On the Role of Mitochondria in the Regulation of Calcium in Motor Nerve Terminals During Repetitive Stimulation

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UNIVERSITY OF MIAMI

ON THE ROLE OF MITOCHONDRIA IN THE REGULATION OF CALCIUM IN MOTOR NERVE TERMINALS DURING REPETITIVE STIMULATION

By

Luis E. García-Chacón

A DISSERTATION

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Coral Gables, Florida

May 2008
ON THE ROLE OF MITOCHONDRIA IN THE REGULATION OF CALCIUM IN MOTOR NERVE TERMINALS DURING REPETITIVE STIMULATION

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During repetitive stimulation of motor nerve terminals, mitochondrial Ca\textsuperscript{2+} uptake limits increases in free cytosolic [Ca\textsuperscript{2+}] and helps ensure faithful neuromuscular transmission. Changes in cytosolic [Ca\textsuperscript{2+}] and in mitochondrial [Ca\textsuperscript{2+}] as well as changes in mitochondrial membrane potential (Ψ\textsubscript{m}) were studied in mouse motor nerve terminals using Ca\textsuperscript{2+} sensitive indicator and potentiometric dyes, respectively. Trains of action potentials (APs) at 50 to 100 Hz produced a rapid increase in mitochondrial [Ca\textsuperscript{2+}] followed by a plateau which usually continued beyond the end of stimulation. After stimulation, mitochondrial [Ca\textsuperscript{2+}] decayed back to baseline over the course of tens of seconds to minutes. Increasing the Ca\textsuperscript{2+} load delivered to the terminal by increasing the number of stimuli (500-2000), increasing bath [Ca\textsuperscript{2+}], or prolonging the AP with 3,4-diaminopyridine (3-4, DAP, 100 µM), prolonged the post-stimulation decay of mitochondrial [Ca\textsuperscript{2+}] without increasing the amplitude of the plateau. Inhibiting openings of the mitochondrial permeability transition pore with cyclosporin A (5 µM) had no significant effect on the decay of mitochondrial [Ca\textsuperscript{2+}]. Inhibition of the mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger with CGP-37157 (50 µM) dramatically prolonged the post-stimulation decay of mitochondrial [Ca\textsuperscript{2+}], reduced post-stimulation residual cytosolic [Ca\textsuperscript{2+}], and reduced the amplitude of end-plate potentials evoked after the end of stimulation. Stimulation-induced mitochondrial Ca\textsuperscript{2+} uptake resulted in Ψ\textsubscript{m}
depolarizations that were small or undetectable at near-physiological temperatures (~30 °C). Their amplitude became larger at lower temperatures (~20 °C), or when AP duration was increased with 3,4-DAP (20 µM). \( \Psi_m \) depolarizations were inhibited by lowering bath \([Ca^{2+}]\) or by blocking P/Q-type \(Ca^{2+}\) channels with \(\omega\)-agatoxin (0.3 µM). Partial inhibition of complex I of the electron transport chain (ETC) with rotenone (50 nM) increased the amplitude of stimulation-induced \(\Psi_m\) depolarizations. These findings suggest that: (1) \(Ca^{2+}\) extrusion from motor terminal mitochondria occurs primarily via the Na\(^+\)-Ca\(^{2+}\) exchanger and helps sustain post-tetanic transmitter release, and (2) that the depolarization of \(\Psi_m\) that accompanies \(Ca^{2+}\) uptake is limited by accelerated proton extrusion via the ETC.
Dedicated to:
Jennifer Lynn Bass, MD, and Daniel Rauch, MD
For their encouragement;
it has meant more than they will ever know

Dedicado a:
Raul de Gasperi, MD
Caballero y erudito, por todo su apoyo
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Finally, I would like to specially thank Rosa Mari Alvarez, Roxanna Braithwaite, Mercedes Dirube, Katrina Hollis, Niurka Ortiz, Ailys Quintana, and Lenora Smith for their friendship and endless encouragement (and for all the Cuban coffee!).
...Anduve como vosotros escarbando
la estrella interminable,
y en mi red, en la noche, me desperté desnudo,
la única presa:
un pez encerrado en el viento.

– Neruda
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<tbody>
<tr>
<td>3,4-DAP</td>
<td>3,4-diaminopyridine</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CCCP</td>
<td>Cyanide m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>fALS</td>
<td>Familial amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotransmitter</td>
</tr>
<tr>
<td>OG-1</td>
<td>Oregon Green BAPTA-1</td>
</tr>
<tr>
<td>OG-5N</td>
<td>Oregon Green BAPTA-5N</td>
</tr>
<tr>
<td>PTP</td>
<td>Post-tetanic potentiation</td>
</tr>
<tr>
<td>Rh123</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
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Chapter 2 consists of a published manuscript* and Chapter 3** of a manuscript in preparation. The data for Fig. 4 in Chapter 2, and for Fig. 5 and Supporting Fig. 2 in Chapter 3 were collected by Khanh T. Nguyen. John N. Barrett collected the data for Supporting Fig. 1 and the $\Psi_m$ data for SOD1 knockout mice in Chapter 3. For some of the figures in Chapter 3, I collected similar data originally, but the records shown in Figs. 1A (upper trace), 3A, 4A, and 5 in Chapter 3 were collected by Gavriel David (who also supervised the data acquisition for all other experiments).

*García-Chacón LE$^1$, Nguyen KT$^2$, David G$^1$ & Barrett EF$^{1,2}$ (2006). Extrusion of Ca$^{2+}$ from mouse motor terminal mitochondria via a Na$^+$-Ca$^{2+}$ exchanger increases post-tetanic evoked release. J Physiol 574, 663-75.

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**García-Chacón LE$^{1,2}$, Nguyen KT$^{1,3}$, Barrett JN$^{2,3}$, Barrett EF$^{2,3}$ & David G$^2$. The mitochondrial depolarization that accompanies mitochondrial Ca$^{2+}$ uptake is greater in pre-symptomatic SOD1-G85R than in wild-type mouse motor terminals. In preparation.

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

A. Mitochondrial calcium uptake under physiological conditions

A principal function of mitochondria is the catabolism of carbohydrates and fatty acids and the use of the energy liberated in these processes to produce adenosine triphosphate (ATP), the “energy currency” of the body. Mitochondria also play a prominent role in programmed cell death, or apoptosis. The mitochondrial function that is the subject of this dissertation is participation in the regulation of intracellular [Ca^{2+}].

In the 1960s it was demonstrated that mitochondria can sequester considerable amounts of free Ca^{2+} (DeLuca & Engstrom 1961; Vasington & Murphy 1962; Rossi & Lehninger, 1963; Lehninger et al. 1967). Isolated mitochondria can sequester Ca^{2+} from the bathing solution and increase their total calcium concentrations up to the millimolar range (reviewed in Gunter & Pfeiffer, 1990). This finding suggested that mitochondria might help regulate Ca^{2+} in living cells.

Alnaes & Rahamimoff (1975) measured neurotransmitter (NT) release at the frog neuromuscular junction (NMJ) in the presence of an agent that inhibited mitochondrial Ca^{2+} uptake (ruthenium red). The result was an increase in spontaneous NT release together with a decrease in the quantal content of evoked release. This finding suggested that mitochondrial Ca^{2+} uptake played a role in the regulation of neurotransmission across the NMJ. However, at the time, there was considerable resistance to the idea of mitochondrial involvement in Ca^{2+} regulation in vivo, due in part to studies on isolated...
mitochondria which had measured an apparent $K_d$ for mitochondrial Ca$^{2+}$ uptake in the micromolar range (see Gunter & Pfeiffer, 1990). Free calcium within living cells was thought to be an order of magnitude lower (~0.1 µM). Investigators believed that if mitochondria did take up any Ca$^{2+}$ in vivo, it was probably under pathological conditions in which cytosolic [Ca$^{2+}$] increased to supra-physiological levels.

However, later studies in cultured neurons from the rat dorsal root ganglion (Thayer & Miller 1990; Werth & Thayer 1994) suggested that mitochondrial Ca$^{2+}$ transport did in fact have an effect on cytosolic [Ca$^{2+}$] under physiological conditions. Mitochondrial sequestration of cytosolic Ca$^{2+}$ loads has been detected in the rat neurohypophysis (Stuenkel, 1994), in bullfrog sympathetic neurons (Friel & Tsien, 1994; Colegrove et al. 2000a), and in rat adrenal chromaffin cells (Herrington et al. 1996; Babcock et al. 1997). Studies in lizard, frog and mouse motor nerve terminals have also detected mitochondrial uptake during stimulation-induced increases in cytosolic [Ca$^{2+}$] (David et al. 1998; David & Barrett 2000; Suzuki et al. 2002). These studies motivated the work described in this dissertation, in which stimulation-induced cytosolic and intra-mitochondrial [Ca$^{2+}$] responses in mouse motor neuron terminals were studied using dyes whose fluorescence increases upon binding Ca$^{2+}$ (Ca$^{2+}$ indicator dyes).

B. Control of cytosolic [Ca$^{2+}$] by mitochondrial Ca$^{2+}$ uptake and buffering of matrix Ca$^{2+}$

The invasion of mouse motor nerve terminals with action potentials (APs) results in the opening of voltage-gated Ca$^{2+}$ channels of the P/Q type (Westenbroek et al. 1998).
Extracellular Ca\(^{2+}\) moves into the cytosol driven by a large electrochemical gradient. Much of the entering Ca\(^{2+}\) is bound by endogenous buffers in the cytosol, resulting in an estimated bound:free ratio of \(~100\) (Neher & Augustine, 1992). Cytosolic endogenous buffers are thought to consist mostly of members of the EF-hand family of proteins and include calbindin, calretinin, and parvalbumin (reviewed in Burnashev & Rozov, 2005). Nevertheless, during stimulation cytosolic [Ca\(^{2+}\)] increases from a resting level of \(~100\) nM (Fiskum, 1985) to the micromolar range. Some of the entering Ca\(^{2+}\) binds to membrane-trafficking synaptotagmin proteins and by doing so triggers NT release (see reviews by Sudhof, 2004, and Rizo et al. 2006).

Entry of Ca\(^{2+}\) into the mitochondrial matrix occurs via a uniporter/channel (Kirichok et al. 2004). Data from some studies have suggested that the uniporter opens when cytosolic [Ca\(^{2+}\)] reaches \(~500\) to \(~600\) nM (Friel & Tsien, 1994; Stuenkel, 1994; Herrington et al. 1996). However, David et al. (1998) reported mitochondrial Ca\(^{2+}\) uptake at cytosolic [Ca\(^{2+}\)] levels as low as 200 nM. In comparison to the studies in isolated mitochondria this suggests a lower K\(_d\) for mitochondrial Ca\(^{2+}\) uptake in vivo. Cytosolic components such as spermine significantly lower the threshold for mitochondrial Ca\(^{2+}\) uptake in vivo (Lenzen et al, 1986; Rustenbeck et al. 1993). The uniporter is activated by Ca\(^{2+}\) itself, with the dependence of the rate of Ca\(^{2+}\) transport on external [Ca\(^{2+}\)] following a simple hyperbolic relation (Kasparinsky & Vinogradov, 1996). Mitochondrial Ca\(^{2+}\) uptake is a passive process which exploits a favorable electrical gradient (\(\Psi_m\)) established by the extrusion of protons by complexes I, III, and IV of the electron transport chain (ETC, reviewed by Gunter & Pfeiffer, 1990; Nicholls &
Chalmers, 2004). Activity of the ETC establishes a potential difference of 150-200 mV (matrix side negative) across the inner mitochondrial membrane.

A number of studies in different types of neurons have recorded changes in cytosolic \([\text{Ca}^{2+}]\) during stimulation. Step depolarization under voltage clamp, high levels of extracellular \([\text{K}^+]\), or trains of APs (at frequencies of 10-100 Hz) result in a rapid increase in cytosolic \([\text{Ca}^{2+}]\) (Stuenkel, 1994; Friel & Tsien, 1994; Wu & Betz, 1996; Ravin et al. 1997; David et al. 1997; David et al. 1998). Shortly after this initial increase, however, the rate of rise of cytosolic \([\text{Ca}^{2+}]\) slows down, stabilizing at a plateau level which is maintained until the end of stimulation. During this plateau, \(\text{Ca}^{2+}\) is still entering the cell, because post-synaptic EPPs, indicative of NT release, can still be recorded (David et al. 1997; David & Barrett, 2003). Therefore, during the observed plateau, the rate of \(\text{Ca}^{2+}\) influx into the terminal must equal the rate of \(\text{Ca}^{2+}\) efflux/sequestration. Calcium efflux could theoretically be occurring across the plasma membrane back into the extracellular space. However, in studies in lizard and frog NMJs, inhibition of two known mechanisms for \(\text{Ca}^{2+}\) efflux across the cell membrane, the \(\text{Na}^+-\text{Ca}^{2+}\) exchanger (inhibited by lithium) and the \(\text{Ca}^{2+}\) ATPase (inhibited by pH ~9), had no significant effect on cytosolic \([\text{Ca}^{2+}]\) during stimulation (David, 1999; Suzuki et al. 2002). It is possible that the smooth endoplasmic reticulum sequesters \(\text{Ca}^{2+}\) during stimulation, but no effects on the cytosolic \([\text{Ca}^{2+}]\) response were observed when endoplasmic reticulum \(\text{Ca}^{2+}\) uptake was inhibited by thapsigargin and cyclopiazonic acid in rat neurohypophysial neurons (Stuenkel, 1994) or by cyclopiazonic acid in lizard and mouse motor terminals (David 1999; David & Barrett, 2003). However, when mitochondrial \(\text{Ca}^{2+}\) uptake was inhibited by either blocking the uniporter/channel
(Stuenkel, 1994), or by dissipating $\Psi_m$ with agents which insert themselves into membranes and allow protons to flow down their electrochemical gradient (proton carriers), the cytosolic $[\text{Ca}^{2+}]$ plateau was abolished and cytosolic $[\text{Ca}^{2+}]$ continued instead to increase throughout stimulation (Friel & Tsien, 1994; Tang & Zucker, 1997; David et al. 1998; David, 1999; David & Barrett, 2000; Suzuki, 2002).

In this laboratory, studies in motor nerve terminals from both lizard (David et al. 1998) and mice (Vila et al. 2003) have measured changes in mitochondrial $[\text{Ca}^{2+}]$ during stimulation by using $\text{Ca}^{2+}$ indicator dyes which are loaded into the mitochondrial matrix with the use of a special protocol (see Methods sections in Chapters 2 & 3). With stimulation at frequencies of 25-100 Hz, mitochondrial $[\text{Ca}^{2+}]$ begins to increase after about 10-15 APs, at about the same time that cytosolic $[\text{Ca}^{2+}]$ begins to plateau. Mitochondrial $[\text{Ca}^{2+}]$ continues to increase, but after a brief time, also reaches a plateau which is usually maintained throughout and even beyond the end of stimulation. This result, together with the marked increase in cytosolic $[\text{Ca}^{2+}]$ measured when mitochondrial $\text{Ca}^{2+}$ uptake is inhibited with proton carriers or inhibitors of mitochondrial ETC complexes, suggest that in motor terminals mitochondria function as powerful $\text{Ca}^{2+}$ buffers of stimulation-induced increases in $\text{Ca}^{2+}$ loads.

But how much $\text{Ca}^{2+}$ can mitochondria sequester? Pivovarova et al. (1999) and Pivovarova et al. (2002) used x-ray microanalysis of rapidly-frozen frog sympathetic neurons to measure stimulation-induced changes in total (i.e. free plus bound) mitochondrial calcium. They calculated that total mitochondrial calcium increases to levels approaching 10 mM during stimulation. This value is several orders of magnitude higher than the mitochondrial $[\text{Ca}^{2+}]$ plateau observed during stimulation, which, in
isolated rat brain mitochondria and in lizard motor terminal mitochondria, has been estimated to range from 1 to 5 µM (David, 1999; Chalmers & Nicholls, 2003; David et al. 2003). Based on these findings, it is evident that the mitochondrial matrix contains powerful mechanisms for buffering increases in mitochondrial [Ca^{2+}], some of which do not appear to saturate under normal physiological conditions. Conventional buffers (maybe in the form of ionized proteins) are probably at work, and the bound:free ratio of matrix Ca^{2+} is estimated to range between 3000 and 4000 (Babcock et al. 1997; Kaftan et al. 2000). However, with continued Ca^{2+} uptake, these conventional buffers would be expected to saturate eventually, and mitochondrial [Ca^{2+}] would then continue to increase, rather than plateau.

Two mechanisms (which are not mutually exclusive) have been proposed to explain the mitochondrion’s powerful Ca^{2+} buffering capacity. One hypothesis (Kaftan et al. 2000) argues that new Ca^{2+} binding sites are made available by the progressive alkalinization of the mitochondrial matrix. Influx of Ca^{2+} into the matrix during stimulation produces a depolarization of Ψm that accelerates ETC activity, which further alkalinizes the matrix, increasing the mitochondrial capacity to buffer Ca^{2+} loads. This hypothesis is based in part on the observation that addition of cyanide m-chlorophenylhydrazone (CCCP, a proton carrier) to acidify the matrix in rat pituitary gonadotropes resulted in a small increase in the baseline level of mitochondrial [Ca^{2+}] (Kaftan et al. 2000). This was interpreted as being due to the liberation of bound Ca^{2+} due to the drop in intra-matrix pH, which led to the observed increase in mitochondrial [Ca^{2+}].
The other hypothesis is based in part on the observation that, in isolated mitochondria, phosphate accompanies the uptake of $\text{Ca}^{2+}$, and that both species precipitate together as a salt in the matrix (e.g. Rossi & Lehninger, 1963). Also, when external phosphate is substituted by acetate (mitochondria are permeant to both anions), isolated mitochondria lose their ability to sequester the large quantities of $\text{Ca}^{2+}$ taken up in the presence of phosphate (Zoccarato & Nicholls, 1982). According to this second hypothesis, an osmotically inactive complex of calcium and phosphate (alone or possibly associated with proteins) reversibly forms in the mitochondrial matrix during stimulation (Pivovarova et al. 1999; David, 1999; Chalmers & Nicholls, 2003; reviewed by Carafoli, 2003) and serves as a “sink” for free $\text{Ca}^{2+}$. After the increase in mitochondrial $[\text{Ca}^{2+}]$ during the beginning of stimulation, the complex begins to form and effectively “clamps” mitochondrial $[\text{Ca}^{2+}]$ at a constant value (the observed mitochondrial $[\text{Ca}^{2+}]$ plateau). The complex continues to grow in mass during stimulation. The inorganic phosphate which forms part of this complex may be part of a large reserve in the mitochondria. It could also by taken up by the mitochondria at an increased rate during stimulation via co-transport with protons or via exchange with dicarboxylates such as malate or succinate (reviewed in Ferreira & Pedersen, 1993).

