Published in final edited form as:

Neurobiol Dis. 2011 June ; 42(3): 381–390. doi:10.1016/j.nbd.2011.01.031.

Repetitive Nerve Stimulation Transiently Opens the Mitochondrial Permeability Transition Pore in Motor Nerve Terminals of Symptomatic Mutant SOD1 Mice

Khanh T. Nguyen^{a,b}, John N. Barrett^{a,b}, Luis García-Chacón^a, Gavriel David^{a,b}, and Ellen F. Barrett^{a,b}

^aDepartment of Physiology and Biophysics, University of Miami Miller School of Medicine, P.O. Box 016430, Miami, FL, 33101, USA

^bNeuroscience Program, University of Miami Miller School of Medicine, P.O. Box 016430, Miami, FL, 33101, USA

Abstract

Mitochondria in motor nerve terminals temporarily sequester large Ca^{2+} loads during repetitive stimulation. In wild-type mice this Ca^{2+} uptake produces a small (<5 mV), transient depolarization of the mitochondrial membrane potential (Ψ_m , motor nerve stimulated with at 100 Hz for 5 s). We demonstrate that this stimulation-induced Ψ_m depolarization attains much higher amplitudes in motor terminals of symptomatic mice expressing the G93A or G85R mutation of human superoxide dismutase 1 (SOD1), models of familial amyotrophic lateral sclerosis (fALS). These large Ψ_m depolarizations decayed slowly and incremented with successive stimulus trains. Additional Ψ_m depolarizations occurred that were not synchronized with stimulation. These large Ψ_m depolarizations were reduced (a) by cyclosporin A (CsA, 1-2 μM), which inhibits opening of the mitochondrial permeability transition pore (mPTP), or (b) by replacing bath Ca^{2+} with Sr^{2+} , which enters motor terminals and mitochondria but does not support mPTP opening. These results are consistent with the hypothesis that the large Ψ_m depolarizations evoked by repetitive stimulation in motor terminals of symptomatic fALS mice result from mitochondrial dysfunction that increases the likelihood of transient mPTP opening during Ca^{2+} influx. Such mPTP openings, a sign of mitochondrial stress, would disrupt motor terminal handling of Ca^{2+} loads and might thereby contribute to motor terminal degeneration in fALS mice. Ψ_m depolarizations resembling those in symptomatic fALS mice could be elicited in wild-type mice following 0.5-1 hr exposure to diamide (200 μM), which produces an oxidative stress, but these depolarizations were not reduced by CsA.

Keywords

motor nerve terminal; superoxide dismutase 1 G93A; superoxide dismutase 1 G85R; mouse models of familial amyotrophic lateral sclerosis; mitochondria; mitochondrial permeability

Corresponding author: Ellen F. Barrett, Department of Physiology and Biophysics, R-430, University of Miami Miller School of Medicine, 1600 N.W. 10th Ave., Miami, FL, 33136, USA, or P.O. Box 016430, Miami, FL, 33101, USA, ebarrett2@med.miami.edu, 305-243-6825 (phone), 305-243-5931 (FAX).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

transition pore; mitochondrial calcium uptake; oxidative stress; mitochondrial membrane potential; motor neuron

Introduction

Motor nerve terminals are small structures that can experience large Ca^{2+} influxes during repetitive stimulation of the motor nerve. Temporary sequestration of these Ca^{2+} loads by abundant terminal mitochondria protects the terminal by limiting the elevation of cytosolic $[\text{Ca}^{2+}]$, and is important for sustaining evoked transmitter release during repetitive stimulation (David et al. 1998; David and Barrett, 2003). Mitochondrial Ca^{2+} uptake, regulated by transporters/channels and sensors (Kirichok et al. 2004; Jiang et al. 2009; Perocchi et al. 2010), occurs down an electrochemical gradient across the inner mitochondrial membrane ($\Delta\Psi_m$, 150-200 mV, inside negative) created by proton extrusion via the electron transport chain (ETC, reviewed by Gunter and Pfeiffer, 1990; Nicholls and Ferguson, 2002). Ca^{2+} entry into the mitochondrial matrix depolarizes Ψ_m , and would thereby be expected to reduce the electrochemical gradient for further Ca^{2+} entry. However, in motor terminals of wild-type (wt) mice the stimulation-induced Ψ_m depolarization measured in motor terminals is very small (<5 mV, Nguyen et al., 2009), probably because the Ψ_m depolarization produced by Ca^{2+} entry is partially offset by depolarization-induced acceleration of H^+ extrusion via the ETC (reviewed by Nicholls and Ferguson, 2002; Johnson-Cadwell et al. 2007; Talbot et al., 2007). This maintenance of Ψ_m , combined with powerful Ca^{2+} buffering within the mitochondrial matrix (reviewed by Nicholls and Chalmers, 2004) permits mitochondrial Ca^{2+} uptake to continue throughout stimulation (David, 1999).

The present study measured stimulation-induced Ψ_m depolarizations in motor terminals of mice that express mutant forms of human superoxide dismutase I (SOD1-G93A and SOD1-G85R). These mice are models of familial amyotrophic lateral sclerosis (ALS), a neurodegenerative disease in which motor neurons die (Rosen et al., 1993); oxidized, misfolded wt SOD1 may also contribute to disease in some cases of sporadic ALS (Bosco et al. 2010; Forsberg et al. 2010). In mutant SOD1 mice, and in at least some patients with sporadic ALS, some of the earliest morphological signs of motor neuron degeneration occur in motor terminals and their mitochondria (Kong and Xu, 1998; Fischer et al. 2004; reviewed by Shi et al. 2010; Murray et al. 2010; Kawamata and Manfredi, 2010). Mitochondria isolated from the spinal cords of these mice exhibit impaired ETC activity (Mattiazzi et al., 2002; Kirkinezos et al., 2005) and reduced Ca^{2+} loading capacity (Damiano et al., 2006). However, in these isolated spinal cord preparations only a fraction of the mitochondria come from motor neurons. Stimulation-induced Ψ_m depolarizations like those measured here have the advantage that the signals originate exclusively from motor neuron mitochondria in situ. Studying motor terminals also yields insight into the function of synaptic mitochondria, which are more sensitive to Ca^{2+} loads than nonsynaptic mitochondria (Brown et al. 2006).

In a previous paper we demonstrated that the stimulation-induced Ψ_m depolarizations recorded from motor terminal mitochondria in presymptomatic SOD1-G93A and SOD1-G85R mice are on average ~ 5 -fold greater than those recorded in wt mice or mice expressing normal human SOD1 (Nguyen et al. 2009). Here we demonstrate that, as the mutant SOD1 mice age and become symptomatic, the stimulation-induced Ψ_m depolarizations become much larger and longer-lasting, and have properties suggesting transient opening of the mitochondrial permeability transition pore (mPTP). We also report that similarly large, long-lasting stimulation-induced Ψ_m depolarizations can be recorded in wt motor terminals exposed to an oxidative stress.

