contribution of spinal and peripheral elements to the output measurement (Fisher et al., 2002).

**Mechanisms of intracortical inhibition and facilitation**

Short interval intracortical inhibition was first reported by Kujirai and colleagues (1993) (Kujirai et al., 1993), who demonstrated that a subthreshold conditioning stimulus could suppress the response to a later suprathreshold test stimulus if the ISI was less than 5 ms. The mechanism of this inhibition was proposed to be cortical in origin, with the conditioning stimulus suppressing the recruitment of descending volleys by the test stimulus. Supportive evidence for such a mechanism was provided by epidural recordings in which descending corticomotoneuron volleys were inhibited during SICI (Nakamura et al., 1997; Di Lazzaro et al., 1998c; Fisher et al., 2002). Specifically, SICI was associated with a reduction in amplitude of I2 and I3 waves, and this suppression of later I waves occurred up to an ISI of 20 ms, the typical duration of the inhibitory postsynaptic potential mediated through GABA<sub>A</sub> receptors (Hanajima et al., 1998b). Neuropharmacological experiments have revealed that GABA<sub>A</sub> receptor agonists, such as benzodiazepines, enhance SICI further supporting the notion that cortical synaptic mechanisms are responsible for SICI (Ziemann et al., 1996b; Di Lazzaro et al., 2000; Ilic et al., 2002b).

Recently, two physiologically distinct phases SICI have been reported (Fisher et al., 2002; Roshan et al., 2003), with the first phase occurring at an ISI of ≤1 ms and second
phase at ISI of 2.5 ms. Although the mechanism of the second phase is believed to be synaptic in origin, there is debate regarding the first phase of SICI. Some have argued that the first phase of SICI reflects local excitability properties of the cortical axon, specifically the relative refractory period coupled with a sensitivity of pyramidal neurons to synchronized synaptic inputs (Fisher et al., 2002). Others have proposed that SICI at ISIs of ≤1 ms is best explained by activation of inhibitory circuits different to that which mediates inhibition at ISI of 2.5 ms (Roshan et al., 2003). Regardless of the exact mechanism, these two distinct phases of SICI were evident in recordings from healthy controls in the present study, and both mechanisms (axonal refractoriness and activation of different inhibitory circuits) may have contributed to this early phase of SICI.

The mechanism of ICF has not been well established, and there are no studies that describe the relationship of descending corticospinal tract volleys to ICF. In the present study, ICF, although present, was less than that established by the constant stimulus method. The reasons for this discrepancy remain unclear. As ICF is highly dependent on the direction of cortical current flow (posterior-anterior) it is possible that technical factors, as yet unknown, were responsible for the relative attenuation of ICF.

**Dissecting contributions of the upper and lower motor neuron**

In order to determine the pattern of upper and lower motor neuron recruitment and to assess the relative contribution of these in cortical excitability, SR curves were generated for both the upper and lower motor neurons. The SR curve describes the increase in MEP or CMAP amplitude in response to an increasing magnetic or electrical stimulus.
respectively. Stimulus-response curves reflect the density of axonal projections onto the target, such that with magnetic stimulation the SR curves reflect the density of corticomotoneuron projections. With both modes of stimulation, the increase in output with stimulus intensity was non-linear (Devanne et al., 1997).

The CSP represents interruption of tonic voluntary EMG activity in the target muscle contralateral to the stimulated motor cortex (Cantello et al., 1992). The mechanisms of CSP generation is complex and is thought to be mediated by inhibition of spinal alpha-motoneurons in the early parts (Cantello et al., 1992; Inghilleri et al., 1993; Ziemann et al., 1993; Chen et al., 1999) and by cortical processes, through GABA_B receptors, in later segments (Connors et al., 1988; Inghilleri et al., 1993; Ziemann et al., 1993; Avoli et al., 1997). The CSP duration has been reported to increase linearly with stimulus intensity (Cantello et al., 1992; Triggs et al., 1993). The present study, however, suggests that the increase in the CSP duration is non-linear and this may be accounted for by the different stimulus intensities employed.

In conclusion, the present study has demonstrated the feasibility of threshold tracking TMS in the assessment of cortical excitability and developed normative values for clinical investigations. Through a combination of threshold tracking TMS and peripheral nerve threshold tracking using electrical stimuli, future studies may be of use in assessing the pattern of involvement of different neuronal processes in neurodegenerative diseases, such as ALS, with the aim of determining the site of disease onset.
Chapter 5

Novel threshold tracking techniques suggest that cortical hyperexcitability is an early feature of ALS
SUMMARY

The dying forward hypothesis of amyotrophic lateral sclerosis (ALS) suggests that corticomotoneurons induce excitotoxic anterior horn cell death, with involvement of the glutamatergic neurotransmitter system. In this chapter, novel threshold tracking transcranial magnetic stimulation (TMS) techniques, described in chapter 4, were applied in conjunction with peripheral nerve excitability studies in ALS patients to further investigate the dying forward hypothesis and possibly determine the site of disease onset. Studies were undertaken in 23 ALS patients using a 90 mm circular coil connected to a BiStim magnetic stimulator for cortical studies and electrical stimulation for peripheral nerve excitability studies. Motor evoked potentials and compound muscle action potentials (CMAP) were recorded from the right abductor pollicis brevis in the same setting. Measures of cortical and peripheral nerve excitability were correlated with clinical and neurophysiological parameters of disease severity. Short-interval intracortical inhibition (SICI) was significantly reduced in ALS patients compared to controls (ALS group 3.6 ± 0.8%; controls 8.5 ± 1.0%, P < 0.001), most prominently in ALS patients with limb-onset disease. Changes in intracortical inhibition were accompanied by alterations in the magnetic stimulus-response curve, cortical silent period duration and resting motor threshold, all indicative of cortical hyperexcitability. Although the reduction in SICI was more pronounced in ALS patients with less severe disease, as assessed by the CMAP amplitude, it remained evident even in ALS patients with advanced disease. Measures of peripheral disease burden, namely the compound muscle action potential amplitude (r = -0.6) and neurophysiologic index (r = -0.6) correlated with cortical hyperexcitability changes, as did the strength-duration time
constant ($r = -0.6$), a peripheral marker of axonal excitability. Simultaneous assessment of central and peripheral nerve excitability established the presence of co-existent upper and lower motor neuron dysfunction, with cortical hyperexcitability an early feature in ALS.
INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder affecting motor neurons in the spinal cord, brainstem, and motor cortex (Desai and Swash, 2002). Although the mechanisms of motor neuronal cell death in ALS remain unknown, cortical hyperexcitability mediated via glutamate excitotoxicity has been proposed as a possible mechanism (Rothstein et al., 1993; Bruijn et al., 1997b; Trotti et al., 1999). This hypothesis is supported by improvement in survival outcome of ALS patients treated with riluzole, an inhibitor of glutamate release (Bensimon et al., 1994; Lacomblez et al., 1996; Miller et al., 2003; Kiernan, 2005).

While the site of disease onset in ALS has not been determined, Eisen and colleagues (Eisen et al., 1992) suggested that ALS was primarily a disorder of corticomotoneurons, with excitotoxic anterograde degeneration of anterior horn cells (AHC) occurring as a secondary process. This “dying forward” hypothesis was based predominantly on the clinical observations that the oculomotor, abducens, and Onuf’s motor nuclei, all lacking direct corticomotorneuron connections, were spared in ALS. Of critical importance pure lower motor neuron forms of ALS remain rare and are usually accompanied by subtle upper motor neuron (UMN) signs. Further support for a “dying forward” hypothesis has been provided by transcranial magnetic stimulation (TMS) studies which have demonstrated increased cortical excitability early in the course of ALS (Caramia et al., 1991; Eisen et al., 1993; Prout and Eisen, 1994; Desiato, 2002; Mills, 2003). Another line of evidence that suggests the presence of cortical excitability in ALS is provided by peristimulus time histogram studies (Kohara et al., 1996b). Neuropathological studies
have also provided support detailing early changes in the motor cortex, including ultrastructural changes in Betz cells, and loss of specific inhibitory cortical interneurons (Leigh and Swash, 1991; Nihei et al., 1993; Eisen and Weber, 2001).

In contrast to these findings, other studies have provided evidence that would argue against a dying forward process. Specifically, neurophysiological studies have suggested that cortical hyperexcitability either increases (Zanette et al., 2002b) or is unrelated to disease duration (Ziemann et al., 1997c), while some neuropathological studies have reported that cortical and lower motor neuron degeneration may occur independently (Kiernan and Hudson, 1991; Pamphlett et al., 1995). In an attempt to evaluate any “dying forward” process in ALS, the present study has combined novel cortical and peripheral nerve excitability studies with functional assessment in ALS patients.

**METHODS**

**Patients**

Studies were undertaken in 23 patients with clinically probable or definite ALS (15 male, 8 female: age range 44-71 years, mean age: 60.7 years) as defined by the revised El Escorial criteria (Brooks et al., 2000a). All patients were clinically staged using the amyotrophic lateral sclerosis functional rating scale-revised (ALSFRS-R) (Cedarbaum et al., 1999), hand strength using the Medical Research Council (MRC) rating scale (Medical Research Council, 1976), hand function score (Triggs et al., 1999), and an upper motor neuron (UMN) score (Turner et al., 2004). This UMN score comprised a sum of pathologically brisk reflexes that included assessment of biceps, supinator,
triceps, finger, knee and ankle reflexes, with plantar responses, facial and jaw jerks, all bilaterally, for a maximum possible score of 16 (Turner et al., 2004).

Patients were classified according to the site of disease onset as either limb-onset or bulbar-onset. Amplitude of the compound muscle action potential (CMAP) was used as a broad marker of disease severity, using CMAP amplitude of < 4 mV as a cut-off for more severe disease involvement, based on previous data obtained from control subjects (see Chapter 4). Most patients (96%) were receiving riluzole treatment.

**Cortical excitability testing**

Cortical excitability testing was performed according to a previously described threshold tracking TMS protocol (see Methodology). In all studies, the motor cortex was stimulated using a 90 mm circular coil, with the resultant MEP response recorded over the abductor pollicis brevis (APB) muscle (see Methodology). The following parameters of cortical excitability were recorded using the previously described cortical excitability protocol (see Methodology): resting motor threshold, magnetic stimulus-response curve, central motor conduction time, short interval intracortical inhibition, intracortical facilitation and cortical silent period duration.

**Peripheral nerve excitability**

In the same sitting, peripheral nerve excitability studies were performed according to a previously described protocol that measures multiple parameters of nerve excitability (Kiernan et al., 2000). In all studies, the median nerve was stimulated electrically at the
wrist, with the resultant CMAP recorded over the APB (see Methods). The following axonal parameters were recorded using the previously described TRONDXM2 multiple excitability protocol (see Methodology): stimulus-response (SR) curves using stimuli of 0.2 and 1 ms duration, rheobase and strength-duration time constant ($\tau_{SD}$), threshold electrotonus to 100 ms polarizing currents, current-threshold relationship, refractoriness, superexcitability and late subexcitability. In addition to calculating parameters of axonal excitability, the neurophysiological index (NI) was derived according to a previously reported formula (de Carvalho and Swash, 2000):

\[
NI = \frac{\text{CMAP amplitude (mV)} \times \text{F-wave frequency}}{\text{Distal motor latency (ms)}}
\]

Where F-wave frequency was expressed as the number of F responses recorded in 20 trials.

Cortical excitability in ALS patients was compared to control data obtained from 34 subjects (16 men; 18 women, aged 23-73 years, mean: 43.1 years) and a sub-group of 17 older age controls (8 men; 9 women, aged 42-73 years, mean age: 54.4). Peripheral nerve excitability parameters were compared to previously reported controls (Kiernan et al., 2000). Peripheral excitability measurements were compensated for age and temperature before statistical analysis, using the relations found in control subjects (Kiernan et al., 2000; Kiernan et al., 2001a). Student’s t-test was used to compare mean differences between ALS patients and controls, and analysis of variance (ANOVA) for multiple comparisons. Correlations between excitability indices and clinical scales were analyzed by Spearman’s rank test. A probability (P) value of < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.
RESULTS

The clinical features and rating scores for 23 ALS patients are summarized in Table 5.1, while conventional neurophysiological indices are detailed in Table 5.2. The motor cortex was inexcitable in 3 ALS patients. These patients had protracted disease duration and in the case of two patients high signal intensity was evident within the region of the corticospinal tract on MRI. In one patient the CMAP response was absent. As such, the TMS findings are those recorded in the remaining 19 ALS patients. Central motor conduction time was prolonged in 3 ALS patients (patient # 6, 9 and 11), but overall was not significantly different in ALS patients as a group when compared to controls (ALS 5.6 ± 0.6 ms; controls 5.1 ± 0.2 ms). In 4 ALS patients the CMCT was at the lower limit of normal. Prolongation of the F-wave latency, secondary to anterior horn cell loss, may partially account for the CMCT spuriously short findings in these patients.