Inorganic phosphate in mitochondria exists as phosphoric acid, dihydrogen phosphate, and monohydrogen phosphate. The $pK_a$s for the dissociation of these acids are, respectively:

\[
\begin{align*}
\text{H}_3\text{PO}_4 & \leftrightarrow \text{H}^+ + \text{H}_2\text{PO}_4^- & pK_a \approx 2.14 \\
\text{H}_2\text{PO}_4^- & \leftrightarrow \text{H}^+ + \text{HPO}_4^{2-} & pK_a \approx 6.86 \\
\text{HPO}_4^{2-} & \leftrightarrow \text{H}^+ + \text{PO}_4^{3-} & pK_a \approx 12.4
\end{align*}
\]
The pH inside the mitochondrial matrix in respiring mitochondria is believed to be ~7.8 (Nicholls, 1974). It is hypothesized that, during repetitive stimulation, the small amount of \( \text{PO}_4^{3-} \) present in the matrix rapidly precipitates with \( \text{Ca}^{2+} \), driving all of the above equations to the right. The overall equation for the formation of the complex is:

\[
\text{Ca}^{2+} + \text{PO}_4^{3-} \leftrightarrow \text{Ca}_3(\text{PO}_4)_2
\]

Alkalization of the matrix due to increased activity of the ETC would thus favor the formation of the complex. The observed plateau level therefore represents that \([\text{Ca}^{2+}]\) at which the solubility product of the complex is reached (\( \text{PO}_4^{3-} \) has the lowest solubility product with \( \text{Ca}^{2+} \) of the three forms of inorganic phosphate). After the end of stimulation, mitochondria begin to extrude \( \text{Ca}^{2+} \) back into the cytosol (see below for mechanisms) and, as matrix \([\text{Ca}^{2+}]\) falls, the calcium-phosphate complex begins to dissolve, releasing free \( \text{Ca}^{2+} \) into the matrix. The plateau of matrix \([\text{Ca}^{2+}]\) would still be observed as long as some intra-matrix calcium remained bound in the complex. Post-stimulation mitochondrial \([\text{Ca}^{2+}]\), as shown in the work presented here, returns back to baseline over the course of minutes.

A diagram detailing some of the features of this hypothesis is shown in Fig. 1.1 (Fig. 7A from David, 1999) showing the cellular and mitochondrial compartments, and the different transport and buffering mechanisms for \( \text{Ca}^{2+} \). During repetitive stimulation, entering \( \text{Ca}^{2+} \) (the current \( I_{\text{Ca}} \)) is bound by conventional buffers in the cytosol (Bc) or is passively taken up into the mitochondrial matrix via the \( \text{Ca}^{2+} \) uniporter. Inside the matrix, \( \text{Ca}^{2+} \) is bound by conventional mitochondrial buffers (Bm) or precipitates with phosphate, which enters mitochondria via a transporter. After stimulation ends, the
calcium-phosphate complex dissolves and the liberated Ca$^{2+}$ is transported out of the matrix into the cytosol via Ca$^{2+}$ extrusion mechanisms (only the mitochondrial Na$^+$-Ca$^{2+}$ exchanger is shown). Cytosolic Ca$^{2+}$ eventually leaves the cell via plasma membrane Ca$^{2+}$ extrusion mechanisms.

According to this "complex formation" hypothesis, the magnitude of the mitochondrial [Ca$^{2+}$] plateau observed during repetitive stimulation would depend on the concentration of available phosphate (in the PO$_4^{3-}$ form) inside the mitochondrial matrix, but not on the Ca$^{2+}$ load delivered to the terminal (i.e. the rate of Ca$^{2+}$ uptake, within physiological ranges), nor on the total amount of Ca$^{2+}$ taken up by mitochondria during stimulation (again within physiological ranges). Increased Ca$^{2+}$ loads, however, would...
be reflected by an increase of the post-stimulation decay of mitochondrial $[\text{Ca}^{2+}]$.

Experiments described in Chapter 2 tested this hypothesis by varying the $\text{Ca}^{2+}$ load delivered to the terminal and measuring the effects on the post-stimulation mitochondrial $[\text{Ca}^{2+}]$ decay.

**C. Mitochondrial $\text{Ca}^{2+}$ extrusion mechanisms**

Sequestered $\text{Ca}^{2+}$ returns to the cytosol via mitochondrial $\text{Na}^{+}$-$\text{Ca}^{2+}$ and $\text{H}^{+}$-$\text{Ca}^{2+}$ exchangers, which exploit favorable inward electrochemical gradients for $\text{Na}^{+}$ and $\text{H}^{+}$, respectively, to extrude $\text{Ca}^{2+}$ from the matrix by secondary active transport (reviewed by Gunter & Pfeiffer, 1990; Bernardi, 1999; Carafoli, 2003). In vertebrate neuronal mitochondria, $\text{Na}^{+}$-$\text{Ca}^{2+}$ exchange (followed by mitochondrial $\text{H}^{+}$-$\text{Na}^{+}$ exchange) appears to be more important than $\text{H}^{+}$-$\text{Ca}^{2+}$ exchange. The mitochondrial $\text{Na}^{+}$-$\text{Ca}^{2+}$ exchanger has a $K_m$ for $\text{Na}^{+}$ of $\sim 9.4$ mM (Carafoli, 1979), which is close to the resting cytosolic $[\text{Na}^{+}]$ of $\sim 8$ mM measured in lizard motor axons (David et al. 1997). Small elevations of cytosolic $[\text{Na}^{+}]$ which might accumulate after $\text{Na}^{+}$ entry into the terminal during repetitive stimulation may help activate the exchanger.

Calcium can also exit mitochondria via openings of the mitochondrial permeability transition pore (MPTP, Crompton et al. 1987), which entails a sudden increase in permeability across the inner mitochondrial membrane to molecules smaller than 1.5 kDa. The MPTP is believed to consist of a number of components, including the adenine nucleotide translocator, the voltage gated anion channel, cyclophilin D, and hexokinase (reviewed in Zoratti & Szabo, 1995; Bernardi, 1999). It can be opened by
several agents, including high levels of inorganic phosphate entering the matrix, reactive oxygen species, agents which cause depletion of matrix adenine nucleotides, fatty acids, as well as high levels of matrix \([\text{Ca}^{2+}]\) (levels approaching 0.3 mM, see comments on Chalmers & Nicholls, 2003, in Chapter 4). A consequence of pore opening is the loss of \(\Psi_m\) together with the efflux of a number of organic molecules, small peptides, and ions, including \(\text{Ca}^{2+}\), from the matrix into the cytosol. Irreversible openings of the pore have been associated with necrotic cell death (Crompton & Costi, 1990) as well as with apoptosis (Cai et al. 1998). However, it has been suggested that \textit{reversible} openings may also occur (Ichas et al. 1997). Such reversible, transient openings of the MPTP, which would be accompanied by brief \(\Psi_m\) depolarizations, might rapidly release relatively large loads of \(\text{Ca}^{2+}\) and thus serve as a mitochondrial \(\text{Ca}^{2+}\) extrusion mechanism in motor terminals during and after repetitive stimulation. In addition, given that high matrix \([\text{Ca}^{2+}]\) is by itself one of the triggers of irreversible openings of the pore, transient openings might serve as a protective mechanism for the mitochondrion.

In the work presented here, the contributions of the mitochondrial \(\text{Na}^+-\text{Ca}^{2+}\) exchanger and of openings of the MPTP to post-stimulation mitochondrial \(\text{Ca}^{2+}\) extrusion were measured by inhibiting these mechanisms with pharmacological agents (CGP-37157, and cyclosporin A, respectively). The \(\text{Na}^+-\text{Ca}^{2+}\) exchanger was found to play a prominent role in post-stimulation mitochondrial \(\text{Ca}^{2+}\) extrusion.
**D. Effects of mitochondrial Ca\(^{2+}\) uptake/extrusion on neurotransmitter release**

Although the participation of mitochondrial Ca\(^{2+}\) transport in the regulation of cytosolic [Ca\(^{2+}\)] during stimulation in vivo has been demonstrated, a remaining question was whether this mechanism was affecting overall motor terminal function. In lizard, frog, and mouse motor terminals, inhibition of mitochondrial Ca\(^{2+}\) uptake during repetitive stimulation with CCCP or with antimycin A1, a blocker of complex III of the ETC, resulted in a much greater elevation of cytosolic [Ca\(^{2+}\)] together with both an increase in asynchronous NT release and marked depression of phasic NT release (Suzuki et al. 2002, David & Barrett, 2003; Talbot et al. 2003). Mitochondrial Ca\(^{2+}\) uptake was thus shown to be essential for ensuring faithful transmission across the NMJ during tetanic stimulation. This provided a strong corroboration of the ideas initially proposed by Alnaes & Rahamimoff (1975).

Extrusion of mitochondrial Ca\(^{2+}\) into the cytosol after the end of a stimulus train might also affect NT release. Following repetitive stimulation, cytosolic [Ca\(^{2+}\)] returns to pre-stimulation levels in two phases: a fast component which brings [Ca\(^{2+}\)] close to baseline followed by a slower component. The second, slower phase of [Ca\(^{2+}\)] decay, also referred to as the post-stimulation cytosolic [Ca\(^{2+}\)] “tail,” is produced by the slow efflux of Ca\(^{2+}\) from mitochondria (Werth & Thayer, 1994; Baron & Thayer, 1997; Colegrove et al. 2000a). A relationship between persisting elevations of cytosolic [Ca\(^{2+}\)] in pre-synaptic terminals and short term synaptic plasticity (e.g. facilitation, augmentation, and post-tetanic potentiation (PTP)) has been proposed (reviewed by Zucker & Regehr, 2002; Millar et al. 2005). Tang & Zucker (1997) suggested that the
post-stimulation cytosolic \([\text{Ca}^{2+}]\) tail mediated PTP, and was the result of mitochondrial \(\text{Ca}^{2+}\) extrusion, based on their finding that at crayfish motor terminals both of these phenomena were abolished by agents that inhibited mitochondrial \(\text{Ca}^{2+}\) uptake and/or extrusion (ruthenium red, tetrphenylphosphonium, CCCP). Similar evidence for a mitochondrial contribution to various forms of synaptic potentiation has been presented in studies of cultured cortical neurons and rat and toad motor nerve terminals (Hubbard & Gage, 1964; Yang et al. 2003; Storozhuk et al. 2005). However, Zhong et al. (2001) found that blocking the mitochondrial \(\text{Na}^+-\text{Ca}^{2+}\) exchanger (also with CGP-37157) had no effect on residual cytosolic \([\text{Ca}^{2+}]\) or on PTP in crayfish motor terminals. This led them to conclude that, in crayfish motor terminals, any mitochondrial contribution to PTP must be via a \(\text{Na}^+\)-independent \(\text{Ca}^{2+}\) efflux mechanism or mechanisms.

In the work presented here, inhibition of mitochondrial \(\text{Na}^+-\text{Ca}^{2+}\) exchange resulted in lower post-stimulation residual cytosolic \([\text{Ca}^{2+}]\) and in decreased amplitudes of post-tetanic EPPs. Thus, mitochondrial \(\text{Ca}^{2+}\) extrusion helps restore NT release following repetitive stimulation in motor nerve terminals.

**E. Effects of stimulation-induced mitochondrial \(\text{Ca}^{2+}\) uptake on \(\Psi_m\)**

Entry of \(\text{Ca}^{2+}\) into the mitochondrial matrix would be expected to depolarize \(\Psi_m\), and this would in turn reduce the gradient driving \(\text{Ca}^{2+}\) uptake. However, in motor nerve terminals, mitochondrial \(\text{Ca}^{2+}\) uptake is maintained during long, repeated stimulus trains (David, 1999). One possible explanation for this paradox is that either a counter ion (e.g. phosphate) moves into the matrix or an ion with the same charge (e.g. protons) are being
extruded. The $\Psi_m$ depolarization produced by Ca$^{2+}$ entry reduces the gradient against which ETC complexes I, III and IV extrude protons, thus allowing for an acceleration of ETC proton extrusion. Such an acceleration would limit the depolarization of $\Psi_m$. In support of this explanation, studies have detected increases in the rate of nicotinamide adenine dinucleotide (NADH) oxidation (indicative of increases in ETC activity) during neuronal stimulation (e.g. dissociated mouse sensory neurons, Duchen 1992; single mouse Purkinje neurons in culture, Hayakawa et al. 2005). A study in lizard motor terminals (Talbot et al. 2007) also detected decreases in NADH simultaneous with increases in mitochondrial [Ca$^{2+}$] during repetitive stimulation. That study proposed that acceleration of ETC activity limits net $\Psi_m$ depolarization, and thereby enables mitochondria to continue taking up Ca$^{2+}$ during prolonged stimulation (see below, and normal side of Fig. 1.2).

In the work described here, this hypothesis was tested in mouse motor terminals. At near physiological temperatures (~30 °C), $\Psi_m$ depolarizations during stimulation were either small or difficult to detect. The amplitude of the depolarizations increased, however, when complex I of the ETC was partially inhibited.

F. Possible early mitochondrial ETC dysfunction in a mouse model for ALS

Aberrant mitochondrial function is thought to contribute to several neurodegenerative diseases (for review see Petrozzi et al. 2007), including amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig’s Disease). ALS usually manifests in the 5th to 7th decades of life and leads unremittingly to the death of upper and lower motor
neurons. The course consists of progressive paralysis of all four limbs and of the respiratory musculature, resulting in death. About 80% of ALS patients die within 5 years of diagnosis. The disease incidence is 1-2 in 100,000 and at present there is no cure (clinical aspects reviewed in Rowland & Schneider, 2001).

About 20% of cases are familial (fALS) and have associations with mutations in a number of different genes. About 5% of fALS patients have one of over 100 mutations in the gene encoding the metalloenzyme (Cu/Zn-dependent) superoxide dismutase-1 (SOD1, Rosen et al. 1993), an anti-oxidant enzyme found in the cytosol of virtually all cell types. Mice transfected with multiple copies of mutant forms of the human gene develop an ALS-like illness and have been used as disease models. Our laboratory has used two of these animal models: SOD1-G93A and SOD1-G85R mice. These animals develop hind limb weakness with less than normal extension, coarse appearance of their coat (because of lack of grooming), and thin flanks. The SOD1-G93A mice, which have a copy number of 25, begin to exhibit these signs at 3-4 months, and die at ~5 months of age (Gurney et al. 1994; Chiu et al. 1995). The SOD1-G85R mice have a copy number of 15, exhibit signs of disease at 8-10 months, and die at 9-11 months (Bruijn et al. 1997). SOD1-G93A retain SOD1 activity, but SOD1-G85R do not (Valentine & Hart, 2003, although heterodimers of normal SOD1 and SOD1-G85R do show enzyme activity, Witan et al. 2008).

Results from one recent study have shown that neuron specific expression of the SOD1-G93A mutation is sufficient to produce motoneuronal degeneration (Jaarsma et al. 2008). However, it is likely that other cell types are intimately involved in the disease pathogenesis. For example, a study which lowered the levels of mutant SOD1-G37R
expression in microglia resulted in a slower progression of the later stage of the disease in mice (Boillée et al. 2006b). In SOD1-G85R mice, astrocytes were found to be activated and contain inclusions of SOD1 early in the course of the disease (Bruijn et al. 1997). It is not clear how expression of mutant SOD1 in muscle contributes to ALS pathogenesis. However, life span in SOD1-G93A mice is extended with moderate exercise (Kirkinezos et al. 2003), with local expression of insulin-like growth factor leading to muscle hypertrophy (Dobrowolny et al. 2005), or a with combination of both (Kaspar et al. 2005).

The association of SOD1 gene mutations with some familial forms indicates that ALS might be caused by a loss of SOD1 function (although this would explain neither all of the familial cases nor any of the sporadic ones). However, deletion of the wild type SOD1 gene in mice does not result in motor neuron death (Reaume et al. 1996). This, plus the dominant pattern of inheritance of the familial forms of the disease, suggests that a toxic gain of function is involved in the pathophysiology.

A number of different mechanisms have been proposed to play a role in the pathogenesis induced by SOD1 mutations (reviewed in Boillée et al. 2006a). Although ALS patients with a mutation in the SOD1 gene retain some SOD1 activity, it has been proposed that any decrease in the enzyme’s function would result in excess superoxide anion production, which could react with nitric oxide to produce peroxynitrite, resulting in protein damage due to tyrosine nitrosylation (Beckman et al. 1993).

Aberrant protein-protein interactions have also been proposed (Shibata et al. 1996), including SOD1 misfoldings leading to abnormal protein aggregates (Bruijn et al. 1998) as well as abnormal accumulations of neurofilaments (Rouleau et al. 1996).
Abnormalities in apoptosis are believed to play a role in the pathogenesis due to the findings of increased susceptibility to activation of the FAS-mediated death receptor pathway in cultured motor neurons from mutant SOD1-G37R, SOD1-G85R, and SOD1-G93A mice (Raoul et al. 2002, 2006; Wengenack et al. 2004), as well as to the detection of lower levels of anti-apoptotic proteins in both neuronal tissue from mutant SOD1-G86R and SOD1-G93A mice (Gonzalez de Aguilar et al. 2000; Vukosavic et al. 2000) and in spinal cord tissue from ALS patients (Ekegren et al. 1999; Mu et al. 1996).

Chronic inflammation has also been proposed to play a role due to findings of increased expression of cyclooxygenase-2 in spinal cord tissue of ALS patients (Yasojima et al. 2001; Yiangou et al. 2006). This finding may be related to the slower disease progression observed when SOD1-G37R expression in microglia is lowered, or to the early increased activation of astrocytes observed in SOD1-G85R mice mentioned above.

Alterations in angiogenesis may be associated with ALS pathogenesis. For example, mutations of the angiogenin gene have been linked to the disease in some ALS patients (Greenway et al. 2006; angiogenin induces vascularization of normal and neoplastic tissues). Deletion of the hypoxia response element in the mouse vascular endothelial growth factor (VEGF) promoter results in an ALS-like disease in mice (Oosthuyse et al. 2001). In one study in SOD1-G93A rats (another rodent model for ALS, see Nagai et al. 2001), reduced levels of VEGF were detected before disease onset, and levels decreased further during disease progression (Lambrechts et al. 2003). In this same study, it was found that certain haplotypes in the human hypoxia response element were associated with reduced levels of circulating VEGF and were more common in
some populations of ALS patients (Belgian, British, and Swedish), although this has not been observed in other populations. Furthermore, a therapeutic role for VEGF was supported by studies in which the delivery of recombinant VEGF, via intracerebroventricular catheter to SOD1-G93A rats or via a lentiviral vector to a number of different muscles in SOD1-G93A mice, prolonged survival in both disease models (Storkebaum et al. 2005; Azzouz et al. 2004). More recently, vascular abnormalities such as evidence of microhemorrhages and breakdown of the blood-spinal cord barrier have been noted in the lumbar spinal cords of SOD1-G37R, SOD1-G85R, and SOD1-G93A mice (Zhong et al. 2008).

Finally, excitotoxicity has also been proposed to play a role in ALS due to the finding of decreased expression of the excitatory amino acid transporter-2 (together with aberrant mRNA molecules coding for this protein) in motor cortex and spinal cord astroglia of sporadic ALS patients (Lin et al. 1998). Lower levels of excitatory amino acid transporter-2 expression were also detected in mutant SOD1 spinal cord tissue of both G93A rats (Howland et al. 2002) and G85R mice (Bruijn et al. 1997). The only FDA-approved therapy for ALS, riluzole (Doble, 1996), is believed to act in part by decreasing the release of glutamate.

