Axotomy Depletes Intracellular Calcium Stores in Primary Sensory Neurons

Marcel Rigaud, M.D.[1,5], **Geza Gemes, M.D.**[1,5], **Paul D. Weyker, B.S.**[6], **James M. Cruikshank**[2], **Takashi Kawano, M.D.**[1], **Hsiang-En Wu, M.D.**[3], and **Quinn H. Hogan, M.D.**[4,7]

[1]Research Fellow, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin

[2]Research Assistant, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin

[3]Assistant Professor, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin

[4]Professor, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin

[5]Resident, Department of Anesthesiology, Medical University of Graz, Graz, Austria

[6]Medical Student, University of Wisconsin, Madison, Wisconsin

[7]Anesthesiologist, Zablocki VA Medical Center, Milwaukee, Wisconsin

Abstract

Background—The cellular mechanisms of neuropathic pain are inadequately understood. Previous investigations have revealed disrupted Ca^{2+} signaling in primary sensory neurons after injury. We therefore examined the effect of injury on intracellular Ca^{2+} stores of the endoplasmic reticulum, which critically regulate the Ca^{2+} signal and neuronal function.

Methods—Intracellular Ca^{2+} levels were measured with Fura-2 or mag-Fura-2 microfluorometry in axotomized fifth lumbar (L5) dorsal root ganglion neurons and adjacent L4 neurons isolated from hyperalgesic rats following L5 spinal nerve ligation, compared to neurons from control animals.

Results—Endoplasmic reticulum Ca^{2+} stores released by the ryanodine-receptor agonist caffeine decreased by 46% in axotomized small neurons. This effect persisted in Ca^{2+}-free bath solution that removes the contribution of store-operated membrane Ca^{2+} channels, and after blockade of both the mitochondrial, sarco-endoplasmic Ca^{2+}-ATPase, and the plasma membrane Ca^{2+} ATPase pathways. Ca^{2+} released by the sarco-endoplasmic Ca^{2+}-ATPase blocker thapsigargin and by the Ca^{2+}-ionophore ionomycin was also diminished by 25% and 41%, respectively. In contrast to control neurons, Ca^{2+} stores in axotomized neurons were not expanded by neuronal activation by K^+ depolarization, and the proportionate rate of refilling by sarco-endoplasmic Ca^{2+}-ATPase was normal. Luminal Ca^{2+} concentration was also reduced by 38% in axotomized neurons in permeabilized neurons. The adjacent neurons of the L4 dorsal root ganglia showed modest and inconsistent changes after L5 spinal nerve ligation.

Conclusions—Painful nerve injury leads to diminished releasable endoplasmic reticulum Ca^{2+} stores and a reduced luminal Ca^{2+} concentration. Depletion of Ca^{2+} stores may contribute to the pathogenesis of neuropathic pain.

Corresponding Author: Quinn H. Hogan, Anesthesiology Research, MEB M4280 Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, Wisconsin 53226; phone: 414 456-5727; fax: 414 456-6507; qhogan@mcw.edu.

Introduction

The primary afferent neuron is the source of all somatic sensory experience, a target of regional anesthetics, the afferent pathway for pain during surgery, and an origin of abnormal activity in chronic pain. Cytoplasmic Ca^{2+} is the dominant second messenger in sensory neurons, and regulates diverse processes including neuronal differentiation, neurotransmitter release, excitability, gene expression, enzymatic activation, and programmed cell death (apoptosis). We have previously shown that painful nerve injury leads to prolonged disruption of the Ca^{2+} signal in primary afferent neurons. Specifically, after transection of their peripheral axons, neurons with their cell bodies in the dorsal root ganglia (DRGs) demonstrate a depressed resting cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$)[1] as well as a diminished temporary increase of $[Ca^{2+}]_c$ that follows neuronal activation (the $[Ca^{2+}]_c$ transient).[2] These findings may in part be attributable to the decrease in activity-related Ca^{2+} entry through the plasmalemma caused by axotomy of DRG neurons. [3-5] However, the cellular machinery that modulates the sensory neuron Ca^{2+} signal is complex,[6] and its function in the context of injury has not been examined.

Sensory neurons possess channels and pumps that extrude Ca^{2+} from the cell, sequester Ca^{2+} into organelles within the cell, and release Ca^{2+} from these stores. Specifically, the plasma membrane Ca^{2+}-ATPase expels cytoplasmic Ca^{2+} from the neuron, as does the Na^+-Ca^{2+} exchange pathway. Cytoplasmic Ca^{2+} is pumped through the action of the sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) into high-concentration subcellular reservoirs, predominantly the endoplasmic reticulum (ER),[6,7] from which it may be released back to the cytoplasm through channels sensitive to ryanodine (the ryanodine receptor, [RyR]) or inositol triphosphate (IP3). The amount of Ca^{2+} accumulated in this store is functionally linked to plasma membrane channels (store-operated Ca^{2+} channels, [SOCCs]) that admit Ca^{2+} into the cytoplasm at a rate inversely related to the store level.[8] Mitochondrial Ca^{2+} uptake driven by the potential across the mitochondrial inner membrane buffers large Ca^{2+} loads by uptake when the $[Ca^{2+}]_c$ rises above a critical threshold, and subsequently releases this Ca^{2+} as the $[Ca^{2+}]_c$ recovers towards baseline levels. All of these processes are tuned by regulatory systems, and may be sites at which neuronal injury perturbs the final Ca^{2+} signal.

The quantity of stored Ca^{2+} critically regulates Ca^{2+} signaling in neurons. This parameter dictates the ability of the sensory neuron to receive Ca^{2+} from the cytoplasm,[9,10] by which the cell limits excessive $[Ca^{2+}]_c$ levels during neuronal activity. Stored Ca^{2+} may also be released into the cytoplasm through the process of Ca^{2+}-induced Ca^{2+} release, by which an initial increase in $[Ca^{2+}]_c$ activates the RyRs to discharge stored Ca^{2+} into the cytoplasm, resulting in amplification of $[Ca^{2+}]_c$ transients. Thus, the ER is at once both a Ca^{2+} sink and source,[11] critically shaping the Ca^{2+} signal.

Disordered homeostasis of the Ca^{2+} store participates in the pathogenesis of various neurological conditions, such as stroke and Alzheimer's disease, as well as painful conditions such as diabetes, human immunodeficiency virus infection, and tissue inflammation.[12-14] The ER stores may likewise play a central role in the pathogenesis of neuropathic pain. Accordingly, the experiments reported here explored the influence of injury upon the size of the intracellular Ca^{2+} store. We employed the spinal nerve ligation (SNL) injury model, since this allows separate evaluation of the axotomized neurons of the fifth lumbar (L5) DRG, for comparison with the L4 neurons that are not directly injured but have axons that transit the sciatic nerve in the company of degenerating distal segments of the L5 neurons, and are thus exposed to mediators of inflammation.[15,16] The relative importance of the contribution to pain genesis of these two populations remains unresolved.[17]

Materials and Methods

All methods and use of animals were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (Milwaukee, Wisconsin).

Injury model

Male Sprague-Dawley (Taconic Farms Inc., Hudson, NY) rats weighing 160 to 180g were subjected to SNL in a manner derived from the original technique.[18] Rats were anesthetized with 2% isoflurane in oxygen and the right paravertebral region was exposed. After removal of the L6 transverse process, the L5 and L6 spinal nerves were ligated with 6-0 silk suture and transected distal to the ligature. The fascia was closed with 4-0 resorbable polyglactin suture and the skin closed with staples. Control animals received anesthesia, skin incision and stapling only. After surgery, the rats were returned to their cages and kept under normal housing conditions with access to pellet food and water *ad lib*.

Sensory testing

Rats underwent sensory testing for hyperalgesic behavior on three different days between 10d and 17d after surgery, as previously described.[1,19] Briefly, right plantar skin was mechanically stimulated 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 incorporates sustained licking, chewing, grooming and elevation of the paw. The frequency of hyperalgesia responses 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.

Neuron isolation and plating

Neurons were dissociated from L4 and L5 DRGs after isoflurane anesthesia and decapitation 21 to 27 days after SNL or skin sham surgery. This interval was chosen since hyperalgesia is fully developed by this time.[19] DRGs were incubated in 0.0625% trypsin (Sigma Aldrich, St. Louis, MO), 0.0125% DNAse (Invitrogen, Carlsbad, CA) and 0.01% blendzyme 2 (Roche Diagnostics, Indianapolis, IN) in Dulbecco's modified Eagle's medium (DMEM)/F12 with glutaMAX (Invitrogen) for 1.5 hours, centrifuged and triturated with fire-polished pipettes in culture medium containing Neural Basal Media A with B27 supplement (Invitrogen), 0.5mM glutamine, 100ng/ml nerve growth factor 7S (Alomone Labs, Jerusalem, Israel) and 0.02mg/ml gentamicin (Invitrogen). Dissociated neurons were plated onto poly-L-lysine coated glass cover slips (Deutsches Spiegelglas, Carolina Biological Supply, Burlington, NC) and maintained at 37°C in humidified 95% air and 5% CO_2 for 2 hours, and were studied no later than 6 hours after harvest.

Solutions and agents

Unless otherwise specified, the bath contained Tyrode's solution (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, Glucose 10, $MgCl_2$ 2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, with an osmolarity of 297-300mOsm and pH 7.40. In some experiments, a Ca^{2+}-free Tyrode's was used that contained (in mM): NaCl 140, KCl 4, Glucose 10, $MgCl_2$ 2 and, HEPES 10, and ethylene glycol tetraacetic acid (EGTA) 0.2. In experiments with permeabilized neurons, a bath solution modeled upon intracellular solution was used that contained (in mM): KCl 120, EGTA 5, $CaCl_2$ 2.25, $MgCl_2$ 2, and HEPES 20. Osmolarity was adjusted to 295 with sucrose, and pH was adjusted to 7.40. This clamped Mg^{2+} and Ca^{2+} at physiologic concentrations[20] of 1.6mM and 39nM respectively, calculated using a web-based EGTA calculator[*]. Adenosine-triphosphate was omitted in order not to trigger release of stored Ca^{2+}. Agents were obtained as follows: caffeine, carbonyl cyanide 4-(trifluoromethoxy)

phenylhydrazone (FCCP), dimethylsulfoxide, lanthanum chloride, thapsigargin from Sigma Aldrich, saponin from Calbiochem EMD (Gibbstown, NJ), Fura-2-AM, mag-Fura-2-AM, ionomycin, and Pluronic F-127 from Invitrogen. Stock solutions of ionomycin, thapsigargin, FCCP, Fura-2-AM, and mag-Fura-2-AM were dissolved in dimethylsulfoxide, and subsequently diluted in the relevant bath solution such that final bath concentration of dimethylsulfoxide was 0.1% or less, which has no effect on $[Ca^{2+}]_c$ (n=20, data not shown). The 500 μl recording chamber was constantly superfused by a gravity-driven bath flow at a rate of 3 ml/min. Agents were delivered by directed microperfusion through a 500 μm diameter hollow quartz fiber 300 μm upstream from the neurons. This flow completely displaced the bath solution, and constant flow was maintained by delivery of bath solution, when specific agents were not being administered, through a computerized valve system. Solution changes were achieved within 200 ms.

