Functional Deficits in Motor Terminals and their Mitochondria in Mouse Models of Amyotrophic Lateral Sclerosis

Khanh Tu Nguyen

University of Miami, knguyen2@med.miami.edu

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FUNCTIONAL DEFICITS IN MOTOR TERMINALS AND THEIR MITOCHONDRIA IN MOUSE MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

By

Khanh Tu Nguyen

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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FUNCTIONAL DEFICITS IN MOTOR TERMINALS AND THEIR MITOCHONDRIA IN MOUSE MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

Khanh Tu Nguyen

Approved:

Ellen F. Barrett, Ph.D.  Terri A. Scandura, Ph.D.
Professor of Physiology and Dean of the Graduate
Biophysics  School

Thomas J. Sick, Ph.D.  Helen Bramlett, Ph.D.
Professor of Neurology  Associate Professor of
Neurological Surgery

Carlos Moraes, Ph.D.  Gavriel David, M.D./Ph.D.
Professor of Neurology  Research Associate
Professor of Physiology and Biophysics
Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease in which the upper and lower motor neurons die. Most studies aimed at elucidating the cause of this disease have focused on the motor neuron cell body. However, recent work has suggested that the disease may begin in motor nerve terminals. The experiments described in Chapters 2-4 of this dissertation studied functional defects in motor nerve terminals of mice expressing mutant human superoxide dismutase 1 (SOD1-G93A, SOD1-G85R), models of familial ALS. In Chapter 2, the proximal hind limbs of SOD1-G93A mice were subjected to varying durations of a tourniquet-induced ischemia/reperfusion injury to determine whether these motor terminals were more vulnerable to this stress than wild-type terminals. Confocal imaging of yellow fluorescent protein (YFP expressed in neurons) and alpha-bungarotoxin (labels acetylcholine receptors on muscle) was used to determine endplate occupancy. In the distal hind limb of SOD1-G93A/YFP terminals innervating fast type muscles (extensor digitorum longus (EDL) and plantaris) were more vulnerable to ischemia/reperfusion injury than those occupying the slow type muscle (soleus). Increased vulnerability to endplate denervation was evident in presymptomatic mice as early as 31 days old.
Experiments in Chapters 3 tested whether mitochondrial handling of Ca\(^{2+}\) loads is altered at presymptomatic stages. These experiments used rhodamine-123 to measure depolarization of the mitochondrial membrane potential (\(\Delta\Psi_m\)) evoked by trains of action potentials delivered to the motor nerve in levator auris longus motor terminals. These \(\Psi_m\) depolarizations depended on Ca\(^{2+}\) entry into motor terminals and were relatively small (~1-2 mV) in wild-type terminals. Consistent with the hypothesis that reduced ability to accelerate the electron transport chain (ETC) activity results in larger stimulation-induced \(\Psi_m\) depolarizations, presymptomatic SOD1-G93A (maintains dismutase activity) and SOD1-G85R (lacks dismutase activity) terminals displayed ~5 times greater depolarizations than wild-type terminals. Expression of normal human SOD1 or knockout of SOD1 did not significantly alter \(\Psi_m\) depolarizations. In the presence of a low concentration of rotenone (inhibits complex 1 of the ETC) wild-type terminals also displayed larger \(\Psi_m\) depolarizations.

Experiments in Chapter 4 studied stimulation-induced \(\Psi_m\) depolarizations in terminals of older, symptomatic SOD1-G93A and SOD1-G85R mice. These depolarizations decayed more slowly than those in wild-type terminals and incremented with successive trains. Asynchronous depolarizations that were not time linked to the stimulus train were also noted. These behaviors were attenuated when opening of the mitochondrial permeability transition pore (mPTP) was inhibited with cyclosporin A or by replacing bath Ca\(^{2+}\) with Sr\(^{2+}\). Incrementing \(\Psi_m\) depolarizations could be elicited in wild-type terminals when subjected to an oxidative stress (diamide-induced depletion of glutathione).
These findings indicate that motor terminals in mutant SOD1 mice display functional deficits even at presymptomatic ages, and that deficits associated with mitochondrial handling of stimulation-induced Ca$^{2+}$ loads increase with age and may contribute to motor terminal degeneration in mutant SOD1 mice.
Dedicated to:

Viet Bach Nguyen

For his love, support, and sense of humor
Acknowledgements

Although my name solely appears on the title page of this dissertation work, many people were involved in its creation and completion. I have been blessed to not have just one mentor or two mentors, but three mentors, each one contributing in a special way to my dissertation project. I would like to thank Ellen Barrett for her patience, understanding and guidance. Thank you for always taking the time to listen, no matter how busy you were. I would like to thank John Barrett for his constant enthusiasm and unconditional optimism. I would like to thank Gavriel David for his technical savvy; he always makes the most difficult experiments look so easy. Thank you to my dissertation committee members: Thomas Sick, Carlos Moraes and Helen Bramlett. Your support, critical analysis and enthusiasm for my project were always welcomed.

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A special thank you to Conrado Freites and Bin Yuan for their technical support, the Physiology and Biophysics staff (Rosa Mari Alvarez, Roxanna Braithwaite, Mercedes Dirube, Katrina Hollis, Niurka Ortiz, Ailys Quintana, and Lenora Smith) and the Neuroscience Program staff (Lisa Babbs, Sean Rinehart, Samone Welch and Judy Harmon) for their assistance and encouragement.
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Publication Note

Chapters 2* and 3** consist of published manuscripts and Chapter 4*** is a manuscript in preparation. The data for Figs. 4 and 6 in Chapter 2, and for Figs. 1A (upper trace), 3A, 4A, and 5 in Chapter 3 were collected by Gavriel David. Luis Garcia-Chacon collected the data for Figs. 1, 2, 3B and 3C in Chapter 3. John N. Barrett collected the data for Supporting Fig. 1 and the $\Psi_m$ data for SOD1 knockout mice in Chapter 3.


1 Department of Physiology and Biophysics and 2 Neuroscience Program, University of Miami Miller School of Medicine, P.O. Box 016430, Miami FL USA.

**Khanh T. Nguyen$^{1,2}$, Luis E. García-Chacón$^{1,3}$, John Barrett$^{2,3}$, Ellen F. Barrett$^{2,3}$, Gavriel David$^{2,3}$ (2009) The $\Psi_m$ depolarization that accompanies mitochondrial Ca$^{2+}$ uptake is greater in mutant SOD1 than in wild-type mouse motor terminals. Proc. Nat. Acad. Sci. 106: 2007-11.

1 These authors contributed equally to this work; 2 Neuroscience Program and 3 Department of Physiology and Biophysics, University of Miami Miller School of Medicine, P.O. Box 016430, Miami FL USA.

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1 Neuroscience Program and 2 Department of Physiology and Biophysics, University of Miami Miller School of Medicine, P.O. Box 016430, Miami FL USA.
Chapter 1

Introduction

The work presented in this dissertation focuses on possible mechanisms of disease progression in mutant superoxide dismutase 1 (SOD1) mouse models of familial amyotrophic lateral sclerosis (fALS). Chapter 2 discusses the effects of ischemia-reperfusion injury on motor terminals innervating slow and fast hind limbs of SOD1-G93A mice. Chapter 3 presents evidence of mitochondrial dysfunction in motor terminals of presymptomatic SOD1-G93A and SOD1-G85R mouse models of fALS. Chapter 4 examines a consequence of this mitochondrial defect in motor nerve terminals of symptomatic SOD1-G93A and SOD1-G85R mice.

The introduction (Chapter 1) will review the structure and function of motor neurons and motor nerve terminals. It will discuss the special role of mitochondria in motor nerve terminals, and how mitochondrial dysfunction can contribute to cell death. Lastly, SOD1 and its link to ALS in humans and in mouse models of the disease will be discussed.
A. Motor neurons

Spinal motor neurons are among the largest neurons in the central nervous system (CNS). Unlike most CNS neurons, they project their axons outside the CNS and innervate skeletal muscle fibers. The work of this dissertation concerns somatic alpha motor neurons that innervate extrafusal muscle fibers.

A motor unit consists of a single motor neuron, its axon, and the muscle fibers it innervates. The number of muscle fibers within each unit is inversely proportional to the muscle’s need for refined motion. In other words, muscles that require more refined motion, such as eye muscles, are innervated by motor units with few muscle fibers. During movements, motor units are generally recruited in the order smallest to largest.

Physiological properties are used to classify motor units into distinct types. The two physiological properties that separate motor units into three categories are (1) the relative resistance to fatigue during stimulation trains (fatigue index, in which high values indicate a resistance to fatigue) and (2) the presence or absence of decline in force output during low-frequency tetanization. Fast-twitch fatiguable or FF type units have a fatigue index of less than or equal to 0.25, while fast-twitch fatigue resistant units (FR type) have a fatigue index of greater or equal to 0.75. Slow or S type units are fatigue resistant but have small force outputs compared to FF and FR units. Table 1.1 summarizes the physiological motor unit properties and muscle fiber histochemistry. The motor unit size,
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muscle fiber diameter and motor neuron soma diameter are larger in FF type muscles compared to S type muscles. However, motor neuron excitability is greater in S type muscles than FF type muscles.

The properties of the spinal motor neurons that integrate the synaptic input influence the discharge pattern. In general, human motor units display two types of firing pattern, characterized by the speed of the force output. During slowly changing force output, motor neurons that are recruited fire at frequencies between 5 and 10 Hz and can fire up to rates of 20 - 40 Hz. As motor neurons are recruited for rapid movements, the firing rates are quite high (> 50 Hz), but for only brief periods. Motor neurons innervating FF type muscles fire at the high rate (Burke, 2004).

In patients with amyotrophic lateral sclerosis (ALS; discussed below), motor neurons innervating FF muscles of the tongue and limbs die first and the motor neurons innervating S muscles of the eye are most resistant to death. In a mouse model of ALS, there is a selective loss of the FF motor units that contain large Type IIB muscle fibers (Hegedus et al., 2008). Chapter 2 provides evidence that motor nerve terminals innervating FF muscles in these mice are also more vulnerable to ischemia/reperfusion injury.

B. Motor nerve terminals

The axons of spinal cord motor neurons project into the periphery and at their terminals synapse with skeletal muscle fibers at the neuromuscular junction. Motor axons are myelinated by Schwann cells until they reach the endplate.
region of the muscle membrane. At this site, the nerve fibers lose their myelin and split into several branches to form terminals which are encircled by perisynaptic Schwann cells. Motor terminals invaginate grooves in the muscle fiber plasma membrane. Each branch is approximately 2 µM in diameter with synaptic boutons formed at the ends of the branch. Each bouton lies over a depression in the muscle membrane where junctional folds form that greatly increase the surface area for synaptic transmission. The innervation site of the muscle membrane is called the endplate and the 20 – 30 nm wide space between the terminal and the fiber membrane is the synaptic cleft.

The machinery required for transmitter release is included in each synaptic bouton: (1) the acetylcholine-filled synaptic vesicles, (2) the active zone, a specialized membrane for transmitter release, and (3) voltage-gated Ca\(^{2+}\) channels. Acetylcholine is synthesized in the cytoplasm, but is quickly packaged into synaptic vesicles. An action potential travels down the terminal axon and spreads over the terminal, opening voltage-gated Ca\(^{2+}\) channels and allowing large quantities of Ca\(^{2+}\) to enter the terminal, reaching localized concentrations estimated at 10-100 µM near release sites. Ca\(^{2+}\) triggers fusion of acetylcholine-filled vesicles with the terminal membrane, and this fusion results in the release of the vesicles’ contents. Each action potential releases several hundred vesicles into the synaptic cleft.

The acetylcholine then binds with the nicotinic acetylcholine receptors located on the post-junctional folds of the muscle. Activation of these nicotinic receptors opens an intrinsic cation-selective channel, causing Na\(^{+}\) to enter and
K⁺ to exit. The electrochemical potential gradient greatly favors Na⁺ entry over K⁺ exit, thus Na⁺ influx dominates over K⁺ efflux. This influx causes the muscle fiber membrane to depolarize, triggering a synaptic potential called the endplate potential. The amplitude of the endplate potential evoked by discharge of the motor neuron is unusually large, up to 70 mV, compared to synaptic potentials of only ~1 mV in the central nervous system (Whitteridge, 1948).

Motor nerve terminals are especially vulnerable following ischemia/reperfusion injury. When rat limbs were subjected to 2 hrs of ischemia in vivo and examined 24 hr – 4 weeks post injury, the axons, Schwann cells and muscle fibers appeared normal. However, electron microscopy revealed that motor nerve terminals displayed signs of degeneration that included disruption of presynaptic membrane, loss of synaptic vesicles and degeneration of mitochondria. The motor nerve terminals of a FF muscle type (extensor digitorum longus) were more degenerated than those of a S type muscle (soleus) (Tombol et al., 2002). Consistent with these findings, Chapter 2 presents further evidence that motor nerve terminals of FF type muscles in a mouse model of familial amyotrophic lateral sclerosis are more vulnerable to ischemia/reperfusion injury than S type muscles. Motor nerve terminals have a large surface to volume ratio and high density of depolarization-activated Ca²⁺ channels. A possible reason for motor nerve terminal vulnerability could be that ischemic injury causes a massive influx of Ca²⁺ into the terminals, thus overloading mitochondria’s ability to continually sequester Ca²⁺ and diminishing the capacity for oxidative phosphorylation.
C. Mitochondria

Work from the lab demonstrates that mitochondrial Ca\(^{2+}\) sequestration contributes importantly to motor nerve terminal function and that mitochondrial dysfunction may contribute to motor nerve terminal death. Fig. 1.1 illustrates a simplified structure of a mitochondrion. Mitochondria contain outer and inner membranes composed of phospholipid bilayers and proteins. The double-membrane organization forms four distinct compartments: (1) the outer mitochondrial membrane, (2) the intermembrane space, (3) the inner mitochondrial membrane, and (4) the matrix. The outer membrane encloses the entire organelle and contains a large number of channels called porins which allow molecules of up to 5000 Da to pass freely. For large proteins to cross the outer membrane, they must be tagged with specific signaling sequences. Unlike the outer membrane, the inner membrane is highly impermeable to most water-soluble molecules. Specialized membrane transporters are needed to transport most ions and molecules to and from the matrix. The inner membrane also contains the proteins that perform the redox reactions of oxidative phosphorylation.

The surface area of the inner membrane is expanded by invaginations called cristae. The increased surface area enhances the mitochondria’s ability to
Fig 1.1. Taken from Celsi et al., 2009. Schematic map of a mitochondrion’s component and its transporters.

produce ATP. ATP synthesis occurs in the matrix with the aid of ATP synthase in the inner membrane. The matrix contains a mixture of enzymes that function in the oxidation of pyruvate and fatty acids and the citric acid cycle.
Mitochondria produce ATP during mitochondrial oxidative phosphorylation by transferring electrons from reduced substrates NAD(P)H and FADH$_2$ to O$_2$ through a chain of respiratory electron transporters. The outward pumping of H$^+$ through complexes I, III, and IV of the chain generates proton and electrical gradients across the inner mitochondrial membrane and establishes an electrochemical membrane potential ($\Delta\Psi_m$) of 150-200 mV (matrix side negative) across the inner mitochondrial membrane. Electrons are transferred from one complex to the other by the water-soluble protein cytochrome c and the lipid-soluble electron carrier coenzyme Q10. The ATP synthase (complex V) then uses the electrochemical energy of this gradient to couple H$^+$ influx down its electrochemical gradient with ADP phosphorylation in the matrix to generate ATP (Nicholls and Ferguson, 2002).

In addition to ATP production, mitochondria function in the regulation of intracellular Ca$^{2+}$ levels in many cell types by temporarily sequestering significant amounts of large stimulation-induced Ca$^{2+}$ loads (Friel et al., 1994; Stuenkel, 1994; Herrington et al., 1996; Babcock et al.; 1997; David et al., 1998; Pivovarova et al., 1999; Kaftan et al., 2000; Suzuki et al., 2002; David et al., 2003). The primary route of Ca$^{2+}$ influx into mitochondria is the uniporter, a highly selective Ca$^{2+}$ channel with a Kd < 2 nM (Kirichok et al., 2004) which is blocked by ruthenium red (Moore, 1971) or a colorless ruthenium red derivative, Ru360 (Reed & Bygrave, 1974). During stimulation, Ca$^{2+}$ enters the cytosol and opens the uniporter in the inner membrane. Ca$^{2+}$ entering the mitochondrial matrix depolarizes $\Psi_m$, and thus reduces the gradient driving Ca$^{2+}$ accumulation.
Evidence supporting strong intramatrix buffering of Ca\(^{2+}\) includes the observation that addition of cyanide m-chlorophenylhydrazone, a proton carrier to acidify the matrix, caused an increase in mitochondrial [Ca\(^{2+}\)] baseline levels (Kaftan et al., 2000). The increase was interpreted to be due to the drop in matrix pH releasing bound Ca\(^{2+}\) and thereby increasing mitochondrial [Ca\(^{2+}\)].

The \(\Psi_m\) across the inner mitochondrial membrane provides the driving force for passive Ca\(^{2+}\) uptake by mitochondria. In motor nerve terminal mitochondria, Ca\(^{2+}\) is continually sequestered by the matrix during prolonged trains of action potentials (David 1999). How is the net \(\Psi_m\) depolarization able to remain small? One possible mechanism is that depolarizations cause the electron transport chain to increase the rate at which it extrudes protons, thus allowing mitochondria to continue taking up Ca\(^{2+}\) with minimal \(\Psi_m\) depolarization (Talbot et al., 2007).

Pivovarova et al., 1999 and Pivovarova et al., 2002 calculated that total (free plus bound) mitochondrial Ca can reach nearly 10 mM during stimulation. The maximal free mitochondrial [Ca\(^{2+}\)] measured during stimulation is only 1-5 \(\mu\)M (David, 1999; Chalmers and Nicholls, 2003; David et al., 2003). The several orders of magnitude difference is additional evidence for a powerful buffering mechanism in the mitochondrial matrix. With conventional buffers at work, the bound to free ratio of matrix Ca is estimated to range between 3000 and 4000 (Babcock et al., 1997). Mitochondria have a phosphate transporter that exchanges matrix phosphate for cytosolic phosphate (Chappell and Crofts, 1965). Phosphate is abundant in the mitochondria and [phosphate] is greatly
increased upon uptake of Ca\(^{2+}\) into isolated mitochondria (Mela and Hess, 1982). Additional evidence for phosphate as a matrix Ca buffer includes the observation that when phosphate is replaced with acetate, the ability of isolated mitochondria to take up large quantities of Ca\(^{2+}\) is lost (Zoccarato and Nicholls, 1982). It is suggested that Ca\(^{2+}\) and phosphate precipitate together as an osmotically inactive, but reversible salt in the alkaline matrix (Pivovarova et al., 1999, David et al., 1999; Chalmers and Nicholls, 2003).

Mitochondrial Na\(^{2+}\)-Ca\(^{2+}\) and H\(^{+}\)-Ca\(^{2+}\) exchangers are the primary routes by which sequestered Ca\(^{2+}\) returns to the cytosol via secondary active transport (reviewed by Gunter & Pfeiffer, 1990). The primary route of Ca\(^{2+}\) extrusion in motor nerve terminal mitochondria occurs is Na\(^{2+}\)-Ca\(^{2+}\) exchanger (García-Chacón et al., 2006).

