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Paclitaxel-induced increase in mitochondrial volume mediates dysregulation of intracellular Ca^{2+} in putative nociceptive glabrous skin neurons from the rat

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Abstract

We have recently demonstrated that in a rat model of chemotherapy-induced peripheral neuropathy (CIPN), there is a significant decrease in the duration of the depolarization-evoked Ca^{2+} transient in small diameter, IB4+, and capsaicin-responsive neurons innervating the glabrous skin of the hindpaw. This change was specific to the transient duration and significantly smaller if not undetectable in neurons innervating the dorsal skin of the hindpaw or the skin of the inner thigh. Given the importance of mitochondria in intracellular Ca^{2+} regulation and the findings of chemotherapy-associated increase in mitotoxicity along the sensory neuron axons, we hypothesized that CIPN is due to both increases and decreases in mitochondria function, with changes manifest in distinct subpopulations of afferents. To begin to test this hypothesis, we used confocal microscopy and Ca^{2+} imaging in combination with pharmacological manipulations to study paclitaxel-induced changes in retrograde tracer-labeled neurons from naïve, vehicle-treated, and paclitaxel-treated rats. Paclitaxel treatment was not associated with decreased mitochondrial membrane potential or increased superoxide levels in the somata of putative nociceptive glabrous skin neurons. However, it was associated with significant increases in the relative contribution of mitochondria to the control of the evoked Ca^{2+} transient duration in putative nociceptive glabrous skin neurons, as well as increases in mitotracker and Tom20 staining which reflected an increase in mitochondrial volume. Furthermore, the relative contribution of the sarco-endoplasmic reticulum Ca^{2+} ATPase to the regulation of the duration of the depolarization evoked Ca^{2+} transient was also increased in this subpopulation of neurons from paclitaxel treated rats. Our results indicate that the paclitaxel-induced decrease in the duration of the evoked Ca^{2+} transient is due to both direct and indirect influences of mitochondria. It remains to be determined if and how these changes contribute to the manifestation of CIPN.

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1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is not only one of the leading dose-limiting side effect of chemotherapy but the primary reason for therapy cessation [1,2]. CIPN generally starts with tingling and numbness in the distal appendages and progresses to a painful neuropathy, severity of which escalates with cumulative dosage [2,3].

We have recently demonstrated in a rat model of paclitaxel-induced CIPN, that the presence of mechanical hypersensitivity is associated with a dysregulation of intracellular Ca^{2+} in a subpopulation of putative nociceptive neurons. Specifically, we observed a significant decrease in the duration of the depolarization-evoked Ca^{2+} transient, with no changes in either the transient peak, or in resting Ca^{2+} [4]. Having recently described an inflammation-induced decrease in the activity of the Na^{+} - Ca^{2+} -exchanger (NCX) that was responsible for the inflammation-induced *increase* in the duration of the evoked Ca^{2+} transient [5] in the same population of neurons in which paclitaxel produced a *decrease* in the evoked transient duration, our initial hypothesis was that an increase in NCX activity was responsible for the paclitaxel-induced changes. However, while paclitaxel does drive changes in NCX activity in a different subpopulation of neurons, we were able to rule out an increase in NCX activity as the mechanism underlying the paclitaxel-induced decrease in transient duration [6]. Thus, in the present study, we have pursued an alternative hypothesis regarding the mechanism(s) underlying the paclitaxel-induced decrease in the duration of the depolarization evoked Ca^{2+} transient.

Given the weight of evidence in support of mitotoxicity as a mechanism responsible for the numbness, tingling, and pain associated with CIPN [7], in combination with both a direct and indirect role for mitochondria in the regulation of intracellular Ca^{2+} , a role for mitochondria in the paclitaxel-induced dysregulation of intracellular Ca^{2+} would seem to be an even more compelling hypothesis than that in support of a role for NCX. Based on several different lines of evidence, Bennett and colleagues proposed that an increase in reactive oxygen species (ROS) combined with the chronic energy deficit associated with chemotherapeutic-induced mitotoxicity are responsible for both spontaneous discharge in afferents with myelinated (tingling) and unmyelinated (pain) axons, and degeneration of peripheral afferent terminals (numbness) [7]. Mitotoxicity has been documented in several different animal models of CIPN, which is characterized by mitochondrial swelling, an increase in the percentage of vacuolated mitochondria, and reduced rates of ATP production and respiration along sensory axons [8–11]. The morphological changes in mitochondria were observed in both small and large diameter sensory but not in motor fibers [9]. In addition, the highest level of paclitaxel accumulation was detected in dorsal root ganglia (DRG) [9], raising the possibility that mitochondria damage is initiated at the sensory neuron cell body. Because longer nerves would be particularly vulnerable to the deleterious impact of mitochondria damage, mitotoxicity has also been suggested to account for the “stocking glove” distribution of CIPN [12].

Mitochondria are able to attenuate increases in intracellular Ca^{2+} directly and indirectly. They do so directly *via* the mitochondrial uniporter (MCU), enabling Ca^{2+} uptake by mitochondria. They do so indirectly by providing the ATP necessary for primary active

transporters including the plasma membrane Ca^{2+} ATPase (PMCA) and sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), which decrease cytosolic Ca^{2+} *via* extrusion and ER uptake, respectively. Mitochondria also influence secondary active transporters such as NCX *via* the ionic gradients maintained by primary active transporters such as the $\text{Na}^{+}/\text{K}^{+}$ -ATPase. Thus, an increase in mitochondria and/or mitochondrial activity could account for the paclitaxel-induced decrease in the evoked Ca^{2+} transient. The problem with this suggestion, however, is that if mitochondria are damaged, there should be an attenuation of mitochondria-dependent Ca^{2+} uptake which should result in an evoked Ca^{2+} transient with a longer [13], not a shorter duration.

We suggest, however, that it is possible for chemotherapeutics to contribute to both gain- and loss-of mitochondrial function, if the changes in mitochondria occur in different subpopulations of neurons. Consistent with this suggestion is the observation that despite being referred to as a “stocking-glove” distribution, more detailed analysis indicates that CIPN symptoms are preferentially localized in the glabrous skin of the hands and feet [14]. We have recently demonstrated this far more restricted manifestation of CIPN in a rat model of paclitaxel-induced CIPN, where mechanical hypersensitivity was detected in the glabrous skin of the hindpaw but not in the dorsal skin of the hindpaw or the inner thigh [4]. Minimally, this observation argues against the axon length-dependence of the manifestation of CIPN, but also supports the suggestion that there is a sensory neuron subpopulation specific vulnerability to the actions of chemotherapeutics.

Furthermore, we and others have not only documented differences among sensory neuron subpopulations with respect to both the magnitude and the duration of the depolarization-evoked Ca^{2+} transients and the underlying Ca^{2+} regulatory pathways [13], but there are now a number of lines of evidence supporting the presence of a sensory neuron subpopulation specific response to injury [15–17]. Most relevant in this regard, the paclitaxel-induced reduction in the duration of the depolarization-evoked Ca^{2+} transient in putative nociceptive afferents was significantly larger in neurons innervating the glabrous skin of the hindpaw in comparison to those innervating the hairy skin of the hind paw or the inner thigh [4]. Similarly, while target of innervation was not determined, morphological evidence of mitotoxicity was only observed in 25–40% of axons [8,9]. Thus, we hypothesize that CIPN is due to both increases and decreases in mitochondria function, with changes manifest in distinct subpopulations of afferents.

To begin to test this hypothesis, we used immunohistochemistry, live confocal microscopy, and Ca^{2+} imaging in combination with pharmacological manipulations to study paclitaxel-induced changes in the subpopulation of putative nociceptive neurons in which we have previously demonstrated that intracellular Ca^{2+} is dysregulated [4,18]. This subpopulation of putative nociceptive glabrous skin neurons was identified with the retrograde tracers, DiI or DiO, had a small cell body diameter, was labeled by isolectin B4 (IB4+), and was responsive to capsaicin. Neurons studied were from naïve, vehicle-treated, and paclitaxel-treated rats. Our results suggest that the paclitaxel-induced decrease in the duration of the evoked Ca^{2+} transient in putative nociceptive glabrous skin neurons is indeed mediated by an increase in mitochondria function, facilitating an increase in the clearance of Ca^{2+} from the cytosol both *via* direct and indirect mechanisms.