Measurement of cytoplasmic Ca^{2+} concentration

Cover slips holding plated neurons were transferred to a room temperature 5 μM solution of Fura-2-AM that contained 0.04% Pluronic F-127 to aid dispersion of the fluorophore. After 30 min, they were washed three times with regular Tyrode's solution and left in a dark environment for deesterification for 30 min and then mounted onto the recording chamber. The fluorophore was excited alternately with 340 nm and 380 nm wavelength illumination (150W Xenon, Lambda DG-4, Sutter, Novato, CA), and images were acquired at 510 nm using a cooled 12-bit digital camera (Coolsnap fx, Photometrics, Tucson, AZ) and inverted microscope (Diaphot 200, Nikon Instruments, Melville, NY) through a 20× or 40× fluor oil-immersion objective. Neurons were visually examined in the brightfield mode and those showing signs of lysis, crenulation or superimposed glial cells were excluded. Recordings from each neuron were obtained as separate regions of interest by appropriate software (MetaFluor, Molecular Devices, Downingtown, PA). After background subtraction, the fluorescence ratio R for individual neurons was determined as the intensity of emission during 340 nm excitation (I_{340}) divided by I_{380}, on a pixel-by-pixel basis. The calcium concentration was then estimated by the formula $[Ca^{2+}]_c = K_d \cdot \beta \cdot (R - R_{min})/(R_{max} - R)$ where $\beta = (I_{380max})/(I_{380min})$. Values of R_{min}, R_{max} and β were determined by in-situ calibrations of 33 neurons from 3 different preparations as described before[1] and were 0.38, 8.49 and 9.54, respectively, and 224 nm was used as K_d.[21]

Determination of Ca^{2+} release

Release of Ca^{2+} from stores was quantified by measuring the size of the resulting $[Ca^{2+}]_c$ transient. Using amplitude to quantify large transients, such as those induced by the action of caffeine, poorly reflects the magnitude of Ca^{2+} release from stores due to the influence of mitochondrial buffering during intervals of high $[Ca^{2+}]_c$. Initial mitochondrial Ca^{2+} uptake depresses the peak $[Ca^{2+}]_c$ during a transient and produces a plateau phase thereafter, during which Ca^{2+} is released from mitochondria into the cytoplasm.[22] Thus, mitochondrial buffering has reciprocal effects on transient amplitude and duration.[23] Further, we have previously shown that transient area is proportionate to cytoplasmic Ca^{2+} load.[2] We therefore chose to quantify caffeine-induced transients by calculating the area of the transient.

In other experiments, application of thapsigargin was used to produce transients, for which the amplitude of the rise from baseline to peak $[Ca^{2+}]_c$ was measured rather than area, since these transients had a more gradual onset without a clearly discernible beginning and endpoint, unlike those triggered by caffeine. Also, such transients do not reach $[Ca^{2+}]_c$ levels that engage mitochondrial buffering, which we estimated from the level of the plateau in large K^+- (316 ± 124 nM; n=115) and caffeine-induced transients (310 ± 128 nM, n=541).[22,24] Amplitude

---

* (http://brneurosci.org/egta.html), last accessed April 18th, 2009

measurement was also employed for transients induced by ionomycin, since this ionophore acts to equilibrate $[Ca^{2+}]$ gradients between subcellular compartments including cytosol, ER, and mitochondria.[25] Calcium was absent from the bath solution during thapsigargin application to remove the contribution of Ca^{2+} entry through SOCCs,[26] and similarly during ionomycin application to avoid entry of Ca^{2+} through the ionophore.

Measurement of ER luminal Ca^{2+} concentration

The low-affinity indicator mag-Fura-2-AM was used to measure the Ca^{2+} concentration contained within the lumina of intracellular compartments ($[Ca^{2+}]_L$).[27,28] Dye loading and recording procedures were similar to those used with Fura-2-AM, although loading took place at 37°C to maximize compartmentalization of the fluorophore in intracellular organelles.[29] Calibration of the mag-Fura-2 signal was performed *in situ* on 26 neurons from 3 different dissociated DRGs, and showed values of R_{min}, R_{max} and β of 0.32, 3.63 and 13.09. A K_d of 53 μM was used for calculation of $[Ca^{2+}]_L$.[30] In protocols requiring removal of cytoplasmic mag-Fura-2, whereas attempts using patch-pipette dialysis proved ineffective, successful permeabilization of neurons was achieved by 75-105 s bath application of 0.01% saponin.[31] Cytoplasmic washout of the fluorophore was confirmed by following the decline in both the fluorescence intensities at 340 nm and 380 nm and a concurrent increase in the ratio R. Only traces in which the decline in intensity during 380 nm illumination exceeded 40% were included for analysis.

Statistical analysis

Preliminary studies showed no differences between neurons dissociated from L4 and L5 ganglia, removed from the same control animals, in Ca^{2+} release by caffeine with normal Tyrode's bath solution, (transient area 14.7 ± 11.3 nM•s $\times 10^3$ in L4, n=19, *vs.* 15.7 ± 11.4 nM•s $\times 10^3$, in L5, n=37, *P*=0.38). We therefore pooled findings from L4 and L5 neurons as the control group. Neurons characterized by size (large > 34 μm, small < 34 μm) were considered separately. Statistical analyses were performed with Statistica (StatSoft Inc, Tulsa, OK). Two-tailed Student's t-test was used for comparing two means. One-way ANOVA was used to detect the influence of injury group on measured parameters. Consideration of additional factors such as time or pharmacologic interventions was achieved using a two-way ANOVA design. An ANCOVA model was used to account for variance due to a covariate after assuring linearity of the influence of the covariate upon the outcome measure. Bonferroni's *post-hoc* test was used to compare relevant means, and a *P* value less than 0.05 was considered significant. Averages are reported ± SD.

Results

Upon needle stimulation, SNL animals (n=69) displayed a hyperalgesia response rate of $35 \pm 21\%$, whereas control animals (n=56) showed a hyperalgesic response $0 \pm 1\%$ (*P*<0.001). Consistent with our previous findings,[1] sensory neuron axotomy depressed resting $[Ca^{2+}]_c$. Specifically, whereas $[Ca^{2+}]_c$ was 66 ± 27 nM in control neurons (n=274), SNL of L5 neurons resulted in a $[Ca^{2+}]_c$ of 46 ± 25 nM (n=157; *P*<0.001 *vs.* control). SNL had no effect on resting $[Ca^{2+}]_c$ of adjacent L4 neurons, in which $[Ca^{2+}]_c$ was 63 ± 26 nM (n=110).

Ryanodine receptor-mediated Ca^{2+} release by caffeine

An estimate of stored Ca^{2+} may be made by measuring the rise of $[Ca^{2+}]_c$ during Ca^{2+} release from stores triggered by application of a high concentration of caffeine (20 mM), which sensitizes the RyR to Ca^{2+} to such an extent that resting levels of Ca^{2+} produce sustained channel opening.[32] For caffeine-induced Ca^{2+} release, we chose to measure transient area rather than amplitude in order to limit the influence of mitochondrial buffering (see methods), and tested assumption in control neurons by comparing transient parameters with and without

blockade of mitochondrial Ca^{2+} buffering with FCCP (1μM).[22] Whereas amplitude of the caffeine-induced transient increased by 108% in the presence of FCCP (from 779 ± 807 nM, n=273, to 1615 ± 2075 nM, n=198), area of the transient increased by only 20% (from 18.2 ± 11.1 nM•s × 10^3, n=268, to 21.9 ± 16.6 M•s × 10^3, n=198).

To measure stores, caffeine was administered continuously until the $[Ca^{2+}]_c$ returned to baseline to assure that the stores were fully emptied, which was confirmed by the observation that reapplication of caffeine 30s after washout produced no further Ca^{2+} release (n=10; data not shown). Sustained application also precluded SERCA function so that this process did not affect the measure of Ca^{2+} release. Application of caffeine (20 mM) in this fashion produced a rapidly rising transient that included a prolonged plateau phase followed by resolution to a level close to resting $[Ca^{2+}]_c$ (fig. 1A). All but one of 542 neurons responded with an increase of at least 50% above resting $[Ca^{2+}]_c$.

DRG neurons are a heterogeneous population representing different sensory modalities. While small neurons are broadly associated with nociceptive sensory modality, neurons with large somata conduct low-threshold sensory information.[33,34] In small neurons, the transient areas evoked by caffeine were significantly decreased by axotomy in L5 neurons after SNL, as well as in adjacent L4 neurons (fig. 1B). Caffeine-induced transients had areas in control large neurons that were smaller than in control small neurons (*P*<0.001). However, there was no effect of injury on large neuron Ca^{2+} release measured this way (fig. 1C).