Mitochondrial abnormalities have been associated with ALS. In studies of both human patients and animal models of ALS, mutant SOD1 proteins have been observed to localize within the matrix and/or inter-membrane 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). Other studies have observed mitochondrial structural and
functional abnormalities in mutant SOD1 mice. For example, Kong & Xu (1998) detected abnormal mitochondrial ultrastructure in motor neurons of SOD1-G93A mice, including swelling, dilated and disorganized cristae, and breakdown of the mitochondrial outer membrane. These abnormalities were detected before the onset of muscle weakness or death of spinal motor neurons. Warita et al. (2001) used immunohistochemical techniques to detect early accumulation of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to nucleic acids, in non-nuclear regions of ventral horn neurons in SOD1-G93A mice, indicative of damage to mitochondrial DNA.

Many of the hypothesized disease mechanisms listed above describe events measured in the central nervous system. However, some studies have suggested that ALS pathology may begin peripherally in motor axons and/or terminals, before detection of any outward signs of disease. Some studies have measured a slowing of axonal transport in pre-symptomatic mutant SOD1-G37R, SOD1-G85R, and SOD1-G93A mice (Zhang et al. 1997; Williamson & Cleveland, 1999). Another study (Fischer et al. 2004) looked for abnormal morphology at three different sites in SOD1-G93A mice: in spinal cord ventral horn, ventral roots, and at NMJs, at different stages of disease. At 28 days, mice showed no abnormalities. In 47 day old asymptomatic mice, 40% of end-plates from gastrocnemius, soleus, and tibialis anterior muscles were found to be denervated, but none of the ventral roots or spinal cord cell bodies examined showed abnormalities. At 80 days, 60% of ventral root axons had been lost while there was still no significant loss of spinal cord cell bodies. Finally, at 100 days, 40% of large neuronal cell bodies in the spinal cord ventral horn had been lost. In addition, this same study examined tissue from a human patient who had unexpectedly died 6 months after being diagnosed with
sporadic ALS. Motor end-plate denervation and axonal sprouting were observed at autopsy even though the morphology of large neuronal cell bodies in the ventral horn of the spinal cord appeared normal.

Kirkinezos et al. (2005) measured a decrease in activity of the ETC in both spinal cord and brain tissue of pre-symptomatic (30-57 day old) SOD1-G93A mice, but not in liver mitochondria. Son et al. (2008) measured decreased complex IV activity in SOD1-G93A mice overexpressing CCS protein, a copper chaperone for SOD1 which appears to accumulate mutant SOD1 proteins in mitochondria. Finally, Damiano et al. (2006) measured a reduced Ca$^{2+}$ uptake capacity in mitochondria from spinal cord (but not liver) of SOD1-G93A mice. These findings suggest not only that mitochondria are involved in the pathogenesis of ALS, but that abnormal mitochondrial function may be present at an early, pre-symptomatic stage (mitochondrial involvement in ALS is reviewed in Manfredi & Xu, 2005). Also, during repetitive stimulation of motor nerve terminals of SOD1-G93A mice, Vila et al. (2003) observed that mitochondrial [Ca$^{2+}$] did not stabilize at a plateau level (see part B, above) but instead ramped up to higher levels. After stimulation ceased, mitochondrial [Ca$^{2+}$] rapidly fell back to baseline with a much shorter time course than that observed in wild type (WT) mice.

In SOD1-G85R and SOD1-G93A mice, motor terminals innervating fast type muscles degenerate earlier than those innervating fatigue-resistant, slow type muscles (Frey et al. 2000; Pun et al. 2006). An increased susceptibility to ischemia (followed by reperfusion) injury was measured in SOD1-G93A mice in motor terminals innervating the extensor digitorum longus and the plantaris, two fast type muscles (David et al. 2007). Neurons innervating these muscle types tend to discharge at higher frequencies
that those innervating slow, fatigue resistant muscles (Hennig & Lømo, 1985). High frequency stimulation would probably lead to higher Ca\textsuperscript{2+} loads on the terminal, and would therefore represent greater demands on the mitochondrion’s Ca\textsuperscript{2+} buffering capacity.

The decrease in ETC activity observed in the Kirkinezos and Son studies suggests that aberrant mitochondria Ca\textsuperscript{2+} handling (as observed in the Vila and Damiano studies) might be due to a reduced ability to accelerate proton extrusion in response to the \(\Psi_m\) depolarization produced by Ca\textsuperscript{2+} uptake. \(\Psi_m\) depolarizations would be expected to be larger than in WT, and this might also translate to less mitochondrial Ca\textsuperscript{2+} uptake and to higher cytosolic [Ca\textsuperscript{2+}] levels during repetitive stimulation. Fig. 1.2 (Fig. 7 from Talbot et al. 2007) shows a flow chart describing the hypothesized effects that repetitive stimulation would have on cytosolic [Ca\textsuperscript{2+}] and \(\Psi_m\) under normal conditions as well as in conditions were mitochondrial metabolism is limited, either artificially with agents which inhibit ETC activity, or in cases of mitochondrial pathology. In work presented in this dissertation, stimulation-induced changes in \(\Psi_m\) were measured in motor terminals from SOD1-G85R and SOD1-G93A mice, WT mice, mice in which endogenous SOD1 had been knocked out, and mice which expressed normal human SOD1 (used as a control). At temperatures near physiological conditions (~30 °C) \(\Psi_m\) depolarizations tended to be larger in SOD1-G85R mice than in WT mice or in mice expressing normal human SOD1.
Figure 1.2 Proposed effects of metabolic limitations on cytosolic [Ca\(^{2+}\)] and on \(\Psi_m\). As Ca\(^{2+}\) influx into mitochondria begins, agents which block ETC activity (e.g. rotenone) or the presence of some underlying pathology that may limit ETC activity (possibly ALS) would result in larger \(\Psi_m\) depolarizations and increased cytosolic [Ca\(^{2+}\)] as diagramed in the box on the lower left. Fig. 7 from Talbot et al. 2007
Chapter 2

Extrusion of Calcium from Mouse Motor Terminal Mitochondria via a Sodium-Calcium Exchanger Increases Post-tetanic Evoked Release

A. Summary

Mitochondria sequester much of the $\text{Ca}^{2+}$ that enters motor nerve terminals during repetitive stimulation at frequencies exceeding 10-20 Hz. We studied the post-stimulation extrusion of $\text{Ca}^{2+}$ from mitochondria by measuring changes in matrix $[\text{Ca}^{2+}]$ with fluorescent indicators loaded into motor terminal mitochondria in the mouse levator auris longus muscle. Trains of APs at 50 Hz produced a rapid increase in mitochondrial $[\text{Ca}^{2+}]$ followed by a plateau, which usually continued after the end of the stimulus train and then slowly decayed back to baseline. Increasing the $\text{Ca}^{2+}$ load delivered to the terminal by increasing the number of stimuli (500-2000) or stimulation frequency (50-100 Hz), by increasing bath $[\text{Ca}^{2+}]$, or by prolonging the AP with 3,4-diaminopyridine (3,4-DAP, 100 $\mu$M), prolonged the post-stimulation decay of mitochondrial $[\text{Ca}^{2+}]$ without increasing the amplitude of the plateau during stimulation. Inhibiting the opening of the mitochondrial MPTP with cyclosporin A (5 $\mu$M) had no significant effect on the decay of mitochondrial $[\text{Ca}^{2+}]$. Inhibition of the mitochondrial $\text{Na}^+\text{-Ca}^{2+}$ exchanger with CGP-37157 (50 $\mu$M) dramatically prolonged the post-stimulation decay of mitochondrial $[\text{Ca}^{2+}]$, reduced post-stimulation residual cytosolic $[\text{Ca}^{2+}]$, and reduced the amplitude of end-plate potentials (EPPs) evoked after the end of a stimulus train in both low and normal bath $[\text{Ca}^{2+}]$. These findings
suggest that Ca\(^{2+}\) extrusion from motor terminal mitochondria occurs primarily via the mitochondrial Na\(^+-\)Ca\(^{2+}\) exchanger and helps to sustain post-tetanic transmitter release at mouse NMJs.

**B. Background**

Mitochondria temporarily sequester large, stimulation-induced calcium loads in neurons and other secretory cells (Friel & Tsien, 1994; Stuenkel, 1994; Herrington et al. 1996; Babcock et al. 1997; David et al. 1998; Pivovarova et al. 1999; David, 1999; Kaftan et al. 2000; Suzuki et al. 2002; David and Barrett, 2003). Mitochondrial Ca\(^{2+}\) uptake during repetitive stimulation of motor nerve terminals limits the increase in cytosolic [Ca\(^{2+}\)] to a plateau level which is maintained until stimulation ceases. Inhibition of mitochondrial Ca\(^{2+}\) uptake results in a much greater elevation of cytosolic [Ca\(^{2+}\)] during stimulation, accompanied by an increase in asynchronous NT release and accelerated depression of phasic transmitter release (David & Barrett, 2000, 2003; Talbot et al. 2003). Thus mitochondrial Ca\(^{2+}\) uptake is important for maintaining neuromuscular transmission during repetitive stimulation. Following stimulation, the slow efflux of Ca\(^{2+}\) from mitochondria contributes to a residual post-stimulation “tail” of elevated [Ca\(^{2+}\)] in the cytosol (Werth & Thayer, 1994; Baron & Thayer, 1997; Colegrove et al. 2000a).

During repetitive stimulation of motor nerve terminals the increase in matrix [Ca\(^{2+}\)] is limited to 1-2 µM, even though mitochondrial Ca\(^{2+}\) uptake continues (David, 1999; David et al. 2003). Powerful buffering of mitochondrial Ca\(^{2+}\) has also been reported in other secretory cells (Kaftan et al. 2000; Warashina, 2006). One major form
of mitochondrial Ca\textsuperscript{2+} buffering appears to involve reversible formation of an insoluble complex containing Ca and phosphate (Pivovarova et al. 1999; Chalmers & Nicholls, 2003; review by Carafoli, 2003). After stimulation ends, matrix [Ca\textsuperscript{2+}] decreases back to baseline over a time course of several minutes (David, 1999). The work reported here determined how the post-stimulation decay of matrix [Ca\textsuperscript{2+}] is affected by varying the amount of Ca\textsuperscript{2+} entering the nerve terminal during a stimulus train (the Ca\textsuperscript{2+} load).

Mitochondria return sequestered Ca\textsuperscript{2+} to the cytosol via several mechanisms. Mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} and H\textsuperscript{+}-Ca\textsuperscript{2+} exchangers exploit a favorable inward electrochemical gradient for Na\textsuperscript{+} and H\textsuperscript{+}, respectively, to extrude Ca\textsuperscript{2+} from the matrix (reviews by Gunter and Pfeiffer, 1990; Bernardi, 1999; Carafoli, 2003). In vertebrate neuronal mitochondria, Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange (followed by H\textsuperscript{+}-Na\textsuperscript{+} exchange) appears to be more important than H\textsuperscript{+}-Ca\textsuperscript{2+} exchange. Ca\textsuperscript{2+} can also exit mitochondria during opening of the MPTP.

We studied mitochondrial Ca\textsuperscript{2+} extrusion mechanisms in mouse motor terminals, and how post-tetanic mitochondrial Ca\textsuperscript{2+} extrusion affects evoked NT release. Many studies have linked short term synaptic plasticity (facilitation, augmentation, PTP) with persisting elevations of cytosolic [Ca\textsuperscript{2+}] in presynaptic terminals (reviewed by Zucker and Regehr, 2002; Millar et al. 2005). Tang and Zucker (1997) suggested that the persisting elevation of cytosolic [Ca\textsuperscript{2+}] that mediates PTP is produced by mitochondrial Ca\textsuperscript{2+} extrusion, based on their finding that at crayfish motor terminals both these phenomena were abolished by agents that inhibit mitochondrial Ca\textsuperscript{2+} uptake and/or extrusion (ruthenium red, tetrathylenephosphonium, and the mitochondrial depolarizing agent CCCP). Similar evidence for a mitochondrial contribution to various forms of
synaptic potentiation has been presented for rat and toad motor nerve terminals and cultured cortical neurons (Hubbard and Gage, 1964; Yang et al. 2003; Storozhuk et al. 2005). However, Zhong et al. (2001) found that CGP-37157, which inhibits the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger, had no effect on residual [Ca\(^{2+}\)] or PTP in crayfish motor terminals. This and other findings led them to conclude that the mitochondrial contribution to PTP in crayfish motor terminals must be via Na\(^+\)-independent Ca\(^{2+}\) efflux mechanisms. We tested the effect of inhibiting the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger in mouse motor nerve terminals, and in contrast to results in crayfish motor terminals, found that CGP-37157 powerfully inhibits mitochondrial Ca\(^{2+}\) extrusion and decreases the amplitude of post-tetanic EPPs. Thus in this mammalian neuromuscular preparation Ca\(^{2+}\) extruded via the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger helps maintain transmitter release following repetitive stimulation.

C. Methods

Preparation and solutions

Experiments used NMJs from the levator auris longus muscle (Angaut-Petit et al. 1987) of WT mice (C57BL/6, Jackson Labs, Bar Harbor, ME) or transgenic mice that express yellow fluorescent protein (YFP) in neurons (B6.Cg-Tg(Thy1-YFP)16Jrs/J, Jackson Labs, Bar Harbor, ME). YFP expression facilitated localization of motor terminals. Mice were sacrificed with 100% CO\(_2\) using a procedure approved by the University of Miami Animal Care and Use Committee. The muscle with attached nerve was pinned down in a chamber with silicon walls constructed atop a No. 1 glass
coverslip. The preparation was bathed in physiological saline containing (in mM): NaCl 137, NaHCO₃ 15, KCl 4, CaCl₂ 1.8, MgCl₂ 1.1, glucose 11.2, and NaH₂PO₄ 0.33. Bath [Ca²⁺] was reduced in Figs. 2.3A and 2.7C,D. Solution pH was kept near 7.4 by gassing with 95% O₂/5% CO₂. Bath temperature (monitored using a thermistor) was maintained between 29-31 ºC by blowing hot air onto the underside of the chamber. Temperature was reduced to 22 ºC in Fig. 2.7B. The motor nerve was stimulated via a suction electrode by applying suprathreshold depolarizing 0.3 ms pulses. Stimulus trains consisted of 200-2000 stimuli delivered at 50-100 Hz. The minimum interval between stimulus trains was ~8 minutes, to allow sufficient time for mitochondrial [Ca²⁺] to return to baseline.

In most imaging experiments (Figs. 2.1-2.6) muscle contractions were blocked using d-tubocurarine (10 mg/L), whose blockade of muscle nicotinic acetylcholine (ACh) receptors prevented entry of Ca²⁺ into the muscle end-plate via ACh receptors or depolarization-activated Ca²⁺ channels. Thus mitochondria in the muscle end-plate did not contribute to the recorded stimulation-induced changes in fluorescence of the indicator for mitochondrial [Ca²⁺] (see below). For Fig. 2.7 in which EPPs were recorded, muscle contractions were blocked with µ-conotoxin GIIIB (2.5-4 µM), which blocks muscle (but not axonal) Na⁺ channels (Hong & Chang, 1989). 3,4-DAP (100 µM), which blocks certain depolarization-activated K⁺ channels in the motor terminal membrane (Tabti et al. 1989; Morita & Barrett, 1990), was used to prolong APs in Fig. 2.3B. The benzothiazepine CGP-37157 (50 µM) was used to inhibit the mitochondrial Na⁺-Ca²⁺ exchanger (Chiesi et al. 1988, Cox and Matlib, 1993); this drug does not block
the plasma membrane Na⁺-Ca²⁺ exchanger. Cyclosporin A (5 µM) was used to inhibit openings of the MPTP (Fournier et al. 1987, Broekemeier et al. 1989).

**Imaging of Ca²⁺ indicator dyes**

Stimulation-induced changes in mitochondrial [Ca²⁺] were measured using Ca²⁺-sensitive fluorescent indicators from the rhod family (rhod-2, Kₐ ~0.6 µM; X-rhod-1, Kₐ ~0.7 µM; or X-rhod-5F, Kₐ ~1.6 µM). These Kₐs are appropriate, since resting mitochondrial [Ca²⁺] is estimated to be 0.05-0.1 µM (reviewed by Gunter and Pfeiffer, 1990) and the maximal increase in mitochondrial [Ca²⁺] under physiological conditions is 1-2 µM (David, 1999; David et al. 2003). Preparations were exposed to the membrane-permeable, acetoxymethylester (AM) forms of these indicators (~25 µg/ml) for ~30 minutes, followed by washout with indicator-free saline for ~30 minutes. The AM moiety is cleaved by cytosolic and intra-mitochondrial esterases, converting the indicator into its charged, Ca²⁺ binding form and effectively trapping it in the compartment where the de-esterification occurred. During washout, the indicator in the cytosol is diluted by diffusion out of the terminal into the myelinated axon, leaving most of the remaining indicator in terminal mitochondria. Morphological and functional criteria used to verify intra-mitochondrial localization of the indicator are described in David (1999). The criterion most relevant to this study is that following the stimulus train the initial decay of intra-mitochondrial [Ca²⁺] is much slower than that of cytosolic [Ca²⁺]. Rhod dyes were excited with a 568 nm argon-krypton laser line (Laser Physics, Salt Lake City, UT) and the emitted light was filtered with a long-pass 590 nm filter (Chroma, Rockingham, VT).
YFP was excited with a 488 nm laser line, and emitted light was filtered with a 535 nm band-pass filter (40 nm bandwidth, Chroma).

Stimulation-induced changes in cytosolic \([\text{Ca}^{2+}]\) were monitored in separate experiments using mice that did not express YFP. The membrane-impermeable, hexapotassium salt forms of Oregon Green 488 BAPTA-1 (OG-1, \(K_d \sim 0.17 \mu\text{M}\)) or Oregon Green 488 BAPTA-5N (OG-5N, \(K_d \sim 50 \mu\text{M}\)) were loaded ionophoretically via an electrode inserted into an axonal internode, using techniques described in David and Barrett (2000). These indicators were excited at 488 nm with emissions monitored at 535 nm.

The experimental chamber was placed on the stage of an inverted microscope in a confocal system that included a Yokogawa spinning disc (Solamere, Salt Lake City, UT), a 60X water immersion lens (NA 1.2, Olympus), and a Photometrics Cascade 512B CCD camera (Roper Scientific, Trenton, NJ) that used on-chip multiplication gain to achieve greater sensitivity with low light intensities. A series of images were obtained before, during and after a train of APs; inter-image intervals ranged from 1 to 3 sec and exposure times ranged from 0.8 to 2 sec. Data were recorded using IP Lab v3.61 software (Scanalytics, Inc., Fairfax, VA) and analyzed on a Pentium computer using V++ software (Digital Micro Optics, Auckland, New Zealand). Variability due to fluctuations in laser light intensity was minimized by ratioing the recorded signal to the simultaneously-recorded signal from a fluorescent bead located close to the dichroic filter in the light path. Net fluorescence \((F_{\text{net}})\) was calculated by averaging fluorescence signals from regions of interest in the terminal, and then subtracting background fluorescence from surrounding non-terminal regions \((F_{\text{net}} = F_{\text{total}} - F_{\text{background}})\). Changes in fluorescence,
representative of changes in mitochondrial or cytosolic \([\text{Ca}^{2+}]\), were plotted as \(\frac{F_{\text{net}}}{F_{\text{rest}}}\) versus time, where \(F_{\text{rest}}\) is the average \(F_{\text{net}}\) for 20 images obtained before stimulation began.