Calcium can also exit mitochondria via openings of the mitochondrial permeability transition pore (mPTP, Crompton et al., 1987). The mPTP is an assembly of proteins from the inner and outer mitochondrial membranes that align to form a large conductance channel permeable to solutes of <1,500 Da. The exact composition of the pore is still debated, but the components believed to regulate the pore include a voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and cyclophilin D. Opening of the mPTP is triggered by high [Ca\(^{2+}\)] in the matrix, by oxidants and by depletion of adenine nucleotide. mPTP opening is inhibited by acidic pH and antioxidants such as reduced glutathione. Cyclosporin A also inhibits pore opening by binding to
cyclophilin D (Woodfield et al., 1998). Inhibitors of pore opening also inhibit cytochrome c release from the intermembrane space (Zamzami et al., 1996).

Once the mPTP is opened, the inner mitochondrial membrane can no longer maintain a barrier to protons, so the proton motive force is dissipated and oxidative phosphorylation becomes uncoupled. Not only are mitochondria prevented from making ATP, but also the proton-translocating ATPase starts operating in reverse, hydrolyzing any ATP available to rebuild the proton electrochemical gradient. The resulting ATP depletion leads to bioenergetic failure of the cell (Halestrap et al., 1998; Halestrap et al., 2004). An additional consequence of mPTP opening is the disruption of metabolic gradients between the mitochondria and cytosol due to the increased permeability of the inner mitochondrial membrane to low molecular weight molecules. These molecules include cofactors and ions such as Ca^{2+}. The matrix compartment swells due to inner membrane cristae unfolding or rupture, leading eventually to rupture of the outer membrane. This releases cytochrome c from the intermembrane space and other pro-apoptotic proteins that may initiate apoptotic cell death (Crompton et al., 2000).

Several findings suggest a role for the mPTP in apoptosis. Pore protein components such as VDAC have been shown to interact with the Bcl family proteins, whose members may be anti-apoptotic or pro-apoptotic (Narita et al., 1998; Shimizu et al., 1999; Tsujimoto & Shimizu et al., 2000). mPTP opening leads to the collapse of ΔΨ_m (a hallmark of apoptosis) and signals the redistribution of Bcl family proteins to form secondary clusters (De Giorgi et al.,
The duration of time that the mPTP opens may determine whether the cell will undergo apoptosis or necrosis. It is argued that apoptosis occurs when the mPTP opens sufficiently to release cytochrome c but then closes again to ensure that ATP concentrations are maintained. However, if the mPTP opens and remains open, then the cell will undergo necrosis (review in Halestrap et al., 2004). A third possibility is that reversible openings may occur, allowing the cell to recover and survive (Ichas et al., 1997). Reversible, transient opening might allow release of relatively large loads of Ca\(^{2+}\) with only brief \(\Psi_m\) depolarizations and not damage the mitochondria.

**D. Amytrophic lateral sclerosis**

The French neurologist Jean-Marie Charcot first introduced the term “la sclérose latérale amyotrophique” (ALS; Charcot and Joffroy, 1869). Similar to other neurodegenerative disorders, ALS appears to be a syndrome rather than a single disease entity. It is characterized by the degeneration and eventual death of both upper and lower motor neurons. Onset of ALS usually occurs in the fourth and fifth decade of life. Although variable, the initial symptoms are usually muscle weakness and paralysis of one limb. The disease rapidly progresses to paralysis of all four limbs, with respiratory failure leading to death 2 - 5 years after diagnosis. The eye muscles are spared in ALS. The incidence of ALS is \(~2\) in 100,000 with no cure at the present time (Rowland and Schneider, 2001).

Some understanding of ALS etiology and pathogenesis comes from patient autopsies. Human ALS is predominantly a sporadic disorder (sALS).
Cytoplasmic shrinkage and lipofuscin granules have been observed in the surviving motor neurons, along with bunina bodies, skein-like and round hyaline inclusions. Bunina bodies are 1-3 µm eosinophilic granular inclusions found within the cytoplasm or dendrites. Skein-like inclusions are filamentous structures that form aggregates of thread-like structures (Kato et al., 2003). Round hyaline inclusions are also eosinophilic inclusions which may or may not form halos around anterior horn cells (Kato et al., 2000). Similar to skein-like inclusions, they have filamentous structure, and immunostain positive for ubiquitin (Kato et al., 2003).

Approximately 5-10% of human ALS cases are familial (fALS) and about 20% of fALS patients have superoxide dismutase 1 (SOD1) gene mutations (Rosen et al., 1993). SOD1 is a ubiquitously expressed metalloenzyme (Cu/Zn-dependent) that converts superoxide, a byproduct of mitochondrial oxidative phosphorylation, into water and hydrogen peroxide. More than 100 different mutations of the SOD1 gene have been linked to fALS (Andersen, 2000). Mutations that cause the disease are found throughout the primary and three-dimensional structure of the protein and all but one mutation (SOD1-D90A) are dominantly inherited (only one copy of the mutant gene is needed to develop the disease).

In addition to the pathological features of sALS, SOD1-mutated fALS patients have Lewy body-like hyaline inclusions in the anterior horn cells throughout the spinal cord. Astrocyte hyaline inclusions were also found in certain long-surviving fALS patients (Kato et al., 1996). These inclusions are 15-
25 nm granule-coated fibrils that are associated with 10 nm normal neurofilaments and have strong SOD1 immunoreactivity, suggesting that the mutated protein is causing aberrant protein aggregation.

Disease onset and progression in fALS patients vary depending on which mutation the patient carries. For example, SOD1-G93A patients usually show rapid disease progression with severe neurological symptoms and death within 4 years. However, the SOD1-H46R has mild neurological signs and a longer disease duration of up to 24 years (reviewed by Kato, 2008). Due to the limitation of not being able to study ALS at each clinical stage from disease onset to death, researchers have come to rely on animal models of ALS. These models are studied with the hope of gaining insight into mechanisms that might suggest possible therapeutic approaches.

Currently, the most suitable animal models of human ALS are transgenic rodents expressing mutant human SOD1. From these animal models, it has been established that mutant SOD1-mediated toxicity is due to gain of toxic function, independent of the levels of SOD1 activity. Evidence against loss of function as a cause for disease development comes from the findings that SOD1 null mice do not develop ALS (Reaume et al., 1996) and that removal of wild-type SOD1 in mice that express the SOD1-G85R mutation does not slow disease onset or progression (Bruijn et al., 1998). Increasing the levels of wild-type SOD1 has no effect on disease onset (Bruijn et al., 1998), nor does it accelerate disease progression (Jaarsma et al., 2000). Also animals expressing dismutase active SOD1-G37R (Wong et al., 1995) and SOD1-G93A (Gurney et al., 1994) or
dismutase inactive SOD1-G85R (Bruijn et al., 1997) develop similar disease pathologies. Thus, the level of SOD1 dismutase activity does not correlate with disease.

Our laboratory studies two animal models of the disease: SOD1-G93A and SOD1-G85R (Table 1.2). In these mice, mutant human SOD1 is ubiquitously expressed at levels equal to or several fold higher than the level of endogenous SOD1. These animals develop tremors and hindlimb weakness and eventually become completely paralyzed at endstage disease. The SOD1-G93A mice have 25 copies of the transgene, begin to exhibit disease symptoms at 3-4 months and die at ~5 months of age (Gurney et al., 1994; Chiu et al., 1995). The SOD1-G85R mice carry 15 copies of the transgene, begin to exhibit disease symptoms at 8-10 months, and die at 9-11 months (Bruijn et al., 1997). SOD1-G93A is catalytically active, but SOD1-G85R is inactive (Valentine & Hart, 2003). However, heterodimers of wild-type SOD1 and SOD1-G85R show enzymatic activity (Witan et al., 2008).

Mutant SOD1 has been expressed in neuronal and non-neuronal cells to study disease mechanisms for fALS. Table 1.3 summarizes findings from mutant SOD1 expression in neuronal and non-neuronal cells. Jaarsma and colleagues (2008) found that selectively expressing SOD1-G93A in motor neurons was sufficient to cause motor neuron degeneration and paralysis. Astrocyte-specific expression of the SOD1-G86R or SOD1-G93A mutations had no effect on mouse development (Gong et al., 2000; Lepore et al., 2008). However, when mutant
<table>
<thead>
<tr>
<th></th>
<th>G93A-high copy number</th>
<th>G93A-low copy number</th>
<th>G85R</th>
<th>SOD1-WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copy number</td>
<td>~25 Copies (Gurney et al., 1997)</td>
<td>8 Copies (Gurney et al., 1997)</td>
<td>15 copies (Bruijn et al., 1997)</td>
<td>8 Copies (Gurney et al., 1997)</td>
</tr>
<tr>
<td>SOD1 activity</td>
<td>active</td>
<td>active</td>
<td>not active</td>
<td>active</td>
</tr>
<tr>
<td>Symptomatic (hindlimb tremor)</td>
<td>symptoms start at 90 days (Frey et al., 2000)</td>
<td>symptoms at 224 days (Sasaki et al., 2004)</td>
<td>symptoms at 210 days (Bruijn et al., 1997 and Gurney et al., 1997)</td>
<td>no fALS symptoms</td>
</tr>
<tr>
<td>Endstage (cannot right itself before 30 sec)</td>
<td>121 days (Gurney et al., 1994)</td>
<td>238 days (Sasaki et al., 2004)</td>
<td>266 days (Bruijn et al., 1997)</td>
<td>normal lifespan</td>
</tr>
<tr>
<td>Loss of motor neurons</td>
<td>100 days (Frey et al., 2000) 80 days (vacuolozaiton) (Fischer et al., 2004)</td>
<td>168 days vacuolization (Sasaki et al., 2004)</td>
<td></td>
<td>some small vacuolar changes in proximal axons of motor neurons (DelCanto &amp; Gurney, 1995)</td>
</tr>
<tr>
<td>Denervation of motor endplate begins</td>
<td>90 days (Pun et al., 2006) 28 days (Fischer et al., 2004) 50 days (Frey et al., 2000)</td>
<td></td>
<td>186 days (Pun et al., 2006)</td>
<td></td>
</tr>
</tbody>
</table>

SOD1, super oxide dismutase 1; WT, wild-type; EDL, extensor digitorum longus; MG, medial gastrocnemius; SOL, soleus; TA, tibialis anterior
<table>
<thead>
<tr>
<th>Study</th>
<th>SOD1 Mutation</th>
<th>Cell type</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gong et al., 2000</td>
<td>G86R</td>
<td>Astrocytes</td>
<td>Mutant SOD1 expression restricted to astrocytes resulted in hypertrophy, increased GFAP reactivity, but normal development of mutant SOD1 mice</td>
</tr>
<tr>
<td>Yamanaka et al., 2008</td>
<td>G37R</td>
<td>Astrocytes</td>
<td>Deleting mutant SOD1 in astrocytes did not affect onset, but delayed microglial activation and sharply slowed later disease progression</td>
</tr>
<tr>
<td>Lepore et al., 2008</td>
<td>G93A</td>
<td>Astrocytes</td>
<td>Neuroadapted sinbis virus (NSV) infected G93A spinal cord did not affect any measures of disease outcome such as animal survival, disease onset, disease duration, hindlimb motor function and motor neuron loss.</td>
</tr>
<tr>
<td>DiGiorgio et al., 2008</td>
<td>G93A</td>
<td>Astrocytes</td>
<td>Human spinal neurons from human embryonic stem cells (hESC), but not interneurons, are selectively sensitive to toxic effects of glial cells</td>
</tr>
<tr>
<td>Marchetto et al., 2008</td>
<td>G37R</td>
<td>Astrocytes</td>
<td>Cocultured hESC-derived motor neurons with SOD1 mutant astrocytes reduced survival of healthy motor neurons by 50%</td>
</tr>
<tr>
<td>Vargas et al., 2008</td>
<td>G93A</td>
<td>Astrocytes</td>
<td>Coculture with upregulated Nrf2 motor neurons reversed toxic effects with mutant astrocytes. Also used two mutant SOD1 mice to cross with Nrf2 mice delayed disease onset and extended survival.</td>
</tr>
<tr>
<td>Miller et al., 2006</td>
<td>G93A</td>
<td>Muscle</td>
<td>Deletion of SOD1 from muscle did not affect onset or survival</td>
</tr>
<tr>
<td>Nagai et al., 2007</td>
<td>G93A, G85R andG37R</td>
<td>Astrocytes</td>
<td>Rodent astrocytes expressing mutated SOD1 kill spinal primary and embryonic mouse stem cell-derived motor neurons</td>
</tr>
<tr>
<td>Dobrowolnny et al., 2008</td>
<td>G93A</td>
<td>Muscle</td>
<td>Transgenic mice expressing SOD1 selectively in skeletal muscle developed progressive muscle atrophy and mitochondrial dysfunction</td>
</tr>
<tr>
<td>Boilée et al., 2008</td>
<td>G37R</td>
<td>Microglia</td>
<td>Diminishing levels in microglia had little effect on the early disease phases but sharply slowed later disease progression</td>
</tr>
<tr>
<td>Jaarsma et al., 2008</td>
<td>G93A</td>
<td>Motor neurons</td>
<td>Neuronal expression of SOD1 sufficient to cause motor neuron degeneration and paralysis</td>
</tr>
<tr>
<td>Lobsiger et al., 2009</td>
<td>G37R</td>
<td>Schwann cells</td>
<td>Diminished synthesis of SOD1 gene accelerates disease progression</td>
</tr>
</tbody>
</table>
SOD1-G37R and mutant SOD1-G93A astrocytes are cocultured with motor neurons, neuronal survival is significantly reduced compared to that of neurons co-cultured with wild-type astrocytes (Marchetto et al., 2008; Nagai et al., 2007). Reducing the levels of mutant SOD1-G37R expression in microglia had no effect on early phases of disease, but did slow disease progression at later stages (Boillee et al., 2006).

When SOD1-G93A protein was expressed solely in muscle, mice developed progressive muscle atrophy and reduced strength (Dobrowolny et al., 2008). However, the complementary experiment, selective deletion of SOD1-G93A from muscle, produced no change in disease onset or survival (Miller et al., 2006). Mutant SOD1 expression in non-neuronal cells is not necessarily detrimental to motor neurons. Surprisingly, diminishing synthesis of SOD1-G37R in Schwann cells accelerates disease progression (Lobsiger et al., 2009).

It has been suggested that altered expression of apoptotic proteins might contribute to spinal cord motor neuron degeneration in ALS. In the spinal cord of ALS patients, there was increased Bax expression (pro-apoptotic) paired with no change or decreased Bcl-2 expression (anti-apoptotic) (Ekegren et al., 1999; Mu et al., 1996). Motor neurons of mutant SOD1-G37R, SOD1-G93A and SOD1-G85R mice were more susceptible to the Fas-triggered cell death pathway than wild-type motor neurons (Raoul et al., 2002, 2006).

Regions of motor neuron loss in ALS patients show strong activation and proliferation of microglia, suggesting inflammation as an immune response to central nervous system damage (Kawamata et al., 1992). Furthermore, mouse
models of ALS express proinflammatory mediators such as TNF-alpha (Elliot, 2001; Hensley et al., 2003) and cyclooxygenase 2 (Almer et al., 2002) prior to onset of motor deficits. Controlling microglial activation in spinal motor neurons, as mentioned above, may be one therapeutic target to slow disease progression in ALS patients.

ALS pathogenesis has also been linked to alterations in angiogenesis. Targeted deletion of the hypoxia-response element in the vascular endothelial growth factor (VEGF) promoter caused ALS-like symptoms and neuropathology in mice (Oosthuyse et al., 2001). Moreover, when SOD1-G93A mice were crossbred with mutant mice that had reduced levels of VEGFa, the hybrid mice died earlier due to more severe motor neuron degeneration. Administration of VEGF protected mice against motor neuron damage due to ischemia/reperfusion injury (Lambrechts et al., 2003). A therapeutic role for VEGF was further supported by studies in which administration of VEGF to motor neurons of SOD1-G93A rats (another rodent model for ALS, Storkebaum et al., 2005) and muscles of SOD1-G93A mice (Azzouz et al., 2004) prolonged survival.

Evidence for glutamate-mediated excitotoxicity in motor neurons stems from the finding that the cerebrospinal fluid of ALS patients has increased glutamate levels (Rothstein et al., 1990, Shaw et al., 1995). Normally glutamate is removed from the synaptic cleft by the excitatory amino acid transporter-2 (EAAT-2; Rothstein et al., 1996). Decreased expression of EAAT-2 was found in motor cortex and spinal cord astroglia of sporadic ALS patients (Lin et al., 1998). Additionally, the discovery that SOD1-G93A rat (Howland et al., 2002) and
SOD1-G85R mouse (Bruijn et al., 1997) spinal cords have lower levels of EAAT-2 supports a role for excitotoxicity in motor neuron death and provides a link between sporadic and SOD1 mutant-mediated ALS. The mechanism behind Riluzole, currently the only FDA-approved therapy for ALS, is decreasing glutamate toxicity. Riluzole acts by blocking sodium channels, thus reducing influx of calcium ions, and indirectly prevents stimulation of glutamate receptors (Doble, 1996). It has been shown to marginally increase ALS patient survival by 3-5 months, but has severe side effects.

Motor neurons and in some cases astrocytes of both ALS patients and mutant SOD1 animal models reveal prominent, cytoplasmic inclusions (Bruijn et al., 1998). These protein aggregates are intensely immunoreactive for ubiquitin (Wang et al., 2003; Watanabe et al., 2001), which labels proteins for proteasomal degradation. Perhaps the misaccumulation of ubiquitin in ALS-associated SOD1 aggregates compromises the ability of the proteasome to remove misfolded proteins. It is unclear whether or how these aggregates damage motor neurons, but several possible mechanisms have been postulated such as aberrant chemistry, loss of protein function through co-aggregation and mitochondrial dysfunction.

Finally, mitochondrial abnormalities have been proposed to play a role in the pathogenesis of ALS. In human patients and animal models of ALS, mutant SOD1 proteins were found to be localized within the matrix and/or intermembrane space of mitochondria (in SOD1-G93A and SOD1-L126Z mice, Deng et al. 2006; in SOD1-G37R, SOD1-G85R, and SOD1-G93A mice, SOD1-G93A
rat, and in spinal cord tissue from a SOD1-G127X human patient, Liu et al. 2004; in SOD1-G93A mice, Mattiazzi et al., 2002). Immunohistochemical analysis of mitochondrial DNA from motor neurons of pre-symptomatic SOD1-G93A mice revealed signs of oxidative damage (Warita et al., 2001) and decreased activity of components of the mitochondrial respiratory chain in ventral horn spinal cord tissue (Borthwick et al., 1999; Jung et al., 2002). Kong and Xu (1998) and Martin et al. (2007) detected swollen mitochondria in SOD1-G93A motor neurons prior to onset of muscle weakness or death of spinal motor neurons.

A decrease in mitochondrial electron transport activity was observed in spinal cord and brain tissue of pre-symptomatic SOD1-G93A mice (Kirkinezos et al., 2005). Overexpression of CCS protein, a copper chaperone for SOD1, in SOD1-G93A mice increases mutant SOD1 levels within spinal cord mitochondria (Son et al., 2007) and decreases complex IV activity (Son et al., 2008). Similar results were observed when CCS protein was overexpressed in SOD1-G37R mice (Son et al., 2009). In addition to the early difference in electron chain transport activity, impaired Ca\(^{2+}\) sequestration was detected in spinal cord mitochondria of pre-symptomatic SOD1-G93A and SOD1-G85R mice (Damiano et al., 2006).