Caffeine release of stored Ca^{2+} triggers influx of Ca^{2+} across the plasmalemma through store-operated Ca^{2+} channels (SOCCs),[35] which could be independently influenced by injury. We therefore performed additional experiments in small neurons eliminating this factor by using Ca^{2+}-free bath solution.[36] Under these conditions, transients triggered by caffeine application showed influences of injury that were congruent with the findings above (fig. 1D). The transient area in all groups was less in the absence of bath Ca^{2+} compared to experiments conducted with bath Ca^{2+}, which likely indicates a contribution by SOCC to the transient generated by caffeine release of Ca^{2+} stores.[35]

To further compare Ca^{2+} stores and injury effect in putative nociceptors *versus* low-threshold afferents, we examined additional small neurons that were further categorized by sensitivity to capsaicin. Sensitivity to capsaicin is typical of peptidergic polymodal nociceptors, while insensitivity is a characteristic of IB4-binding mechanosensitive nociceptors.[37] In a separate preliminary study (data not shown), the EC_{50} for capsaicin necessary to achieve a 50% increase in $[Ca^{2+}]_c$ was 7.9 nM (total n=175 neurons, used only for this determination). Using 10 nM, 45/85 (53%) of control neurons responded to capsaicin by this criterion (fig. 1E), comparable to the typical proportion of nociceptive neurons.[38] In control neurons, $[Ca^{2+}]_c$ transient areas were significantly larger in capsaicin-responsive cells than in nonresponsive ones (*P*<0.001; fig. 1F), as reported previously. SNL decreased the proportion of neurons sensitive to capsaicin in the L4 group (10/37, 27%) and especially the L5 group (8/53, 15%; *P*<0.001), as expected from previous findings that axonal injury causes loss of the capsaicin receptor, transient receptor potential vanilloid-1.[1,39] In both capsaicin-responsive and non-responsive populations, axotomy reduced the area of the caffeine-induced transient (fig. 1F), indicating loss of Ca^{2+} stores. Specifically, in capsaicin-responsive neurons, the average area under the transient curve was decreased by 59% in axotomized compared to control neurons, and axotomy of capsaicin-nonresponsive neurons decreased the area by 33%. This indicates that both subgroups of small nociceptive sensory neurons have decreased releasable Ca^{2+} stores after injury.

Ca^{2+} release during inhibition of sequestration and extrusion

In order to examine Ca^{2+} release in the absence of the buffering by uptake into mitochondria, we applied caffeine (20 mM) during the continued presence of FCCP (1μM; fig. 2A). Peak of the transient in L4 neurons was not different from controls, while the axotomized L5 neurons decreased by 51% compared to controls (fig. 2B). This indicates that smaller caffeine-induced [Ca^{2+}]$_c$ transients in axotomized neurons are not due to greater mitochondrial buffering in this neuronal group.

We additionally eliminated the potential influence of variable Ca^{2+} reuptake by the SERCA, Ca^{2+} extrusion *via* the plasma membrane Ca^{2+}-ATPase and Ca^{2+}-Na$^+$ exchange mechanism, and Ca^{2+} influx by inducing Ca^{2+} release with caffeine (20 mM) in the context of multiple pharmacological blockers (FCCP, 1μM; thapsigargin, 1μM to block SERCA, administered only with and after caffeine in order to avoid an initial thapsigargin-induced store release; and La^{3+}, 1mM to block plasma membrane Ca^{2+}-ATPase, Ca^{2+}-Na$^+$ exchange, and Ca^{2+} entry pathways, discontinued during caffeine in order to avoid precipitation). This resulted in transients in which an initial increase of [Ca^{2+}]$_c$ was not followed by recovery (fig. 2C). Injury had a significant effect on the caffeine-induced transient amplitude (fig. 2D), in which the L4 group was unchanged but the L5 group showed 30% less Ca^{2+} release compared to control.

Constitutive Ca^{2+} release revealed by thapsigargin

It is unclear whether ryanodine receptor-sensitive Ca^{2+} stores fully overlap with Ca^{2+} stores sensitive to inositol-3P.[6] For this reason, and because receptor-mediated Ca^{2+} release may be influenced by altered expression of the receptor after injury without actually affecting Ca^{2+} stores *per se*, we additionally determined the effect of injury on Ca^{2+} stores using techniques that do not rely on receptor activation. Thapsigargin provides such an alternate method by blocking SERCA, which exposes a constitutive Ca^{2+} leak from stores. This effect is dose related,[26] and although a prior study using 20 nM thapsigargin failed to produce transients in sensory neurons,[40] we observed slow [Ca^{2+}]$_c$ transients with application of 1μM thapsigargin in all neurons (fig. 3A). Full emptying of RyR-sensitive stores by thapsigargin was demonstrated by reduction of the subsequent caffeine-induced Ca^{2+} release transient area in control neurons to 165 ± 353 nM•s (n=20), which is 1% of caffeine-induced Ca^{2+} release reported above. However, thapsigargin-sensitive Ca^{2+} stores encompass more than just the RyR-sensitive pool, since a thapsigargin-induced transient (amplitude 23 ± 17 nM, n=19) persists after caffeine-induced Ca^{2+} release, which constitutes 22% of the thapsigargin-induced transient in the absence of preceding caffeine (107 ± 69, n=85). An effect of injury is evident in the thapsigargin-sensitive pool, since the thapsigargin-induced transient in SNL L5 neurons had a 25% lower transient amplitude than control or SNL L4 neurons (fig. 3B).

The rise of [Ca^{2+}]$_c$ during thapsigargin application was reliably a straight line (fig. 3A inset) indicative of a zero order process, consistent with the observation that the leak rate from Ca^{2+} stores is independent of [Ca^{2+}]$_L$ until the stores are nearly exhausted.[10] In order to evaluate the rate of constitutive release from thapsigargin-sensitive stores, we measured the slope of this rising phase of the thapsigargin-induced [Ca^{2+}]$_c$ transient. Leak rate measured this way showed a tight dependence upon total releasable Ca^{2+} content as indicated by amplitude of the thapsigargin release transient ($R^2 = 0.85$ for all groups together, $P<0.001$; n=95 neurons, fig 3C). For this reason, an analysis of covariance (ANCOVA) model was used to examine the average leak rate, which was depressed in SNL L5 neurons compared to control and SNL L4 neurons (fig. 3D). This was attributable to the effect of the depressed releasable Ca^{2+} in the L5 neurons, as demonstrated by the comparable relationship between leak rate and releasable Ca^{2+} in control and L5 neurons (fig. 3C). However, L4 neurons had a slightly depressed relationship of leak *versus* level of releasable Ca^{2+}.

Ca^{2+} release by ionomycin

Application of the Ca^{2+} ionophore ionomycin (1μM) is an additional technique that releases stored Ca^{2+} without requiring receptor activation[12] and eliminates the influence of mitochondria.[25] A Ca^{2+}-free bath solution was used for 3min preceding ionomycin application and thereafter, in order to avoid the influence of Ca^{2+} entry through the plasmalemma. Transients showed an exponential resolution that lacked the plateau attributable to mitochondria (fig. 4A). After SNL, ionomycin released more Ca^{2+} in the adjacent L4 neurons, but releasable Ca^{2+} was reduced in axotomized L5 neurons compared to control (fig. 4B).

SERCA-dependent recovery of Ca^{2+} stores at rest

One possible reason that Ca^{2+} stores cannot be filled in injured neurons may be dysfunction of the SERCA mechanism that pumps Ca^{2+} into the ER. Repeat application of caffeine (20 mM) produces a diminished Ca^{2+} release until adequate time has elapsed to allow refilling of the stores (fig. 5A). We therefore tested the pace at which neurons in the different groups recovered from caffeine-induced depletion of stored Ca^{2+}. Complete release of stored Ca^{2+} was achieved by sustained application of caffeine (20 mM), which was followed by a 1, 3 or 10-minute recovery period, and then caffeine was applied again. Each neuron was used to test only one time interval. After 10min recovery time, the area of the second transient in control neurons had recovered to $79 \pm 23\%$ of the first (fig. 5B), and there was no difference in the proportionate rate of recovery after injury, normalized to the initial baseline Ca^{2+} release. We confirmed that replenishment during rest was SERCA-dependent, as thapsigargin applied during the refilling interval reduced 10-min recovery to $0.3 \pm 1.4\%$ (n=20). These findings indicate that SERCA function is adequate following neuronal injury to provide recovery to the original baseline level of stores.

Role of activity in regulating Ca^{2+} stores

Ca^{2+} enters sensory neurons predominantly through voltage-gated Ca^{2+} channels during neuronal activity. After SNL, axotomized L5 neurons receive no afferent activity generated from natural stimulation of the receptive field, and this loss may thereby lead to depleted Ca^{2+} stores. We therefore examined whether membrane depolarization with K$^+$, which in turn leads to Ca^{2+} influx across the plasmalemma and store repletion, might increase Ca^{2+} stores in axotomized neurons to the levels of control neurons. Priming transients were generated by 5s application of 50 mM K$^+$ followed 3min later by sustained application of 20 mM caffeine. Compared to caffeine-induced transients without prior *in vitro* activation, caffeine-induced transients that followed activation by K$^+$ (fig. 6) were larger by 94% in control neurons and by 182% in SNL L4 neurons, whereas activation produced no expansion (0%) of the caffeine-induced transient in axotomized L5 neurons. However, the K$^+$-induced transient in SNL L5 neurons was also smaller ($13.0 \pm 12.6 \times 10^3$ nM•s, n=24) than in control neurons ($31.4 \pm 27.9 \times 10^3$ nM•s, n=40) or SNL L4 neurons ($44.1 \pm 23.5 \times 10^3$ nM•s, n=14), indicating that these groups may have received a greater Ca^{2+} influx and boost in stores than the SNL L5 population. Application of K$^+$ lasting only 2 s resulted in K$^+$-induced transients in control neurons ($10.2 \pm 7.2 \times 10^3$ nM•s, n=30) that were comparable in area to those produced by 5 s K$^+$ activation in SNL L5 neurons, but these similar Ca^{2+} loads still showed a greater priming effect in control neurons ($28.7 \pm 20.1 \times 10^3$ nM•s, n=29; 58% greater than baseline caffeine-induced transient) than in axotomized L5 neurons (0%). These observations indicate that the deficit in Ca^{2+} stores following injury cannot be repaired by activity, and therefore is not due to neuronal disuse.

Direct measurement of [Ca^{2+}]$_L$

Pharmacological control of Ca^{2+} release, sequestration and extrusion is inevitably imperfect, due to incomplete drug specificity and efficacy. We therefore devised a technique for directly measuring Ca^{2+} concentration in the subcellular stores using the low-affinity Ca^{2+} fluorophore

mag-Fura-2.[31] Although this approach defines the concentration of unbound Ca^{2+} in the membrane delimited Ca^{2+} stores rather than their net mass, it can be assumed that the concentration of free luminal Ca^{2+} is directly related to total stored Ca^{2+}.