Analysis was restricted to terminals whose average fluorescence during stimulation exceeded twice the standard deviation of pre-stimulation values, and whose fluorescence responses remained relatively stable during repeated stimulus trains. The time integral of the post-stimulation decay of mitochondrial \([\text{Ca}^{2+}]\) (decay integral) was calculated from normalized data. Integration was stopped when fluorescence fell to pre-stimulation values, or (for very prolonged decays) when imaging stopped. Other measures of the decay of post-stimulation fluorescence, such as the time to decay to baseline or the half-decay time, were less useful due to signal noise. Most responses did not exhibit a simple exponential decay.

**Electrophysiology**

EPPs were recorded with a 3 M KCl-filled microelectrode inserted into the muscle fiber near the end-plate region, using standard intracellular recording techniques. EPP amplitudes were measured using Clampfit (Axon Instruments, Union City, CA, USA).

**Reagent sources**

\(\mu\)-conotoxin GIIIB was from Alomone Labs (Jerusalem, Israel), CGP-37157 was from Tocris (Ellisville, MO), and the \(\text{Ca}^{2+}\)-indicator dyes were from Invitrogen (Carlsbad, CA). Other reagents came from Sigma (St. Louis, MO).
D. Results

*Increasing the Ca^{2+} load prolongs the post-stimulation decay of mitochondrial [Ca^{2+}].*

Fig. 2.1A shows a fluorescence micrograph of a YFP-labeled motor nerve terminal (green) superimposed on a phase-contrast image. Fig. 2.1B shows regions in which the fluorescence of the mitochondrially-loaded Ca^{2+} indicator X-rhod-5F increased in this terminal during 50 Hz stimulation of the motor nerve (red). The overlaid fluorescence images in Fig. 2.1C show that the regions in which X-rhod-5F fluorescence increased were within the YFP-labeled motor terminal. Fig. 2.1D plots the increase in X-rhod-5F fluorescence ($F_{net}/F_{rest}$) recorded in this terminal during and following a 50 Hz, 20 sec train. During stimulation, mitochondrial [Ca^{2+}] increased to a plateau value that persisted for ~50 sec after the end of stimulation and then decayed back to baseline over a time course of several minutes.
Figure 2.1 Localization of stimulation-induced increases in X-rhod-5F fluorescence within a YFP-expressing mouse motor nerve terminal. A, overlay of phase and YFP (green) images. B, overlay of phase and X-rhod-5F difference (red) images, illustrating regions in which fluorescence increased in response to 50 Hz stimulation for 20 sec. The difference image was created by subtracting the average pre-stimulation fluorescence (20 frames) from the average fluorescence during stimulation (6 frames). C, overlay of YFP image and X-rhod-5F difference image. Calibration bar: 50 µm. D, time course of stimulation-induced X-rhod-5F fluorescence changes (plotted as $F/F_{rest} = F_{net}/F_{rest}$) in this terminal. The dashed horizontal line indicates the baseline measured from pre-stimulation images. Dashed vertical lines indicate the duration of stimulation. For this record, as well as those in Figs. 2.2-2.6, muscle contraction was blocked using d-tubocurarine (10 µg/ml). The plateau amplitude observed during and immediately following the stimulus train in this and subsequent figures is not an artifact due to dye saturation. As noted in the Introduction, the maximal stimulation-induced increase in matrix $[Ca^{2+}]$ in normal motor terminals is only 1-2 µM above a resting level estimated as 0.05-0.10 µM, and none of the rhod dyes used here would saturate over this range. Also, a similar stimulation-induced plateau of matrix $[Ca^{2+}]$ is evident with a very low affinity indicator (rhod-5N, $K_d \sim 300$ µM, David et al. 2003).
Fig. 2.2A compares F/F_{rest} changes recorded in a different terminal during and following trains of 1000 and 2000 stimuli delivered at 50 Hz. Records were aligned so that time 0 corresponds to the end of stimulation. Increasing the train duration prolonged the post-tetanic decay.

The time course of decay of mitochondrial [Ca^{2+}] following a given stimulus train varied greatly from terminal to terminal. Thus to assess the effect of varying the Ca^{2+} load, we analyzed responses recorded sequentially from single terminals (as in Fig. 2.2A). Fig. 2.2B compares the time integral of the decay of mitochondrial [Ca^{2+}] recorded after 500 stimuli to that recorded after 1000 or 2000 stimuli for 5 different terminals. These comparisons showed that increasing the number of stimuli always prolonged the post-stimulation decay of mitochondrial [Ca^{2+}], but the percentage increase was variable. On average, increasing the stimulus train from 500 to 1000 stimuli or to 2000 stimuli increased the decay integral by 60% and 200%, respectively (Table 2.1). Similarly, Warashina (2006) reported that the decay of matrix [Ca^{2+}] in chromaffin cells was prolonged by increasing the duration of a high [K^+] -induced depolarization.

Figure 2.2 (following page) Increasing the duration of a 50 Hz stimulus train prolongs the post-stimulation decay of mitochondrial [Ca^{2+}], measured by changes in the fluorescence of X-rhod-5F. A, Two trains (1000 and 2000 stimuli) were delivered sequentially to a single terminal. Curves drawn through the data points were calculated as the weighted average of the 5 nearest neighbors. The dotted vertical lines mark (from left to right) the beginning of the 2000 stimulus train, the beginning of the subsequent 1000 stimulus train, and the end of stimulation for both trains. B, Pairwise comparison of the time integrals of the post-train decays of mitochondrial [Ca^{2+}] measured after 1000 stimuli (n = 4 terminals) or 2000 stimuli (n = 3 terminals), normalized to that measured in the same terminal after 500 stimuli.
The Ca\(^{2+}\) load delivered to the terminal was also varied by changing bath [Ca\(^{2+}\)]. Fig. 2.3A shows that decreasing bath [Ca\(^{2+}\)] from the normal 1.8 mM to 0.4 mM shortened the decay of mitochondrial [Ca\(^{2+}\)]. Table 2.1 quantifies how changing bath [Ca\(^{2+}\)] affected the decay integral.

An additional manipulation to vary the Ca\(^{2+}\) load was addition of 3,4-DAP (100 \(\mu\)M). This drug prolongs the duration of APs in motor terminals and thereby prolongs the amount of time that voltage-gated Ca\(^{2+}\) channels are open (Tabti et al. 1989; Morita & Barrett, 1990; Robitaille & Charlton, 1992). Fig. 2.3B shows that 3,4-DAP prolonged the post-stimulation decay of mitochondrial [Ca\(^{2+}\)] (also see Table 2.1). Note that neither reducing nor increasing the stimulation-induced Ca\(^{2+}\) load altered the plateau value of matrix [Ca\(^{2+}\)] recorded during the stimulus train, a reflection of the powerful Ca\(^{2+}\) buffering system in the mitochondrial matrix.

Figure 2.3 (following page) The post-stimulation decay of mitochondrial [Ca\(^{2+}\)] is accelerated by reducing bath [Ca\(^{2+}\)] (A) and slowed by 3,4-DAP (B). A, Responses to trains of 500 stimuli at 50 Hz measured in a motor terminal in 1.8 and 0.4 mM bath [Ca\(^{2+}\)]. Similar changes were seen in another terminal (not shown) in which bath [Ca\(^{2+}\)] was changed from 2.4 to 0.4 mM. B, Responses to trains of 1000 stimuli at 50 Hz measured in another terminal before and 24 minutes after adding 100 \(\mu\)M 3,4-DAP. The constant amplitude of the peak increase in mitochondrial [Ca\(^{2+}\)] in different bath [Ca\(^{2+}\)] and after addition of 3,4-DAP is consistent with the hypothesized formation of an insoluble Ca-containing complex that limits the increase in matrix [Ca\(^{2+}\)] (see Introduction). Both experiments used X-rhod-1.
Table 2.1 Increasing the Ca\(^{2+}\) load increases the time integral of the post-tetanic decay of mitochondrial [Ca\(^{2+}\)]

<table>
<thead>
<tr>
<th>Manipulation</th>
<th>Ratio of decay integrals (post/pre)</th>
<th>% of expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 → 1000 stimuli</td>
<td>1.6 ± 0.4 (4)</td>
<td>79*</td>
</tr>
<tr>
<td>500 → 2000 stimuli</td>
<td>3.0 ± 1.0 (3)</td>
<td>74*</td>
</tr>
<tr>
<td>0.4 → 1.8 mM Ca(^{2+})</td>
<td>3.5</td>
<td>78#</td>
</tr>
<tr>
<td>0.4 → 2.4 mM Ca(^{2+})</td>
<td>3.9</td>
<td>65#</td>
</tr>
<tr>
<td>Add 3,4-DAP</td>
<td>3.0 ± 0.2 (3)</td>
<td>—</td>
</tr>
</tbody>
</table>

Number of tested terminals indicated in parenthesis.  *Calculated assuming that the Ca\(^{2+}\) load is proportional to the number of stimuli in the train.  #Calculated assuming that the Ca\(^{2+}\) load is proportional to bath [Ca\(^{2+}\)].

Fig. 2.4 shows that increasing the stimulation frequency also prolonged the post-stimulation decay of mitochondrial [Ca\(^{2+}\)]. Increasing frequency while keeping the total number of stimuli constant would not be expected to change the total influx of Ca\(^{2+}\) into the terminal. However, delivering the same Ca\(^{2+}\) load in a shorter time increases the elevation of cytosolic [Ca\(^{2+}\)] during stimulation (David & Barrett, 2000), which would increase the driving force for Ca\(^{2+}\) entry into mitochondria. Ca\(^{2+}\) influx via the mitochondrial uniporter is activated in a supralinear manner by increases in cytosolic [Ca\(^{2+}\)] (reviewed by Gunter and Pfeiffer, 1990). Thus one would expect a greater accumulation of mitochondrial Ca\(^{2+}\) at higher stimulation frequencies, consistent with the observed prolongation of the post-stimulation decay of mitochondrial [Ca\(^{2+}\)].
Figure 2.4 Increasing the frequency of stimulation prolongs the post-stimulation decay of mitochondrial [Ca$^{2+}$], measured using rhod-2. Six trains of 1000 stimuli were delivered sequentially to a single terminal, 3 at 50 Hz and 3 at 100 Hz, alternating the frequencies. Records plot the average of each set of 3 trains.

The mitochondrial Na$^+$-Ca$^{2+}$ exchanger is the principal pathway for mitochondrial Ca$^{2+}$ extrusion.

How is the Ca$^{2+}$ taken up by mitochondria during repetitive stimulation returned to the cytosol after stimulation? Fig. 2.5A shows that inhibiting the mitochondrial Na$^+$-Ca$^{2+}$ exchanger with 50 µM CGP-37157 markedly prolonged the decay of mitochondrial [Ca$^{2+}$]. CGP-37157 has also been demonstrated to inhibit Ca$^{2+}$ extrusion by mitochondria in other vertebrate preparations including rat heart (Cox and Matlib, 1993), rat chromaffin cells (Babcock et al. 1997; Warashina, 2006) and multiple types of neurons and other secretory cells (Babcock et al. 1997; Baron and Thayer, 1997; White and
Reynolds, 1997; Colegrove et al. 2000a). In contrast, Fig. 2.5B shows that inhibiting openings of the MPTP with 5 µM cyclosporin A had little effect on the post-stimulation decay of mitochondrial [Ca^{2+}]. Thus the mitochondrial Na^{+}-Ca^{2+} exchanger is a more important Ca^{2+} extrusion pathway for mouse motor terminal mitochondria than cyclosporin-inhibitable openings of the transition pore.

Figure 2.5 (following page) The mitochondrial Na^{+}-Ca^{2+} exchanger contributes more to mitochondrial Ca^{2+} extrusion than openings of the MPTP. A, Responses to 500 stimuli at 50 Hz recorded in a single terminal before and 22 minutes after addition of 50 µM CGP-37157, which blocks the mitochondrial Na^{+}-Ca^{2+} exchanger. B, Responses to 1000 stimuli at 50 Hz recorded in a different terminal before (open circles) and 18 minutes after (filled circles) addition of 5 µM cyclosporin A, which inhibits some openings of the MPTP. Both experiments used X-rhod-1.
Ca\(^{2+}\) extruded from mitochondria contributes to the slowly-decaying tail of elevated cytosolic [Ca\(^{2+}\)] that follows stimulation. Thus one would predict that inhibition of the mitochondrial Na\(^+-Ca^{2+}\) exchanger with CGP-37157 would reduce post-train residual [Ca\(^{2+}\)]. This prediction is supported by the measurements in Fig. 2.6 showing changes in cytosolic [Ca\(^{2+}\)] produced during and after 50 Hz stimulation, monitored as F/F\(_{\text{rest}}\) of ionophoretically-injected OG-5N (K\(_d\) ~50 µM, Fig. 2.6A) or OG-1 (K\(_d\) ~0.17 µM, Fig. 2.6B,C). For the 500 stimulus trains in Fig. 2.6A,B CGP-37157 reduced post-train [Ca\(^{2+}\)] but had little effect on [Ca\(^{2+}\)] during the train, similar to the results of David (1999) in lizard motor terminals (see also Colegrove et al. 2000a). The low-affinity indicator OG-5N in Fig. 2.6A allows a good estimate of the relative magnitude of the [Ca\(^{2+}\)] elevations during and following stimulation. The high-affinity indicator OG-1 yields a better signal-to-noise ratio, but is more saturated with Ca\(^{2+}\) during the train than the low-affinity OG-5N. Thus the OG-1 records in Fig. 2.6B,C overestimate the ratio of post-train to intra-train [Ca\(^{2+}\)]. In Fig. 2.6C the number of stimuli was increased to 1000 to improve resolution of post-train [Ca\(^{2+}\)]. Single exponentials fit to these data (2-70 sec post-train) had magnitudes and time constants of (respectively) 0.5 and 25 sec in control, 0.19 and 29 sec in CGP-37157. Thus this drug mainly affected the magnitude of post-train [Ca\(^{2+}\)]. In Fig. 2.6C CGP-37157 slightly reduced the elevation of cytosolic [Ca\(^{2+}\)] during the latter half of the stimulus train. This effect is expected if mitochondrial Na\(^+-Ca^{2+}\) exchange occurs during as well as after the stimulus train (see modeling study of Colegrove et al. 2000b). Activation of this exchanger, which increases cytosolic [Ca\(^{2+}\)], would be expected to increase over the course of the train as mitochondrial [Ca\(^{2+}\)] and intra-axonal [Na\(^+\)] increase. Thus the reduction in cytosolic [Ca\(^{2+}\)] produced by
inhibiting this exchanger with CGP-37157 would be expected to become greater over the course of the train.

Figure 2.6  Inhibiting the mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger with CGP-37157 reduces the post-tetanic tail of residual cytosolic [Ca\textsuperscript{2+}], monitored as F/F\textsubscript{rest} for OG-5N (A) or OG-1 (B,C) loaded ionophoretically into the axon. Each record shows the average of 3-4 stimulus trains recorded in the same terminal before (open circles) and 15-80 minutes after (closed circles) addition of 50 µM CGP-37157. 500 stimuli in A,B; 1000 stimuli in C. A, B and C came from different terminals, all in 1.8 mM bath Ca\textsuperscript{2+}. Error bars indicate SEM.

*Inhibition of the mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger reduces evoked transmitter release following high-frequency stimulation.*

During post-tetanic stimulation, the residual tail of cytosolic Ca\textsuperscript{2+} sums with AP-induced Ca\textsuperscript{2+} influx, thus increasing post-tetanic evoked transmitter release (reviewed by Zucker and Regehr, 2002). To test whether mitochondrial Ca\textsuperscript{2+} extrusion increases post-tetanic release, we measured EPP amplitudes before, during and after 50 Hz stimulus trains in the presence and absence of CGP-37157 (Fig. 2.7). In these experiments muscle contractions were blocked using µ-conotoxin GIIIB rather than d-tubocurarine, since
during repetitive stimulation there is less depression of transmitter release in µ-conotoxin GIIIB than in d-tubocurarine (Hong & Chang, 1989). Changes in mitochondrial [Ca\(^{2+}\)] during and after a 500 stimulus train delivered in µ-conotoxin GIIIB were similar to those recorded in d-tubocurarine, i.e. attainment of a plateau during stimulation and a prolonged (> 1 minute) post-stimulation decay.

Fig. 2.7A shows that in physiological saline prior to addition of CGP-37157, post-tetanic EPP amplitudes returned to pre-stimulation values within less than 5 s; no augmentation or potentiation was evident. Hubbard et al. (1971) also reported minimal PTP in rat motor terminals subjected to similar stimulus trains at 30 °C (their Fig. 9). This lack of a post-tetanic increase in EPP amplitudes may have occurred because augmentation and potentiation were obscured by depression, as described by Kalkstein and Magleby (2004) in frog motor terminals. CGP-37157 markedly slowed the post-tetanic recovery of EPP amplitude, consistent with the hypothesis that mitochondrial extrusion of sequestered Ca\(^{2+}\) into the cytosol is important for accelerating the recovery of evoked transmitter release following repetitive stimulation. The time course of the difference between post-stimulation EPP amplitudes recorded in the presence and absence of CGP-37157 is plotted in the lower right corner of Fig. 2.7A. This difference decayed with a time constant of ~10 sec (dotted line).

In Fig. 2.7A, CGP-37157 also increased the depression of EPP amplitudes recorded during the latter part of the tetanus. This result, considered together with the cytosolic [Ca\(^{2+}\)] record in Fig. 2.6C, is consistent with the idea that mitochondrial Ca\(^{2+}\) extrusion via the Na\(^+\),Ca\(^{2+}\) exchanger can in some terminals increase cytosolic [Ca\(^{2+}\)] and evoked release during as well as after the train. Another possible explanation of the
increased depression in CGP-37157 is that this drug increased the initial (baseline) level of evoked release. This explanation seems unlikely, because CGP-37157 had no significant effect on either the muscle resting potential or the initial EPP amplitude.

We also tested the effects of CGP-37157 on tetanic and post-tetanic EPP amplitudes under two non-physiological experimental conditions designed to minimize tetanic depression and increase post-tetanic release. For the experiment of Fig. 2.7B the duration of the stimulus train was reduced from 500 to 200 stimuli and the temperature was reduced from 30 to 22 °C. In control solution there was a pronounced and prolonged post-tetanic increase in EPP amplitudes, consistent with Hubbard et al.’s (1971) demonstration that lowering the temperature enhances post-tetanic release in rat motor terminals. This post-tetanic increase was abolished by CGP-37157. Under these conditions CGP-37157 had no effect on EPP amplitudes during the stimulus train, indicating that this drug’s inhibition of post-tetanic release can be seen independent of any inhibition of release during the tetanus.

For the experiment of Fig. 2.7C,D bath [Ca^{2+}] was reduced from the normal 1.8 mM to 0.4 mM. Under this condition of reduced release, EPPs increased (rather than decreased) during the train, and remained above control levels for tens of sec following the train (reviewed by Magleby, 2004). CGP-37157 abolished the post-tetanic increase in release, and the increase in EPP amplitudes during the stimulus train was less marked.

