Published in final edited form as:

Brain Res. 2014 November 17; 1589: 112–125. doi:10.1016/j.brainres.2014.09.040.

Divergent Effects of Painful Nerve Injury on Mitochondrial Ca^{2+} Buffering in Axotomized and Adjacent Sensory Neurons

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Abstract

Mitochondria critically regulate cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$), but the effects of sensory neuron injury have not been examined. Using FCCP (1 μM) to eliminate mitochondrial Ca^{2+} uptake combined with oligomycin (10 μM) to prevent ATP depletion, we first identified features of depolarization-induced neuronal $[\text{Ca}^{2+}]_c$ transients that are sensitive to blockade of mitochondrial Ca^{2+} buffering in order to assess mitochondrial contributions to $[\text{Ca}^{2+}]_c$ regulation. This established the loss of a shoulder during the recovery of the depolarization (K^+)-induced transient, increased transient peak and area, and elevated shoulder level as evidence of diminished mitochondrial Ca^{2+} buffering. We then examined transients in Control neurons and neurons from the 4th lumbar (L4) and L5 dorsal root ganglia after L5 spinal nerve ligation (SNL). The SNL L4 neurons showed decreased transient peak and area compared to control neurons, while the SNL L5 neurons showed increased shoulder level. Additionally, SNL L4 neurons developed shoulders following transients with lower peaks than Control neurons. Application of FCCP plus oligomycin elevated resting $[\text{Ca}^{2+}]_c$ in SNL L4 neurons more than in Control neurons. Whereas application of FCCP plus oligomycin 2s after neuronal depolarization initiated mitochondrial Ca^{2+} release in most Control and SNL L4 neurons, this usually failed to release mitochondrial Ca^{2+} from SNL L5 neurons. For comparable cytoplasmic Ca^{2+} loads, the releasable mitochondrial Ca^{2+} in SNL L5 neurons was less than Control while it was increased in SNL L4 neurons. These findings show

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Conflict of Interest

The authors have no conflicts of interest relevant to the work in this report.

Authors' Contributions

Participated in research design: Hogan, Bienengraeber, Pan, Wu.

Conducted experiments: Sprick, Guo, Mueller, Wu.

Performed data analysis: Hogan, Wu.

Wrote or contributed to the writing of the manuscript: Hogan, Wu.

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diminished mitochondrial Ca^{2+} buffering in axotomized SNL L5 neurons but enhanced Ca^{2+} buffering by neurons in adjacent SNL L4 neurons.

Keywords

Neuropathic pain; mitochondrial Ca^{2+} ; spinal nerve ligation; sensory neuron function

1. Introduction

Pain following peripheral nerve injury is in part the result of elevated sensory neuron excitability due to disordered Ca^{2+} signaling. Specifically, axonal trauma is followed by diminished Ca^{2+} influx through voltage-gated Ca^{2+} channels (Hogan et al., 2000; McCallum et al., 2006), in part attributable to reduced activation of Ca^{2+} -calmodulin-dependent protein kinase II (Kawano et al., 2009; Kojundzic et al., 2010; Tang et al., 2012). The cytoplasmic Ca^{2+} signal initiated by Ca^{2+} influx is further shaped in sensory neurons by the simultaneous processes of Ca^{2+} extrusion, sequestration, and release from stores. We have identified dysfunction of these processes in axotomized neurons following painful nerve injury, including elevated function of the plasma membrane Ca^{2+} -ATPase (PMCA) (Gemes et al., 2012b), accompanied by decreased resting cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_c$) (Fuchs et al., 2005), reduced function of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) (Duncan et al., 2013) that reduces ER Ca^{2+} stores (Gemes et al., 2009; Rigaud et al., 2009), with resulting elevation of store-operated Ca^{2+} entry (SOCE) (Gemes et al., 2011) and diminished release of Ca^{2+} from stores upon neuronal activity through the process of Ca^{2+} -induced Ca^{2+} release. Together, these disturbances of Ca^{2+} signaling contribute to elevated generation and transmission of high-frequency trains of action potentials in the injured sensory neurons (Gemes et al., 2009; Gemes et al., 2012a; Hogan et al., 2008; Lirk et al., 2008; Sapunar et al., 2005; Tang et al., 2012).

Mitochondria are also recognized as a critical element in intracellular Ca^{2+} management (Nicholls, 2005). In sensory neurons, mitochondria serve as a nonsaturable buffer with a clearance rate in neurons that exceeds the PMCA and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Herrington et al., 1996). Mitochondrial sequestering of Ca^{2+} during periods of high $[\text{Ca}^{2+}]_c$ after neuronal activation buffers peak $[\text{Ca}^{2+}]_c$, while the subsequent slow release of Ca^{2+} as the $[\text{Ca}^{2+}]_c$ falls prolongs $[\text{Ca}^{2+}]_c$ recovery and produces a characteristic shoulder in the Ca^{2+} transient (Werth and Thayer, 1994). Thus, mitochondrial Ca^{2+} buffering modulates the duration and peak levels of activity-induced Ca^{2+} transients, and protects the neuron from excessive elevations of $[\text{Ca}^{2+}]_c$. More recent findings indicate constitutive operation of mitochondrial Ca^{2+} cycling even at low levels of $[\text{Ca}^{2+}]_c$ in resting sensory neurons (Colegrove et al., 2000; Kang et al., 2008). The ability of mitochondria to sequester Ca^{2+} is tied to their energy state, since Ca^{2+} influx through the mitochondrial Ca^{2+} uniporter is driven by the potential difference across the inner mitochondrial membrane (Ψ_m). In a reciprocal fashion, mitochondrial energy production in sensory neurons is upregulated by Ca^{2+} loading (Duchen, 1999), which provides a link between cell activity and energy production through elevations of $[\text{Ca}^{2+}]_c$.

There has been minimal exploration of mitochondrial function in sensory neurons subjected to models of painful traumatic neuropathy. We have previously noted a decrease in the incidence of a shoulder (also called a plateau) in the depolarization-induced transient trace of axotomized fifth lumbar (L5) neurons in the spinal nerve ligation (SNL) model of peripheral nerve injury (Fuchs et al., 2005), which suggests a deficit in mitochondrial function after injury. Furthermore, there is a growing recognition of a contributing role of mitochondrial dysfunction in painful neuropathy (Chu et al., 2011; Flatters and Bennett, 2006; Joseph and Levine, 2006). Accordingly, we hypothesized that nerve injury disrupts Ca^{2+} buffering by mitochondria in axotomized sensory neurons. Ideally, mitochondrial buffering of activity-induced Ca^{2+} influx would be gauged by isolating this process through blocking SERCA and PMCA, but this leads to inability of the neuron to maintain stable $[\text{Ca}^{2+}]_c$ under resting conditions (Duncan et al., 2013). We therefore adopted the strategy of first identifying measurable features of mitochondrial buffering revealed by their sensitivity to ψ_m elimination with FCCP. These features were then compared in injured and control neurons, including the L4 population of neurons that are intact after SNL but are exposed to inflammation that accompanies Wallerian degeneration of the degenerating L5 axonal fragments. Overall, our findings confirm a fundamental dependence of sensory neuron Ca^{2+} homeostasis on mitochondrial activity. Furthermore, we have identified extensive disruption of this role in divergent patterns for both axotomized (L5) and intact (L4) neuronal populations after SNL, such that the axotomized L5 neurons exhibit features suggesting reduced mitochondrial Ca^{2+} buffering while the L4 population shows amplified mitochondrial buffering activity.