Materials and Methods

Preparation

This study used two types of familial ALS mice, SOD1-G93A (B6.Cg-Tg(SOD1-G93A)1Gur/J, founders from Jackson Laboratories, Bar Harbour, ME) and hSOD1-G85R (Tg(SOD1-G85R)148Dwc, founders from Dr. Don Cleveland, University of California San Diego). Some experiments used wt mice (C57BL/6, founders from Jackson Laboratories) or mice expressing normal human SOD1 (hSOD1-wt; B6SJL-Tg(SOD1)2Gur/J, founders from Dr. Cleveland). The hSOD1-G93A mice have a higher copy number of the mutant gene, and exhibit disease onset and end-stage/death earlier than the hSOD1-G85R mice (Gurney et al., 1994; Chiu et al. 1995; Bruijn et al., 1997). All mice were maintained in the C57BL/6 background for at least 10 generations; hSOD1-G93A, hSOD1-G85R and hSOD1-WT mice were housed separately. Between postnatal days 18-22, 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 (as in Vila et al., 2003). The mutant SOD1 mice used in this study were symptomatic (4-5 month hSOD1-G93A (n=7); 10-12 month hSOD1-G85R (n=9)), with symptoms ranging from hindlimb tremor (an early sign of disease) to inability to right within 30 s (late stage disease, Wooley et al., 2005).

Experiments used neuromuscular junctions in the levator auris longus (LAL), a thin muscle in the head that is well suited for functional imaging of living motor terminals (Angaut-Petit et al., 1987). LAL contains mostly fast fibers (Erzen et al., 2000). In mutant SOD1 mice motor terminals that innervate fast muscle fibers in the limb degenerate earlier than those innervating slow muscle fibers (Frey et al. 2000; Schaefer et al. 2005; Pun et al. 2006; Hegedus et al. 2008). Motor terminals in LAL are also affected by disease (Vila et al. 2003; Nguyen et al. 2009, and data presented here), but the changes occur at later ages than in fast limb muscles, such that many LAL endplates remain innervated in symptomatic mice.

Mice were sacrificed using cervical dislocation under deep isoflurane anesthesia (4%) or with 100% CO₂, procedures approved by the University of Miami Animal Care and Use Committee. The muscle, dissected with attached nerve, was pinned flat in a chamber made from silicon on a No. 1 glass coverslip. The physiological saline solution contained (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.

Measurement of stimulation-evoked Ψ_m depolarizations

In neuromuscular preparations measurements of resting respiration are dominated by the abundant muscle mitochondria. To isolate the measurements to mitochondria within motor terminals, we measured changes in Ψ_m evoked by stimulating the motor nerve, using rhodamine 123 (Rh-123; Sigma Aldrich, St. Louis, MO, USA). Rh-123 is a cell-permeant, cationic, potentiometric fluorescent indicator that is readily sequestered by viable mitochondria. Action potentials were evoked in the motor nerve terminal 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 s, were repeated at ≥ 8 min intervals to allow time for mitochondrial [Ca²⁺] to return to baseline (David, 1999). Muscle contractions were blocked using 15 μ M *d*-tubocurarine, an inhibitor of muscle nicotinic acetylcholine receptors. Blocking activation of these receptors ensured that the recorded stimulation-induced changes in Ψ_m did not arise from muscle mitochondria.

The preparation was incubated with 3 μ M Rh-123 for 30 min, which allowed sufficient uptake of dye into the matrix to self-quench. When Ψ_m becomes depolarized, Rh-123 leaks

out of the mitochondrial matrix into the cytosol. Here the concentration of Rh-123 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 Rh-123 was reduced to a maintenance concentration ($\sim 0.3 \mu\text{M}$) that allowed sustained recordings of the large Ψ_m depolarizations in these mice. Control experiments in wt mice showed that stimulation-induced fluorescence signals remained stable for at least 2 h, suggesting that this maintenance concentration did not cause the time-dependent increases in Ψ_m depolarizations reported in Results.

Rh-123 was excited at 485 nm 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/s using IP Lab v3.61 software (Scanalytics, Inc., Fairfax, VA, USA). Data were analyzed using ImageJ software (rsbweb.nih.gov/ij/). Net fluorescence (F_{net}) was calculated by subtracting background fluorescence 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 cumulative fluorescence were plotted as $F_{\text{net}}/F_{\text{rest}}$ vs. time, where F_{rest} is the mean of 30 F_{net} values recorded before the first stimulus train was delivered. Cumulative peak amplitude was calculated by subtracting F_{rest} from the mean of the 3 largest F_{net} values recorded during the stimulus train. The change in fluorescence produced by an individual stimulus train was calculated by subtracting the mean of the 3 F_{net} values recorded just prior to that train from the 3 largest F_{net} values recorded during the train (Fig. 1C illustrates the difference between cumulative and individual peak amplitudes).

Tests of statistical significance (ANOVA, t-tests) were performed using Prism (version 3, Graph Pad Software, San Diego, CA). Reagents came from Sigma (St. Louis, MO) and Invitrogen (Carlsbad, CA).

Results

Motor terminals of symptomatic mutant SOD1 mice display large stimulation-induced Ψ_m depolarizations

Figure 1A shows Ψ_m depolarizations, measured by increases in Rh-123 fluorescence, evoked in a motor terminal of a mouse expressing normal human SOD1 (hSOD1-WT) by stimulation with 3 trains of action potentials (each 100 Hz, 5 s). In these mice the average stimulation-induced increase in Rh-123 fluorescence was 1% or less, similar to the value recorded in wt mice (Nguyen et al., 2009). The average fluorescence change increased to 5-6% in presymptomatic mutant SOD1 mice (Nguyen et al., 2009). Figure 1B shows that in the older, symptomatic mutant SOD1 mice studied here, these stimulation-evoked Ψ_m depolarizations were often much larger. (Note that the vertical scale in Figure 1B is compressed compared to that in Figure 1A; the hSOD1-WT record in Figure 1B is the same as that in Figure 1A). At least three factors contributed to the increased Ψ_m depolarizations in terminals of symptomatic mutant SOD1 mice. First, responses in symptomatic mutant SOD1 terminals often became much larger with successive trains, i.e., Ψ_m depolarizations in response to the 2nd and 3rd trains were usually larger than that to the 1st train. We defined an increment index by dividing the peak response to train #3 by the peak response to train #1 (response defined as $(F_{\text{net}}/F_{\text{rest}}) - 1$). The mean increment index was 2.05 ± 0.32 (SEM, $n=17$ terminals), a value significantly greater than 1.0 (one sample t test). Second, responses in symptomatic terminals returned to baseline more slowly, suggesting slower repolarization of Ψ_m (see Figure 1 of Supplementary Materials). Third, stimulated symptomatic terminals showed additional Ψ_m depolarizations that were not synchronized with the stimulus train (arrows in Figure 1B). As a result of these changes, the cumulative depolarization recorded during the 3rd stimulus train was often much greater in terminals of symptomatic mutant

SOD1 mice than in terminals of the wt, hSOD1-wt and presymptomatic mutant SOD1 mice described in Nguyen et al. (2009).

Figure 1C illustrates two methods used to calculate the change in fluorescence following each train. Cumulative train responses were calculated as the peak fluorescence increase during the train divided by the average fluorescence before the first train. Individual train responses were calculated as the peak fluorescence increase during the train divided by the average fluorescence just prior to that train. The latter measure corrects for the residual fluorescence increase remaining from preceding trains. By either measure, the response to the 2nd and 3rd trains exceeded that to the 1st train in terminals of symptomatic mutant SOD1 mice.