The observations mentioned above focus on mitochondrial abnormalities that occur within the motor neuron cell body. However, there is building evidence that motor neuron damage in fALS disease models begins peripherally in motor axons and/or motor terminals. In 30 day old SOD1-G93A mice, thinning of motor terminal branches innervating fast-fatiguable leg muscle fibers was detected.
There was extensive local denervation of these fibers by day 50 (Frey et al., 2000). Muscle endplates from SOD1-G85R fast-fatiguable fibers (but not slow type fibers) were also denervated before symptom onset (Pun et al., 2006). In another study, the spinal cord ventral horn, ventral roots and the neuromuscular junctions of SOD1-G93A mice were examined for abnormal morphology. At 47 days old (pre-symptomatic age), 40% of end-plates were found to be denervated from gastrocnemius, soleus, and tibialis anterior muscles, but the ventral roots or spinal cord cell bodies did not have abnormalities. Ventral root axon loss (60%) was not detected until 80 days and loss of large neuronal cell bodies in the spinal cord ventral horn (40%) was not detected until 100 days (Fischer et al., 2004). In addition, the same study examined tissue from a sporadic ALS patient who died unexpectedly 6 months after being diagnosed. In post-mortem examination, denervation of motor endplates and axonal sprouting were observed, but large neuronal cell bodies in the ventral horn appeared normal.

Presymptomatic mutant SOD1 motor nerve terminal mitochondria had evidence of swelling (Kong and Xu, 1998) or vacuolization (Gould et al., 2006). Repetitive stimulation of symptomatic SOD1-G93A motor terminals caused mitochondrial [Ca\(^{2+}\)] to ramp up to higher levels, instead of stabilizing at a plateau level (Vila et al., 2003), suggesting a defect in matrix Ca\(^{2+}\) buffering. The “Trojan horse” hypothesis proposed by Martin and colleagues (2009) suggests that retrograde transport of toxic mitochondria from motor terminals could contribute to motor neuron degeneration. Work described in Chapters 3 and 4 offer evidence of mitochondrial dysfunction in presymptomatic and symptomatic
mutant SOD terminals. These findings describing early stage of mitochondrial
dysfunction progressing to more severe dysfunction with age also suggest a role
for motor terminal mitochondria in ALS disease pathogenesis.
Chapter 2

Early Vulnerability to Ischemia/Reperfusion Injury in Motor Terminals Innervating Fast Muscles of SOD1-G93A Mice

A. Summary

In mouse models of familial amyotrophic lateral sclerosis (fALS) motor neurons are especially vulnerable to oxidative stresses in vitro. To determine whether this increased vulnerability also extends to motor nerve terminals in vivo, we assayed the effect of tourniquet-induced ischemia/reperfusion (I/R) injury on motor terminals innervating fast and slow hindlimb muscles in male G93A-SOD1 mice and their wild-type littermates. These mice also expressed yellow fluorescent protein (YFP) in motor neurons. We report that in SOD1-G93A/YFP mice the motor terminals innervating two predominantly fast muscles, extensor digitorum longus (EDL) and plantaris, were more vulnerable to I/R injury than motor terminals innervating the predominantly slow soleus muscle. The mean duration of EDL ischemia required to produce a 50% reduction in endplate innervation in SOD1-G93A/YFP mice was 26 min, compared to 45 min in YFP-only mice. The post-I/R destruction of EDL terminals in SOD1-G93A mice was rapid (< 2 hr) and was not duplicated by cutting the sciatic nerve at the tourniquet site. The increased sensitivity to I/R injury was evident in EDL muscles of SOD1-G93A/YFP mice as young as 31 days, well before the onset of motor neuron
death at ~90 days. This early vulnerability to I/R injury may correlate with the finding (confirmed here) that in fALS mice motor nerve terminals innervating fast hindlimb muscles degenerate before those innervating slow muscles, at ages that precede motor neuron death. Early vulnerability of fast motor terminals to I/R injury thus may signal, and possibly contribute to, early events involved in motor neuron death.

B. Background

Most studies of mechanisms underlying motor neuron death in the neurodegenerative disease ALS have focussed on changes occurring within the spinal cord. However in animal models of familial ALS (e.g. mice expressing mutant forms of human superoxide dismutase I, SOD1) there is structural and functional evidence for damage to motor axons and motor nerve terminals that precedes the onset of paralysis and motor neuron death (Chiu et al., 1995; Azzouz et al., 1997; Kennel et al., 1996; Kong and Xu, 1998; Frey et al., 2000; Fischer et al., 2004; Sasaki et al., 2004; Wooley et al., 2005; Pun et al., 2006; Boillée et al., 2006; Gould et al., 2006). Fischer et al. (2004) presented evidence for more extensive peripheral than central morphological damage in a patient diagnosed with sporadic ALS who died prematurely. Early distal defects have also been demonstrated in hereditary canine spinal motor atrophy (Rich et al., 2002). This distal damage contributes to motor symptoms and might also accelerate motor neuron death, an hypothesis supported by findings that certain
types of mechanical injury to peripheral nerves can accelerate disease progression in SOD1-G93A mice (Mariotti et al., 2002; Sharp et al., 2005).

Due to their peripheral location, motor nerve terminals may be subjected to stresses not normally experienced by the parent motoneuron, such as transient partial ischemia during intense (anaerobic) muscle activity or I/R injury following temporary interruption of limb blood supply. When hindlimbs of wild-type rodents are subjected to I/R injury, motor nerve terminals are the most vulnerable neuromuscular component. For example, electron microscopic studies of rat limbs examined 1 day – 4 weeks following 2 hr tourniquet ischemia detected no changes in axons, Schwann cells or muscle fibers, but multiple signs of degeneration in motor nerve terminals, including disruption of presynaptic membrane, degeneration of mitochondria and the presence of vacuoles (Tombol et al., 2002). Makitie and Teravainen (1977) detected swelling of motor terminal mitochondria after a similar stress. Functional measurements also show that the neuromuscular junction is a major site of I/R injury (e.g. Eastlack et al., 2004). Mechanisms underlying the special vulnerability of motor terminals to I/R stress have not yet been determined.

The present study tested whether motor terminals in the G93A-SOD1 mouse model of familial ALS are more vulnerable to I/R injury than wild-type motor terminals, and if so, when this extra vulnerability first becomes evident. We also tested whether motor terminals that innervate different muscle types are differentially sensitive to I/R injury, since Frey et al. (2000) and Pun et al. (2006) demonstrated in SOD1-G93A mice that the hindlimb motor terminals that
innervate fast (types IIa and IIb) muscle fibers degenerate earlier than those innervating slow, fatigue-resistant fibers (type I). We show that in SOD1-G93A mice the motor terminals innervating two predominantly-fast hindlimb muscles are more vulnerable to tourniquet-induced I/R injury than those innervating a slow hindlimb muscle. This increased vulnerability becomes evident more than a month before the onset of paralysis or motor neuron death.

C. Methods

Mice

SOD1-G93A/YFP mice were generated by crossing two transgenic strains obtained from Jackson Labs (Bar Harbor, ME): B6.Cg-TgN(SOD1-G93A)1Gur/J (stock #4435), which express a high copy number of this mutant human SOD1 in all cells, and B6.Cg-Tg(Thy1-YFP)16Jrs/J (stock number #3709), which express YFP in many neurons, including spinal motor neurons, but not in muscle or Schwann cells. YFP expression facilitates assessment of motor terminal innervation for large populations of endplates and does not reduce the viability of SOD1-G93A mice, as also reported by Schaefer et al. (2005). Tail clips obtained at postnatal days 18–20 (P18–20) were used to identify SOD1-G93A mice by RT-PCR (as described in Vila et al., 2003), and to identify YFP expression (in sensory axons) by fluorescence microscopy. A genotype identification code was tattooed on each mouse (AIMS Tattoo System, Hornell, NY). Since most SOD1-G93A mice were studied before signs of weakness/paralysis became apparent, PCR was repeated at the time of the experiment to confirm genotype. We used
male mice (ages P24–121) which expressed both SOD1-G93A and YFP and their YFP-only littermates. Only male mice were used to ensure more uniform results, since disease progression is reported to be slower in female than in male SOD1-G93A mice (Veldink et al., 2003, Miana-Mena et al, 2005), and in wild-type mice females are more resistant to CNS I/R injury than males (Sampei et al, 2000; Bramlett, 2005).

**Ischemia/reperfusion (I/R) protocol**

Mice were anesthetized by subcutaneous injection of 5 μl/g of a solution containing ketamine (5 mg/ml) and xylaxine (0.5 mg/ml), and placed on a heating pad maintained at 38 °C (Harvard Apparatus, Holliston, MA). Tourniquet ischemia was applied to one hindlimb by tying an elastic rubber band (3 mm width) around the thigh at the level of the greater trochanter. There was no direct pressure on EDL, plantaris or soleus muscles, which are below the femorotibial joint. The tension of the tourniquet was adjusted to be sufficient to interrupt the arterial pulse in the limb, monitored using a homemade optical pulse meter (footpad placed between a light-emitting diode and a photoresistor) that converted the pulse wave into voltage changes on an oscilloscope screen. Interruption of the arterial blood supply was indicated by disappearance of the pulse waves and reduction of the footpad skin temperature by ~2 °C.

Reperfusion following tourniquet removal was indicated by reappearance of the pulse waves. The ischemic period lasted 15–60 min; anesthetic was re-injected for ischemic durations >30 min. Mice were sacrificed by inhalation of 100% CO₂
6 – 24 hr following the onset of reperfusion, except as noted. All procedures were approved by the Animal Care and Use Committee of the University of Miami.

**Muscle dissection and staining of endplates**

Control and post-I/R hindlimbs were removed by cutting below the ilio-femoral joint. EDL and soleus (and in 2 cases plantaris) muscles were exposed by rapidly (~5 min) removing the skin and dissecting away the overlying muscles in normal mouse saline (NMS) containing (in mM): NaCl 137, NaHCO$_3$ 15, KCl 4, CaCl$_2$ 1.8, MgCl$_2$ 1.1, NaH$_2$PO$_4$ 0.33, and glucose 11.2, equilibrated with a 95% O$_2$/5%CO$_2$ gas mixture, pH 7.4 Limbs were then fixed at room temperature in freshly prepared 4% paraformaldehyde (in phosphate-buffered saline) for 40 min and rinsed in NMS for 30 min. Muscles were removed by cutting their tendons. Endplate acetylcholine (ACh) receptors were labeled with 25 μg/ml α-bungarotoxin (α-BgTx) Alexa Fluor 594 conjugate (Invitrogen, Carlsbad, CA) for 30 min and rinsed with NMS for 40 min. This is a good marker for endplates because junctional ACh receptors turn over very slowly, even after denervation (Bevan and Steinbach, 1983). Each muscle was separated into 4–5 longitudinal segments and mounted between #1 glass coverslips (Thomas Scientific) with hard mounting medium (Vectashield, Vector Laboratories, Burlingame, CA). EDL and soleus muscles have different fiber type compositions. In the C57BL/6J strain studied here, soleus contains predominantly type I fibers (slow, oxidative, 34–40%) and type IIa fibers (fast, fatigue-resistant, mixed oxidative and glycolytic, 55–59%, Totsuka et al., 2003; Luedeke et al., 2004). In contrast, EDL
contains mainly type IIb (fast, fatiguable, glycolytic, 78%) and intermediate (type IIx, 20%) fibers (Luedeke et al., 2004). Like EDL, plantaris contains mainly types IIb and IIx fibers (83%, Waters et al., 2004). Muscle fiber type is determined mainly by the activity pattern of the innervating motor neuron; type IIb fibers are innervated by FF motor neurons that innervate fast, fatiguable motor units; type IIa fibers are innervated by FR motor neurons that innervate fast, fatigue-resistant motor units; and type I fibers are innervated by S motor neurons that innervate slow, fatigue-resistant motor units (reviewed by Burke, 2004). Thus EDL and plantaris are innervated mainly by FF (and perhaps FR) motor neurons, and soleus is innervated by FR and S motor neurons. Expression of SOD1-G93A may alter these fiber type distributions as the disease progresses (Frey et al., 2000; Derave et al., 2003; Atkin et al., 2005), but is unlikely to have any major effect at the earliest (P30–P40) presymptomatic stages assayed here.

Imaging and calculation of endplate occupancy

Images were obtained with a confocal system that included a Yokogawa spinning disk (Solamere, Salt Lake City, UT), and a Cascade 512B camera (Roper Scientific, Tucson, AZ) mounted on a Nikon TE2000E microscope (Nikon, Melville, NY). Images were obtained using a 60X water immersion lens (NA 1.2, Olympus, Melville, NY) and a 4X lens (NA 0.2, Nikon). YFP and α-BgTx were excited at 488 or 568 nm, respectively (argon/krypton laser, Laser Physics, West Jordan, UT). Emitted light was filtered with a 535 nm band pass filter (40 nm bandwidth) for YFP or a long-pass 590 nm filter for α-BgTx (both filters from Chroma, Rockingham, VT). Images were acquired using IPLAB (v. 3.6,
Scanalytics Inc, Fairfax, VA) using 0.5 s exposures. Muscle fields containing endplates were identified by their α-BgTx labeling, and 30–50 endplates were imaged for each muscle. For each field YFP and α-BgTx image pairs were acquired for 15–25 Z sections separated by 1 μm in the vertical dimension, using custom-written scripts in IPLAB.

Measurements of endplate and motor terminal areas were made using custom programs written in V-Pascal with V++ imaging software (Digital Optics, Auckland, New Zealand). For each endplate one YFP and one α-BgTx image were generated from the series of Z sections by creating a maximal projection image onto the XY plane. Endplate area was calculated from outlines traced around the α-BgTx image (see Fig. 2.1 A, B). The corresponding motor terminal area was calculated after tracing the outlines of the largest YFP-filled structure contiguous with the preterminal axon. Endplate occupancy was calculated in Excel as the ratio of terminal and endplate areas. Since in YFP-only mice the area of the α-BgTx projection image usually exceeded that of the corresponding YFP projection image, the mean endplate occupancy calculated for a fully innervated, non-stressed muscle was usually less than 1, with a mean of 0.88 (see e.g.Fig. 2.3).

To estimate the ischemia duration at which endplate occupancy was reduced to 50% of maximal (ischemia duration 50, ID$_{50}$), plots of mean endplate occupancy as a function of ischemia duration (Fig. 2.3) were fitted with curves using a sigmoidal equation:

$$Q = Q_{\text{min}} + (Q_{\text{max}} - Q_{\text{min}})/(1 + (ID_{50}/t)^s),$$
where \( O = \) mean occupancy, \( O_{\text{min}} \) is the minimal measured mean occupancy (0), \( O_{\text{max}} \) is maximal occupancy measured in non-stressed muscles, \( t \) is the duration of ischemia in min, and \( s \) is slope. The best-fitting value of \( s \) (−8.0) was used for all plotted curves.

**Statistics**

Plots and histograms were created using Prism (version 3, Graph Pad Software, San Diego, CA), which was also used to perform tests of statistical significance. Non-parametric tests (Mann-Whitney, Kruskal-Wallis with Dunn’s post-test) were used for non-Gaussian distributions.

**D. Results**

*Motor terminals in EDL muscle of SOD1-G93A mice are highly sensitive to ischemia-reperfusion (I/R) injury, even at presymptomatic ages*

Motor terminals in EDL muscle of SOD1-G93A mice are highly sensitive to ischemia-reperfusion (I/R) injury, even at presymptomatic ages. Fig 2.1Ab shows micrographs of EDL muscles from young (P31–P33) YFP-only (A) and SOD1-G93A/YFP (B) mice whose right hindlimbs were subjected to tourniquet-induced ischemia for 30 min followed by 6 hr reperfusion in vivo (Ab, Bb). Non-stressed contralateral EDLs served as controls (Aa, Ba). All endplates in the non-stressed YFP-only muscle appear yellow-orange, indicating that all the \( \alpha \)-BgTx-labelled
Fig. 2.1 (following page)  An ischemia/reperfusion (I/R) stress denervates most endplates in the EDL of SOD1-G93A/YFP (B,D), but not YFP-only mice (A,C). One hindlimb in each of 2 male mice (P33 YFP-only; P31 SOD1-G93A/YFP) was subjected to 30 min of tourniquet-induced ischemia followed by 6 hr of reperfusion in vivo. The non-stressed contralateral hindlimb served as a control. The upper, low-power micrographs (Aa-b, Ba-b) are digital overlays of the fluorescence of YFP (green, delineating motor axons and terminals) and Alexa 594-labelled α-BgTx (red, marking endplate ACh receptors) in stressed and non-stressed EDLs. Innervated endplates are yellow-orange; denervated endplates are red. Middle and lower rows show at higher magnification the separate α-BgTx (Ac-d, Bc-d) and YFP (Ae-f, Be-f) images corresponding to the regions indicated by the dotted boxes in Aa-b and Ba-b. Endplates marked by α-BgTx staining were outlined (dotted lines) and the motor terminal innervating each endplate was outlined in the YFP image, to determine endplate occupancy (see Materials and Methods). Histograms of endplate occupancy in Ca,b and Da,b show results pooled from EDLs of all analyzed YFP-only and SOD1-G93A/YFP mice (P31–P101) subjected to 30 min ischemia followed by 6–24 hr reperfusion. Each histogram is based on measurements from 152–291 terminals in 5–8 mice. Analysis of cumulative histograms compiled from these data (Cc, Dc) indicated that the I/R-induced reductions in endplate occupancy were significant for both SOD1-G93A/YFP and YFP-only mice (p<0.01 or better, Mann-Whitney 2-tailed test; Kruskal-Wallis test). We found no significant differences in vulnerability to I/R stress among endplates associated with the 4 different heads of the EDL muscle, perhaps because EDL’s fiber type composition is quite uniform (98% type IIb or type IIx).
endplates (red) were innervated by a YFP-labelled motor terminal (green). All endplates in the non-stressed SOD1-G93A/YFP muscle were also innervated, consistent with previous studies demonstrating no significant motor terminal degeneration in these mice at one month (Chiu et al., 1995; Frey et al., 2000; Pun et al., 2006). Following the I/R stress, most YFP-only endplates remained innervated, but endplates in SOD1-G93A/YFP EDL appeared mostly red, indicating widespread denervation.

The higher-magnification images in Fig 2.1 show separate α-BgTx (Ac-d, Bc-d) and YFP (Ae-f, Be-f) images. Dotted lines illustrate how the endplate region was outlined in the α-BgTx image, and the corresponding motor terminal (if present) was outlined in the YFP image. These outlined areas were used to calculate the percentage of the endplate area that was occupied by the motor terminal (endplate occupancy). Fig. 2.1C,D shows histograms (a,b) and cumulative histograms (c) of endplate occupancy for each of the experimental conditions illustrated in Fig. 2.1A,B, compiled from data pooled from EDLs from mice aged P31–P101. Histograms in non-stressed (control) show a single cluster of endplates with occupancies ranging from ~70–100%. Muscles subjected to the 30 min I/R stress showed this cluster plus another cluster with less than 10% occupancy. Very few terminals exhibited intermediate occupancies. Low-occupancy endplates were rare in stressed YFP-only EDLs, but constituted the majority of endplates in the EDLs of SOD1-G93A/YFP mice. Thus a 30 min I/R stress that produced only minor denervation of EDL endplates in a YFP-only
mouse produced nearly complete denervation of EDL endplates in SOD1-G93A/YFP mice, even at young ages.