Cortical excitability

Resting motor threshold, defined as the unconditioned stimulus intensity required to produce and maintain the target MEP response, was reduced in ALS patients (ALS 56.5 ± 1.9%; controls 60.7 ± 1.5%, P = 0.05). However, the RMT was only significantly reduced in a subset of ALS patients with limb-onset disease (ALS limb-onset 55.2 ± 2.1%; controls 60.7 ± 1.5%, P < 0.05). There was significant correlation between RMT and UMN score (r = 0.6), with the RMT being significantly increased in ALS patients with severe UMN signs (ALS group with UMN score [10-16], 62.3 ± 3.0%; ALS group with UMN score ≤ 9, 54.3 ± 2.2%, P < 0.05).
<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (years)/Sex</th>
<th>ALS onset</th>
<th>Disease duration (months)</th>
<th>ALSFRS-R</th>
<th>Triggs hand score</th>
<th>MRC</th>
<th>FVC (%)</th>
<th>UMN score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69,M</td>
<td>UL</td>
<td>6</td>
<td>45</td>
<td>1</td>
<td>4</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>64,M</td>
<td>UL</td>
<td>26</td>
<td>36</td>
<td>2</td>
<td>4</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>63,M</td>
<td>UL</td>
<td>53</td>
<td>36</td>
<td>2</td>
<td>4</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>67,F</td>
<td>UL</td>
<td>16</td>
<td>40</td>
<td>2</td>
<td>4</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>69,M</td>
<td>UL</td>
<td>17</td>
<td>40</td>
<td>2</td>
<td>5</td>
<td>67</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>44,F</td>
<td>UL</td>
<td>13</td>
<td>46</td>
<td>2</td>
<td>4</td>
<td>79</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>50,M</td>
<td>UL</td>
<td>24</td>
<td>42</td>
<td>0</td>
<td>5</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>71,M</td>
<td>UL</td>
<td>99</td>
<td>42</td>
<td>1</td>
<td>4</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>70,M</td>
<td>UL</td>
<td>8</td>
<td>42</td>
<td>1</td>
<td>4</td>
<td>106</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>57,M</td>
<td>UL</td>
<td>12</td>
<td>29</td>
<td>1</td>
<td>4</td>
<td>90</td>
<td>13</td>
</tr>
<tr>
<td>11</td>
<td>60,F</td>
<td>UL</td>
<td>8</td>
<td>46</td>
<td>1</td>
<td>4</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>53,F</td>
<td>UL</td>
<td>12</td>
<td>39</td>
<td>2</td>
<td>4</td>
<td>91</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>58,F</td>
<td>UL</td>
<td>10</td>
<td>39</td>
<td>2</td>
<td>4</td>
<td>86</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>56,M</td>
<td>UL</td>
<td>17</td>
<td>37</td>
<td>1</td>
<td>4</td>
<td>92</td>
<td>16</td>
</tr>
<tr>
<td>15</td>
<td>55,M</td>
<td>LL</td>
<td>41</td>
<td>34</td>
<td>2</td>
<td>4</td>
<td>98</td>
<td>4</td>
</tr>
<tr>
<td>16</td>
<td>60,M</td>
<td>LL</td>
<td>7</td>
<td>40</td>
<td>1</td>
<td>4</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>17</td>
<td>69,M</td>
<td>LL</td>
<td>29</td>
<td>36</td>
<td>0</td>
<td>4</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>46,M</td>
<td>LL</td>
<td>9</td>
<td>42</td>
<td>1</td>
<td>5</td>
<td>62</td>
<td>4</td>
</tr>
<tr>
<td>19</td>
<td>53,F</td>
<td>Bulbar</td>
<td>50</td>
<td>44</td>
<td>0</td>
<td>5</td>
<td>57</td>
<td>7</td>
</tr>
<tr>
<td>20</td>
<td>66,M</td>
<td>Bulbar</td>
<td>10</td>
<td>38</td>
<td>2</td>
<td>4</td>
<td>79</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>68,F</td>
<td>Bulbar</td>
<td>21</td>
<td>36</td>
<td>0</td>
<td>5</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>22</td>
<td>62,F</td>
<td>Bulbar</td>
<td>10</td>
<td>40</td>
<td>1</td>
<td>4</td>
<td>98</td>
<td>14</td>
</tr>
<tr>
<td>23</td>
<td>65,M</td>
<td>Bulbar</td>
<td>14</td>
<td>42</td>
<td>1</td>
<td>4</td>
<td>81</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>61</td>
<td></td>
<td>22.3</td>
<td>39.6</td>
<td>1.2</td>
<td>4.2</td>
<td>83.0</td>
<td>6.4</td>
</tr>
<tr>
<td>SEM</td>
<td>1.6</td>
<td></td>
<td>4.5</td>
<td>0.9</td>
<td>0.2</td>
<td>0.1</td>
<td>4.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 5.1.** Clinical details for the 23 patients with amyotrophic lateral sclerosis (ALS). The site of disease onset was classified as upper limb (UL), lower limb (LL) or bulbar. Disease duration refers to the period from symptom onset to date of testing. The patients were clinically graded using the ALS functional rating scale revised (ALSFRS-R), with a maximum score of 48 when there is no disability. The ALSFRS-R is comprised of 4 sub-scores; bulbar (maximum score 12), fine motor (maximum score 8), gross motor (maximum score 16), and respiratory (maximum score 12). Muscle strength was clinically assessed using the Medical Research Council (MRC) for the abductor pollicis brevis, as this muscle was utilized for excitability testing. Forced vital capacity (FVC) was assessed in each patient. Upper motor neuron (UMN) involvement was graded according to the UMN score with a maximum score of 16.
<table>
<thead>
<tr>
<th>Patients</th>
<th>CMAP amplitude (mV)</th>
<th>NI (MSO%)</th>
<th>RMT (%)</th>
<th>MEP amplitude (mV)</th>
<th>CMCT (ms)</th>
<th>MEP/CMAP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.6</td>
<td>0.6</td>
<td>46</td>
<td>3.2</td>
<td>4.4</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>6.5</td>
<td>1.3</td>
<td>57</td>
<td>2.9</td>
<td>7.0</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>1.2</td>
<td>45</td>
<td>4.6</td>
<td>4.9</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>3.5</td>
<td>0.5</td>
<td>53</td>
<td>2.1</td>
<td>6.3</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>9.4</td>
<td>2.0</td>
<td>48</td>
<td>5.8</td>
<td>6.1</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>6.8</td>
<td>0.8</td>
<td>57</td>
<td>1.0</td>
<td>7.6</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>6.9</td>
<td>0.2</td>
<td>54</td>
<td>4.5</td>
<td>6.0</td>
<td>0.7</td>
</tr>
<tr>
<td>8</td>
<td>2.2</td>
<td>0.1</td>
<td>52</td>
<td>0.6</td>
<td>5.2</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td>3.1</td>
<td>0.1</td>
<td>64</td>
<td>0.7</td>
<td>14.7</td>
<td>0.2</td>
</tr>
<tr>
<td>10</td>
<td>1.7</td>
<td>0.2</td>
<td>74</td>
<td>0.4</td>
<td>5.9</td>
<td>0.2</td>
</tr>
<tr>
<td>11</td>
<td>9.8</td>
<td>1.7</td>
<td>57</td>
<td>2.8</td>
<td>7.5</td>
<td>0.9</td>
</tr>
<tr>
<td>12</td>
<td>2.9</td>
<td>0.5</td>
<td>43</td>
<td>1.4</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>13</td>
<td>3.3</td>
<td>0</td>
<td>IE</td>
<td>0</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>15</td>
<td>4.2</td>
<td>0.2</td>
<td>IE</td>
<td>0</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>5.4</td>
<td>0.6</td>
<td>IE</td>
<td>0</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>4.0</td>
<td>0.2</td>
<td>55</td>
<td>1.8</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>18</td>
<td>7.8</td>
<td>0.3</td>
<td>59</td>
<td>2.1</td>
<td>3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>19</td>
<td>7.5</td>
<td>0.8</td>
<td>71</td>
<td>1.6</td>
<td>4.3</td>
<td>0.2</td>
</tr>
<tr>
<td>20</td>
<td>3.6</td>
<td>0</td>
<td>57</td>
<td>0.8</td>
<td>NR</td>
<td>0.2</td>
</tr>
<tr>
<td>21</td>
<td>4.9</td>
<td>0.9</td>
<td>63</td>
<td>0.7</td>
<td>4.1</td>
<td>0.2</td>
</tr>
<tr>
<td>22</td>
<td>4.6</td>
<td>0.6</td>
<td>55</td>
<td>1.2</td>
<td>3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>23</td>
<td>1.9</td>
<td>0.1</td>
<td>67</td>
<td>1.0</td>
<td>3.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Mean</td>
<td>4.9</td>
<td>0.5</td>
<td>56.5</td>
<td>1.8</td>
<td>5.6</td>
<td>0.4</td>
</tr>
<tr>
<td>SEM</td>
<td>0.5</td>
<td>0.1</td>
<td>1.9</td>
<td>0.3</td>
<td>0.6</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**Table 5.2.** Summary of clinical neurophysiologic indices corresponding for the 23 ALS patients detailed in Table 5.1. The compound muscle action potential (CMAP) amplitude was recorded from abductor pollicis brevis (APB). The CMAP response was absent in one patient and this precluded further studies (NR, non-recordable). The neurophysiological index (NI) was calculated according to an established formula (see Methods). Resting motor threshold (RMT) was defined as the unconditioned stimulus intensity, expressed as percentage of maximum stimulator output (MSO%), required to maintain a target of 0.2 mV (see Methods). In 3 ALS patients the cortex was inexcitable (IE). The magnetic evoked potential (MEP) amplitude was also recorded from the APB. Central motor conduction time (CMCT) was calculated according to an established method (see Methods). As the F-wave responses were absent in 3 patients, the CMCT could not be calculated in these patients. Furthermore, the cortex was inexcitable in 3 patients (RMT = 100%), and as such calculation of CMCT was not possible. In 2 patients with an inexcitable cortex, the F-wave responses were absent.
To generate stimulus-response curves for TMS studies the MEP amplitude was expressed as a percentage of the CMAP amplitude recorded following electrical stimulation. The MEP:CMAP ratio was significantly increased in ALS patients as a group when compared to normal controls over stimulus intensities from 110 to 150% RMT (ANOVA, P < 0.01, Fig. 5.1A). Sub-group analysis revealed that this increase in the MEP:CMAP ratio was only significant in ALS patients with limb-onset disease (Fig. 5.1B). This MEP:CMAP ratio was increased in ALS patients independent of CMAP amplitude, used as a marker of disease severity (Fig. 5.1C).

**Intracortical inhibition**

Short-interval intracortical inhibition, as reflected by an increase in the conditioned stimulus intensity required to track a constant target MEP of 0.2 mV (see Chapter 4), was significantly reduced in ALS patients as a group when compared to controls (P < 0.001, Fig. 5.2A). The initial phase of SICI previously documented for normal controls at ISI ≤ 1 ms (see Chapter 4) was completely absent in ALS patients (Fig. 5.2A), while peak SICI occurring at ISI of 3 ms was also significantly reduced in ALS patients. Sub-group analysis revealed that while SICI was reduced in ALS patients with bulbar-onset disease, the biggest reduction of SICI occurred in ALS patients with limb-onset disease (Fig. 5.2B). Furthermore, although the reduction in SICI was most prominent in ALS patients with limited peripheral disease burden, as reflected by the CMAP amplitude, this reduction of SICI was also evident in patients with advanced disease (Fig. 5.2C).

Following SICI, a period of intracortical facilitation (ICF) followed, marked by a decrease in the test stimulus intensity required to maintain the target MEP of 0.2 mV.
ICF was significantly increased in ALS patients as a group compared to controls over a period of testing from 10-30 ms (Fig. 5.2A).

**Figure 5.1.** Stimulus-response curves obtained following transcranial magnetic stimulation (TMS) stimulation in 19 amyotrophic lateral sclerosis (ALS) patients and 34 normal controls, with the motor evoked potential (MEP) expressed as a percentage of compound muscle action potential (CMAP) amplitude. (A) MEP amplitude was significantly increased in ALS compared to controls over stimulus intensities from 110-150% (P < 0.05) of resting motor threshold (RMT). (B) The MEP:CMAP ratio was significantly increased in ALS patients with limb onset disease compared to controls and ALS patients with bulbar-onset disease. (C) The increase in the MEP:CMAP ratio was equally evident in ALS patients with less severe disease (CMAP > 4 mV) compared to those with advanced disease (CMAP < 4 mV). *P < 0.05.
Figure 5.2. (A) Short interval intracortical inhibition (SICI), defined as the stimulus intensity required to maintain a target output of 0.2 mV (see Methods) was reduced in amyotrophic lateral sclerosis (ALS) patients as a group when compared to normal controls (P < 0.001). Furthermore, the early phase of SICI, occurring at interstimulus intervals (ISI) ≤ 1 ms, was absent in ALS patients. (B) The reduction in SICI, although evident in all ALS phenotypes, was most prominent in ALS patients with limb-onset disease. (C) The reduction of SICI was evident in ALS patients with less severe (compound muscle action potential [CMAP] amplitude > 4 mV) and advanced disease (CMAP amplitude < 4 mV). ***P < 0.0001; *P < 0.05.
In a contracting muscle, the MEP is followed by a period of electrical silence that interferes with ongoing EMG activity, known as the cortical silent period (CSP). In the present series, the increase in the CSP recruitment curve was non-linear in ALS patients similar to the relationship established previously for control subjects. The mean CSP duration increased from 0 ms to 178.8 ± 12.8 ms as stimulus intensity increased from 60 to 150% RMT in ALS patients and was significantly reduced in ALS patients compared to normal controls (ANOVA, P < 0.0001, Fig. 5.3).

![Graph showing CSP duration vs stimulus intensity]

**Figure 5.3.** (A) Cortical silent period (CSP) duration, measured from the onset of the motor evoked potential to the beginning of EMG activity, lengthens in a non-linear manner with increasing stimulus intensity. There was a significant reduction of CSP duration in amyotrophic lateral sclerosis (ALS) patients as a group compared to controls (P < 0.05) over stimulus intensities from 80-150 % of resting motor threshold (RMT).

**Peripheral nerve excitability**

Stimulus-response curves obtained following electrical stimulation of the median nerve at the wrist were shifted to the right in ALS patients for stimuli of both 0.2- and 1-ms
duration relative to controls, indicating that axons in ALS patients were of higher
threshold. CMAP amplitude was significantly reduced in ALS patients when compared
to controls (ALS 4.9 ± 0.5 mV; controls 9.8 ± 0.5 mV; P < 0.0005) and correlated with the
ALSFRS-R fine motor sub-score (r = 0.7, P < 0.01) and MRC score (r = 0.7, P < 0.01).
The NI was significantly reduced in ALS patients compared to controls (ALS 0.6 ± 0.1;
controls 2.5 ± 0.2, P < 0.0001) and correlated with the CMAP amplitude (r = 0.6, P <
0.05). Overall, there was no difference in the CMAP amplitude between ALS patients
with limb-onset compared to those with bulbar-onset disease (ALS limb-onset 5.0 ± 2.7
mV; ALS bulbar-onset 4.5 ± 0.9 mV, P = 0.3), nor was there a significant difference in
the NI between the two groups (ALS limb-onset 0.6 ± 0.2; ALS bulbar-onset 0.5 ± 0.2
mV, P = 0.2).

Strength-duration time constant, which reflects nodal persistent Na⁺ conductance
(Bostock and Rothwell, 1997) and rheobase, defined as the threshold current for a
stimulus of infinitely long duration (Bostock et al., 1998) were calculated from SR curves
according to Weiss’s formula (Weiss, 1901; Bostock, 1983; Mogyoros et al., 1996a). As
previously reported (Mogyoros et al., 1998a), mean τ_{SD} was longer in ALS patients (ALS
group 0.46 ± 0.03; controls 0.42 ± 0.02 ms), and rheobase was increased (ALS group 3.3
± 1.1 mA; controls 3.1 ± 1.1 mA) although these differences were not significant.

The type I abnormality of threshold electrotonus, in which there is a greater change in
response to a sub-threshold depolarizing pulse (Bostock et al., 1995), is depicted for an
illustrative ALS patient (Fig. 5.4A), and was evident in 20% of ALS patients from the
present series. The type II abnormality, in which there is a sudden decrease in membrane excitability marked by an abrupt increase in threshold (Bostock et al., 1995), was not evident in any of the ALS patients studied. Overall, threshold electrotonus established greater changes in threshold to depolarizing and hyperpolarizing sub-threshold conditioning pulses in ALS patients (Fig. 5.4 B-D) similar to the “fanned out” response that occurs with membrane hyperpolarization. These findings in threshold electrotonus were accompanied by a significant increase in superexcitability as recorded during the recovery cycle of excitability (Fig. 5.5 A, B).

Figure 5.4. (A) The figure illustrates a recording from one patient with amyotrophic lateral sclerosis (ALS) superimposed on mean data ± standard error of mean from 29 controls. This patient exhibited the type I response in which there is a greater threshold reduction to sub-threshold depolarizing currents. Group data revealed a significant increase in threshold to (B) sub-threshold depolarizing currents at 40-60 ms, TEd (40-60 ms), (C) sub-threshold depolarizing currents at 90-100 ms, TEd (90-100 ms), and (D) sub-threshold hyperpolarizing currents at 90-100 ms, TEh (90-100 ms) in ALS patients compared to controls. **P < 0.01; ***P < 0.001.
There was no significant change in other parameters of the recovery cycle including the relative refractory period (ALS 3.1 ± 1.0 ms; controls 3.2 ± 1.0, P = 0.6) and subexcitability (ALS 15.2 ± 0.9%; controls 14.9 ± 0.7%, P = 0.8). In addition, there was no significant difference in the hyperpolarizing current/voltage slope between ALS patients and controls (ALS 0.38 ± 0.04; controls 0.37 ± 0.01).

Figure 5.5. (A) Illustration of the recovery cycle in a single patient with amyotrophic lateral sclerosis (ALS) superimposed on mean data ± standard error of mean from 29 controls. Relative refractory period (RRP, ms), refers to the first intercept on the \(y\)-axis. Supercitability (%) is expressed as the percentage change in the threshold current. Late subexcitability (%) refers to the largest increase in threshold following superexcitability. Superexcitability was increased in this representative patient. (B) Group data revealed a significant increase in superexcitability in ALS patients compared to normal controls. **P < 0.01.
Combining measures of cortical and peripheral excitability, clinical assessment and disease severity, it was evident that SICI correlated with measures of peripheral disease burden, particularly CMAP amplitude (Fig. 5.6A) and NI (Fig. 5.6B). Furthermore, the MEP: CMAP ratio correlated with a peripheral marker of axonal excitability, namely $\tau_{SD}$ which increases with peripheral nerve degeneration (Fig. 5.6C). Together, these correlations suggest that cortical hyperexcitability is most evident in early ALS, when peripheral disease burden is small, and that cortical hyperexcitability may be an underlying mechanism of neurodegeneration, supporting a dying forward process.

**DISCUSSION**

The present study, using a combination of novel threshold tracking techniques to explore central and peripheral neuronal excitability in ALS patients, has established widespread abnormalities throughout the neural axis. Cortical hyperexcitability was evident in ALS patients with reductions in the resting motor threshold, short-interval intracortical inhibition, and cortical silent period duration, increases in the maximum MEP:CMAP ratio and in the cortical stimulus-response curve gradient. The increase in cortical excitability correlated with traditional measures of peripheral nerve function such as the CMAP amplitude and neurophysiological index, and with axonal excitability parameters, including strength-duration time constant. Together, these findings confirm co-existent dysfunction in upper and lower motor neuron systems in ALS patients with evidence of cortical hyperexcitability as an early feature in ALS when peripheral disease burden is small.
Figure 5.6. Correlation studies illustrating an inverse relationship between peak short interval intracortical inhibition (SICI) and measures of peripheral disease burden, namely (A) compound muscle action potential (CMAP) amplitude and (B) neurophysiological index in 14 amyotrophic lateral sclerosis (ALS) patients with upper limb onset disease. (C) Maximum motor evoked potential (MEP):CMAP ratio correlated inversely with the strength-duration time constant in the same ALS patients, with the exception of two patients in whom the strength-duration time constant was not recorded.
Mechanisms mediating abnormalities of intracortical inhibition and facilitation

Short-interval intracortical inhibition was first reported by Kujirai and colleagues (Kujirai et al., 1993), who demonstrated that a subthreshold conditioning stimulus could suppress the response to a later suprathreshold test stimulus when the ISI was less than 5 ms. Recently, two physiologically distinct phases SICI have been reported with the first phase occurring at an ISI of ≤1 ms and second phase at ISI of 2.5-3 ms (Fisher et al., 2002; Roshan et al., 2003). Although there is debate regarding the first phase of SICI with some arguing that it reflects local excitability properties of the cortical axon (Fisher et al., 2002), and others activation of inhibitory circuits different to that which mediates inhibition at ISI of 2.5-3 ms (Roshan et al., 2003), the mechanism of the second phase of SICI (ISI 2.5-3 ms) is believed to be cortical in origin and mediated by GABA-secreting inhibitory cortical interneurons via GABA\(_A\) receptors (Ziemann et al., 1996b; Ziemann, 2004b; Ziemann, 2004a).