Figure 2 shows that the stimulation-induced Ψ_m depolarizations in both symptomatic mutant SOD1 (A) and hSOD1-wt (B) terminals are Ca^{2+} -dependent, similar to findings previously reported for wt terminals (Nguyen et al., 2009). These findings are consistent with the hypothesis that these depolarizations are caused by Ca^{2+} influx into mitochondria. Because the Ψ_m depolarizations in hSOD1-wt terminals are normally so small (near the noise level), the records illustrated Figure 2B were recorded at a cooler temperature (25 °C), which increases the amplitude of stimulation-induced Ψ_m depolarizations.

Transient opening of mitochondrial permeability transition pore (mPTP) accounts for a portion of the increased Ψ_m depolarization in mutant SOD1 mice

One possible mechanism for the large, incrementing Ψ_m depolarizations recorded in terminals of symptomatic mutant SOD1 mice is opening of the mitochondrial permeability transition pore (mPTP), a poly-protein transmembrane channel formed at contact sites between the inner and outer mitochondrial membranes. We tested this idea using CsA, which inhibits mPTP opening by binding to the cyclophilin D component of the pore (Woodfield et al., 1998). Figure 3A shows that addition of 2 μM CsA to a mutant SOD1 terminal with large, incrementing Ψ_m depolarizations reduced the depolarization evoked by the 2nd and 3rd trains, with a less marked effect on the depolarization to the 1st train. CsA also blocked asynchronous Ψ_m depolarizations. CsA also inhibits calcineurin activity (Liu et al., 1991), so we tested the effect of FK506, which inhibits calcineurin but does not inhibit mPTP opening. Figures 3B,C show that FK506 did not reduce the Ψ_m depolarizations, regardless of whether amplitudes were plotted as cumulative depolarization (Figure 3B) or as the response to individual trains (Figure 3C). This result suggests that the effects of CsA were due to inhibition of mPTP opening rather than to inhibition of calcineurin activity.

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 (Figure 2 in Supplementary Material). This finding is consistent with experiments in isolated mitochondria showing that CsA increases the threshold for mPTP opening in response to Ca^{2+} loads, rather than blocking mPTP opening entirely (e.g. Nicholls and Chalmers, 2004).

To test further the idea that the incrementing Ψ_m depolarizations in terminals of symptomatic mutant SOD1 mice reflect mPTP opening, we substituted Sr^{2+} for bath Ca^{2+} . Sr^{2+} permeates voltage-dependent Ca^{2+} channels readily (Forsythe et al. 1998) and passes through the mitochondrial uniporter as readily as Ca^{2+} (Kushnareva and Sokolove, 2000), but does not support mPTP opening (Bernardi et al., 1992). Figures 4A,B show that when Sr^{2+} (2 mM) was substituted for bath Ca^{2+} , the Ψ_m depolarization in response to the 1st train continued (as noted also by Talbot et al., 2007), but the Ψ_m depolarizations to the 2nd and 3rd trains no longer incremented. Results in Figs. 2-4 thus support the hypothesis that the large, incrementing Ψ_m depolarizations evoked in motor terminals of symptomatic mutant SOD1 mice involve mPTP opening in response to stimulation-induced Ca^{2+} influx into

mitochondria. This finding in terminals of symptomatic mutant SOD1 mice contrasts with results in presymptomatic terminals of these mice, where the more modest increase in Ψ_m depolarization was largely insensitive to CsA (Nguyen et al., 2009; see also Discussion and Supplementary Material).

The mPTP openings in terminal mitochondria were likely transient, because when stimulation stopped, the fluorescence returned to baseline (Supplementary Figure 1), as would be expected if repolarizing mitochondria re-accumulated and thereby quenched Rh-123. Experiments like that in Figure 5 further tested the hypothesis that the stimulation-evoked mPTP openings are transient. Black and white images in Figure 5A show Rh-123 fluorescence in an SOD1-G93A terminal subdivided into 5 adjoining compartments (a-e). In each compartment the Ψ_m depolarization in response to the 3rd stimulus train was larger than that to the first two trains. In addition there were two asynchronous depolarizations (marked by arrows), one after the 2nd train localized to adjoining subregions d and e, another after the 3rd train localized to adjoining subregions a and b. Pseudocolor images in Figure 5B use a color scale to depict the fluorescence increases in this terminal at rest (blue indicates no change), during the 2nd and 3rd stimulus trains, and during the labeled asynchronous depolarizations; these images demonstrate that the intra-terminal heterogeneity evident in the compartmental Ψ_m depolarizations in Figure 5A was not simply an artifact dependent on how compartment boundaries were drawn. This intra-terminal heterogeneity suggests that only a fraction of the mitochondria in this mutant SOD1 terminal exhibited transition pore opening, at least during the asynchronous depolarizations.

If mPTP openings in a given compartment were irreversible, then mitochondria in that compartment would remain depolarized, unable to re-accumulate Rh-123 and respond to subsequent stimulation. Thus a compartment that exhibited a large Ψ_m depolarization during an initial triplet train (suggesting mPTP opening) would not be expected to exhibit a large Ψ_m depolarization during a subsequent triplet train. If instead mPTP openings are transient, then a large response to the first triplet train would not preclude a large response to subsequent stimulation. Figure 5C shows Ψ_m depolarizations evoked by two consecutive triplet trains in compartment c of Figure 5A, showing that the response to the 3rd train was also large in the second triplet. Figure 5D is a scatter plot of the peak response to the 3rd train in two consecutive triplet trains; this plot includes the compartments of the G93A terminal in Figure 5A-B as well as compartments for 3 G85R terminals, normalized in each case to the maximal response recorded from that terminal. In some terminals responses to the first and second triplet trains were positively correlated; in other terminals (e.g. that in Figure 5A) there was no significant correlation. For the summed data plotted in Figure 5D there was a modest positive correlation ($r=0.43$, $p<0.05$). The finding that the correlation was not negative, i.e. that compartments that exhibited a large responses during the 1st triplet train could also exhibit a large response during a subsequent triplet train, is consistent with the hypothesis that the stimulation-evoked mPTP openings in symptomatic mutant SOD1 terminals were transient.

The intra-terminal heterogeneity in Ψ_m depolarizations evident in Figure 5A-B was related to expression of *mutant* SOD1, not simply to increased expression of human SOD1. Figure 3 in Supplementary Materials shows representative data from an hSOD1-wt tg terminal. Since Ψ_m depolarizations in hSOD1-wt terminals are normally small (close to the noise level), this terminal was treated with 3,4-diaminopyridine (DAP, 0.1 mM) to make the amplitude of the stimulation-induced response comparable to that in the mutant SOD1 terminal of Fig. 5A-C. This drug prolongs action potential duration by blocking certain depolarization-activated K^+ channels in motor axons and terminals (Tabti et al. 1989; Morita and Barrett, 1990; David et al. 1995), thereby increasing Ca^{2+} influx. The pseudocolor images recorded during

stimulation were similar all along this hSOD1-wt terminal, and there were no asynchronous depolarizations during the interval between stimulus trains.