The expanded time scale in Fig. 2.7D shows that this latter effect of CGP-37157 developed during the course of stimulation, similar to the delayed onset in Fig. 2.7A.

Thus under all the experimental conditions tested in Fig. 2.7, inhibition of mitochondrial Ca^{2+} extrusion with CGP-37157 reduced post-tetanic release.
Figure 2.7 Inhibition of mitochondrial Ca\(^{2+}\) extrusion with CGP-37157 reduces post-tetanic evoked release in near-physiological conditions (A), with reduced temperature and fewer stimuli (B), and in low bath [Ca\(^{2+}\)] (C). A,B,C plot EPP amplitudes (normalized to pre-train EPP amplitude) during and after 50 Hz stimulation. Error bars indicate SD. Insets in the lower right of A and C plot the difference between post-tetanic EPP amplitudes recorded in the absence and presence of CGP-37157. Monoexponential decays (dotted line) fitted to these data had time constants of 10.5 sec in A (95% confidence interval 8.8-12.1 sec) and 22.6 sec in C (95% confidence interval 9.8-35 sec). D plots tetanic EPPs from C on an expanded time scale to show effects of CGP-37157 during the train. Traces are drawn through data points, each of which is the weighted average of the 9 nearest neighbors. In all experiments muscle contractions were blocked with 2.5-4 µM µ-conotoxin-GIIIB. Data in A were averaged from 17 trains recorded from 12 end-plates in control (open circles), and from 12 trains in 10 terminals after addition of CGP-37157 (filled circles). Data in B are the average of 3 control (open circles) and 6 post-drug trains (closed circles) from the same terminal. Data in C,D are the average of 12 trains from 12 terminals in 4 animals (control, open circles, light gray) and 10 trains from 10 terminals (drug, closed circles, black).
E. Discussion

The post-tetanic decay of mitochondrial $[\text{Ca}^{2+}]$ depends on the mitochondrial $\text{Na}^+\text{-Ca}^{2+}$ exchanger and the $\text{Ca}^{2+}$ load.

Results presented here demonstrate that following 500-2000 APs delivered at 50-100 Hz, the $[\text{Ca}^{2+}]$ within mouse motor terminal mitochondria decays back to baseline over a time course of several minutes. This decay is greatly prolonged by inhibiting the mitochondrial $\text{Na}^+\text{-Ca}^{2+}$ exchanger with CGP-37157, consistent with evidence from isolated mitochondria and other intact neurons that this exchanger is the dominant mechanism for $\text{Ca}^{2+}$ extrusion from vertebrate neuronal mitochondria (see Introduction). The increase in mitochondrial $[\text{Ca}^{2+}]$ during stimulation occurs rapidly, consistent with ion flux through a channel driven by a large electrochemical gradient. The slow post-stimulation decay of mitochondrial $[\text{Ca}^{2+}]$ is consistent with the slower turnover rate of transporters. Like matrix $[\text{Ca}^{2+}]$, intra-terminal $[\text{Na}^+]$ remains elevated for several minutes following high-frequency stimulation, as assessed by a fluorescent $\text{Na}^+$ indicator (Zhong et al. 2001) or by the duration of the $\text{Na}^+,\text{K}^+$-ATPase-mediated post-tetanic axonal hyperpolarization (Morita et al. 1993; Kiernan et al. 2004). Thus after a stimulus train, $\text{Ca}^{2+}$ extrusion from the matrix is favored by sustained elevations of both matrix $[\text{Ca}^{2+}]$ and cytosolic $[\text{Na}^+]$.

The post-stimulation decay of matrix $[\text{Ca}^{2+}]$ was minimally affected by cyclosporin A, suggesting that under these experimental conditions $\text{Ca}^{2+}$ extrusion from the mitochondrial matrix does not occur via transient opening of the mitochondrial transition pore. This conclusion is consistent with the finding of minimal depolarization
of the mitochondrial membrane potential during or after stimulation in lizard motor nerve
terminals (David, 1999). Also, Chalmers and Nicholls (2003) found no evidence for
opening of the transition pore in isolated brain mitochondria until matrix [Ca\(^{2+}\)] exceeded
3-5 µM, whereas the elevation in matrix [Ca\(^{2+}\)] in stimulated motor terminals is limited to
1-2 µM (David, 1999; David et al. 2003).

In all motor terminals mitochondrial [Ca\(^{2+}\)] reached a plateau level during the
stimulus train whose amplitude was not changed by manipulations designed to vary the
Ca\(^{2+}\) load. This finding is consistent with the powerful matrix buffering described in the
Introduction. Assuming little change in matrix [inorganic phosphate], matrix [Ca\(^{2+}\)]
would be expected to remain “clamped” at this plateau level as long as the (hypothesized)
insoluble Ca-phosphate complexes remained. Thus one might expect that after
stimulation stopped, matrix [Ca\(^{2+}\)] would remain near the plateau level until all of the
complex formed during stimulation had dissolved. Consistent with this idea, matrix
[Ca\(^{2+}\)] in many motor terminals exhibited an initial continuation of the plateau during the
post-stimulation decay. The larger the Ca\(^{2+}\) load delivered to the terminal, the more
likely the persistence of this post-stimulation plateau phase (e.g. compare 2000 and 1000
stimulus trains in Fig. 2.2A).

However, in other terminals matrix [Ca\(^{2+}\)] began decaying immediately after
stimulation stopped (e.g. control record in Fig. 2.3B). We do not know why some
terminals whose matrix [Ca\(^{2+}\)] exhibited a clear plateau during stimulation did not sustain
that plateau during the initial phase of the post-stimulation decay. One possible explanation
is that in these mitochondria the rate of Ca\(^{2+}\) efflux via the Na\(^{+}\)-Ca\(^{2+}\) exchanger exceeded
the rate at which the hypothesized Ca-phosphate complex could dissolve. Pivovarova et
al.’s (1999) energy-dispersive X-ray microanalysis measurements of total Ca within the matrix of high [K+]-stimulated sympathetic ganglion neurons disclosed marked inter- and intra-mitochondrial heterogeneity. Their results suggest that the hypothesized Ca-phosphate complexes are heterogeneously distributed among and within mitochondria.

Since strong buffering limits the increase in matrix [Ca\(^{2+}\)] during stimulation, measurements of matrix [Ca\(^{2+}\)] cannot be used to assess total mitochondrial Ca\(^{2+}\) uptake. However, since increasing the stimulation-induced Ca\(^{2+}\) influx into the terminal prolonged the post-stimulation decay of matrix [Ca\(^{2+}\)], we tested whether the time integral of this decay might be proportional to the (presumed) mitochondrial Ca\(^{2+}\) load. Table 2.1 indicates that the percentage increase in the time integral of post-stimulation matrix [Ca\(^{2+}\)] decay was less than the percentage increase in the (presumed) Ca\(^{2+}\) load. The ratio of the average percentage increases in the decay integral and the estimated Ca\(^{2+}\) load was similar (65-79%) whether the extra Ca\(^{2+}\) load was delivered without an extra Na\(^{+}\) load (as with increases in bath [Ca\(^{2+}\)]), or with an extra Na\(^{+}\) load (as with increases in the number of stimuli). Thus changes in the post-train time integral of matrix [Ca\(^{2+}\)] probably underestimated the percentage change in mitochondrial Ca\(^{2+}\) load.

**Ca\(^{2+}\) extrusion from mitochondria helps sustain post-tetanic transmitter release.**

Kamiya and Zucker (1994) demonstrated that decreasing cytosolic [Ca\(^{2+}\)] in crayfish motor terminals by photo-activating a Ca\(^{2+}\) chelator at various times after tetanic stimulation decreased facilitation, augmentation and potentiation. However, the decrease in potentiation was only transient, suggesting that the elevated cytosolic [Ca\(^{2+}\)] that contributed to potentiation arose from a non-cytosolic source that could be replenished.
One of these sources is mitochondria (Tang and Zucker, 1997). Another source is Ca\(^{2+}\) influx from the bath via reverse action of a plasmalemmal Na\(^+\)-Ca\(^{2+}\) exchanger (Zhong et al. 2001).

In crayfish motor terminals Ca\(^{2+}\) extrusion from mitochondria is Na\(^+\)-independent; inhibiting the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger with CGP-37157 had no effect on residual [Ca\(^{2+}\)] or PTP (Zhong et al. 2001). In contrast, in mouse motor terminals CGP-37157 prolonged the post-tetanic decay of mitochondrial [Ca\(^{2+}\)], reduced post-tetanic residual cytosolic [Ca\(^{2+}\)], and reduced post-tetanic transmitter release at both normal and low quantal contents. These results demonstrate that in mouse motor terminals the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger contributes importantly to sustaining evoked release during the post-tetanic period. This drug offers a “cleaner” method for testing the hypothesis of mitochondrial involvement in post-tetanic release than some previous strategies because (at least in the short term) it does not block mitochondrial Ca\(^{2+}\) uptake or interfere with mitochondrial energy production. Previously used agents that inhibit mitochondrial Ca\(^{2+}\) uptake (e.g. CCCP or inhibitors of the uniporter or ETC) also reduce post-tetanic evoked release, but interpretation of this effect is complicated because inhibition of mitochondrial Ca\(^{2+}\) uptake results in greater elevations of cytosolic [Ca\(^{2+}\)] during the tetanus, resulting in high levels of asynchronous release and faster depression of phasic release during the tetanus (Talbot et al. 2003; David and Barrett, 2003). The effects of CGP-37157 are easier to interpret because it did not increase cytosolic [Ca\(^{2+}\)] during tetanic stimulation, and (in certain experimental conditions) its inhibition of post-tetanic evoked release could be observed without increased depression during the tetanus.
Under near-physiological conditions (normal bath \([\text{Ca}^{2+}]\), 30 °C) the time constant of the post-tetanic release component that was inhibited by CGP-37157 was \(~10\) sec (Fig. 2.7A), similar to time constants reported for augmentation. Since mitochondrial \([\text{Ca}^{2+}]\) extrusion under these conditions continued for at least 80 sec, it likely also affected potentiation. Indeed, under altered experimental conditions designed to increase PTP, CGP-37157 abolished all post-tetanic enhancement of release (Fig. 2.7B,C).

The contrasting physiology of crayfish and mouse motor nerve terminals may be related to the mechanisms by which they sustain the elevation of cytosolic \([\text{Ca}^{2+}]\) following tetanic stimulation. At crayfish exciter-opener NMJs there is marked potentiation of synaptic transmission during and following tetanic stimulation under physiological conditions, and this potentiation is physiologically important because contractions of the underlying muscle are graded with EPP amplitude. Thus it seems appropriate that the elevated residual \([\text{Ca}^{2+}]\) that sustains this potentiation comes both from mitochondria and from reverse operation of a plasmalemmal \(\text{Na}^+\)-\(\text{Ca}^{2+}\) exchanger (Zhong et al. 2001). In contrast, at mouse and rat NMJs neither tetanic nor PTP is prominent under physiological conditions; rather the net result is usually depression (as in Fig. 2.7A; also Halstead et al. 2005, and in vivo Argaw et al. 2004). Augmentation and potentiation dominate only under non-physiological conditions (e.g. low bath \([\text{Ca}^{2+}]\)). Since contraction of the underlying muscle fiber is all-or-nothing, the physiologically important task is not to increase the amplitude of the post-tetanic EPP to supra-control levels, but rather simply to restore EPP amplitude to a value suprathreshold for muscle contraction as quickly as possible. Evidence presented in this paper demonstrates that
mitochondrial extrusion of previously-sequestered Ca\textsuperscript{2+} via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is a major mechanism for accomplishing this task.

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Chapter 3

The Mitochondrial Depolarization that Accompanies Mitochondrial Calcium Uptake is Greater in Pre-symptomatic SOD1-G85R than in Wild Type Mouse Motor Terminals

A. Summary

Mitochondrial Ca\(^{2+}\) uptake depends on the mitochondrial membrane potential (\(\Psi_m\)) established by ETC activity, and helps to limit the increase in cytosolic [Ca\(^{2+}\)] induced by repetitive stimulation of motor nerve terminals. Using rhodamine-123 (Rh123) we determined the effect of repetitive nerve stimulation (100 Hz) on \(\Psi_m\) in motor terminals innervating mouse levator auris muscles. Stimulation-induced \(\Psi_m\) depolarizations in WT terminals were small (~2 mV), but increased when the temperature was lowered from 30 to 20 °C. \(\Psi_m\) depolarizations depended on Ca\(^{2+}\) influx into motor terminals since they were inhibited when P/Q-type Ca\(^{2+}\) channels were blocked with \(\omega\)-agatoxin, and enhanced by increasing AP frequency (25 to 100 Hz) or duration (3,4-DAP). 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 nM), suggesting that acceleration of ETC proton extrusion normally limits the magnitude of \(\Psi_m\) depolarization during mitochondrial Ca\(^{2+}\) uptake, thereby facilitating continued Ca\(^{2+}\) sequestration. Compared to WT, stimulation-induced \(\Psi_m\) depolarizations were larger in motor terminals from pre-symptomatic mice expressing the G85R mutation of human SOD1. \(\Psi_m\) depolarizations were not significantly altered by
expression of WT human SOD1 or knockout of SOD1. We suggest that an early functional consequence of SOD1-G85R association with motoneuronal mitochondria is reduced capacity of the ETC to restrict Ca\(^{2+}\)-induced \(\Psi_m\) depolarization, and that this impairment contributes to disease progression in SOD1-G85R motor terminals.

B. Background

Mitochondria sequester significant amounts of stimulation-induced Ca\(^{2+}\) loads in many cell types (Friel & Tsien, 1994; Stuenkel, 1994; Herrington et al. 1996; David et al. 1998; Pivovarova et al. 1999; Kaftan et al. 2000; Suzuki et al. 2002; David & Barrett, 2003). For example, in rat chromaffin cells, Babcock et al. (1997) estimated that mitochondrial Ca\(^{2+}\) uptake accounted for ~70% of cytosolic Ca\(^{2+}\) removal after stimulation-induced increases in cytosolic [Ca\(^{2+}\)]. 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 (reviewed by Gunter & Pfeiffer, 1990; Nicholls & 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 stimulus trains (e.g. 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. As proposed for lizard motor terminals by Talbot et al. (2007), this acceleration would limit
the net $\Psi_m$ depolarization, thereby allowing mitochondria to continue taking up Ca$^{2+}$ even during prolonged stimulation.

We tested this hypothesis in mouse motor terminals, and found that at near-physiological temperatures (30 °C) the $\Psi_m$ depolarizations produced by repetitive stimulation at 50-100 Hz were small (or undetectable) and reversible. Partially inhibiting ETC activity with low concentrations of rotenone (25-50 nM) or low temperature (20 °C) increased the amplitude of these depolarizations. These $\Psi_m$ depolarizations required Ca$^{2+}$ influx into the motor terminal.

We also tested motor terminals in transgenic mice expressing a mutant version of human SOD1 (G85R) that produces a familial form of ALS (fALS). 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 the cytoplasmic face of spinal cord mitochondria (Vande Velde et al. 2008), and Kirkinezos et al. (2005) reported impaired ETC activity in the spinal cord of mice expressing another fALS-inducing SOD1 mutation (G93A). Damiano et al. (2006) demonstrated an early reduction in Ca$^{2+}$ loading capacity in mitochondria from spinal cord (but not liver) in both SOD1-G93A and SOD1-G85R mice. We hypothesized that this reduced ability to sequester Ca$^{2+}$ loads might be due to 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 stimulation-induced $\Psi_m$ depolarizations at 30 °C are greater in SOD1-G85R mice than in WT mice.

C. Results

$\Psi_m$ depolarization is small during repetitive stimulation at near-physiological temperatures

Fig. 3.1A shows representative changes in cytosolic [Ca$^{2+}$], mitochondrial [Ca$^{2+}$], and $\Psi_m$ recorded in WT motor nerve terminals (levator auris longus muscle) stimulated with three trains of APs (100 Hz, 5 sec) at 30 °C. The topmost trace shows the elevation of cytosolic [Ca$^{2+}$], measured as the normalized increase in fluorescence ($F/F_{\text{rest}}$) of OG-1 that had been ionophoretically injected into the axoplasm. The initial rapid increase at the onset of stimulation is followed by a slower rate of rise during the remainder of the train. The reduction in the rate of rise is 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 followed by a slower decrease. The slowly-decaying phase is due in part to Ca$^{2+}$ extrusion from mitochondria via a Na$^+$-Ca$^{2+}$ exchanger (García-Chacón et al. 2006).

The second trace in Fig. 3.1A shows the stimulation-induced increase in mitochondrial [Ca$^{2+}$], measured as the fluorescence of mitochondrially-loaded X-rhod-1 (see Materials and Methods). Compared to cytosolic [Ca$^{2+}$], mitochondrial [Ca$^{2+}$] exhibits a slower rate of rise and a slower decay, as described by David et al. (1998) and García-Chacón et al. (2006). Because of this slower decay, the mitochondrial [Ca$^{2+}$]
responses evoked by the second and third stimulus trains begin during the decaying phase of the response to previous trains. In spite of this overlap, the level of the peak fluorescence evoked by subsequent trains does not exceed that evoked by the first train. This “cap” on the maximal increase in mitochondrial \([\text{Ca}^{2+}]\) is not due to reduced \(\text{Ca}^{2+}\) entry during subsequent trains, because the cytosolic \([\text{Ca}^{2+}]\) increases produced by each train are similar. The cap is not due to saturation of the indicator dye, because similar caps on increases in mitochondrial \([\text{Ca}^{2+}]\) are also measured using indicators with much lower affinities than X-rhod-1 (e.g. rhod-5N, \(K_d \sim 320 \mu\text{M}\), David et al. 2003). Instead it appears that the cap represents a limitation on the maximal increase in matrix \([\text{Ca}^{2+}]\). It is thought that this cap represents the concentration of free \(\text{Ca}^{2+}\) in equilibrium with an insoluble complex containing \(\text{Ca}\) and phosphate that (reversibly) sequesters matrix \(\text{Ca}^{2+}\) (reviewed by e.g. Chalmers & Nicholls, 2003). When the \(\text{Ca}^{2+}\) load is applied gradually (e.g. during physiological stimulation), the maximal increase in mitochondrial \([\text{Ca}^{2+}]\) is only \(\sim 1-2 \mu\text{M}\) (David, 1999; Chalmers & Nicholls, 2003).