In the present series, SICI was significantly reduced in ALS patients in keeping with previous studies (Hanajima et al., 1996; Yokota et al., 1996; Ziemann et al., 1997c; Sommer et al., 1999; Stefan et al., 2001; Zanette et al., 2002b). One possible mechanism for reduction of SICI is loss of inhibitory cortical interneurons. Evidence for this is provided by neuropathological studies that have revealed a loss of parvalbumin-positive inhibitory cortical interneurons in ALS patients (Nihei et al., 1993).

Loss of inhibitory cortical interneurons is not the only mechanism mediating SICI reduction, as SICI may be rapidly restored in ALS patients treated with pharmacological
agents such as riluzole (Stefan et al., 2001; Ziemann, 2004b). This would suggest that glutamate mediated down-regulation of SICI may be another mechanism mediating the reduction of SICI in ALS. In addition to the effects on SICI, riluzole also decreases intracortical facilitation and inhibits persistent Na\(^+\) currents (Liepert et al., 1997; Kuo et al., 2005). As such, the neurophysiological findings may have been even more severely affected in patients from the present study had they not been treated with riluzole.

Another possible explanation for reduction in SICI and the concomitant increase in MEP:CMAP ratio, may be related to reduced phase cancellation of corticomotoneuron discharges in ALS. However, peristimulus time histogram studies have established that corticomotoneuron volleys to the anterior horn cell are desynchronised to a greater extent in ALS compared to normal controls (Kohara et al., 1996b; Weber and Eisen, 2000; Weber et al., 2000a). Furthermore, methods such as the triple stimulation technique, which resynchronise the MEP (Magistris et al., 1998), would not be as sensitive at detecting upper motor neuron dysfunction in ALS compared to conventional TMS techniques were the corticomotoneuron volley to be less desynchronised in ALS (Komissarow et al., 2004).

The reduction in SICI, along with other TMS changes suggestive of cortical hyperexcitability, although uniform across all clinical ALS phenotypes, were most evident in ALS patients with limb-onset disease. While it remains possible that cortical hyperexcitability is less prominent in ALS patients with bulbar-onset disease, more likely the difference lies in the choice of target muscles. That is, all studies from ALS patients
in the present series were undertaken using a peripheral target muscle. Desiato and colleagues (Desiato, 2002) have reported on the presence of cortical hyperexcitability in ALS patients with bulbar-onset disease, with reduction of CSP duration when recordings were undertaken directly from affected bulbar muscles.

In addition to reduction of SICI, the CSP duration was uniformly reduced in all clinical ALS phenotypes. Given that the CSP duration is mediated by inhibition of anterior horn cells in the early phase (Cantello et al., 1992; Inghilleri et al., 1993; Chen et al., 1999; Ziemann, 2004a) and cortical processes, through GABAβ receptors, in later segments (Connors et al., 1988; Avoli et al., 1997; Werhahn et al., 1999; Ziemann, 2004a), this finding is in keeping with previous studies in ALS patients documenting both disinhibition of anterior horn cells (Raynor and Shefner, 1994; Drory et al., 2001) and dysfunction of cortical inhibitory interneurons acting via GABAβ receptors (Zanette et al., 2002b).

**Is there a dying forward process in ALS?**

In the present study, coincidental dysfunction of upper and lower motor neuron systems was evident in ALS patients. While the study design does not lend itself to absolutely determining the site of disease onset, the fact that cortical hyperexcitability inversely correlated with measures of peripheral disease burden, particularly CMAP amplitude and NI, would suggest that cortical hyperexcitability is an early feature of ALS, thereby providing indirect support for a dying forward process. Furthermore, the fact that cortical
hyperexcitability was evident in ALS patients with advanced disease (CMAP \(< 4\) mV, Fig. 3.2C and 3.3C) suggests that cortical hyperexcitability is persistent and ongoing.

Further indirect support for a “dying forward” process, which proposes that corticomotor neurons drive anterior horn cell loss (Eisen et al., 1992), may be derived by the inverse correlation between the \(\tau_{\text{SD}}\) and MEP:CMAP ratio. Given that the prolongation of \(\tau_{\text{SD}}\) has been previously linked to the processes of axonal regeneration and sprouting (Kanai et al., 2003), this correlation may suggest that cortical hyperexcitability is responsible for motor neuron loss in ALS, although compensatory upregulation of the upper motor neuron system attempting to overcome lower motor neuron dysfunction cannot be excluded.

In conclusion, the present study has established the presence of cortical hyperexcitability in all ALS phenotypes, being most prominent in patients with limb-onset disease. Furthermore, correlation studies suggest that cortical hyperexcitability is an early feature of ALS that persists throughout the disease process, possibly serving as a mechanism driving neuronal dysfunction in ALS.
Chapter 6

Abnormalities in cortical and peripheral excitability in flail-arm variant ALS
SUMMARY

The neurogenic variant of “man-in-the barrel” syndrome, termed flail-arm syndrome, is considered to be an unusual pure lower motor neuron variant of amyotrophic lateral sclerosis (ALS) by some. Others have argued that the neurogenic “man-in-the-barrel” syndrome represents a sub-type of progressive muscular atrophy. In this chapter, the clinical phenotype of flail-arm syndrome is further defined and the pathophysiology of this disease is investigated through application of threshold tracking techniques. Clinical data, structural imaging and conventional neurophysiological studies were collected in flail-arm patients, in addition to simultaneous assessment of cortical and peripheral nerve excitability. Eleven flail-arm patients were identified, representing 17% of our amyotrophic lateral sclerosis (ALS) database. Mean age at disease-onset (60.3 years) was similar to the remaining ALS patients (58.3 years), with a strong male predominance (male:female; flail-arm 10:1; ALS 1.5:1, P < 0.05), and prolonged disease duration (flail-arm 62.5; ALS 15.8 months, P < 0.05) in flail-arm patients. Lower-limb symptoms developed in 18%, bulbar in 27% and respiratory symptoms in 18% of flail-arm patients. Peripheral nerve excitability studies demonstrated upregulation in persistent Na+ currents and reduction of slow K+ conductances. Flail-arm patients exhibited cortical hyperexcitability, with reduction in short interval intracortical inhibition (-0.8 ± 0.6%; controls 8.5 ±1.0%, P < 0.0001) and resting motor threshold (53.4 ± 2.8%; controls 60.7 ± 1.5%, P < 0.05), along with an increase in motor evoked potential amplitude (49.5 ± 9.0%; controls 25.8 ± 2.8%, P < 0.05). Flail-arm syndrome is an unusual ALS phenotype, with male predominance and long survival, which appears to exhibit a similar pathophysiology, i.e. cortical hyperexcitability, as typical ALS.
INTRODUCTION

The “man-in-the barrel” syndrome is clinically characterized by severe bilateral weakness of shoulder girdle muscles, originally reported in the setting of watershed territory infarction between the middle and anterior cerebral arteries (Sage and Van Uitert, 1986). A neurogenic variant of “man-in-the barrel” syndrome resulting from cervical anterior horn cell loss has been reported (Hu et al., 1998; Gamez et al., 1999; Katz et al., 1999; Sasaki and Iwata, 1999; Couratier et al., 2000; Czaplinski et al., 2004). Hu and colleagues (Hu et al., 1998) suggested that this neurogenic “man-in-the barrel” syndrome, which they termed “flail-arm syndrome”, was a variant of amyotrophic lateral sclerosis (ALS) since the majority of patients ultimately developed bulbar and generalized limb involvement.

Others have argued that the neurogenic “man-in-the-barrel” syndrome represents a subtype of progressive muscular atrophy (PMA) (Katz et al., 1999). Katz and colleagues (Katz et al., 1999) reported an absence of bulbar and upper motor neuron signs in a group of patients with the neurogenic “man-in-the-barrel” phenotype, which they termed brachial amyotrophic diplegia (BAD). Indeed, the flail-arm syndrome or “BAD” may be clinically differentiated from classic ALS by the unique pattern of muscle weakness, absence of upper motor neuron signs in the affected upper limbs and prolonged survival (Hu et al., 1998; Gamez et al., 1999; Katz et al., 1999; Sasaki and Iwata, 1999; Couratier et al., 2000; Czaplinski et al., 2004).
The diagnosis of ALS relies on the presence of a combination of upper and lower motor neuron features in the same region of involvement with evidence of disease spread over time (Desai and Swash, 2002; Kiernan, 2003). Clinical evidence of upper motor neuron dysfunction may be elusive (Triggs et al., 1999), particularly in the early stages of ALS, or when upper motor neuron (UMN) dysfunction is obscured by severe motor neuron cell loss. Given the uncertainties of the neurogenic “man-in-the-barrel” syndrome, the aim of the present study was to expand the clinical series, further define the clinical phenotype, and to implement novel cortical threshold tracking transcranial magnetic stimulation (TMS) techniques to further explore disease pathophysiology.

METHODS

Patients

Clinical, radiological, and conventional neurophysiological data were collected from 11 patients with flail-arm variant of ALS identified from the Prince of Wales Hospital Multidisciplinary motor neuron disease clinic. The criteria for inclusion included the presence of progressive muscle weakness confined to the proximal aspects of the upper limbs for a period of 24 months with neurophysiological evidence of lower motor neuron dysfunction. Patients with evidence of weakness in the neck, bulbar, and respiratory muscles before 24 months were excluded from the study.

All flail-arm patients were staged using the amyotrophic lateral sclerosis functional rating scale-revised (ALSFRS-R) (Cedarbaum et al., 1999), muscle strength using the Medical Research Council (MRC) rating scale (Medical Research Council, 1976), hand function
score (Triggs et al., 1999), and an upper motor neuron (UMN) score (Turner et al., 2004). Muscle strength in the proximal upper limbs was graded by summing the Medical Research Council (MRC) score for 12 different movements (i.e. shoulder abduction, adduction, internal and external rotation, and elbow flexion and extension bilaterally) for a maximum score of 60, while the strength in the distal upper limbs was also assessed for 12 different movements (i.e. wrist flexion and extension, finger and thumb abduction, finger extension and flexion bilaterally) for a maximum score of 60. The UMN score comprised a sum of pathologically brisk reflexes that included the assessment of biceps, supinator, triceps, finger, knee and ankle reflexes, with plantar responses, facial and jaw jerks, all bilaterally, for a maximum possible score of 16 (Turner et al., 2004).

Cortical excitability testing
Cortical excitability was measured by applying the threshold tracking TMS technique (see Methodology). In all studies, the motor cortex was stimulated using a 90 mm circular coil, with the resultant MEP response recorded over the abductor pollicis brevis (APB) muscle (see Methodology). The following parameters of cortical excitability were recorded using the previously described cortical excitability protocol (see Methodology): resting motor threshold, magnetic stimulus-response curve, central motor conduction time, short interval intracortical inhibition, intracortical facilitation and cortical silent period duration.
Peripheral nerve excitability

Peripheral nerve excitability studies were performed in the same sitting according to a previously described protocol that measures multiple parameters of nerve excitability including (see Methodology): SR curves for stimuli of 0.2 and 1-ms duration; strength-duration time constant (τSD); threshold electrotonus using prolonged sub-threshold polarizing currents of 100 ms duration, set to + 40% (depolarizing) and - 40% (hyperpolarizing) of controlled threshold current; and recovery cycle of axonal membrane excitability (Kiernan et al., 2000). Skin temperature was maintained at 32°C.

In all studies, the median nerve was stimulated at the wrist and the resultant CMAP was recorded using surface electrodes positioned over the APB (see Methodology). In addition to calculating parameters of axonal excitability, the neurophysiological index (NI) was derived according to a previously reported formula (de Carvalho and Swash, 2000) (see Chapter 5).

Statistical Analysis

Cortical excitability in flail-arm patients was compared to other ALS phenotypes (limb and bulbar onset patients, N = 23) and normal control data obtained from 39 subjects (20 men; 19 women, aged 23-73 years, mean: 43.4 years). Peripheral nerve excitability parameters were compared to previously reported controls and were compensated for age and temperature before statistical analysis (Kiernan et al., 2000; Kiernan et al., 2001a; Kiernan et al., 2001c). Student’s t-test and Chi square (χ²) testing were used to compare differences between groups, and analysis of variance (ANOVA) was used for multiple
comparisons. A probability (P) value of < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.

RESULTS

Clinical Features: Clinical features of 11 flail-arm patients are summarized in Table 6.1 and four illustrative case histories are described.

Case histories

Patient #1 (Table 6.1, Fig. 6.1A, B), a 59-year old male, presented 4-years prior to the present study with right shoulder girdle muscle weakness, fasciculations, wasting and cramps. One year later the patient reported weakness and wasting in the left shoulder girdle muscles. Recently, the patient developed exertional dyspnea and marked orthopnea requiring non-invasive pressure support ventilation. A six-month course of intravenous immunoglobulin (IVIg, 0.4 mg/kg/month) was unsuccessful. Examination demonstrated widespread fasciculations in the upper limb muscles. There was marked wasting and weakness of shoulder girdle musculature (deltoid, biceps brachii, and triceps). Reflexes in the upper limbs were reduced. Electrophysiological examination demonstrated ongoing and chronic neurogenic changes in upper limb muscles with absence of motor conduction block. Brain and whole spine MRI were normal. The patient's condition progressed and he died 57 months after symptom onset.

Patient #2 (Table 6.1, Fig. 6.1C, D), a 50-year old male, developed right shoulder girdle muscle weakness, fasciculations, wasting and cramps 2-years prior to the present studies.
Twelve months later, he developed left shoulder muscle weakness. Examination demonstrated widespread fasciculations in the upper limb muscles. There was marked wasting and weakness of shoulder girdle muscles (deltoid, supra- and infraspinatus, biceps brachii, and triceps) bilaterally. Reflexes in the upper limbs were absent, while the lower limb reflexes were brisk with flexor plantar responses. Electrophysiological examination demonstrated chronic neurogenic changes in proximal upper limb muscles with absence of motor conduction block.

**Patient #4** (Table 6.1, Fig. 6.1E, F), a 67-year old male, presented with left shoulder girdle muscle weakness and wasting 4-years before the present investigation. Two years later he developed dysphagia followed by dysarthria, neck flexion weakness, fatigue and respiratory insufficiency requiring non-invasive pressure support ventilation. Examination demonstrated widespread fasciculations in the upper limb muscle, along with marked wasting and weakness of shoulder girdle muscles. Reflexes in the upper limbs were absent. Electrophysiological examination demonstrated chronic neurogenic changes in proximal upper limb muscles with absence of motor conduction block. Brain and whole spine MRI were normal.

**Patient #5** (Table 6.1, Fig. 6.1 G, H), a 69-year old male, presented 8-months prior to the present study with bilateral shoulder girdle muscle weakness, fasciculations, wasting and cramps. Examination demonstrated widespread fasciculations in the upper and lower limb muscles, with marked wasting and weakness of shoulder girdle muscles bilaterally. Reflexes in the upper limbs were absent. Electrophysiological examination demonstrated
fasciculations and chronic neurogenic changes in upper limb muscles with absence of motor conduction block.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (years)/Sex</th>
<th>Disease duration (months)</th>
<th>ALSFRS-R</th>
<th>Triggs hand score</th>
<th>MRC UL Prox. Score</th>
<th>MRC UL Distal Score</th>
<th>UMN score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59,M</td>
<td>57</td>
<td>30</td>
<td>2</td>
<td>24</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>50,M</td>
<td>24</td>
<td>42</td>
<td>0</td>
<td>40</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>71,M</td>
<td>96</td>
<td>38</td>
<td>0</td>
<td>40</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>67,M</td>
<td>48</td>
<td>40</td>
<td>2</td>
<td>48</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>69,M</td>
<td>10</td>
<td>42</td>
<td>2</td>
<td>40</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>52,M</td>
<td>180</td>
<td>44</td>
<td>1</td>
<td>40</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>68,M</td>
<td>74</td>
<td>15</td>
<td>2</td>
<td>36</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>73,M</td>
<td>24</td>
<td>32</td>
<td>2</td>
<td>24</td>
<td>42</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>67,F</td>
<td>96</td>
<td>28</td>
<td>2</td>
<td>36</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>42,M</td>
<td>31</td>
<td>46</td>
<td>0</td>
<td>30</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>56,M</td>
<td>36</td>
<td>40</td>
<td>0</td>
<td>46</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>61.3</td>
<td>61.5</td>
<td>36.1</td>
<td>1.4</td>
<td>36.7</td>
<td>47.1</td>
<td>2.2</td>
</tr>
<tr>
<td>SEM</td>
<td>3.7</td>
<td>14.7</td>
<td>2.7</td>
<td>0.3</td>
<td>2.4</td>
<td>2.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 6.1. Clinical details for the 11 patients with flail-arm syndrome. Disease duration refers to the period from symptom onset to date of testing or last review in the amyotrophic lateral sclerosis (ALS) clinic. The patients were clinically graded using the ALS functional rating scale revised (ALSFRS-R), with a maximum score of 48 when there is no disability. The ALSFRS-R is comprised of 4 sub-scores; bulbar (maximum score 12), fine motor (maximum score 8), gross motor (maximum score 16), and respiratory (maximum score 12). Muscle strength was clinically assessed using the Medical Research Council (MRC) scale. The strength in the proximal (Prox.) upper limb (UL) was graded by summating the MRC score for 12 different movements (see Methods) giving a maximum score of 60. The strength in the distal ULs was also assessed for 12 different movements (see Methods) for a maximum score of 60. Upper motor neuron (UMN) involvement was graded according to the UMN score with a maximum score of 16 (see Methods). Four patients were graded as having an UMN score of 6 due to hyperreflexia in the lower limbs.
Flail-arm patients comprised 17% of patients in our ALS database. Mean age at disease onset was similar for flail-arm patients and other ALS phenotypes (flail-arm 60.3 ± 3.0 years; other ALS phenotypes 58.3 ± 1.8 years). There was a significant male predominance in the flail-arm patients compared to other ALS phenotypes (flail-arm 10:1; other ALS phenotypes 1.5:1, P < 0.05), and the mean duration of illness was significantly longer in the flail-arm patients (flail-arm 62.5 ± 15.6 months; other ALS phenotypes 15.8 ± 2.3 months, P < 0.05).