We initially determined $[Ca^{2+}]_L$ in nonpermeabilized neurons.[41] Although such neurons contain mag-Fura-2 in the cytoplasm, the conditions of loading (see methods) were optimized to preferentially accumulate mag-Fura-2 in organelle compartments. Under these conditions, calculated $[Ca^{2+}]_L$ was found to be significantly lower in L4 neurons and especially L5 neurons after SNL compared to control (fig. 7A). Caffeine (20mM) was applied to the loaded neurons to validate the location of mag-Fura-2 in the stored Ca^{2+} compartment. This produced a decrease of calculated $[Ca^{2+}]_L$ by a least 10% in 107/131 (82%) of the neurons, although this may be an underestimate due to the influences of caffeine.[42] If the principal source of fluorescence emission were in fact cytoplasmic rather than compartmentalized mag-Fura-2, release of Ca^{2+} stores would have produced an increase in the calculated $[Ca^{2+}]$ value, so these findings confirm that the fluorophore is preferentially located in the compartment from which Ca^{2+} is released by caffeine.

It has been argued the determination of $[Ca^{2+}]_L$ may be vitiated by even small amounts of residual mag-Fura-2 in the relatively low Ca^{2+} concentration environment of the cytoplasm. [28] This view is supported by our observation of $[Ca^{2+}]_L$ in these intact neurons that are lower than the range reported by others.[43,44] We therefore permeabilized the neurons to provide a more accurate estimation of $[Ca^{2+}]_L$. Using this measurement technique (fig. 7B), $[Ca^{2+}]_L$ measurements were higher than in non-permeabilized neurons. A luminal origin of the signal was confirmed by demonstration of decreased $[Ca^{2+}]_L$ upon ionomycin application (1μM) in a subset of neurons (fig. 7B), during which $[Ca^{2+}]_L$ decreased by $42 \pm 16\%$ (n=6, $P<0.01$). This incomplete equilibration of Ca^{2+} between stores and cytoplasm may be due to either an equilibration rate slower than our technique could observe, or the existence of sub-compartments that ionomycin failed to affect. Other studies using the same ionomycin concentration have shown comparable levels of store depletion.[35,45] Small neurons again showed a marked decrease after SNL in L5 neurons compared to control (fig. 7C). These $[Ca^{2+}]_L$ findings, combined with observations on Ca^{2+} release, reveal a deficit of stored Ca^{2+} following injury of sensory neurons.

As has been done in other studies,[43,44] we included Mg^{2+} in the bath to maintain a natural intracellular concentration[20] following permeabilization. However, entry of bath Mg^{2+} acting on residual cytoplasmic mag-Fura-2 could contribute to our observed increase of $[Ca^{2+}]_L$ during permeabilization, and bias our determination of $[Ca^{2+}]_L$. This is an unlikely explanation, since this would also cause fluorescence intensities to increase during permeabilization, whereas we observed them to decrease. However, to additionally test this factor, permeabilization was performed in a set of control neurons using Mg^{2+}-free bath solution, which produced a $[Ca^{2+}]_L$ (132 ± 90 μM, n=28) that was minimally different from experiments in which the bath solution contained Mg^{2+} (105 ± 69 μM, n=94; P=0.16).

Discussion

The somata of sensory neurons are known to be substantially affected by injury of their peripheral axons.[46] Our present findings extend this recognition by showing that a fundamental feature, the intracellular Ca^{2+} store, is depleted after injury. Specifically, the amount of Ca^{2+} that can be released from stores through a variety of pathways is diminished after axotomy, and the concentration of Ca^{2+} in the luminal compartment is also reduced as estimated by direct imaging.

Intracellular Ca^{2+} stores in neurons reside in several organelles. The best characterized is the ER store, where Ca^{2+} also critically regulates the luminal processes of protein assembly and folding.[47,48] Additional sites of Ca^{2+} storage include the nuclear envelope[49,50] and the Golgi apparatus.[51] The contribution of storage in secretory vesicles[52] and so-called calciosomes[53] remains poorly defined. Recent evidence indicates the dominant site for the Ca^{2+} pool is in the ER lumen.[7] Although subcellular localization may play a role in directing released Ca^{2+} to particular targets,[54] current techniques do not allow these stores to be functionally distinguished.

We employed standard techniques to identify the magnitude of the Ca^{2+} store.[11] Caffeine freely diffuses through the cell membrane and, by activating the RyR, completely discharges Ca^{2+} from the stores available to this release pathway. Although caffeine interacts with Ca^{2+} sensitive fluorophores, these effects are minor when using ratiometric indicators such as in the present studies.[42,55] Stored Ca^{2+} measured this way is affected selectively for specific subgroups of neurons. Axotomy by SNL has no effect on caffeine-releasable Ca^{2+} in large neurons that mostly transduce and transmit low threshold sensory information. However, axotomy substantially depresses caffeine-releasable stores in the small neuron population, especially in capsaicin-sensitive putative nociceptors. Capsaicin non-responsive small neurons, which probably represent a population of nonpeptidergic, isolectin B4-binding mechanosensitive nociceptors,[37] also have depleted stores after injury, but are less affected than capsaicin sensitive neurons. Loss of Ca^{2+} store in only certain neuron subtypes after axotomy may be due to ER damage selectively in these neurons, as is explored in the companion paper.

Sensory neurons possess additional intracellular Ca^{2+} stores that may be released through activation of an IP3-sensitive channel. There is not full agreement whether the Ca^{2+} pools sensitive to IP3 and caffeine overlap in peripheral sensory neurons,[6,56] although experiments using sequential activation of these pathways mostly support the view that the pools are one and the same.[57,58] We did not measure Ca^{2+} release by IP3 because injury of peripheral sensory neurons directly affects IP3 signaling,[59,60] and membrane permeant or caged IP3 compounds are not readily available. We therefore turned to an alternate strategy of discharging stores indiscriminately either by the Ca^{2+} ionophore ionomycin or by constitutive leak from stores during SERCA inhibition by thapsigargin. Observations using both of these techniques support the findings from caffeine experiments and show an axotomy-induced deficit of Ca^{2+} in stores that is independent of the release mechanism.

The Ca^{2+} transient during release of stores is supplemented by Ca^{2+} influx through SOCCs.[26] Therefore, a loss of this capacitative Ca^{2+} entry process due to injury could create the appearance of a deficit in stores measured by release. However, we observed that the depressant effect of axotomy on store release persists in Ca^{2+}-free bath solution, so this explanation is unlikely. Also, we considered whether neuronal inactivity might have led to store depletion after axotomy. However, cytoplasmic Ca^{2+} loads provided by activation of voltage-gated Ca^{2+} channels minimally expanded stores in axotomized neurons compared to control neurons. Together, our findings support the conclusion that the capacity of the intracellular Ca^{2+} storage reservoir is compromised after axotomy in sensory neurons.

The amount of stored Ca^{2+} is dictated by the concentration of Ca^{2+} in the store, $[Ca^{2+}]_L$, the bound Ca^{2+} in equilibrium with this free fraction, and the extent of the compartment. Therefore, although mag-Fura-2 microfluorimetry reflects $[Ca^{2+}]_L$, it cannot indicate the total mass of stored Ca^{2+} *per se*. Although the concentration of the free fraction should be proportionate with the total Ca^{2+} store, this method cannot account for changes in the amount or performance of various Ca^{2+} binding proteins in the compartment, which could alter also the mass of stored Ca^{2+} without changing $[Ca^{2+}]_L$. Our findings with mag-Fura-2 reveal a decrease in resting

$[Ca^{2+}]_L$ associated with axotomy. Since caffeine produces a fall in $[Ca^{2+}]_L$ measured by mag-Fura-2, it is likely that the mag-Fura-2 technique quantifies the state of the same Ca^{2+} pool as does caffeine-induced release. The combined results of these two approaches point to a model in which axotomy decreases the ability of the storage compartment, predominantly the ER, to concentrate Ca^{2+} within its lumen. This level of $[Ca^{2+}]_L$ is set by the dynamic balance between SERCA and constitutive Ca^{2+} leakage from the ER.[10] Although resting axotomized neurons show no delay in fractional recovery of Ca^{2+} stores back to their baseline following release, this is nonetheless compatible with compromised SERCA function since baseline store levels are comparatively depleted. We showed that the relationship between constitutive Ca^{2+} leak rate and Ca^{2+} store level is normal in axotomized neurons, which points to a normal leak pathway. This leaves compromised SERCA function as the cause of decreased $[Ca^{2+}]_L$, which could be due to altered regulation by kinases.[41] Our data do not reveal whether injury also diminishes the extent of the stores, which is addressed in the companion paper.

The effect of painful conditions on neuronal Ca^{2+} stores has previously been examined only in models of diabetes mellitus. Those studies found that Ca^{2+} transients induced by ionomycin[12] or caffeine[61,62] are decreased in diabetic animals, similar to our observations after peripheral nerve trauma. However, a substantially different pathogenic mechanism in diabetes is suggested by differences in other aspects of the Ca^{2+} signaling pathway in sensory neurons, including an increased resting $[Ca^{2+}]_c$[12,61,63,64] and prolonged activity-induced transients,[61,62,64] which contrast with decreased resting $[Ca^{2+}]_c$ and decreased activity-induced transients after axotomy.[1,2,65] Like diabetes, painful peripheral inflammation is associated with elevated resting $[Ca^{2+}]_c$ and transients in DRG neurons,[14] but the state of the Ca^{2+} stores in this condition has not been determined.

The SNL model provides the opportunity to separately evaluate neurons in the L5 DRG that are axotomized, *versus* the adjacent L4 neurons. Following SNL, features of Ca^{2+} storage and release in these two populations are divergent. In contrast to axotomized L5 neurons, ionomycin release of stored Ca^{2+} is elevated in L4 neurons, although this may represent increased release from the mitochondrial component of stored Ca^{2+} released. The L4 neurons also show an expanded Ca^{2+} storage capacity, as shown by a supranormal level of releasable Ca^{2+} after cytoplasmic Ca^{2+} loading. Finally, whereas axotomy diminishes Ca^{2+} release by caffeine or thapsigargin and decreases $[Ca^{2+}]_L$ measured by mag-Fura-2 in permeabilized L5 neurons, these are unaffected in the L4 population. These divergent findings may result from the difference in predominant influences of SNL on the L5 versus L4 neurons. Whereas the SNL results in axotomy of the L5 population, the peripheral axons of the adjacent L4 neurons remain intact but are exposed to inflammatory mediators triggered by Wallerian degeneration of detached L5 axonal segments.[15,16,66] Other studies have shown divergent influences on Ca^{2+} handling in inflammation compared to axotomy. Specifically, models of inflammatory pain reveal increased activity-induced Ca^{2+} transients in DRG neurons,[14] whereas the opposite occurs after axotomy.[2] Our present findings also suggest that Ca^{2+} stores are also differentially regulated by axotomy and inflammation. A degree of inconsistency in our L4 findings may be attributable to a variable admixture of axotomy among the L4 neuronal population following L5 SNL.[67]

Disruption of the level of Ca^{2+} stores in neuronal subcellular compartments is a fundamental pathogenic factor in a variety of diseases including ischemia, acquired immunodeficiency syndrome, and Alzheimer's disease.[6] In addition to storing Ca^{2+}, the ER is the site assembly and post-translational modification of proteins by glycosylation and folding. Depletion of luminal Ca^{2+} triggers highly conserved stress responses involving accumulation of unfolded protein, global suppression of protein synthesis, and activation of a variety of transcription factors, resulting in neuronal dysfunction and death.[68] For instance, Ca^{2+} store depletion by caffeine or thapsigargin exposure suppresses protein synthesis and induces neuronal apoptosis

independent of increased $[Ca^{2+}]_c$.[69,70] The present findings suggest that similar processes may take place in sensory neurons after peripheral nerve injury, and that they may contribute to long-term changes associated with chronic pain.