The third trace in Fig. 3.1A shows stimulation-induced depolarizations of \(\Psi_m\), recorded as increases in Rh123 fluorescence. In this motor terminal each stimulus train reversibly increased fluorescence by \(\sim 2\%\). The bottom trace shows these same data on a different vertical scale, along with the much larger increase in Rh123 fluorescence \(\sim 140\%) measured in this terminal during the near-complete \(\Psi_m\) depolarization produced by a high concentration of the proton carrier carbonyl CCCP (2 \(\mu\text{M}\)). See Supporting Information for information about a quantitative relation between Rh123 fluorescence and \(\Psi_m\).
Fig. 3.1B shows stimulation-induced changes in mitochondrial \([Ca^{2+}]\) and \(\Psi_m\) recorded simultaneously, demonstrating that in some motor terminals there was no detectable increase in Rh123 fluorescence, even though the increase in mitochondrial \([Ca^{2+}]\) verified that nerve stimulation had reached the terminal. In physiological saline at temperatures \(\geq 28 \, ^\circ\text{C}\), the mean increase in Rh123 fluorescence after 500 stimuli at 100 Hz was only \(1.16 \pm 0.27\%\) (SEM, range 0-4.5%, \(n = 14\) terminals). These measurements included only terminals in which nerve conduction was verified by (1) clear, reversible and repeatable increases in Rh123 fluorescence during stimulation as in Fig. 3.1A, (2) the appearance of stimulation-evoked \(\Psi_m\) depolarizations when experimental conditions were changed as described below, and/or (3) simultaneous measurement of increases in mitochondrial \([Ca^{2+}]\) as in Fig. 3.1B. Calculations described in item #1 of Supporting Information suggest that the average fluorescence increase of 1.16% corresponds to a \(\Psi_m\) depolarization of at most 1.7 mV.
1A

Time (sec)

F/Frest OG-5N

1.0

1.1

1.2

1.3

100 Hz

F/Frest X-rhod-1

1.0

1.1

1.2

1.3

F/Frest Rh 123

1.0

1.1

1.2

1.3

2 µM CCCP

0 40 80 120 160

F/Frest Rh 123

1.0

1.1

1.2

1.3

2.0

2.2

2.4

0.98

1.00

1.02

1.04

3.0

3.2

3.4

4.0
Figure 3.1 (this and previous page) Stimulation at 100 Hz increases cytosolic and mitochondrial $[\text{Ca}^{2+}]$ and depolarizes $\Psi_m$ in WT mouse motor terminals at 30 °C. A (previous page), Upper trace: elevation of cytosolic $[\text{Ca}^{2+}]$ in response to 3 stimulus trains at 100 Hz, monitored as normalized increases ($F/F_{\text{rest}}$) in the fluorescence of intra-axonally injected OG-1 (vertical lines indicate duration of stimulation). Second trace: elevations of mitochondrial matrix $[\text{Ca}^{2+}]$ in a different terminal, monitored as increases in the fluorescence of mitochondrially-loaded X-rhod-1 (mean of 5 traces). Third trace: depolarization of $\Psi_m$ in a different terminal, monitored as increases in the fluorescence of Rh123. 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 µM). B, Simultaneous imaging of X-rhod-1 and Rh123 fluorences (mean of 3 traces). In this terminal, 100 Hz stimulation produced a clear increase in mitochondrial $[\text{Ca}^{2+}]$ (verifying nerve conduction), but any $\Psi_m$ depolarization was below the limit of detection. WT mice in A,B were 64-88 days old.
Stimulation-induced $\Psi_m$ depolarizations are $\text{Ca}^{2+}$-dependent

In order to study mechanisms underlying stimulation-induced $\Psi_m$ depolarizations, we needed to increase the magnitude of the recorded Rh123 signal. Fig. 3.2A demonstrates that signal magnitude increased when the temperature was reduced, or when AP duration was prolonged using 3,4-DAP (20 µM), which blocks certain depolarization-activated $\text{K}^+$ channels in motor axons and terminals (Tabti et al. 1989; Morita & Barrett, 1990; David et al. 1995).

Fig. 3.2B shows that stimulation-induced $\Psi_m$ depolarizations in mouse motor terminals are $\text{Ca}^{2+}$-dependent. These depolarizations were reversibly abolished by replacing bath $\text{Ca}^{2+}$ with $\text{Mg}^{2+}$, and were also inhibited by $\omega$-agatoxin TK (0.6 µM), which blocks the P/Q-type $\text{Ca}^{2+}$ channels that mediate most $\text{Ca}^{2+}$ entry into mouse motor terminals (Teramoto et al. 1993; Westenbroek et al. 1998). These findings demonstrate that the $\text{Na}^+$ influx associated with axonal AP propagation, which continues in low $[\text{Ca}^{2+}]$ and $\omega$-agatoxin, is not by itself sufficient to produce stimulation-induced $\Psi_m$ depolarizations. Rather, these depolarizations require $\text{Ca}^{2+}$ influx into motor terminals.

Figure 3.2 (following page) The stimulation-induced $\Psi_m$ depolarization increases with cooling or addition of 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 AP with 20 µM 3,4-DAP (lower right). B, $\Psi_m$ depolarizations (whose magnitudes were enhanced by both cooling to 20 °C and 3,4-DAP, open circles) were reduced by replacing bath $\text{Ca}^{2+}$ with $\text{Mg}^{2+}$ (filled circles, left) or (in a different terminal) by adding 0.6 µM $\omega$-agatoxin (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,B is the mean of 2-9 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 experiments of Fig. 3.3 examined how the stimulation-induced $\Psi_m$ depolarization was affected by the rate of stimulation, i.e., the rate at which $\text{Ca}^{2+}$ entered the terminal. The upper trace superimposes stimulation-induced elevations of cytosolic $[\text{Ca}^{2+}]$ in a terminal given 1000 stimuli at frequencies ranging from 10-100 Hz; elevations were greater at higher stimulation frequencies, as previously reported by David et al. (1998). In isolated mitochondria $\text{Ca}^{2+}$ influx through the uniporter/channel exhibits a greater-than-linear dependence on the $[\text{Ca}^{2+}]$ surrounding mitochondria (Gunter & Pfeiffer, 1990), so one would predict faster $\Psi_m$ depolarization with higher stimulation frequencies. The lower two traces of Fig. 3.3 show a test of this prediction in a motor terminal subjected to 1500 stimuli, delivered either as 3 separate trains at 100 Hz (middle trace), or as a single 60 sec train at 25 Hz (lower trace). The three $\Psi_m$ depolarizations seen in the middle trace resulted in increases in fluorescence of 1 to 2%, similar to the % average increase in fluorescence seen in Fig. 3.1A (third trace) before the addition of CCCP. As predicted, $\Psi_m$ depolarized more rapidly during the higher-frequency stimulation.

Figure 3.3 (following page) 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 motor terminal given 1000 stimuli at the indicated frequencies. B, $\Psi_m$ depolarizations in a different terminal produced by 3 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 3-4 traces. Other experiments (not shown) 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).
3A

100 Hz
50 Hz
25 Hz
10 Hz

F/Frest OG-1
Time (sec)

3B

100 Hz
25 Hz

F/Frest Rh 123
Time (sec)
Another possible cause of stimulation-induced $\Psi_m$ depolarizations is transient opening of the MPTP. However, cyclosporin A (10 µM), which inhibits pore opening, did not reduce the magnitude of stimulation-induced $\Psi_m$ depolarizations (see item #2 in Supplemental Material). Our findings differ from those reported by Chalmers and McCarron (2008) for mitochondria in smooth muscle cells: they detected no $\Psi_m$ depolarization following Ca$^{2+}$ influx or store release, but did detect (under stress conditions) spontaneous $\Psi_m$ depolarizations inhibited by cyclosporin A. Such spontaneous depolarizations were not seen in motor terminal mitochondria.

Partial inhibition of ETC complex I increases the stimulation-induced $\Psi_m$ depolarization

Results in Figs. 3.2 and 3.3 link stimulation-induced $\Psi_m$ depolarizations to Ca$^{2+}$ entry into motor terminals, but do not prove a linkage to Ca$^{2+}$ influx into mitochondria. It is difficult to block mitochondrial Ca$^{2+}$ influx selectively, because Ru360, which blocks the mitochondrial uniporter/channel (Matlib et al. 1998), also reduces Ca$^{2+}$ influx across neuronal plasma membranes (Duchen, 1992; David, 1999). Fig. 3.4 provides indirect evidence linking 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 nM) increases the $\Psi_m$ depolarization (lower traces) evoked by 100 Hz stimulation. The average change in Rh123 fluorescence increased from 0.96 ± 0.22% in control medium to 4.13 ± 1.18% in rotenone (p < 0.05). This result is consistent with the hypothesis outlined in the Introduction, which predicts 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. Barrientos and Moraes (1999)
found that in isolated mitochondria this concentration of rotenone decreased complex I activity by ~85%, but had minimal effect on \( \Psi_m \). Likewise, we found that the rotenone concentrations used here had no significant effect on resting Rh123 fluorescence, suggesting little or no effect on resting \( \Psi_m \) polarization (see item #3 in Supplemental Material).

Greater \( \Psi_m \) depolarization would be predicted to reduce the electrical gradient that permits \( \text{Ca}^{2+} \) influx into mitochondria, and thus increase the elevation of cytosolic \( [\text{Ca}^{2+}] \) that accompanies stimulation. This prediction is supported by the increased elevations of cytosolic \( [\text{Ca}^{2+}] \) in rotenone (Fig. 3.4, upper trace). Likewise, Talbot et al. (2007) demonstrated increases in stimulation-induced \( \Psi_m \) depolarization and elevation of cytosolic \( [\text{Ca}^{2+}] \) in lizard motor terminals when complex I activity was partially inhibited with amytal.

Figure 3.4 (following page) A low concentration of rotenone increases stimulation-induced elevations of cytosolic \( [\text{Ca}^{2+}] \) and \( \Psi_m \) depolarizations. Cytosolic \( [\text{Ca}^{2+}] \) (upper trace) and \( \Psi_m \) depolarizations (lower trace) produced by 3 trains at 100 Hz before (open circles in both graphs) and after (closed circles in both graphs) addition of a low concentration of rotenone (50 nM). [\( \text{Ca}^{2+} \)] and \( \Psi_m \) traces came from different terminals. Histogram summarizes paired \( \Psi_m \) data from 8 terminals studied before and after rotenone exposure in WT mice and transgenic mice expressing normal human SOD1 (ages 50-375 days). * indicates significant difference from control, \( p < 0.05 \). Only measurements from the initial 100 Hz train were included in the averages. Duration of rotenone exposure was 17-30 minutes. With higher rotenone concentrations stimulation-induced changes in \( \Psi_m \) polarization became impossible to detect, perhaps because \( \Psi_m \) was now significantly depolarized at rest.
Stimulation-induced $\Psi_m$ depolarizations are enhanced in mice expressing mutant human superoxide dismutase 1

As summarized in the Introduction, it has been reported that spinal cord ETC activity is diminished in mice expressing the fALS-linked G93A mutation of human SOD1. If motor terminal mitochondria in SOD1-G85R mice have a similar limitation that hinders their ability to accelerate ETC activity in response to the $\Psi_m$ depolarization caused by Ca$^{2+}$ influx, then one would predict a greater stimulation-induced $\Psi_m$ depolarization. Fig. 3.5A presents phase and resting Rh123 fluorescence images of a terminal in a pre-symptomatic SOD1-G85R mouse, along with a difference image highlighting those regions in which Rh123 fluorescence increased during stimulation. This difference image shows signals specific to motor terminal mitochondria. Fig. 3.5B shows Rh123 recordings from this pre-symptomatic SOD1-G85R mouse, contrasted with similar recordings from a WT terminal. The histogram in Fig. 3.5B shows that at 30 °C SOD1-G85R motor terminals displayed larger stimulation-induced $\Psi_m$ depolarizations than WT terminals (5.6 ± 1.6% SEM vs. 1.16 ± 0.27%, p < 0.05). Resting values of Rh123 fluorescence were similar between WT and SOD1-G85R terminals, and there was no significant correlation between resting Rh123 fluorescence and the stimulation-induced increase in fluorescence (see item #4 in Supplemental Material).
Figure 3.5 (following page) Stimulation-induced $\Psi_m$ depolarizations are increased in pre-symptomatic SOD1-G85R mice. A, Phase (left) and Rh123 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. Temperature was 30 °C. SOD1-G85R mice were studied at pre-symptomatic ages (4-5.5 months), when all motor end-plates remained innervated, and no motor terminal sprouts were seen (not shown). B, Side-by-side comparison of representative stimulation-induced $\Psi_m$ depolarizations evoked by repeated brief 100 Hz trains in a 71 day old male (mean of 2) WT and a 121 day old male SOD1-G85R mouse, both at 30 °C. The histogram plots data averaged from 18 terminals in WT mice (50-135 days old) and 39 terminals in pre-symptomatic SOD1-G85R mice (121-161 days old). Only measurements from the first 100 Hz train were included in the averages. The difference between $\Psi_m$ depolarizations in WT and SOD1-G85R mice was significant ($p < 0.05$) at 30 °C [Mann Whitney, 2 tailed], as illustrated, but not at lower temperatures.
18 terminals from 14 animals

39 terminals from 4 animals

Max % Increase Rh 123

WT hSOD1 G85R
SOD1 helps defend against oxidative damage. SOD1-G85R lacks SOD1 enzymatic activity (though heterodimers of G85R and WT SOD1 may have activity). To test whether the greater $\Psi_m$ depolarizations recorded in these mice were linked to reduced SOD1 activity, similar experiments were conducted on mice lacking SOD1 activity altogether, and on mice with excess SOD1 activity due to expression of normal human SOD1 in addition to mouse SOD1. Stimulation-induced $\Psi_m$ depolarizations were not significantly different from those recorded in WT terminals ($2.2 \pm 0.33\%$ for SOD1 knockout, $0.67 \pm 0.31\%$ for normal human SOD1, $n = 5\text{--}19$ terminals in $3\text{--}4$ animals). Thus, reduced SOD1 activity in the SOD1-G85R mice was not the sole cause of their increased $\Psi_m$ depolarizations.

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 the motor nerve terminal, and has a rising time course similar to that expected for Ca$^{2+}$ influx into motor terminal mitochondria. This $\Psi_m$ depolarization is small, reversible and repeatable. These latter characteristics contrast with results from studies that applied large Ca$^{2+}$ loads to isolated mitochondria and recorded large $\Psi_m$ depolarizations (Vergun & Reynolds, 2005). The difference in the magnitude and reversibility of the Ca$^{2+}$-induced $\Psi_m$ depolarization is likely due to the magnitude of the Ca$^{2+}$ load and the rate at which it is presented. Chalmers and Nicholls (2003) demonstrated that isolated mitochondria could take up
larger amounts of Ca\(^{2+}\) with minimal \(\Psi_m\) depolarization when the Ca\(^{2+}\) load was presented slowly than when the Ca\(^{2+}\) load was presented as a bolus. The small, reversible \(\Psi_m\) depolarizations recorded here are thus consistent with the gradual delivery of Ca\(^{2+}\) loads produced by repetitive nerve stimulation.

To determine whether the magnitude of the recorded stimulation-induced \(\Psi_m\) depolarizations are consistent with current 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. 3.1A). 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 (see item #6 in Supplemental Material). These calculations yielded a predicted \(\Psi_m\) depolarization of \(\sim 2\) mV, similar to the magnitude of the \(\Psi_m\) depolarization estimated from the magnitude of the stimulation-induced Rh123 fluorescence change (item #1 in Supplemental Material). The studies of Johnson-Cadwell et al. (2007) suggest that \(\Psi_m\) depolarizations of this magnitude are sufficient to accelerate ETC activity significantly.

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 the 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 during partial blockade of complex I with rotenone. (In the electrical model, rotenone would increase ETC resistance.) Partial ETC inhibition has a greater effect on $\Psi_m$ in stimulated than in resting terminals (Talbot et al. 2007). The fact that Ca$^{2+}$ entry increased rotenone’s effect on $\Psi_m$ recalls Votyakova and Reynolds’ (2005) finding of synergistic effects of Ca$^{2+}$ and low concentrations of rotenone on release of free radicals from isolated rat brain mitochondria.

We cannot be certain of the mechanism(s) by which lowering the temperature increased the magnitude of the recorded $\Psi_m$ depolarization. The stimulation-induced elevation of cytosolic [Ca$^{2+}$] is greater at lower temperatures, but the elevation of cytosolic [Ca$^{2+}$] becomes less sensitive to CCCP and the elevation of mitochondrial [Ca$^{2+}$] is reduced (David and Barrett, 2003; Vila et al. 2003). Thus the increase in $\Psi_m$ depolarization recorded at low temperatures seems more likely due to reduced ETC activity than to increased Ca$^{2+}$ influx into mitochondria.

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

As mentioned in the Introduction, there is morphological evidence for early mitochondrial damage in motor terminals and motor neurons of SOD1 mutant mouse models of fALS, as well as functional evidence for reduced activity of certain ETC complexes and reduced capacity for Ca$^{2+}$ uptake in the ventral horn of the spinal cord. Given these findings, and the fact that rotenone mimicked the effect of the fALS mutation, the most logical explanation for the larger stimulation-induced $\Psi_m$ depolarizations recorded in motor terminals of SOD1-G85R mice is that their
mitochondria have a reduced ability to accelerate ETC activity in response to the depolarization produced by Ca\textsuperscript{2+} entry. This is the first demonstration of altered handling of Ca\textsuperscript{2+} loads in mitochondria localized exclusively within motor neurons (as distinct from surrounding glial cells), and is consistent with Jaarsma et al.’s (2008) finding that neuron-specific expression of mutant SOD1 is sufficient to produce disease.

Deficits in mitochondrial Ca\textsuperscript{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\textsuperscript{2+} loads 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).

In summary, mitochondria in motor nerve terminals have multiple features that permit them to sequester the Ca\textsuperscript{2+} loads introduced by repetitive stimulation: (1) initiation of Ca\textsuperscript{2+} uptake with relatively small elevations of cytosolic [Ca\textsuperscript{2+}], (2) a powerful sequestration mechanism that limits the increase in matrix [Ca\textsuperscript{2+}], and (3) acceleration of ETC activity in response to the depolarization produced by Ca\textsuperscript{2+} entry, hence preserving the electrical gradient that favors Ca\textsuperscript{2+} entry. Evidence presented here suggests that the Ca\textsuperscript{2+}-dependent Ψ_m depolarization produced by repetitive stimulation is only ~2 mV in WT terminals, but increases in mitochondria of pre-symptomatic SOD1-G85R terminals. Ψ_m depolarizations would be expected to increase in older mutant SOD1 mice as ETC function deteriorates. Increased Ψ_m depolarization reduces mitochondrial ability to synthesize ATP and sequester [Ca\textsuperscript{2+}] loads. The resulting increased elevation of cytosolic [Ca\textsuperscript{2+}] during repetitive stimulation may contribute to the early motor terminal damage documented in fALS mice.
E. Materials and methods

Preparation, solutions, stimulation

Most experiments used NMJs from the levator auris longus muscle of WT (C57BL/6, bred from mice obtained from Jackson Laboratories, Bar Harbour, ME, USA) and hSOD1-G85R mice (Tg(SOD1-G85R)148Dwc) bred from mice kindly supplied by Dr. Don Cleveland (Ludwig Institute for Cancer Research, University of California at San Diego). Some experiments used mice that express normal human SOD1 (hSOD1-WT; B6SJL-Tg(SOD1)2Gur/J, also supplied by Dr. Cleveland) and mice lacking SOD1 (B6,129S7-SOD1^tm1Leb/J002972, Jackson Laboratories). The hSOD1-G85R mice have a copy number of 15, and exhibit disease onset at 8-10 months and end-stage/death 9-11 months (Bruijn et al. 1997).