Lower limb symptoms developed in 18% of patients, 58.8-months after symptom onset. Bulbar symptoms developed in 27% of patients, 36-months after symptom onset and respiratory symptoms occurred in 18% of patients, 31-months after symptom onset and these patients required ongoing non-invasive ventilatory support.
Figure 6.1. Anterior (A, C, E, G), posterior (B, D) and lateral (F, H) views in four patients with flail-arm syndrome attempting shoulder abduction. There is marked wasting of anterior and posterior shoulder girdle muscles, with an inability to abduct the shoulders, with a resultant “man-in-the-barrel” syndrome. Despite the presence of marked wasting and weakness of upper limb muscles, all patients are standing freely.
Cortical excitability

In total, 72% of flail-arm patients underwent TMS studies. The central motor conduction time (CMCT) in flail-arm patients was not significantly different when compared to other ALS phenotypes (flail-arm 5.9 ± 0.4 ms; other ALS phenotypes 5.1 ± 0.5 ms) or normal control subjects (controls 5.1 ± 0.3 ms).

Resting motor threshold, defined as the unconditioned stimulus intensity required to produce and maintain the target MEP response, was significantly reduced in flail-arm patients compared to controls, but not when compared to other ALS phenotypes (flail-arm 53.4 ± 2.8%; other ALS phenotypes 56.6 ± 1.8%; controls 60.7 ± 1.5%, P < 0.05). The MEP amplitude, expressed as a percentage of the CMAP amplitude recorded following electrical stimulation was increased in flail-arm patients as a group when compared to normal controls over stimulus intensities from 110 to 150% RMT (P < 0.05, Fig. 6.2). Further, the maximal MEP amplitude, expressed as a percentage of the CMAP response, was significantly increased in flail-arm patients when compared to controls (flail-arm 49.5 ± 9.0%; controls 25.8 ± 2.4%, P < 0.05), but not when compared to other ALS phenotypes (flail-arm 49.5 ± 9.0%; other ALS phenotypes 43.5 ± 6.8%).

Short interval intracortical inhibition is reflected by an increase in the conditioned stimulus intensity required to track a constant target MEP of 0.2 mV. There was a significant reduction of SICI in both flail-arm patients (averaged SICI from 1-7 ms, -0.8 ± 0.6%, P < 0.0001) and other ALS phenotypes (averaged SICI from 1-7 ms, 4.1 ± 1.1%, P < 0.01) when compared to normal controls (averaged SICI from 1-7 ms, 8.5 ± 1.1%,
Fig. 6.3A, B). The reduction of SICI was greater in flail arm patients when compared to other ALS phenotypes (P < 0.001, Fig 6.3 B).

Following SICI, a period of intracortical facilitation (ICF) followed, reflected by a decrease in the test stimulus intensity required to maintain the target MEP of 0.2 mV. ICF was significantly increased in flail-arm patients compared to controls (averaged ICF 10-30 ms, flail-arm -3.2 ± 0.7%; controls -0.9 ± 0.4%, P < 0.05, Fig 6.3A), similar to other ALS phenotypes (averaged ICF 10-30 ms other ALS phenotypes -2.5 ± 0.3%, P = 0.2).

**Figure 6.2.** Stimulus-response (SR) curves obtained following transcranial magnetic stimulation in 8 flail-arm amyotrophic lateral sclerosis (ALS) patients and 39 normal controls, with the motor evoked potential (MEP) expressed as a percentage of compound muscle action potential (CMAP) amplitude recorded following electrical stimulation. The MEP amplitude was significantly increased in flail-arm syndrome compared to controls over stimulus intensities from 110-150 % (P < 0.05) of resting motor threshold (RMT).
Figure 6.3. (A) Short interval intracortical inhibition (SICI), defined as the stimulus intensity required to maintain a target output of 0.2 mV, was reduced in flail-arm amyotrophic lateral sclerosis patients (ALS, \( N = 8, P < 0.0001 \)) compared to normal controls (\( N = 39 \)). (B) The reduction in SICI was significantly more prominent in flail-arm patients compared to ALS (\( N = 23 \)) and controls. ***\( P < 0.001 \), ****\( P < 0.0001 \) compared to controls.
Peripheral nerve excitability

Peripheral nerve excitability studies were undertaken in 81% of flail-arm patients. Stimulus-response curves obtained following electrical stimulation of the median nerve at the wrist were shifted to the right in flail-arm patients for stimuli of both 0.2- and 1-ms duration, indicating that axons were of higher threshold relative to controls. CMAP amplitude (flail-arm 3.6 ± 1.1 mV; controls 9.8 ± 0.5 mV; P < 0.001) and neurophysiological index (flail-arm 0.5 ± 0.1; controls 2.5 ± 0.1; P < 0.0001) were significantly reduced in flail-arm patients.

Strength-duration time constant, which reflects nodal persistent Na⁺ conductance, and the rheobase, defined as the threshold current for a stimulus of infinitely long duration, were again calculated from SR curves according to Weiss’s formula (Weiss, 1901; Bostock, 1983; Mogyoros et al., 1996a). As previously reported in the typical ALS patients (Mogyoros et al., 1998a; Kanai et al., 2006), the mean \( \tau_{SD} \) was longer in flail-arm patients compared to controls (flail-arm 0.44 ± 0.07; controls 0.42 ± 0.02 ms), and the rheobase was increased (flail-arm 4.2 ± 1.3 mA; controls 3.1 ± 1.1 mA), although these differences were not significant.

The type I abnormality of threshold electrotonus (Bostock et al., 1995), in which there is a greater change in response to a sub-threshold depolarizing pulse is depicted for an illustrative flail-arm patient (Fig. 6.4A). Mean data revealed the presence of the type I abnormality in flail-arm patients (Fig. 6.4B, C). The type II abnormality (Bostock et al., 1995), in which there is a sudden decrease in membrane excitability marked by an abrupt
increase in threshold, was not evident in any of the flail-arm patients. There was no significant change in any of the parameters of the recovery cycle including the relative refractory period (flail-arm 3.4 ± 1.1 ms; controls 3.2 ± 1.0, P = 0.15), superexcitability (flail-arm 28.4 ±1.2%; controls 24.7 ± 1.0%, P = 0.12) and subexcitability (flail-arm 16.1 ± 2.1%; controls 14.9 ± 0.7%, P = 0.53).

**DISCUSSION**

The present study has further defined the flail-arm phenotype of ALS, which is clinically characterized by proximal upper limb weakness, male predominance and prolonged survival. Disease progression to other regions, including the lower limbs, bulbar, and respiratory muscles, was documented in the flail-arm patients, 18% of whom required non-invasive pressure ventilation. Although upper motor neuron signs were clinically absent, cortical excitability studies established upper motor neuron dysfunction, as indicated by reduction in resting motor threshold and short-interval intracortical inhibition, together with increases in the MEP amplitude and cortical stimulus-response curve gradient. Changes in cortical excitability were accompanied by abnormalities of axonal excitability, including an increase in persistent Na⁺ and reduction in slow K⁺ conductances. Together, these changes in central and peripheral nerve excitability are similar to those recently reported in ALS, providing further support to the hypothesis that the flail-arm syndrome is a variant of ALS.
Figure 6.4. Threshold electrotonus refers to changes in membrane excitability in response to long duration polarizing currents. Threshold reduction with depolarization is represented in an upward direction and hyperpolarization in a downward direction. (A) The figure illustrates a recording from one flail-arm patient superimposed on mean data ± standard error of mean from 29 controls (curve with error bars). This patient exhibited the type I response in which there is a greater threshold reduction to sub-threshold depolarizing currents. Group data revealed a significant increase in threshold to (B) sub-threshold depolarizing currents at 40-60 ms, TEd (40-60 ms), and (C) sub-threshold depolarizing currents at 90-100 ms, TEd (90-100 ms), in flail-arm patients compared to controls. ***P < 0.001.
Prior to consideration of the diagnosis of a specific flail-arm variant of ALS, a number of secondary disorders that can result in the same clinical phenotype clearly need to be excluded. Specifically, lesions localized to the cortex (Sage and Van Uitert, 1986; Moore and Humphrey, 1989; Wilck et al., 1996), pons (Alberca et al., 1985; Paulin et al., 2005), and spinal cord, including cervical spondylosis (Dorsen and Ehni, 1979; Kameyama et al., 1998), spinal cord infraction (Berg et al., 1998), and postradiation myelopathy (Lalu et al., 1998) are important differential diagnoses that may mimic the flail-arm syndrome phenotype. Demyelinating motor neuropathies, including multifocal motor neuropathy with conduction block (Parry and Clarke, 1988), may also present with bilateral upper limb weakness. All of these conditions were excluded in the present study on the basis of clinical investigation, including neuroimaging of the brain and cervical spine, neurophysiological studies, and laboratory testing.

Evidence for upper motor neuron dysfunction in flail-arm patients from the present series was provided by novel threshold tracking TMS studies that were undertaken to assess cortical excitability. These studies demonstrated a significant reduction in SICI, indicative of cortical hyperexcitability and were in keeping with previous studies that investigated the more typical ALS phenotypes (Yokota et al., 1996; Ziemann et al., 1997c; Hanajima and Ugawa, 1998; Sommer et al., 1999; Stefan et al., 2001; Zanette et al., 2002b) (see Chapter 5). The mechanism of SICI is believed to be cortical in origin, mediated by GABA-secreting inhibitory cortical interneurons via GABA-A receptors (Ziemann, 2004b). Loss of these inhibitory cortical interneurons partly accounts for the
reduction in SICI in flail-arm syndrome, as was previously established in ALS (Nihei et al., 1993).

In addition to cortical hyperexcitability, peripheral nerve excitability studies revealed an increase in depolarizing threshold electrotonus (TEd 90-100 ms, the type I response) (Bostock et al., 1995) and strength-duration time constant, much as recently established in larger cohorts of ALS patients (Bostock et al., 1995; Mogyoros et al., 1998a; Kanai et al., 2006) (see Chapter 1). Mathematical modeling of these excitability changes in ALS patients, suggested that the changes in nerve excitability were best explained by reduction in slow nodal and internodal K⁺ channel conductances, in addition to increases in nodal leak and persistent Na⁺ conductances (Kanai et al., 2006). The changes in peripheral axonal excitability established for flail-arm patients in the present series are entirely in keeping with the modeled hypothesis, suggesting the presence of a similar peripheral pathophysiological process in flail-arm and the more typical ALS patients.

While the flail-arm phenotype was first described in ALS patients by Mulder (Mulder, 1957), it was Hu and colleagues (Hu et al., 1998) who reported that the flail-arm phenotype comprised 10% of their ALS population and suggested that this phenotype was an unusual variant of ALS. Katz and colleagues (Katz et al., 1999) argued that the flail-arm syndrome included patients with classic ALS, and reported a series of 10 patients with pure lower motor neuron disease, who had failed to progress despite an 11 year follow-up, and suggested that the flail-arm syndrome was a specific phenotype of progressive muscular atrophy, termed bibrachial amyotrophic diplegia. The clinical
phenotype described in the present series is in keeping with previously reported series (Hu et al., 1998; Gamez et al., 1999; Katz et al., 1999; Sasaki and Iwata, 1999; Couratier et al., 2000; Czaplinski et al., 2004). However, by demonstrating evidence of upper motor neuron dysfunction and documenting disease progression to other regions, including respiratory and bulbar, the present study provides further support for the argument that the flail-arm syndrome is a variant of ALS, rather than a form of progressive muscular atrophy.

The notion that the flail-arm syndrome is a variant of ALS may influence the design of future ALS drug trials. Specifically, flail-arm patients may be diagnosed with clinically probable and laboratory supported ALS on the basis of finding lower motor neuron signs in the arms, with upper motor neuron signs in one region (lower limbs), as was the case in 4 of the flail-arm patients in the present series (see Table 6.1). Given the natural history of the flail-arm syndrome, the inclusion of flail-arm patients in therapeutic clinical trials may thereby influence outcome measures in terms of disease survival. As such, future ALS trials should ideally identify flail-arm patients, apportion them equally to each intervention arm, and the outcome results of these patients should be analyzed separately from the more typical ALS patients.

What underlies the difference in clinical phenotype between flail-arm and the more typical ALS patients?

Given that neurophysiological studies revealed similar abnormalities of cortical and axonal excitability between flail-arm and more typical ALS patients, it is unclear as to
what accounts for the significant differences in the clinical phenotype, particularly related to survival. Focal amyotrophy syndromes, including Hirayama’s disease in which there is an initial rapid loss of motor neurons followed by an arrest of the disease process, is postulated to result from compression and ischemia of the cervical spinal cord with particular involvement of anterior horn cells (Hirayama et al., 1963; Kiernan et al., 1999; Hirayama and Tokumaru, 2000). In flail-arm patients, although lower motor neuron dysfunction remains localized to the cervical myotomes for an extended period, there is invariably evidence of disease progression to other regions, arguing against the mechanisms implicated in Hirayama’s disease.

The greater male predominance in flail-arm patients may suggest that either hormonal or genetic factors linked to the male sex underlie the clinical phenotype. In the central nervous system, expression levels of the androgen receptor (AR) are relatively high in spinal motor neurons (Katsuno et al., 2006). Trinucleotide triplet repeat (CAG) expansions in the AR have been well documented in Kennedy’s disease, a male predominant, slowly progressive hereditary neurodegenerative disorder of the motor neurons (La Spada et al., 1991). While the clinical and neurophysiological features in flail-arm patients are clearly different to Kennedy’s disease, and the triplet repeat (CAG) expansion in the AR gene was excluded in all flail-arm patients from the present series, studies investigating the function of the AR and its agonists, such as testosterone, may yet prove useful in unlocking this clinical mystery.
Chapter 7

Cortical excitability in Kennedy’s disease
SUMMARY

It has been argued that the increase in cortical excitability evident in amyotrophic lateral sclerosis (ALS) patients (Chapter 5 and 6) represents a compensatory down-regulation of inhibitory processes in response to anterior horn cell loss (AHC), rather than an excitotoxic process underlying motor neuron degeneration. To determine whether cortical hyperexcitability is a primary event in ALS, in this chapter cortical excitability studies were undertaken in Kennedy’s disease (KD), a disease control. Kennedy’s disease is an X-linked inherited neurodegenerative disorder characterized by prominent fasciculations, bulbar and limb weakness. Although these clinical features result from AHC degeneration, the mechanisms underlying AHC degeneration in KD remain undefined. Cortical excitability studies were undertaken in 7 KD patients and compared to 55 controls and 38 ALS patients, using a 90 mm circular coil connected to a BiStim device. Motor responses were recorded over abductor pollicis brevis. Short-interval intracortical inhibition (SICI) in KD was similar compared to controls (KD 6.0 ± 1.2%; controls 8.4 ± 1.1%, P = 0.08), but significantly greater when compared to ALS (ALS 0.7 ± 0.7%, P<0.0001). The magnetic stimulus-response curve gradient, motor evoked potential amplitude and cortical silent period duration in KD were similar to controls. These findings would argue against the presence of cortical hyperexcitability in KD and re-affirm the presence of cortical hyperexcitability in ALS. Together, the threshold tracking TMS techniques would seem to argue against the possibility that cortical hyperexcitability evident in ALS represents simple down-regulation of inhibitory processes in response to AHC loss.
INTRODUCTION

Spinobulbomuscular atrophy, or Kennedy’s disease (KD), is a slowly progressive X-linked inherited neurodegenerative disorder of motor and sensory neurons (Kennedy et al., 1968). The disease is caused by an expansion of a trinucleotide (CAG) repeat in the N-terminal domain of the androgen receptor (AR) gene on the X chromosome, at Xq11-12 (La Spada et al., 1991).