Acknowledgments

References

1. Fuchs A, Lirk P, Stucky C, Abram SE, Hogan QH. Painful nerve injury decreases resting cytosolic calcium concentrations in sensory neurons of rats. Anesthesiology 2005;102:1217–25. [PubMed: 15915036]

2. Fuchs A, Rigaud M, Hogan QH. Painful nerve injury shortens the intracellular Ca2+ signal in axotomized sensory neurons of rats. Anesthesiology 2007;107:106–16. [PubMed: 17585222]

3. Hogan QH, McCallum JB, Sarantopoulos C, Aason M, Mynlieff M, Kwok WM, Bosnjak ZJ. Painful neuropathy decreases membrane calcium current in mammalian primary afferent neurons. Pain 2000;86:43–53. [PubMed: 10779659]

4. McCallum JB, Kwok WM, Mynlieff M, Bosnjak ZJ, Hogan QH. Loss of T-type calcium current in sensory neurons of rats with neuropathic pain. Anesthesiology 2003;98:209–16. [PubMed: 12502999]

5. McCallum JB, Kwok WM, Sapunar D, Fuchs A, Hogan QH. Painful peripheral nerve injury decreases calcium current in axotomized sensory neurons. Anesthesiology 2006;105:160–8. [PubMed: 16810008]

6. Verkhratsky A. Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. Physiol Rev 2005;85:201–79. [PubMed: 15618481]

7. Albrecht MA, Colegrove SL, Hongpaisan J, Pivovarova NB, Andrews SB, Friel DD. Multiple modes of calcium-induced calcium release in sympathetic neurons I: Attenuation of endoplasmic reticulum Ca2+ accumulation at low [Ca2+](i) during weak depolarization. J Gen Physiol 2001;118:83–100. [PubMed: 11429446]

8. Lewis RS. The molecular choreography of a store-operated calcium channel. Nature 2007;446:284–7. [PubMed: 17361175]

9. Usachev YM, Thayer SA. Ca2+ influx in resting rat sensory neurones that regulates and is regulated by ryanodine-sensitive Ca2+ stores. J Physiol 1999;519(Pt 1):115–30. [PubMed: 10432343]

10. Mogami H, Tepikin AV, Petersen OH. Termination of cytosolic Ca2+ signals: Ca2+ reuptake into intracellular stores is regulated by the free Ca2+ concentration in the store lumen. EMBO J 1998;17:435–42. [PubMed: 9430635]

11. Friel DD, Tsien RW. A caffeine- and ryanodine-sensitive Ca2+ store in bullfrog sympathetic neurones modulates effects of Ca2+ entry on [Ca2+]i. J Physiol 1992;450:217–46. [PubMed: 1432708]

12. Kruglikov I, Gryshchenko O, Shutov L, Kostyuk E, Kostyuk P, Voitenko N. Diabetes-induced abnormalities in ER calcium mobilization in primary and secondary nociceptive neurons. Pflugers Arch 2004;448:395–401. [PubMed: 15048576]

13. Medina I, Ghose S, Ben-Ari Y. Mobilization of intracellular calcium stores participates in the rise of [Ca2+]i and the toxic actions of the HIV coat protein GP120. Eur J Neurosci 1999;11:1167–78. [PubMed: 10103113]

14. Lu SG, Gold MS. Inflammation-induced increase in evoked calcium transients in subpopulations of rat dorsal root ganglion neurons. Neuroscience 2008;153:279–88. [PubMed: 18367340]

15. Sommer C, Schafers M. Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: Differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. Brain Res 1998;784:154–62. [PubMed: 9518588]

16. Ramer MS, French GD, Bisby MA. Wallerian degeneration is required for both neuropathic pain and sympathetic sprouting into the DRG. Pain 1997;72:71–8. [PubMed: 9272789]

17. Gold MS. Spinal nerve ligation: What to blame for the pain and why. Pain 2000;84:117–20. [PubMed: 10666515]

18. Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 1992;50:355–63. [PubMed: 1333581]

19. Hogan Q, Sapunar D, Modric-Jednacak K, McCallum JB. Detection of neuropathic pain in a rat model of peripheral nerve injury. Anesthesiology 2004;101:476–87. [PubMed: 15277932]

20. Henrich M, Buckler KJ. Effects of anoxia, aglycemia, and acidosis on cytosolic Mg2+, ATP, and pH in rat sensory neurons. Am J Physiol Cell Physiol 2008;294:C280–94. [PubMed: 17977942]

21. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50. [PubMed: 3838314]

22. Friel DD, Tsien RW. An FCCP-sensitive Ca2+ store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in [Ca2+]i. J Neurosci 1994;14:4007–24. [PubMed: 8027759]

23. Friel DD. Mitochondria as regulators of stimulus-evoked calcium signals in neurons. Cell Calcium 2000;28:307–16. [PubMed: 11115370]

24. Thayer SA, Miller RJ. Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones *in vitro*. J Physiol 1990;425:85–115. [PubMed: 2213592]

25. Abramov AY, Duchen MR. Actions of ionomycin, 4-BrA23187 and a novel electrogenic Ca2+ ionophore on mitochondria in intact cells. Cell Calcium 2003;33:101–12. [PubMed: 12531186]

26. Kwan CY, Takemura H, Obie JF, Thastrup O, Putney JW Jr. Effects of MeCh, thapsigargin, and La3+ on plasmalemmal and intracellular Ca2+ transport in lacrimal acinar cells. Am J Physiol 1990;258:C1006–15. [PubMed: 2360617]

27. Hofer AM, Schlue WR, Curci S, Machen TE. Spatial distribution and quantitation of free luminal [Ca] within the InsP3-sensitive internal store of individual BHK-21 cells: Ion dependence of InsP3-induced Ca release and reloading. Faseb J 1995;9:788–98. [PubMed: 7601343]

28. Hofer AM, Schulz I. Quantification of intraluminal free [Ca] in the agonist-sensitive internal calcium store using compartmentalized fluorescent indicators: Some considerations. Cell Calcium 1996;20:235–42. [PubMed: 8894270]

29. Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P. A comparison of fluorescent Ca2+ indicator properties and their use in measuring elementary and global Ca2+ signals. Cell Calcium 2000;28:213–23. [PubMed: 11032777]

30. Raju B, Murphy E, Levy LA, Hall RD, London RE. A fluorescent indicator for measuring cytosolic free magnesium. Am J Physiol 1989;256:C540–8. [PubMed: 2923192]

31. Solovyova N, Verkhratsky A. Monitoring of free calcium in the neuronal endoplasmic reticulum: An overview of modern approaches. J Neurosci Methods 2002;122:1–12. [PubMed: 12535760]

32. Neering IR, McBurney RN. Role for microsomal Ca storage in mammalian neurones? Nature 1984;309:158–60. [PubMed: 6717595]

33. Lawson SN. Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp Physiol 2002;87:239–44. [PubMed: 11856969]

34. Gold MS, Dastmalchi S, Levine JD. Co-expression of nociceptor properties in dorsal root ganglion neurons from the adult rat *in vitro*. Neuroscience 1996;71:265–75. [PubMed: 8834408]

35. Hofer AM, Fasolato C, Pozzan T. Capacitative Ca2+ entry is closely linked to the filling state of internal Ca2+ stores: A study using simultaneous measurements of ICRAC and intraluminal [Ca2+]. J Cell Biol 1998;140:325–34. [PubMed: 9442108]

36. Usachev Y, Shmigol A, Pronchuk N, Kostyuk P, Verkhratsky A. Caffeine-induced calcium release from internal stores in cultured rat sensory neurons. Neuroscience 1993;57:845–59. [PubMed: 8309540]

37. Dirajlal S, Pauers LE, Stucky CL. Differential response properties of IB(4)-positive and -negative unmyelinated sensory neurons to protons and capsaicin. J Neurophysiol 2003;89:513–24. [PubMed: 12522198]

38. Holzer P. Capsaicin: Cellular targets, mechanisms of action, and selectivity for thin sensory neurons. Pharmacol Rev 1991;43:143–201. [PubMed: 1852779]

39. Michael GJ, Priestley JV. Differential expression of the mRNA for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. J Neurosci 1999;19:1844–54. [PubMed: 10024368]

40. Shmigol A, Kostyuk P, Verkhratsky A. Dual action of thapsigargin on calcium mobilization in sensory neurons: Inhibition of Ca2+ uptake by caffeine-sensitive pools and blockade of plasmalemmal Ca2 + channels. Neuroscience 1995;65:1109–18. [PubMed: 7617166]

41. Usachev YM, Marsh AJ, Johanns TM, Lemke MM, Thayer SA. Activation of protein kinase C in sensory neurons accelerates Ca2+ uptake into the endoplasmic reticulum. J Neurosci 2006;26:311–8. [PubMed: 16399701]

42. Muschol M, Dasgupta BR, Salzberg BM. Caffeine interaction with fluorescent calcium indicator dyes. Biophys J 1999;77:577–86. [PubMed: 10388782]

43. Solovyova N, Fernyhough P, Glazner G, Verkhratsky A. Xestospongin C empties the ER calcium store but does not inhibit InsP3-induced Ca2+ release in cultured dorsal root ganglia neurones. Cell Calcium 2002;32:49–52. [PubMed: 12127062]

44. Solovyova N, Veselovsky N, Toescu EC, Verkhratsky A. Ca(2+) dynamics in the lumen of the endoplasmic reticulum in sensory neurons: Direct visualization of Ca(2+)-induced Ca(2+) release triggered by physiological Ca(2+) entry. Embo J 2002;21:622–30. [PubMed: 11847110]

45. Morgan AJ, Jacob R. Ionomycin enhances Ca2+ influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. Biochem J 1994;300(Pt 3):665–72. [PubMed: 8010948]

46. Devor, M. Response of nerves to injury in relation to neuropathic pain, Wall and Melzack's Textbook of Pain. 5th. McMahon, S.; Koltzenburg, M., editors. London: Churchill Livingston; 2006. p. 905-27.