Diamide-induced oxidative stress induces increased depolarizing responses to stimulation-induced Ca^{2+} loads in wt terminals

To investigate possible mechanisms underlying the large Ψ_m depolarizations recorded in terminals of symptomatic mutant SOD1 mice, we applied stresses to terminals of wt or hSOD1-wt mice, in an attempt to induce similar behavior. One approach was to increase the magnitude of the Ψ_m depolarization using 3,4-DAP (20-100 μM) to increase Ca^{2+} influx into the motor terminal (as also done in Figure 5D,E). Figure 6A shows that 3,4-DAP (0.1 mM) increased the amplitude of the Ψ_m depolarization in a wt terminal to levels comparable to those evoked in symptomatic mutant SOD1 terminals, but these depolarizations continued to decay rapidly and did not increase in amplitude with successive stimulus trains. Also, CsA did not reduce the amplitude of the 3,4-DAP-enhanced Ψ_m depolarizations (Figure 6B). Thus simply increasing Ca^{2+} influx and/or the magnitude of the Ψ_m depolarization was not sufficient to make normal SOD1 terminals exhibit behavior resembling that in terminals of symptomatic mutant SOD1 mice.

One mechanism proposed for the deleterious effects of mutant SOD1 is production of an oxidative stress (reviewed by e.g. Barber and Shaw, 2010). Figure 7A shows the effect of adding diamide (0.2 mM) to a wt terminal. Diamide produces an oxidative stress via multiple mechanisms, including depletion of reduced glutathione (Shen et al., 2005). Following exposure to diamide, Ψ_m depolarized more after each stimulus train, the depolarizations decayed more slowly, and the magnitude of the depolarization increased with successive stimulus trains. These effects increased with longer exposure to diamide. However, the large Ψ_m depolarizations in diamide were not blocked by CsA (Figure 7B, see Discussion). Thus the stimulation-induced Ψ_m depolarizations produced in wt terminals following a diamide-induced oxidative stress mimicked some, but not all, features of the Ψ_m depolarizations recorded in symptomatic mutant SOD1 mice.

Discussion

Mitochondrial dysfunction increases with disease progression in motor terminals of mutant SOD1 mice

As mentioned previously, even at early, presymptomatic ages SOD1-G93A and SOD1-G85R motor terminal mitochondria show increased Ψ_m depolarizations in response to stimulation-induced Ca^{2+} loads (Nguyen et al., 2009). The present work demonstrates that as these mutant SOD1 mice become older and symptomatic, the peak amplitude of these stimulation-induced Ψ_m depolarizations become progressively larger with repeated stimulus trains. The large Ψ_m depolarizations in symptomatic mice also repolarized more slowly than those in presymptomatic mice, and were sensitive to CsA. In essence, treatment with CsA made Ψ_m depolarizations in terminals from symptomatic mutant SOD1 mice resemble those in presymptomatic mice. The fact that findings at presymptomatic and symptomatic stages were similar in both G93A and G85R terminals suggests that alterations in dismutase activity were not involved, since the G93A mutation retains dismutase activity, whereas the G85R mutant does not (Valentine et al., 2005).

These findings suggest that at least two components contribute to the increased stimulation-induced Ψ_m depolarizations recorded in motor terminals of symptomatic mutant SOD1 mice: a CsA-insensitive component that is also evident in presymptomatic mice, and a CsA-sensitive component evident mainly in symptomatic mice. The CsA-insensitive component might be due to a reduced ability to accelerate H^+ extrusion via the ETC in response to the

Ψ_m depolarization produced by Ca^{2+} influx (Talbot et al. 2007; Nguyen et al. 2009). Reduced ability to accelerate ETC activity might result from a reduction in the number and/or function of ETC complexes due to association of misfolded mutant SOD1 with mitochondria (reviewed in Shi et al. 2010; also Israelson et al. 2010; Li et al. 2010).

The CsA-sensitive component of the stimulation-induced Ψ_m depolarization in symptomatic mice is likely due to mPTP opening. These mPTP openings appeared to be transient rather than irreversible. Studies applying different techniques to mitochondria from other tissues (mainly liver and heart) have presented evidence for several modes of mPTP opening, including high- and low-conductance states, both of which are favored by Ca^{2+} and inhibited by CsA. It has been reported that in these mitochondria openings to the high-conductance state are irreversible and inhibited by Sr^{2+} , whereas openings to the low-conductance state are reversible and favored by Sr^{2+} (reviewed by Ichas and Mazat, 1998). Using this categorization it is difficult to classify the CsA-sensitive Ψ_m depolarizations in mutant SOD1 motor terminals, which were reversible (suggesting low-conductance state), but not present when Sr^{2+} replaced bath Ca^{2+} (suggesting high-conductance state). However, others have reported rapid, spontaneous, CsA-inhibited fluctuations in Ψ_m in individual mitochondria, both isolated and in situ (Hüser et al. 1998; Hüser and Blatter, 1999), suggesting that brief openings to a high-conductance state can be reversible.

While it is clear that irreversible opening of the mPTP leads to loss of matrix metabolites, mitochondrial swelling and cellular damage, it is as yet unclear whether transient openings like those suggested by our data are also harmful. Some have suggested that transient mPTP openings might protect mitochondria from oxidative stress by facilitating efflux of Ca^{2+} from the matrix and reducing generation of endogenous oxygen radicals (e.g. Hüser and Blatter, 1999; Saotome et al. 2009). On the other hand, mPTP opening might render the terminal more susceptible to damage from subsequent 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. These changes might eventually lead to irreversible mPTP opening, and degeneration of the motor terminal. Retrograde transport of damaged motor terminal mitochondria might also speed degeneration of the motor neuron cell body. Evidence from the literature also suggests that mPTP opening is detrimental in mutant SOD1 mice (reviewed by Martin, 2010). For example, Karlsson et al. (2004) and Kirkinezos et al. (2004) found that intracerebroventricular injections of CsA into presymptomatic SOD1-G93A mice delayed the onset of hind limb weakness and slowed disease progression. Also, Martin et al. (2009) found that genetic deletion of cyclophilin D delayed disease onset and extended lifespan in SOD1-G93A mice, though a preliminary report by Parone et al. (2010) found no effect, suggesting the importance of additional factors such as mouse genetic background. Naga et al. (2007) suggest that the high concentration of cyclophilin D in synaptic mitochondria renders them especially sensitive to mPTP opening.

Asynchronous Ψ_m depolarizations in symptomatic mutant SOD1 mice may be triggered by Ca^{2+} release from mitochondria and/or endoplasmic reticulum

Stimulation of terminals in symptomatic mutant SOD1 mice also elicited transient Ψ_m depolarizations that were not synchronized with stimulation. These asynchronous depolarizations were localized to particular subregions of the terminal. This finding, and Vila et al.'s (2003) report that in symptomatic SOD1-G93A mice the stimulation-induced elevations of mitochondrial $[\text{Ca}^{2+}]$ varied in different regions of the motor terminal, suggest that individual mitochondria within motor terminals can exhibit varied responses to Ca^{2+} loads. The asynchronous Ψ_m depolarizations recorded in motor terminals may resemble the localized Ψ_m depolarizations recorded e.g. by Chalmers and McCarron (2008) in smooth muscle cells subjected to Ca-overload. One possible origin for the asynchronous Ψ_m

depolarizations in motor terminals is that mitochondria undergoing permeability transition might release the Ca^{2+} they took up during nerve stimulation, thereby elevating cytosolic $[\text{Ca}^{2+}]$ and initiating Ca^{2+} uptake and Ψ_m depolarization in neighboring mitochondria (e.g. Nizami et al. 2010). Another possibility is that the asynchronous Ψ_m depolarizations might be elicited by Ca^{2+} released from endoplasmic reticulum (ER). The literature documents many examples of ER-mitochondrial interactions with respect to Ca^{2+} handling in other cells (reviewed by e.g. Spät et al. 2008; Giorgi et al. 2009). For the stimulation patterns used here, blocking Ca^{2+} uptake into ER has only a minimal effect on cytosolic $[\text{Ca}^{2+}]$ elevations and transmitter release in wt motor terminals (David and Barrett, 2003), suggesting that motor terminal mitochondria can take up Ca^{2+} from the cytosol without the need for an ER intermediary. However it is possible that ER might assume a more prominent role in Ca^{2+} handling in symptomatic mutant SOD1 mice.