2. Experimental procedures

2.1. Animals

Adult (250–320 g) male Sprague-Dawley rats (Envigo, Indianapolis, IN) were used for all experiments. While preliminary results suggested that there is a sex difference in the development of chemotherapy-induced mechanical sensitivity in rats, only male were used in this study for two major reasons. First, male rats were used on all our previous studies with this model. Second, the study was already very large, with groups defined by target of innervation and treatment. Thus, while potentially interesting and important, we felt this potential sex difference should be pursued in future studies. Rats were housed two per cage in a temperature and humidity controlled, Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited animal housing facility on a 12 h:12 h light:dark schedule. Food and water were available *ad libitum*. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals in research.

2.2. Retrograde labeling

1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) or 3,3'-Diocadecyloxacarbocyanine Perchlorate (DiO) was injected intradermally at one of three different locations so as to identify subpopulations of cutaneous afferents based on the target of innervation. These sites included the glabrous skin of the hind paw, the hairy skin on the dorsal side of the hind paw, and the hairy skin of the upper inner thigh. The hair covering the thigh was removed with an electrical shaver before retrograde labeling with DiI or DiO. The retrograde tracer was injected bilaterally under isoflurane (Abbott Laboratories, North Chicago, IL) anesthesia with a 30 g needle at 3–5 sites per target for a total volume of 10 µL in the dorsal and glabrous hindpaw and 20 µL in the thigh. Only one tracer was injected into each animal.

2.3. Paclitaxel treatment

One week following the DiI or DiO injection, rats were anesthetized with isoflurane and injected into the tail vein with 2 mg/kg paclitaxel or its vehicle (1:1:23, cremophor EL:ethanol:0.9% saline). The tail vein injection was repeated three more times every other day for a total of four injections.

2.4. Behavioral assessment

All behavioral data were collected in the Rodent Behavior Analysis Core of the University of Pittsburgh, School of Medicine by an experimenter blinded to the test group. Rats were habituated to the testing procedure, equipment, and the experimenter for two to three days before the collection of baseline data. For the cold hypersensitivity test, rats were placed individually in acrylic clear boxes (10 cm × 20 cm), separated by opaque dividers. The boxes were set on a 1/8 inch-thick glass platform. The barrel of a 3 mL syringe filled with powdered dry ice, was placed on the surface of the glass platform precisely beneath the footpad of the hindpaw. Paw withdrawal latency was recorded three times per paw, with an

interval of at least five min for each paw. The cold hypersensitivity test was conducted three times per week.

For assessment of changes in mechanical sensitivity in the glabrous skin, the same clear acrylic boxes were used, except for this test, the boxes were placed on an aluminum mesh. Four g and 15 g von Frey filaments were applied to the center foot pad of the hind paw at a right angle from below the mesh as previously described [8]. Each filament was applied five times per paw (10 times total per rat), at a frequency of 3–5 min. Paw withdrawal response probability was calculated as, $P = \text{number of withdrawal responses}/10 \text{ trials}$. Mechanical assessments were conducted once per week on a day that the rats were not tested for cold sensitivity.

2.5. Sensory neuron isolation

Rats were deeply anesthetized with an intraperitoneal injection (1 mL/kg) of an anesthetic cocktail containing ketamine (55 mg/kg), xylazine (5.5 mg/kg) and acepromazine (1.1 mg/kg). L4 and L5 DRGs were removed bilaterally, enzymatically treated, and mechanically dissociated. DRG neurons were plated on laminin (Thermo-Fisher, 1 mg/mL) and poly-L-ornithine (Sigma-Aldrich, 1 mg/mL) coated glass coverslips as previously described [13]. For immunocytochemistry experiments, chamber slides (EMD Millipore, Billerica, MA, USA) were used instead of glass coverslips. All subsequent experiments were performed within 8 h of tissue harvest.

2.6. Fura-2, Rhod-2, and TMRM Imaging

Fura-2 was used to monitor changes in free cytosolic Ca^{2+} , Rhod-2 was used to monitor relative changes in mitochondrial Ca^{2+} [19], and TMRM was used to assess the mitochondrial membrane potential [20]. Neurons from DiI or DiO-labeled rats were loaded with Fura-2 AM ester (2.5 μM) with 0.025% Pluronic F-127 for 20 min at room temperature. For mitochondrial Ca^{2+} experiments, neurons from DiO-labeled rats were loaded with Rhod-2 AM ester (500 nM) in combination with Fura-2 AM ester + Pluronic F-127 for 20 min at room temperature. For mitochondrial membrane potential experiments, neurons were loaded with TMRM 50 nM with Fura-2 AM ester + Pluronic F-127 for 60 min at room temperature, and this TMRM concentration was maintained in solutions used throughout imaging experiments. Following loading with Ca^{2+} indicators or TMRM, neurons were incubated with FITC, or Alexa Fluor 647-conjugated IB4 (10 $\mu\text{g/mL}$) for 10 min at room temperature. They were then placed in a recording chamber and continuously superfused with a 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)-buffered bath solution (HBS) consisting of (in mM): 130 NaCl, 3 KCl, 2.5 CaCl_2 , 0.6 MgCl_2 , 10 HEPES, 10 glucose, pH 7.4, osmolality 325 mOsm. Fluorescence data were acquired on a PC running Metafluor software (Molecular Devices, Sunnyvale, CA) via an EMCCD camera (Photometrics, Tucson, AZ; model QuantEM 512SC). For Fura-2 imaging, the ratio (R) of fluorescence emission (510 nm) in response to 340/380 nm excitation (controlled by a DG-4 (Sutter Instrument, Novato, CA)) was acquired at 1 Hz during application of KCl or capsaicin, which were applied through a computer-controlled, piezo-driven perfusion system (switching time <20 ms; Warner Instruments, Hamden, CT, USA, Fast-Step Model SF-77B). $[\text{Ca}^{2+}]_i$ was determined

from Fura-2 ratio according to the equation $[Ca^{2+}]_i \text{ (nM)} = K_d (S_{f2}/S_{b2}) ((R-R_{min})/(R_{max}-R))$ following *in situ* calibration as described previously [21], where K_d is the dissociation constant for Fura-2 for Ca^{2+} at room temperature; S_{f2}/S_{b2} is the fluorescence ratio of the emission intensity excited with the 380 nm wavelength in the absence of Ca^{2+} to that in the presence of saturating Ca^{2+} ; R_{min} and R_{max} are the minimal and maximal fluorescence ratios, respectively. S_{f2}/S_{b2} , R_{min} and R_{max} were determined empirically with a calibration protocol as described previously [22], run every ~six weeks throughout the data collection period. The signal intensity of Rhod-2 or TMRM at 580 nm in response to excitation at 555 nm was acquired simultaneously with Fura-2 acquisition with a custom dichroic enabling excitation at 340, 380 and 555, and a band pass emission filter enabling detection of light emitted at wavelengths from 470 to 530 nm and from 580 to 640 nm (Chroma Technology, Bellows Fall, VT). Rhod-2 intensity values were plotted as a change in fluorescence over baseline ($\Delta F/F$). TMRM fluorescence in mitochondria (F_{mito}) was estimated by the decrease in fluorescence observed following application of CCCP/oligomycin. TMRM fluorescence in the cytosol (F_{cyto}) was estimated by the decrease in fluorescence observed following application of 30 mM K^+ .

Neurons from a single field were studied on each coverslip. Resting Ca^{2+} or Rhod-2 intensity were determined prior to stimulation, where the average of the 30 s just prior to stimulation (with high K^+ (30 mM)) was used for this value. The magnitude of the evoked Ca^{2+} transient was determined as the difference between resting and the peak of the evoked Ca^{2+} transient. The duration of the evoked Ca^{2+} transient was determined as the time for the transient to decay to 25%, 50%, 75%, and 90% of the peak (T_{25} , T_{50} , T_{75} , and T_{90} , respectively). For experiments involving the application of test compounds, a vehicle control group was always included. Neurons with a small cell body diameter ($<30 \mu m$), responsive to the capsaicin (500 nM), and labeled with the isolectin IB4, determined under epifluorescence illumination, are referred to as putative nociceptors. Cell body diameter was determined with a calibrated eye-piece reticle. Capsaicin sensitivity was assessed at the end of every experiment and neurons were considered capsaicin sensitive if application of capsaicin resulted in an increase in $[Ca^{2+}]_i$ greater than 20% above baseline. Neurons in which the plasma membrane was clearly defined by epifluorescence were considered IB4+.