Clinically, Kennedy’s disease is characterized by widespread fasciculations, especially prominent in the face, and muscle cramping. Subsequently, other neuromuscular symptoms such as muscle weakness and wasting, postural hand tremor, dysarthria and dysphagia develop (Kennedy et al., 1968; Harding et al., 1982; Olney et al., 1991; Gallo, 2004). These motor symptoms develop as a result of anterior horn cell (AHC) degeneration (Kennedy et al., 1968; Sobue et al., 1989; Li et al., 1995). The mechanisms underlying AHC degeneration in KD remain as yet undefined (Gallo, 2004).

In amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder of motor neurons that may appear clinically similar to KD (Parboosingh et al., 1997; Desai and Swash, 2002; Kiernan, 2003), AHC degeneration was hypothesized to result from anterograde excitotoxicity mediated by corticomotoneurons (Eisen et al., 1992). Support for such a process was provided by transcranial magnetic stimulation (TMS) studies demonstrating that corticomotoneuronal hyperexcitability is a prominent and early feature of ALS (Caramia et al., 1991; Eisen et al., 1993; Mills and Nithi, 1997; Desiato, 2002; Mills, 2003) (see Chapter 5). In Kennedy’s disease, TMS studies have revealed conflicting
results. While some studies have reported normal corticospinal tract function, thereby
distinguishing KD from ALS (Weber and Eisen, 1999; Eisen, 2001; Attarian et al., 2005),
recent studies have suggested the converse, namely corticomotoneuronal
hypercexcitability in KD (Attarian et al., 2006; Pachatz et al., 2007).

A possible explanation for these discordant TMS findings in KD may relate to marked
variability in the motor evoked potential (MEP) amplitude with consecutive stimuli
resulting from spontaneous fluctuations in the resting threshold of cortical neurons (Kiers
et al., 1993). In order to overcome this potential limitation, a threshold tracking technique
was recently adapted for assessment of cortical excitability and normative data
established (see Chapter 4). Subsequently, the threshold tracking TMS technique
established that cortical hyperexcitability was an early and prominent feature of ALS (see
Chapter 5). Consequently, the present study applied novel threshold tracking TMS
techniques to clarify the variable findings in relation to changes in cortical excitability
and to assess the utility of this novel technique in distinguishing ALS from KD.

METHODS

Patients
Studies were undertaken on 7 patients with Kennedy’s disease (aged 35-68, mean 53
years). The diagnosis of KD was confirmed by genetic screening (La Spada et al., 1991),
with the size of the CAG expansion varying from 40-46 (normal < 38)(La Spada et al.,
1991). Patients were clinically assessed using the Medical Research Council (MRC)
rating scale (Medical Research Council, 1976).
Cortical excitability testing

Cortical excitability was assessed by means of a 90 mm circular coil oriented to induce current flow in a posterior-anterior direction with magnetic evoked potential (MEP) responses recorded over the abductor pollicis brevis (APB) muscle. Using the threshold tracking paired-pulse TMS technique (see Methodology and Chapter 4) the following parameters of cortical excitability were recorded; resting motor threshold, magnetic stimulus-response curve, central motor conduction time, short interval intracortical inhibition, intracortical facilitation and cortical silent period duration.

Peripheral nerve studies

For peripheral studies the median nerve was stimulated electrically at the wrist with the resultant CMAP recorded over the APB and measured from peak to peak (see Methodology). The neurophysiological index (NI) was calculated according to a previously reported formula (de Carvalho and Swash, 2000) (see Chapter 5):

Statistical Analysis

Cortical excitability in KD patients was compared to normal controls (28 men; 27 women, aged 23-73 years, mean: 46 years) and ALS patients, as a disease control group (22 men; 16 woman, aged 44-78 years, mean 59 years). All ALS patients fulfilled the revised El Escorial criteria for definite or probable ALS (Brooks et al., 2000b). Student’s t-test and analysis of variance (ANOVA) was used to compare differences between groups. Correlations between cortical excitability indices and clinical and neurophysiological scales of disease severity were analyzed by Spearmen’s rank test. In
order to assess the utility of threshold tracking TMS in differentiating KD from ALS, the highest positive and lowest negative likelihood ratios (LR) were calculated for averaged SICI between 1-7 ms. A probability (P) value < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.

RESULTS

Clinical features

The clinical findings for the seven KD patients is summarised in Table 7.1. Mean age at disease onset was 47 years (range, 36-51 years). All patients were males and the mean disease duration was 10.2 ± 2.3 years. All patients reported peri-oral contraction-fasciculations, along with fasciculations in the limbs and muscle cramps. Dysarthria and dysphagia was reported by 6 patients (86%), while facial weakness was evident in 5 (71%) patient. Proximal weakness of lower limb muscles was evident in 5 (71%) while distal lower limb weakness was evident in 4 (57%) patients. Weakness of proximal upper limb muscles was noted in 3 (43%) patients, while weakness of distal upper limb muscles was evident in 4 (57%) patients. Deep tendon reflexes in the lower limbs (knee and ankle) were absent in 5 (71%) patients, while in 2 (29%) patients the deep tendon reflexes were absent in the upper limbs. Upper limb postural tremor was present in 2 (29%) patients. Although only 2 patients reported numbness and paraesthesia, the sural nerve sensory responses were absent in 5 (71%) patients and reduced in one (14%) patient. Gynaecomastia was present in 4 (57%) patients.
Peripheral nerve studies

The CMAP amplitude was reduced in KD patients compared to controls (KD, 6.8 ± 0.5 mV; controls, 10.4 ± 0.7 mV, P < 0.001), but was similar to the ALS (6.1 ± 3.6 mV) and flail arm variant ALS (5.7 ± 1.1 mV) patients in the present series. Further, the NI was reduced in KD, ALS and flail arm variant ALS patients compared to controls (ALS, 0.8 ±

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Triplet (CAG) repeat length</th>
<th>Disease duration (months)</th>
<th>UMN signs</th>
<th>Thenar muscle strength MRC</th>
<th>Peri-oral contraction-fasciculations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>46</td>
<td>168</td>
<td>Absent</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>46</td>
<td>240</td>
<td>Absent</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>46</td>
<td>84</td>
<td>Absent</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>44</td>
<td>180</td>
<td>Absent</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>44</td>
<td>60</td>
<td>Absent</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>40</td>
<td>72</td>
<td>Absent</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>45</td>
<td>48</td>
<td>Absent</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Mean</td>
<td>56.0</td>
<td>45.0</td>
<td>121.7</td>
<td></td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>4.2</td>
<td>0.4</td>
<td>27.9</td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.1. Clinical details for 7 Kennedy’s disease (KD) patients. The diagnosis of KD was confirmed by genetic testing in all cases by revealing an increase in the triplet repeat (CAG) expansion in the androgen receptor gene on the X chromosome (> 38 repeats abnormal). Hand strength was assessed using the Medical Research Council score (MRC) from the tested muscle, abductor pollicis brevis. All patients exhibited the characteristic peri-oral contraction-fasciculations. Upper motor neuron signs (UMN), such as overactive deep tendon reflexes, positive Hoffman sign or extensor plantar responses, were absent in all KD patients.
indicating that the degree of peripheral disease burden was comparable in KD and the two pathological control groups.

**Cortical excitability in KD compared to ALS**

The motor cortex was excitable in all KD patients. Central motor conduction time was not significantly different in KD patients when compared to controls (KD 5.6 ± 0.8 ms; controls 5.1 ± 0.2 ms) or ALS patients (ALS 4.9 ± 0.3 ms). Further, the resting motor threshold, defined as stimulus intensity required to produce and maintain the target MEP response of 0.2 mV, in KD patients was similar to both controls (KD 53.3 ± 6.4%; controls 60.5 ± 1.2%, P = 0.15) and ALS patients (58.1 ± 2.1%).

**Short interval intracortical inhibition**

Short interval intracortical inhibition (SICI) is defined as an increase in the test stimulus intensity required to track a constant target MEP of 0.2 mV. In normal controls it has been established that with threshold tracking TMS SICI develops between ISIs of 1-7 ms, peaking at an ISI of 3 ms (see Chapter 4). Peak SICI (at ISI 3ms) in KD patients was similar to controls (KD 10.4 ± 3.3%; controls 13.2 ± 1.4%, P = 0.2), but was significantly greater compared to ALS (ALS 3.3 ± 1.4%, P < 0.05, Fig. 7.1B). Average SICI, between ISIs of 1-7 ms, was again similar to controls (KD 6.0 ± 1.2%; controls 8.4 ± 1.1%, P = 0.08), but markedly greater when compared to ALS patients (ALS 0.7 ± 0.7%, P < 0.0001, Fig. 7.1A).
Following SICI, a period of intracortical facilitation (ICF) develops between an ISI of 10-30 ms, marked by a decrease in the test stimulus intensity required to maintain the target MEP of 0.2 mV, and peaks at an ISI 15 ms (see Chapter 4). Peak ICF was similar in KD patients compared to controls (KD -1.0 ± 0.8; controls -1.7 ± 1.3%, Fig. 7.2), but significantly smaller when compared to ALS (ALS -5.0 ± 1.2%, P < 0.01).

Figure 7.1. Short interval intracortical inhibition (SICI) is defined as the stimulus intensity required to maintain a target output of 0.2 mV (see Methods). In normal controls SICI occurs between interstimulus intervals (ISI) of 1-7 ms. (A) The peak SICI in Kennedy’s disease (KD) patients was similar to controls, but greater compared to amyotrophic lateral sclerosis (ALS) patients. (B) Averaged SICI, between ISIs 1-7 ms, in KD patients was similar to controls, but significantly greater compared to ALS patients. *P < 0.05; *** P < 0.001
The MEP amplitude, expressed as a percentage of the CMAP amplitude recorded following electrical stimulation, in KD patients was similar when compared to controls (KD 28 ± 6.0%; controls 26 ± 4.0%), but significantly reduced when compared to ALS patients (ALS, 42 ± 5.0%, P < 0.05). Further, the SR curve gradient in KD was again similar to controls, but significantly reduced when compared to ALS patients over stimulus intensities from 110 to 150% RMT (ANOVA, P < 0.01).

**Figure 7.2.** Intracortical facilitation (ICF), as indicated by a decrease in the test stimulus intensity required to maintain the target MEP of 0.2 mV, in Kennedy’s disease (KD) patients was similar to controls, but significantly smaller compared to amyotrophic lateral sclerosis patients (ALS). **P < 0.01**
Cortical Silent Period

The cortical silent period (CSP), refers to a period of electrical silence induced by a magnetic stimulus in a contracting muscle that interferes with background muscle activity. The increase in the CSP recruitment curve was non-linear in KD patients, similar to the relationship established previously for controls and ALS (see Chapter 4 and Chapter 5). The mean CSP duration increased from 2.2 ± 2.0 ms to 207.7 ± 6.1 ms, as stimulus intensity increased from 60 to 150% RMT. This increase in CSP duration in KD patients was similar to controls, but significantly longer compared to ALS (ANOVA, P < 0.001, Fig. 7.3).

![Figure 7.3](image-url)

**Figure 7.3.** The cortical silent period (CSP) duration, defined as electrical silence in a surface electromyography signal imparted by a magnetic stimulus, is measured from the onset of the motor evoked potential to the beginning of EMG activity. The CSP duration lengthens in a non-linear manner with increasing stimulus intensity. The CSP duration in Kennedy’s disease (KD) patients was similar to normal controls, but significantly longer compared to amyotrophic lateral sclerosis patients (ALS). P < 0.001
Comparing cortical excitability in KD to flail arm variant ALS

Given that ALS is a disorder of upper and lower motor neurons, comparing cortical excitability in KD patients to ALS could have introduced statistical bias, as ALS patients with upper motor neuron features would be expected to show evidence of cortical hyperexcitability. As such, cortical excitability changes in KD were compared to flail-arm variant ALS, a clinically pure lower motor neuron form of ALS (see chapter 6).

Averaged SICI was significantly increased in KD patients compared to flail arm variant ALS (-0.8 ± 0.7%, P < 0.0001). In addition, peak SICI was also significantly greater in KD patients compared to flail-arm variant ALS (-0.1 ± 4.2%, P < 0.05). Intracortical facilitation was smaller in KD patients compared to flail-arm variant ALS (-4.3 ± 2.3%, P < 0.05).

The MEP amplitude in KD patients was significantly reduced when compared to flail-arm variant ALS patients (49.9 ± 7.4%, P < 0.05). The SR curve gradient in KD was significantly reduced when compared to flail arm variant ALS patients over stimulus intensities from 110 to 150% RMT (ANOVA, P < 0.01). Further, the RMT in KD patients was similar to flail-arm variant ALS (53.3 ± 2.8%, P = 0.5). The CSP duration was longer in KD patients compared to and flail-arm variant ALS, although this difference was not significant (ANOVA, P = 0.06).
Diagnostic utility of cortical excitability testing

The utility of threshold tracking TMS to differentiate KD from ALS was assessed by calculating positive and negative likelihood ratios. The highest positive likelihood ratio of 4.4 was established for an averaged SICI of $\leq 2.5\%$, indicating that the finding of an averaged SICI value of $\leq 2.5\%$ is 4.4 times more likely to be seen in ALS as opposed to KD patients (or controls). The corresponding negative likelihood ratio was 0.45, indicating that if the averaged SICI was $\leq 2.5\%$ then the likelihood that this could be attributed to KD was reduced by 55%.

DISCUSSION

Using threshold tracking TMS techniques, the present study has established normal corticomotoneuron function in Kennedy’s disease. Specifically, short interval intracortical inhibition, cortical silent period duration, maximum MEP:CMAP ratio and the cortical stimulus-response curve gradient were statistically indistinguishable from controls. In contrast, cortical hyperexcitability was apparent in ALS and flail-arm variant ALS patients with reduction in short-interval intracortical inhibition and cortical silent period duration, along with increases in the maximum MEP:CMAP ratio and cortical stimulus-response curve gradient compared to KD. Together, these findings indicate that upper motor neuron function remains unaffected in KD, re-affirm that the presence of cortical hyperexcitability in ALS, thereby suggesting that the presence of corticomotoneuron dysfunction is a useful criterion in differentiating KD from the ALS phenotypes. The present studies might also suggest that different neurodegenerative processes are responsible for KD compared to ALS. Through differentiation of these two
neurodegenerative disorders on the basis of cortical excitability testing, threshold tracking TMS techniques may be a useful clinical neurophysiological aid.

Short-interval intracortical inhibition reflects an increase in suprathreshold test stimulus intensity required to maintain and produce a target MEP response of 0.2 mV following a subthreshold conditioning stimulus (Fisher et al., 2002) (Chapter 4). SICI is believed to be cortical in origin and mediated by GABA-secreting inhibitory cortical interneurons acting via GABA_A receptors (Ziemann et al., 1996b; Ziemann, 2004b; Ziemann, 2004a). In the present series, SICI in KD patients was similar to controls, both being significantly different to the reduction observed in ALS patients (Yokota et al., 1996; Ziemann et al., 1997c; Hanajima and Ugawa, 1998; Sommer et al., 1999; Stefan et al., 2001; Zanette et al., 2002b). The possible mechanisms mediating the reduction of SICI in ALS include loss of parvalbumin-positive inhibitory cortical interneurons (Nihei et al., 1993) and glutamate mediated down-regulation of SICI (Sommer et al., 1999; Ziemann, 2003; Ziemann, 2004b). Findings from the present series would suggest that in KD there is normal functioning of the GABA-secreting inhibitory cortical interneurons, arguing against upper motor neuron involvement. These TMS findings are in keeping with previous peristimulus time histogram studies in KD patients that established normal corticomotoneuron volleys to the anterior horn cells (Weber and Eisen, 1999; Eisen, 2001).

Some have argued that the reduction of SICI in ALS is not a primary event, but rather a compensatory down-regulation of inhibitory control over the motor cortex (M1) in order
to compensate for anterior horn cell loss (Zanette et al., 2002b). Support for this notion is provided by functional imaging studies, in the form of PET and f-MRI, which have revealed an expansion of the upper limb output zone, as indicated by greater and more widespread activation of the contralateral cerebral cortex in response to hand movement (Kew et al., 1993; Kew et al., 1994; Brooks et al., 2000a). The findings in the present study, that SICI was significantly reduced in phenotypically-matched flail arm variant ALS patients when compared to KD, would argue against the notion that SICI reduction solely represents a compensatory down-regulation of inhibitory control in response to neurodegeneration.

Recently, corticomotoneuron dysfunction was documented in Kennedy’s disease, as reflected by an increase in the amplitude of single motor units using the peristimulus time histogram technique (Attarian et al., 2006). This finding was attributed to sprouting of corticospinal axons innervating the surviving motor neurons. Given that SICI is mediated by cortical interneurons (Di Lazzaro et al., 1998c), this parameter is not sensitive in detecting abnormalities at the motor neuron level. The stimulus response curve gradient and MEP:CMAP ratio, however, reflect the density of corticomotoneuron projections onto the motor neuron (Ziemann, 2004a) and as such would be sensitive to any changes in the sprouting of corticospinal axons. In the present series, the SR curve gradient and MEP:CMAP ratio in KD patients were similar to controls arguing against significant sprouting of corticospinal axons in KD patients.
In addition to the SR curve gradient and MEP:CMAP ratio, differences in the cortical silent period duration between KD and ALS patients were established in the present series. The CSP duration is mediated by local inhibition of anterior horn cells in the early phase (Cantello et al., 1992; Inghilleri et al., 1993; Chen et al., 1999; Ziemann, 2004a) and by cortical processes, through GABA<sub>B</sub> receptors, in later segments (Connors et al., 1988; Cantello et al., 1992; Inghilleri et al., 1993; Chen et al., 1999; Ziemann, 2004a). The CSP duration in KD patients was similar to controls but significantly longer compared to ALS patients. These findings provide further evidence against upper motor neuron dysfunction, at both the cortical and spinal level, in KD patients.