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% CO\textsubscript{2}, 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 cover slip. The physiological saline contained (in mM) NaCl 137, NaHCO\textsubscript{3} 15, KCl 4, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 1.1, glucose 11.2 and NaH\textsubscript{2}PO\textsubscript{4} 0.33. In some experiments CaCl\textsubscript{2} was replaced with equimolar MgCl\textsubscript{2}. The pH of the solution was kept near 7.4 by gassing the inside of the chamber with 5% CO\textsubscript{2} in 95% O\textsubscript{2}. Temperature was monitored with a thermistor. Unless otherwise stated, experiments were conducted at 30 °C, achieved by blowing hot air onto the underside of the chamber,
and monitored with a thermistor. This was the warmest temperature at which the
dissected preparation could remain functional for several hours.

The motor nerve was stimulated via a suction electrode by applying trains of
suprathreshold 0.3 ms depolarizing pulses (500-2000 at 50-100 Hz). Muscle contractions
were blocked using d-tubocurarine (7-15 µM), which blocks muscle nicotinic
acetylcholine (ACh) receptors and thereby prevents entry of Ca\(^{2+}\) into the muscle end-
plate via ACh receptor channels or depolarization-activated Ca\(^{2+}\) channels.

**Imaging of stimulation-induced changes in cytosolic and mitochondrial \([Ca^{2+}]\) and \(\Psi_m\)**

Stimulation-induced changes in cytosolic \([Ca^{2+}]\) were monitored using Oregon
Green 488 BAPTA-1 (OG-1, \(K_d\) 0.17 µM) or Oregon Green BAPTA 5N (OG-5N, \(K_d\) 60
µM) excitation 488 nm, emission 535 nm. The membrane-impermeable hexapotassium
salt form was loaded ionophoretically into an internodal axon (as described in David &
Barrett, 2003), and subsequently diffused into motor terminals.

Stimulation-induced changes in mitochondrial \([Ca^{2+}]\) were measured using the
indicator X-rhod-1 (\(K_d\) 0.7 µM, excitation 568 nm, emission monitored with Chroma
long-pass 590 nm filter). This \(K_d\) is appropriate because resting mitochondrial \([Ca^{2+}]\) is
estimated to be 0.05-0.1 µM (reviewed by Gunter & Pfeiffer, 1990) and the maximal
increase in mitochondrial \([Ca^{2+}]\) in repetitively stimulated motor terminals is \(\sim 2 \mu M\)
(David, 1999; David et al. 2003). After a 30 minute exposure to 25 µg/ml of the
membrane-permeable acetoxymethylester (AM) form of X-rhod-1, the preparation was
washed with indicator-free saline. The AM moiety is cleaved by cytosolic and intra-
mitochondrial esterases, converting the indicator into its charged, Ca\(^{2+}\)-binding form, and
trapping it within the compartment in which the de-esterification occurred. During washout from 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).

Changes in $\Psi_m$ were measured using Rh123, a cell-permeant, cationic, fluorescent dye that is sequestered by polarized mitochondria. Rh123 was excited at 488 nm, with emissions monitored using a 535 nm filter (bandwidth 40 nm, Chroma, Rockingham, VT, USA). Loading for 30 minutes with 3 µg/ml (followed by rinsing with indicator-free saline) produced sufficient uptake of Rh123 into the mitochondrial matrix to self-quench. Depolarization of $\Psi_m$ causes Rh123 to leak out of mitochondria into the cytosol where Rh123 becomes unquenched, producing an increase in fluorescence (reviewed by Nicholls & Ward, 2000).

The experimental chamber was placed on the stage of an inverted microscope in a confocal system that included a Yokogawa spinning disc (Solamere, Salt Lake City, UT, USA), a 60X water immersion lens (NA 1.2, Olympus), and a high-sensitivity Photometrics Cascade 512B CCD camera (Roper Scientific, Trenton, NJ, USA) 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, USA). A series of images were obtained before, during and after a train of APs. Inter-image intervals were 1-3 sec and exposure times were 0.8-2 sec. Data were recorded using IP Laboratory v3.61 software (Scanalytics, Inc., Fairfax, VA, USA) and analyzed on a Pentium computer using V++ software (Digital Micro
Optics, Auckland, NZ) or ImageJ software (version 1.33u, public domain, http://rsb.info.nih.gov/ij/). Variability due to 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. Net fluorescence ($F_{\text{net}}$) was calculated by averaging the total fluorescence ($F_{\text{total}}$) signals from regions of interest in the terminal, and then subtracting background fluorescence ($F_{\text{background}}$) from surrounding non-terminal regions ($F_{\text{net}} = F_{\text{total}} - F_{\text{background}}$). Changes in fluorescence were plotted as $F_{\text{net}}/F_{\text{rest}}$ (abbreviated as $F/F_{\text{rest}}$ in figures), where $F_{\text{rest}}$ is the average $F_{\text{net}}$ for 20-30 images obtained before stimulation began.

Reagent sources

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

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F. Supporting information

1. The quantitative relationship between changes in Rh123 fluorescence and $\Psi_m$ depolarization is complex, but may be approximately linear for small depolarizations from rest (Huang et al. 2007). Making the following simplifying assumptions:

- that the relationship is linear,
- that the ~140% increase in Rh123 fluorescence produced by 2 µM CCCP represents complete depolarization of $\Psi_m$, and
- that the resting value of $\Psi_m$ is -200 mV,

then the measured average stimulation-induced change in Rh123 fluorescence (1.2%) would correspond to a depolarization of $(1.2/140)(200 \text{ mV}) = 1.7 \text{ mV}$. This value would be an overestimate if the relationship between Rh123 fluorescence and $\Psi_m$ is curvilinear, if the CCCP-induced depolarization of $\Psi_m$ is incomplete, and/or if the resting value of $\Psi_m$ is more depolarized.
2. Figure 3.6 Stimulation-induced $\Psi_m$ depolarizations probably do not depend on transient openings of the MPTP. The amplitude of $\Psi_m$ depolarizations (measured as change in Rh123 fluorescence) increased following addition of 3,4-DAP (50 µM), but did not decrease following the subsequent addition of cyclosporin A (1 µM). Average of 2 terminals in an 11 month old SOD1 knockout mouse. Similar results were obtained in an experiment in which the cyclosporin A concentration was increased to 10 µM (not shown).

3. Summing together results from both WT mice and mice expressing normal human SOD1, the mean value of resting Rh123 fluorescence was $378 \pm 73$ SEM (arbitrary fluorescence units) before rotenone application and $358 \pm 52$ after rotenone ($n = 8$ terminals in 7 animals, not significantly different by a paired t-test). These results suggest that the low concentrations of rotenone used here (25-50 nM) had no significant effect on pre-stimulation values of $\Psi_m$. 
4. Pre-stimulation (resting) values of Rh123 fluorescence measured in WT mice 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 terminal in 14 WT mice, 5 terminals in 4 mice expressing normal human SOD1, and 39 terminals in 4 mice expressing SOD1-G85R). There were no significant differences among these values (Kruskal-Wallis test). Also, there was no significant correlation between the % change in Rh123 fluorescence and the resting fluorescence value. These findings suggest that the differential $\Psi_m$ responses of these mice to nerve stimulation were not due to differences in resting $\Psi_m$.

5. Figure 3.7 G85R end-plates are fully innervated at ages at which terminals display abnormally large stimulation-induced $\Psi_m$ depolarizations (as shown in Fig. 3.5B). Shown are images of (A) $\alpha$-bungarotoxin labeled, (B) YFP-filled, and (C) overlay of both images of an SOD1-G85R terminal from a 88 day old animal. A total of 253 end-plates from the levator auris muscle of 3 pre-symptomatic SOD1-G85R mice (ages 88-157 days, including the one represented in above images) were all found to be fully occupied.

6. The following summarizes a method to calculate the $\Psi_m$ depolarization predicted from the estimated rate of $\text{Ca}^{2+}$ entry into motor terminal mitochondria. Calculations used
values calculated for 1 mg of mitochondria, with an estimated free water volume of 0.6-1 µL (Gunter and Pfeiffer, 1990).

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

To calculate the predicted voltage drop, this current was injected into a simplified electrical circuit of the mitochondrial inner membrane (for 1 mg protein, see Fig. 8 in Talbot et al. 2007) which includes the resistance of the ETC (estimated at ~10 Ω in normally respiring mitochondria, Nicholls, 1974) in series with an electron motive force (200 mV), in parallel with the resistance of the mitochondrial inner membrane (3000 Ω,
Magnus & Keizer, 1997). Calculations using this simplified circuit yielded a predicted $\Psi_m$ depolarization of ~2 mV.

In this model, inhibition of ETC complexes (e.g. by rotenone) increases the resistance of the ETC and thereby increases the calculated $\Psi_m$ depolarization.
Chapter 4

General Discussion

Work described in this dissertation studied the effects of mitochondrial Ca\(^{2+}\) uptake and extrusion on matrix and cytosolic [Ca\(^{2+}\)], \(\Psi_m\) and NT release. The principal conclusions include: (1) increases in the Ca\(^{2+}\) load to motor terminals during stimulation prolong the decay of post-stimulation mitochondrial matrix [Ca\(^{2+}\)], (2) during the post-stimulation decay of matrix [Ca\(^{2+}\)], Ca\(^{2+}\) extrusion occurs principally via the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger, and this extrusion of Ca\(^{2+}\) helps re-establish pre-stimulation EPP amplitudes, (3) Ca\(^{2+}\) entry into the matrix during repetitive stimulation results in \(\Psi_m\) depolarizations that are either small or undetectable, (4) the mechanism by which mitochondria are able to sequester significant amounts of cytosolic Ca\(^{2+}\) without large \(\Psi_m\) depolarizations probably involves an increase in the ETC’s proton pumping activity, and (5) this increase in ETC activity might be compromised in the early pathogenesis in fALS mice.

A. Post-stimulation mitochondrial [Ca\(^{2+}\)] decays are prolonged with increasing Ca\(^{2+}\) loads

The “complex formation” hypothesis (see Chapter 1) predicts that varying the Ca\(^{2+}\) load on motor terminals during stimulation should have little effect on the amplitude of the mitochondrial [Ca\(^{2+}\)] plateau. The calcium-phosphate complex is expected to begin to form when a critical matrix [Ca\(^{2+}\)] is reached and, as long as the complex is

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present, matrix $[\text{Ca}^{2+}]$ should remain “clamped” at this plateau level. Also, according to this hypothesis, increases in the $\text{Ca}^{2+}$ load during stimulation would be reflected as a prolongation of the post-stimulation mitochondrial $\text{Ca}^{2+}$ decay (measured as the decay integral, see Chapter 2). Both of these predicted results were observed in the work presented here (Figs. 2.2A, 2.3). Table 2.1 shows that 2-fold and 4-fold increases in stimulus train duration as well as 4.5-fold and 6-fold increases in bath $[\text{Ca}^{2+}]$ resulted in increases in decay integrals that were lower than expected. This result could be due to mitochondrial $\text{Ca}^{2+}$ extrusion occurring during the stimulus train, with consequently less $\text{Ca}^{2+}$ being extruded during the decay. Extrusion would be favored by high cytosolic $[\text{Na}^+]$ during stimulation, since this would help drive the mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchanger in favor of $\text{Na}^+$ transport into and $\text{Ca}^{2+}$ transport out of the matrix. Pivovarova et al. (1999) detected an increase in total mitochondrial matrix sodium in bullfrog sympathetic ganglia during stimulation, presumably reflecting $\text{Na}^+$ influx coupled to $\text{Ca}^{2+}$ efflux. In some terminals studied here, cytosolic $[\text{Ca}^{2+}]$ during stimulation was lower when the mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchanger was inhibited with CGP-37157 (Fig. 2.6C). This suggests that in some terminals, the mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchanger may be extruding $\text{Ca}^{2+}$ not only after but also during tetanic stimulation (see next section for more discussion on the role of the mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchanger in $\text{Ca}^{2+}$ extrusion).
B. The mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger is the principal Ca\(^{2+}\) extrusion mechanism for motor terminal mitochondria

Inhibition of the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger resulted in a dramatic prolongation of the post-stimulation decay of matrix [Ca\(^{2+}\)] in mouse motor nerve terminals (Fig. 2.4A). Previous studies that also used CGP-37157 to block the exchanger observed similar results in isolated mitochondria (Cox and Matlib, 1993), rat chromaffin cells (Babcock et al. 1997), and rat pituitary gonadotropes (Kaftan et al. 2000). The results presented here also show that the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger is a more important Ca\(^{2+}\) extrusion mechanism in comparison with openings of the MPTP (Fig. 2.4B). A study by Chalmers and Nicholls (2003) using isolated rat mitochondria did not detect openings of the MPTP until a steady infusion of 85 nmol Ca\(^{2+}\) per mg mitochondrial protein per minute resulted in the accumulation of 800 nmol total calcium per mg mitochondrial protein in the matrix. Assuming a free water volume of 1.0 µl per mg mitochondrial protein (Gunter & Pfeiffer, 1990) and a bound:free ratio of 3000 for intra matrix calcium (Kaftan et al. 2000), this corresponds to an intra-mitochondrial [Ca] of ~0.27 mM, which greatly exceeds the 1-2 µM estimate for the maximum increase in matrix [Ca\(^{2+}\)] in stimulated lizard motor terminals (David et al. 2003). It is therefore unlikely that the levels of matrix [Ca\(^{2+}\)] reached during stimulation in this study were sufficient to trigger openings of the pore. Also, one would expect to detect large \(\Psi_m\) depolarizations if the MPTP was activated, but any \(\Psi_m\) depolarizations occurring during stimulation were either small or undetectable in near-physiological conditions (see Fig. 3.1), and no decrease in \(\Psi_m\) depolarizations was observed when openings of the MPTP
were inhibited with cyclosporin A (Fig. 3.6). However, it is possible that reversible, asynchronous openings of the MPTP occurred.

C. Post-stimulation mitochondrial Ca\(^{2+}\) extrusion contributes to cytosolic [Ca\(^{2+}\)] and to evoked NT release

Mitochondrial Ca\(^{2+}\) extrusion into the cytosol during and after repetitive stimulation would increase cytosolic [Ca\(^{2+}\)] and therefore influence Ca\(^{2+}\)-dependent processes in the terminal. A number of studies have measured cytosolic [Ca\(^{2+}\)] \textit{during} stimulation in the presence of CGP-37157 in various cell types. Some of these investigations found no significant difference in stimulation-induced peak cytosolic [Ca\(^{2+}\)] levels with CGP-37157 (David, 1999 in lizard motor nerve terminals, Colegrove et al. 2000a, in bullfrog sympathetic neurons). In other studies, the addition of CGP-37157 resulted in decreased peak cytosolic [Ca\(^{2+}\)] levels (Baron & Thayer, 1997 in cultured rat dorsal root ganglion cells; Scanlon et al. 2000, in rat forebrain neurons). The authors for the two latter studies interpreted their results as being due to an inhibitory effect of CGP-37157 on plasmalemmal voltage-gated Ca\(^{2+}\) channels, but this result could also reflect decreased extrusion of mitochondrial Ca\(^{2+}\) into the cytosol by the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger. Based on the data presented here (compare Figs. 2.6A & B with 2.6C) it is not clear if the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchange is always active \textit{during} stimulation in mouse motor terminals. Variability in cytosolic [Na\(^+\)] near individual mitochondria (which might depend on the relative distance of individual mitochondria from plasma
membrane Na\(^+\) channels) may explain these differences, since the exchanger exploits the favorable inward gradient for Na\(^+\) (into the mitochondrial matrix) to extrude Ca\(^{2+}\).

Previous studies have detected decreases in the post-stimulation residual tail of cytosolic [Ca\(^{2+}\)] when mitochondrial Ca\(^{2+}\) extrusion via the Na\(^+\)-Ca\(^{2+}\) exchanger was inhibited with CGP-37157 (Babcock et al. 1997; Baron & Thayer, 1997; White & Reynolds, 1997; David, 1999; Colegrove et al. 2000a). In mouse motor terminals, inhibition of mitochondrial Ca\(^{2+}\) extrusion also resulted in a decrease in post-stimulation residual [Ca\(^{2+}\)] (Fig. 2.6), as well as a slowed recovery of EPP amplitude (Fig. 2.7A) after the tetanus. Because the depression observed during stimulation under physiological conditions might be masking other processes which tend to increase NT release (i.e. facilitation and potentiation, see Kalkstein & Magleby, 2004), experiments were carried out in low bath [Ca\(^{2+}\)] in order to decrease quantal content (Fig. 2.7C). These conditions enabled PTP to be directly measured. In control, EPP amplitudes normalized to pre-stimulation values (EPP/EPPo) reached a maximum of ~1.5 within the first 5 sec after the end of stimulation and returned to baseline by ~25 sec. These results are comparable to experiments which measured PTP in other mammalian tissues (rat diaphragm, Gage & Hubbard, 1966; mouse diaphragm, Tsai et al. 1989). In mouse motor terminals, PTP was abolished when mitochondrial Ca\(^{2+}\) extrusion via the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger was blocked (Fig. 2.7C). The time constant of the post-tetanic release component inhibited by blocking the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger was ~10 sec, similar to the time constant reported for augmentation of NT release (Magleby & Zengel, 1976).

In conclusion, at mouse motor nerve terminals, mitochondrial Ca\(^{2+}\) transport regulates NT release in important ways. Mitochondrial Ca\(^{2+}\) uptake during stimulation
helps sustain NT release (David & Barrett, 2003), and mitochondrial Ca\(^{2+}\) extrusion after stimulation aids in the restoration of EPP amplitudes to pre-stimulation levels. Both of these mechanisms contribute to the “safety factor” at NMJs, i.e. under normal physiological conditions, more than enough NT is usually released by each nerve AP to elicit an AP in the post-synaptic muscle. Given the results presented here, one could speculate that, due to their prominent role in Ca\(^{2+}\) handling, mitochondria might modulate NT release at synapses other than the NMJ. Lee et al. (2007) have recently shown that inhibition of mitochondrial Ca\(^{2+}\) extrusion by blocking the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger (also with CGP-37157) results in both lower post-stimulation residual cytosolic [Ca\(^{2+}\)] and in a decrease in PTP at hippocampal mossy fiber boutons in the rat.

D. Estimates of the time constant of the inner mitochondrial membrane

As in other biological membranes, an equivalent circuit for the inner mitochondrial membrane consists of resistances and capacitances in parallel. This section uses the circuit model of the inner mitochondrial membrane proposed in Talbot et al. (2007) (modified to include capacitance, see Fig. 4.1) together with data from studies in isolated mitochondria to calculate the magnitude and time course of the voltage change produced by the current associated with Ca\(^{2+}\) entry during repetitive stimulation, and the magnitude of this current.
Figure 4.1  Simplified equivalent circuit of the mitochondrial inner membrane.  $\Delta \Psi_m$ represents the voltage across the inner mitochondrial membrane.  The battery electromotive force (Emf) drives proton (H$^+$) extrusion by the ETC across $R_{ETC}$, the resistance of all proton-extruding complexes of the ETC (I, III, and IV).  H$^+$ current into the matrix occurs across $R_m$, representing the sum of the resistance across all ATPsynthase complexes with the resistance associated with the proton leak current.  $C_m$ is the capacitance associated with the inner mitochondrial membrane. $R_{Ca}$ together with the switch represents the resistance across the mitochondrial Ca$^{2+}$ uniporter which is activated (switch is closed) when cytosolic [Ca$^{2+}$] increases during nerve stimulation.  Modified from Talbot et al. 2007 Fig. 8A.

