Peripheral and central oxidative stress in chemotherapy-induced neuropathic pain

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is an adverse side effect of many anti-cancer chemotherapeutic treatments. CIPN often causes neuropathic pain in extremities, and oxidative stress has been shown to be a major contributing factor to this pain. In this study, we determined the site of oxidative stress associated with pain (specifically, mechanical hypersensitivity) in cisplatin- and paclitaxel-treated mouse models of CIPN and investigated the neurophysiological mechanisms accounting for the pain. C57BL/6N mice that received either cisplatin or paclitaxel (2 mg/kg, once daily on four alternate days) developed mechanical hypersensitivity to von Frey filament stimulations of their hindpaws. Cisplatin-induced mechanical hypersensitivity was inhibited by silencing of Transient Receptor Potential channels V1 (TRPV1)- or TRPA1-expressing afferents, whereas paclitaxel-induced mechanical hypersensitivity was attenuated by silencing of Aβ fibers. Although systemic delivery of phenyl N-tert-butylnitrone, a reactive oxygen species scavenger, alleviated mechanical hypersensitivity in both cisplatin- and paclitaxel-treated mice, intraplantar phenyl N-tert-butylnitrone was effective only in cisplatin-treated mice, and intrathecal phenyl N-tert-butylnitrone, only in paclitaxel-treated mice. In a reactive oxygen species-dependent manner, the mechanosensitivity of Aδ/C fiber endings in the hindpaw skin was increased in cisplatin-treated mice, and the excitatory synaptic strength in the spinal dorsal horn was potentiated in paclitaxel-treated mice. Collectively, these results suggest that cisplatin-induced mechanical hypersensitivity is attributed to peripheral oxidative stress sensitizing mechanical nociceptors, whereas paclitaxel-induced mechanical hypersensitivity is due to central (spinal) oxidative stress maintaining central sensitization that abnormally produces pain in response to Aβ fiber inputs.

Keywords

Oxidative stress, chemotherapy, chemotherapy-induced peripheral neuropathy, neuropathic pain, paclitaxel, cisplatin

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Introduction

A frequent side effect of many cancer chemotherapeutics is peripheral neuropathy manifesting sensory symptoms including spontaneous pain and mechanical/cold allodynia in both hands and feet (a "stocking and glove" distribution).[1,2] The pain due to chemotherapy-induced peripheral neuropathy (hereafter, termed CIPN pain) impedes the anti-cancer treatments by limiting treatment options (e.g., dose reduction, a switch to less efficacious agents, cessation of treatment, etc.).[3] Furthermore, CIPN pain may persist well beyond the cessation of treatment,[4,5] negatively impacting cancer survivors' quality of life in the long term.

Substantial research efforts have been made to elucidate the mechanisms of CIPN, and oxidative stress—the imbalance between the production of reactive oxygen species (ROS) and the ability to detoxify its harmful effect—is identified as one of the important pathogenic factors damaging peripheral sensory neurons.[6] Indeed, antioxidant chemicals, such as phenyl-N-tert-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethyl-piperidine1-oxyl (TEMPOL), temporarily reduce

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already developed CIPN pain in animal models,[7–9] demonstrating that reducing oxidative stress is a promising CIPN pain therapy. However, because ROS is also required for normal body functions,[10,11] it will be necessary to take strategic approaches for reducing oxidative stress to be "pain-specific." One strategy would be to identify the site of pain-associated oxidative stress and specifically target the site with antioxidant compounds. Therefore, the purpose of this study is to determine such sites using two mouse models of CIPN, cisplatin- and paclitaxel-induced CIPN, and understand the neurophysiological mechanisms accounting for CIPN pain. The hallmark behavioral sign of CIPN in rodent models is mechanical hypersensitivity to tactile stimulation, reflecting mechanical allodynia in human CIPN patients.[12] Here, we report that peripheral and central oxidative stress play a differential role in mechanical hypersensitivity induced by the two chemotherapeutics.

Materials and methods

Animals

Male C57BL/6N mice (7–12 weeks, Charles River, Houston, TX) were used throughout this study. The mice were housed on a 12–12 h light–dark cycle with standard bedding and free access to food and water in animal facility accredited by the Association for the Assessment and Accreditation of Laboratory and Care International. All experimental procedures using animals were done according to the guidelines of the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Induction of CIPN

Cisplatin and paclitaxel (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ml. The solution was mixed with an equal volume of Tween-80 and then diluted in sterile saline to 2 mg/mL just before injection. Each chemical (2 mg/kg) was injected intraperitoneally on four alternate days (days 0, 2, 4, and 6) to induce CIPN. Control animals were injected with the same volume of vehicle (0.4% DMSO and 0.4% Tween-80 in saline). The body weight and behavioral mechanosensory responses were measured before and at various time points after the injections of chemotherapeutics.