Diamide-induced oxidative stress increases Ψ_m depolarizations in response to stimulation

To gain insight into mechanisms underlying the aberrant response to stimulation-induced Ca^{2+} loads in terminals of symptomatic mutant SOD1 mice, we imposed stresses on wt terminals in an attempt to induce similar aberrant behavior. Increasing stimulation-induced Ca^{2+} influx with 3,4-DAP increased the amplitude of the Ψ_m depolarization, but these depolarizations did not increment with successive trains, were not longer-lasting, and were not reduced by CsA. This result suggests that the aberrant behavior of mutant SOD1 mitochondria in response to Ca^{2+} loads (1) is not due simply to an increase in the magnitude of the stimulation-induced Ca^{2+} influx into the nerve terminal, (2) is not due simply to a paucity of mitochondria in the terminal, resulting in a larger Ca^{2+} load per mitochondrion, and (3) is not due simply to increasing the magnitude of the Ψ_m depolarization.

Another possible reason for the impaired mitochondrial response to Ca^{2+} loads in mutant SOD1 mice is oxidative damage, which can damage the electron transport chain (ETC). Damage to ETC complexes (especially complex I) can cause increased production of reactive oxygen species (ROS, e.g. Votyakova and Reynolds, 2005). Partial inhibition of complex I with low concentrations of rotenone increases stimulation-induced Ψ_m depolarizations in wt terminals (Nguyen et al. 2009). Oxidation of even normal SOD1 can induce toxic properties (Kabashi et al., 2007; Ezzi et al., 2007), and spinal cords from symptomatic SOD1-G93A mice display increased ROS production (Wu et al. 2006). Oxidative stress might increase the probability of opening pre-existing mPTP complexes, e.g. via ROS-induced reduction in the ability of the ETC to accelerate H^+ extrusion, thereby increasing the Ψ_m depolarization during Ca^{2+} influx. Oxidative stress might also increase assembly of mPTP complexes, e.g. by modifying the adenine nucleotide transporter (ANT), cyclophilin D and/or the phosphate carrier (all components/modifiers of the mPTP) or by increasing the binding of cyclophilin D and ANT (McStay et al., 2002).

We found that, as predicted, exposure of wt terminals to an oxidative stress (~0.5-1 hr in 0.2 mM diamide) increased the amplitude and duration of stimulation-induced Ψ_m depolarizations. This finding is consistent with demonstrations that glutathione depletion (an effect of diamide) inhibits complex I activity (e.g. Jha et al. 2000) and that diamide reduces mitochondrial respiratory capacity (e.g. Hill et al. 2010); both effects would reduce the ability of the ETC to accelerate H^+ extrusion during Ca^{2+} influx. The large Ψ_m depolarizations in diamide-treated wt terminals lacked the sensitivity to CsA evident in mutant SOD1 terminals. Perhaps the stimulation-induced Ψ_m depolarizations in diamide did not involve mPTP opening. Another possibility is that thiol crosslinking by diamide modified ANT in a manner that increased mPTP (Costantini et al. 2000) and also rendered it CsA-insensitive; however, this effect cannot by itself explain our data, since diamide did not produce a detectable steady-state mitochondrial depolarization, and the stimulation-induced Ψ_m depolarizations in diamide were at least partially reversible. The increased tendency for

mPTP opening following stimulation of mutant SOD1 terminals may require more prolonged exposure to oxidative stress, exposure to a different type of oxidative stress, and/or exposure to an additional type of stress. Oxidative stress likely contributes to at least part of the damage caused by mutant SOD1, because, for example, 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) slows disease progression and thereby prolongs survival of mutant SOD1 mice. Our finding that one type of oxidative stress can reproduce in wt terminals several features of the aberrant response to Ca^{2+} loads found in mutant SOD1 terminals suggests that one benefit of reducing oxidative stress might be reduced damage to motor terminal mitochondria.

Conclusions

The mitochondrial dysfunction in motor nerve terminals of SOD1-G93A and SOD1-G85R mice increases as the disease progresses. In the symptomatic mutant SOD1 mice studied here Ca^{2+} loads delivered by repetitive nerve stimulation elicited Ψ_m depolarizations that were larger and longer-lasting than those elicited in wt or presymptomatic terminals. These large evoked Ψ_m depolarizations, as well as additional, asynchronous Ψ_m depolarizations, were reduced by CsA, suggesting that the large Ψ_m depolarizations recorded in symptomatic mice were due in part to transient openings of the mPTP. In wt terminals the combination of Ca^{2+} influx with a diamide-induced oxidative stress elicited large Ψ_m depolarizations that mimicked some aspects of the behavior recorded in terminals of symptomatic mutant SOD1 mice. Thus oxidative stress might contribute to the aberrant mitochondrial response to stimulation in motor terminals of these mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Muscular Dystrophy Association (MDA 4344 JB, MDA 4348 & 112102 GD), the Amyotrophic Lateral Sclerosis Association (ALSA 1348 EB), and the National Institutes of Health (R01NS 58888 GD, R01 NS12404 EB, fellowship 1F31NS054606 LGC). Dr. Nguyen's graduate studies were supported by training grants T32 NS 07044 and T32 NS 007459. We thank Dr. Janet Talbot and Ms. Doris Nonner for help with genotyping, and Dr. Don Cleveland for providing founder transgenic mice.