2.7. Live cell confocal microscopy

All live cell confocal imaging experiments were carried using a Nikon A1R upright confocal microscope system, controlled with the Nikon (NIS) Elements software. Dissociated neurons retrogradely labeled with DiI or DiO depending on the mitochondria reagent used were loaded with one or when appropriate with various combinations with Mitotracker Green (200 nM in HBS), Mitotracker Deep Red (200 nM in HBS), and/or Mitosox (250 nM in HBS) in the dark for 30–60 min at room temperature. At the same time, they were labeled with IB4-Alexa Fluor 647 (1 $\mu g/mL$ in HBS). Neurons were then washed 10 min in HBS and placed in a live cell chamber on the microscope stage containing HBS. All experiments were carried out at room temperature. For Mitotracker green studies, images were collected with a $60\times$ water immersion objective ($NA = 1$) in a series of $3 \mu m$ -thick optical sections extending through the entire neuron at a 512×512 pixel resolution and a $1/2$ frame/sec rate. For all other experiments, a single $3 \mu m$ optical section obtained at a 2048×2048 -pixel

resolution and a 1/8 frame/sec rate was collected with the same 60× objective, with the optical section oriented at the center of each retrograde tracer-labeled neuron. Excitation/detection settings were determined with preliminary experiments using neurons from naïve rats with each fluorophore to yield an image that was not saturated. The same settings were applied for all conditions (naïve, vehicle, paclitaxel). For all experiments neurons from paclitaxel- and vehicle-treated rats, as well as naïve rats were tested the same day. Nikon (NIS) Elements and ImageJ software were used for quantification of fluorescence signals. Neurons from a single field were studied for TMRM and mitoxox experiments within three min of placement into the live cell chamber to minimize variability.

2.8. Fluorescence immunocytochemistry

Neurons plated on chamber slides were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature and then washed three times in PBS. Slides first were incubated in blocking buffer (0.2% Triton X-100, 3% normal donkey serum in PBS) for one hour at room temperature and then incubated with anti-Tom20 primary antibody [23,24] at a 1:2000 dilution in the blocking buffer overnight at 4 °C. Slides were washed three times for 10 min each in Tris-buffered saline with Tween 20 (TBST) at room temperature and then incubated with an AlexaFluor 488-conjugated donkey anti-rabbit secondary antibody at 1:200 dilution in the blocking buffer for one hour at room temperature. Slides were washed two times 10 min each in TBST and incubated in TBST containing 1 µg/mL AlexaFluor 647-conjugated IB4 and 1:1000 DAPI. Slides were washed one last time in TBST for 10 min and subsequently coverslipped using Fluoromount (Sigma-Aldrich). The Tom20 images were collected with a 100× oil immersion objective (NA = 1.45) in a series of 0.125 µm-thick optical sections extending through the entire neuron at a 2048 × 2048 pixel resolution and a 1/8 frame/sec rate using Nikon (NIS) Elements software. The same settings were applied for all neurons. The images in the stacks were deconvolved using the blind configuration for 20 iterations with Nikon (NIS) Elements software. Following deconvolution, the same 3D threshold setting was applied to all neurons to enable estimation of the volume and number of immunolabeled structures.

2.9. Chemicals

Paclitaxel (Sigma-Aldrich, St Louis, MO, USA), was dissolved at 25 mg/mL in 1:1 Cremophor EL: ethanol and freshly diluted 1:12.5 in 0.9% sterile saline prior to injections. DiI (Thermo-Fisher, Waltham, MA, USA), was dissolved at 170 mg/mL in anhydrous dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted 1:10 in 0.9% sterile saline for a final concentration of 17 mg/mL. DiO (Thermo-Fisher), was dissolved at a concentration of 10% (wt/vol) in anhydrous DMSO and diluted 1:10 in 0.9% sterile saline for a final concentration of 1%. Both DiI and DiO were freshly prepared before injections. As DiO is more susceptible to precipitation after the addition of saline, a small amount of fresh DiO stock was diluted in saline immediately before injection for each paw. FITC-conjugated Isolectin B4 (IB4, Sigma-Aldrich) was dissolved in dH₂O as a stock solution of 1 mg/mL, and then diluted to a final concentration of 5 µg/mL in HEPES bath solution (HBS) the day of use. Alexa Fluor 647-conjugated IB4 (Thermo-Fisher) was dissolved in dH₂O 0.5 mM stock solution and diluted to a final concentration of 1 µg/mL in HBS. Fura-2 AM ester (TEF Laboratories, Austin, TX, USA) was dissolved in DMSO as a 2.5 mM stock solution

and diluted to a final concentration of 2.5 μM in HBS. Pluronic F-127 (TEF Laboratories) was dissolved in DMSO as a 25% stock solution and diluted to 0.025% in HBS. Rhod-2 AM ester (Thermo-Fisher) was dissolved in DMSO as a 5 mM stock solution and diluted to a final concentration of 500 nM in HBS. Capsaicin (Sigma-Aldrich) was dissolved in ethanol as a 10 mM stock solution and diluted to 500 nM in HBS. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and Cyclopiazonic acid (CPA) (both from Sigma-Aldrich) were dissolved in DMSO as 100 mM stocks and diluted to 10 μM in HBS. Oligomycin A (Tocris, Bristol, UK) was dissolved in DMSO as a 10 mM stock and diluted to 10 μM in HBS. Mitotracker Green FM and Mitotracker Deep Red FM (both from Thermo-Fisher) were dissolved in DMSO as 1 mM stocks and diluted to 200 nM in HBS. Mitosox (Thermo-Fisher) was dissolved in DMSO as a 1 mM stock and diluted to 250 nM.

Tetramethylrhodamine Methyl Ester Perchlorate (TMRM, Thermo-Fisher) was dissolved in DMSO as a 10 mM stock and diluted to 50 nM. Rabbit polyclonal anti-Tom20 antibody (Cat # sc-11415, Santa Cruz, Dallas, TX, USA: Antibody Registry: AB 2207533) was diluted in blocking buffer (0.2% Triton X-100, 3% normal donkey serum in phosphate buffer solution (PBS)) to 1:2000. Alexa Fluor 488-conjugated donkey anti-rabbit antibody (Jackson Immuno Research, West Grove, PA, USA) was diluted 1:200 in blocking buffer. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Thermo-Fisher) was diluted in TBST to 1:1000.

2.10. Statistical analysis

Data are expressed as mean $\pm$ s.e.m. One and two-way ANOVA were used for analysis of more than two groups with the Holm-Sidak test used for post-hoc analysis. Statistical significance was assessed at $p < 0.05$.