Behavioral test

Mechanical sensitivity was determined by examining the response rates of paw withdrawals from 10 repeated stimuli applied to the paw using a von Frey filament delivering 0.98 mN (Stoelting, Wood Dale, IL). All experiments were conducted by an experimenter blinded to the treatment groups. Mice were placed in a plastic box on a metal grid floor and acclimated for 15 to 20 min prior to testing. The von Frey filament was applied perpendicularly to the skin for 2 to 3 s on the hindpaw with enough force to bend it slightly. An abrupt withdrawal of the foot during or immediately after stimulation was regarded as a positive response. Response rates were calculated as a percentage of the number of positive responses per 10 stimuli.

Silencing of specific types of sensory fibers in vivo

Capsaicin (Sigma-Aldrich) or allyl isothiocyanate (AITC, Sigma-Aldrich) was freshly prepared in 10% ethanol, 10% Tween-80, and 80% saline containing QX-314 (Sigma-Aldrich; the final concentration of QX-314 was 2%). Flagellin (Sigma-Aldrich) was dissolved in saline containing 2% QX-314. QX-314 alone, QX-314 with capsaicin (0.1%), QX-314 with AITC (0.1%), and QX-314 with flagellin (0.9 μg) were intradermally injected (5 μL) at von Frey filament stimulation sites. QX-314, at 2% concentration, was shown to block capsaicin-induced mechanical/heat hypersensitivity development[13] and Aβ fiber excitation[14] when injected with the abovementioned doses of capsaicin and flagellin, respectively. Likewise, we chose 2% QX-314 with 0.1% AITC based on our pilot experiment showing a complete blockade of mechanical hypersensitivity development that is normally induced by 0.1% AITC alone.

Treatment of a ROS scavenger in vivo

To locate the pain-associated oxidative stress sites, effects of the ROS scavenger PBN (Sigma-Aldrich; dissolved in sterile saline) was determined after injecting the drug via three different routes: intraperitoneal (i.p., 100 mg/kg), intradermal (i.d., 100 μg in 5 μL), and intrathecal (i.t., 100 μg in 5 μL) injections under isoflurane anesthesia (3% for induction and 2% for maintenance in a flow of O_2). The i.p. and i.t. doses of PBN were based on our previous studies demonstrating their inhibitory effects on spinal nerve ligation- and paclitaxel-induced mechanical hypersensitivity without causing sedation.[7,15,16] For i.d. injection, PBN was injected at von Frey filament stimulation sites, and for i.t. injection, the scavenger was injected by the lumbar puncture method.[17,18]

Single afferent unit recording in ex vivo skin-nerve preparations

The hindpaw glabrous skin from the ankle to the tips of the toes was dissected with the tibial nerve attached. The skin was placed corium side up in an organ bath superfused with an oxygen-saturated, warmed (34°C) artificial interstitial fluid (in mM: NaCl, 123; KCl, 3.5; $MgSO_4$,

0.7; $CaCl_2$, 2.0; Na gluconate, 9.5; NaH_2PO_4, 1.7; glucose, 5.5; sucrose, 7.5; and HEPES, 10; pH 7.4) at a flow rate of 15 mL/min. The tibial nerve was placed in a separate, mineral oil-filled chamber and teased into small bundles. The small bundles were placed onto a platinum recording electrode to detect a single fiber activity (i.e., action potential (AP) firing). The signal was amplified using a differential amplifier (DAM80, World Precision Instruments, Sarasota, FL) and recorded through CED1401 interface and Spike2 software (CED Ltd., Cambridge, UK). Mechanosensitive fibers were identified initially by probing the skin with a blunt glass rod. Only units responding to this search stimulus were studied in detail. The conduction velocity (CV) of each unit was determined from the latency of the AP triggered by monopolar electrical stimulation (0.3–3.0 ms duration, 0.02–1.0 mA) of the receptive field and the distance between the stimulation and the recording electrodes. Fibers with CV $\leq$1.3 m/s were regarded as C fibers, CV $\geq$13.3m/s as Aβ fibers, and CV between the two values as Aδ fibers.[19]

Ramp (10 mN/s for 20 sec, from 20 mN) mechanical stimulation was applied to the receptive field using a dual mode lever system (Aurora Scientific Inc., Ontario, Canada). The compression probe for mechanical stimulation was 0.7 mm in diameter. The force eliciting the first AP upon the ramp stimulation was considered activation threshold for the unit. To determine the unit's stimulation intensity–response magnitude profile, the number of APs was measured from 20 mN to a given stimulation intensity. Mechanical stimulation was applied before and after PBN (1 mM) application onto the receptive field for 30 min.