In SOD1-G93A/YFP mice motor terminals innervating fast muscles are more sensitive to I/R injury than motor terminals innervating a slow muscle. In other hindlimb muscles of SOD1-G93A mice (triceps surae, lateral gastrocnemius) evidence has been presented that motor terminals innervating fast type IIb muscle fibers degenerate faster than motor terminals innervating other muscle fiber types (Frey et al., 2000; Pun et al., 2006). Since the major fiber type in EDL is IIb (see Materials and Methods), we hypothesized that increased motor terminal vulnerability to I/R injury would develop sooner in EDL than in soleus, a slow muscle with few (if any) type IIb fibers. Fig. 2.2 plots mean endplate occupancy following a 30 min I/R stress as a function of age in non-stressed and stressed EDL (Fig. 2.2A) and soleus (Fig. 2.2B) of YFP-only and SOD1-G93A/YFP mice. Consistent with the above-cited studies, in non-stressed SOD1-G93A/YFP the mean endplate occupancy declined slowly with age in EDL, but not in soleus (compare open circles in Fig. 2.2Ab, Bb). In stressed SOD1-G93A/YFP limbs, mean endplate occupancy fell sharply with age in EDL, but did not fall in soleus (with one exception; compare filled circles in Fig. 2.2Ab, Bb). The marked increase in vulnerability to I/R injury in SOD1-G93A/YFP EDL was evident in some muscles as early as P31, and was evident in all sampled muscles older than P40. There was a tendency toward less I/R-induced damage in SOD1-G93A/YFP EDL younger than P30, but we did not examine animals younger than P24. In YFP-only mice the mean endplate occupancy of both non-
In SOD1-G93A/YFP mice, the increased vulnerability of EDL motor terminals to I/R injury begins within the first month; soleus (SOL) motor terminals are more resistant to this stress. Graphs plot mean endplate occupancy as a function of age for YFP-only and SOD1-G93A/YFP EDL (A) and soleus (B) muscles. Filled circles indicate the post-I/R limb; open circles indicate the non-stressed (control) limb. Mean endplate occupancy decreased slowly with age in non-stressed EDLs of SOD1-G93A/YFP mice (dashed line, slope = $-0.004 \pm 0.0007$, significantly different from zero, linear trend test). In these mice the I/R stress greatly decreased EDL occupancy beginning at ~P30; the exponential fit to these points (solid line) had a time constant of 9.8 days. In contrast, in SOD1-G93A/YFP soleus and in all YFP-only muscles, the slopes of fitted linear regression lines (continuous for stressed, dashed for non-stressed) were not significantly different from zero, indicating maintenance of innervation throughout the sampled age range. In all cases the ischemic stress lasted 30 min; reperfusion times varied from 6–24 hr. Each symbol indicates the mean endplate occupancy computed from 30–80 endplates in the indicated muscle from an individual mouse. Data came from 11 SOD1-G93A/YFP and 16 YFP-only mice aged 24–121 days.
stressed and stressed EDL and soleus remained high (>70%) for all ages examined (P24–P120, Fig. 2.2Aa, Ba).

Thus over the tested age range, most motor terminals in SOD1-G93A/YFP soleus showed no apparent damage following an I/R stress that denervated most endplates in EDL. The increased vulnerability to I/R damage in EDL motor terminals of SOD1-G93A/YFP mice became evident weeks before the onset of significant endplate denervation in the contralateral non-stressed EDL, suggesting that increased vulnerability to I/R injury may be an early functional sign of impending motor terminal degeneration.

To test whether increased vulnerability to I/R injury is a general feature of SOD1-G93A hindlimb fast muscles or just a peculiar feature of EDL terminals, we performed two additional experiments that included analysis of plantaris, another predominantly fast hindlimb muscle rich in type IIb fibers (SOD1-G93A mice, P50–52, 46–99 endplates sampled in each muscle). In one mouse the 30 min I/R stress denervated 96% of endplates in EDL, 90% in plantaris, and 7% in soleus. In the other mouse this stress denervated 22% of endplates in EDL, 33% in plantaris, and none in soleus. Denervation in non-stressed limbs was 1%. The different magnitudes of endplate denervation measured in these two mice probably reflects the fact that a 30 min I/R stress falls near the steepest and most variable region of the relationship between ischemia duration and endplate occupancy for EDL (see Fig. 2.3A, described below). But in both mice the magnitude of the I/R-induced damage in plantaris was similar to that in EDL and more extensive than that in soleus. This result suggests that increased
Fig. 2.3  Effect of ischemia duration on endplate occupancy in EDL (A) and soleus (B) of SOD1-G93A/YFP and YFP-only mice following I/R. Each data set was fitted with a sigmoidal equation (see Materials and Methods) to calculate the ischemia duration at which mean endplate occupancy would decrease to 50% of maximal (ID$_{50}$). ID$_{50}$ values (± SEM) for EDL were 45 ± 0.5 min in YFP-only vs. 26 ± 1 in SOD1-G93A/YFP; comparable values for soleus were 44 ± 0.4 and 48 ± 1. All of the following differences were significant at p<0.05 or better (t-test): SOD1-G93A/YFP vs. YFP-only for both EDL and soleus, and YFP-only EDL vs. YFP-only soleus. All curves used maximal and minimal occupancy values of 0.88 and 0, respectively, and a slope of -8.0; the R$^2$ for all fits exceeded 0.98. Values for 0 min ischemia came from non-stressed muscles. Each point was derived from measurements in 2–14 muscles, except for the 55 min point from a single animal in which the tourniquet was removed after 45 min ischemia, but limb blood flow did not return until 10 min later. More mice were used for ischemia durations that straddled the transitions between maintained innervation and complete denervation, where variability was greatest. In some cases the SEM was smaller than the plotting symbol.

Vulnerability to I/R stress is a general property of fast hindlimb muscles in SOD1-G93A mice. Fig. 2.3 plots how the duration of ischemia (0–60 min) affected mean endplate occupancy. Results were fitted with a sigmoidal curve that enabled estimation of the ischemia duration expected to reduce mean endplate occupancy to 50% of that measured in non-stressed muscles (ID$_{50}$, see Materials and Methods). For EDL the ID$_{50}$ fell from 45 min in YFP-only to 26 min in SOD1-G93A/YFP, a 42% decrease (Fig. 2.3A). For soleus the SOD1-G93A mutation
had only a small effect on the ID$_{50}$ (48 min in YFP-only, 44 min in SOD1-G93A/YFP, Fig. 3B). These measurements thus suggest that in SOD1-G93A/YFP mice >P35, motor terminals innervating fast EDL have much less tolerance for I/R stress than those innervating slow soleus. In contrast, the stress tolerance of these fast and slow muscles is similar in YFP-only mice, resembling that measured in SOD1-G93A/YFP soleus.

*Post-I/R denervation of SOD1-G93A endplates is not correlated with endplate area, and is not duplicated by axotomy at the tourniquet site*

Endplate areas in EDL varied over a wide range, from <100 to >600 um$^2$. To test whether the area of an endplate influenced its tendency to lose innervation following an I/R stress, we plotted endplate occupancy vs. area for all post-I/R SOD1-G93A/YFP endplates. The slope of the regression line fitted to these data was not significantly different from zero, suggesting that endplate area did not influence post I/R denervation.

Fig. 2.4 shows results of an experiment to test whether the damage to EDL SOD1-G93A/YFP motor terminals illustrated in Figs. 2.1-2.3 might have been due to mechanical injury of axons at the tourniquet site rather than to I/R injury. The sciatic nerve innervating one hindlimb was cut at the level of the sciatic notch; the other hindlimb was subjected to the standard tourniquet-
Fig. 2.4  Axotomy does not duplicate the effects of I/R on EDL endplate occupancy in a SOD1-G93A/YFP mouse. Cumulative histograms of endplate occupancies were measured in EDL muscles of a P85 mouse in which one hindlimb was subjected to 30 min ischemia followed by 6 hr reperfusion (I/R, filled circles), and the other hindlimb underwent axotomy at the sciatic notch (open circles), which is near the upper hindlimb region where tourniquets were applied. 30–37 endplates were analyzed in each muscle. Comparable measurements for soleus in both axotomized and post-I/R limbs overlapped with those for post-axotomy EDL (not shown).

induced 30 min ischemia followed by 6 hr reperfusion. The cumulative histogram occupancy shows that in the axotomized limb EDL retained a high mean occupancy, in contrast to the almost complete denervation of EDL after the I/R stress. Thus the rapid post-I/R denervation of EDL endplates in SOD1-G93A/YFP mice did not result from axonal injury at the tourniquet site.
Post-I/R denervation of EDL SOD1-G93A endplates develops rapidly

Fig. 2.5 shows results from an experiment to estimate the time course of motor terminal degeneration following the onset of reperfusion. EDLs from SOD1-G93A/YFP and YFP-only mice were subjected to 30 min ischemia in vivo, then reperfused, dissected, transferred to oxygenated mouse saline and imaged over time. Thus in this experiment reperfusion began in vivo, and re-oxygenation continued in vitro during and following dissection. The YFP-only terminal showed no signs of degeneration 35 or 100 min after reperfusion began (compare a and b in Fig. 2.5A), but over this same time interval the SOD1-G93A/YFP terminal fragmented and lost continuity with the preterminal axon (Fig. 2.5Ba,b). Filled circles in Fig. 2.5Bc plot the post-ischemic time course of the decay of total fluorescence in the SOD1-G93A/YFP terminal, demonstrating the greatest loss between ~80–120 min following the onset of reperfusion. The post-I/R terminal degeneration in the in vivo experiments of Figs. 2.1-2.4 was likely also complete within this interval or even sooner, because post-I/R mean endplate occupancies measured in SOD1-G93A/YFP terminals showed no significant correlation with in vivo reperfusion times ranging from 1–24 hr (1 hr, n = 2 muscles; 6 hr, n = 7; 24 hr, n = 2; Pearson correlation test, two-tailed, p = 0.16).
Fig. 2.5  SOD1-G93A/YFP EDL motor terminal degenerates rapidly following post-ischemic reperfusion/reoxygenation. Fluorescent micrographs of motor terminals from P76 YFP-only (A) and P69 SOD1-G93A/YFP (B) EDLs that were subjected to 30 min ischemia in vivo. After tourniquet removal, muscles were dissected and placed in physiological saline equilibrated with 95% O₂/5% CO₂ at 32–34 °C. Images taken 35 min and 100 min following conclusion of the ischemic stress show that the YFP-only terminal remained intact (Aa,b), whereas the SOD1-G93A/YFP terminal broke apart (Ba,b). Dotted lines in Ba outline the preterminal axon at 35 min; this region had disappeared by 100 min (Bb). Arrowheads in B indicate a section of the terminal that was continuous at 35 min, but became fragmented by 100 min. Bc, filled circles show the time course of fluorescence decay in the SOD1-G93A/YFP terminal; open circles show that the fluorescence of a neighboring background region did not change. The plot begins at 30 min, the time required to dissect and mount the muscle. Note that this graph measures total YFP fluorescence (arbitrary units); YFP measurements made for all other figures included only those sections of the motor terminal that remained connected to the preterminal axon. Similar results were obtained in 2 additional post-I/R SOD1-G93A/YFP terminals and 3 additional post-I/R YFP-only terminals.
EDL motor terminals in SOD1-G93A mice can resprout following I/R injury

Pun et al. (2006) reported that after a proximal peripheral nerve crush in SOD1-G93A mice, axons could reinnervate fast muscle fibers if the crush occurred at P30, but not if the crush occurred at P38, suggesting that fast motor axons lose their ability to resprout at an early age. Thus we wondered whether endplates in SOD1-G93A/YFP EDL could become reinnervated following an I/R stress sufficient to denervate endplates. Fig. 2.6 shows results from an experiment in which the EDL in one hindlimb of a P65 SOD1-G93A/YFP mouse underwent a 30 min ischemic stress followed by 10 days of reperfusion. Almost all endplates in the post-I/R EDL were innervated, and at least some of this innervation represented re-innervation of formerly denervated endplates because a 30 min I/R stress denervates many EDL endplates in SOD1-G93A mice (Fig. 2.3A). Another sign of reinnervation are the numerous nerve sprouts extending beyond the α-BgTx-delimited endplate region in the post-I/R EDL (arrowheads, e.g. Brown and Ironton, 1978; Brown et al. 1980). Thus at least some of the SOD1-G93A motor axons innervating this fast muscle can resprout following I/R-induced degeneration of their motor terminals.

To determine whether post-I/R reinnervation could also occur in EDLs from older SOD1-G93A/YFP mice, we administered the standard 30 min I/R stress to one hindlimb in each of two P89 littermates, and measured endplate innervation in 10 days later at P99. The fraction of fully occupied EDL endplates in the stressed limb was 47 ± 5.5% (SEM), significantly less than that in the non-stressed limb (93 ± 1.4%, p < 0.001, Dunn’s multiple comparison test applied to
measurements from endplates in 15–20 microscope fields, each containing 5–20 endplates). Sprouts were present in 14 ± 1.9% of the post-I/R endplates, compared to 5.3 ± 1.8% in the non-stressed limb (p < 0.001). These data suggest that even at this relatively late age, at least some EDL axons in SOD1-G93A mice remained capable of reinnervating denervated endplates. However, this reinnervation was incomplete compared to the near complete reinnervation measured in the younger mouse of Fig. 2.6.
Fig. 2.6  EDL endplates in a SOD1-G93A/YFP mouse are reinnervated 10 days following a 30 min I/R stress. Micrographs are overlays of α-Bgtx-labelled endplates (red) and YFP-labelled motor axons (green) from non-stressed (left) and stressed (right) hindlimb. Ischemia was applied at P65. Arrowheads indicate terminal sprouting that extended beyond labelled endplates.

E. Discussion

In SOD1-G93A mice the motor terminals innervating fast hindlimb muscles become especially vulnerable to ischemia/reperfusion (I/R) injury at an early age.

Our results demonstrate that in male mice expressing the G93A mutation of SOD1 the motor terminals innervating two predominantly fast hindlimb muscles, EDL and plantaris, were more vulnerable to I/R injury than terminals in the same muscles of wild-type mice. In contrast, the vulnerability of SOD1-G93A motor terminals innervating soleus muscle was similar to wild-type. The differential vulnerability of motor terminals innervating fast and slow muscles was associated with the SOD1-G93A mutation, because in wild-type mice the sensitivity to I/R injury was similar for EDL and soleus motor terminals. This increased vulnerability of EDL motor terminals to I/R injury in male SOD1-G93A mice began as early as P31, well before the onset of motor neuron death.

The increased vulnerability to I/R injury developed before the onset of degeneration of motor terminals innervating non-stressed EDL muscles (Fig. 2.2Ab), and thus might reflect a stress-induced acceleration of the disease-related degeneration of motor terminals in fast muscles. This motor terminal degeneration is consistent with the early, selective decline in the number of the most forceful (type IIb) motor units in fast-twitch hindlimb muscles reported in
SOD1-G93A mice (Hegedus et al. 2006). Differential denervation of endplates may account for Derave et al.’s (2003) finding of atrophy in EDL but not soleus of SOD1-G93A mice.

The literature suggests several possible reasons why the G93A mutation of SOD1 might make motor terminals innervating fast muscles more vulnerable than those innervating slow muscles. Fast-fatiguable (FF) motor neurons might be more vulnerable because they innervate more muscle fibers than other motor neuron types (Fahim et al., 1984). The fast muscle fibers specified by FF innervation have lower basal blood flows (review by Burke, 2004) and a lower basal expression of vascular endothelial growth factor (VEGF, an angiogenic and motor neuron trophic factor, Annex et al., 1998; Lambrechts et al., 2003; Storkebaum et al., 2005). Fast muscles release more H₂O₂ than slow muscles (Capel et al., 2004), and SOD1-G93A expression increases production of toxic hydroxyl radicals from H₂O₂ (Yim et al., 1996, 1999). Fast muscles also exhibit higher concentrations of neuronal nitric oxide synthase than slow muscles (Grozdanovic, 2001). Expression of SOD1-G93A sensitizes cultured spinal motor neurons to nitric oxide-induced death (Raoul et al., 2002, 2006).

Could stress-induced damage to motor nerve terminals contribute to motor neuron death in SOD1-G93A mice?

The I/R stress applied here did not kill all EDL motor neurons in SOD1-G93A mice, because EDL endplates were reinnervated within 10 days after a stress administered at P65 (Fig. 2.6). But repeated bouts of I/R stress might permanently damage motor neurons, since evidence from developmental and
reinnervation studies in wild-type rodents indicates that motor neurons become especially sensitive to stress-induced damage during axonal regeneration (reviewed in Sharp et al., 2005). These authors found that crushing a hindlimb nerve, an injury also followed by regeneration of motor terminals, sped the time course of motor neuron death in SOD1-G93A mice. Even if motor neurons remain alive, a peripheral I/R stress could hasten functional disease progression if motor axons fail to reinnervate previously denervated endplates. We found reduced reinnervation of EDL terminals in SOD1-G93A mice stressed at an older age (P89).

If I/R injury to motor terminals can contribute to motor neuron death and/or persisting functional denervation in fALS mice, then one might predict that a history of intense, anaerobic exercise might correlate with more rapid disease progression. Consistent with this idea, in SOD1-G93A mice intense exercise is reported to accelerate disease progression (Mahoney et al. 2004), though moderate exercise tends to slow disease progression (e.g. Kirkinezos et al. 2003; results in males and females sometimes differ). Another circumstance that might predispose to peripheral I/R stress is inability to increase blood flow to exercising muscle. Consistent with this idea, there is selective motor neuron degeneration in mice lacking the hypoxia response element in the VEGF gene (Oosthuyse et al. 2001), and crossing these mice with SOD1-G93A speeds disease progression (Lambrechts et al. 2003). Murakami et al. (2003) found that SOD1-G93A expression impairs hypoxia-induced VEGF expression in presymptomatic mice. Reperfusion following ischemia imposes an oxidative stress, and muscles in
SOD1-G93A mice exhibit upregulation of antioxidant enzymes, which might reflect compensation for oxidative stress (Mahoney et al., 2006).

If motor terminals are a significant site of motor neuron damage in fALS mice, then disease onset and/or progression might be slowed by protecting motor terminals. The protective effects of neurotrophic factors expressed by viral constructs injected into SOD1-G93A skeletal muscles have traditionally been attributed to retrograde transport of the trophic factor to motor neuron somata rather than to locally-mediated effects on motor terminals (e.g. Kaspar et al. 2003), but local effects on motor terminals might contribute to the findings that glial-derived neurotrophic factor is more protective when expressed in muscle than when expressed in astrocytes (Li et al. 2006), and that muscular expression of non-retrogradely transported VEGF is also neuroprotective (Lafuste et al. 2006). Also consistent with the idea that peripheral events contribute to disease onset/progression in fALS mice is the finding that protection of motor neuron somata (via deletion of the apoptotic protein BAX) is not sufficient to prevent death of SOD1-G93A mice (Gould et al. 2006).