3. Results

3.1. The paclitaxel-induced decrease in the duration of the depolarization evoked Ca^{2+} transient was fully resolved at a time point at which the paclitaxel-induced hypersensitivity had resolved as well

We previously described the paclitaxel-induced decrease in the duration of the depolarization evoked Ca^{2+} transient in putative nociceptive neurons that were isolated during a period of peak mechanical hypersensitivity (*i.e.*, one week after the last paclitaxel injection) [4]. To further assess the association between the paclitaxel-induced decrease in the transient duration and pain behavior, we assessed the resting and evoked Ca^{2+} transient in neurons from rats in which paclitaxel-induced hypersensitivity was no longer detectable. So that behavioral data could be collected in a blinded fashion, paclitaxel and vehicle treated rats were run in parallel. The probability of paw withdrawal in response to stimulation of the glabrous skin with 4 g and 15 g von Frey filaments increased within the first week of treatment and resolved by day 39 (Fig. 1A and B, $p > 0.05$). For the same group of rats, cold sensitivity was detectable in paclitaxel-treated rats ($n = 6$) after the first day of treatment (Fig. 1C), with withdrawal latencies significantly ($p < 0.01$) shorter than those in vehicle treated rats ($n = 6$). This difference persisted through day 28 post-treatment. While we have observed a significant decrease in the duration of the evoked Ca^{2+} transient in putative nociceptive glabrous neurons from 27 different cohorts of rats one week after the last

paclitaxel injection [4,6], by six weeks after the last paclitaxel injection, there was no detectable difference between neurons from paclitaxel ($n = 12$ neurons from 4 rats) and vehicle ($n = 12$ neurons from 4 rats) treated rats with respect to the duration of evoked Ca^{2+} transients ($p > 0.05$, Fig. 1D and E). Nor were there differences between these groups with respect to resting $[\text{Ca}^{2+}]_i$, (138.6 ± 6.04 (vehicle) and 148.8 ± 7.63 (paclitaxel)) or the magnitude of the evoked Ca^{2+} transient (260.6 ± 27.6 (vehicle) and 288.2 ± 21.9 (paclitaxel)).

3.2. Mitochondrial $[\text{Ca}^{2+}]_i$ buffering was increased in putative nociceptive glabrous skin neurons from paclitaxel-treated rats

Evoked Ca^{2+} transients in putative nociceptive neurons is generally bi-phasic, with a rapidly decaying phase followed by a more slowly decaying phase that results in the emergence of a “shoulder” at 30–40% of the peak increase (Fig. 2A). Mitochondrial “buffering” of Ca^{2+} is thought to contribute to this unique decay profile: The initial decay of the evoked transient is thought to be due to the rapid uptake of cytosolic Ca^{2+} by mitochondria *via* the mitochondrial Ca^{2+} uniporter (MCU), while the “shoulder” would be due to the slow release of Ca^{2+} from mitochondria *via* the mitochondrial $\text{Na}^+/\text{Li}^+-\text{Ca}^{2+}$ -exchanger (NLCX) [25,26]. One prediction of this model is that if mitochondrial Ca^{2+} uptake is increased, the rapid phase would make up a greater proportion of the transient and the slow-decaying phase would become even slower. To test this prediction, we performed a more detailed analysis of the evoked Ca^{2+} transient decay in putative nociceptive glabrous skin neurons from vehicle- and paclitaxel-treated rats. In this and all subsequent experiments, neurons were harvested one week after the last IV injection, at a time point at which hypersensitivity was fully manifest and well before the onset of recovery. Results of this analysis indicated that while there was no detectable influence of paclitaxel on the relative contribution of the rapid component to the overall decay of the evoked Ca^{2+} transient (which was $29.44 \pm 2.04\%$ and $29.91 \pm 2.10\%$ in neurons from vehicle and paclitaxel treated rats, respectively), the duration of the evoked transient was significantly (one-way repeated measures ANOVA, $p < 0.01$) shorter throughout the decay of the evoked transient in neurons ($n = 18$) from paclitaxel treated rats compared to neurons from vehicle treated rats ($n = 18$, Fig. 2A inset). To more clearly illustrate the decrease in duration throughout the decay of the transient, data in Fig. 2A were analyzed as a percent change from vehicle. The percent decrease in duration at each time-point throughout the decay were comparable (one-way ANOVA $p > 0.05$, Fig. 2B).

To more directly assess the contribution of mitochondria to the decay of the evoked Ca^{2+} transient we assessed the impact of pharmacologically blocking mitochondrial Ca^{2+} buffering in neurons from vehicle- and paclitaxel-treated rats on the evoked Ca^{2+} transient. Furthermore, based on previous data indicating that the impact of paclitaxel is significantly greater in glabrous skin neurons, than in neurons innervating structures such as the dorsal skin of the hindpaw or the inner thigh, where there was no detectable changes in mechanical sensitivity, we also assessed the impact of blocking mitochondrial Ca^{2+} uptake in neurons innervating these structures from vehicle- and paclitaxel-treated rats. Mitochondrial Ca^{2+} uptake depends on an intact mitochondrial membrane potential and uncouplers such as CCCP and FCCP are widely used to diminish the Ca^{2+} uptake into these organelles [27]. To block mitochondrial Ca^{2+} uptake without ATP depletion due to the membrane potential loss-

induced reversal of the ATP synthase [28], we applied the uncoupler CCCP (10 μ M) together with ATP synthase inhibitor oligomycin (10 μ M) (Fig. 2C). Application of CCCP/oligomycin caused an increase in the duration of evoked Ca^{2+} transient in all putative nociceptors (Fig. 2D). However, the CCCP/oligomycin-induced increase in the transient duration was significantly ($p < 0.01$, two-way ANOVA, with the Holm-Sidak post-hoc test) larger in putative nociceptive glabrous skin neurons from paclitaxel-treated rats ($n = 13$) when compared to all other groups included in the analysis. These included unlabeled neurons from the same coverslips ($n = 15$), glabrous skin neurons from vehicle treated rats ($n = 13$), and dorsal skin neurons of the hindpaw ($n = 10$, and 11) or inner thigh neurons ($n = 12$ and 8) from vehicle- and paclitaxel-treated rats (Fig. 2D). To determine whether there were differences between groups of putative nociceptive neurons defined by target of innervation with respect to the CCCP/oligomycin-induced increase in the duration of the evoked Ca^{2+} transient, data from paclitaxel treated rats were analyzed as a change relative to the mean increase in the respective subpopulation of neurons from vehicle treated rats. Analysis of group mean data with a one-way ANOVA indicated that there was a significant difference between groups ($p < 0.01$). Post-hoc analysis showed that while there was no difference among unlabeled, hairy hindpaw, and thigh skin neurons with respect to the CCCP/oligomycin-induced increase in T50, the increase in the duration of the evoked transient in putative nociceptive glabrous skin neurons was significantly greater than that in other groups of neurons (Fig. 2E).

3.3. While there were differences between putative nociceptive and non-nociceptive DRG neurons with respect to resting mitochondrial Ca^{2+} levels, paclitaxel was not associated with an increase in mitochondrial Ca^{2+} uptake in putative nociceptive glabrous skin neurons

Because our results with CCCP/oligomycin suggested that a relative increase in mitochondrial Ca^{2+} uptake contributed to the observed decrease in the duration of the evoked Ca^{2+} transient in putative nociceptive glabrous skin neurons, we next sought to determine whether this influence of mitochondria was due to an increase in the amount of Ca^{2+} taken up by mitochondria. To address this issue, mitochondrial Ca^{2+} levels were assessed in DiO-labeled neurons with Rhod-2 in conjunction with Fura-2 imaging (Fig. 3A). Since Rhod-2 at concentrations greater than 2 μ M is associated with reduced specificity and mitochondrial damage [29], we used a low concentration of Rhod-2 (500 nM). Furthermore, because Rhod-2 in the cytosol will contaminate the mitochondrial signal, we first confirmed that depolarization-evoked Rhod-2 transients reflected changes in mitochondria Ca^{2+} levels by assessing the impact of CCCP/oligomycin on the high K^{+} -evoked Rhod-2 transient. Treating neurons with CCCP/oligomycin for three min, eliminated the majority of Rhod-2 transient (Fig. 3B and 3C). Interestingly, in neurons from naïve rats, resting Rhod-2 levels were significantly ($p < 0.05$) higher in putative non-nociceptive glabrous skin neurons ($n = 6$, large cell body diameter, IB4(-), capsaicin unresponsive), than in putative nociceptive glabrous skin neurons ($n = 17$, Fig. 3D). In addition, the magnitude of the depolarization evoked Rhod-2 transient was significantly smaller ($p < 0.01$) in putative non-nociceptive neurons (Fig. 3E and F), although there was no difference between these two groups with respect to the duration ($p > 0.05$) of the depolarization evoked Rhod-2 transient (Fig. 3G). More importantly, there was no detectable influence of paclitaxel on resting Rhod-2 levels,

or on either the magnitude or duration of the depolarization-induced increase in Rhod-2 levels, in putative nociceptive glabrous skin neurons ($n = 17$), compared to putative nociceptive glabrous skin neurons from vehicle treated rats ($n = 15$, Fig. 3H–K).