Patch-clamp recording of dorsal horn neurons in ex vivo spinal cord slices

Spinal cord slices were prepared as previously described.[20] Briefly, the spinal cord was sliced transversely at a thickness of 350 μm using a vibratome (Leica VT1200S, Buffalo Grove, IL) in cold ($\sim$4°C) NMDG (N-methyl-D-glucamine) solution (in mM: 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$ and 0.5 $CaCl_2$, pH 7.4), saturated with 95% O_2 and 5% CO_2.

Whole-cell recordings were made on random neurons in lamina II in artificial cerebrospinal fluid (ACSF in mM: 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 5 HEPES, 12.5 glucose, 2 $MgSO_4$, and 2 $CaCl_2$, pH 7.4) using Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), DigiDATA (Molecular Devices), and pClamp software (version 10.6. Molecular Device) at a 10 kHz sampling rate and a 2 kHz filtering rate. The patch-pipettes (4–8 MΩ) were filled with internal solution (in mM: 120 K-gluconate, 10 KCl, 2 Mg-ATP, 0.5 Na-GTP, 0.5 EGTA, 20 HEPES, and 10 phosphocreatine, pH 7.3). After making whole-cell recording configuration, the miniature excitatory postsynaptic currents (mEPSC) were recorded for 100 s at -65 mV with 10 mM lidocaine (Sigma-Aldrich) in ACSF. For pharmacological scavenging of ROS, 1 mM PBN (Sigma-Aldrich) was superfused for 5 min during recordings; the concentration of PBN was based on previous electrophysiological studies.[16,21]

Data analysis

All data are expressed as the mean±standard error of the mean (SEM) with n, the number of samples. For multiple comparison tests, we predetermined pairwise comparison groups (an *a priori* approach). For behavioral data at multiple time points after drug treatments, nonparametric Friedman test followed by Dunn's test was first used to examine a difference between the pre- and post-drug treatment values. If the behavioral dataset passed a normality test (Shapiro–Wilk test) and an equal variance test (Brown–Forsythe test), the dataset was analyzed using one-way repeated measure (RM) analysis of variance (ANOVA) followed by Holm–Sidak multiple comparison test. Electrophysiological data were analyzed using Spike2 (CED Ltd.) and Clampfit software (Molecular Devices). Events were detected using the template event detection method. These electrophysiological data were statistically analyzed using either one-way ANOVA or two-way RM ANOVA followed by Holm–Sidak multiple comparison tests. Detection frequency (i.e., relative proportion of each fiber type in three mouse groups) was analyzed by Chi-square test. In all tests, $p < 0.05$ was considered significant.

Results

Afferent types mediating chemotherapy-induced mechanical hypersensitivity

Mice treated with vehicle or paclitaxel steadily gained body weight, whereas cisplatin-treated mice lost their weight by $\sim$10% by the end of the chemotherapy (Figure 1(a)) and then regained body weight afterward as previously reported.[22] During and after the chemotherapy regimen, mice gradually developed mechanical hypersensitivity, which manifested as increased hindpaw withdrawals from von Frey filament stimulation that normally did not evoke the nocifensive behavior in the baseline (i.e., before the chemotherapy) (Figure 1(b)). Vehicle of the chemotherapeutics did not produce such mechanical hypersensitivity over time.