2. Results

2.1 FCCP plus oligomycin for blockade of mitochondrial Ca^{2+} buffering

As an overall strategy for evaluating injury effects on mitochondrial Ca^{2+} buffering, we first sought to characterize which quantifiable features of the depolarization-induced Ca^{2+} transient (Fig. 1A and B) reliably reflect mitochondrial Ca^{2+} uptake and release. This requires selective blockade of mitochondrial Ca^{2+} buffering, for which we chose the protonophore FCCP (1 μM) since this prevents Ca^{2+} accumulation by elimination of ψ_m . However, protonophores also deplete ATP through reversal of ATP synthase function (Budd and Nicholls, 1996), which may disrupt ATP-dependent SERCA and PMCA pathways, resulting in nonspecific inhibition of Ca^{2+} sequestration and extrusion. We therefore co-administered the ATP synthase blocker oligomycin (10 μM), which has been shown to prevent ATP depletion in dissociated neurons (Budd and Nicholls, 1996). To further confirm preservation of ATP levels in our dissociated sensory neurons, we assayed the level of ATP available for driving physiological functions by measuring the performance of the ATP-driven Ca^{2+} pump PMCA as an indicator of ATP availability (Schatzmann and Vincenzi, 1969), quantified as the time constant of recovery for transients during SERCA blockade (thapsigargin 1 μM , Fig. 1C) (Gemes et al., 2012b). This was unaffected by this combination of FCCP and oligomycin (FCCP/Oligo, Fig. 1D), which confirms prior findings that FCCP/Oligo does not deplete ATP during the timeframe of our experiments (Budd and Nicholls, 1996). Using TMRM as an indicator of ψ_m , we additionally confirmed the efficacy of

FCCP/Oligo in collapsing ψ_m (Fig. 1E), thereby validating FCCP/Oligo application as a means of blocking mitochondrial Ca^{2+} buffering.

2.2 Effect of mitochondrial blockade on Ca^{2+} transients in Control and SNL neurons

To identify measurable traits of mitochondrial buffering, we compared Ca^{2+} transients in Control neurons before and after elimination of mitochondrial Ca^{2+} uptake through application of FCCP/Oligo (Fig. 2). Two durations of K^+ depolarization were used, 0.5s and 1.3s, in order to encompass a range of Ca^{2+} loads, and findings were compared to Control neurons that received the same depolarization protocol but with application of only vehicle solution during the second pair of depolarizations. A shoulder during the transient recovery was evident in 20 of 41 (49%) baseline transients that followed depolarization by 0.5s K^+ (hereafter referred to as “0.5s transients”), which decreased to 7 of 41 (17%, $P < 0.01$) after FCCP/Oligo. At baseline, 1.3s transients showed a shoulder phase in 39 of 41 neurons (95%), which decreased to 5 of 41 (12%, $P < 0.001$) after FCCP/Oligo. In contrast, vehicle controls showed that all 0.5s transients with shoulders at baseline ($n=3$) retained shoulders on repeat 0.5s transients, as did all but one of 1.3s transients with shoulders ($n=41$). These observations confirm the findings of others (Svichar et al., 1997; Thayer and Miller, 1990; Werth and Thayer, 1994) that blocking mitochondrial Ca^{2+} accumulation with FCCP interferes with the formation of a shoulder, from which we can infer that the shoulder is an indicator of prior neuronal mitochondrial Ca^{2+} buffering.

Mitochondrial regulation of cytoplasmic Ca^{2+} accumulation during activation of Control neurons was tested by determining the fold change of the depolarization-induced peak $[\text{Ca}^{2+}]_c$ caused by FCCP/Oligo application, normalized against the baseline peak (Table 1). Whereas the peak of 0.5s transients was not predictably affected by FCCP/Oligo (i.e. the one-sample Wilcoxon test showed that the fold change was not significantly different than 1), transient peaks during 1.3s transients were more than doubled by FCCP/Oligo, which indicates that mitochondrial buffering regulates the extent of $[\text{Ca}^{2+}]_c$ rise, confirming prior findings in sensory neurons (Gover et al., 2007; Lu et al., 2006; Svichar et al., 1997; Thayer and Miller, 1990). A lack of a similar change in vehicle controls (Table 1) shows that the increased transient peak is not attributable to repetitive stimulation. To identify if injury affects mitochondrial regulation of the 1.3s transient peak, we compared the responses to FCCP/Oligo application of SNL L4 and L5 neurons that were studied concurrently with the Control neurons. This showed a significant main effect of Group, with a pattern of a greater increase for SNL L4 neurons and decrease for SNL L5 neurons, (Table 1), although paired comparisons were not significant.

Transient area (Table 1) was increased upon FCCP/Oligo application at both depolarization durations, but statistical comparison (Mann-Whitney) to the changes induced by vehicle application showed that only 1.3s transients were more affected by FCCP/Oligo than by vehicle alone. The selective effects of FCCP/Oligo further highlight the preferential influence of mitochondrial buffering on large Ca^{2+} loads. Comparing the effect of FCCP/Oligo on 1.3s transient area in SNL L4 and L5 neurons to Control showed that there was no effect of group on the regulation of this parameter by mitochondrial buffering.

Duration of the transient, measured as the time to achieve 80% recovery back to resting level (T80, Table 2), was prolonged in the second depolarizations during FCCP/Oligo for both 0.5s and 1.3s transients, but these prolongations were not different from the effects of vehicle alone. This indicates that T80 does not reflect mitochondrial function, and it was not examined further.

Since the application of FCCP/Oligo did not fully eliminate the shoulder of the depolarization-induced transient in some neurons, we examined whether FCCP/Oligo treatment affected the level of the shoulder, which represents the set-point of mitochondrial Ca^{2+} uptake and release. While the separate 0.5s and 1.3s data for the low number of neurons with shoulders both before and after FCCP/Oligo showed no significant effect (Table 2), combining these (using only a single averaged value for neurons that had shoulders for both 0.5 transients and 1.3 transients) showed a 1.51-fold elevation of the shoulder which was not evident in vehicle controls, which suggests that incomplete blockade of mitochondrial buffering results in an elevation of the shoulder.