3.4. Paclitaxel was associated with an increase in mitochondrial volume in putative nociceptive glabrous skin neurons

Our results with CCCP/oligomycin and Rhod-2 raised the possibility that the apparent increase in mitochondrial Ca^{2+} buffering in neurons from paclitaxel treated rats was due to an increase in the number of mitochondria in these neurons. Three additional experiments were used to test this possibility. The first of these involved the use of mitotracker green, a fluorescent compound that binds to thiol groups on mitochondria, enabling an estimation of total mitochondria volume/area from the relative intensity of the mitotracker fluorescence [30]. Putative nociceptive neurons targeting either the glabrous, dorsal hindpaw, or inner thigh skin from naïve, vehicle-, and paclitaxel-treated rats were studied. The neurons from each group were analyzed in parallel with the same confocal settings for image acquisition. The average fluorescence intensity per unit area was determined for each neuron, by dividing the cumulative background-subtracted intensity over the entire cell area minus the area of the nucleus (Fig. 4A). Representative images (Fig. 4A) suggested that mitotracker levels were higher in glabrous neurons from paclitaxel treated rats (Fig. 4A). Pooled data confirmed this impression, as the fluorescence per μm^2 in glabrous neurons from paclitaxel treated rats ($n = 50$) was significantly ($p < 0.01$, Holm-Sidak post-hoc test), higher than that in glabrous neurons from vehicle or naïve rats ($n = 26$ (naïve) and 41 (vehicle)), or from dorsal hindpaw ($n = 20, 30, 36$) or thigh skin ($n = 23, 29, 31$) neurons from naïve, vehicle treated, or paclitaxel treated rats, respectively (Fig. 4B and C).

Because an increase in mitotracker fluorescence may reflect an increase in the thiol groups the dye binds to rather than an increase in mitochondria volume/area, we performed a second complimentary experiment using the antibody against the 20 kDa mitochondrial protein, translocase of outer membrane 20 (Tom20). The specificity of this antibody has been confirmed elsewhere [31]. Only small diameter IB4(+) glabrous skin neurons from naïve, vehicle-treated and paclitaxel-treated rats were studied. As with mitotracker, there appeared to be more Tom20 staining in neurons from paclitaxel treated rats, than from vehicle treated rats (Fig. 4D). Group mean data again confirmed this impression, as the fluorescence per μm^2 , in neurons from paclitaxel treated rats ($n = 19$) was significantly higher than that in neurons from vehicle ($n = 19$) rats (Fig. 4E, t -test, $p < 0.01$).

Next, to confirm that the increase in Tom20 staining was due to an increase in mitochondria, neurons from each group were analyzed with higher resolution imaging techniques combined with image deconvolution post-processing, to facilitate resolution of individual mitochondria in each neuron. With post-processing in which the same settings were used to analyze the Tom20 staining pattern, it was possible to estimate the relative volume, size, and number of mitochondria in each neuron (Fig. 5A). Group mean data indicate that total mitochondrial volume analyzed as a percent of the total cytosolic volume was significantly ($p < 0.05$) greater in putative nociceptive glabrous skin neurons ($19.41 \pm 5.53\%$, $n = 9$) from paclitaxel-treated rats than that in neurons ($6.96 \pm 2.16\%$, $n = 11$) from vehicle treated rats

(Fig. 5B). This increase in volume did not appear to be due to increase in number of mitochondria in neurons from paclitaxel treated rats, which was, in fact, significantly smaller in neurons from paclitaxel treated rats than vehicle treated rats (Fig. 5C, $p < 0.01$). Rather, the difference in mitochondrial volume appeared to be due to formation of so called “megamitochondria” [32] which presented itself as large networks of interconnected mitochondria and thus fewer smaller mitochondria.

Finally, to determine whether the paclitaxel-induced increase in mitochondria is an artifact of the dissociated neuron preparation, we conducted a preliminary analysis of Tom20 staining intensity of DiI-labeled glabrous skin neurons on 16 μm -thick L5 DRG sections taken from one vehicle- and one paclitaxel-treated rat. The results of this experiment suggest that the changes in mitochondria observed in isolated DRG neurons were not an artifact of the dissociation or culturing process (Fig. 6).

3.5. Paclitaxel did not reduce the health and functionality of mitochondria in putative nociceptive glabrous skin neurons

Studies have shown that bath application of paclitaxel to a variety of cell types is capable of causing damage to mitochondria [33,34]. As noted above, there is also ample evidence that mitochondria in sensory neuron axons are damaged by paclitaxel treatment [8,9]. Therefore, it was possible that the increase in mitochondrial volume in putative nociceptive glabrous skin neurons was a reflection of some of this damage, such as swelling. Two additional experiments were performed to address this possibility. First, because the membrane potential is necessary for essential functions such as ATP synthesis and Ca^{2+} uptake, mitochondrial membrane potential is indicative of mitochondrial functionality [27,35]. We therefore used live imaging of neurons labeled with the mitochondrial membrane potential (V_{mito})-dependent dye TMRM. However, because the intensity of TMRM staining correlates with both plasma membrane potential (V_{m}) and V_{mito} , it was necessary to determine the relative contribution cytosolic and mitochondrial TMRM fluorescence, $F_{\text{mito}}/F_{\text{cyto}}$ [20]. Neurons were continuously superfused with solutions containing TMRM to ensure a stable cell loading and to enable background subtraction (Fig. 7A). To estimate F_{cyto} , 30 mM K^{+} was used to depolarize neurons to ~ -20 mV [36], based on the assumption that difference between neurons with respect to high K^{+} evoked decrease in TMRM fluorescence would reflect differences in V_{m} . Similarly, the change in TMRM fluorescence in response to CCCP/oligomycin was used to estimate F_{mito} [35]. Interestingly, when high K^{+} was applied before CCCP/oligomycin, there was a small but significant increase in TMRM fluorescence ($\sim 5\%$) that was not different between neurons from naïve, vehicle or paclitaxel treated rats ($p > 0.05$). However, TMRM fluorescence decreased by $80.8 \pm 2.4\%$ ($n = 11$), $87.8 \pm 2.4\%$ ($n = 10$), $81.6 \pm 2.6\%$ ($n = 12$) in neurons from naïve, vehicle- and paclitaxel-treated rats, respectively, in response to the subsequent application of CCCP/oligomycin in combination with high K^{+} (data not shown).

The V_{m} -dependent drop in TMRM fluorescence may have been masked by an increase in mitochondrial TMRM secondary to hyperpolarization of V_{mito} in response to the increased demand for ATP associated with the depolarization of V_{m} and the concomitant increase in cytosolic Ca^{2+} . Therefore, we repeated this experiment, by first applying CCCP/oligomycin

followed by CCCP/oligomycin + high K^+ application. Performed in this way, there were significant drops in TMRM fluorescence in response to both CCCP/oligomycin and CCCP/oligomycin + high K^+ application (Fig. 7B). The $[Ca^{2+}]_i$ changed as expected in response to both CCCP/oligomycin, and CCCP/oligomycin + high K^+ (Fig. 7B and C). Using the changes in TMRM fluorescence to calculate F_{mito}/F_{cyto} , there was no significant difference ($p > 0.05$) between neurons from naïve ($n = 5$), vehicle ($n = 5$) and paclitaxel ($n = 4$) treated rats (Fig. 7D). The trend toward an increase in F_{mito}/F_{cyto} in neurons from paclitaxel treated rats in the absence of the opposite trend in F_{cyto} , is consistent with the suggestion that V_{mito} in neurons from paclitaxel treated rats was even more hyperpolarized than that in control neurons.