When the chemotherapy-induced mechanical hypersensitivity fully developed (i.e., four to five weeks after
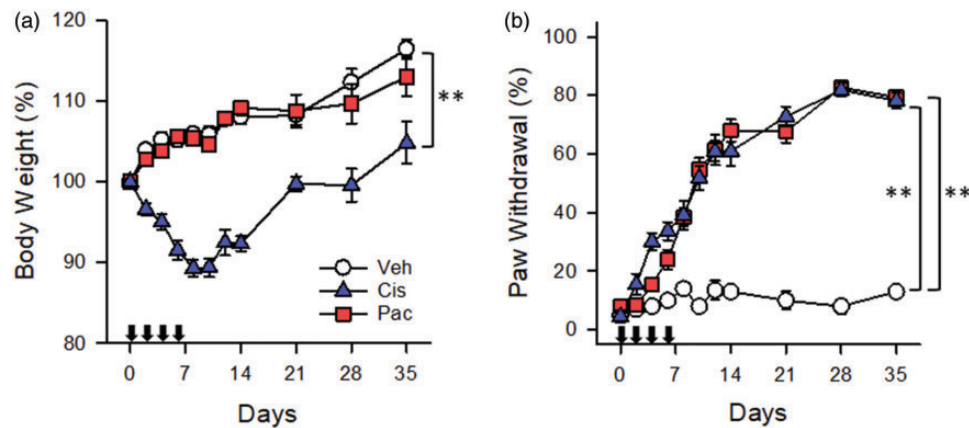
Figure 1. Effects of cisplatin and paclitaxel on the body weight and mechanical hypersensitivity. The two chemotherapeutics were intraperitoneally injected once daily on four alternate days (days 0, 2, 4, and 6; black arrows); on the injection days, the body weight and withdrawal behaviors were measured before the injection. (a) Mice lost their body weight during the cisplatin (Cis, n = 11) treatment and then regained the weight afterwards. Paclitaxel (Pac, n = 15) had no effect on the body weight. (b) Both Cis and Pac induced hypersensitive response to normally innocuous von Frey filament stimulations, producing increased withdrawals from the mechanical stimulation. **p < 0.01 versus vehicle (Veh, n = 10) by two-way RM ANOVA.

the chemotherapy initiation), we examined the types of sensory fibers mediating the hypersensitivity. To this end, we took advantage of the approaches using QX-314, a membrane impermeable lidocaine analog, together with Transient Receptor Potential Channel V1 (TRPV1) agonists, TRPA1 agonists, or Toll-like receptor 5 (TLR5) agonists to selectively silence TRPV1-expressing, TRPA1-expressing, or Aβ fibers, respectively.[13,14,23] As shown in Figure 2, QX-314 (2%, 5 μL) injected alone at the von Frey filament stimulation site did not affect the chemotherapy-induced mechanical hypersensitivity. Co-injection of QX-314 with the TRPV1 agonist capsaicin (0.1%, 5 μL) or the TRPA1 agonist AITC (0.1%, 5 μL) significantly inhibited the hypersensitivity in cisplatin-treated mice but not in paclitaxel-treated mice. By contrast, co-injection of QX-314 with the TLR5 agonist flagellin alleviated the hypersensitivity in paclitaxel-treated mice but not in cisplatin-treated mice, collectively suggesting that TRPV1/TRPA1-expressing afferents mediate cisplatin-induced mechanical hypersensitivity, whereas Aβ fibers mediate paclitaxel-induced mechanical hypersensitivity.

The sites of oxidative stress associated with chemotherapy-induced mechanical hypersensitivity

We then examined whether oxidative stress commonly contributes to cisplatin- and paclitaxel-induced mechanical hypersensitivities despite that they are mediated by different types of afferents. Systemic application of the ROS scavenger PBN (100 mg/kg, i.p.) partially but significantly inhibited both the cisplatin- and paclitaxel-induced mechanical hypersensitivity (Figure 3).

To determine whether the site of this pain-associated oxidative stress is at the periphery or at the level of the spinal cord, PBN was given either intradermally (100 μg, 5 μL at the von Frey filament stimulation site) or intrathecally (100 μg, 5 μL into the lumbar cistern), respectively. Intradermal PBN significantly attenuated cisplatin-induced mechanical hypersensitivity but had no effect on the paclitaxel counterpart. On the contrary, i.t. PBN significantly inhibited paclitaxel-induced mechanical hypersensitivity without affecting the cisplatin counterpart. These results suggest that the site of mechanical hypersensitivity-associated oxidative stress is at the periphery in cisplatin-treated mice and at the level of the spinal cord in paclitaxel-treated mice. Therefore, in the next experiments, we examined whether the two chemotherapeutics alter peripheral and spinal sensory neuronal excitability, and if so, whether oxidative stress maintains the alteration(s).