Together, these effects of FCCP/Oligo show that mitochondrial function modulates the transient peak, area, and shoulder, and that injury affects the extent of FCCP/Oligo-sensitive mitochondrial activity.

2.3 Effect of neuronal injury on transient features regulated by mitochondrial Ca^{2+} buffering

We next asked which of these features are affected by injury in a fashion indicative of over or underperformance of mitochondrial Ca^{2+} buffering. We first examined the occurrence of a shoulder as an indicator of mitochondrial involvement in handling cytoplasmic Ca^{2+} loads induced by neuronal activity. Since injury may influence Ca^{2+} influx during depolarization of sensory neurons (Hogan et al., 2000; McCallum et al., 2006), we analyzed shoulder occurrence in a manner designed to account for varying Ca^{2+} loads. Specifically, we exposed each neuron to increasing K^+ durations of 0.3s, 0.5s, 0.8s, 1.3s, and 2.5s to test the occurrence of a shoulder across a range of Ca^{2+} loads (Fig. 3A), and identified the lowest transient peak that was adequate to generate a shoulder in each neuron as an indication of the threshold for mitochondrial control of the transient (Fig. 3B). This revealed that SNL L4 neurons developed shoulders associated with lower peak $[\text{Ca}^{2+}]_c$ compared to Control and SNL L5 neurons, which suggests that mitochondrial sensitivity to cytoplasmic Ca^{2+} is enhanced in the SNL L4 population.

We next determined whether injury affects the peak during 1.3s transients, which was shown to reflect mitochondrial function in the FCCP/Oligo experiments above. Neurons from SNL L4 ganglia developed lower transient peaks (Fig. 4A), representing a change opposite that produced by FCCP/Oligo. Since our FCCP/Oligo data also showed that transient area for 1.3s transients is regulated by mitochondrial Ca^{2+} buffering, we compared that measure between injury groups (Fig. 4B), but this was not affected by injury. Finally, comparison of the level of the shoulder between groups showed that 1.3s transients had higher shoulders in the SNL L5 neurons compared to Control and SNL L4 neurons (Fig. 4C). This shows release of Ca^{2+} from mitochondria at a higher $[\text{Ca}^{2+}]_c$ in axotomized L5 neurons than in Control neurons, which duplicates the effect of FCCP/Oligo.

2.4 Mitochondrial regulation of resting $[Ca^{2+}]_c$ and the effect of injury

In the present data, peripheral nerve injury (SNL) depressed the resting $[Ca^{2+}]_c$ in the SNL L5 population (Fig. 5A), as we have noted before (Fuchs et al., 2005). It is unlikely that an injury-induced shift in mitochondrial buffering alone accounts for this effect as we have previously found that injury also alters performance of PMCA and SOCE (Duncan et al., 2013; Gemes et al., 2011), which contribute to setting the resting $[Ca^{2+}]_c$. Therefore, to directly test the role of mitochondrial Ca^{2+} buffering in controlling resting $[Ca^{2+}]_c$, we examined resting $[Ca^{2+}]_c$ before and after FCCP/Oligo administration to resting neurons (Fig. 5B). FCCP/Oligo elevated $[Ca^{2+}]_c$ of Control neurons significantly greater than the effect of vehicle (Fig. 5C), indicating a role of mitochondria in regulating resting $[Ca^{2+}]_c$. Comparison of injury groups (Fig. 5C) showed a heightened effect of FCCP/Oligo on resting $[Ca^{2+}]_c$ in SNL L4 neurons, suggesting enhanced $[Ca^{2+}]_c$ buffering in resting neurons of the SNL L4 population.

2.5 Effect of nerve injury on mitochondrial Ca^{2+} stores

Calcium buffering by mitochondria is most strongly engaged during periods of high $[Ca^{2+}]_c$, such as those that follow intense neuronal activation. To test the effect of injury on mitochondrial Ca^{2+} sequestration under these peak conditions, we applied FCCP/Oligo 2s after the termination of a 5s K^+ application to neurons of the various injury groups (Fig. 6A). To confirm that this triggered release of Ca^{2+} stored in mitochondria and was independent of Ca^{2+} influx into the neuron, we compared the response to FCCP/Oligo in neurons bathed in normal Tyrode's solution (2mM Ca^{2+}) to neurons bathed in Ca^{2+} -free solution, which showed that the rate of measurable Ca^{2+} release (Fig. 6B, left panel) and the amplitude of the Ca^{2+} transient triggered by the release (Fig. 6B, right panel) were the same. Whereas most of the Control and SNL L4 neurons showed measureable FCCP/Oligo-induced Ca^{2+} release, axotomized SNL L5 neurons typically lacked this response (Fig. 6C, left panel). Analysis of the neurons with a measurable release showed that the amplitude of the response in the SNL L5 group was diminished compared to the other groups (Fig. 6C, right panel). However, it is known that axotomy results in reduced Ca^{2+} influx through voltage-gated Ca^{2+} channels in sensory neurons (Hogan et al., 2000; McCallum et al., 2006), which was again demonstrated in the present data by lower depolarization-induced transient peaks (Fig. 6D). Since reduced Ca^{2+} influx may have contributed to reduced mitochondrial Ca^{2+} uptake in SNL L5 neurons, we separately examined neurons with a restricted range of K^+ -transient peaks (200–800nM), but this did not change the reduced incidence or amplitude of FCCP/Oligo Ca^{2+} release (data not shown). We additionally examined the relationship between each neuron's depolarization-induced transient peak, which reflects the Ca^{2+} influx ((Duncan et al., 2013), also Fig. 3C) and the amount of releasable Ca^{2+} in the mitochondrial stores for the entire population of neurons. Comparison of the regression relationship between cytoplasmic Ca^{2+} load (represented by the peak of the K^+ -induced transient) and mitochondrial Ca^{2+} release (represented by the FCCP/Oligo transient amplitude) in the various injury groups (Fig. 6E) revealed that, under comparable loading conditions, mitochondria in SNL L4 neurons accumulate more releasable Ca^{2+} while mitochondria in SNL L5 neurons accumulate less. Together, these findings indicate that mitochondria of axotomized SNL L5 neurons are functionally deficient and often fail to sequester Ca^{2+} ,

while mitochondria of adjacent SNL L4 neurons show hyperactive mitochondrial Ca^{2+} buffering.