**What is the mechanism of neurodegeneration in KD?**

The mechanisms underlying neurodegeneration in KD remain as yet undefined. It has been suggested that increased triplet repeat expansion (CAG) in the androgen receptor gene, which is abundantly expressed in motor neurons (Sar and Stumpf, 1977; Yu and McGinnis, 1986; Simerly et al., 1990; Menard and Harlan, 1993), may result in dysfunctional proteins, that, when translocated to the nucleus result in dysregulation of gene transcription (Gallo, 2004) and ultimately motor neuron degeneration. In ALS, there is evidence that cortical hyperexcitability underlies the development of neurodegeneration (Yokota et al., 1996; Ziemann et al., 1997c; Hanajima and Ugawa, 1998; Sommer et al., 1999; Stefan et al., 2001; Zanette et al., 2002b). Given some of the clinical similarities between KD and ALS (Desai and Swash, 2002; Kiernan, 2003), the present study established normal cortical excitability in KD, in the setting of a comparable peripheral disease burden as measured by CMAP amplitude, NI and
peripheral markers of axonal excitability, thereby arguing against a significant contribution of corticomotoneuronal dysfunction to neurodegeneration in KD.
Chapter 8

Cortical hyperexcitability precedes the
development of familial ALS
SUMMARY

To determine whether cortical hyperexcitability, established in sporadic amyotrophic lateral sclerosis (ALS) patients in Chapters 5 and 6, precedes the development of clinical features of ALS and thereby underlies motor neurodegeneration, longitudinal studies were undertaken in familial ALS (FALS). FALS is an inherited neurodegenerative disorder of the motor neurons in the motor cortex, brain stem and spinal cord, with 10-15% of cases related to mutations in the copper/zinc superoxide-dismutase-1 gene (SOD-1) gene. Although SOD-1 gene mutations result in toxic gain of function, the mechanisms underlying motor neuron loss remain unknown. Cortical hyperexcitability, mediated via the glutamatergic neurotransmitter system, has been proposed as a potential mechanism of neurodegeneration in ALS. In this chapter, novel threshold tracking TMS techniques were applied in 17 asymptomatic carriers of the SOD-1 mutation, followed longitudinally for three-years. Cortical excitability findings were compared to 50 sporadic ALS patients, 7 clinically affected FALS patients and 55 normal controls. Motor evoked and compound muscle action potentials were recorded from the right abductor pollicis brevis. There was evidence of cortical hyperexcitability in clinically affected SOD-1 FALS and sporadic ALS patients as indicated by a marked reduction in short-interval intracortical inhibition (SICI) (averaged SICI affected FALS, -1.2 ± 0.6%; SALS, 0.8 ± 1.2%) when compared to asymptomatic carriers or normal controls (asymptomatic SOD-1 mutation carriers, 9.8 ± 1.5%; controls, 8.5±1.1%, P < 0.00001). In two pre-symptomatic SOD-1 mutation carriers, assessed prior to developing ALS, SICI was absent 1-3 months before symptom development (patient 1, -3.2%; patients 2, -1.3%). In a third pre-symptomatic patient, there was a 32% reduction in SICI prior to
symptom onset. Reduction of SICI was accompanied by an increase in intracortical facilitation, motor evoked potential amplitude and magnetic stimulus-response curve gradient, all indicative of cortical hyperexcitability. The present study confirms that cortical hyperexcitability develops before the onset of clinical features of ALS, thereby suggesting that cortical hyperexcitability underlies the basis of motor neurodegeneration in familial ALS.
INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disorder of the motor neurons in the cortex, brain stem and spinal cord (Borasio and Miller, 2001; Rowland and Shneider, 2001). Degeneration of motor neurons results in progressive paresis of limb, bulbar and respiratory muscles leading to death typically in 3-5 years after symptom onset (Cudkowicz et al., 2004).

Ten percent of all ALS cases are familial (FALS), in which two or more family members are clinically affected (Andersen, 2006a). Autosomal dominant and recessive modes of inheritance have been reported and eight genetic loci have been identified in FALS to date (Andersen, 2006a). Mutations in one of these genes, the copper/zinc superoxide-dismutase-1 gene (SOD-1), results in classical ALS, called ALS 1 (Rosen et al., 1993).

The SOD-1 gene is located on chromosome 21 q22.1, spans 11 kilobases of genomic DNA, comprises five exons and four introns (Levanon et al., 1985). The main function of the SOD-1 enzyme, which constitutes 0.5-1% of soluble protein in the brain and spinal cord, is in free radical scavenging whereby the enzyme catalyses the conversion of the superoxide anion to molecular oxygen and hydrogen peroxide, which in turn is reduced to water by glutathione peroxidase and catalase (Fridovich, 1986). To date over 122 different SOD-1 mutations have been reported in FALS, the majority being missense mutations with autosomal dominant mode of inheritance evident in most mutations (Andersen, 2006a; Gros-Louis et al., 2006).
Mutations in the SOD-1 gene result in acquisition of new cytotoxic activity by the SOD-1 enzyme, referred to as a “toxic gain of function” (Andersen, 2006b). Support for such a process is provided by the transgenic SOD-1 mouse model. In this model, SOD-1 gene “knock out” mice do not develop ALS (Reaume et al., 1996) while mice overexpressing the mutated SOD-1 gene develop an ALS-like syndrome despite normal or elevated SOD-1 enzyme activity (Wong et al., 1995). Although the mechanisms by which the SOD-1 enzyme overactivity results in neurodegeneration remains as yet undefined, there is increasing evidence of glutamate-mediated excitotoxicity from the transgenic SOD-1 mouse model (Van Den Bosch et al., 2006). Specifically, impaired glutamate clearance and increased glutamate concentration within the motor cortex have been reported in the SOD-1 (G93A) mice (Roy et al., 1998). The presence of SOD-1 mutations also increases motor neuron sensitivity to glutamatergic excitotoxicity by post-synaptic Ca\(^{2+}\)-dependent mechanisms (Van Den Bosch et al., 2006). Of further relevance, SOD-1 mutations may result in mitochondrial dysfunction, further increasing the susceptibility of motor neurons to glutamate mediated cell death (Hand and Rouleau, 2002).

Glutamate, the principle excitatory neurotransmitter in the brain, exerts its action in part by binding to ionotrophic receptors and leading to an influx of Ca\(^{2+}\) into cells (Hand and Rouleau, 2002; Stys, 2005). Excessive glutamate activity results in an increase in intracellular Ca\(^{2+}\) concentration and thereby activation of Ca\(^{2+}\)-dependent enzyme systems leading to neurodegeneration (Stys, 2005). Of clinical relevance, cortical excitability may be assessed by transcranial magnetic stimulation (TMS) techniques (Kujirai et al., 1993) (see Chapter 4). TMS studies in sporadic ALS patients have
provided evidence for increased excitability early in the course of disease (Eisen et al., 1993; Prout and Eisen, 1994; Mills and Nithi, 1997) (see Chapter 5). Conversely, in SOD-1 FALS patients the TMS findings have been contradictory, with some reporting cortical hyperexcitability (Weber et al., 2000b; Turner et al., 2005b), while others have reported normal cortical excitability (Stewart et al., 2006).

One method to clarify these discordant findings in FALS, while at the same time to further investigate the site of onset of ALS, would be to undertake longitudinal studies in asymptomatic carriers of the SOD-1 mutation and thereby chart development of the disease. Consequently, to determine whether cortical hyperexcitability precedes the development of clinical symptoms, the present study employed novel threshold tracking TMS techniques to assess longitudinal changes in asymptomatic SOD-1 mutation carriers, with the results compared to FALS patients manifesting the disease and to sporadic ALS patients.

**METHODS**

**Patients**

Subjects were recruited from the SOD-1 database at the Molecular Genetic Laboratory, Concord Hospital and from the Multidisciplinary ALS clinic at Prince of Wales Hospital (Sydney, Australia). Seventeen asymptomatic SOD-1 mutation carriers (7 male, 10 female: age range 23-60 years, mean age: 40 years) were followed longitudinally for three years, in addition to 7 clinically definite familial ALS patients as per the revised El Escorial criteria (Brooks et al., 2000b) who also expressed SOD-1 mutation (4 males, 3
females: age range 25-70 years, mean age 47 years). Families with three different SOD-1 mutations were studied including: Valine to Glycine mutation in exon 5 at codon position 148 (V148G); Isoleucine to Threonine mutation in exon 4 at codon position 113 (I113T); Glutamic acid to Glycine mutation in exon 4 at codon position 100 (E100G). Fifty sporadic ALS patients were also studied (32 males, 18 females: age range 26-78, mean age 58 years).

ALS patients were clinically reviewed on a regular basis through the multidisciplinary ALS clinic at Prince of Wales Hospital. This incorporated assessment using the ALS function rating scale revised (ALSFRS-R) (Cedarbaum et al., 1999). Strength was assessed using the Medical Research Council (MRC) rating scale (Medical Research Council, 1976), and a separate hand function score was also recorded (Triggs et al., 1999). Asymptomatic SOD-1 mutation carriers also underwent clinical examination at the time of formal neurophysiological assessment. The results of cortical excitability for familial and sporadic ALS patients were compared to 55 healthy controls (28 men; 27 women, aged 23-83 years, mean: 47 years).

**Peripheral nerve testing**

The median nerve was stimulated electrically at the wrist in all studies. The resultant CMAP was recorded from the abductor pollicis brevis (APB) and was measured from baseline to negative peak (see Methodology). From peripheral nerve conduction studies, the NI was derived according to a previously reported formula (de Carvalho and Swash, 2000) (see Chapter 5).
Cortical excitability

Cortical excitability was assessed by applying TMS to the motor cortex by means of a 90 mm circular coil oriented to induce current flow in a posterior-anterior direction (see Methodology). Using the threshold tracking paired-pulse TMS techniques the following parameters of excitability were determined; RMT, magnetic SR curve, central motor conduction time, short interval intracortical inhibition, intracortical facilitation and cortical silent period (see Methodology).

Statistical analysis

Student’s t-test was used to compare mean differences between groups, and analysis of variance (ANOVA) was used for multiple comparisons. A probability (P) value of < 0.05 was considered statistically significant. Results are expressed as mean ± standard error of the mean.

RESULTS

The clinical and genetic features for 17 asymptomatic SOD-1 mutation carriers and 7 clinically affected FALS patients are summarized in Table 8.1. The asymptomatic SOD-1 mutation carriers underwent annual TMS testing at which time they were clinically reviewed by the same examiner. The physical examination in all the asymptomatic SOD-1 mutation carriers, including the three pre-symptomatic carriers was normal. Specifically, there were no upper motor neuron features, such as increased muscle tone, hyper-reflexia, extensor plantar responses, positive Hoffman sign or the presence of a jaw-jerk in any of the subjects at the time of testing. Three SOD-1 mutation carrier
subjects were pre-symptomatic, i.e. asymptomatic at the time of TMS, and their case histories are outlined.

**Case histories**

**Case # 1 (Table 1)** is a 43-year old male who underwent initial threshold tracking TMS in June 2005. At the time of testing, the subject was asymptomatic and formal neurological examination was normal. Further, the CMAP amplitude (9.7 mV) and neurophysiological index (2.2) were within normal limits. Although surface EMG, including testing over the target muscle was unremarkable, formal needle EMG testing was not undertaken at time of cortical excitability testing. In September of 2005, this subject developed fasciculations, muscle cramps and weakness of the right foot and hip. Subsequently, he developed wasting of the muscles in the right leg, including calf, anterior leg and thigh muscles. By December 2006, he developed fasciculations in the proximal upper limb muscles along with weakness of the shoulder girdle muscles. Neurophysiological testing in September 2005, established features of denervation in the lower limbs accompanied by fasciculations.

**Case # 2 (Table 1)** is a 34 year-old male who underwent threshold tracking TMS testing in June 2006. Subsequently, he reported generalized fasciculations and difficulty running. Although the neurological examination was normal at the time of TMS testing, re-assessment at the time of symptom onset disclosed widespread fasciculations with a generalized hyperreflexia. At the time of cortical excitability testing, the CMAP amplitude (9.1 mV) and NI (2.3) were unremarkable. Formal needle EMG testing was
not undertaken at the time of cortical excitability testing. However, subsequent needle EMG testing disclosed fasciculations of complex morphology, with associated neurogenic abnormalities.

**Case # 3 (Table 1)** is a 41 year-old female who underwent TMS testing in May 2005 and April 2006. Peripheral neurophysiological testing, in the form of CMAP amplitude and NI, in May 2005 (CMAP amplitude, 13 mV; NI, 3.3) and April 2006 (CMAP amplitude, 12.8 mV; NI, 3.0) were normal, as was surface EMG sampling. In December 2006, the subject reported lower limb weakness manifesting as difficulty with walking and standing her toes. By late January 2007, the subject developed exertional dyspnea developed, left hand weakness (MRC grade 4, see Table 1) and generalized fasciculations were evident. Neurophysiological testing, nerve conduction studies and needle electromyography (EMG), disclosed widespread neurogenic changes consistent with ALS. The subject underwent further TMS testing in April 2007. Currently, the patient is wheelchair-dependent, with global weakness, and requires nocturnal pressure support ventilation.

**Peripheral nerve studies**

The CMAP amplitude (symptomatic FALS 6.1 ± 1.6 mV; sporadic ALS 5.9 ± 0.6 mV; controls 10.3 ± 0.5 mV, P < 0.001) and NI (symptomatic FALS 1.3 ± 0.4; sporadic ALS, 0.8 ± 0.1; controls 2.6 ± 0.2, P < 0.005), both quantitative markers of peripheral disease burden, were significantly reduced in symptomatic FALS and sporadic ALS patients compared to controls.
### Table 8.1.

Clinical details for the 17 asymptomatic superoxide dismutase-1 (SOD-1) mutation carriers and 7 clinically affected FALS patients with SOD-1 mutation. Three different SOD-1 mutations were present in the patients studied; GTA to GGA sequence change resulting in valine to glycine substitution in exon 5 at the 148 position (V148G), GAA to GGA sequence change resulting in a glutamic acid to glycine substitution in exon 4 at the 100 position (E100G), and ATT to ACT sequence change resulting in an isoleucine to threonine substitution in exon 4 at the 113 position (I113T). Site of disease onset was upper limb (UL), lower limb (LL) or bulbar. Disease duration refers to the period from symptom onset to date of testing. Time to ALS onset refers to development of clinical features of ALS after last testing. Three SOD-1 mutation carriers developed ALS after cortical excitability testing (*). The patients were graded using the ALS functional rating scale revised (ALSFRS-R). Muscle strength was assessed using the Medical Research Council (MRC) score for the abductor pollicis brevis.