Evidence suggests that 1–2% of oxygen consumed is converted into superoxide due to transfer of electrons that have leaked from the electron transfer chain to oxygen molecules during oxidative phosphorylation [37]. Moreover, under certain conditions, such as cellular stress or mitochondrial damage, there is a surge in the number of leaked electrons, resulting in an increase in the generation of mitochondrial superoxide [38]. Thus, to further assess the presence of a subpopulation of damaged mitochondria in neurons from paclitaxel treated rats, we tested in sister coverslips the mitochondrial superoxide indicator, MitoSox Red. Antimycin A, which was used as a positive control for driving an increase in mitochondrial superoxide generation [39], produced an increase in MitoSox Red staining in DRG neurons (Fig. 8A). However, there was no detectable difference between putative nociceptive neurons innervating the glabrous skin from naïve ($n = 13$), vehicle- ($n = 14$), and paclitaxel-treated ($n = 14$) rats, with respect to the intensity of MitoSox Red staining (Fig. 8B and C, $p > 0.05$).

3.6. Paclitaxel increased ATP-dependent Ca^{2+} uptake into endoplasmic reticulum

In addition to NCX, there are two other major regulators of the duration of evoked Ca^{2+} transients, SERCA and PMCA [13,40,41]. Both of these proteins are membrane bound Ca^{2+} pumps that utilize the energy released from ATP hydrolysis to reduce the $[Ca^{2+}]_i$ [42]. Because an increase in total, particularly healthy mitochondrial volume should be associated with an increase in ATP [43], it is possible that the paclitaxel-induced change in mitochondria also contributes to the attenuation of the evoked Ca^{2+} transient indirectly by increasing Ca^{2+} ATPase activity. We tested this possibility indirectly with Fura-2 imaging before and after blocking SERCA activity with the SERCA inhibitor CPA. Inhibition of SERCA resulted in significant increases in the duration of the evoked Ca^{2+} transient in neurons from both vehicle- and paclitaxel-treated rats (Fig. 9A), confirming that SERCA contributes to the regulation of the duration of the evoked transient in putative nociceptive glabrous skin neurons. However, the relative increase in the Ca^{2+} transient duration after CPA application was significantly ($p < 0.05$) larger in neurons ($n = 31$) from paclitaxel-treated rats than that in neurons ($n = 27$) from vehicle treated rats (Fig. 9B).

4. Discussion

The purpose of this study was to begin to test the hypothesis that CIPN is due to both increases and decreases in mitochondria function, with changes manifest in distinct subpopulations of afferents. We started by further testing the association between the

changes in Ca^{2+} regulation and the changes in nociceptive behavior: changes in Ca^{2+} regulation were no longer detectable at a time point when paclitaxel-induced hypersensitivity had fully resolved. A detailed time course analysis of depolarization-evoked Ca^{2+} transients revealed that while the duration of the evoked transient was significantly shorter throughout the transient decay in neurons from paclitaxel-treated rats, the overall shape of the transient, thought to reflect mitochondrial Ca^{2+} uptake and release, was unchanged. Nevertheless, the relative impact of pharmacologically blocking mitochondrial Ca^{2+} uptake resulted in a significantly larger increase in the duration of the evoked Ca^{2+} transient in putative nociceptive glabrous skin neurons from paclitaxel treated rats than that in glabrous skin neurons from vehicle treated or naïve rats, or from hindpaw hairy skin or inner thigh skin neurons from paclitaxel treated or control rats. However, there was no detectable difference in the relative amount of Ca^{2+} taken up by mitochondria in neurons from paclitaxel-treated rats. In contrast, staining intensities of mitochondrial markers were significantly higher in putative nociceptive glabrous skin neurons from paclitaxel-treated rats than in neurons from control rats or those innervating hairy hindpaw or thigh skin. Higher resolution analysis of mitochondria in sensory neurons revealed an increase in mitochondrial volume. Furthermore, while there were differences between putative nociceptive and non-nociceptive neurons with respect to mitochondria resting membrane potential, there was no evidence of mitochondria with a low resting membrane potential in putative nociceptive glabrous skin neurons from paclitaxel treated rats. In addition, neurons from paclitaxel-treated rats had levels of mitochondrial superoxide comparable to controls. Finally, in addition to an increase in a direct role for mitochondria in the attenuation of the evoked Ca^{2+} transient, we obtained evidence of an increase in an indirect role, with at least a significant increase in the contribution of SERCA to the regulation of the evoked Ca^{2+} transient in putative nociceptive glabrous skin neurons from paclitaxel treated rats. Taken together our results support at least half of our initial hypothesis that CIPN is due to an increase in mitochondria function in a distinct subpopulations of afferents.

The resolution of the paclitaxel-induced decrease in the evoked Ca^{2+} transient duration in neurons from rats in which and cold/mechanical hypersensitivity had also resolved does not mean that these two phenomena are causally linked. However, the persistence of the Ca^{2+} dysregulation in neurons from rats in which hypersensitivity had resolved would have suggested that the changes in Ca^{2+} regulation are not sufficient to maintain hypersensitivity. That said, given the importance of the tight regulation of $[\text{Ca}^{2+}]_i$ for numerous cellular processes and that the observation of the paclitaxel-induced decrease in the evoked Ca^{2+} transient duration is limited to a specific subpopulation of neurons, establishing a causal link between the decrease in the evoked Ca^{2+} transient and pain associated with CIPN will not be easy. A more detailed analysis of the time-course of the changes in Ca^{2+} regulation may help in this regard, if only to enable rejection of the hypothesis. The use of IB4-saporin, to eliminate the neurons in which the changes are manifest, as we have done previously [5], would also help in this regard. It may also be possible to acutely manipulate Ca^{2+} levels in sensory neurons with Ca^{2+} ionophores, or Ca^{2+} uptake with blockers, as was done previously in a model of CIPN [44]. With respect to the specific role for mitochondrial Ca^{2+} , it would be possible to target the uniporter pharmacologically, where a block of MCU would be expected to normalize pain behavior. While genetic strategies may also enable a more

targeted approach, we suggest that the first step should be to determine where in the sensory neuron the changes in Ca^{2+} regulation are manifest, and now that we have identified a potential mechanism underlying these changes, such experiments should be possible.

The time course of the paclitaxel-induced mechanical sensitivity observed in the present study was consistent with that previously described [45]. However, there is evidence that mechanical hypersensitivity may persist for considerably longer [8]. While additional factors may contribute to the relative duration of the hypersensitivity, we suggest one likely factor is the route of paclitaxel administration: it was administered IV in the present study and IP in the study in which a more persistent hypersensitivity was observed. Given the impact of route of administration on pharmacokinetic and pharmacodynamics of the drug administered [46], combined with evidence that the signs and symptoms of CIPN are dose and treatment duration dependent [2,3], it will be important to determine whether the more persistent hypersensitivity reflects the same underlying mechanisms. In this regard, it was interesting to note that there was a difference in the resolution of cold and mechanical hypersensitivity, suggesting that there are sensory neuron subpopulation specific differences in the response to same dose and route of administered paclitaxel.

While we employed a variety of different analyses and approaches in this study, all were internally consistent and pointed to a direct role of mitochondria in the paclitaxel-induced decrease in the duration of the depolarization-evoked Ca^{2+} transient due to an increase in mitochondria volume, and consequently buffering capacity, rather than a change in functionality. The CCCP/oligomycin data were consistent with an increase in the relative contribution of mitochondria to the control of the duration of the evoked Ca^{2+} transient in neurons from paclitaxel treated rats. The results of the detailed analysis of the decay of the Ca^{2+} transient were consistent with an increase in the total mitochondrial buffering capacity rather than a shift in functionality, as changes to either uptake or release should have resulted in changes to the fast and/or slow components of the transient, and/or the relative proportion of the decay accounted for by either component. Consistent with this interpretation, the TMRM data argued against a change in mitochondrial resting membrane potential, where hyperpolarization could have contributed to faster Ca^{2+} uptake and/or a slower release. Conversely, the presence of even a subpopulation of damaged mitochondria with a low resting membrane potential should have resulted in a decrease in average TMRM signal. The absence of a change in mitochondrial resting membrane potential was confirmed with the Rhod-2 data, which indicated that there was no influence of paclitaxel on either the magnitude of the mitochondrial Ca^{2+} uptake, or the rate of release. These data also argue against paclitaxel-induced changes in either MCU or NCLX activity. Similarly, the absence of a detectable influence of paclitaxel on mitoxox staining, argues against the presence of even a subpopulation of injured mitochondria. On the other hand, the increase in mitotracker and Tom20 staining both point to an increase in mitochondria, where the higher resolution of the Tom20 data confirmed the increase in total mitochondria volume. Importantly, the presence of an increase in Tom20 in cutaneous afferent somata in situ, suggests that the changes observed in the present study are not an artifact of cell culture. Thus, in stark contrast to the chemotherapeutic-induced mitotoxicity described in sensory axons, the chemotherapeutic-induced changes in Ca^{2+} regulation in the cell body, appear to be due, at least in part, to an increase in otherwise healthy mitochondria.