3. Discussion

The cytoplasmic Ca^{2+} signal is a fundamental regulator of most neuronal functions. The ability of mitochondria to transiently sequester massive amounts of Ca^{2+} indicates a pivotal involvement in normal neuronal function and in the management of potentially toxic Ca^{2+} loads. Therefore, we sought evidence of a possible role of mitochondria in contributing to disrupted Ca^{2+} signaling of sensory neurons after peripheral nerve injury. We chose to examine mitochondrial function in the setting of depolarization-induced Ca^{2+} loading since Ca^{2+} influx is a critical link between membrane activation and processes that control neuronal excitability (Lirk et al., 2008; Tang et al., 2012) and synaptic transmission (Kann and Kovacs, 2007). Our initial examination with FCCP/Oligo identified mitochondrial participation in generating a shoulder during transient recovery and limiting transient peak and area after entry of an adequate Ca^{2+} load (1.3s depolarization), as well as regulating the shoulder level. Using these markers, heightened mitochondrial Ca^{2+} buffering in L4 neurons after SNL was suggested by several lines of evidence, including development of a shoulder after exposure of the mitochondria to lower cytoplasmic Ca^{2+} concentrations, lower transient peaks, an accentuated effect of FCCP/Oligo (on transient peak, shoulder level, and resting $[\text{Ca}^{2+}]_c$), and greater activity-induced loading of Ca^{2+} into the mitochondria. In contrast, axotomized SNL L5 neurons showed changes that suggest diminished mitochondrial Ca^{2+} buffering, including elevated shoulder level, reduced sensitivity of peak and shoulder level to FCCP/Oligo, and diminished activity-induced mitochondrial Ca^{2+} loading (Fig. 7).

Despite retaining axonal continuity, L4 neurons in the SNL model are exposed to inflammation from Wallerian degeneration of axotomized neurons (Shamash et al., 2002; Uceyler and Sommer, 2006), which results in functional alterations such as elevated TRPV1 expression (Hudson et al., 2001) and increased sensitivity to tumor necrosis factor- α (Schafers et al., 2003). Although our prior investigations of Ca^{2+} signaling in the SNL model have showed that injury effects are preferentially expressed in the axotomized SNL L5 population (e.g. depressed resting $[\text{Ca}^{2+}]_c$ (Fuchs et al., 2005), loss of ER Ca^{2+} stores (Rigaud et al., 2009), elevated SOCE (Gemes et al., 2011), increased PMCA (Gemes et al., 2012b), and depressed SERCA function (Duncan et al., 2013)), our present study offers evidence that injury also alters Ca^{2+} handling in the intact L4 population, in this case via amplified mitochondrial buffering. The underlying mechanism for this functional shift could involve greater Ca^{2+} affinity of the Ca^{2+} uniporter, elevated electrochemical drive for Ca^{2+} uptake through the mitochondrial inner membrane, or a higher buffering capacity within the mitochondrial matrix. Even the resting $[\text{Ca}^{2+}]_c$ of SNL L4 neurons was found to be more sensitive to FCCP/Oligo than Control or SNL L5 neurons. This finding might be considered unexpected since mitochondrial Ca^{2+} buffering has often been viewed as operating only at high $[\text{Ca}^{2+}]_c$, but recent reports demonstrate constitutive Ca^{2+} cycling at low $[\text{Ca}^{2+}]_c$ in resting neurons (Colegrove et al., 2000; Kang et al., 2008).

In contrast to L4 neurons, L5 neurons after SNL are subject to axonal transection and loss of target-derived neurotrophins. In parallel with differing pathophysiology, L5 neurons showed effects on mitochondrial Ca^{2+} buffering that were distinct from the L4 population. Specifically, L5 neurons lacked the features indicative of accelerated Ca^{2+} buffering acquired by L4 neurons after SNL, but instead developed a deficient ability to accumulate Ca^{2+} , which was evident as reduced or absent releasable mitochondrial Ca^{2+} despite large cytoplasmic Ca^{2+} loads. This is possibly due to reduced Ca^{2+} uniporter function, inadequate Ψ_m due to uncoupling or defective electron transport chain, a loss of Ca^{2+} buffering capacity in the mitochondrial matrix, or a more general structural defect. Additionally, mitochondria of SNL L5 neurons show early Ca^{2+} release during the transient recovery phase and have a higher shoulder level as a result. This indicates a shifted balance of mitochondrial Ca^{2+} uptake and release, and implicates disordered control of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger release pathway. Since this process is coupled to Na^+/H^+ exchange across the inner mitochondrial membrane, diminished respiratory chain function and the resulting deficit in H^+ gradient and Ψ_m would be expected to delay release of intra-mitochondrial Ca^{2+} during the shoulder phase in injured neurons (Montero et al., 2001) in contrast to our findings. Replication of this finding with FCCP/Oligo application nonetheless suggests loss of Ψ_m as a contributing factor. Additionally, the mitochondrial Ca^{2+} release mechanism is regulated by $[\text{Ca}^{2+}]_c$ (Bathori et al., 2006; Montero et al., 2001), so a shift in this sensitivity could cause a higher shoulder after injury.

Upstream triggers for mitochondrial dysfunction after axotomy are undefined. However, several possibilities can be considered on the basis of recent observations on the sensory neuronal response to injury. It is increasingly recognized that mitochondria reside at the hub of a complex system of reciprocal interactions that requires close structural proximity (Szabadkai and Duchen, 2008) near regions of high local $[\text{Ca}^{2+}]_c$ such as plasmalemmal Ca^{2+} channels (Pivovarov et al., 1999) and ER Ca^{2+} release channels (Hayashi et al., 2009). This allows mitochondrial participation in Ca^{2+} buffering despite relatively low Ca^{2+} affinity of the uniporter intake path. We have observed extensively disordered ER ultrastructure after axotomy (Gemes et al., 2009), so a possible consequence is the loss of the particular physical interactions that underlie mitochondrial colocalization and the coordinated physiological function of these ultrastructural elements. In support of this, we have noted a decrease in ER profile lengths in SNL L5 neurons, but an increase in the L4 population (Gemes et al., 2009). Close mitochondrial association with ER provides for bidirectional exchange of Ca^{2+} . On the one hand, since mitochondria provide an important source of Ca^{2+} for refilling ER Ca^{2+} stores (Arnaudeau et al., 2001; Malli et al., 2005), deficient mitochondrial Ca^{2+} uptake in axotomized neurons may in part explain our prior finding of depleted ER Ca^{2+} stores (Rigaud et al., 2009). On the other hand, mitochondria also receive Ca^{2+} from ER (Montero et al., 2001). Structural association of ER and mitochondria fails when $[\text{Ca}^{2+}]_c$ is low (Wang et al., 2000), so the reduced resting $[\text{Ca}^{2+}]_c$ in axotomized sensory neurons may interrupt subcellular interactions of mitochondrial with ER and contribute to loss of mitochondrial Ca^{2+} buffering. However, evidence that Ca^{2+} released from ER is not buffered by mitochondria in sensory neurons (Scheff et al., 2013; Svichar et al., 1997) raises a question whether mitochondria receive Ca^{2+} from closely associated ER in the same fashion as adrenal chromaffin cells (Montero et al., 2001).