Peripheral oxidative stress affecting mechanosensitivity of Aδ/C fibers in the skin

In this experiment, we determined the mechanosensitivity of sensory fibers in the tibial nerve innervating the hindpaw skin. The detection frequencies of Aβ, Aδ, and C fibers were not significantly different between vehicle-, cisplatin-, and paclitaxel-treated mice (Table 1). As for Aβ fibers, neither mechanical thresholds before/after PBN ($F_{(2,11)} = 1.393$, $p = 0.289$ by two-way RM ANOVA) nor CV ($F_{(2,13)} = 0.285$, $p = 0.757$ by one-way ANOVA) differed between the three mouse groups. Because of the limited numbers of slowly
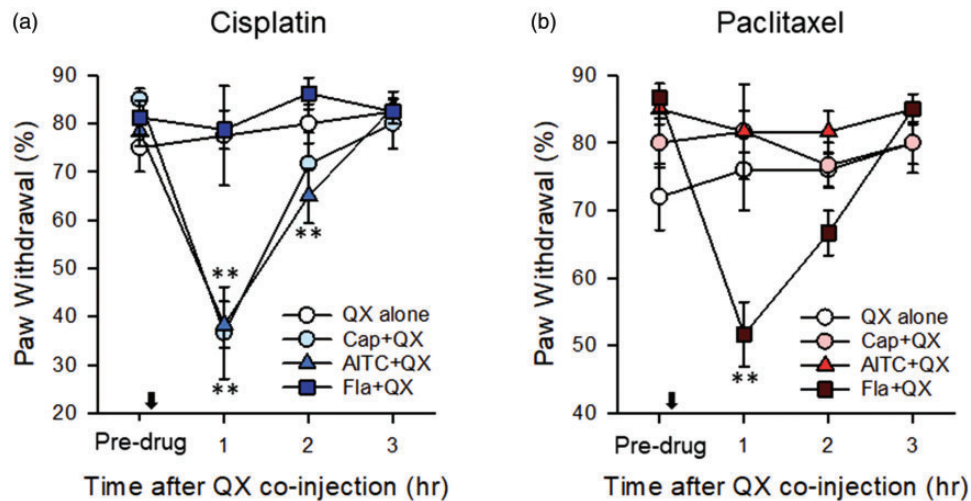
Figure 2. Afferent types mediating the chemotherapy-induced mechanical hypersensitivity. When cisplatin (Cis)- and paclitaxel (Pac)-induced mechanical hypersensitivity fully developed (i.e., four to five weeks after the first injection of the chemotherapeutics), mice received (indicated by a black arrow) QX-314 (QX), a membrane-impermeable lidocaine analogue, together with the Transient Receptor Potential channel V1 (TRPV1) agonist capsaicin (Cap), the TRPA1 agonist allyl isothiocyanate (AITC), or the Toll-like receptor 5 (TLR5) agonist flagellin (Fla) at their hindpaw to selectively silence TRPV1-expressing, TRPA1-expressing, and Aβ sensory fibers. (a) Cis-induced mechanical hypersensitivity was significantly inhibited by QX+Cap (n = 6) and QX+AITC (n = 6) but not by QX+Fla (n = 8). (b) By contrast, Pac-induced mechanical hypersensitivity was inhibited only by QX+Fla (n = 8) but neither by QX+Cap (n = 6) nor QX+AITC (n = 6). QX alone had no effect in both mouse models (n = 4 each) of chemotherapy-induced neuropathic pain. **$p < 0.01$ versus pre-drug (before QX+drug injections) by Holm–Sidak multiple comparison test following one-way RM ANOVA in (a) and by Dunn's test following Friedman test in (b).
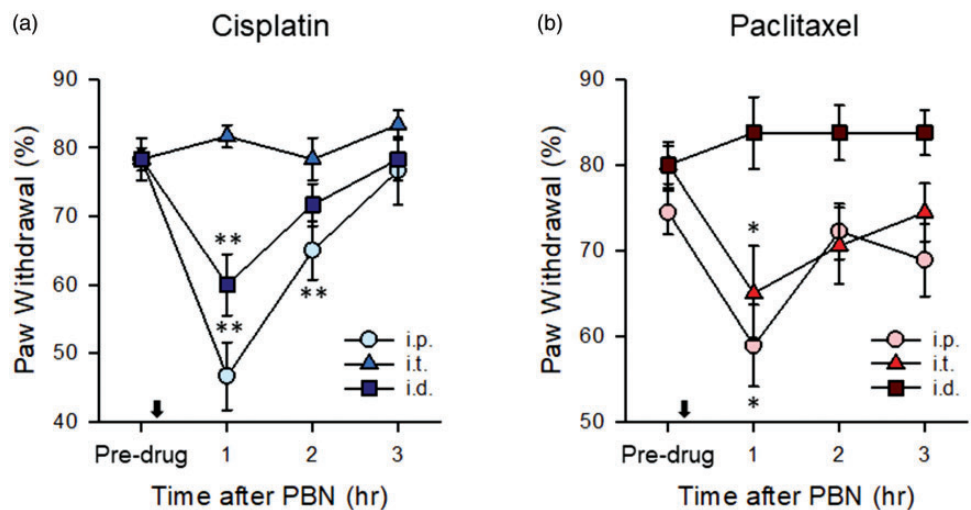
Figure 3. Sites of pain-associated oxidative stress in chemotherapy-induced neuropathic pain. When cisplatin (Cis)- and paclitaxel (Pac)-induced mechanical hypersensitivity fully developed, the free radical scavenger phenyl-N-tert-butylnitrone (PBN) was given (indicated by a black arrow) systemically (i.e., i.p. injection), at the level of the spinal cord (i.e., i.t. injection), or at the hindpaw (i.e., i.d. injection). (a) Cis-induced mechanical hypersensitivity was significantly inhibited by PBN given via i.p. (n = 6) and i.d. (n = 6) routes but not i.t. (n = 6) route. (b) By contrast, Pac-induced mechanical hypersensitivity was inhibited by PBN given via i.p. (n = 9) and i.t. (n = 9) routes but not i.d. (n = 8) route. **$p < 0.01$ versus pre-drug (before PBN injections) by Holm–Sidak multiple comparison test following one-way RM ANOVA in (a); *$p < 0.05$ by Dunn's test following Friedman test in (b).