References

- Angaut-Petit D, Molgo J, Connold AL, Faille L. The levator auris longus muscle of the mouse: a convenient preparation for studies of short- and long-term presynaptic effects of drugs or toxins. *Neurosci Lett*. 1987; 82:83–8. [PubMed: 2962016]
- Barber SC, Shaw PJ. Oxidative stress in ALS: Key role in motor neuron injury and therapeutic target. *Free Radic Biol Med*. 2010; 48:629–41. [PubMed: 19969067]
- Bernardi P, Vassanelli S, Veronese P, Colonna R, Szabo I, Zoratti M. Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations. *J Biol Chem*. 1992; 267:2934–9. [PubMed: 1737749]
- Bosco DA, Morfini G, Karabacak NM, Song Y, Gros-Louis F, Pasinelli P, et al. Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. *Nature Neuroscience*. 2010; 13:1396–403.
- Brown MR, Sullivan PG, Geddes JW. Synaptic mitochondria are more susceptible to Ca^{2+} overload than nonsynaptic mitochondria. *J Biol Chem*. 2006; 281:11658–68. [PubMed: 16517608]
- Bruijn LI, Becher MW, Lee MK, Anderson KL, Jemkins NA, Copeland NG, et al. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron*. 1997; 18:327–38. [PubMed: 9052802]

- Chalmers S, McCarron JG. The mitochondrial membrane potential and Ca^{2+} oscillations in smooth muscle. *J Cell Sci.* 2008; 121:75–85. [PubMed: 18073239]
- Chiu AY, Zhai P, Dal Canto MC, Peters TM, Kwon YW, Prattis SM, et al. Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Mol Cell Neurosci.* 1995; 6:349–62. [PubMed: 8846004]
- Costantini P, Belzacq AS, Vieira HLA, Larochette N, de Pablo MA, Zamzami N, et al. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene.* 2000; 19:307–14. [PubMed: 10645010]
- Damiano M, Starkov AA, Petri S, Kipiani K, Kiaei M, Mattiazzi M, et al. Neural mitochondrial Ca^{2+} capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice. *J Neurochem.* 2006; 96:1349–61. [PubMed: 16478527]
- David G. Mitochondrial clearance of cytosolic Ca^{2+} in stimulated lizard motor nerve terminals proceeds without progressive elevation of mitochondrial matrix $[\text{Ca}^{2+}]$. *J Neurosci.* 1999; 19:7495–506. [PubMed: 10460256]
- David G, Barrett EF. Mitochondrial Ca^{2+} uptake prevents desynchronization of quantal release and minimizes depletion during repetitive stimulation of mouse motor nerve terminals. *J Physiol (Lond).* 2003; 548:425–38. [PubMed: 12588898]
- David G, Barrett JN, Barrett EF. Evidence that mitochondria buffer physiological Ca^{2+} loads in lizard motor nerve terminals. *J Physiol (Lond).* 1998; 509:59–65. [PubMed: 9547381]
- David G, Modney B, Scappaticci KA, Barrett JN, Barrett EF. Electrical and morphological factors influencing the depolarizing after-potential in rat and lizard myelinated axons. *J Physiol.* 1995; 489:141–57. [PubMed: 8583398]
- Erzen I, Cvetko E, Obreza S, Angaut-Petit D. Fiber types in the mouse levator auris longus muscle: a convenient preparation to study muscle and nerve plasticity. *J Neurosci Res.* 2000; 59:692–7. [PubMed: 10686598]
- Ezzi SA, Urushitani M, Julien JP. Wild-type superoxide dismutase acquires binding and toxic properties of ALS-linked mutant forms through oxidation. *J Neurochem.* 2007; 102:170–8. [PubMed: 17394546]
- Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, et al. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp Neurol.* 2004; 185:232–40. [PubMed: 14736504]
- Forsberg K, Jonsson PA, Andersen PM, Bergemalm D, Graffmo KS, Hultdin M, Jacobsson J, Rosquist R, Marklund SL, Brannstrom T. Novel antibodies reveal inclusions containing non-native SOD1 in sporadic ALS patients. *PLoS One.* 2010; 5:e11552.
- Forsythe ID, Tsujimoto T, Barnes-Davies M, Cuttle MF, Takahashi T. Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. *Neuron.* 1998; 20:797–807. [PubMed: 9581770]
- Frey D, Schneider C, Xu L, Borg J, Spooren W, Caroni P. Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *J Neurosci.* 2000; 20:2534–42. [PubMed: 10729333]
- Giorgi C, De Stefani D, Bononi A, Rizzuto R, Pinton P. Structural and functional link between the mitochondrial network and the endoplasmic reticulum. *Int J Biochem Cell Biol.* 2009; 41:1817–27. [PubMed: 19389485]
- Gunter TE, Pfeiffer DR. Mechanisms by which mitochondria transport calcium. *Am J Physiol.* 1990; 258:C755–86. [PubMed: 2185657]
- Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, et al. Motor neuron degeneration in mice that express a human Cu/Zn superoxide dismutase mutation. *Science.* 1994; 264:1772–5. [PubMed: 8209258]
- Harraz MM, Marden JJ, Zhou W, Zhang Y, Williams A, Sharov VS. SOD1 mutations disrupt redox-sensitive Rac regulation of NADPH oxidase in a familial ALS model. *J Clin Invest.* 2008; 118:659–70. [PubMed: 18219391]
- Hegedus J, Putman CT, Tyreman N, Gordon T. Preferential motor unit loss in the SOD1G93A transgenic mouse model of amyotrophic lateral sclerosis. *J Physiol (Lond).* 2008; 586:3337–51. [PubMed: 18467368]

- Hill BG, Higdon AN, Dranka BP, Darley-USmar VM. Regulation of vascular smooth muscle cell bioenergetic function by protein glutathiolation. *Biochim Biophys Acta*. 2010; 1797:285–95. [PubMed: 19925774]
- Hüser J, Blatter LA. Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. *Biochem J*. 1999; 343:311–7. [PubMed: 10510294]
- Hüser J, Rechenmacher CE, Blatter LA. Imaging the permeability pore transition in single mitochondria. *Biophys J*. 1998; 74:2129–37. [PubMed: 9545072]
- Ichas F, Mazat JP. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim Biophys Acta*. 1998; 1366:33–50. [PubMed: 9714722]
- Israelson A, Arbel N, Da Cruz S, Ilieva H, Yamanaka K, Shoshan-Barmatz V, Cleveland DW. Misfolded mutant SOD1 directly inhibits VDAC1 conductance in a mouse model of inherited ALS. *Neuron*. 2010; 67:575–87. [PubMed: 20797535]
- Jha N, Jurma O, Lalli G, Liu Y, Pettus EH, Greenamyre JT, et al. Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity. Implications for Parkinson's disease. *J Biol Chem*. 2000; 275:26096–101. [PubMed: 10846169]
- Jiang D, Zhao L, Clapham DE. Genome-wide RNAi screen identifies Letm1 as a mitochondrial $\text{Ca}^{2+}/\text{H}^{+}$ antiporter. *Science*. 2009; 326:144–7. [PubMed: 19797662]
- Johnson-Cadwell LI, Jekabsons MB, Wang A, Polster BM, Nicholls DG. 'Mild uncoupling' does not decrease mitochondrial superoxide levels in cultured cerebellar granule neurons but decreases spare respiratory capacity and increases toxicity to glutamate and oxidative stress. *J Neurochem*. 2007; 101:1619–31. [PubMed: 17437552]
- Kabashi E, Valdmanis PN, Dion P, Rouleau GA. Oxidized/misfolded superoxide dismutase-1: the cause of all amyotrophic lateral sclerosis? *Ann Neurol*. 2007; 62:553–9. [PubMed: 18074357]
- Karlsson J, Fong KS, Hansson MJ, Elmer E, Csiszar K, Keep MF. Life span extension and reduced neuronal death after weekly intraventricular cyclosporin injections in the G93A transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosurg*. 2004; 101:128–37. [PubMed: 15255263]
- Kawamata H, Manfredi G. Mitochondrial dysfunction and intracellular calcium dysregulation in ALS. *Mech Ageing Develop*. 2010 in press.
- Kirichok Y, Krapivinsky G, Clapham DE. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature*. 2004; 427:360–4. [PubMed: 14737170]
- Kirkinezos IG, Bacman SR, Hernandez D, Oca-Cossio J, Arias LJ, Perez-Pinzon MA, et al. Cytochrome c association with the inner mitochondrial membrane is impaired in the CNS of G93A-SOD1 mice. *J Neurosci*. 2005; 25:164–72. [PubMed: 15634778]
- Kirkinezos IG, Hernandez D, Bradley WG, Moraes CT. An ALS mouse model with a permeable blood-brain barrier benefits from systemic cyclosporine A treatment. *J Neurochem*. 2004; 88:821–6. [PubMed: 14756802]
- Kong J, Xu Z. Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *J Neurosci*. 1998; 18:3241–50. [PubMed: 9547233]
- Kushnareva YE, Sokolove PM. Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. *Arch Biochem Biophys*. 2000; 376:377–88. [PubMed: 10775426]
- Li Q, Van de Velde C, Israelson A, Xie J, Bailey AO, Dong MQ, Chun SJ, Roy T, Winer L, Yates JR, Capaldi RA, Cleveland DW, Miller TM. ALS-linked mutant superoxide dismutase I (SOD1) alters mitochondrial protein composition and decreases protein import. *Proc Nat Acad Sci USA*. 2010; 107:21146–51. [PubMed: 21078990]
- Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*. 1991; 66:807–15. [PubMed: 1715244]
- Marden JJ, Harraz MM, Williams AJ, Nelson K, Luo M, Paulson H, et al. Redox modifier genes in amyotrophic lateral sclerosis in mice. *J Clin Invest*. 2007; 117:2913–9. [PubMed: 17853944]
- Martin LJ. The mitochondrial permeability transition pore: A molecular target for amyotrophic lateral sclerosis therapy. *Biochim Biophys Acta*. 2010; 1802:186–97. [PubMed: 19651206]