The functional implications of disordered mitochondrial Ca^{2+} handling could be extensive. Mitochondrial energy production is sensitive to matrix Ca^{2+} levels via regulation of rate limiting enzymes of the citric acid cycle, so neuronal ATP/ADP ratios may be affected. Inability of mitochondria to prevent excessive $[\text{Ca}^{2+}]_c$ could contribute to excitotoxicity and neuronal apoptosis after injury. Mitochondrial Ca^{2+} release in presynaptic terminals regulates synaptic function, although the evidence from various tissues is divergent, showing both mitochondrial support for presynaptic $[\text{Ca}^{2+}]_c$ (Tang et al., 2012) or suppression of presynaptic $[\text{Ca}^{2+}]_c$ (Shutov et al., 2013; Wan et al., 2012). The role of mitochondria in dorsal horn neurotransmission of nociceptive traffic will be an important area of investigation. Depolarized Ψ_m develops in sensory neurons of a rat model of diabetes (Huang et al., 2003) and in sensory neurons exposed to cisplatin or paclitaxel (Melli et al., 2008), all of which produce clinical neuropathic pain. Combined with our findings, these reports support our suspicion of a mitochondrial role in pain pathophysiology. Since both axotomized neurons and adjacent inflamed neurons may contribute to generating pain after nerve injury (Gold, 2000), we cannot speculate about the relative importance of the changes we have found in L4 and L5 neurons after SNL, although a disease phenotype due to excessive mitochondrial Ca^{2+} buffering has not been described before now.

Several important limitations of our data should be mentioned. Mitochondrial Ca^{2+} cycling is temperature sensitive. Whereas our recordings were performed at 25°C , greater buffering may be expected at higher temperatures (David and Barrett, 2000; Kang et al., 2008). It is unknown whether this would alter our intergroup comparisons. Additionally, while our recording of Ca^{2+} provides a view of averaged cytoplasmic Ca^{2+} levels, it nonetheless neglects important microdomains where mitochondrial effects may differ. Also, we examined only small to medium sized sensory neurons. However, the observations by Lu *et al.* (Lu et al., 2006) that elimination of mitochondrial Ca^{2+} buffering affected all size groups of sensory neurons and effects were least in small sensory neurons suggest that our findings may be representative of the full population of sensory neurons. Finally, our approach of tracking FCCP/Oligo-sensitive features after SNL suffers from a lack of specificity since we did not evaluate mitochondrial Ca^{2+} buffering in isolation from other Ca^{2+} sequestration and extrusion processes. For instance, compensatory increase of PMCA and SERCA function driven by relatively higher $[\text{Ca}^{2+}]_c$ during mitochondrial blockade may have resulted in underestimating the role of mitochondrial Ca^{2+} buffering in the present experiments. However, functional examination of isolated mitochondria would not reflect their operation in the natural context of the intact neuron, whereas our approach reveals the integrated physiological consequences of altered mitochondrial Ca^{2+} cycling at the cellular level.

We conclude that peripheral nerve trauma substantially affects mitochondrial performance in both directly injured neurons as well as neurons with intact fibers. These populations show a divergent response in which mitochondria in axotomized neurons have a deficient ability to buffer Ca^{2+} following intense neuronal activity, while the adjacent inflamed neurons show enhanced buffering of cytoplasmic Ca^{2+} . Since mitochondrial Ca^{2+} cycling is functionally linked with numerous other organelles and membrane pumps, these shifts in Ca^{2+} management support a view that mitochondrial dysfunction contributes to neuropathic pain.

4. Experimental Procedures

4.1 Animals

All methods and use of animals were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Taconic Farms Inc., Hudson, NY) were housed in pairs in a room maintained at $22 \pm 0.5^\circ\text{C}$ and constant humidity ($60 \pm 15\%$) with an alternating 12hr light-dark cycle. Food and water were freely available throughout the experiments.

4.2 Injury model

Rats weighing 150 to 180g were subjected to SNL modified from the original technique (Kim and Chung, 1992). Specifically, rats were anesthetized with 2% isoflurane in oxygen and the right paravertebral region was exposed. The L6 transverse process was removed, after which the L5 and L6 spinal nerves were ligated with 6-0 silk suture and transected distal to the ligation. To minimize non-neural injury, no muscle was removed, muscles and intertransverse fascia were incised only at the site of the two ligations, and articular processes were not removed. The muscular fascia was closed with 4-0 resorbable polyglactin sutures and the skin closed with staples. Control animals received skin incision and closure only.

4.3 Sensory testing

We measured the incidence of a pattern of hyperalgesic behavior that we have previously documented to be associated with conditioned place avoidance (Hogan et al., 2004; Wu et al., 2010). Briefly, on 3 different days between 10d and 17d after surgery, right plantar skin was touched (10 stimuli/test) with a 22G spinal needle with adequate pressure to indent but not penetrate the skin. Whereas control animals respond with only a brief reflexive withdrawal, rats following SNL may display a complex hyperalgesia response that includes licking, chewing, grooming and sustained elevation of the paw. The average frequency of hyperalgesia responses over the 3 testing days was tabulated for each rat. After SNL, only rats that displayed a hyperalgesia-type response after at least 20% of stimuli were used further in this study.

4.4 Neuron isolation and plating

Neurons were rapidly harvested from L4 and L5 DRGs during isoflurane anesthesia and decapitation 21 to 28 days after SNL or skin sham surgery. This interval was chosen since hyperalgesia is fully developed by this time (Hogan et al., 2004). Ganglia were incubated in 0.5mg/ml Liberase TM (Roche, Indianapolis, IN) in DMEM/F12 with glutaMAX (Life Technologies) for 30min at 37°C , followed with 1 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO) and 150 Kunitz units/ml DNase (Sigma-Aldrich) for another 10min. After addition of 0.1% trypsin inhibitor (Type II, Sigma-Aldrich), tissues were centrifuged, lightly triturated in neural basal media (1X) (Life Technologies) containing 2% (v:v) B27 supplement (50x) (Life Technologies), 0.5mM glutamine (Sigma-Aldrich), 0.05mg/ml gentamicin (Life Technologies) and 10 ng/ml nerve growth factor 7S (Alomone Labs Ltd., Jerusalem, Israel). Cells were then plated onto poly-L-lysine (70–150kDa, Sigma-Aldrich) coated glass cover

slips (Deutsches Spiegelglas, Carolina Biological Supply, Burlington, NC) and incubated at 37°C in humidified 95% air and 5% CO₂ for 2hr and were studied 3–8hr after dissociation.