It is known that mitochondria form a complex reticulum in many cell types [47–49], however axonal mitochondria are separated from this network and observed as individual entities of typically 1–3 μm in length [49]. Our higher resolution imaging results suggest the paclitaxel-induced increase in mitochondrial volume was reflected as an enlargement of the interconnected mitochondrial network and reduction of the number of individual smaller mitochondria. However, we cannot rule out the possibility that paclitaxel-induced increase in mitochondrial volume is due to an increase in the number of mitochondria in very close proximity that is below the resolution limit of imaging techniques employed. Additional higher-resolution techniques may be required to determine the nature of the paclitaxel-induced changes in mitochondrial volume.

There are several mechanisms that could account for the paclitaxel-induced increase in the total volume of mitochondria in putative nociceptors. First, it might be a reaction to a more distal insult associated with paclitaxel treatment, where an increase in mitochondrial volume may enable the neurons to respond to the increased demands associated with injury. Similarly, if mitochondria in the axons are damaged, the increase in mitogenesis in the soma may enable the neuron to compensate for the loss of energy production. Such a mechanism has been described in the *Lepr^{db/db}* diabetic neuropathy mouse model, where mitochondria are increased in DRG neurons and the dorsal root [50], which was suggested to compensate for the decrease in ATP production [7,50]. Moreover, a large network of mitochondria that are either interconnected or in close proximity that facilitates fusion/fission may be a mechanism to sustain mitochondrial health as fusion/fission kinetics of mitochondria are closely linked to mitochondrial health and function. Fusion is thought to protect mitochondrial function by providing an opportunity for mitochondria to mix their contents, which in turn enables efficient repairing and complementation of damaged components and functions and sharing of a larger pool of structural/functional proteins and metabolites [32,51]. Moreover, mitochondria resume normal structure and function after the response to an insult is resolved [52].

Another mechanism that could account for the paclitaxel-induced increase in mitochondrial volume is a reduction in axonal transport [12], which may result in accumulation of mitochondria in the cell bodies. If paclitaxel treatment increases the volume of mitochondria by facilitating fusion, these organelles may also be too large or heavy to be transported along the axons.

There are at least two possibilities that can account for the apparent increase in healthy mitochondria observed in the present study in the face of all the previous data pointing to chemotherapy-induced mitotoxicity. First, it is possible that the increase in healthy mitochondria in the cell body and mitotoxicity along the axons occur in separate subpopulations of afferents. As noted previously, morphological evidence of paclitaxel-induced mitotoxicity was only present in 25–40% of the small and large-diameter fibers [8,9], and we have studied an even smaller proportion of the neurons contributing axons to the peripheral nerve. That we failed to detect a change in Ca^{2+} regulation or mitochondria in putative non-nociceptive afferents argues against a role for the changes observed in putative nociceptive glabrous skin neurons in either the numbness or tingling associated with CIPN. And to the extent to which numbness is due to the chemotherapeutic-induced decrease in

intra-epidermal nerve fiber density and associated loss of Merkel cell innervation, the presence of mitotoxicity in myelinated fibers, puts this mechanism in the right population to mediate this effect. This begs the question, however, as to whether there is a subpopulation of nociceptive afferents in which mitotoxicity also accounts for the CIPN-induced pain, or whether the pain is due to the changes described in the present study.

The second possibility is that both the increase in mitochondrial volume and the mitotoxicity occur in the same subpopulation of neurons. The increase in mitochondrial volume may then be a consequence or/response to mitotoxicity in the axons as discussed above. This possibility would also beg the question as to which, if either of the two changes contribute to the pain associated with CIPN. Given the presence of Ca^{2+} -dependent K^{+} channels in putative nociceptive glabrous skin neurons [53], and the role for these channels in controlling the excitability of these neurons [53], a paclitaxel-induced decrease in Ca^{2+} could contribute to an increase in neuronal excitability and pain secondary to the decrease in K^{+} current. Minimally, however, the presence of both an increase in healthy mitochondria in the cell body and damaged mitochondria in the axons would suggest that the mitochondria damage is not initiated within the cell body as previously suggested [9].

It is possible that paclitaxel influences SERCA activity *via* a mechanism independent of the changes in mitochondria. However, given that SERCA activity is ATP-dependent and that mitochondria and ER are structurally and functionally linked, especially in the context of Ca^{2+} regulation [54–57], we suggest that it is most-likely that the apparent increase in SERCA activity is secondary to the paclitaxel-induced increase in mitochondrial volume. This may be due to an associated increase in resting ATP levels and/or a more tightly coupled mitochondria and ER. Such an increase in ATP should also increase PMCA activity which may also contribute to the paclitaxel-induced decrease in evoked Ca^{2+} transient duration [58]. Similarly, such an increase in ATP levels could also contribute to an increase in the excitability of putative nociceptive neurons secondary to an increased Na^{+} - K^{+} -ATPase-dependent membrane hyperpolarization. The increase in excitability would be due to the increase in voltage-gated Na^{+} channels able to contribute to action potential generation following hyperpolarization-induced relief of channel inactivation.

Taken together, our study provides evidence that paclitaxel-induced decrease in evoked Ca^{2+} transient duration in putative nociceptive glabrous skin neurons is mediated by mitochondria, whose structure and volume, but not health and functionality is greatly affected by this treatment. Our results argue against the notion that mitotoxicity is manifest throughout the sensory neuron, and raise the possibility that mitotoxicity is present in a subpopulation of neurons that do not contribute to the pain associated with CIPN. In addition, since these large mitochondrial networks are still functional, it may explain why clinical trials with acetyl-L-carnitine treatment that aimed to improve mitochondrial function resulted in worsening of the symptoms [59]. Moreover, our results suggest that SERCA, and by association PMCA activity also are important for paclitaxel-induced decrease in the evoked Ca^{2+} transient duration in putative nociceptors innervating the glabrous skin. Understanding the contribution of these changes in Ca^{2+} regulation as well as increased mitochondria volume to the pain associated with CIPN, may provide novel therapeutic

strategies to limit the most debilitating side effects of some of the most effective cancer treatments.

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Fig. 1.

Paclitaxel (black circles) administered at 2 mg/kg every other day for a total of 4 injections (pink column) induced mechanical allodynia (A), hyperalgesia (B), and cold sensitivity (C), as assessed with 4 g and 15 g Von Frey filaments, and the dry ice test, respectively. No changes in nociceptive behavior were observed in vehicle treated (grey circles) animals. Following resolution of behavioral sensitivity (at week 6), acutely dissociated DRG neurons retrogradely labeled from the glabrous skin of the hindpaw were studied with Ca²⁺ imaging. High K⁺ (30 mM, 4 s) evoked transients in putative nociceptive neurons (IB4⁺, capsaicin sensitive, small diameter) from vehicle- (gray trace) and paclitaxel-treated (black trace) rats were comparable (D). Group mean data (E) confirmed the absence of a significant difference in the duration of evoked Ca²⁺ transients, quantified as the time to decay 50 percent of the magnitude of the evoked Ca²⁺ transient. The number of neurons in each group is in parenthesis in each bar.

Fig. 2.