adapting Aβ fibers (n = 2–3) in our samples, AP numbers in this fiber type were not statistically analyzed.

As for Aδ fibers, CV was significantly slower in cisplatin- and paclitaxel-treated mice than in vehicle-treated mice (Table 1), suggesting that this fiber type is undergoing axonal or demyelinating damages in our experimental CIPN setting. Although mechanical thresholds of Aδ fibers did not significantly differ

Table 1. Detection frequencies and CV of Aβ, Aδ, and C fibers in vehicle-, cisplatin-, and paclitaxel-treated mice.

	Vehicle (n = 26)	Cisplatin (n = 25)	Paclitaxel (n = 18)
Aβ			
n	3 (12%)	5 (20%)	6 (33%)
CV (m/s)	15.8 ± 0.7	16.9 ± 1.4	16.3 ± 0.9
Aδ			
n	17 (65%)	12 (48%)	7 (39%)
CV (m/s)	8.2 ± 0.9	5.0 ± 0.7[†]	4.4 ± 0.7[††]
C			
n	6 (23%)	8 (32%)	5 (28%)
CV (m/s)	0.7 ± 0.3	0.9 ± 0.3	0.7 ± 0.2

Note: [†] $p < 0.05$; [††] $p < 0.01$ versus vehicle group by Holm–Sidak multiple comparison test following one-way ANOVA. CV: conduction velocity.

between the three mouse groups before PBN application (Figure 4(b)) ($F_{(2,33)} = 0.507$, $p = 0.607$ by two-way RM ANOVA), the number of APs discharged during the ramp stimulation was significantly greater in cisplatin-treated mice than in vehicle-treated mice (Figure 4(c)). The increased mechanosensitivity of Aδ fibers in cisplatin-treated mice was inhibited by applying PBN (1 mM) onto their receptive endings (Figure 4(e)). Furthermore, PBN significantly increased their mechanical thresholds (Figure 4(b)), while having no effect on mechanosensitivity of Aδ fibers in vehicle- and paclitaxel-treated mice (Figure 4(b), (d), and (f)).

The CV of C fibers was not affected by cisplatin or paclitaxel treatment (Table 1) ($F_{(2,16)} = 1.575$, $p = 0.238$ by one-way ANOVA). However, their mechanical thresholds were significantly decreased by the two chemotherapeutics (Figure 5(a)). The number of APs discharged during the ramp stimulation was significantly greater only in cisplatin-treated mice than in vehicle-treated mice (Figure 5(b)), which was inhibited by PBN application onto their receptive endings (Figure 5 (d)). There was also a trend ($p = 0.078$ by Holm–Sidak test following two-way RM ANOVA) toward an increase in C fiber mechanical thresholds after PBN application in cisplatin-treated mice. By contrast, in vehicle- and paclitaxel-treated mice, PBN had no effect on C fiber mechanical thresholds and their stimulation intensity–response magnitude profiles (Figure 5(a), (c), and (e)).