4.5 Solutions and agents

Unless otherwise specified, the bath contained Tyrode's solution (in mM): NaCl 140, KCl 4, CaCl₂ 2, Glucose 10, MgCl₂ 2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, with an osmolarity of 297–300mOsm and pH 7.40. Fura-2-AM was obtained from Invitrogen (Carlsbad, CA), tetramethylrhodamine methyl ester (TMRM) was from Molecular Probes (Waltham, MA), antimycin, oligomycin, and the SERCA blocker thapsigargin (TG) were from Sigma Aldrich (St. Louis, MO), and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) was from Tocris (Minneapolis, MN). Stock solutions of TG, FCCP, Fura-2-AM, and TMRM were dissolved in DMSO. Subsequently dilution in the relevant bath solution produced final bath concentrations of DMSO of 0.1% for TG-containing solution, 0.002% for TMRM, and 0.01% for FCCP-containing solutions. Oligomycin stock was dissolved in ethanol, and subsequent dilution produced bath concentrations containing 0.05% ethanol, which does not effect [Ca²⁺]_c (data not shown, and (Avery and Johnston, 1997)). The 500μl recording chamber was superfused by gravity-driven flow at a rate of 3ml/min. Agents were delivered by directed microperfusion controlled by a computerized valve system (ALA Scientific Instruments, Farmingdale, NY) through a 500μm diameter hollow quartz fiber 300μm upstream from the neurons. This flow completely displaced the bath solution (demonstrated by imaging dye flow, data not shown) such that neurons in the imaging field were exposed to undiluted microperfusion fluid, and constant flow was maintained through this microperfusion pathway by delivery of bath solution when specific agents were not being administered. Dye imaging shows that solution changes were achieved within 200ms. Neurons were activated by depolarization produced by microperfusion application of 50mM K⁺ for various durations as specified below. Repeat depolarizations were initiated only after 60s had elapsed following recovery of the prior transient.

4.6 Fluorimetric imaging

To measure [Ca²⁺]_c, neurons plated on cover slips were exposed to Fura-2-AM (5μM) for 30min at room temperature in a solution that contained 2% bovine albumin to aid dispersion of the fluorophore, washed 3 times with regular Tyrode's solution, and given 30min for de-esterification. Neurons showing signs of lysis, crenulation or superimposed glial cells under brightfield illumination were excluded. For [Ca²⁺]_c recording, the fluorophore was excited alternately with 340nm and 380nm wavelength illumination (150W Xenon, Lambda DG-4, Sutter, Novato, CA), and images were acquired at 510nm using a cooled 12-bit digital camera (Coolsnap fx, Photometrics, Tucson, AZ) and inverted microscope (Diaphot 200, Nikon Instruments, Melville, NY) through a 20x objective. Recordings from each neuron were obtained as separate regions (MetaFluor, Molecular Devices, Downingtown, PA) at a rate of 1Hz. After background subtraction, the fluorescence ratio R for individual neurons was determined as the intensity of emission during 340nm excitation (I₃₄₀) divided by I₃₈₀, on a pixel-by-pixel basis. The Ca²⁺ concentration was then estimated by the formula $K_d \cdot \beta \cdot (R - R_{\min}) / (R_{\max} - R)$ where $\beta = (I_{380\max}) / (I_{380\min})$. Values of R_{min}, R_{max} and β

were determined by in-situ calibration (Fuchs et al., 2005; Gemes et al., 2011) and were 0.85, 11.10 and 5.28, and K_d was 224nM (Grynkiewicz et al., 1985).

Data were derived only from neurons with stable resting R traces, which were analyzed using Axograph X 1.1 (Axograph Scientific, Sydney, Australia). Transient amplitude was determined as the difference between the peak $[Ca^{2+}]_c$ and the resting level just before depolarization (Fig. 1A). To characterize transient duration, it is conventional to choose a level, such as 20% of the transient amplitude above resting $[Ca^{2+}]_c$ (i.e. 80% recovery, T80), at which to measure the duration. However, mitochondrial blockade may have divergent influences on transient duration when measured below *versus* above the level of the plateau (e.g. eliminating the plateau but prolonging the phase above the plateau), which combined with variance in the level of the plateau makes this type of measurement highly arbitrary. We therefore additionally evaluated the area of the transient, which incorporates both amplitude and duration without the need to record duration at a specific level. Presence of a shoulder was determined by differentiation of the trace of $[Ca^{2+}]_c$ vs. time. A shoulder was considered present when the differentiated trace showed two negative maxima (Fig. 1B). When the transient had a shoulder, its level was measured at the time specified by the inflection point, i.e. when the slope of the $[Ca^{2+}]_c$ vs. time trace during the recovery phase of the transient shifted from becoming less negative to becoming more negative (Fig. 1B). Recovery of transients that lacked a shoulder was fit by a mono-exponential curve from which the time constant (τ) was derived as a measure of the pace of recovery.

To indicate trends in ψ_m , TMRM was used in a non-quenched mode, in which falling ψ_m is accompanied by loss of mitochondrial fluorophore to the cytoplasm and hence out of the neuron, resulting in diminished total neuron fluorescence (Ward, 2010). The fluorophore was applied in a similar fashion as FURA-2 but at a concentration of 20nM (i.e. a concentration too low to permit auto-quenching within the mitochondria (Ward, 2010)), and loading the dye was performed at 37°C for 60min. TMRM was present in the bath solution during imaging at 25°C, using 550nm wavelength excitation and image acquisition at 570nm.

4.7 Statistical analysis

Sensory neuron somatic diameter is broadly associated with specific sensory modalities (Ma et al., 2003). In this study, we examined small to medium sized neurons (diameter $\geq 34\mu m$), which represent predominantly nociceptive neurons, and are the population for which Ca^{2+} signaling is preferentially affected by injury (Duncan et al., 2013; Rigaud et al., 2009). Neurons were grouped separately as Control neurons from sham-operated animals, and axotomized L5 neurons and neighboring L4 neurons from SNL animals.

Statistical analysis was performed in collaboration with the Medical College of Wisconsin Statistical Consultation Service. Prism (version 4.1, GraphPad Software, Inc., San Diego, CA) and Statistica (StatSoft Inc, Tulsa, OK) were used to perform statistical evaluations. Incidence of features was evaluated by Fisher's exact test for two groups or Chi-square for 3 groups. When a significant influence of group was shown by Chi-square, paired comparisons were performed by Fischer's exact test and corrected by the Bonferroni method. Influence of blockade of mitochondrial Ca^{2+} buffering upon Ca^{2+} transient

parameters was evaluated by nonparametric analysis since the data distribution was frequently non-Gaussian. Either one-sample Wilcoxon test for Control neurons alone, Mann-Whitney for comparing two groups, or Kruskal-Wallis test followed by Dunn's Multiple Comparison Test for comparing the effect of mitochondrial blockade in three groups. A *P* value less than 0.05 was considered significant. Data are reported as Median and interquartile range (IQR) represented as first and third quartiles (as [Q₁, Q₃]), and plots of data show median and Q₁, Q₃ (box plot) with whiskers denoting the lowest datum still within 1.5 IQR of Q₁ and the highest datum still within 1.5 IQR of Q₃ (i.e. Tukey boxplot). Log plots were used for data that was highly skewed toward lower values.