Consistent with previous results, and in marked contrast to the evoked Ca²⁺ transients 6 weeks after the last paclitaxel injection, the duration of the Ca²⁺ transient evoked with high K⁺ (30 mM, 4 s (hiK⁺)) was significantly shorter in putative nociceptive neurons from paclitaxel treated rats, one week after the last paclitaxel injection (A). This appeared to be true throughout the transient decay. When analyzed as the time to decay 25 (T25), 50 (T50), 75 (T75), and 90 (T90) percent of the peak of the evoked Ca²⁺ transient (as indicated in A), group mean data confirmed that the duration was significantly reduced at each of these time points compared to neurons from vehicle treated rats ($p < 0.01$, two-way ANOVA). Data plotted were calculated as a percent change from neurons from vehicle treated rats (B). (C) Representative hiK⁺ evoked Ca²⁺ transients from putative nociceptive glabrous skin neurons from vehicle (top) and paclitaxel (bottom) treated rats before and after application CCCP (10 μ M) and oligomycin (10 μ M). (D) The increase in the duration of the evoked Ca²⁺ transient in the presence of CCCP/oligomycin, was analyzed as a percent change from baseline at T50. Analysis of group mean data from putative nociceptive glabrous skin (Glab), unlabeled (Unl), dorsal hindpaw skin (Dor), and inner thigh skin (Thi) neurons from vehicle (gray bars) and paclitaxel (black bars) treated rats revealed a significant interaction between paclitaxel treatment and target of innervation. (E) To determine whether the relative increase observed in glabrous skin neurons from paclitaxel treated rats was significant, data were analyzed as a percent change from vehicle. The difference between groups was significant (p

< 0.01, one-way ANOVA). The number of neurons in each group is in parenthesis in each bar. ** is $p < 0.01$.

Fig. 3.

(A) Images of a DiO-labeled neuron from a Rhod-2/Fura-2 experiment. Images were taken prior to high K^+ application, with the regions of interest used for data acquisition shown with white circles in the “merged” panel. (B) To confirm that Rhod-2 fluorescence reflected changes in mitochondrial Ca^{2+} levels, high K^+ (30 mM, 4 s) was used to evoke a Ca^{2+} transients in a naïve neuron before and after application of CCCP/oligomycin. Similar results were obtained in the 10 other neurons tested in this manner. (C) Data showing CCCP/oligomycin induced changes in Ca^{2+} transient amplitudes ($p < 0.01$). (D) Distribution of resting Rhod-2 intensity levels in arbitrary units, in the absence of stimulation in small diameter IB4+ capsaicin responsive (sm IB4+ cap+, solid traces) and large diameter IB4- neurons unresponsive to capsaicin (lg IB4- cap-, dotted traces) from naïve rats. Solid bars indicate the mean of each group, which were significantly different ($p < 0.01$). (E) Typical high K^+ (30 mM, 4 s) evoked increases in Rhod-2 fluorescence a small diameter IB4+ capsaicin+ neuron and a large diameter IB4- capsaicin- neuron from a naïve rat. Both the magnitude and the time-course of decay appear to be different. Group mean data confirmed this impression for magnitude (F) but not duration (G) when analyzed as a T50. However, the T25 and the T90 were different between these subpopulations of neurons. (H) Typical high K^+ (30 mM, 4 s) evoked increases in Rhod-2 fluorescence (blue traces) recorded simultaneously with Fura-2 (dotted black traces), in putative nociceptive glabrous skin neurons from vehicle (left) and paclitaxel (right) treated rats. Grouped resting (I), and evoked (magnitude (J) and T50 duration (K)) Rhod-2 data from glabrous skin neurons from vehicle and paclitaxel treated rats. Note the absence of a significant difference between groups, even for duration, in contrast to the significant decrease in the duration of the fura-2 signal in neurons from paclitaxel treated rats. The number of neurons in each group is in parenthesis in each bar. ** is $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

(A) Live-cell confocal microscopy composite (top; green = mitotracker green, orange-red = DiI, blue = IB4) and mitotracker green (bottom) images of neurons from naïve, vehicle, and paclitaxel treated rats innervating the glabrous skin of the hindpaw, and dorsal hindpaw and inner thigh skin from paclitaxel treated rats. (B) Pooled mitotracker green intensity data from neurons from naïve (white bars), vehicle treated (gray bars), and paclitaxel treated (black bars) rats innervating the glabrous hindpaw, dorsal hindpaw, and inner thigh skin. There was a significant ($p < 0.05$, two-way ANOVA) interaction between paclitaxel treatment and target of innervation. (C) As in Fig. 2, to determine whether the change in mitotracker staining was significant between groups, data were analyzed as a percent change from control. In this case, because there were no detectable differences between neurons from naïve and vehicle treated rats, data from these two groups of neurons were pooled for each target of innervation. The difference between groups defined by target of innervation was significant ($p < 0.01$, one-way ANOVA). Composite (top; green = Tom20, orange-red = DiI, DAPI = blue, light gray = IB4) and Tom20 (bottom) images (D) and group mean data (E) from glabrous skin neurons from vehicle and paclitaxel treated rats. Scale bars are 20 μ m for all panels in (A) and 25 μ m for both panels in (D). The number of neurons in each group is in parenthesis in each bar. ** is $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

(A) Confocal images of Tom20 staining (top is mid-section on the z-axis, bottom is mid-section on the y-axis) in neurons from vehicle (left) and paclitaxel (right) treated rats. Images were post-processed with 20 deconvolution iterations, to enable resolution of individual mitochondria. Predicted mitochondria as determined with deconvolution are outlined in red. Pooled data from 8 neurons in each group analyzed as in A, for percent cytoplasmic volume comprised of mitochondria (B) and number of distinct mitochondria units per neuron (C). * is $p < 0.05$ and ** is $p < 0.01$.

Fig. 6. Confocal microscopy composite images of Tom20 (green) and DiI (red) staining in DRG slices from vehicle (left) and paclitaxel (right) treated rats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

(A) Images of a DiO-labeled neuron from a TMRM/Fura-2 experiment. (B) Fura-2 (top traces) and TMRM (bottom traces) fluorescence from neurons in which CCCP/oligomycin was applied following establishment of a stable baseline. When the decay in TMRM fluorescence had stabilized in the presence of CCCP/oligomycin, a combination of CCCP/oligomycin and 30 mM K^+ (hiK) was applied. Data are from putative nociceptive glabrous skin neurons from naïve, vehicle, and paclitaxel treated rats. (C) Grouped fura-2 data for neurons from naïve, vehicle, and paclitaxel treated rats. Resting Ca^{2+} was determined in the 30 s prior to the application of CCCP/oligomycin. The magnitude of the response to both CCCP/oligomycin and CCCP/oligomycin/hiK were calculated as the difference between the peak increase and the resting Ca^{2+} concentration prior to application of test agents. (D) Grouped TMRM data from neurons from naïve, vehicle, and paclitaxel treated rats. F_{mito} was estimated from the drop in TMRM fluorescence following the application of CCCP/oligomycin. F_{cyto} was estimated from the subsequent drop in TMRM fluorescence following the application of hiK in combination with CCCP/oligomycin. There were no significant differences between groups. The scale bar in A is 25 μm .

Fig. 8.

(A) To confirm that it was possible to detect an increase in mitoxox staining in neurons in which there was an increase in superoxide, neurons were studied with live confocal imaging before (top) and after (bottom) a 30 min application of antimycin A to induce mitochondrial superoxide production. Mitoxox Red (left) and mitotracker far red (right), were monitored in the same neurons. (B) Composite (top; red = mitoxox, blue = mitotracker far red, green = DiO) and mitoxox (bottom) images from neurons from naïve (left), vehicle treated (middle), paclitaxel treated (right) rats. (C) Pooled data from glabrous skin neurons from the three groups of rats shown in B. The number of neurons in each group is in parenthesis in each bar. Scale bars are 20 μm for all panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9.

(A) Fura-2 was used to assess high K⁺ (30 mM, 4 s) evoked Ca²⁺ transients in glabrous skin neurons from vehicle and paclitaxel treated rats before and after blocking SERCA with CPA (10 μM). To assess the impact of CPA on the duration of the evoked Ca²⁺ transient, data obtained in the presence of CPA were analyzed as a percent change from baseline T50. (B) Grouped data from neurons analyzed in this way indicated that the relative increase in putative nociceptive glabrous skin neurons from paclitaxel treated rats was significantly greater than that in vehicle treated rats. The number of neurons in each group is in parenthesis in each bar. * is p < 0.05.