### Central oxidative stress affecting excitatory synaptic strength in the spinal cord

In ex vivo spinal cord slices collected from the three mouse groups, we recorded miniature excitatory postsynaptic currents (mEPSC) to assess the strength of excitatory synapses in the superficial dorsal horn. As shown in Figure 6, mEPSC frequency was significantly higher in cisplatin- (0.36 ± 0.07 Hz, n = 8; $p = 0.004$ versus vehicle by Holm–Sidak multiple comparison test) and paclitaxel-treated mice (0.45 ± 0.07 Hz, n = 7; $p < 0.001$ versus vehicle by Holm–Sidak multiple comparison test) than in vehicle-treated mice (0.11 ± 0.04 Hz, n = 8). Superfusion of PBN (1 mM) into the recording chamber decreased the mEPSC frequency only in paclitaxel-treated mice (0.16 ± 0.07 Hz, n = 7; $p < 0.001$ versus before PBN by Holm–Sidak multiple comparison test). The amplitudes of mEPSC were neither different between mouse groups ($F_{(2,20)} = 0.357$, $p = 0.704$ by two-way RM ANOVA) nor changed by PBN application ($F_{(1,20)} = 0.184$, $p = 0.672$ by two-way RM ANOVA).

Discussion

This study demonstrates that peripheral and central (spinal) oxidative stress differentially contributes to cisplatin- and paclitaxel-induced mechanical hypersensitivity in the mouse. Specifically, cisplatin-induced mechanical hypersensitivity was found to be associated with increased mechanosensitivity of Aδ/C fibers at the periphery ex vivo, which accounts for the attenuation of cisplatin-induced mechanical hypersensitivity by silencing of mostly unmyelinated TRPV1/TRPA1-expressing afferents[24] in vivo. The fact that the increased mechanosensitivity of Aδ/C fibers was reduced by a ROS scavenger at the receptive fields suggests that the increase is reversibly maintained by ongoing peripheral oxidative stress. By contrast, we found no statistically significant change in the stimulation intensity–response magnitude profiles of Aδ/C fibers in paclitaxel-treated mice despite a decrease in C fiber mechanical threshold, suggesting that paclitaxel-induced mechanical hypersensitivity may not be primarily due to sensitization of mechanical nociceptors. Supporting this notion, silencing of Aβ fibers, not TRPV1/TRPA1-expressing afferents, effectively alleviated paclitaxel-induced mechanical hypersensitivity. Although potential interference with non-neuronal cells, such as peripheral immune cells expressing TRPV1, TRPA1, and TLR5,[25,26] can be a confounding factor in the identification of afferents mediating mechanical hypersensitivity by using QX-314 with the channel/receptor's agonist, our findings are aligned well with previous studies reporting that blocking the activity of Aβ sensory neurons inhibits mechanical hypersensitivity in paclitaxel-induced CIPN model,[14] whereas in cisplatin/oxaliplatin-induced CIPN models, TRPV1/TRPA1 expression is upregulated in sensory ganglia, and impairing these channels attenuates mechanical/cold/heat hypersensitivity.[27,28]