Acknowledgments

We are grateful for the statistical consultation provided by Dr. Aniko Szabo PhD, supported in part by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant Number 8UL1TR000055. This study was funded by NIH grants NS042150 to QH and DA-K01-024751 to H-EW.

Abbreviations

ψ_m	inner mitochondrial membrane potential
[Ca ²⁺] _c	cytoplasmic Ca ²⁺ concentration
DRG	dorsal root ganglion
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
L4	4 th lumbar
PMCA	plasma membrane Ca ²⁺ -ATPase
SERCA	sarcoplasmic/endoplasmic reticulum Ca ²⁺ -ATPase
SOCE	store-operated Ca ²⁺ entry
SNL	spinal nerve ligation
TMRM	tetramethylrhodamine methyl ester

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Highlights

- Mitochondrial Ca^{2+} buffering was examined in injured sensory neurons.
- Mitochondria regulate resting $[\text{Ca}^{2+}]_c$ and K^+ -induced transient shoulder and peak.
- Neurons axotomized show evidence of deficient Ca^{2+} buffering.
- Adjacent neurons exposed to inflammation show enhanced cytoplasmic Ca^{2+} buffering.

Figure 1.

Measures and protocol validation. (A) Measured parameters for K^+ -induced Ca^{2+} transients are shown for resting level, amplitude, peak, shoulder level, and time for 80% recovery to resting level. (B) To determine shoulder level, $[Ca^{2+}]_c$ traces (top panel) were differentiated (bottom panel, with the first positive and first negative maxima clipped), and the presence of two negative maxima (indicated by *) was considered as evidence of a shoulder. The level of the shoulder was measured at the inflection point (dashed arrows), identified as time during the recovery phase at which the slope of the $[Ca^{2+}]_c$ vs. time trace shifted from becoming less negative to becoming more negative. (C) To test adequacy of ATP levels, plasma membrane Ca^{2+} ATPase (PMCA) activity is measured as the time constant (τ) for the exponential recovery of $[Ca^{2+}]_c$ from small Ca^{2+} loads. The top panel is an example of repeated depolarization during application of Tyrode's solution (Tyr) and after vehicle application in Tyrode's. The bottom panel shows response to co-administration of FCCP (1 μ M) and oligomycin (10 μ M). (D) Average data show PMCA activity is unchanged. (E) Application of FCCP successfully reduces mitochondrial Ψ_m recorded by TMRM fluorescence, normalized as percent of baseline.

Figure 2.

Demonstration traces of the response of neuronal $[Ca^{2+}]_c$ to depolarizations induced by K^+ application of 0.5s and 1.3s duration in Tyrode's solution (Tyr) and 1.5 minutes after switching to a bath solution containing either vehicle (**A**) or FCCP/Oligo (**B**). Response to FCCP/Oligo application is shown for neurons from Control dorsal root ganglion and from the fourth lumbar (L4) and L5 ganglia after L5 spinal nerve ligation (SNL). The second depolarization was initiated only after recovery from the preceding depolarization. During FCCP/Oligo application, the amplitude of the 1.3s transient increases by 2.10-fold for Control, 2.25-fold for SNL L4, and 1.53-fold for SNL L5, compared to baseline (Tyr).

Figure 3.

Repeated depolarization protocol and determination of Ca²⁺ load adequate to produce a shoulder. **(A)** Cytoplasmic Ca²⁺ concentration trace (top) and its differentiation trace (bottom) in a control neuron, showing the protocol for sequential depolarizations, and the identification of a shoulder (indicated by dashed arrows) during the recovery phase of transients with larger Ca²⁺ loads. **(B)** Determination for each neuron of the lowest peak associated with the appearance of a shoulder shows that neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4, n=24) developed shoulders associated with lower transient peaks than control (C, n=50) or SNL L5 (n=71) groups. This and other boxplots show median and first and third quartiles (Q₁, Q₃) with whiskers denoting the lowest datum still within 1.5 times the interquartile range of Q₁ and the highest datum still within 1.5 times the interquartile range of Q₃*P < 0.05.

Figure 4.

Comparison of FCCP/Oligo-sensitive dimensions of transients induced by 1.3s K^+ depolarization in Control neurons compared to neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4) and SNL L5. (A) Determination of the transient peak. (B) Determination of the transient area. (C) Determination of the level of the shoulder during transient recovery. C, $n=56$; SNL L4, $n=28$; SNL L5, $n=77$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 5.

Effect of nerve injury on mitochondrial regulation of resting $[Ca^{2+}]_c$. **(A)** Resting $[Ca^{2+}]_c$ in Control neurons (C, $n=279$), neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4, $n=76$), and SNL L5 ($n=113$). **(B)** Sample traces showing the effect of co-administration of FCCP (1 μ M) and oligomycin (10 μ M, FCCP/Oligo) after application of Tyrode's solution (Tyr). **(C)** Summary data comparing the response of resting $[Ca^{2+}]_c$ in neurons from Control animals to vehicle (Veh, $n=63$), and the response to FCCP/Oligo of neurons from Control (C, $n=94$), SNL L4 ($n=38$) and SNL L5 ($n=25$) ganglia. ** $P < 0.01$, *** $P < 0.001$.

Figure 6.

Effect of nerve injury on mitochondrial Ca^{2+} stores following neuronal activation. **(A)** Sample traces from control neurons, neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4), and SNL L5, showing response of $[\text{Ca}^{2+}]_c$ to K^+ depolarization (5s) and to application of FCCP (1 μM) and oligomycin (10 μM , FCCP/Oligo) following bath superfusion of Tyrode's solution. **(B)** The left panel shows the frequency of measurable Ca^{2+} release triggered by application of FCCP/Oligo to uninjured neurons bathed either in normal Tyrode's solution ($n=46$) or to matched neurons in which the bath was switched to Ca^{2+} -free Tyrode's solution 5s before the application of FCCP/Oligo (in Ca^{2+} -free vehicle) ($n=36$). The right panel shows amplitude of the $[\text{Ca}^{2+}]_c$ transient induced by the application of FCCP/Oligo, excluding data from neurons in with no measurable Ca^{2+} release. In both cases, there was no difference between groups normal Ca^{2+} bath ($n=34$) and Ca^{2+} -free conditions ($n=27$). **(C)** The left panel shows summary data for the frequency of measurable Ca^{2+} release triggered by application of FCCP/Oligo to control neurons (C, $n=135$), SNL L4 ($n=52$), and SNL L5 ($n=52$). The right panel shows amplitude of the $[\text{Ca}^{2+}]_c$ transient induced by the application of FCCP/Oligo, excluding data from neurons in with no measurable Ca^{2+} release. Groups include control neurons (C, $n=95$), neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4, $n=45$), and SNL