47. Meldolesi J. Rapidly exchanging Ca2+ stores in neurons: Molecular, structural and functional properties. Prog Neurobiol 2001;65:309–38. [PubMed: 11473791]

48. Michalak M, Robert Parker JM, Opas M. Ca2+ signaling and calcium binding chaperones of the endoplasmic reticulum. Cell Calcium 2002;32:269–78. [PubMed: 12543089]

49. Gerasimenko O, Gerasimenko J. New aspects of nuclear calcium signalling. J Cell Sci 2004;117:3087–94. [PubMed: 15226390]

50. Petersen OH, Gerasimenko OV, Gerasimenko JV, Mogami H, Tepikin AV. The calcium store in the nuclear envelope. Cell Calcium 1998;23:87–90. [PubMed: 9601603]

51. Pinton P, Pozzan T, Rizzuto R. The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca2 + store, with functional properties distinct from those of the endoplasmic reticulum. Embo J 1998;17:5298–308. [PubMed: 9736609]

52. Petersen OH. Can Ca2+ be released from secretory granules or synaptic vesicles? Trends Neurosci 1996;19:411–3. [PubMed: 8888514]

53. Rossier MF, Putney JW Jr. The identity of the calcium-storing, inositol 1,4,5-trisphosphate-sensitive organelle in non-muscle cells: Calciosome, endoplasmic reticulum … or both? Trends Neurosci 1991;14:310–4. [PubMed: 1719679]

54. Berridge MJ. Neuronal calcium signaling. Neuron 1998;21:13–26. [PubMed: 9697848]

55. Nohmi M, Hua SY, Kuba K. Basal Ca2+ and the oscillation of Ca2+ in caffeine-treated bullfrog sympathetic neurones. J Physiol 1992;450:513–28. [PubMed: 1432716]

56. Thayer SA, Perney TM, Miller RJ. Regulation of calcium homeostasis in sensory neurons by bradykinin. J Neurosci 1988;8:4089–97. [PubMed: 3183714]

57. Crawford JH, Wootton JF, Seabrook GR, Scott RH. Activation of Ca2+-dependent currents in dorsal root ganglion neurons by metabotropic glutamate receptors and cyclic ADP-ribose precursors. J Neurophysiol 1997;77:2573–84. [PubMed: 9163377]

58. Solovyova N, Verkhratsky A. Neuronal endoplasmic reticulum acts as a single functional Ca2+ store shared by ryanodine and inositol-1,4,5-trisphosphate receptors as revealed by intra-ER [Ca2+] recordings in single rat sensory neurones. Pflugers Arch 2003;446:447–54. [PubMed: 12764616]

59. Bradbury EJ, Burnstock G, McMahon SB. The expression of P2X3 purinoreceptors in sensory neurons: Effects of axotomy and glial-derived neurotrophic factor. Mol Cell Neurosci 1998;12:256–68. [PubMed: 9828090]
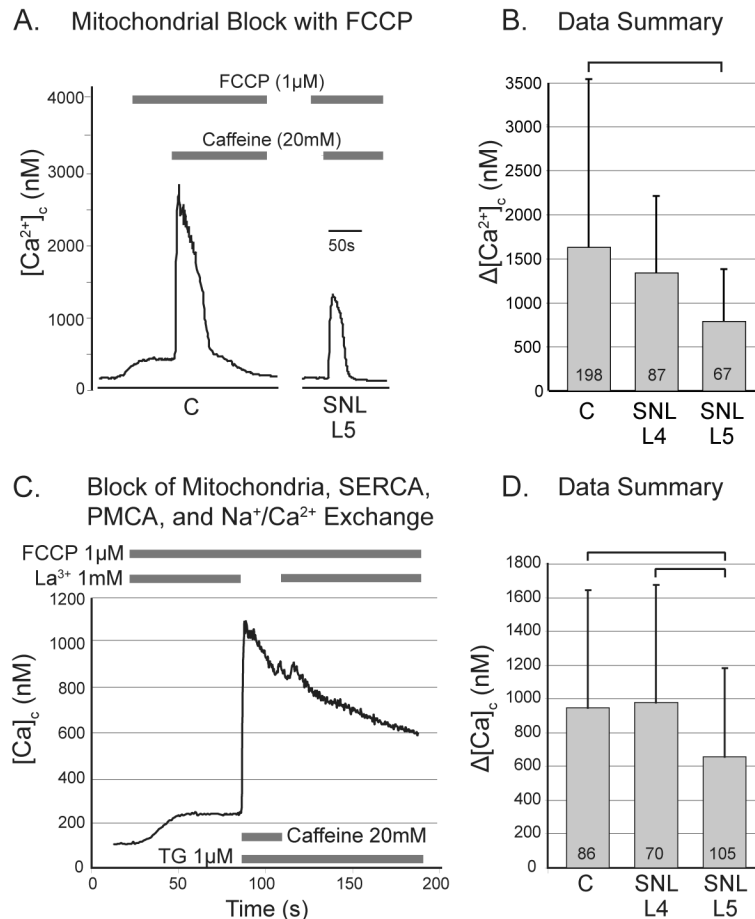
60. Tsuzuki K, Kondo E, Fukuoka T, Yi D, Tsujino H, Sakagami M, Noguchi K. Differential regulation of P2X(3) mRNA expression by peripheral nerve injury in intact and injured neurons in the rat sensory ganglia. Pain 2001;91:351–60. [PubMed: 11275393]

61. Huang TJ, Sayers NM, Fernyhough P, Verkhratsky A. Diabetes-induced alterations in calcium homeostasis in sensory neurones of streptozotocin-diabetic rats are restricted to lumbar ganglia and are prevented by neurotrophin-3. Diabetologia 2002;45:560–70. [PubMed: 12032634]

62. Li F, Obrosova IG, Abatan O, Tian D, Larkin D, Stuenkel EL, Stevens MJ. Taurine replacement attenuates hyperalgesia and abnormal calcium signaling in sensory neurons of STZ-D rats. Am J Physiol Endocrinol Metab 2005;288:E29–36. [PubMed: 15585600]

63. Kostyuk E, Svichar N, Shishkin V, Kostyuk P. Role of mitochondrial dysfunction in calcium signalling alterations in dorsal root ganglion neurons of mice with experimentally-induced diabetes. Neuroscience 1999;90:535–41. [PubMed: 10215157]

64. Kostyuk E, Voitenko N, Kruglikov I, Shmigol A, Shishkin V, Efimov A, Kostyuk P. Diabetes-induced changes in calcium homeostasis and the effects of calcium channel blockers in rat and mice nociceptive neurons. Diabetologia 2001;44:1302–9. [PubMed: 11692179]

65. Fuchs A, Rigaud M, Sarantopoulos CD, Filip P, Hogan QH. Contribution of calcium channel subtypes to the intracellular calcium signal in sensory neurons: the effect of injury. Anesthesiology 2007;107:117–27. [PubMed: 17585223]

66. Sheth RN, Dorsi MJ, Li Y, Murinson BB, Belzberg AJ, Griffin JW, Meyer RA. Mechanical hyperalgesia after an L5 ventral rhizotomy or an L5 ganglionectomy in the rat. Pain 2002;96:63–72. [PubMed: 11932062]

67. Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN. Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. J Neurosci 2006;26:1281–92. [PubMed: 16436616]

68. Paschen W. Dependence of vital cell function on endoplasmic reticulum calcium levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. Cell Calcium 2001;29:1–11. [PubMed: 11133351]

69. Wei H, Wei W, Bredesen DE, Perry DC. Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. J Neurochem 1998;70:2305–14. [PubMed: 9603195]