In sum, our demonstration that motor terminals innervating fast hindlimb muscles in male SOD1-G93A mice become especially susceptible to I/R injury at a young age, combined with other findings cited above, suggests that damage to motor terminals deserves serious consideration as a source of motor neuron damage in fALS mice and possibly in human ALS.
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A. Summary

The electrical gradient across the mitochondrial inner membrane ($\Psi_m$) is established by electron transport chain (ETC) activity and permits mitochondrial Ca$^{2+}$ sequestration. Using rhodamine-123, we determined how repetitive nerve stimulation (100 Hz) affects $\Psi_m$ in motor terminals innervating mouse levator auris muscles. Stimulation-induced $\Psi_m$ depolarizations in wild-type (WT) terminals were small (<5 mV at 30 °C) and reversible. These depolarizations depended on Ca$^{2+}$ influx into motor terminals, as they were inhibited when P/Q-type Ca$^{2+}$ channels were blocked with $\omega$-agatoxin. Stimulation-induced $\Psi_m$ depolarization and elevation of cytosolic [Ca$^{2+}$] both increased when complex I of the ETC was partially inhibited by low concentrations of rotenone (25–50 nmol/l). This finding is consistent with the hypothesis that acceleration of ETC proton extrusion normally limits the magnitude of $\Psi_m$ depolarization during mitochondrial Ca$^{2+}$ uptake, thereby permitting continued Ca$^{2+}$ uptake. Compared with WT, stimulation-induced increases in rhodamine-123 fluorescence were ≈5 times larger in motor terminals from presymptomatic mice expressing mutations of human superoxide dismutase I (SOD1) that cause familial amyotrophic lateral sclerosis (SOD1-G85R, which lacks dismutase activity; SOD1-G93A, which
retains dismutase activity). $\Psi_m$ depolarizations were not significantly altered by expression of WT human SOD1 or knockout of SOD1 or by inhibiting opening of the mitochondrial permeability transition pore with cyclosporin A. We suggest that an early functional consequence of the association of SOD1-G85R or SOD1-G93A with motoneuronal mitochondria is reduced capacity of the ETC to limit Ca$^{2+}$-induced $\Psi_m$ depolarization, and that this impairment contributes to disease progression in mutant SOD1 motor terminals.

B. Background

Mitochondria sequester significant amounts of stimulation-induced Ca$^{2+}$ loads in many cell types (Friel and Tsien, 1994; Stuenkel et al., 1994; Herrington et al., 1996; Babcock et al., 1997; David et al., 1998; Pivovarova et al., 1999; Kaftan et al., 2000; Suzuki et al., 2002; David and Barrett, 2003). This mitochondrial Ca$^{2+}$ uptake occurs via a Ca$^{2+}$ uniporter/channel (Kirichok et al., 2004) down a potential gradient ($\Psi_m$, 150–200 mV, matrix negative) established by ETC activity (Gunter and Pfeiffer, 1990; Nicholls and Chalmers, 2004). Entry of Ca$^{2+}$ into mitochondria depolarizes $\Psi_m$, which would be expected to reduce the gradient driving Ca$^{2+}$ uptake. However, in motor nerve terminals, mitochondrial Ca$^{2+}$ uptake continues throughout prolonged trains of action potentials (David, 1999). One possible explanation for this apparent paradox is that the $\Psi_m$ depolarization produced by Ca$^{2+}$ entry reduces the gradient against which ETC complexes I, III and IV extrude protons, thus accelerating ETC proton
extrusion (Nicholls and Ferguson, 2002). This acceleration would limit the net $\Psi_m$ depolarization, thereby allowing mitochondria to continue taking up $\text{Ca}^{2+}$ even during prolonged stimulation (Talbot et al., 2007). We tested this hypothesis in mouse motor terminals, and found that the $\Psi_m$ depolarizations produced by repetitive stimulation at 50–100 Hz were $\text{Ca}^{2+}$ dependent and reversible, and were small (or undetectable) at near-physiological temperatures (30 °C). Partially inhibiting ETC activity with low concentrations of rotenone or low temperature increased the amplitude of these depolarizations.

We also tested motor terminals in transgenic mice expressing mutant versions of human SOD1 (G85R, G93A) that produce familial amyotrophic lateral sclerosis (fALS) (Gurney et al., 1994; Chiu et al., 1995; Bruijn et al., 1997). In these and other transgenic mice expressing fALS-inducing SOD1 mutations, motor terminals begin to degenerate well before motor neuron somata in the spinal cord begin to die, and some of the earliest reported morphological changes occur in motor terminal mitochondria (Kong and Xu, 1998; Frey et al., 2000; Fischer et al., 2004; Schaefer et al., 2005; Gould et al., 2006; Pun et al., 2006). Misfolded fALS-linked mutant SOD1s associate with mitochondria (cytoplasmic face, intermembrane space, inner membrane, matrix) (Mattiazzi et al., 2002; Higgins et al., 2003; Liu et al., 2004; Sasaki et al., 2004; Vijayvergiya et al., 2005; Ferri et al., 2006; Goldsteins et al., 2008; Kawamata et al., 2008; Ahtoniemi et al., 2008; Vande Velde et al., 2008), and impaired ETC activity has been reported in the spinal cord of mice expressing SOD1-G93A (Jung et al., 2002; Mattiazzi et al., 2002; Kirkinezos et al., 2005; Goldsteins et al., 2008; Son
et al., 2008). Damiano et al. (2006) demonstrated a reduction in Ca$^{2+}$ loading capacity in spinal cord mitochondria from both SOD1-G93A and SOD1-G85R mice. We hypothesized that this reduced ability to sequester Ca$^{2+}$ loads might be caused by reduced ability to accelerate mitochondrial proton extrusion in response to the $\Psi_m$ depolarization produced by Ca$^{2+}$ entry. Consistent with this hypothesis, we demonstrate that at 30 °C stimulation-induced $\Psi_m$ depolarizations are greater in SOD1-G85R and SOD1-G93A mice than in WT mice, and that this increase does not depend on dismutase activity. This is the first demonstration in presymptomatic fALS mice of an altered response to Ca$^{2+}$ loads in mitochondria located exclusively within motor neurons (as distinct from surrounding glial cells).

C. Results

*During repetitive stimulation at near-physiological temperatures, $\Psi_m$ depolarization is small.*

Fig. 3.1 shows representative changes in cytosolic [Ca$^{2+}$], mitochondrial [Ca$^{2+}$], and $\Psi_m$ recorded in WT motor nerve terminals stimulated with three trains of action potentials (100 Hz, 5 seconds). The topmost trace shows the elevation of cytosolic [Ca$^{2+}$], measured as the normalized increase in fluorescence (F/F$_{rest}$) of Oregon Green 488 BAPTA-5N (OG-5N) that had been ionophoretically injected into the axoplasm. After an initial rapid increase at the onset of stimulation, the rate of rise slows, due mainly to Ca$^{2+}$ sequestration by mitochondria (David et al., 1998; David and Barrett, 2003). When stimulation stops, cytosolic [Ca$^{2+}$] shows a rapid initial decrease. Measurements of cytosolic
[Ca$^{2+}$] made with a higher affinity Ca$^{2+}$ indicator than that used here reveal an additional slowly-decaying phase caused in part by Ca$^{2+}$ extrusion from mitochondria via a Na$^+$/Ca$^{2+}$ exchanger (Garcia-Chacon et al., 2006).

Fig. 3.1. Stimulation at 100 Hz increases cytosolic and mitochondrial [Ca$^{2+}$] and depolarizes $\Psi_m$ in WT mouse motor terminals at 30 °C. (A) Upper trace: Elevation of cytosolic [Ca$^{2+}$] in response to three stimulus trains at 100 Hz, monitored as normalized increases (F/F$_{rest}$) in the fluorescence of intra-axonally injected OG-5N (vertical lines indicate duration of stimulation). The average increase in cytosolic [Ca$^{2+}$] is 1.0 μmol/l above an assumed resting value of 0.1 μmol/l (David et al., 2000). Second trace: Elevations of mitochondrial matrix [Ca$^{2+}$], monitored as increases in the fluorescence of mitochondrially-loaded X-rhod-1 (mean of five traces). The average increase in mitochondrial [Ca$^{2+}$] is $\approx$1–2 μmol/l above an assumed resting value of 0.05–0.1 μmol/l (David, 1999; David
et al., 2003; Chalmer and Nicholls, 2003). Third trace: Depolarization of $\Psi_m$, monitored as increases in the fluorescence of Rh-123. The line is a smoothed moving bin average of three neighboring points. Lower trace shows (using a different vertical scale) these same data, along with the much larger $\Psi_m$ depolarization produced in this terminal by the proton carrier CCCP (2 μmol/l). Cytosolic [Ca$^{2+}$], mitochondrial [Ca$^{2+}$], and $\Psi_m$ were recorded from different preparations, aged 64–88 days.

The second trace in Fig. 3.1 shows the stimulation-induced increase in mitochondrial [Ca$^{2+}$], measured as the fluorescence of mitochondrially-loaded X-rhod-1. Mitochondrial [Ca$^{2+}$] exhibits a slower rate of rise and a slower decay than cytosolic [Ca$^{2+}$] (David et al., 1998; Garcia-Chacon et al., 2006). Mitochondrial [Ca$^{2+}$] responses evoked by the second and third stimulus trains begin during the decaying phase of the response to previous trains, but the peak fluorescence evoked by subsequent trains does not exceed that evoked by the first train. This “cap” on the maximal increase in mitochondrial [Ca$^{2+}$] is not due to saturation of the indicator dye (David et al., 2003), and is unlikely to be caused by decreased mitochondrial Ca$^{2+}$ entry during subsequent trains, because both the sustained elevation of cytosolic [Ca$^{2+}$] and recordings of transmitter release indicate that action potentials continue to admit Ca$^{2+}$ into the cytosol throughout the train (David and Barrett, 2003; Garcia-Chacon et al., 2006), and the increase in cytosolic [Ca$^{2+}$] produced by each train is similar. Also, if mitochondrial Ca$^{2+}$ sequestration is blocked, cytosolic [Ca$^{2+}$] rises progressively during stimulation (David et al., 1998; David and Barrett, 2003). Rather, the maximal increase in matrix [Ca$^{2+}$] is limited by formation of an insoluble complex containing Ca and phosphate that reversibly sequesters matrix Ca$^{2+}$ (Chalmers and Nicholls, 2003).
The maximal increase in mitochondrial [Ca^{2+}] is only ≈1–2 μmol/l (David, 1999; David et al., 2003; Chalmers and Nicholls, 2003).

The third trace in Fig. 3.1 shows stimulation-induced depolarizations of \( \Psi_m \), recorded as increases in rhodamine 123 (Rh-123) fluorescence. In this motor terminal, each stimulus train reversibly increased fluorescence by ≈2%.

The bottom trace shows these same data on a different vertical scale, along with the much larger increase in Rh-123 fluorescence (≈140%) measured during the near-complete \( \Psi_m \) depolarization produced by the proton carrier carbonyl cyanide \( m \)-chloro phenyl hydrazone (CCCP, 2 μmol/l). At 28–30 °C the mean increase in Rh-123 fluorescence after 500 stimuli at 100 Hz was only 0.92% ± 0.13% (SEM, range 0–4.5%, \( n = 47 \) terminals). These measurements are described further in SI Text Item #1 and Fig. S3.1), including analysis suggesting that this fluorescence increase corresponds to a \( \Psi_m \) depolarization of 3–5 mV.

**Stimulation-induced \( \Psi_m \) depolarizations are Ca^{2+} dependent.**

To study mechanisms underlying stimulation-induced \( \Psi_m \) depolarizations, we needed to increase the magnitude of the recorded Rh-123 signal. Fig. 3.2A demonstrates that signal magnitude increased when the temperature was reduced, or when action potential duration (and thus Ca^{2+} entry) was prolonged using 3,4-diaminopyridine (3,4-DAP, 20 μmol/l), which blocks certain depolarization-activated K^+ channels in motor axons and terminals (Tabti et al., 1989; Morita and Barrett, 1990; David et al., 1995).
Fig. 3.2B shows that stimulation-induced $\Psi_m$ depolarizations were inhibited both by replacing bath Ca$^{2+}$ with Mg$^{2+}$ and by $\omega$-agatoxin TK (0.6 μmol/l), which blocks the P/Q-type (Cav2.1) Ca$^{2+}$ channels that mediate most Ca$^{2+}$ entry into motor terminals in mice (Teramoto et al., 1993; Westenbroek et al., 1998). Thus the Na$^+$ influx associated with axonal action potential propagation, which continues in low [Ca$^{2+}$] and $\omega$-agatoxin, is not by itself sufficient to produce stimulation-induced $\Psi_m$ depolarizations. Rather, these depolarizations require Ca$^{2+}$ influx into motor terminals.
Fig. 3.2. The stimulation-induced \( \Psi_m \) depolarization increases with cooling or addition of 3,4-diaminopyridine (3,4-DAP) and requires \( \text{Ca}^{2+} \) entry through plasma membrane \( \text{Ca}^{2+} \) channels. (A) The \( \Psi_m \) depolarization produced by 100 Hz stimulation at 30 °C (left) was increased by cooling to 18 °C (upper right) or by prolonging the action potential with 20 \( \mu \text{mol/l} \) 3,4-DAP (lower right). (B) \( \Psi_m \) depolarizations (the magnitudes of which were enhanced by both cooling to 20 °C and 3,4-DAP, open circles) were reduced by omitting \( \text{Ca}^{2+} \) from the bath (filled circles, left) or (in a different terminal) by adding 0.6 \( \mu \text{mol/l} \) \( \omega \)-agatoxin-TK (filled circles, right). The effects of low bath \( [\text{Ca}^{2+}] \) were readily reversible; reversal of agatoxin effects was slow and incomplete. Each record in (A) and (B) is the mean of two to nine traces. The effects of cooling, 3,4-DAP, and removal of bath \( \text{Ca}^{2+} \) were observed in 10, 5, and 4 additional terminals, respectively. Exposures to 3,4-DAP and agatoxin were 7–62 minutes and 60 minutes, respectively.

The elevation of cytosolic \( [\text{Ca}^{2+}] \) increases with increasing frequencies of stimulation (David et al., 1998) (Fig. S3.2A). In isolated mitochondria, \( \text{Ca}^{2+} \) influx through the uniporter/channel exhibits a greater-than-linear dependence on external \( [\text{Ca}^{2+}] \) (Gunter and Pfeiffer, 1990). Thus one would predict faster \( \Psi_m \) depolarization with higher stimulation frequencies. As predicted, \( \Psi_m \) depolarized more rapidly during 100 Hz than during 25 Hz stimulation (Fig. S3.2B).

Partial inhibition of ETC complex I increases the stimulation-induced \( \Psi_m \) depolarization.

Results in Fig. 3.2 link stimulation-induced \( \Psi_m \) depolarizations to \( \text{Ca}^{2+} \) entry into motor terminals but do not prove a linkage to \( \text{Ca}^{2+} \) influx into mitochondria. It is difficult to block mitochondrial \( \text{Ca}^{2+} \) influx selectively, because Ru360, which blocks the uniporter/channel (Matlib et al., 1998), has limited permeability across plasma membranes and appears to reduce \( \text{Ca}^{2+} \) influx across motor terminal membranes (David, 1999) (Fig. S3.3). Fig. 3.3B and 3.3C link stimulation-induced \( \Psi_m \) depolarizations to mitochondrial function by
demonstrating that partial inhibition of complex I of the ETC with low concentrations of rotenone (25–50 nmol/l) produced a 4-fold increase in the Rh-123 signal evoked by 100 Hz stimulation. This result is consistent with the prediction that reducing mitochondrial ability to accelerate ETC activity in response to stimulation-induced Ca\(^{2+}\) influx will increase the magnitude of the resulting \(\Psi_m\) depolarization. In cell lines, neurons, and isolated nerve terminals, these low rotenone concentrations have been reported to decrease complex I activity by 20–85% but to have little effect on resting \(\Psi_m\) (Higgins and Greenamyre et al., 1996; Barrientos and Moraes et al., 1999; Sipos et al., 2003; Li et al., 2003; Kweon et al., 2004; Kilbride et al., 2008). Likewise, we found that these low rotenone concentrations had no significant effect on resting Rh-123 fluorescence, suggesting little or no effect on resting \(\Psi_m\) (SI Text Item #2).

Greater \(\Psi_m\) depolarization during stimulation would be predicted to reduce the electrical gradient that permits Ca\(^{2+}\) influx into mitochondria and thus increase the elevation of cytosolic [Ca\(^{2+}\)]. Fig. 3.3A demonstrates the predicted increase of cytosolic [Ca\(^{2+}\)] in rotenone.

**Fig. 3.3** (following page) A low concentration of rotenone increases stimulation-induced elevations of cytosolic [Ca\(^{2+}\)] and \(\Psi_m\) depolarizations. Cytosolic [Ca\(^{2+}\)] (A) and \(\Psi_m\) depolarizations (B) produced by three trains at 100 Hz before and after addition of rotenone (50 nM). [Ca\(^{2+}\)] and \(\Psi_m\) traces came from different terminals. (C) Paired data from eight terminals studied before and after rotenone exposure show that in mice expressing normal SOD1 (WT or human) rotenone increased the average change in Rh-123 fluorescence from 0.96% ± 0.22% (SEM) in control medium to 4.13% ± 1.18% in rotenone (* \(P < 0.05\)). Only measurements from the initial 100 Hz train were included in the averages. The duration of rotenone exposure was 17–30 minutes. Mice were 50–375 days old.
Stimulation-induced $\Psi_m$ depolarizations are enhanced in mice expressing mutant human superoxide dismutase 1.

If motor terminal mitochondria in presymptomatic SOD1-G85R and SOD1-G93A mice have a reduced ability to accelerate ETC activity (see Introduction), then one would predict a greater stimulation-induced $\Psi_m$ depolarization. Fig. 3.4A presents phase and resting Rh-123 fluorescence images of a terminal in a presymptomatic SOD1-G85R mouse. The difference image highlights those regions in which Rh-123 fluorescence increased during stimulation, showing signals specific to motor terminal mitochondria. Fig. 3.4B shows Rh-123
recordings from this SOD1-G85R mouse, as well as representative traces from presymptomatic SOD1-G93A and WT terminals. The bar graph in Fig. 3.4B shows that the average stimulation-induced increase in Rh-123 fluorescence in SOD1-G85R and SOD1-G93A motor terminals was ≈5-fold greater than that in WT terminals.

**Fig. 3.4.** Stimulation-induced \( \Psi_m \) depolarizations are increased in presymptomatic SOD1-G85R and SOD1-G93A mice. (A) Phase (left) and Rh-123 fluorescence (middle) images show a resting motor terminal in a 121-day-old SOD1-G85R mouse. At right is a difference image of the same region, calculated by subtracting the resting fluorescence from the fluorescence during 100 Hz stimulation. (B) Representative stimulation-induced \( \Psi_m \) depolarizations evoked by repeated brief 100 Hz trains in a WT mouse (mean of two traces), a mouse lacking SOD1 (SOD1-KO), an “overexpressor” mouse with both normal mouse and normal human SOD1 (SOD1-OX), the SOD1-G85R terminal in (A), and an
SOD1 helps to defend against oxidative damage by catalyzing the conversion of superoxide to hydrogen peroxide. SOD1-G93A retains SOD1 enzymatic activity, but SOD1-G85R lacks this activity (Valentine et al., 2005), although heterodimers of G85R and wt SOD1 show activity (Witan et al., 2008). The fact that both types of mutant SOD1 terminals showed increased $\Psi_m$ depolarizations suggests that this increase did not depend on the level of SOD1 activity. This suggestion was tested further by conducting similar experiments on mice lacking SOD1 activity altogether, and on mice with excess SOD1 activity due to expression of normal human SOD1. Fig. 3.4B shows that stimulation-induced $\Psi_m$ depolarizations in these knockout and overexpressing mice were not significantly different from those recorded in WT terminals.

Another possible mechanism underlying the increased stimulation-induced $\Psi_m$ depolarizations measured in mutant SOD1 mice is transient opening of the mitochondrial permeability transition pore (MPTP). However, Fig. 3.5 shows that...
cyclosporin A, which inhibits pore opening, did not reduce the magnitude of these depolarizations in SOD1-G85R mice.