L5 (n=14). *** $P < 0.001$. **(D)** Peak of the $[Ca^{2+}]_c$ transient induced by application of K^+ (5s). Groups include control neurons (C, n=305), neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4, n=52), and SNL L5 (n=52). *** $P < 0.001$. **(E)** Relationships between the peak of the K^+ -induced transient and the FCCP/Oligo-induced transient for each neuron, separately considered by group. Groups include control neurons (C, n=135), neurons from the fourth lumbar dorsal root ganglion after spinal nerve ligation (SNL L4, n=52), and SNL L5 (n=52). Data points for neurons with no measurable Ca^{2+} release (0 value for Y-axis) are displayed as a group below the plot, separated vertically for clarity. The regression lines represent a linear fit for the log transform of all data in that group, including non-responding neurons, for which the value of 5 (half of the lowest non-zero value in the data set) was substituted for the purpose of log transform and fitting the regression line. Comparison of the regression lines showed that the slope for SNL L4 was greater than for SNL L5 ($P < 0.05$), the Y-intercept for SNL L4 was greater than for Control ($P < 0.01$), and the Y-intercept for Control was greater than for SNL L5 ($P < 0.001$).

Figure 7.

A summary of possible underlying functional changes that could explain nerve injury effects on mitochondrial function. Cycling of Ca^{2+} through the mitochondria is illustrated as uptake by the Ca^{2+} uniporter and release by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Axotomized neurons accumulate less Ca^{2+} in the mitochondrial matrix and retain Ca^{2+} less well. In contrast, the adjacent neurons that are intact but share peripheral nerve fascicles with degenerating segments of the axotomized neurons develop exaggerated Ca^{2+} accumulation without evidence of an altered release process.

Table 1

Fold change in Peak and Area induced by FCCP/Oligo

Depolarization	Peak						Area					
	0.5s			1.3s			0.5s			1.3s		
	Vehicle	FCCP/Oligo	Vehicle	C	L4	L5	Vehicle	FCCP/Oligo	Vehicle	C	L4	L5
Group	C	C	C	41	33	26	61	44	66	44	38	28
n	66	41	70									
Median	1.25	1.10	1.05	2.04	2.47	1.76	1.84	2.38	1.30	2.92	4.30	2.71
IQR	1.08–1.52	0.59–1.61	0.93–1.14	1.56–2.84	1.69–4.20	1.24–3.10	1.18–2.72	1.07–3.98	0.99–1.60	1.86–5.56	2.55–6.26	1.70–5.70
1-sample Wilcoxon	<0.001	0.692	0.07	<0.001			<0.001	<0.001	<0.001	<0.001		
Vehicle vs. FCCP/Oligo								0.202				
Group Main Effect					0.046						0.273	
Paired Comparisons												

Measured parameters include the peak and area of the transients produced by depolarization with K^+ . Depolarizations were of either 0.5s or 1.3s duration. Agents included either vehicle or FCCP μM combined with oligomycin $10\mu M$ (FCCP/Oligo). Groups included Control (C), and neurons from the 4th lumbar (L4) and L5 dorsal root ganglia after spinal nerve ligation. n is the number of neurons. The median and interquartile range (IQR) are shown for the fold change of the measured parameter produced by FCCP/Oligo application, calculated as the ratio of the second depolarization (following FCCP/Oligo) divided by the first depolarization (baseline) for each neuron. 1-sample Wilcoxon is the *P* of an effect of vehicle or FCCP/Oligo in that group using a one-sample Wilcoxon test (compared to a value of 1). Vehicle vs. FCCP/Oligo is the *P* for the comparison of vehicle vs. FCCP/Oligo in control neurons using Mann-Whitney test. Group Main Effect *P* is the probability of a main effect comparing the three groups (C, L4, L5) during FCCP/Oligo application, using Kruskal-Wallis. Paired Comparisons gives the *P* for significant paired comparisons between these groups (blank when none) using Dunn's test.

* *P* < 0.05.
 * *P* < 0.05,
 ** *P* < 0.01.

Table 2

Fold change in T80 and τ induced by FCCP/Oligo

Depolarization Agent	T80				Shoulder Level					
	0.5s		1.3s		0.5s		1.3s		Combined	
	Vehicle	FCCP/Oligo	Vehicle	FCCP/Oligo	Vehicle	FCCP/Oligo	Vehicle	FCCP/Oligo	Vehicle	FCCP/Oligo
Group	C	C	C	C	C	C	C	C	C	C
n	61	44	66	44	3	7	19	5	22	9
Median	1.39	1.32	1.33	1.78	1.73	1.26	1.06	2.27	1.06	1.51
IQR	1.01–1.98	0.88–2.46	1.07–1.91	1.00–8.09	0.05–2.69	0.96–2.33	0.86–1.33	1.00–7.24	0.85–1.36	0.91–2.54
1-sample Wilcoxon	<0.001	0.002	<0.001	<0.001	0.75	0.109	0.251	0.125	0.206	0.027
Vehicle vs. FCCP/Oligo	0.647		0.105		0.500		0.044		0.042	
Group Main Effect									0.009	
Paired Comparisons									L4 vs. L5*	

Measured parameters include the transient duration measured as the time for 80% recovery back to baseline from the peak, and the $[Ca^{2+}]_c$ level of the shoulder during the recovery phase of transients produced by depolarization with K^+ of either 0.5s or 1.3s duration. Agents included either vehicle or FCCP 1 μ M combined with oligomycin 10 μ M (FCCP/Oligo). Groups included Control (C), and neurons from the 4th lumbar (L4) and L5 dorsal root ganglia after spinal nerve ligation. n is the number of neurons. The median and interquartile range (IQR) are shown for the fold change of the measured parameter produced by FCCP/Oligo application, calculated as the ratio of the second depolarization (following FCCP/Oligo) divided by the first depolarization (baseline) for each neuron. 1-sample Wilcoxon is the *P* of an effect of vehicle or FCCP/Oligo in that group using a one-sample Wilcoxon test (compared to a value of 1). Vehicle vs. FCCP/Oligo is the *P* for the comparison of vehicle vs. FCCP/Oligo in control neurons using Mann-Whitney test. Group Main Effect *P* is the probability of a main effect comparing the three groups (C, L4, L5) during FCCP/Oligo application, using Kruskal-Wallis. Paired Comparisons gives the *P* for significant paired comparisons between these groups (blank when none) using Dunn's test.

* *P* < 0.05.