<table>
<thead>
<tr>
<th>Asymptomatic SOD-1</th>
<th>Age (years)/Sex</th>
<th>SOD-1 mutation</th>
<th>ALS onset</th>
<th>Time to ALS onset (months)</th>
<th>ALSFRS-R</th>
<th>Triggs hand score</th>
<th>MRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>43,M</td>
<td>V148G</td>
<td>UL/LL</td>
<td>3</td>
<td>46</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2*</td>
<td>34,M</td>
<td>E100G</td>
<td>UL/LL</td>
<td>3</td>
<td>47</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>3*</td>
<td>41,F</td>
<td>V148G</td>
<td>UL/LL</td>
<td>8</td>
<td>44</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>25,F</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60,F</td>
<td>I113T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>47,F</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>37,M</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25,F</td>
<td>I113T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>44,F</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>43,F</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>49,M</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>23,F</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>45,F</td>
<td>E100G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>57,M</td>
<td>I113T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>31,M</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>54,M</td>
<td>I113T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>31,F</td>
<td>V148G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>4.7</td>
<td>47.6</td>
<td>0</td>
<td>4.9</td>
</tr>
<tr>
<td>SEM</td>
<td>3.1</td>
<td></td>
<td></td>
<td>1.7</td>
<td>0.3</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Symptomatic SOD1 patients</th>
<th>SOD-1 mutation</th>
<th>ALS onset</th>
<th>Disease duration (months)</th>
<th>ALSFRS-R</th>
<th>Triggs hand score</th>
<th>MRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>70,F</td>
<td>I113T</td>
<td>UL</td>
<td>9</td>
<td>41</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>35,M</td>
<td>V148B</td>
<td>LL</td>
<td>33</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>49,F</td>
<td>E100G</td>
<td>LL</td>
<td>14</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>44,M</td>
<td>V148G</td>
<td>LL</td>
<td>6</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>53,M</td>
<td>I113T</td>
<td>UL</td>
<td>7</td>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>23</td>
<td>50,M</td>
<td>V148G</td>
<td>LL</td>
<td>13</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>25,F</td>
<td>V148G</td>
<td>LL</td>
<td>1.5</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>11.9</td>
<td>42.4</td>
<td>0.6</td>
</tr>
<tr>
<td>SEM</td>
<td>5.4</td>
<td></td>
<td></td>
<td>3.9</td>
<td>1.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 8.1. Clinical details for the 17 asymptomatic superoxide dismutase-1 (SOD-1) mutation carriers and 7 clinically affected FALS patients with SOD-1 mutation. Three different SOD-1 mutations were present in the patients studied; GTA to GGA sequence change resulting in valine to glycine substitution in exon 5 at the 148 position (V148G), GAA to GGA sequence change resulting in a glutamic acid to glycine substitution in exon 4 at the 100 position (E100G), and ATT to ACT sequence change resulting in an isoleucine to threonine substitution in exon 4 at the 113 position (I113T). Site of disease onset was upper limb (UL), lower limb (LL) or bulbar. Disease duration refers to the period from symptom onset to date of testing. Time to ALS onset refers to development of clinical features of ALS after last testing. Three SOD-1 mutation carriers developed ALS after cortical excitability testing (*). The patients were graded using the ALS functional rating scale revised (ALSFRS-R). Muscle strength was assessed using the Medical Research Council (MRC) score for the abductor pollicis brevis.
There were no differences in the CMAP amplitude (asymptomatic SOD-1 mutation carriers, 9.9 ± 0.8 mV; controls 10.3 ± 0.5 mV) and neurophysiological index (asymptomatic SOD-1 mutation carriers, 2.3 ± 0.3; controls 2.6 ± 0.2) between asymptomatic SOD-1 mutation carriers and normal controls. Central motor conduction time was not significantly different in FALS and sporadic ALS patients compared to asymptomatic SOD-1 mutation carriers and controls (symptomatic FALS 5.0 ± 0.5 ms; ALS 5.1 ± 0.3 ms; asymptomatic SOD-1 mutation carriers 5.2 ± 0.4 ms; controls 5.1 ± 0.2 ms).

Cortical excitability

The full sequence of cortical excitability was well tolerated and successfully completed in all asymptomatic SOD-1 mutation carriers, FALS and sporadic ALS patients. Short-interval intracortical inhibition, as reflected by an increase in the conditioned stimulus intensity required to track a constant target MEP of 0.2 mV, was significantly reduced in symptomatic FALS (-1.2 ± 0.6%) and sporadic ALS (0.8 ± 1.2%) patients compared to asymptomatic SOD-1 mutation carriers (9.8 ± 1.5%) and controls (8.5 ± 1.1%, P < 0.001, Fig. 8.1A, B). In two pre-symptomatic SOD-1 mutation carriers who developed clinical features of ALS within 3-months of assessment of cortical excitability, short interval intracortical inhibition disappeared (patient #1, -3.2%; patient #2, -1.3%, Table 1, Fig. 8.2A), in sharp contrast to the remaining asymptomatic SOD-1 mutation carriers (Fig. 8.3). In one pre-symptomatic SOD-1 mutation carrier (patient #3), there was a 32% reduction in SICI prior to the development of clinical features of ALS and SICI was reduced in the motor cortex contralateral to side of development of muscle weakness.
As part of a subsequent diagnostic work-up, this patient underwent a muscle biopsy which revealed the presence of groups of atrophic muscle fibers with mixed-type fibers, a finding distinctive of motor neuron disease (Fig. 8.2D) (Baloh et al., 2007).

**Figure 8.1.** (A) Short interval intracortical inhibition (SICI), defined as the stimulus intensity required to maintain a target output of 0.2 mV (see Methods) was reduced in clinically affected familial amyotrophic lateral sclerosis (FALS) and sporadic ALS patients when compared to normal controls (P < 0.001). (B) Averaged SICI, between interstimulus interval (ISI) 1-7 ms was also reduced in FALS and sporadic ALS patients compared to asymptomatic superoxide dismutase-1 (SOD-1) mutation carriers and controls. ***P < 0.001.
Short-interval intracortical inhibition is followed by a period of intracortical facilitation (ICF), marked by a decrease in the test stimulus intensity required to maintain the target MEP of 0.2 mV. ICF was increased in symptomatic FALS (-2.5 ± 0.7%) and sporadic ALS patients (-3.0 ± 1.2%) when compared to asymptomatic SOD-1 mutation carriers.
(-0.1 ± 0.5%) and controls (-1 ± 0.3%) over a period of testing from 10-30 ms (Fig. 8.4A, ANOVA P < 0.05). ICF increased in two pre-symptomatic SOD-1 mutation carriers prior to the development of ALS (patient #1, -9.3%; patient #2, -6.1%) compared to remaining asymptomatic SOD-1 mutation carriers and controls (Fig. 8.4B). In the third pre-symptomatic SOD-1 mutation carrier (patient #3), ICF again increased in the motor cortex contralateral to the side of muscle weakness (Fig 8.4C, D).

**Figure 8.3.** Averaged short interval intracortical inhibition (SICI) is depicted for two pre-symptomatic subjects (case #1 and #2, from table 1) and 14 asymptomatic superoxide dismutase-1 (SOD-1) mutation carriers (subjects 4-17, table 8.1). Averaged SICI was absent in two pre-symptomatic subjects, but was present in all the asymptomatic SOD-1 mutation carriers. The numbers on the x-axis, correspond to the number ascribed to each subject in table 1. The data from the third pre-symptomatic subject (subject #3, table 1) is depicted separately in Fig. 8.2B.
Another measure of cortical excitability, the MEP amplitude which is expressed as a percentage of the CMAP response, reflects the density of corticospinal projection onto the motor neuron. The MEP amplitude was increased in both clinically affected FALS patients and sporadic ALS patients compared to asymptomatic SOD-1 mutation carriers and controls (symptomatic FALS 42.8 ± 13.0%; ALS 37.7 ± 4.5%; asymptomatic SOD-1 mutation carriers 20.6 ± 4.2%; controls 26.2 ± 2.4%, P < 0.05). The stimulus-response

Figure 8.4. (A) Intracortical facilitation (ICF), which develops at interstimulus intervals (ISI) of 10-30 ms, was increased in clinically affected familial amyotrophic lateral sclerosis (FALS) and sporadic amyotrophic lateral sclerosis (SALS) patients compared to asymptomatic SOD-1 mutation carriers and controls. (B) In two pre-symptomatic superoxide dismutase-1 (SOD-1) mutation carriers (patient #1 and #2), ICF was increased compared to the remaining asymptomatic superoxide dismutase-1 (SOD-1) mutation carriers and controls prior to development of clinical features of ALS. (C) In a further pre-symptomatic SOD-1 mutation carrier (patient #3), ICF increased prior to the development of ALS. (D) Intracortical facilitation was increased in the right motor cortex, which was contralateral to the side of development of muscle weakness. *P < 0.05
curve gradient, where the MEP amplitude was expressed as a percentage of the CMAP amplitude recorded following electrical stimulation, was steeper in symptomatic FALS patients compared to asymptomatic SOD-1 mutation carriers and controls (ANOVA P < 0.05, Fig. 8.5).

![Stimulus intensity (% RMT) vs. MEP amplitude (%CMAP)](image)

**Figure 8.5.** The motor evoked potentials (MEP) amplitude and the gradient of the stimulus-response curve was increased in clinically affected familial amyotrophic lateral sclerosis (FALS) and sporadic ALS (SALS) patients when compared to asymptomatic superoxide dismutase-1 (SOD-1) mutation carriers and normal controls (ANOVA, P < 0.05).

Resting motor threshold, defined as the unconditioned stimulus intensity required to produce and maintain a target MEP response, was not significantly different between the groups (symptomatic FALS 59.2 ± 5.1%; sporadic ALS 57.9 ± 1.6%; asymptomatic SOD-1 mutation carriers, 59.4 ± 2.2%; controls, 60.6 ± 1.3%).
Changes in the cortical silent period (CSP), defined as a period of electrical silence that interferes with ongoing EMG activity following an MEP in a contracting muscle, was also measured. In the present series, the increase in the CSP recruitment curve were non-linear in FALS patients similar to the relationship established previously for control subjects (see Chapter 4). The mean CSP duration increased from 0 to 199.6 ± 6.2 ms in affected FALS and from 0 to 208.9 ± 4.2 ms in asymptomatic SOD-1 mutation carriers as stimulus intensity increased from 60 to 150% RMT, and was not significantly different when compared to controls (0 to 209.0 ± 4.7 ms). However in three pre-symptomatic SOD-1 mutation carriers, CSP duration was reduced compared to controls and other asymptomatic SOD-1 mutation carriers, but comparable to sporadic ALS patients, prior to the development of clinical features of ALS (patient #1 CSP duration, 0-180 ms; patient #2 0-192 ms; patient #3 0-141 ms; sporadic ALS 0-177 ± 8.5 ms).

DISCUSSION

The present longitudinal studies using novel threshold tracking TMS techniques to explore cortical excitability in familial SOD-1 positive ALS and sporadic ALS patients, have established the presence of cortical hyperexcitability in both symptomatic FALS and sporadic ALS patients. Reduction in short-interval intracortical inhibition, associated with increases in intracortical facilitation and cortical stimulus-response curve gradient were all indicative of cortical hyperexcitability. In contrast, in asymptomatic SOD-1 mutation carriers cortical excitability was normal. However, an increase in cortical excitability in three pre-symptomatic SOD-1 mutation carriers preceded the development
of clinical features of ALS. Together, these findings confirm that cortical hyperexcitability develops prior to clinical symptoms in FALS and suggests that cortical hyperexcitability underlies the development of neurodegeneration in FALS.

**Where does ALS begin?**

Given that cortical hyperexcitability precedes the development of clinical features of ALS, at least in SOD-1 mutation carriers, it would suggest that the initial abnormality in ALS occurs within the corticomotoneurons. Such findings would be in keeping with flumazenil PET studies that demonstrated cortical abnormalities in two pre-symptomatic SOD1 patients (Turner et al., 2005a), and with studies in a SOD-1 (G93A) mouse model suggesting that degeneration of spinal cord motor neurons is secondary to dysfunction within CNS motor pathways, i.e. within the primary motor cortex, corticospinal tract and bulbospinal pathways (Browne et al., 2006).

Evidence for cortical hyperexcitability in the present study is provided by a significant reduction of SICI in both clinically affected SOD-1 and sporadic ALS patients, and in longitudinal studies in three pre-symptomatic SOD-1 mutation carriers who contracted ALS within months of developing cortical hyperexcitability. The mechanisms underlying reduction of SICI are complex and involve both degeneration of GABA secreting inhibitory cortical interneurons and glutamate mediated down-regulation of SICI (Nihei et al., 1993; Stefan et al., 2001). In addition, dysfunction of GABAergic synapses, whereby they become excitatory, may be another mechanism underlying the reduction of SICI (Ben-Ari et al., 2004). Specifically, in the neonatal brain, GABAergic
synapses are excitatory. Conversion from excitatory to inhibitory transmission is dependent on the establishment of the chloride electrochemical gradient by expression of a specific K⁺/Cl⁻ co transporter (KCC2) on post-synaptic membranes (Ben-Ari et al., 2004). Studies assessing the function of KCC2 in FALS patients may yet confirm this potential mechanism of SICI reduction.

Previous cortical excitability studies in familial SOD-1 positive ALS patients have reported contradictory findings (Weber et al., 2000b; Turner et al., 2005b; Stewart et al., 2006). While normal cortical excitability was reported in patients with the D90A (Weber et al., 2000b; Turner et al., 2005b) and I113T mutations (Stewart et al., 2006), abnormalities of corticomotoneuronal function have been recently reported in patients with the A4V mutations (Stewart et al., 2006). The present findings establish the presence of cortical hyperexcitability in patients with the V148G, E100G, and I113T SOD-1 mutations, in keeping with the findings in the A4V mutation. The homozygous D90A mutation is associated with a slowly progressive phenotype, probably related to co-inheritance of protective or modifying genes with the D90A alleles (Simpson and Al-Chalabi, 2006). The inheritance of these protective or modifying genes may have been responsible for normal cortical hyperexcitability in the D90A SOD-1 positive patients.

If other investigators reproduce the present study, the findings will also have implications for the diagnosis of ALS. At present, there are no diagnostic tests for ALS, with the diagnosis relying on clinical criteria that are too restrictive and lack sensitivity, particularly in the early stages of disease when patients could benefit most from
therapeutic intervention (Winhammar et al., 2005). Earlier treatment with neuroprotective agents, such as the anti-glutamatergic agent riluzole, may result in a slower disease progression and prolonged survival, particularly given contemporary changes recently documented in ALS outcomes (Czaplinski et al., 2006).
SUMMARY

AND

CONCLUSIONS
The studies comprising the present thesis have explored the pathophysiological mechanisms underlying neurodegeneration in amyotrophic lateral sclerosis (ALS). A novel threshold tracking TMS technique, combined with axonal excitability studies, were applied to normal controls and ALS patients. These studies demonstrated that cortical hyperexcitability was an early and prominent feature in sporadic ALS and preceded the development of clinical features in familial ALS (FALS), thereby suggesting that cortical hyperexcitability underlies the neurodegeneration evident in ALS. Studies in Kennedy’s disease (KD) patients, a pathological control group that clinically resembles ALS, confirmed the presence of normal cortical excitability, thereby suggesting that cortical hyperexcitability is a primary event in ALS, rather than a compensatory down-regulation of cortical inhibitory processes in response to motor neuron loss.

In chapter 1, axolemmal ion channel function was assessed in ALS patients, using a previously reported threshold tracking protocol that measures multiple indices of axonal excitability (Kiernan et al., 2000). These studies demonstrated an increase in strength-duration time constant (τSD), coupled with abnormalities of threshold electrotonus characterized by greater threshold change to both depolarizing and hyperpolarizing conditioning stimuli, similar to the “fanned out” appearance that occurs with membrane hyperpolarization. Further, there was a shift of the stimulus–response curves in a hyperpolarizing direction along with increased superexcitability. Together, the changes were consistent with widespread dysfunction in axonal ion channel conduction, including increased persistent Na⁺ channel conduction, and abnormalities of fast paranodal K⁺ and internodal slow K⁺ channel function. It was postulated that such changes in axonal
excitability might predispose axons of ALS patients to generation of fasciculations and cramps. Furthermore, these changes in axonal excitability provided insight into mechanisms responsible for motor neuron loss in ALS.

In chapter 2, axonal excitability studies were applied to patients with KD in order to investigate whether the mechanisms underlying fasciculations in KD were similar to ALS. The axonal excitability studies revealed an increase in $\tau_{SD}$, which correlated with the compound muscle action potential (CMAP) amplitude and the fasciculation frequency. The increase in $\tau_{SD}$ was accompanied by an increase the hyperpolarizing current/threshold gradient, greater changes in threshold electrotonus in response to a sub-threshold depolarizing current and changes in refractoriness, superexcitability and late subexcitability, all consistent with axonal hyperpolarization. Together, the axonal excitability changes in KD suggested that upregulation of persistent Na$^+$ conductances is the primary event, contributing to the development axonal hyperexcitability in KD and generation of fasciculations, while the alterations in other excitability parameters were probably secondary to ectopic activity.

While studies in chapter 1 demonstrated significant abnormalities of axonal excitability in ALS patients, the mechanisms underlying fatigue, a major symptom in ALS, remained unclear with dysfunction of central and peripheral nervous systems independently reported as contributing factors. Further, it had also been suggested that reduced function of the Na$^+/\text{K}^+$ pump may in part be responsible for motor neurone loss in ALS. This possibility was investigated by recording the excitability changes that occurred following
a maximum voluntary contraction. Activity resulted in a significantly greater increase of threshold in ALS patients compared to controls that was associated with reduced $\tau_{so}$ and increased superexcitability, consistent with axonal hyperpolarisation, arguing against any significant dysfunction in the axonal $\text{Na}^+/\text{K}^+$ pump in ALS. The increase in threshold was more pronounced in ALS patients with predominantly lower motor neuronal involvement. Together, these findings suggested that higher firing rates of surviving motor axons attempting to compensate for neurogenic weakness was a likely explanation for the greater activity-dependent changes in ALS and that dysfunction of the peripheral nervous system was a significant contributing factor in the development of fatigue in ALS.

In chapter 4, the threshold tracking was adapted for assessing cortical excitability using the paired-pulse transcranial magnetic stimulation (TMS) technique. These studies demonstrated that the threshold tracking protocol, previously validated in electrical studies of peripheral axons in normal controls and in the investigation of disease pathophysiology, could be successfully applied to study cortical excitability. Normative data were established with recordings over the abductor pollicis brevis muscle, and the technique appeared to be a reliable and reproducible measure of cortical excitability.