Fig. 3.5. Stimulation-induced $\Psi_m$ depolarizations in SOD1-G85R mice are not reduced by cyclosporin A. (A) Representative traces show the Rh-123 fluorescence response evoked by a 100-Hz stimulus train before and 56 minutes after exposure to 5 $\mu$mol/l cyclosporin. (B) Graph plots peak amplitudes (normalized to resting fluorescence) before and during drug exposure for this terminal. Similar experiments on three additional SOD1-G85R mice (130–150 days) also showed no significant change in $\Psi_m$ depolarizations in cyclosporin A (paired Wilcoxon signed rank test, $P > 0.50$). Similar experiments in additional mouse types (e.g., SOD1 knockout treated with 50 $\mu$mol/l 3,4-diaminopyridine) confirmed the lack of effect of 8–10 $\mu$mol/l cyclosporin A.
D. Discussion

Determinants of the stimulation-evoked $\Psi_m$ depolarization in mouse motor terminals.

Data presented here demonstrate that the $\Psi_m$ depolarization produced by repetitive stimulation depends on Ca$^{2+}$ influx into motor terminals. In WT mice this $\Psi_m$ depolarization is small, reversible, and repeatable, in contrast to the large $\Psi_m$ depolarizations measured after applying large Ca$^{2+}$ loads to isolated mitochondria (Vergun and Reynolds, 2005). Some of the difference in the magnitude and reversibility of the Ca$^{2+}$-induced $\Psi_m$ depolarization may be due to the fact that the Ca$^{2+}$ load was delivered physiologically via repetitive nerve stimulation to mitochondria in situ. Isolated mitochondria can take up larger amounts of Ca$^{2+}$ with minimal $\Psi_m$ depolarization when the Ca$^{2+}$ load is presented in increments rather than as a bolus (Chalmers and Nicholls, 2003).

To determine whether the magnitude of the stimulation-induced $\Psi_m$ depolarization is consistent with electrical models of mitochondrial function, we estimated the current entering mitochondria using the initial slope of the mitochondrial [Ca$^{2+}$] response to stimulation (as in Fig 3.1). The depolarization expected from this current was then estimated using an electrical model of the mitochondrial inner membrane consisting of the resistance of the ETC (in series with a battery representing the electron motive force) in parallel with the resistance of the mitochondrial membrane (SI Text Item #4). These calculations yielded a predicted $\Psi_m$ depolarization of $\approx$2 mV, similar to the value estimated independently from the magnitude of the stimulation-induced change in Rh-123.
fluorescence (SI Text Item #1). $\Psi_m$ depolarizations of this magnitude are sufficient to accelerate ETC activity significantly (Johnson-Cadwell et al., 2007).

According to this electrical model of the mitochondrial membrane, the magnitude of the stimulation-induced $\Psi_m$ depolarization can be varied in at least two ways. The first is to alter the magnitude of the Ca$^{2+}$ current that enters mitochondria: this is the likely explanation for the changes in the magnitude of the $\Psi_m$ depolarization produced by reducing (with $\omega$-agatoxin) or increasing (with 3,4-DAP) the magnitude of the stimulation-induced Ca$^{2+}$ influx into the motor terminal. The second way is to change ETC activity; this is the likely mechanism underlying the increase in the stimulation-induced $\Psi_m$ depolarization produced by partial blockade of complex I with rotenone. Partial ETC inhibition produces a greater $\Psi_m$ depolarization in stimulated than in resting terminals (Talbot et al., 2007). Synergistic damaging effects of partial complex I inhibition and Ca$^{2+}$ challenges have also been documented in other preparations (Votyakova and Reynold, 2005; Kwong et al., 2007).

The larger stimulation-induced $\Psi_m$ depolarizations in SOD1-G85R and SOD1-G93A motor terminals likely reflect reduced ability to accelerate ETC activity after Ca$^{2+}$ entry.

The Introduction section cited morphological evidence for early mitochondrial damage in motor terminals and motor neurons of mutant SOD1 models of fALS, as well as functional evidence for reduced activity of certain ETC complexes and reduced capacity for Ca$^{2+}$ uptake in spinal cord mitochondria. Work presented here demonstrates that rotenone (low concentration) mimics the
effect of fALS mutations on stimulation-induced $\Psi_m$ depolarizations, and that these depolarizations are not reduced by cyclosporin A. Given these past and present findings, a logical explanation for the larger $\Psi_m$ depolarizations recorded in motor terminals of SOD1-G85R and SOD1-G93A mice is that their mitochondria have a defect in the ETC (or its regulation) that reduces their ability to accelerate ETC activity in response to the depolarization produced by Ca$^{2+}$ entry. Synaptic mitochondria are more sensitive than somatic mitochondria to both complex I inhibition and Ca$^{2+}$ overload (Davey et al., 1997; Brown et al., 2006). This might explain the finding that, in fALS mice, motor terminal damage precedes damage to the motor neuron cell body.

Deficits in mitochondrial Ca$^{2+}$ handling would be expected to be most evident in motor terminals innervating fast muscles, whose tendency to discharge in high frequency bursts (Burke, 2004) would expose them to larger Ca$^{2+}$ loads and $\Psi_m$ depolarizations than motor terminals innervating slow muscle. Consistent with this idea, motor terminals innervating fast limb muscles are the earliest to degenerate in fALS mice (Frey et al., 2000; Pun et al., 2006; Hegedus et al., 2008).

In summary, mitochondria have multiple features that permit them to sequester the Ca$^{2+}$ loads introduced by repetitive stimulation, including powerful Ca$^{2+}$ buffering within the matrix, and acceleration of ETC activity in response to the depolarization produced by Ca$^{2+}$ entry, hence preserving the electrical gradient that favors Ca$^{2+}$ entry. Evidence presented here suggests that the Ca$^{2+}$-dependent $\Psi_m$ depolarization produced by repetitive stimulation is $<5$ mV in WT
motor terminals, but increases in mitochondria of presymptomatic SOD1-G85R and SOD1-G93A terminals. These $\Psi_m$ depolarizations would be expected to increase in terminals of older mutant SOD1 mice as ETC function deteriorates, impairing both mitochondrial $\text{Ca}^{2+}$ sequestration and ATP synthesis.

**E. Materials and Methods**

Experiments used neuromuscular junctions from the levator auris longus muscle of WT, hSOD1-G85R, and hSOD1-G93A mice, as well as mice that express normal human SOD1 and mice lacking SOD1. Most experiments were conducted at 30 °C, the warmest temperature at which the dissected preparation could remain functional for several hours. Action potentials were evoked in the motor nerve by applying suprathreshold 0.3 ms depolarizing pulses (500–2000 at 50–100 Hz) via a suction electrode. Muscle contractions were blocked with d-tubocurarine (7–15 $\mu$mol/l).

Stimulation-induced changes in cytosolic $[\text{Ca}^{2+}]$ were monitored using OG-5N ($K_d$ 60 $\mu$mol/l) injected ionophoretically into an internodal axon (David and Barrett, 2000). Stimulation-induced changes in mitochondrial $[\text{Ca}^{2+}]$ were measured using the indicator X-rhod-1, loaded as described (Garcia-Chacon et al., 2006). Changes in $\Psi_m$ were measured using Rh-123, as described (Talbot et al., 2007). The preparation was imaged using a spinning disk confocal microscope system designed to use low excitation light intensities. Spatially averaged changes in fluorescence were plotted as $F/F_{\text{rest}}$, where $F_{\text{rest}}$ is the
average net, background-subtracted fluorescence calculated from 20–30 images obtained before stimulation began. SI Text Item #5 contains additional methodological details.

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F. Supporting Information

Item #1. Measurements included only terminals in which nerve conduction was verified by (i) clear, reversible, and repeatable increases in Rh-123 fluorescence during stimulation as in Fig. 3.1,(ii) the appearance of stimulation-evoked $\Psi_m$ depolarizations when experimental conditions (temperature, 3,4-DAP) were changed, and/or (iii) simultaneous measurement of increases in mitochondrial [Ca$^{2+}$] as illustrated in Fig. S3.1.

The quantitative relationship between changes in Rh-123 fluorescence and $\Psi_m$ depolarization was studied by Huang et al. (2007). Their Fig. 3.3A shows that between the resting $\Psi_m$ (~200 mV) and complete $\Psi_m$ depolarization Rh-123 fluorescence increases by 1.4- to 2.8 fold, depending on dye concentration. The increase in fluorescence we recorded after CCCP-
induced $\Psi_m$ depolarization falls within this range. Using their curves, the ~1% average increase in fluorescence we recorded during 500 stimuli at 100 Hz corresponds to a $\Psi_m$ depolarization of 3–5 mV.

**Fig. S3.1.** Simultaneous imaging of X-rhod-1 and Rh-123 fluorescences (mean of three traces) in a young WT mouse. In this terminal, 100 Hz stimulation produced a clear increase in mitochondrial $[\text{Ca}^{2+}]_{\text{mit}}$, but during the first 10 seconds any $\Psi_m$ depolarization was below the limit of detection.

**Item #2.** Summing together results from both WT mice and mice expressing normal human SOD1, the mean value of resting Rh-123 fluorescence was 378 ± 73 SEM (arbitrary fluorescence units) before rotenone application and 358 ± 52 after rotenone ($n = 8$ terminals in seven animals, not significantly different by a paired $t$ test). These results suggest that the low concentrations of rotenone used here (25–50 nmol/l) had no significant effect on prestimulation values of $\Psi_m$, consistent with findings in other neuronal preparations (Kweon et al., 2004). The partial inhibition of complex I produced by these low rotenone concentrations may have little effect on $\Psi_m$ because when mitochondria begin to
be depolarized they use ATP to rebuild $\Psi_m$, reversing the normal direction of the reaction catalyzed by the F1, F0-ATPase (complex V) (Nicholls and Ferguson, 2002). Also, block of complex I by rotenone might be partially bypassed by metabolites entering at complex II of the ETC. A caveat is that any small, slow depolarization produced by rotenone would have been difficult to distinguish from baseline noise.

With rotenone concentrations $>0.5 \mu$mol/l stimulation-induced changes in $\Psi_m$ became impossible to detect, probably because $\Psi_m$ was depolarized at rest, causing loss of Rh-123 from mitochondria.

We did not test the effects of rotenone on stimulation-induced changes in mitochondrial $[Ca^{2+}]$ because in motor terminals the peak amplitude of the mitochondrial $[Ca^{2+}]$ response appears to have no clear quantitative relationship to the magnitude of mitochondrial $Ca^{2+}$ entry (amplitude shows little or no variation with stimulation frequency, or over a range of non-zero bath $[Ca^{2+}]$) (David et al., 2003; Garcia-Chacon et al., 2006). The amplitude of the mitochondrial $[Ca^{2+}]$ plateau appears to depend more on processes associated with $Ca^{2+}$ buffering within the matrix, which involves formation of complexes containing $Ca$ and phosphate in an alkaline environment (Nicholls and Chalmers, 2004).

**Item #3.** Motor terminal areas and resting (prestimulation) values of spatially-averaged Rh-123 fluorescence were similar among the tested mouse groups. Areas were measured from projection images as described in David *et al.* (2007). Resting average Rh-123 fluorescence averaged 567 ± 136 (SEM,
arbitrary fluorescence units) in WT terminals, 368 ± 86 in mice expressing normal human SOD1, and 322 ± 31 in SOD1-G85R mice (n = 18 terminals in 14 WT mice, five terminals in four mice expressing normal human SOD1, and 39 terminals in four mice expressing SOD1-G85R). These values were not significantly different (Kruskal-Wallis test). There was no significant correlation between resting Rh-123 fluorescence and the stimulation-induced increase in fluorescence. These findings suggest that presymptomatic mutant SOD1 mice retain a normal complement of mitochondria, and that their different \( \Psi_m \) responses to nerve stimulation were not caused by differences in resting \( \Psi_m \).

**Item #4.** The following summarizes a method to calculate the \( \Psi_m \) depolarization predicted from the estimated rate of Ca\(^{2+} \) entry into motor terminal mitochondria. Calculations used values calculated for 1 mg of mitochondrial protein, with an estimated volume of 0.6–1 µl (Gunter and Pfeiffer, 1990).

The magnitude of the Ca\(^{2+} \) current entering motor terminal mitochondria during 100 Hz stimulation was estimated from the finding (Fig. 3.1) that mitochondrial [Ca\(^{2+} \)] increased to its plateau value (~1 µmol/l above resting value) (4, 8) within ~3 seconds. Even though mitochondrial [Ca\(^{2+} \)] remained at a plateau value during the remainder of the stimulation, we assume that Ca\(^{2+} \) continued to enter mitochondria because (i) sustained phasic evoked transmitter release suggests continued Ca\(^{2+} \) entry into the terminal (David, 1999; David and Barrett, 2003; Garcia-Chacon et al., 2006), and (ii) the fact that cytosolic [Ca\(^{2+} \)] remained at a plateau value throughout stimulation (David et al., 1998) (Fig. 3.1)
suggests that much of this entering Ca\(^{2+}\) was sequestered within mitochondria. Using a Ca\(^{2+}\) buffering capacity of 3000 (bound/free ratio) estimated for conventional (nonphosphate) matrix buffers (Babcock et al., 1997; Kaftan et al., 2000), a 1-µmol/l increase in matrix free [Ca\(^{2+}\)] within 3 seconds corresponds to a rate of increase of total mitochondrial Ca of \((3 \times 10^{-3} \text{ mol/l})/(3 \text{ s}) = 1 \times 10^{-3} \text{ mol/l/s}\). In a 1-µl volume, this rate of increase in Ca would correspond to \(1 \times 10^{-3} \text{ mol/l/s} \times 10^{-6} \text{ L} = 1 \times 10^{-9} \text{ moles/s}\). Multiplying by the Faraday (96,500 charges/mole) and by 2 for a divalent cation yields \(\sim 2 \times 10^{-4} \text{ coulombs/s} = 2 \times 10^{-4} \text{ amperes}\).

To calculate the predicted voltage drop, this current was applied to a simplified equivalent electrical circuit of the mitochondrial inner membrane (for 1 mg protein, see figure 8 in Talbot et al. (2007), which includes the resistance of the ETC (estimated at \(\sim 10 \Omega\) in normally respiring mitochondria, ref. 7) in series with an electron motive force (200 mV), in parallel with the resistance of the mitochondrial inner membrane (3000 \(\Omega\)) (Magnus and Keizer, 1997). Calculations using this simplified circuit yielded a predicted \(\Psi_m\) depolarization of \(\sim 2 \text{ mV}\).

In this model, the increased membrane conductance and \(\Psi_m\) depolarization produced by opening the uniporter will increase current through the ETC. Inhibition of ETC complexes (e.g., by rotenone) increases the resistance of the ETC and thereby increases the calculated \(\Psi_m\) depolarization.

**Item #5.** Methodological details: WT mice (C57BL/6) were bred from mice obtained from Jackson Laboratories (Bar Harbor, ME). hSOD1-G85R mice
(Tg(SOD1-G85R)148Dwc) were bred from mice kindly supplied by Don Cleveland (Ludwig Institute for Cancer Research, University of California at San Diego). Mice expressing normal human SOD1 (hSOD1-WT; B6SJLTg (SOD1)2Gur/J, were also supplied by Don Cleveland. The hSOD1-G93A mice (B6.Cg-Tg(SOD1-G93A)1Gur/J) and mice lacking SOD1 (B6.129S7-SOD1tm1Leb/J002972) were bred from founders obtained from Jackson Laboratories; the SOD1 knockout mice were kindly supplied by Carlos Moraes of the University of Miami. hSOD1-G85R mice have a copy number of 15, and exhibit disease onset at 8–10 months and end-stage/death at 9–11 months (Bruijn et al., 1997). hSOD1-G93A mice have a copy number of 25, and exhibit disease onset at 3–4 months and end-stage/death at 5 months (Gurney et al., 1994; Chiu et al., 1995). All SOD1 mutants were maintained in the C57BL/6 background by breeding with C57BL/6 mice for at least 10 generations.

The thin levator auris longus neuromuscular preparation is comprised mostly of fast muscle fibers (Angaut-Petit et al., 1987; Erzen et al., 2000). Mice were killed with 100% CO₂ using a procedure approved by the University of Miami Animal Care and Use Committee. The muscle with attached nerve was dissected and pinned flat in a chamber with silicon walls atop a No. 1 glass coverslip. The physiological saline contained (in mmol/l) NaCl 137, NaHCO₃ 15, KCl 4, CaCl₂ 1.8, MgCl₂ 1.1, glucose 11.2, and NaH₂PO₄ 0.33. For an experiment in Fig. 2B CaCl₂ was replaced with MgCl₂. The pH of the solution was kept near 7.4 by gassing the inside of the chamber.
with 5% CO₂ in 95% O₂. Temperature was adjusted by blowing hot air onto the underside of the chamber, and monitored with a thermistor.

D-Tubocurarine blocks muscle nicotinic acetylcholine (ACh) receptors and thereby prevents entry of Ca²⁺ into the muscle endplate via ACh receptor channels or depolarization-activated Ca²⁺ channels.

Oregon Green BAPTA-5N (OG-5N, Kd 60 µmol/l) and Oregon Green 488 BAPTA-1 (OG-1, Kd 0.17 µmol/l, Figs. S3.2 and S3.3) both have excitation/emission wavelengths of 488 nm and 535 nm, respectively. The ionophoretically injected, membrane-impermeable hexapotassium salt form diffused from the injected internodal axon into motor terminals.
Fig. S3.2. Increasing the stimulation frequency increases both the elevation of cytosolic $[\text{Ca}^{2+}]$ and the rate of $\Psi_m$ depolarization. (A) Superimposed cytosolic $[\text{Ca}^{2+}]$ elevations in a WT motor terminal given 1000 stimuli at frequencies ranging from 10–100 Hz. The $K_d$ of Oregon Green 488 BAPTA-1 (OG-1) is 0.17 µmol/l. (B) $\Psi_m$ depolarizations in a different terminal produced by three trains of stimulation at 100 Hz (upper) and by the same number of stimuli delivered at 25 Hz (lower). Each record in (B) is the mean of three to four traces. Other experiments demonstrate that mitochondrial $[\text{Ca}^{2+}]$ exhibits a faster rate of rise at higher frequencies, but its peak magnitude exhibits little or no dependence on stimulation frequency (David et al., 2003).
Ru360 reduces stimulation-induced elevations of cytosolic [Ca\textsuperscript{2+}] in mouse motor terminals (similar to its effect in lizard motor terminals) (8). (A) Stimulation-induced elevations in cytosolic [Ca\textsuperscript{2+}] before (blue) and after (red) exposure to 10 µm Ru360 in a WT terminal. Traces are the mean of four repetitions in control solution and during the second hour of exposure to Ru360, which was prepared from fresh stock kept on ice, and reapplied at ~15-minute intervals. Error bars indicate SEM. (B) Time course of changes in peak amplitude after exposure to Ru360. The observed decrease in cytosolic [Ca\textsuperscript{2+}] is opposite to the increase in cytosolic [Ca\textsuperscript{2+}] that would be predicted if the main effect of Ru360 had been to reduce mitochondrial Ca\textsuperscript{2+} uptake, and suggests instead that Ru360 reduced Ca\textsuperscript{2+} influx through the terminal’s P/Q-type channels. It should be noted that the control record in (A) illustrates both fast and slow components of the decay of cytosolic [Ca\textsuperscript{2+}]. We also tested the effects of Ru360 on Rh-123 and mitochondrial X-rhod-1 responses to stimulation. There were no detectable effects of nanomolar concentrations of Ru360, even after 2–4-hour incubations. Prolonged (2-hour) incubation with 2 µmol/l Ru360 reduced the Rh-123 signal by about 50% (not shown), but it was impossible to determine whether this effect was due to reduced Ca\textsuperscript{2+} influx into the terminal and/or to reduced Ca\textsuperscript{2+} influx into mitochondria.