Studies with respiring, isolated mitochondria exposed to Ca$^{2+}$ loads have measured reversible increases in total matrix [Ca] of up to 3 µmol per mg mitochondrial protein (equivalent to a total matrix [Ca] of 3 M, since the volume of water associated with 1 mg mitochondrial protein is ~1 µl, reviewed in Gunter & Pfeiffer, 1990).  Using x-ray microanalysis, Pivovarova et al. (1999) estimated an increase in total [Ca] of up to ~9 mM in mitochondria from intact frog sympathetic neurons exposed to 50 mM K$^+$.  As explained in the introduction, most of this calcium is buffered upon entering the matrix, and the bound:free ratio of intra matrix calcium has been estimated to be ~3000 (Kaftan et al. 2000).  Therefore, the resting matrix [Ca$^{2+}$], estimated to be ~100 nM (Di Lisa et al. 1993, also reviewed in Gunter & Pfeiffer, 1990) would correspond to a total [Ca] of 300 µM.  Repetitive stimulation of lizard motor terminals increased matrix [Ca$^{2+}$] to ~1 µM (David 1999; David et al, 2003), which corresponds to a total [Ca] of 3 mM, a value of the same order of magnitude as the concentrations noted above for total calcium from the
Pivovarova study. Because of the mitochondrion’s powerful phosphate-dependent buffering system, it is likely that the value for total matrix calcium underestimates the actual amount of calcium that motor terminal mitochondria can accumulate during stimulation.

The time constant can be calculated by using the capacitance for a single mitochondrion and the equivalent resistance of the circuit in Fig. 4.1. Given a capacitance of 1 \( \mu \text{F/cm}^2 \) (Gentet et al. 2000) and a surface area of 1.41 \( \mu \text{m}^2 \) (Safiulina et al. 2006), the capacitance of a single mitochondrion can be calculated to be 14.1 \( \times 10^{-9} \) \( \mu \text{F} \). The total resistance of the circuit is:

\[
R_{\text{total}} = \frac{(R_{\text{ETC}})(R_m)(R_{\text{Ca}})}{(R_m)(R_{\text{Ca}}) + (R_{\text{ETC}})(R_{\text{Ca}}) + (R_{\text{ETC}})(R_m)}
\]

where \( R_{\text{ETC}} \) and \( R_m \) are as described in Fig. 4.1, and \( R_{\text{Ca}} \) is the resistance of the Ca\(^{2+}\) uniporter at half maximal conductance. Using 1.6 \( \times 10^{11} \) \( \Omega \), 4.9 \( \times 10^{13} \) \( \Omega \), and 1.2 \( \times 10^{13} \) \( \Omega \) for \( R_{\text{ETC}} \), \( R_m \), \( R_{\text{Ca}} \) respectively (Magnus & Keiser 1997, Nicholls, 1974, and converted from resistance per mg mitochondrial protein to resistance for a single mitochondrion), \( R_{\text{total}} \) is: 1.6 \( \times 10^{11} \) \( \Omega \). With a capacitance of 14.1 \( \times 10^{-9} \) \( \mu \text{F} \), the time constant (RC) is calculated to be \( \sim 2.3 \) ms. This value is short compared to the duration of the stimulus trains used here and can therefore be ignored.
E. Estimates of total matrix Ca and $\Psi_m$ depolarization

By using the circuit model described in Fig. 4.1, an estimate of the magnitude of the inward Ca$^{2+}$ current entering the mitochondrial matrix can be used to calculate the expected increase in total matrix calcium during repetitive stimulation. Finally, using the same circuit model, $\Psi_m$ can be calculated both in the presence and absence of the Ca$^{2+}$ current in order to estimate how much $\Psi_m$ would be expected to depolarize during repetitive stimulation.

Again, using a $R_{Ca}$ of 700 $\Omega$ per mg mitochondrial protein for the Ca$^{2+}$ uniporter (resistance at half maximal conductance, Magnus & Keizer, 1997), a battery electromotive force of 200 mV would drive a current of $\sim$2.86 x $10^{-4}$ amps per mg mitochondrial protein. After about 2 sec this current elevates free matrix [Ca$^{2+}$] to a plateau level (Fig. 3.1). The charge entering in 2 sec, 5.72 x $10^{-4}$ coulombs, corresponds to 2.97 nmol total Ca per mg mitochondrial protein. With a volume of water of 1.0 µl per mg mitochondrial protein (Gunter & Pfeiffer, 1990), this would increase the total matrix calcium concentration by 2.97 mM. However, as explained above, the majority of this Ca$^{2+}$ is buffered upon entry into the matrix. With a bound:free ratio of 3000 (Kaftan et al. 2000), the increase in total calcium would correspond to an increase in matrix [Ca$^{2+}$] from a resting level of $\sim$100 nM to $\sim$0.99 µM, which is close to the 1 to 2 µM stimulation-induced increase in matrix [Ca$^{2+}$] measured in lizard motor nerve terminals (David et al. 2003).
In order to calculate the change in $\Psi_m$, Kirchoff’s and Ohm’s laws can be applied to the circuit model to derive an equation for $\Psi_m$ as a function of the Emf and the different resistances of the circuit (see Fig. 4.1):

$$\Psi_m = \frac{\text{Emf}}{1 + R_{\text{ETC}}(R_m^{-1} + R_{\text{Ca}}^{-1})}$$

With values of 10 and 3000 Ω per mg mitochondrial protein for $R_{\text{ETC}}$ and $R_m$, respectively (Magnus & Keizer, 1997), and in the absence of the Ca$^{2+}$ current at rest (that is, with $R_{\text{Ca}}^{-1}$ approaching zero), $\Psi_m$ equals ~199 mV. During repetitive stimulation (Ca$^{2+}$ current present) and $R_{\text{Ca}}$ equal to 700 Ω per mg mitochondrial protein, $\Psi_m$ equals ~197 mV. Therefore, mitochondrial Ca$^{2+}$ uptake during repetitive stimulation would be expected to depolarize mitochondria by ~2 mV.

How does this value compare to depolarizations measured in near-physiological conditions in this study? Huang et al. (2007) have modeled a relation between Rh123 fluorescence (in quenching concentrations) and $\Psi_m$. For $\Psi_m$ values of -60 to -200 mV, the relationship between $\Psi_m$ and changes in rhodamine fluorescence is close to linear (their Fig. 3A). In their curves, depolarizations from -200 mV to zero increased Rh123 fluorescence 1.4 to 2.8-fold. In close agreement with this range, addition of 2 µM CCCP increased F/F$_{\text{rest}}$ 2.3-fold in Fig. 3.1A. By using their Rh123 intensity-$\Psi_m$ relation, the depolarization corresponding to a 1.024-fold increase in fluorescence, averaged from the three stimulation-induced $\Psi_m$ depolarizations in Fig. 3.1A, had a magnitude of 3 to 5 mV. This is in close agreement with the value calculated based on Ca$^{2+}$ entry (~2 mV, see above).
The depolarization caused by Ca\textsuperscript{2+} influx is masked by the inward movement of negative charges, or outward movement of positive charges. One candidate for a negative charge moving into the matrix during Ca\textsuperscript{2+} uptake is phosphate. However, both mechanisms for phosphate entry into mitochondria (co-transport with protons or exchange with dicarboxylates such as malate or succinate) are electroneutral (reviewed in Ferreira & Pedersen, 1993). As for the possibility of positive charge moving out of the matrix, the hypothesis outlined in Talbot et al. (2007) posits that mitochondria are able to keep $\Psi_m$ depolarizations to a minimum because as Ca\textsuperscript{2+} enters the matrix, protons are pumped across the inner membrane by increased activity of the ETC (also observed in Duchen, 1992; Mironov & Richter, 2001; Schuchmann et al. 2000; Kann et al. 2003; Hayakawa et al, 2005; Warashina 2006). This idea is supported by the finding that partial inhibition of ETC complex I by rotenone (Fig. 3.4) led to an increase in stimulation-evoked $\Psi_m$ depolarizations. That is, a decrease in proton extrusion (which in the equivalent circuit would be simulated by an increase in $R_{ETC}$) resulted in a greater $\Psi_m$ depolarization during stimulation-induced Ca\textsuperscript{2+} entry.

Any Ca\textsuperscript{2+} entry into the matrix should result in some depolarization of $\Psi_m$. As physiological conditions are approached, however, the increase in ETC activity is rapid enough to maintain $\Psi_m$ depolarizations small and/or short-lived. Therefore, it is possible that the inability to detect depolarizations in some experiments in near physiological conditions (Fig. 3.1) was due to an insufficient signal-to-noise ratio and not to the complete absence of any $\Psi_m$ depolarization. Also, elevations in matrix [Ca\textsuperscript{2+}] increase the activities of pyruvate dehydrogenase, isocitrate dehydrogenase, and $\alpha$-ketoglutarate dehydrogenase of the citric acid cycle (reviewed in McCormack & Denton, 1990). This
results in a greater availability of reducing equivalents (e.g. NADH) for consumption by the ETC, which would increase ETC activity and possibly hyperpolarize $\Psi_m$. However, in lizard motor terminal mitochondria, an increase in the activities of the citric acid cycle dehydrogenases did not occur, since a decrease in NADH was observed with stimulation (Talbot, et al. 2007).

In a previous study using isolated rat brain mitochondria, $\Psi_m$ depolarizations due to Ca$^{2+}$ loads were often large and irreversible (Vergun & Reynolds, 2005). However, most studies in isolated mitochondria have added Ca$^{2+}$ in relatively large boluses. In contrast, Chalmers & Nicholls (2003) added Ca$^{2+}$ to isolated mitochondria in the form of multiple, small boluses during which no depolarizations were detected. It was only after several minutes that a large, irreversible $\Psi_m$ depolarization was measured. The protocol used in their study is analogous to the one used here in that both consisted of the gradual presentation of small amounts of Ca$^{2+}$ to mitochondria (in isolation or in intact tissue, respectively), as occurs with AP trains arriving at motor nerve terminals. The large $\Psi_m$ depolarization due to Ca$^{2+}$ overload observed by Chalmers & Nicholls (due presumably to opening of the MPTP) did not occur here. During repetitive stimulation, the driving force for Ca$^{2+}$ entry remains high due to (1) increased ETC activity, which maintains the electrical gradient for Ca$^{2+}$ entry, and due to (2) the powerful Ca$^{2+}$ buffering mechanisms in the mitochondrial matrix (including the precipitation of a Ca-P complex acting as a Ca$^{2+}$ sink) which maintains the chemical gradient for Ca$^{2+}$ entry.
F. Colder temperatures may reduce the mitochondrial ability to sequester Ca$^{2+}$ loads

In previous studies with mouse neuromuscular preparations, repetitive stimulation of motor terminal mitochondria at lower temperatures (~20 °C) resulted in higher levels of cytosolic [Ca$^{2+}$] (David & Barrett 2000), and lower levels of mitochondrial [Ca$^{2+}$] (Vila et al. 2003) than those measured at ~30 °C. Here, repetitive stimulation at lower temperatures resulted in higher amplitude $\Psi_m$ depolarizations (Fig. 3.3A). One mechanism that can explain all three observations is that at lower temperatures the ETC’s ability to maintain $\Psi_m$ is compromised, and that mitochondria are consequently less efficient buffers of increases in cytosolic [Ca$^{2+}$]. A decrease in oxidative phosphorylation (Smith, 1973; Dufour et al, 1996) and a decrease in the activities of specific ETC components (Lenaz et al, 1972; Paradies et al. 1994) have indeed been measured in isolated mitochondria at lower temperatures. This would explain the larger $\Psi_m$ depolarizations observed. Larger $\Psi_m$ depolarizations would in turn decrease the driving force for mitochondrial Ca$^{2+}$ uptake, resulting in lower levels of matrix [Ca$^{2+}$] and in higher levels of cytosolic [Ca$^{2+}$].

However, changes in temperature affect many processes. It is possible that the increase in cytosolic [Ca$^{2+}$] at lower temperatures is due to increased Ca$^{2+}$ transport across the cell membrane into the terminal due to increased AP duration as well as amplitude, and/or due to lower rates of Ca$^{2+}$ extrusion (Helmchen et al. 1997). On the other hand, low temperatures can slow the activation kinetics and decrease the open probability of ion channels (Nobile et al. 1990), which would lead to less Ca$^{2+}$ entering
the cytosol during repetitive stimulation resulting in lower cytosolic \([\text{Ca}^{2+}]\) levels. Also, the lower levels of matrix \([\text{Ca}^{2+}]\) observed at lower temperatures do not necessarily reflect decreased mitochondrial \(\text{Ca}^{2+}\) uptake, since they may not reflect changes in the rate of mitochondrial \(\text{Ca}^{2+}\) uptake, or the amount of total \(\text{Ca}^{2+}\) taken up.

The multiple effects of changes in temperature also make the larger \(\Psi_m\) depolarizations seen here with lower temperatures difficult to interpret. For example, the proton leak current has been shown to decrease at low temperatures (Canton et al. 1995), and this phenomenon (corresponding to an increase in resistance \((R_m)\) across the inner membrane) could alone explain the larger depolarizations observed with \(\text{Ca}^{2+}\) entry. Also, slowed kinetics of the \(\text{Ca}^{2+}\) uniporter/channel might account for a decrease in total \(\text{Ca}^{2+}\) uptake. On the other hand, lower temperatures could decrease the solubility product of the Ca-P complex. A lower solubility product would result in lower levels of matrix \([\text{Ca}^{2+}]\).

G. Metabolic impairment diminishes mitochondrial ability to sequester cytosolic \(\text{Ca}^{2+}\)

As explained in the introduction, mitochondria have been implicated in the pathogenesis of fALS. A number of studies have detected abnormalities in ETC function in both animal models and human tissue. A study which transferred mitochondrial DNA from tissues of ALS patients into mitochondrial DNA-depleted human neuroblastoma cells measured a decrease in ETC function in these cybrid cells when compared with controls (Swerdlow et al. 1998). Other studies using the mutant hSOD1 mouse models
have also measured decreased activity of ETC components when compared to WTs (Menzies et al. 2002; Wiedeman et al. 2002; Jung & Higgins 2002; Kirkinezos et al. 2005). Son et al. (2008) found impairment of complex IV function in hSOD1-G93A mice overexpressing CCS protein, a copper chaperone for SOD1 which appears to accumulate mutant hSOD1 proteins in the mitochondrial matrix. However, some studies that measured activity of ETC components in mitochondria isolated from post mortem brains of fALS patients reported a marked increase in complex I and III activities (Bowling et al. 1993; Browne et al. 1998).

At near physiological temperatures (~30 °C), in pre-symptomatic SOD1-G85R mice, significantly larger stimulation-induced $\Psi_m$ depolarizations were detected compared to WT mice (Fig. 3.5B). In terminals from pre-symptomatic SOD1-G93A mice, the average 100 Hz stimulation-induced $\Psi_m$ depolarizations at ~30 °C were higher than those measured in WTs in similar conditions, but the difference did not reach statistical significance (unlike the results in G85R mice). (The maximum % increase in Rh123 fluorescence was 2.4 ± 1.8 in the SOD1-G93As (ages 43-85 days, 5 terminals from 3 mice) versus 1.2 ± 1.1 in WTs (ages 50-135 days, 18 terminals from 14 mice)). Interestingly, in a 141 day old male SOD1-G93A mice with signs of disease I observed $\Psi_m$ depolarizations of over 200% increase in Rh123 fluorescence in response to 50 Hz stimulation, greater than any $\Psi_m$ depolarization measured in pre-symptomatic SOD1 G85R mice (Fig. 4.2). We hypothesize that stimulation-induced $\Psi_m$ depolarizations in near physiological conditions become progressively larger as mutant SOD mice age and demonstrate signs of disease.
Figure 4.2 Large stimulation-induced $\Psi_m$ depolarization in an older mutant SOD1 mouse. A train of 2000 stimuli at 50 Hz was delivered to the levator auris muscle of a 141 day SOD1-G93A male mouse in which hind limb weakness and muscle atrophy were evident. Temperature was $\sim$28 °C.

In isolated brain and spinal cord mitochondria from SOD1-G93A mice, Damiano et al. (2006) found a decreased capacity to take up sequential Ca$^{2+}$ loads when compared to age-matched non-transgenic littermates. This difference became significant at age 65 days, before any signs of disease had developed in the SOD1-G93A mice. The same study reported little or no differences in oxidative phosphorylation capacity in mitochondria from SOD1-G93A or SOD1-G85R mice when compared to WTs, although oxidative phosphorylation capacity was measured without the stress of repeated Ca$^{2+}$ loading. It is probable that in pre-symptomatic SOD1-G93A and SOD1-G85R mice, as in WTs in which complex I activity is partially inhibited, ETC activity is not diminished
at rest, but becomes affected only when challenged with the entry of a large \( \text{Ca}^{2+} \) current into the matrix or with an increased demand for ATP (see Talbot et al. 2007, Fig. 8). If this were true, it would explain the lack of a decrease in oxidative phosphorylation capacity observed in the Damiano study.

It therefore seems plausible that dysfunction of ETC components may be a factor in the pre-symptomatic pathogenesis of ALS. This could affect the capacity for mitochondria to act as buffers of increases in cytosolic \([\text{Ca}^{2+}]\) during repetitive stimulation, and could result in both acute and chronic elevations of cytosolic \([\text{Ca}^{2+}]\). Such deregulation of cytosolic \([\text{Ca}^{2+}]\) might contribute to motor terminal degeneration and/or motoneuronal death.

**H. Future directions**

In this dissertation, measurements of changes in cytosolic \([\text{Ca}^{2+}]\), mitochondrial \([\text{Ca}^{2+}]\), EPP amplitudes, and \( \Psi_m \), at motor nerve terminals during repetitive activity were obtained. Of these different types, measurements of NT release might be highly sensitive to the changes that may be occurring in the early pathogenesis of ALS (small differences in cytosolic \([\text{Ca}^{2+}]\) have a profound effect on NT release). Subtle changes in NT release from motor terminals in mutant hSOD1 mice may, over time, result in dysfunction. Changes in EPP/EPPo during and after stimulus trains in young, pre-symptomatic SOD1-G85R and SOD1-G93A, and in WT mice could also be obtained under different stresses (e.g. oxygen and/or glucose deprivation), in the presence of agents known to decrease ETC activity (inhibitors of the different complexes) or in the presence of increased loads
of oxygen radicals (e.g. in the presence of high \([H_2O_2]\)). The results of related experiments in which the morphological effects of ischemia/reperfusion injury on motoneurite terminals were measured in SOD1-G93A versus WT mice have already been published (more denervation was observed in the SOD1-G93A mice, David et al. 2007). If differences in NT release were to be found, more experiments measuring changes in \(\Psi_m\), as well as in cytosolic and mitochondrial \([Ca^{2+}]\), would probably be needed. The results could help illuminate the nature of the dysfunction, and might pinpoint the specific dysfunction to mitochondria.


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