70. Doutheil J, Gissel C, Oschlies U, Hossmann KA, Paschen W. Relation of neuronal endoplasmic reticulum calcium homeostasis to ribosomal aggregation and protein synthesis: implications for stress-induced suppression of protein synthesis. Brain Res 1997;775:43–51. [PubMed: 9439827]
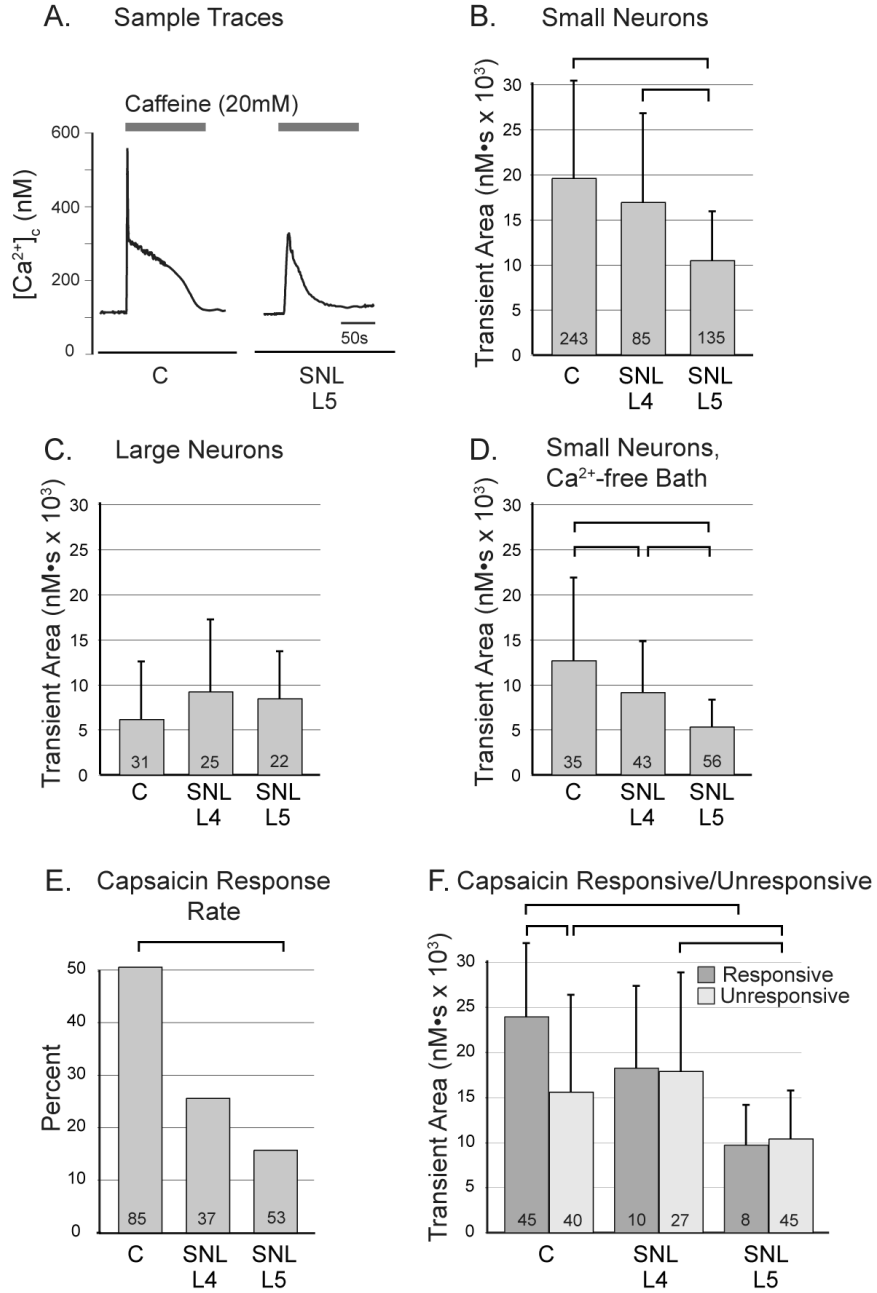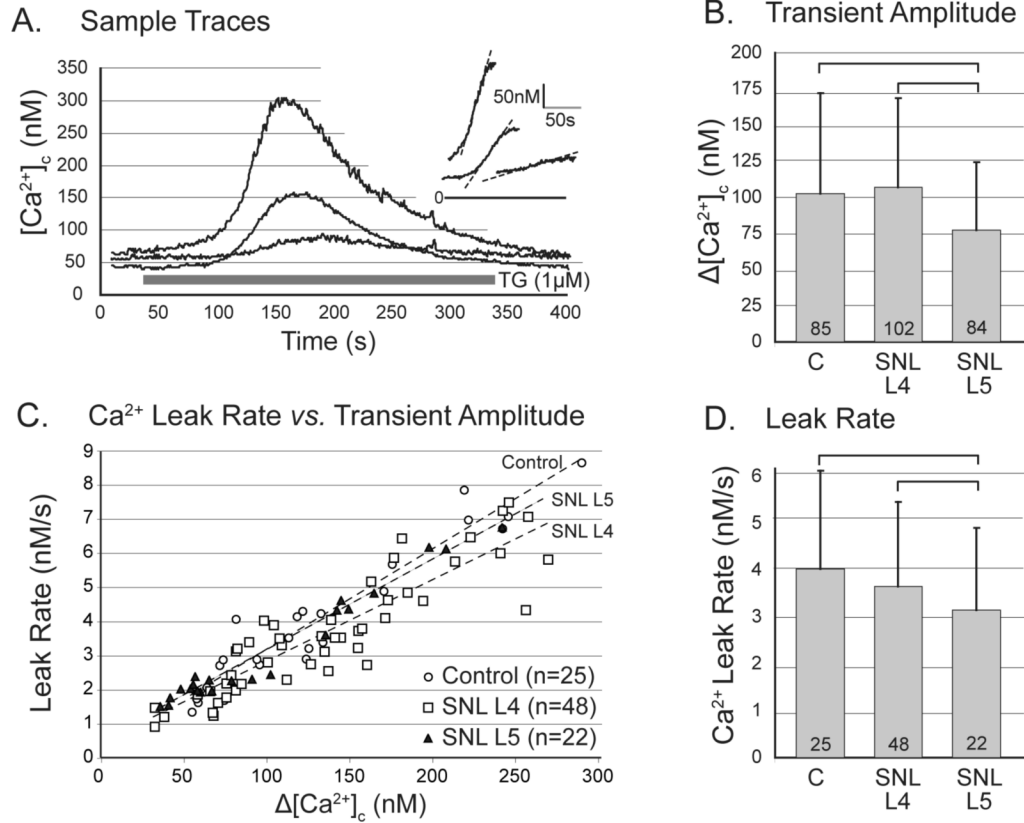
Figure 1.
Response of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) in sensory neurons to application of caffeine (20mM). A. Characteristic transients show a rapid peak and sustained plateau, with diminished size after spinal nerve ligation (SNL) in axotomized small neurons of the fifth lumbar (L5) dorsal root ganglion. B. In small neurons (diameter less than 34μm), transient area is decreased in L5 neurons compared to control (C). C. In large neurons, there are no differences between injury groups. D. When Ca^{2+} is removed from the bath solution, transients in small neurons are reduced in size compared to Ca^{2+}-containing solution (panel B.), but injury still reduces transient in L5 neurons, as well as in L4 neurons. E. The frequency of neuronal sensitivity to capsaicin (10nM) is decreased in L5 neurons after SNL. F. The area of caffeine-

induced transients is less in neurons insensitive to capsaicin than in responsive ones in control neurons but not in L4 or L5 neurons after SNL. Transient area is reduced in L5 neurons compared to control in both capsaicin sensitive and insensitive populations. For panels B-E, one-way ANOVA with Bonferroni *post-hoc* testing was used. For panel F, two-way ANOVA with Bonferroni post-hoc testing was used. For all panels, numbers in the bars indicate n for number of neurons, brackets above bars connect groups that are significantly different (*P*<0.05), and error bars show standard deviation.

Ca^{2+} Release during Extrusion/Sequestration Block

A. Mitochondrial Block with FCCP

B. Data Summary

C. Block of Mitochondria, SERCA, PMCA, and Na$^+$/Ca^{2+} Exchange
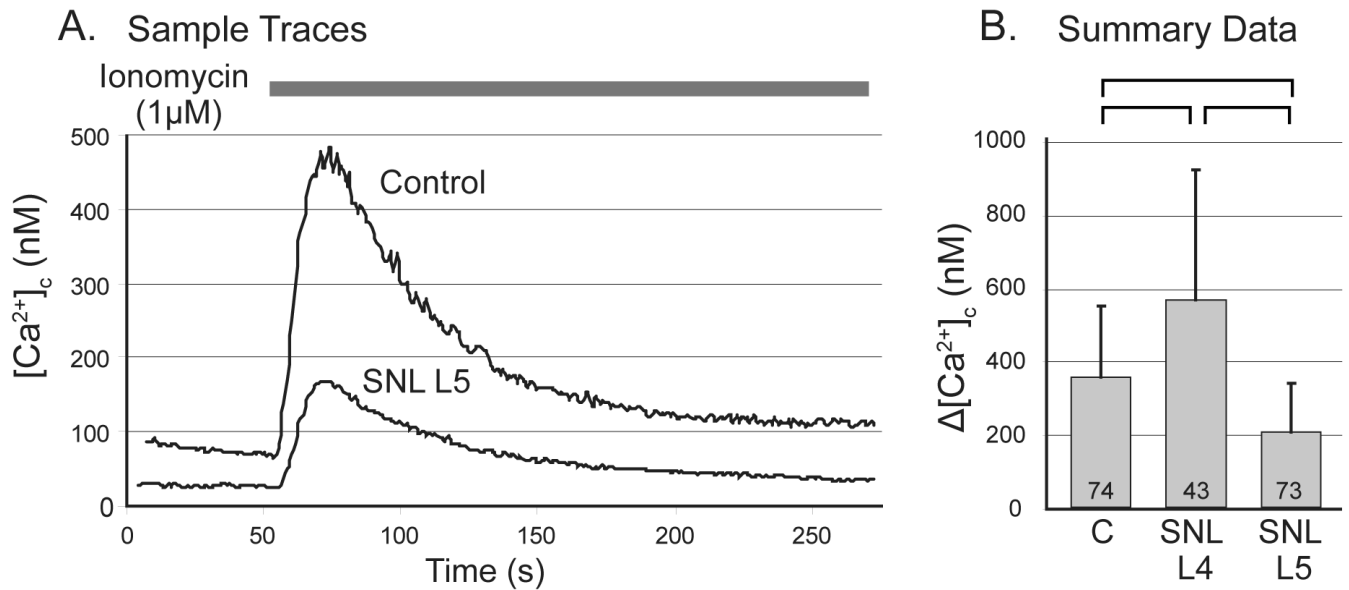
D. Data Summary

Figure 2.
Response of cytoplasmic Ca^{2+} concentration ([Ca^{2+}]$_c$) in sensory neurons to application of caffeine (20mM) during pharmacological blockade of processes that extrude and sequester Ca^{2+}. A. Typical traces of caffeine-induced Ca^{2+} release in the presence of the mitochondrial protonophore p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP), which eliminates buffering of cytoplasmic Ca^{2+} by mitochondria, and increases amplitude of the transient while eliminating the plateau phase. B. Spinal nerve ligation (SNL) depresses the amplitude of caffeine-induced transients during FCCP in the axotomized neurons of the fifth lumbar (L5) dorsal root ganglion. C. Typical traces of caffeine-induced Ca^{2+} transients in the presence of FCCP, and thapsigargin (TG), which blocks sequestration of Ca^{2+} by the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), as well as La^{3+}, which blocks extrusion of Ca^{2+} from the neuron by the plasma membrane Ca^{2+} ATPase (PMCA) and the Na$^+$/Ca^{2+} exchanger. D. SNL depresses amplitude of the resulting caffeine-induced transient in the L5 population. For panels B and D, one-way ANOVA with Bonferroni *post-hoc* testing was used, numbers in the bars indicate n for number of neurons, and brackets above bars connect groups that are significantly different (*P*<0.05), and error bars show standard deviation.

Thapsigargin-induced Ca²⁺ Release

Figure 3.

Response of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) in sensory neurons to application of thapsigargin (TG). A. Sample traces from control (C) neurons demonstrate variable amplitude of response but a linear rising limb of the transient (inset). B. Spinal nerve ligation (SNL) depresses the response in axotomized neurons of the fifth lumbar (L5) dorsal root ganglion. (One-way ANOVA with Bonferroni *post-hoc* testing.) C. The rate of Ca^{2+} leak during TG application, measured as the slope of the rising limb, is linearly dependent on the transient amplitude for all injury groups. D. Leak rate is depressed in L5 neurons. For panels C and D, an ANCOVA model with Bonferroni *post-hoc* testing was employed. For panels B and D, numbers in the bars indicate n for number of neurons, brackets above bars connect groups that are significantly different (*P*<0.05), and error bars show standard deviation.