The Kd of X-rhod-1 (0.7 µmol/l, excitation 568 nm, emission monitored with long-pass 590 nm filter, Chroma, Rockingham, VT) is appropriate because resting mitochondrial [Ca\textsuperscript{2+}] is estimated to be 0.05–0.1 µmol/l (Gunter and Pfeiffer, 1990) and the maximal increase in mitochondrial [Ca\textsuperscript{2+}] in repetitively stimulated motor terminals is ~1–2 µmol/l (David et al., 2003; David, 1999). After a 30-minute exposure to 25 µg/ml of the membrane-permeable acetoxy methane ester (AM) form of X-rhod-1, the preparation was washed with indicator-free saline solution. The AM moiety is cleaved by cytosolic and intramitochondrial esterases, converting the indicator into its charged, Ca\textsuperscript{2+}-binding form, and trapping it within the compartment in which the de-esterification occurred. When indicator is washed out of the bath, the indicator in terminal cytosol is diluted by diffusion out of the terminal into the
myelinated axon, so that most of the indicator remaining in the terminal is contained within mitochondria. Criteria used to verify mitochondrial localization of the indicator are described in (David, 1999).

Rh-123 is a cell-permeant, cationic, fluorescent dye that is sequestered by polarized mitochondria (excitation 488 nm, emissions monitored using a 535-nm filter, bandwidth 40 nm, Chroma). Loading for 30 minutes with 3 µg/ml (followed by rinsing with indicator-free saline) produced sufficient uptake of Rh-123 into the mitochondrial matrix to self-quench. Depolarization of $\Psi_m$ causes Rh-123 to leak out of mitochondria into the cytosol where Rh-123 becomes unquenched, producing an increase in fluorescence (Nicholls and Ward, 2000).

The experimental chamber was placed on the stage of an inverted microscope in a confocal system that included a Yokogawa spinning disk (Solamere, Salt Lake City, UT), a 60X water immersion lens (NA 1.2, Olympus), and a high-sensitivity Photometrics Cascade 512B CCD camera (Roper Scientific, Trenton, NJ) that enabled use of low excitation light intensities. Light sources were an argon-krypton laser with 488 and 568 nm excitation lines, and a monochromator (Photon Technology International, Birmingham, NJ). Series of images were obtained before, during, and after a train of action potentials. Interimage intervals were 1–3 seconds and exposure times were 0.8–2 seconds. Data were recorded using IP Laboratory v3.61 software (Scanalytics, Inc., Fairfax, VA) and analyzed using V++ software (Digital Micro Optics, Auckland, NZ) or ImageJ software. Variability caused by fluctuations in laser light intensity was minimized by correcting the recorded signal based on the simultaneously-
recorded signal from a fluorescent bead located close to the dichroic filter in the light path.

For all imaging experiments, the spatial average of fluorescence over the region of interest was determined by summing the fluorescence of pixels within the terminal (identified from difference images as in Fig. 4A) and dividing by the number of pixels; thus measurements were independent of the area of the region of interest/terminal.

Fluorescent indicator dyes were from Molecular Probes/Invitrogen (Carlsbad, CA. The ω-agatoxin-TK was from Alomone Labs (Jerusalem, Israel). Other reagents were from Sigma (St. Louis, MO).
A. Summary

Mitochondria in motor nerve terminals temporarily sequester large amounts of Ca\(^{2+}\) during repetitive stimulation. Previous work demonstrated that stimulation of the motor nerve (5 s at 100 Hz) produces a transient, Ca\(^{2+}\)-dependent depolarization of the mitochondrial membrane potential (\(\Psi_m\)) in motor terminals that is small (~1-2 mV) in wild-type mice. This stimulation-induced depolarization is ~5 times larger in presymptomatic mice that express the G93A or G85R mutation of human superoxide dismutase 1 (SOD1), models of familial amyotrophic lateral sclerosis (fALS). We report here that in older, symptomatic fALS mice the amplitude of this \(\Psi_m\) depolarization (measured using rhodamine 123) increases with successive stimulus trains. In these symptomatic mice large \(\Psi_m\) depolarizations also occurred that were not synchronized with stimulation.

Both synchronous and asynchronous \(\Psi_m\) depolarizations were reduced (a) by cyclosporin A (1 uM), which inhibits opening of the mitochondrial permeability transition pore (mPTP), and (b) by replacing bath Ca\(^{2+}\) with Sr\(^{2+}\), which enters motor terminals and mitochondria but does not support mPTP opening.

Stimulation-induced \(\Psi_m\) depolarizations resembling those in symptomatic fALS mice could be elicited in wild-type mice when treated with diamide (200 µM), which produces an oxidative stress. These results are consistent with the
hypothesis that the large, stimulation-induced $\Psi_m$ depolarizations in motor terminals of symptomatic fALS mice result from a mitochondrial deficiency that increases the likelihood of mPTP opening, perhaps when $\text{Ca}^{2+}$ influx is combined with oxidative stress. Such mPTP openings might contribute to motor terminal degeneration in fALS mice.

**B. Background**

Mitochondria temporarily sequester the large $\text{Ca}^{2+}$ loads that enter motor nerve terminals during stimulation at $\geq$25 Hz (David et al., 1998). This mitochondrial $\text{Ca}^{2+}$ sequestration protects the terminal by limiting the elevation of cytosolic $[\text{Ca}^{2+}]$, and helps to sustain evoked transmitter release by limiting the increase in asynchronous release (David and Barrett, 2003). The driving force for mitochondrial uptake of $\text{Ca}^{2+}$ is provided by the electron transport chain (ETC) activity that establishes an electrochemical gradient ($\Delta\Psi_m$) across the inner mitochondrial membrane. $\text{Ca}^{2+}$ entry into the mitochondrial matrix depolarizes $\Psi_m$, which would reduce the gradient favoring further $\text{Ca}^{2+}$ entry. However, we have presented evidence that in wild-type (WT) motor terminals this depolarization is very small (1-2 mV), probably because the depolarization produced by $\text{Ca}^{2+}$ entry is partially offset by acceleration of ETC activity (Nguyen et al., 2009). With only a small depolarization, mitochondrial $\text{Ca}^{2+}$ uptake can continue throughout stimulation.

If this idea is true, then $\Psi_m$ depolarization would be expected to be greater in diseases that impair mitochondrial ability to accelerate respiration. Our lab has
demonstrated that in presymptomatic mouse models of familial ALS (fALS, hSOD1-G85R and hSOD1-G93A), motor nerve terminal mitochondria depolarize more in response to stimulation, suggesting impaired ability to handle Ca\textsuperscript{2+} loads (Nguyen et al., 2009). This finding is consistent with previous work demonstrating that isolated spinal cord mitochondria from young, presymptomatic hSOD1-G93A mice show impaired ETC activity (Mattiazzi et al., 2002; Kirkinezos et al., 2005) as well as reduced Ca\textsuperscript{2+} loading capacity (Damiano et al., 2006). If ability to accelerate ETC activity continues to decline as the disease progresses, it would be expected that stimulation-induced $\Psi_m$ depolarization will become greater as fALS mice age and become symptomatic. We report here that $\Psi_m$ depolarizations become much greater in motor terminals of symptomatic fALS mice, and that these large depolarizations are substantially reduced by treatments that inhibit mPTP opening.

C. Methods

Preparation

Experiments used neuromuscular junctions from the levator auris longus muscle from wild-type (WT; C57BL/6, Jackson Laboratories, Bar Harbour, ME), mutant hSOD1-G85R (G85R; Tg(SOD1-G85R)148Dwc, gift of Dr. Don Cleveland, University of California San Diego) and mutant hSOD1-G93A mice (G93A; B6.Cg-Tg(SOD1-G93A), Jackson Laboratories). The hSOD1-G85R mice have a copy number of 15, and exhibit disease onset at 8-10 months and end-stage/death at 9-12 months (Bruijn et al., 1997). The hSOD1-G93A mice have a
copy number of 25, and exhibit disease onset at 3-4 months and end-stage/death at 5 months (Gurney et al., 1994). Mice had been bred with the C57BL/6 background for at least 10 generations. Between P18 and P22, mouse tails were tattooed with an identification code (AIMS Tattoo System, Hornell, NY) and clipped for PCR analysis to determine presence of the hSOD1 transgene (Vila et al., 2003). hSOD1-G85R and hSOD1-G93A mice were housed separately.

This study used symptomatic mice (10-12 month hSOD1-G85R (n=9), 4-5 month hSOD1-G93A (n=7)) with symptoms ranging from hindlimb tremor (an early sign of disease) to inability to right itself within 30 sec (late stage disease, Wooley et al., 2005).

The thin levator auris longus muscle contains mostly fast fibers (Angaut-Petit et al., 1987; Erzen et al., 2000). Mice were killed in a container filled with 100% CO₂. The muscle, dissected with attached nerve, was pinned down in a chamber made from silicon on a No. 1 glass coverslip. The preparation was submerged in physiological saline solution containing (in mM): NaCl 137, NaHCO₃ 15, KCl 4, CaCl₂ 1.8, MgCl₂ 1.1, glucose 11.2 and NaH₂PO₄ 0.33. pH was maintained at ~7.4 by aerating with 95% O₂/5% CO₂. Temperature (monitored with a thermistor) was maintained between 27 - 30°C by blowing hot air onto the underside of the experimental chamber. Action potentials used to stimulate the motor nerve terminal were achieved by applying suprathreshold 0.3 ms depolarizing pulses to the proximal end of the motor nerve via a suction electrode. Three sets of stimulus trains, each consisting of 500 stimuli at 100 Hz, applied every 30 sec were repeated at ≥ 8 min intervals to allow time for
mitochondrial [Ca\textsuperscript{2+}] to return to baseline (David 1999). Muscle contractions were blocked using 15 µM \textit{d}-tubocurarine, an inhibitor of muscle nicotinic acetylcholine receptors. Blocking Ca\textsuperscript{2+} influx into muscle ensured that the recorded stimulation-related in \(\Psi_m\) did not arise from muscle mitochondria.

\textit{Measurement of changes in \(\Psi_m\) depolarizations}

Stimulation-induced changes in \(\Psi_m\) were measured using rhodamine 123 (Rh123; Sigma Aldrich, St. Louis, MO, USA), a cell-permeant, cationic, potentiometric fluorescent indicator that is readily sequestered by viable mitochondria. The preparation was incubated with 3 µM Rh123 for 30 min, which allowed sufficient uptake of dye into the matrix to self-quench. When \(\Psi_m\) becomes depolarized, Rh123 leaks out of the mitochondrial matrix into the cytosol. Here the concentration of Rh123 is much smaller, so the dye becomes unquenched, producing an increase in fluorescence (reviewed by Nicholls and Ward, 2000). After 30 min, the bath concentration of Rh123 was reduced to a maintenance concentration (~300 nM) that allowed sustained recordings of the large \(\Psi_m\) depolarizations in these mice.

Excitation of Rh123 at 485 nm was achieved using a monochromator (Delta Ram V, Photon Technology International, Birmingham, NJ, USA). Emissions were detected using a 40 nm bandpass filter centered at 535 nm and recorded at 1 image per second using IP Lab v3.61 software (Scanalytics, Inc., Fairfax, VA, USA). Data were analyzed using ImageJ software (rsbweb.nih.gov/ij/). To calculate the net fluorescence (\(F_{\text{net}}\)) of a signal,
fluorescence signals from background were subtracted from the total fluorescence of a region of interest encompassing a motor nerve terminal \( (F_{\text{net}} = F_{\text{total}} - F_{\text{background}}) \). Changes in fluorescence were plotted as \( F_{\text{net}}/F_{\text{rest}} \) versus time, where \( F_{\text{rest}} \) is the average of the \( F_{\text{net}} \) values before the first stimulus train was delivered.

D. Results

Motor terminals of symptomatic mutant SOD1 mice display large stimulation-induced \( \Psi_m \) depolarizations

Fig. 4.1A shows \( \Psi_m \) depolarizations, measured by increases in Rh 123 fluorescence, evoked in WT terminals stimulated with three trains of action potentials (each 100 Hz, 5 sec). The average stimulation-induced increase in Rh123 fluorescence is \( \sim 1\sim 2\% \), consistent with values for WT terminals reported by Nguyen et al., 2009. Fig. 4.1B compares the WT trace of Figure 4.1A (black, reduced scale) to fluorescence changes evoked by this stimulation pattern in motor terminals of a symptomatic G85R (blue) and G93A (red) mouse. The mutant SOD1 terminals showed larger depolarizations in response to the 2\textsuperscript{nd} and 3\textsuperscript{rd} trains than to the 1\textsuperscript{st} train. The ratio of the peak responses to the 3\textsuperscript{rd} and 1\textsuperscript{st} stimulus trains in symptomatic mice was greater than the comparable ratio for presymptomatic mice (\( p<0.05 \), two tailed Mann-Whitney test). Responses in symptomatic terminals were also slower to return to baseline after stimulation. Symptomatic terminals also showed additional responses not synchronized to the stimulus train (indicated by arrows). As a result, the cumulative depolarization recorded during the 3\textsuperscript{rd} stimulus train was much greater in
terminals of symptomatic mutant SOD1 mice than in the terminals of presymptomatic mutant SOD1 and wild-type mice described by Nguyen et al. (2009). Fig. 4.1C illustrates two methods used to calculate the change in fluorescence for each train. Cumulative train responses (solid bars) were calculated as the average of the peak fluorescence change during the train divided by the average fluorescence before the first train. Individual train responses (dashed lines) were calculated as the average of the peak fluorescence change during the train divided by the average fluorescence just prior to that train. This measure corrects for the residual fluorescence increase remaining from preceding trains. By either measure, the response to the 2\textsuperscript{nd} and 3\textsuperscript{rd} trains exceeded that to the 1\textsuperscript{st} train.

Fig. 4.2 shows that these stimulation-induced $\Psi_m$ depolarizations were partially inhibited when bath $[\text{Ca}^{2+}]$ was reduced from 2 to 0.5 mM. These data are consistent with the finding that stimulation-induced $\Psi_m$ depolarizations are $\text{Ca}^{2+}$-dependent in WT terminals (Nguyen et al., 2009).
Fig 4.1 Stimulation at 100 Hz depolarizes $\Psi_m$ in symptomatic mutant SOD1 motor terminals more than in WT motor terminals. Each panel shows depolarization of $\Psi_m$ in response to three stimulus trains at 100 Hz, measured as the fluorescence increase in Rh 123. (A) Depolarizations in WT terminals (160 days old). (B) Superimposed $\Psi_m$ depolarizations in WT terminals of A (black), G93A terminal (132 days, red), and G85R terminal (227 days, blue). Matching colored arrows indicate asynchronous depolarizations in mutant SOD1 terminals. Similar results were found in 3 G93A terminals, 3 G85R terminals and 5 WT terminals of separate animals. (C) Example of how magnitudes for cumulative (solid line) and individual (dotted line) responses to stimulation trains were calculated.

![Graph showing depolarization response to Ca^2+ concentration](image)

Fig 4.2 The $\Psi_m$ depolarization produced by 100 Hz stimulation in a G85R terminal was reduced when bath [Ca^{2+}] was reduced from 2 mM (filled circles) to 0.5 mM (open circles). G85R mouse (273d). Similar findings were seen in two additional G85R terminals.

$\Psi_m$ depolarizations are reduced by cyclosporin A (CsA) or by substituting Sr^{2+} for bath Ca^{2+}

One possible mechanism for the large, incrementing depolarizations recorded in symptomatic mutant SOD1 mice is opening of the mitochondrial permeability transition pore (mPTP). To test this idea, we added CsA, which
inhibits the mPTP opening by binding to the cyclophilin D component of the pore (Woodfield et al., 1998). CsA has no effect on stimulation-induced \( \Psi_m \) depolarization in WT terminals or on \( \Psi_m \) depolarizations to the first train in presymptomatic terminals (Nguyen et al., 2009). However, Fig. 4.3 shows that addition of 1 \( \mu \)m CsA to a symptomatic terminal with large, incrementing depolarizations reduced the depolarization evoked by trains #2 and #3, with a less marked effect on the depolarization to train #1. CsA also blocked asynchronous depolarizations (not shown). CsA also inhibits calcineurin activity (Liu et al., 1991), so we tested whether FK506, which also inhibits calcineurin, but does not inhibit mPTP opening, would have a similar effect on the incrementing depolarizations. Fig. 4.3A, B shows that FK506 did not reduce the \( \Psi_m \) depolarizations, regardless of whether data were plotted as cumulative depolarization (Fig. 4.3A) or as the response to individual trains (Fig. 4.3B).

In some cases, the effects of CsA were temporary, with an initial decrease in the response to later trains, followed by rebuilding of the depolarization (Fig. 4.3C). This finding is consistent with experiments loading Ca\(^{2+}\) into isolated mitochondria, which demonstrated that CsA increases the threshold for mPTP opening rather than blocking mPTP opening entirely (Nicholls and Chalmers, 2004).
Fig. 4.3  The large $\Psi_m$ depolarizations evoked by trains 2 and 3 in symptomatic mutant SOD1 terminals are inhibited by cyclosporin A (1 $\mu$M), but not by FK 506 (1 $\mu$M). In each panel, lines connect data points from trains 1, 2, and 3 in each triplet. (A) Cumulative responses from a G85R mouse (288d). (B) Individual train responses from terminal A. (C) Incrementing depolarizations were only transiently diminished by CsA. CsA had a similar effect in terminals never exposed to FK 506. Data are representative of two additional G85R and two G93A terminals.
To further test the idea that the incrementing $\Psi_m$ depolarizations in symptomatic mutant SOD1 mice reflect mPTP opening, we substituted $\text{Sr}^{2+}$ for bath $\text{Ca}^{2+}$. $\text{Sr}^{2+}$ permeates voltage-dependent $\text{Ca}^{2+}$ channels readily (Wakamori et al. 1998) and passes through the mitochondrial uniporter as readily as $\text{Ca}^{2+}$ (Kushnareva and Sokolove, 2000), but does not support mPTP opening (Bernardi et al., 1992). Fig. 4.4 shows that when $\text{Sr}^{2+}$ [2 mM] was substituted for bath $\text{Ca}^{2+}$, the $\Psi_m$ depolarization in response to train #1 continued (as noted by Talbot et al., 2007), but the $\Psi_m$ depolarizations to trains #2 and #3 did not increment. Thus, the effects of CsA and $\text{Sr}^{2+}$ support the hypothesis that the large, incrementing depolarizations evoked in motor terminals of symptomatic mutant SOD1 mice involve mPTP opening in response to $\text{Ca}^{2+}$ influx into mitochondria.