Subsequently, the threshold tracking TMS technique was applied to sporadic ALS patients (Chapter 5) and to patients with a pure lower motor neuron form ALS (Chapter 6), called flail-arm variant ALS. In sporadic ALS patients, cortical hyperexcitability was evident with reductions in the resting motor threshold, short-interval intracortical
inhibition and cortical silent period duration, accompanied by increases in the maximum
MEP:CMAP ratio and in the magnetic stimulus-response curve gradient. The increase in
cortical excitability correlated with traditional measures of peripheral nerve function such
as the CMAP amplitude and neurophysiological index, and with axonal excitability
parameters, including the $\tau_{SD}$. Together, these findings confirmed co-existent
dysfunction of upper and lower motor neuron systems in ALS patients and suggested that
cortical hyperexcitability was an early feature in ALS and possibly underlying
neurodegeneration in ALS.

In patients with the flail arm variant ALS (Chapter 6), threshold tracking TMS techniques
confirmed the presence of cortical hyperexcitability as indicated by reduction in resting
motor threshold and short-interval intracortical inhibition, together with increases in the
MEP amplitude and magnetic stimulus-response curve gradient, despite the absence of
clinical upper motor neuron signs. These changes in cortical excitability were similar to
those reported in ALS, providing support to the hypothesis that the flail-arm syndrome is
a variant of ALS. The notion that the flail-arm syndrome is a variant of ALS may
influence the design of future ALS drug trials. Specifically, given the natural history of
flail-arm syndrome patients, the inclusion of flail-arm patients in therapeutic clinical
trials may influence outcome measures in terms of disease survival. As such, ALS trials
should apportion flail-arm patients equally to each intervention arm, and the outcome
results should be analyzed separately from the more typical ALS patients.
Some have argued that the reduction of SICI in ALS is not a primary event, but rather a compensatory down-regulation of inhibitory control over the motor cortex in response to anterior horn cell loss. Therefore, in chapter 7 threshold tracking TMS studies were applied to KD patients, an inherited disorder of the motor neurons and sensory axons that clinically resembles ALS. These studies established normal cortical excitability in KD as indicated by the fact that short interval intracortical inhibition, cortical silent period duration, maximum MEP:CMAP ratio and the magnetic stimulus-response curve gradient were statistically indistinguishable from controls. The KD patients could be clearly distinguished from both ALS and flail arm variant ALS patients, on the basis of cortical excitability findings, thereby suggesting that the presence of cortical hyperexcitability may be a useful criterion in differentiating KD from ALS. Further, given the comparable peripheral disease burden in KD and ALS patients, as measured by the CMAP amplitude and NI recorded over the target muscle, it appeared that cortical hyperexcitability is a primary event rather than a compensatory down-regulation in response to motor neuron loss.

In order to determine whether cortical hyperexcitability, established in sporadic ALS patients in Chapters 5 and 6, preceded the development of clinical features of ALS and thereby was responsible for motor neurodegeneration, longitudinal studies were undertaken in a group of familial ALS (FALS) patients caused by mutations in the copper/zinc superoxide-dismutase-1 gene (SOD-1) gene. In chapter 8, threshold tracking TMS studies were applied to clinically affected FALS patients and to asymptomatic carriers of the SOD-1 mutation. There was evidence of cortical hyperexcitability in
clinically affected SOD-1 FALS patients, comparable to findings in sporadic ALS patients described in Chapter 5 and 6, as indicated by a marked reduction in SICI along with increases in intracortical facilitation, motor evoked potential amplitude and magnetic stimulus-response curve gradient. In asymptomatic SOD-1 mutation carrier subjects, longitudinal studies established that there was absence of SICI in two pre-symptomatic SOD-1 mutation carriers and a 32% reduction of SICI in a third pre-symptomatic subject prior to symptom onset. These findings confirmed that cortical hyperexcitability developed before the onset of clinical features of ALS, thereby suggesting that cortical hyperexcitability underlies motor neurone degeneration in familial ALS.

In conclusion, the findings in the present thesis suggest that cortical hyperexcitability develops prior to the onset of clinical features of FALS, consistent with the hypothesis that ALS is initially a disorder of corticomotoneurons, with cortical hyperexcitability underlying the ensuing motor neuron degeneration. Further, the present thesis suggests that the threshold tracking TMS technique may become a useful neurodiagnostic investigation in establishing an early diagnosis of ALS.

**Perspectives for future research**

Some epidemiological studies have reported an increased incidence of ALS amongst high performance athletes, such as soccer players (Chio et al., 2005; Vanacore et al., 2006; Taioli, 2007; Wicks et al., 2007). The mechanisms underlying this increased incidence of ALS in high performance sportsmen remain unclear. Recently, significant changes in
cortical inhibitory processes have been reported during and after fatiguing exercise (Benwell et al., 2006; Benwell et al., 2007). Future studies will measure changes in cortical excitability during and after fatiguing exercise in ALS patients. Furthermore, cortical excitability will be assessed in other high performance athletes. Together, these studies may shed light on whether cortical hyperexcitability underlies the development of neurodegeneration in high performance athletes.

The present thesis has established that cortical excitability is an early feature in sporadic ALS (Chapter 5), preceding the onset of clinical features in FALS (Chapter 8). Some longitudinal studies, however, have suggested that cortical hyperexcitability develops with disease progression (Zanette et al., 2002a). In order to address this issue, longitudinal studies will be undertaken in sporadic ALS patients to document the nature of cortical excitability changes with disease progression. Clarification of this issue will bear importance for disease pathogenesis and may have diagnostic and therapeutic implications.

Although the increase in cortical excitability was a uniform finding in ALS patients (Chapter 5, 6 and 8), this was most prominent in ALS patients with limb-onset disease. While it remains possible that cortical hyperexcitability is less prominent in ALS patients with bulbar-onset disease, more likely the difference lies in the choice of target muscles, as previously reported (Desiato et al., 2002). Future studies will be undertaken from both limb and bulbar muscles in ALS patients to address this issue.
# Glossary of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>Activity-dependent hyperpolarisation</td>
</tr>
<tr>
<td>AHC</td>
<td>Anterior horn cell</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ALSFRS-R</td>
<td>Amyotrophic lateral sclerosis functional rating scale-revised</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Aminopyridine</td>
</tr>
<tr>
<td>APB</td>
<td>Abductor pollicis brevis</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Brachial amyotrophic diplegia</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cl$^{-}$</td>
<td>Chloride</td>
</tr>
<tr>
<td>CMAP</td>
<td>Compound muscle action potential</td>
</tr>
<tr>
<td>CMCT</td>
<td>Central motor conduction time</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSP</td>
<td>Cortical silent period</td>
</tr>
<tr>
<td>D-waves</td>
<td>Direct waves</td>
</tr>
<tr>
<td>DAP</td>
<td>Depolarising afterdepolarization</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAAT2</td>
<td>Excitatory amino acid transporter type 2</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation contraction coupling</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>FALS</td>
<td>Familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Gamma amino butyric acid receptor type A</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;</td>
<td>Gamma amino butyric acid receptor type B</td>
</tr>
<tr>
<td>GluR2</td>
<td>Glutamate receptor type 2</td>
</tr>
<tr>
<td>I-waves</td>
<td>Indirect waves</td>
</tr>
<tr>
<td>I</td>
<td>Intermediate channel</td>
</tr>
<tr>
<td>ICF</td>
<td>Intracortical facilitation</td>
</tr>
<tr>
<td>I&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Inward rectifying current</td>
</tr>
<tr>
<td>I&lt;sub&gt;Kf1&lt;/sub&gt;</td>
<td>Fast potassium current type 1</td>
</tr>
<tr>
<td>I&lt;sub&gt;Kf2&lt;/sub&gt;</td>
<td>Fast potassium current type 2</td>
</tr>
<tr>
<td>I&lt;sub&gt;NaP&lt;/sub&gt;</td>
<td>Persistent sodium current</td>
</tr>
<tr>
<td>ITPR2</td>
<td>Inositol 1,4,5-triphosphate receptor 2</td>
</tr>
<tr>
<td>I/V</td>
<td>Current/threshold relationship</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Potassium</td>
</tr>
<tr>
<td>KCC2</td>
<td>Potassium chloride co transporter type 2</td>
</tr>
<tr>
<td>KD</td>
<td>Kennedy’s disease</td>
</tr>
<tr>
<td>LL</td>
<td>Lower limb</td>
</tr>
<tr>
<td>LMN</td>
<td>Lower motor neuron</td>
</tr>
<tr>
<td>MEP</td>
<td>Motor evoked potential</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MFI</td>
<td>Multidimensional fatigue inventory</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>MND</td>
<td>Motor neurone disease</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>MSO</td>
<td>Maximum stimulator output</td>
</tr>
<tr>
<td>MT</td>
<td>Motor threshold</td>
</tr>
<tr>
<td>MVC</td>
<td>Maximal voluntary contraction</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NCS</td>
<td>Nerve conduction study</td>
</tr>
<tr>
<td>NI</td>
<td>Neurophysiological index</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PLS</td>
<td>Primary lateral sclerosis</td>
</tr>
<tr>
<td>PMA</td>
<td>Primary muscular atrophy</td>
</tr>
<tr>
<td>RMT</td>
<td>Resting motor threshold</td>
</tr>
<tr>
<td>RRP</td>
<td>Relative refractory period</td>
</tr>
<tr>
<td>S</td>
<td>Slow channel</td>
</tr>
<tr>
<td>SALS</td>
<td>Sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>SICI</td>
<td>Short interval intracortical inhibition</td>
</tr>
<tr>
<td>SOD-1</td>
<td>Superoxide dismutase-1</td>
</tr>
<tr>
<td>SR</td>
<td>Stimulus response</td>
</tr>
<tr>
<td>τ&lt;sub&gt;SD&lt;/sub&gt;</td>
<td>Strength-duration time constant</td>
</tr>
<tr>
<td>TE</td>
<td>Threshold electrotonus</td>
</tr>
<tr>
<td>TMS</td>
<td>Transcranial magnetic stimulation</td>
</tr>
<tr>
<td>UL</td>
<td>Upper limb</td>
</tr>
<tr>
<td>UMN</td>
<td>Upper motor neuron</td>
</tr>
</tbody>
</table>
REFERENCES

Intracortical inhibition and facilitation are abnormal in Huntington's disease: a

Abdeen MA, Stuchly MA. Modeling of magnetic field stimulation of bent neurons. IEEE
Transactions on Biomedical Engineering 1994; 41: 1092-5.

Abe K, Aoki M, Ikeda M, Watanabe M, Hirai S, Itoyama Y. Clinical characteristics of
familial amyotrophic lateral sclerosis with Cu/Zn superoxide dismutase gene

Abe K, Pan LH, Watanabe M, Konno H, Kato T, Itoyama Y. Upregulation of protein-
tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis.

Abrahams S, Goldstein LH, Simmons A, Brammer M, Williams SC, Giampietro V,
Leigh PN. Word retrieval in amyotrophic lateral sclerosis: a functional magnetic

Abrahams S, Goldstein LH, Suckling J, Ng V, Simmons A, Chitnis X, Atkins L,
Williams SC, Leigh PN. Frontotemporal white matter changes in amyotrophic

Aggarwal A, Nicholson G. Age dependent penetrance of three different superoxide
dismutase 1 (sod 1) mutations. Interntaional Journal of Neuroscience 2005; 115:
1119-30.

Al-Chalabi A, Andersen PM, Chioza B, Shaw C, Sham PC, Robberecht W, Matthijs G,
Camu W, Marklund SL, Forsgren L, Rouleau G, Laing NG, Hurse PV, Siddique


homozygosity for Asp90Ala CuZn-superoxide dismutase mutation. A clinical and

Andersen PM, Nilsson P, Ala-Hurula V, Keränen ML, Tarvainen I, Haltia T, Nilsson L,
Binzer M, Forsgren L, Marklund SL. Amyotrophic lateral sclerosis associated
with homozygosity for an Asp90Ala mutation in CuZn-superoxide dismutase.

LO, Gredal O, Marklund SL. Phenotypic heterogeneity in motor neuron disease
patients with CuZn-superoxide dismutase mutations in Scandinavia. Brain 1997;
120: 1723-37.

Andersen PM, Sims KB, Xin WW, Kiely R, O'Neil G, Ravits J, Pioro E, Harati Y,
novel mutations in the Cu/Zn superoxide dismutase gene in amyotrophic lateral
sclerosis: a decade of discoveries, defects and disputes. Amyotrophic Lateral

Andrew SE, Goldberg YP, Hayden MR. Rethinking genotype and phenotype correlations
in polyglutamine expansion disorders. Human Molecular Genetics 1997; 6: 2005-
10.

Andrus PK, Fleck TJ, Gurney ME, Hall ED. Protein oxidative damage in a transgenic
mouse model of familial amyotrophic lateral sclerosis. Journal of Neurochemistry

Angstadt JD, Calabrese RL. A hyperpolarization-activated inward current in heart


Banks MI, Pearce RA, Smith PH. Hyperpolarization-activated cation current (Ih) in neurons of the medial nucleus of the trapezoid body: voltage-clamp analysis and enhancement by norepinephrine and cAMP suggest a modulatory mechanism in the auditory brain stem. Journal of Neurophysiology 1993; 70: 1420-32.


Bruijn LI, Beal MF, Becher MW, Schulz JB, Wong PC, Price DL, Cleveland DW. Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked superoxide dismutase 1 mutant. Proceedings of the National Academy of Sciences USA 1997a; 94: 7606-11.


Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annual Reviews of Neuroscience 2004; 27: 723-49.

Burke D, Mogyoros I, Vagg R, Kiernan MC. Quantitative description of the voltage
dependence of axonal excitability in human cutaneous afferents. Brain 1998; 121:
1975-83.


Canton T, Pratt J, Stutzmann JM, Imperato A, Boireau A. Glutamate uptake is decreased

Cappelen-Smith C, Kuwabara S, Lin CS, Mogyoros I, Burke D. Activity-dependent
hyperpolarization and conduction block in chronic inflammatory demyelinating

Cappelen-Smith C, Kuwabara S, Lin CS, Mogyoros I, Burke D. Membrane properties in

Excitability changes of muscular responses to magnetic brain stimulation in
patients with central motor disorders. Electroencephalography and Clinical

Carpentier A, Duchateau J, Hainaut K. Motor unit behaviour and contractile changes
during fatigue in the human first dorsal interosseus. Journal of Physiology

modulation of slow, activity-dependent alterations in sodium channel availability
endows neurons with a novel form of cellular plasticity. Neuron 2003; 39: 793-
806.


Choe CU, Ehrlich BE. The inositol 1,4,5-trisphosphate receptor (IP3R) and its regulators: sometimes good and sometimes bad teamwork. Sci STKE 2006; 2006: re15.


Filippov AK, Couve A, Pangalos MN, Walsh FS, Brown DA, Moss SJ. Heteromeric assembly of GABA(B)R1 and GABA(B)R2 receptor subunits inhibits Ca(2+) current in sympathetic neurons. Journal of Neuroscience 2000; 20: 2867-74.


from neuropsychological investigation and event-related potentials. Brain

Hanajima R, Ugawa Y. Impaired motor cortex inhibition in patients with ALS: evidence

Hanajima R, Ugawa Y, Terao Y, Sakai K, Furubayashi T, Machii K, Uesugi H,
Mochizuki H, Kanazawa I. Cortico-cortical inhibition of the motor cortical area
projecting to sternocleidomastoid muscle in normals and patients with spasmodic
torticollis or essential tremor. Electroencephalography and Clinical

Hanajima R, Ugawa Y, Terao Y, Ogata K, Kanazawa I. Ipsilateral cortico-cortical
inhibition of the motor cortex in various neurological disorders Journal of

magnetic stimulation of the human motor cortex: differences among I waves.

Compound heterozygous D90A and D96N SOD1 mutations in a recessive


Harding AE, Thomas PK, Baraitser M, Bradbury PG, Morgan-Hughes JA, Ponsford JR.
X-linked recessive bulbospinal neuronopathy: a report of ten cases. Journal of
Haverkamp LJ, Appel V, Appel SH. Natural history of amyotrophic lateral sclerosis in a


Hebert T, Drapeau P, Pradier L, Dunn RJ. Block of the rat brain IIA sodium channel alpha subunit by the neuroprotective drug riluzole. Molecular Pharmacology 1994; 45: 1055-60.


Isom LL. Sodium channel beta subunits: anything but auxiliary. Neuroscientist 2001; 7: 42-54.


single motor unit study using transcranial magnetic stimulation.


Mohler H. GABA(A) receptor diversity and pharmacology. Cell Tissue Research 2006; 326: 505-16.


Ritchie JM, Straub RW. Effects of metabolic inhibitors, potassium and lithium ions on the post-tetanic hyperpolarization in mammalian non-medullated fibres. Journal of Physiology (London) 1957; 135: 6-7P.


Rossini PM, Barker AT, Berardelli A, Caramia MD, Caruso G, Cracco RQ, et al. Non-invasive electrical and magnetic stimulation of the brain, spinal cord and roots:


Weiss G. Sur la possibilité de rendre comparables entre eux les appareils servant l'excitation électrique. Archives Italiennes de biologie 1901; 35: 413-446.