- Martin LJ, Gertz B, Pan Y, Price AC, Molkentin JD, Chang Q. The mitochondrial permeability transition pore in motor neurons: involvement in the pathobiology of ALS mice. *Exp Neurol*. 2009; 218:333–46. [PubMed: 19272377]
- Mattiazzi M, D'Aurelio M, Gajewski CD, Martushova K, Kiaei M, Beal MF, et al. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J Biol Chem*. 2002; 277:29626–33. [PubMed: 12050154]
- McStay GP, Clarke SJ, Halestrap AP. Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. *Biochem J*. 2002; 367:541–8. [PubMed: 12149099]
- Morita K, Barrett EF. Evidence for two calcium-dependent potassium conductances in lizard motor nerve terminals. *J Neurosci*. 1990; 10:2614–25. [PubMed: 1696981]
- Murray LM, Talbot K, Gillingwater TH. Review: Neuromuscular synaptic vulnerability in motor neurone disease: amyotrophic lateral sclerosis and spinal muscular atrophy. *Neuropathol Appl Neurobiol*. 2010; 36:133–56. [PubMed: 20202121]
- Naga KK, Sullivan PG, Geddes JW. High cyclophilin D content of synaptic mitochondria results in increased vulnerability to permeability transition. *J Neurosci*. 2007; 27:7469–75. [PubMed: 17626207]
- Nguyen KT, García-Chacón LE, Barrett JN, Barrett EF, David G. The Ψ_m depolarization that accompanies mitochondrial Ca^{2+} uptake is greater in mutant SOD1 than in wild-type mouse motor terminals. *Proc Natl Acad Sci U S A*. 2009; 106:2007–11. [PubMed: 19174508]
- Nicholls DG, Chalmers S. The integration of mitochondrial calcium transport and storage. *J Bioenerg Biomembr*. 2004; 36:277–81. [PubMed: 15377857]
- Nicholls DG, Ferguson SJ. *Bioenergetics 3*. London: Academic Press; 2002.
- Nicholls DG, Ward MW. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci*. 2000; 23:166–74. [PubMed: 10717676]
- Nizami S, Lee VWY, Davies J, Long P, Jovanovic JN, Sihra TS. Presynaptic roles of intracellular Ca^{2+} stores in signalling and exocytosis. *Biochem Soc Trans*. 2010; 38:529–35. [PubMed: 20298216]
- Parone, PA.; Da Cruz, S.; Garcia, ML.; Forte, MA.; Bernardi, P.; Cleveland, DW. Program No 656/13/N7. 2010 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience; 2010. Increasing mitochondrial calcium buffering capacity systemically in mice expressing ALS-linked mutations does not alter disease course. Online
- Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK. MICU1 encodes a mitochondrial EF hand protein required for Ca^{2+} uptake. *Nature*. 2010; 467:291–6. [PubMed: 20693986]
- Pun S, Santos AF, Saxena S, Xu L, Caroni P. Selective vulnerability and pruning of phasic motoneuron axons in motoneuron disease alleviated by CNTF. *Nat Neurosci*. 2006; 9:408–19. [PubMed: 16474388]
- Rosen DR, Siddique T, Patterson DA, Figlewicz P, Sapp A, Hentati D, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*. 1993; 362:59–62. [PubMed: 8446170]
- Saotome M, Katoh H, Yaguchi Y, Tanaka T, Urushida T, Satoh H, et al. Transient opening of mitochondrial permeability transition pore by reactive oxygen species protects myocardium from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2009; 296:H1125–32. [PubMed: 19202002]
- Schaefer AM, Sanes JR, Lichtman JW. A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. *J Comp Neurol*. 2005; 490:209–19. [PubMed: 16082680]
- Shen D, Dalton TP, Nebert DW, Shertzer HG. Glutathione redox state regulates mitochondrial reactive oxygen production. *J Biol Chem*. 2005; 280:25305–12. [PubMed: 15883162]
- Shi P, Gal J, Kwinter DM, Liu X, Zhu H. Mitochondrial dysfunction in amyotrophic lateral sclerosis. *Biochim Biophys Acta*. 2010; 1802:45–51. [PubMed: 19715760]
- Spät A, Szanda G, Csordás G, Hajnóczky. High and low calcium-dependent mechanisms of mitochondrial calcium signalling. *Cell Calcium*. 2008; 44:51–63. [PubMed: 18242694]

- Tabti N, Bourret C, Mallart A. Three potassium currents in mouse motor nerve terminals. *Pflugers Arch.* 1989; 413:395–400. [PubMed: 2928092]
- Talbot J, Barrett JN, Barrett EF, David G. Stimulation-induced changes in NADH fluorescence and mitochondrial membrane potential in lizard motor nerve terminals. *J Physiol.* 2007; 579:783–98. [PubMed: 17218351]
- Valentine JS, Doucette PA, Zittin Potter S. Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. *Annu Rev Biochem.* 2005; 74:563–93. [PubMed: 15952898]
- Vila L, Barrett EF, Barrett JN. Stimulation-induced mitochondrial $[Ca^{2+}]$ elevations in mouse motor terminals: comparison of wild-type with SOD1G93A. *J Physiol (Lond).* 2003; 549:719–28. [PubMed: 12717010]
- Votyakova TV, Reynolds IJ. Ca^{2+} -induced permeabilization promotes free radical release from rat brain mitochondria with partially inhibited complex I. *J Neurochem.* 2005; 93:526–37. [PubMed: 15836612]
- Woodfield KA, Ruck AD, Brdiczka D, Halestrap AP. Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. *Biochem J.* 1998; 336:287–90. [PubMed: 9820802]
- Wooley CM, Sher RB, Kale A, Frankel WN, Cox GA, Seburn KL. Gait analysis detects early changes in transgenic SOD1(G93A) mice. *Muscle Nerve.* 2005; 32:43–50. [PubMed: 15880561]
- Wu DC, Ré DB, Nagai M, Ischiropoulos H, Przedborski S. The inflammatory NADPH oxidase enzyme modulates motor neuron degeneration in amyotrophic lateral sclerosis mice. *Proc Natl Acad Sci USA.* 2006; 103:12132–7. [PubMed: 16877542]