![Graph showing calcium and strontium responses](image)

**Fig. 4.4** Substitution of $\text{Sr}^{2+}$ for bath $\text{Ca}^{2+}$ reduces the $\Psi_m$ depolarizations evoked by trains 2 and 3 in mutant SOD1 terminals. The duration of $\text{Sr}^{2+}$ exposure was 20-30 min. (A) Symptomatic G93A (164d) (B) Symptomatic G85R (321d). Similar findings were recorded in 2 G93A and 2 G85R terminals.
Stimulation-induced large, \( \Psi_m \) depolarizations are not uniform throughout a terminal

Fig. 4.5A shows \( \Psi_m \) depolarizations recorded within different sub-regions of a G85R terminal. Some regions (e.g. b, f) show relatively large increases in the \( \Psi_m \) depolarization from the first to the third stimulus train, whereas other regions (e.g. d, g) show similar responses to all stimulus trains. This intraterminal non-uniformity might be expected if only a fraction of the mitochondria open their transition pore during the later trains. If mPTP openings are indeed occurring in some terminal mitochondria, the records suggest that these openings are transient, because the fluorescence returns to baseline when stimulation stops. As a further test of this idea, we compared the cumulative response to the 3\(^{rd}\) train of two consecutive triplet trains, illustrated by the solid lines in Fig. 4.5B and 4.5C, and plotted these values for each sub-region in Fig. 4.5D. If mPTP openings in a given sub-region were irreversible, then a region that yielded a large depolarization during the first triplet train might expected to be unable to yield a large depolarization during subsequent stimulation. If instead mPTP openings are transient, then a large response to the first triplet train would not preclude a large response to subsequent stimulation. Fig. 4.5D shows that regions gave larger responses during the first triplet train could also give large response during a subsequent train, consistent with the idea that any mPTP openings are transient.
Fig. 4.5 (below) Intra-terminal heterogeneity of stimulation-induced $\Psi_m$ depolarizations in a G85R motor terminal. (A) Terminal area was outlined in white and depolarizations from arbitrarily drawn sub-regions A-G are plotted. (B and C) Individual traces of two subsequent trains from sub-region B. Solid line indicates the cumulative amplitude responses measured for the third train of each triplet. (D) Scatter plot of cumulative responses to 3rd train of 1st triplet (x axis) vs. response to 3rd train of 2nd triplet (y axis). The calculated values for sub-region B are circled ($r = 0.8414$). Symptomatic G85R mouse (335d). Data were repeated in 2 additional G85R terminals. Correlation coefficient values for G85R terminals were $r = 0.8796$ and $r = 0.8459$. 

A
In WT terminals, an oxidative stress can produce stimulation-induced \( \Psi_m \) depolarizations resembling those in symptomatic mutant SOD1 terminals

To test possible mechanisms underlying the large \( \Psi_m \) depolarizations in terminals of symptomatic mutant SOD1 mice, we tried to induce similar behavior in WT terminals. Fig. 4.6A shows the effect of adding 3,4-diaminopyridine, which prolongs action potential duration by blocking depolarization-activated \( K^+ \) channels in motor axons and terminals. Since action potential duration is increased, more \( \text{Ca}^{2+} \) enters the motor terminal. This drug increased the amplitude of \( \Psi_m \) depolarizations to levels similar to those seen in symptomatic terminals, but the response to each stimulus train remained fairly constant, without the incrementing depolarizations observed in symptomatic mutant SOD1 terminals. Thus, simply increasing the amplitude of the depolarization was not sufficient to make WT terminals behave like those in symptomatic mice.

Fig. 4.6B shows the effect of adding diamide, which produces an oxidative stress via multiple mechanisms, including depletion of the antioxidant glutathione by oxidizing the thiol group, thereby converting glutathione to glutathione disulfide (Shen et al., 2005). In diamide, \( \Psi_m \) depolarized more after each train, the depolarizations decayed more slowly, and the magnitude of the depolarization increased with successive trains and with longer exposure to diamide (Fig. 4.6B). This finding is consistent with the hypothesis that the diamide-induced decrease in glutathione caused an increase in ROS, which increased the stimulation induced \( \Psi_m \) depolarization by reducing the ability of the ETC to accelerate during \( \text{Ca}^{2+} \) influx. Since the \( \Psi_m \) depolarizations in Fig. 4.6B mimic many aspects of the depolarizations in symptomatic mutant SOD1
terminals, one mechanism underlying the depolarizations in mutant SOD1 terminals might be increased ROS concentration occurring simultaneously with high Ca^{2+} entry into mitochondria.

**Fig. 4.6** Effects of 3,4-diaminopyridine (A) and diamide (B) on stimulation-induced $\Psi_m$ depolarization in WT terminals. (A) 3, 4-diaminopyridine (DAP) increases the amplitude of $\Psi_m$ depolarizations. (B) Diamide increases and prolongs stimulation-induced $\Psi_m$ depolarizations. Terminal was exposed to 200 μM diamide for 26 min (red) to 49 min (blue). WT mice were 50 (A) and 164 (B) days. These findings were repeated in 3 (A) and 2 (B) WT terminals.
E. Discussion

Stimulation induces transient mPTP openings in motor terminals of symptomatic fALS mice

Work presented here demonstrates that repetitive nerve stimulation evokes large Ca\(^{2+}\)-dependent \(\Psi_m\) depolarizations in motor terminals of symptomatic SOD1-G93A and SOD1-G85R mice. The cumulative \(\Psi_m\) depolarizations in symptomatic mice are considerably greater than those reported in pre-symptomatic fALS mice (Nguyen et al., 2009), suggesting that an increase in stimulation-induced \(\Psi_m\) depolarizations correlates with disease progression. Since SOD1-G93A retains dismutase activity, but SOD1-G85R does not (Valentine et al., 2005), the finding of similarly large \(\Psi_m\) depolarizations in motor terminals from both types of fALS mouse suggests that the large \(\Psi_m\) depolarizations are not related to differences in mutant SOD1 dismutase activity.

In symptomatic mice the cumulative \(\Psi_m\) depolarization evoked by 3 brief stimulus trains displayed at least 3 features different than those in WT mice. First, the peak amplitude of the Rh-123 signal during each stimulus train often increased with successive trains. Second, during the intervals between stimulus trains the Rh-123 signal usually decayed more slowly than in WT terminals, suggesting a slower repolarization of \(\Psi_m\) that allowed the \(\Psi_m\) depolarization evoked by later stimulus trains to sum with the residual \(\Psi_m\) depolarization remaining from preceding trains. Third, additional \(\Psi_m\) depolarizations occurred that were not synchronized with stimulation (asynchronous depolarizations).

mPTP opening likely contributed to the large stimulation-induced \(\Psi_m\) depolarizations in symptomatic fALS mice, because the cumulative \(\Psi_m\)
depolarization was greatly reduced by cyclosporin A, which blocks the cyclophilin D component of the transition pore. (This effect was not replicated by FK506, indicating that cyclosporin A’s effects were not due to inhibition of calcineurin activity.) Also, the cumulative $\Psi_m$ depolarization became smaller when bath $Ca^{2+}$ was replaced by $Sr^{2+}$, which enters cells and mitochondria as readily as $Ca^{2+}$, but does not support mPTP opening. In cyclosporin A or $Sr^{2+}$ the stimulation-evoked $\Psi_m$ depolarizations in motor terminals of symptomatic mice remained larger than those in wild-type terminals, but their peak amplitude did not cumulate with successive trains, and asynchronous $\Psi_m$ depolarizations were absent. Thus in the presence of these agents, the $\Psi_m$ depolarizations recorded in symptomatic terminals resembled those recorded in younger, pre-symptomatic mutant SOD1 mice.

Stimulation-associated mPTP openings in terminals of symptomatic mice were likely transient rather than irreversible, because the Rh-123 signal usually declined toward baseline following each stimulus train. Also, sub-regions of motor terminals that displayed the largest $\Psi_m$ depolarizations during one set of stimulus trains remained able to produce large signals during subsequent stimulation. Thus a large $\Psi_m$ depolarization occurring in one sub-region of a terminal did not render that sub-region refractory to subsequent stimulation, as would have been predicted if mPTP opening were irreversible.

While it is clear that irreversible opening of the mPTP damages cells, it is as yet unclear whether transient openings like those suggested by our data are harmful to the terminal or might even be beneficial. Saotome et al. (2009)
suggest that transient mPTP openings might protect mitochondria from oxidative stress by speeding the efflux of Ca\(^{2+}\) from the matrix. On the other hand, mPTP opening might render the terminal more susceptible to damage from stimulation-induced Ca\(^{2+}\) influx, both by reducing mitochondrial uptake of Ca\(^{2+}\) loads and by reducing production of the ATP needed to extrude Ca\(^{2+}\) from the cytoplasm.

Evidence from the literature also suggests that mPTP opening is detrimental in mutant SOD1 mice. For example, Karlsson et al. (2004) and Kirkinezos et al. (2004) found that intracerebroventricular injections of cyclosporin A into presymptomatic SOD1-G93A mice delayed the onset of hind limb weakness and slowed disease progression. Also, genetic deletion of cyclophilin D delayed disease onset and extended lifespan in SOD1-G93A mice (Martin et al., 2009).

Oxidative stress produced by diamide can induce large stimulation-evoked \(\Psi_m\) depolarizations in wild-type terminals

To probe mechanisms underlying stimulation-evoked \(\Psi_m\) depolarizations in symptomatic mutant SOD1 terminals, we tested whether application of stresses could induce similar depolarizations in wild-type terminals. Increasing Ca\(^{2+}\) influx with 3,4-DAP increased the amplitude of the stimulation-evoked \(\Psi_m\) depolarization to levels comparable to that recorded in symptomatic terminals, but these depolarizations continued to decay rapidly and did not increase in amplitude with successive stimulus trains. Thus simply increasing Ca\(^{2+}\) influx and/or the magnitude of the \(\Psi_m\) depolarization was not sufficient to induce behavior resembling that in symptomatic SOD1 terminals. However, stimulation of wild-type terminals following prolonged exposure to diamide did evoke large,
incrementing, slowly-decaying $\Psi_m$ depolarizations. This finding that mitochondria in wild-type terminals needed both Ca$^{2+}$ influx (from repetitive nerve stimulation) and an oxidative stress is consistent with the Brookes et al.’s (2004) “two hit” hypothesis for mPTP opening. Spinal cords from symptomatic SOD1-G93A mice display increased production of reactive oxygen species (ROS, Wu et al. 2006), and reduction of ROS production via genetic ablation of NADPH oxidase (Marden et al. 2007) or inhibition of NADPH oxidase with apocynin (Harraz et al. 2008) slow disease progression and thereby prolong survival of mutant SOD1 mice. Further work is needed to determine whether these treatments also preserve motor terminal function in these mice.

In sum, mitochondria within motor terminals of symptomatic SOD1-G93A and SOD1-G85R mice display large, cumulating $\Psi_m$ depolarizations during repetitive nerve stimulation, as well as asynchronous depolarizations. The marked reduction of these $\Psi_m$ depolarizations by cyclosporin A suggests that they are due in part to opening of the mPTP. The repeatability of these $\Psi_m$ depolarizations suggests that the mPTP openings are reversible. Experiments in which similar behavior was evoked in wild-type terminals suggest that the large, cumulating $\Psi_m$ depolarizations in motor terminals of symptomatic mutant SOD1 mice may be caused by a combination of Ca$^{2+}$ influx and oxidative stress. These large $\Psi_m$ depolarizations may contribute to motor terminal degeneration and hence to disease progression in mutant SOD1 mice.
A. Future directions

Work described in Chapter 2 studied the effects of ischemia/reperfusion injury on motor terminals innervating fast and slow muscle types. Evidence was presented that motor nerve terminals innervating fast muscles of SOD1-G93A/YFP mice are more vulnerable to ischemia/reperfusion injury than WT terminals even at young, presymptomatic ages. This finding raises the possibility that stress to motor nerve terminals in the periphery might contribute to disease progression.

Possible experiments to follow up this finding might address the following questions: (1) Do fALS terminals have greater vulnerability to repetitive nerve stimulation? *In vivo* experiments in which terminals innervating both fast and slow type limb muscles are subjected to prolonged trains of action potentials over several days would determine if fALS endplates were denervated more than WT endplates

(2) Does nerve stimulation/muscle activation increase ROS production more in fALS mice than in wild-type mice? To test this idea, stimulation-induced superoxide production would be measured in motor nerve terminal mitochondria using MitoSOX Red fluorescent dye. This reagent is permeable to living cells and is selectively targeted to mitochondria. Once in the mitochondria, the dye is oxidized by superoxide and emits a red fluorescence. Increased ROS production
in mutant SOD1 terminals or muscles might increase susceptibility to terminal damage in these mice.

An interesting result was that motor axon sprouting and reinnervation of endplates occurred 10 days post ischemia/reperfusion stress even in mutant SOD1 mice. This compensatory mechanism may eventually not be able to keep up with the increasing number of dying terminals in mutant SOD1 mice. Experiments in which terminal morphology and function are examined up to several months after ischemia-induced endplate denervation would test the long-term stability of regenerated mutant SOD1 terminals to sprout.

Saxena et al. (2009) reported that terminals innervating fast-type muscles in SOD1-G93A mice could not sprout after multiple nerve crushes. To determine if this idea would also be true for ischemia/reperfusion injury, mutant SOD1 terminals would be subjected to two or more episodes of ischemic stress and left to recover for 10 days. Endplate occupancy would be calculated to determine the ability of mutant SOD1 terminals to sprout after multiple exposures to ischemia-induced stress.

Chapters 3 and 4 described the effects of repetitive nerve stimulation on the membrane potential of motor terminal mitochondria. Chapter 3 demonstrated that stimulation-induced $\Psi_m$ depolarizations, as measured by rhodamine-123 fluorescence, were ~5 times greater in motor terminal mitochondria of presymptomatic mouse models of fALS. Chapter 4 presented findings that older, symptomatic fALS terminals displayed stimulation-induced $\Psi_m$ depolarizations that cumulated to much larger amplitudes and were attenuated by cyclosporin A.
One follow up experiment would be to determine if the larger $\Psi_m$ depolarizations in fALS terminals disrupt Ca$^{2+}$ handling and transmitter release. Greater cytosolic [Ca$^{2+}$] in mutant SOD1 terminals compared to wild-type terminals would suggest an impaired ability for mitochondria to buffer Ca$^{2+}$ (David and Barrett, 2000). Based on the findings of David et al. 2003, increases in stimulation-induced cytosolic [Ca$^{2+}$] are predicted to cause greater asynchronous neurotransmitter release and an inability to sustain phasic release during tetanic stimulation. Further experiments that measure cytosolic [Ca$^{2+}$] and transmitter release in young, presymptomatic mutant SOD1 terminals would help determine how early these terminals demonstrated aberrant Ca$^{2+}$ handling.

Results from symptomatic studies of mutant SOD1 motor terminal mitochondria strongly suggest that mPTP opening as one mechanism underlying the greater $\Psi_m$ depolarizations. A follow-up experiment would be to determine whether mPTP opening can be produced in wild-type terminal mitochondria. In wild-type terminals, we were able to induce incrementing stimulation-induced $\Psi_m$ depolarizations with diamide. We need to test whether the larger $\Psi_m$ depolarizations in diamide can be blocked by CsA.

Together, these results are consistent with the hypothesis that stress to motor nerve terminals might contribute to disease progression. This findings that motor nerve terminals and their mitochondria degenerate before cell bodies die (Kong and Xu, 1998; Frey et al., 2000; Fischer et al., 2004; Schaefer et al., 2005; Gould et al., 2006; Pun et al., 2006) and that damaged mitochondria retrogradely transported from terminals can be found in motor neuron cell body (Martin et al.,
2009) support the possibility that disease may begin at motor nerve terminals. But these findings do not rule out the possibility that disease originating in the cell body might first manifest itself in motor nerve terminals. Regardless of where disease originates, treatments that specifically protect the motor terminal might thus help slow disease progression, or even prevent disease onset.

**B. Possible non-mitochondrial mechanisms of motor neuron/motor terminal death in fALS mice**

The endoplasmic reticulum (ER) is another organelle that might contribute to ALS disease progression. Inability to fold mutant SOD1 proteins properly might lead to an accumulation of unfolded proteins in the ER (ER stress). This accumulation initiates the unfolded protein response (UPR) pathway, decreases protein synthesis and protects the cell from apoptosis. Saxena et al. (2009) identified a population of motor neurons in SOD1-G93A and SOD1-G85R mice that not only demonstrated disease-related genetic alterations, but were also prone to ER stress. UPR expression peaked around P30 in the vulnerable motor neurons, before terminals began to degenerate. UPR expression peaked again around P85, when clinical symptoms appeared. The investigators suggested that with age and disease progression, the motor neurons could no longer manage the increasing number of misfolded proteins, leading to motor neuron soma and motor terminal death. They were able to slow motor neuron death and denervation with salubrinal, which protects from ER stress by inducing the phosphorylation of P1-elf2α, a component of the UPR. Salubrinal also helped
SOD1-G93A terminals following multiple nerve crushes. These terminals were able to reinnervate their endplates in the presence of salubrinal as vigorously as wild-type terminals (Saxena et al., 2009).

Brennan and colleagues (2009) presented evidence for a non-mitochondrial source of superoxide, also activated by Ca$^{2+}$ influx, to be a membrane-bound enzyme complex, NADPH oxidase (NOX), which transfers an electron from NADPH to molecular oxygen to generate superoxide. Exposing cultured neurons and mouse hippocampal slices to glutamate caused an increase in superoxide production, followed by neuronal death. Genetic ablation of functional NOX or inhibition of its activation with apocynin limits superoxide production and increased viability of neurons exposed to glutamate (Brennan et al., 2009). This data are consistent with findings that treatment of fALS mice at a young, presymptomatic age with apocynin increased the lifespan and slowed disease progression in a dose-dependent manner (Harraz et al., 2008).

Overexpression of mitochondrial manganese superoxide (MnSOD) in these neurons did not blunt the production of superoxide (Brennan et al., 2009), suggesting that mitochondria are not the source of superoxide generation. The precise organelle that houses NOX is unknown, but the ER is a possible location since mitochondria are tethered to the ER by specific adaptor proteins (de Brito and Scorrano, 2008).

These studies focused on mechanisms at the neuron cell body, so further work is needed to examine ER function at motor nerve terminals. Direct application of salubrinal and apocynin to motor nerve terminals of mutant SOD1
mice would be one method to test whether reducing ER stress would prolong terminal survival.

C. Challenges to clarifying disease mechanisms by studying motor nerve terminals

Mitochondria isolated from spinal cord come mostly from astrocytes, microglia, and interneurons, rather than motor neurons. Further, with disease progression, more and more motor neurons die, resulting in an even smaller number of mitochondria isolated from motor neurons. Thus, functional measurements of motor neuron mitochondria are quite limited.

There are some disadvantages in using motor nerve terminals to study possible fALS disease mechanisms. Motor terminals are small structures that constitute a very small percentage of tissue in neuromuscular preparations. Thus, conventional biochemical techniques are not possible. However, since neurodegeneration in fALS mice may begin at the motor terminal, it is important to study this structure when considering possible therapeutic options. This dissertation has provided evidence for early dysfunction of motor terminal mitochondria in fALS mice. Although we are not able to measure the resting state of mitochondria in terminals, we are able to measure stimulation-induced mitochondrial responses to repeatable, graded Ca^{2+} challenge in situ. Additionally, we are confident that the stimulation-evoked responses come strictly from mitochondria in motor nerve terminals.

Motor neuron connection to muscles via motor nerve terminals is critical to retain voluntary movement, and also to ensure transport of muscle-derived
trophic factors back to motor neuron cell body for survival. To date, only one single-drug therapy has shown limited effectiveness in ALS patients, and this drug’s effects are directed to the CNS. Combination drug therapies will likely be needed, and this combination will need to include treatments focused on preserving motor nerve terminals.
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