Ionomycin-induced Ca^{2+} Release

A. Sample Traces

Ionomycin
(1μM)

Control

SNL L5

$[Ca^{2+}]_c$ (nM)

Time (s)

B. Summary Data

$\Delta[Ca^{2+}]_c$ (nM)

| 74 | 43 | 73 |

C   SNL   SNL
     L4   L5

Figure 4.
Response of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) in sensory neurons to application of ionomycin in bath solution not containing Ca^{2+}. A. Sample traces from a control (C) neuron and another from the fifth lumbar (L5) dorsal root ganglion after spinal nerve ligation (SNL). B. Application of ionomycin produces transients with reduced amplitude in L5 neurons, but increased amplitude in L4 neurons (one-way ANOVA with Bonferroni *post-hoc* testing). Numbers in the bars indicate n for number of neurons, brackets above bars connect groups that are significantly different ($P<0.05$), and error bars show standard deviation.

Restoration of Releasable Ca²⁺ Stores
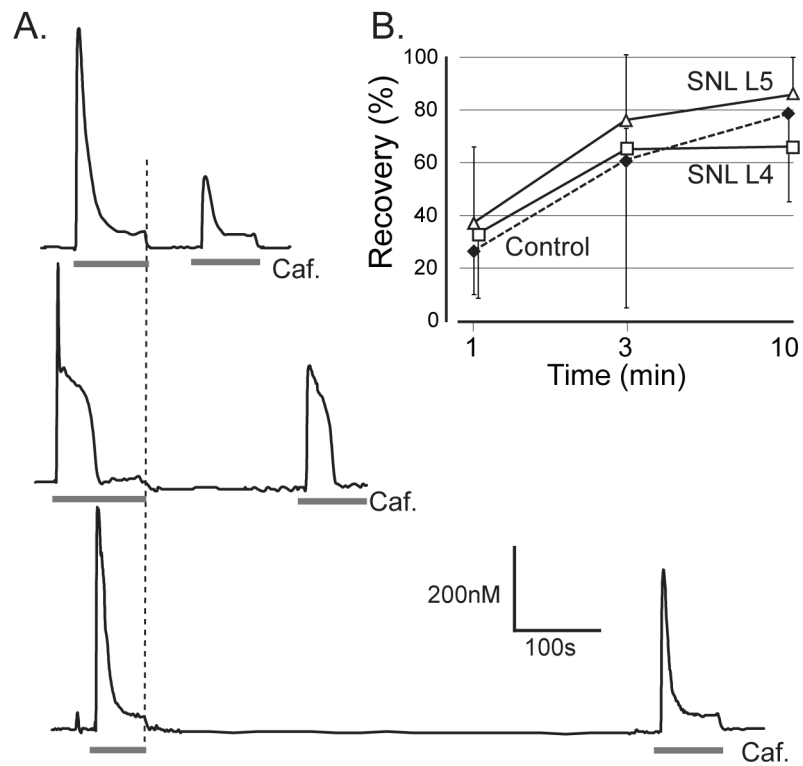
Figure 5.
Recovery of caffeine-induced Ca^{2+} release during rest. A. A second application of caffeine (Caf., 20mM) at intervals of 1, 3, and 10min following the offset of the previous application (dotted line) shows progressive recovery of transient area in three different neurons. B. Proportionate recovery is comparable in control neurons and in fourth lumbar (L4) and L5 neurons after spinal nerve ligation (SNL). Two-way ANOVA shows a significant main effect for time interval, but not for injury. Groups at each data point include 4 to 14 neurons. Error bars show standard deviation.

Ca^{2+} Release: Effect of Activity
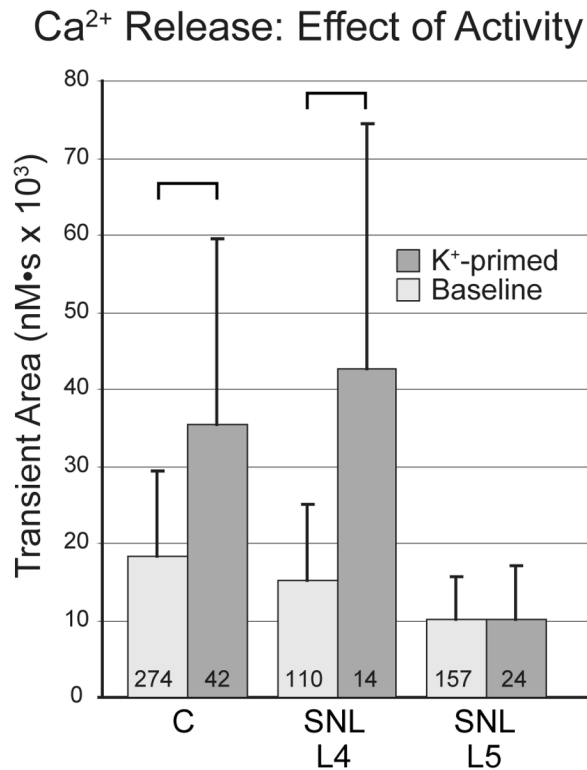
Figure 6.
Recovery of caffeine-induced Ca^{2+} release after Ca^{2+} loading during activation with depolarization by application of K$^+$ (50mM, 5s) compared to transients without prior activation (same data as Fig. 1B). Although a priming Ca^{2+} load increases releasable Ca^{2+} in control (C) neurons and fourth lumbar (L4) neurons after spinal nerve ligation (SNL), L5 neurons after SNL show no increased Ca^{2+} release after priming by activation. Two-way ANOVA with Bonferroni *post-hoc* testing, numbers in the bars indicate n for number of neurons, brackets above bars connect groups that are significantly different ($P<0.05$), and error bars show standard deviation. After K$^+$ priming, transient area is reduced in L5 neurons compare to both control and L4 neurons (significance bar not shown).

ER Luminal Ca^{2+} Measurement by Mag-Fura-2

A. Intact Neurons

B. Membrane Permeabilization

C. Permeabilized Neurons
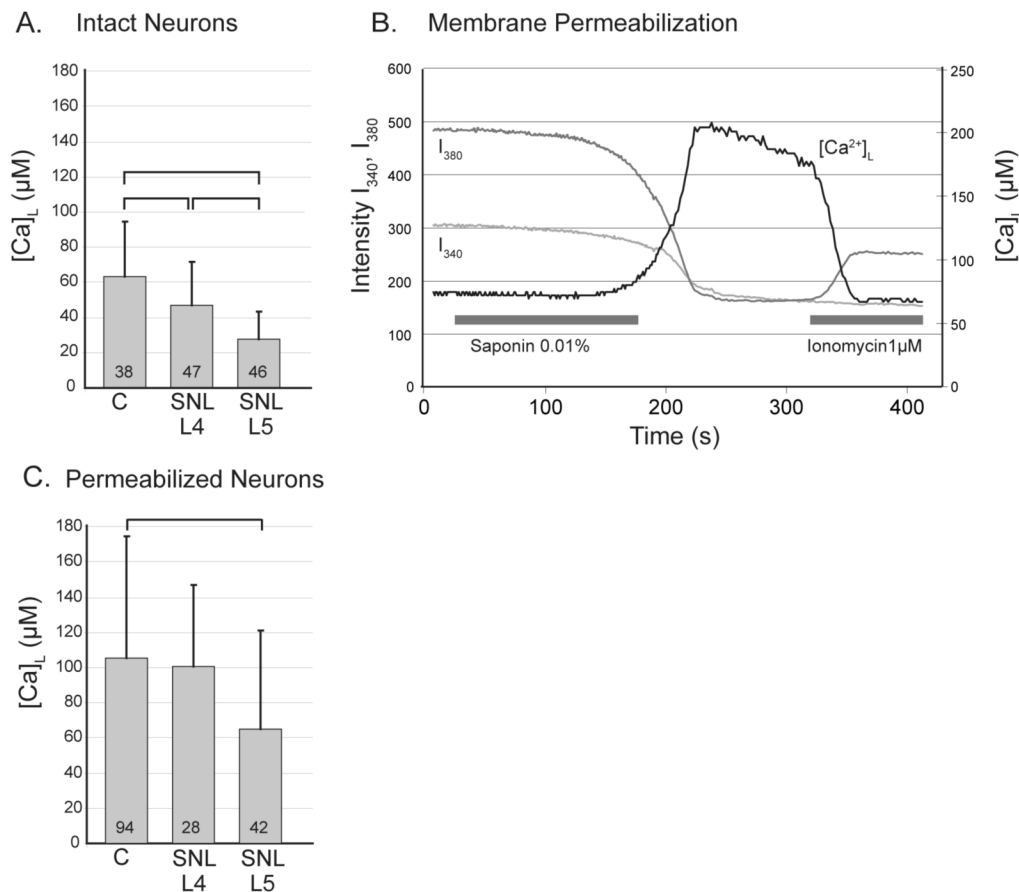
Figure 7.
Determination of the Ca^{2+} concentration in the endoplasmic reticulum (ER) lumen ($[Ca^{2+}]_L$) using the low affinity fluorophore mag-Fura-2. A. In non-permeabilized neurons, the estimated $[Ca^{2+}]_L$ is lower than control in neurons from the fourth lumbar (L4) dorsal root ganglion after spinal nerve ligation (SNL), and lower in L5 neurons than both other groups. B. Application of saponin in a bath solution resembling intracellular solution (free $[Ca^{2+}] \sim 39$ nM) allows washout of cytoplasmic mag-Fura-2, indicated by a decrease in intensity of fluorescence emission during both 380nm (I_{380}) and 340nm (I_{340}) excitation. The resulting $[Ca^{2+}]_L$ calculated from the I_{340}/I_{380} ratio increased to a peak indicative of $[Ca^{2+}]_L$. Subsequent application of ionomycin releases Ca^{2+} from the stores, represented by decreased $[Ca^{2+}]_L$. C. The $[Ca^{2+}]_L$ in permeabilized neurons is depressed in axotomized L5 neurons after SNL compared to control. For panels A and C, one-way ANOVA with Bonferroni *post-hoc* testing was used, numbers in the bars indicate n for number of neurons, brackets above bars connect groups that are significantly different ($P<0.05$), and error bars show standard deviation.