Abbreviations

CsA	cyclosporin A
DAP	diaminopyridine
ETC	electron transport chain
fALS	familial amyotrophic lateral sclerosis
LAL	levator auris longus muscle
mPTP	mitochondrial permeability transition pore
Rh-123	rhodamine 123
SOD1	Cu/Zn superoxide dismutase 1
Ψ_m	membrane potential across inner mitochondrial membrane

Fig. 1.

During stimulation at 100 Hz motor terminals in symptomatic mutant SOD1 mice exhibit large, incrementing Ψ_m depolarizations. Panels A-C show Ψ_m depolarizations evoked by 3 trains of 100 Hz stimulation, measured as increased fluorescence of Rh-123. (A) Small, non-incrementing Ψ_m depolarizations in terminals of an hSOD1-wt mouse (270 days old). (B) Superimposed Ψ_m depolarizations recorded in the hSOD1-wt terminal of A (using a different amplitude scale), an SOD1- G93A terminal (132 days old), and an SOD1-G85R terminal (227 days old). Arrows indicate asynchronous Ψ_m depolarizations in the mutant SOD1 terminals. These results are representative of those recorded in the terminals of 3 symptomatic G93A and 3 symptomatic G85R mice. (C) Arrows indicate methods used to calculate peak amplitudes of cumulative and individual responses to stimulation trains, applied to the 3rd train in the G85R record of B (also see Materials and Methods).

Fig. 2.

The stimulation-induced Ψ_m depolarization is Ca^{2+} -dependent. A, Sequential traces show the response to 100 Hz stimulus trains in a symptomatic G93A terminal before (left), during removal of bath Ca^{2+} with addition of the Ca^{2+} buffer BAPTA (2 mM, middle) and after restoration of Ca^{2+} (right). B, Similar records from an hSOD1-wt terminal, cooled to 22 °C to increase the amplitude of Ψ_m depolarizations. Similar Ca^{2+} dependence was also documented in three symptomatic G85R terminals.

Fig. 3. The incrementing, stimulation-induced Ψ_m depolarizations in symptomatic mutant SOD1 terminals are inhibited by cyclosporin A (CsA, 1-2 μ M), but not by FK 506 (1 μ M). (A) Rh-123 fluorescence changes evoked by three successive “triplet train” stimulation protocols in a motor terminal from a symptomatic G93A mouse. Prior to addition of CsA, the fluorescence response to successive trains incremented (as also seen in Fig. 1B). CsA markedly reduced the amplitude of these depolarizations. (B and C) Time course of cumulative (B) and individual (C) response amplitudes in a G85R terminal before and after addition of FK506, followed by CsA. Lines connect the peak amplitudes evoked by train #1, #2, and #3 in each triplet. Times are relative to addition of FK506 at time zero. The

technique for measuring response amplitudes is illustrated in Fig. 1C. Similar results were obtained in terminals of four additional symptomatic mice (2 G85R, 2 G93A).

Fig. 4. Substitution of Sr^{2+} for bath Ca^{2+} reduces the Ψ_m depolarizations evoked by the 2nd and 3rd trains in mutant SOD1 terminals. Each panel superimposes the response to triplet train stimulation before and after bath Ca^{2+} was replaced by Sr^{2+} in symptomatic G93A (A) and G85R (B) terminals. Similar findings were recorded in 2 additional G93A and 2 additional G85R terminals. The duration of Sr^{2+} exposure was 20-30 min.

Fig. 5. Stimulation-induced mPTP openings are transient, and some Ψ_m depolarizations are localized to subregions in an SOD1-G93A terminal. (A) Images show Rh-123 fluorescence in a G93A terminal (top), divided into the indicated 5 compartments (a-e, bottom). (The bright region at the top was excluded from analysis because its fluorescence did not change with stimulation; it probably represents a perisynaptic Schwann cell.) Traces at right show Ψ_m depolarizations measured from each of the indicated compartments during triplet stimulus trains. Arrows indicate asynchronous depolarizations (one between the 2nd and 3rd trains, another after the 3rd train), which were localized to different adjoining compartments. (B) Intra-terminal heterogeneity demonstrated in pseudocolor images of the terminal in A in

which the magnitude of the fluorescence change was color-coded on a blue→red scale (blue = rest). Images are shown at rest, during the 2nd and 3rd stimulation trains, and during the asynchronous depolarizations that arose following the 2nd and 3rd trains. The color scale was identical for all images in the terminal, and was normalized to the maximal response measured in that terminal. (C) Superimposed sequential triplet stimulus trains recorded in compartment c. Arrows indicate the peak amplitude of the response to the 3rd train in each triplet; these peak amplitudes, and those from other similarly analyzed subregions, are plotted in D. (D) Scatter plot of cumulative response to the 3rd train of the 1st triplet (x axis) and the 3rd train of the 2nd triplet (y axis). Amplitudes were normalized to the peak amplitude for that terminal. Circles plot data for the compartments outlined in A; other symbols came from similar compartmental analyses of 3 G85R terminals. Compartments that showed large responses during the 1st triplet train also tended to show large responses during the 2nd triplet train ($r=0.43$, $p<0.05$).

Fig. 6.

Application of 3,4-diaminopyridine (DAP) to wt or hSOD1-wt terminals increases the stimulation-evoked Ψ_m depolarization, but this increased depolarization is not reduced by CsA. (A) Superimposed response to triplet train stimulation in a wt terminal before and after application of 3,4-DAP (20 μ M). (B) Cumulative response to each of the 3 stimulus trains in the terminal of a hSOD1-wt mouse before and after addition of 100 μ M 3,4-DAP, with subsequent addition of 1 μ M CsA. Note in both A and B that the responses in 3,4-DAP did not increment with successive stimulus trains, i.e. the responses to 2nd and 3rd trains had amplitudes and time courses similar to that evoked by the 1st train. Similar findings were recorded in 3 non-fALS terminals.

Fig. 7.

Wt terminals show incrementing stimulation-evoked Ψ_m depolarizations following prolonged exposure to the oxidizing agent diamide, but these depolarizations are not blocked by CsA. (A) Superimposed responses to stimulation before and at the indicated times after exposure to 200 μ M diamide. Responses in diamide had larger, incrementing amplitudes, and decayed more slowly. (B) Cumulative response to each of 3 stimulus trains before and after addition of 200 μ M diamide, with subsequent addition of 1 μ M CsA. Similar findings were recorded in 3 (A) and 2 (B) additional terminals.