Because Aβ fibers are normally unable to activate the nociceptive sensory pathway, central processing of their inputs must be altered for Aβ fibers to mediate
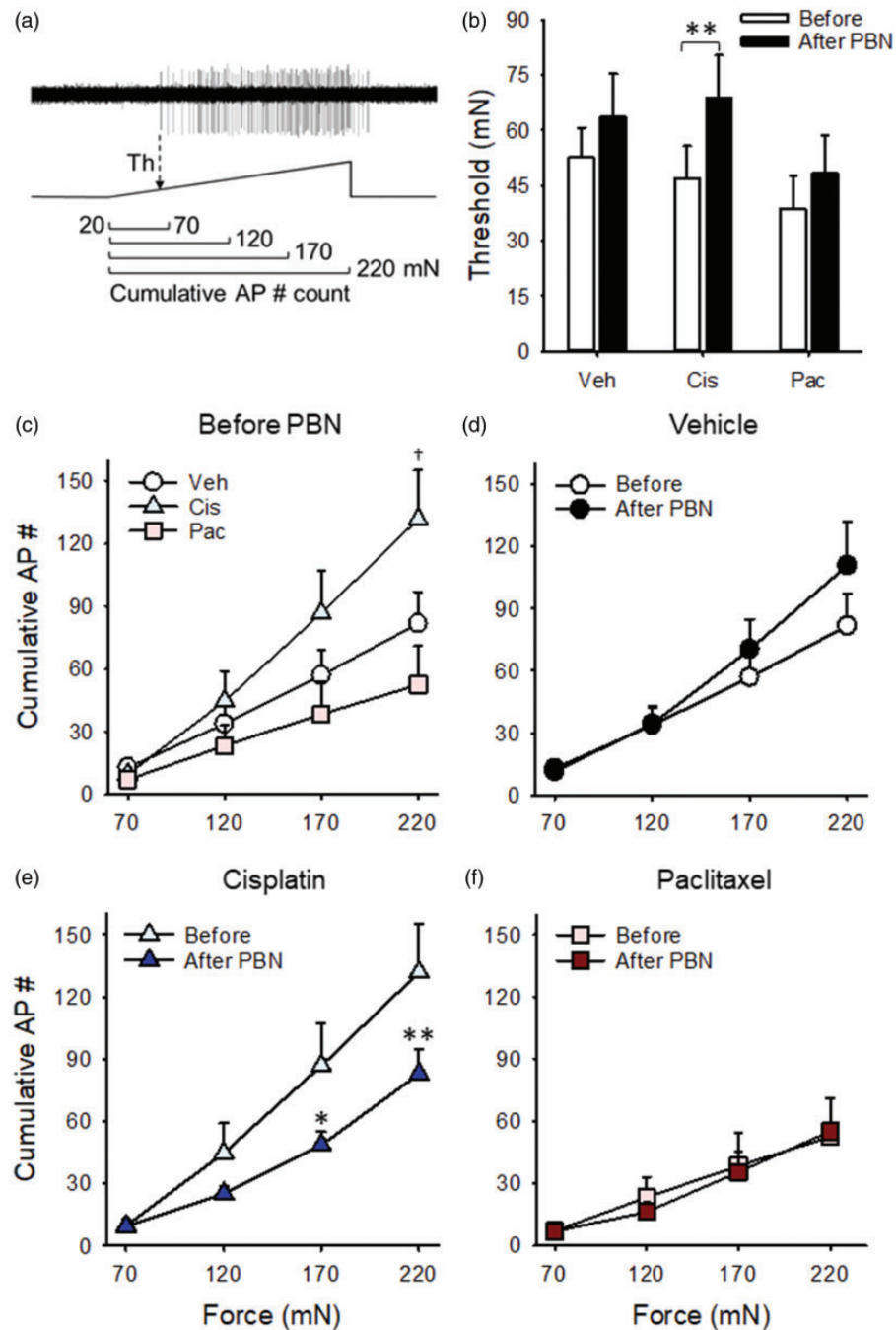
Figure 4. Mechanosensitivity of cutaneous Aδ fibers in mouse models of chemotherapy-induced neuropathic pain. (a) Representative traces of single fiber recording in an ex vivo hindpaw skin-tibial nerve preparation. Mechanosensitive Aδ fibers were stimulated by ramp stimulation (20 to 220 mN, 10 mN/s). The force evoking the first AP firing was regarded as the unit's mechanical threshold (Th) and the number of APs was cumulatively counted. (b) Mechanical thresholds of Aδ fibers before and after PBN application onto their receptive endings in vehicle (Veh, n = 17)-, cisplatin (Cis, n = 12)-, and paclitaxel (Pac, n = 7)-treated mice. (c) Stimulus intensity–response magnitude relationship of Aδ fibers in the three mouse groups before PBN application. (d, e, and f) Effects of PBN on the relationship in the three mouse groups. *p < 0.05; **p < 0.01 versus "before" in each mouse group; †p < 0.05 versus Veh by Holm–Sidak multiple comparison test following two-way RM ANOVA. PBN: phenyl-N-tert-butylnitrone.

mechanical hypersensitivity. One potential mechanism is an increase in excitatory synaptic transmission in spinal dorsal horn nociceptive circuits, constituting central sensitization.[29] In our previous study, a mouse model of traumatic peripheral neuropathy showed increased mEPSC frequency in spinal dorsal horn neurons, which was reversibly maintained by upregulated mitochondrial superoxide.[20] In this study, we also observed
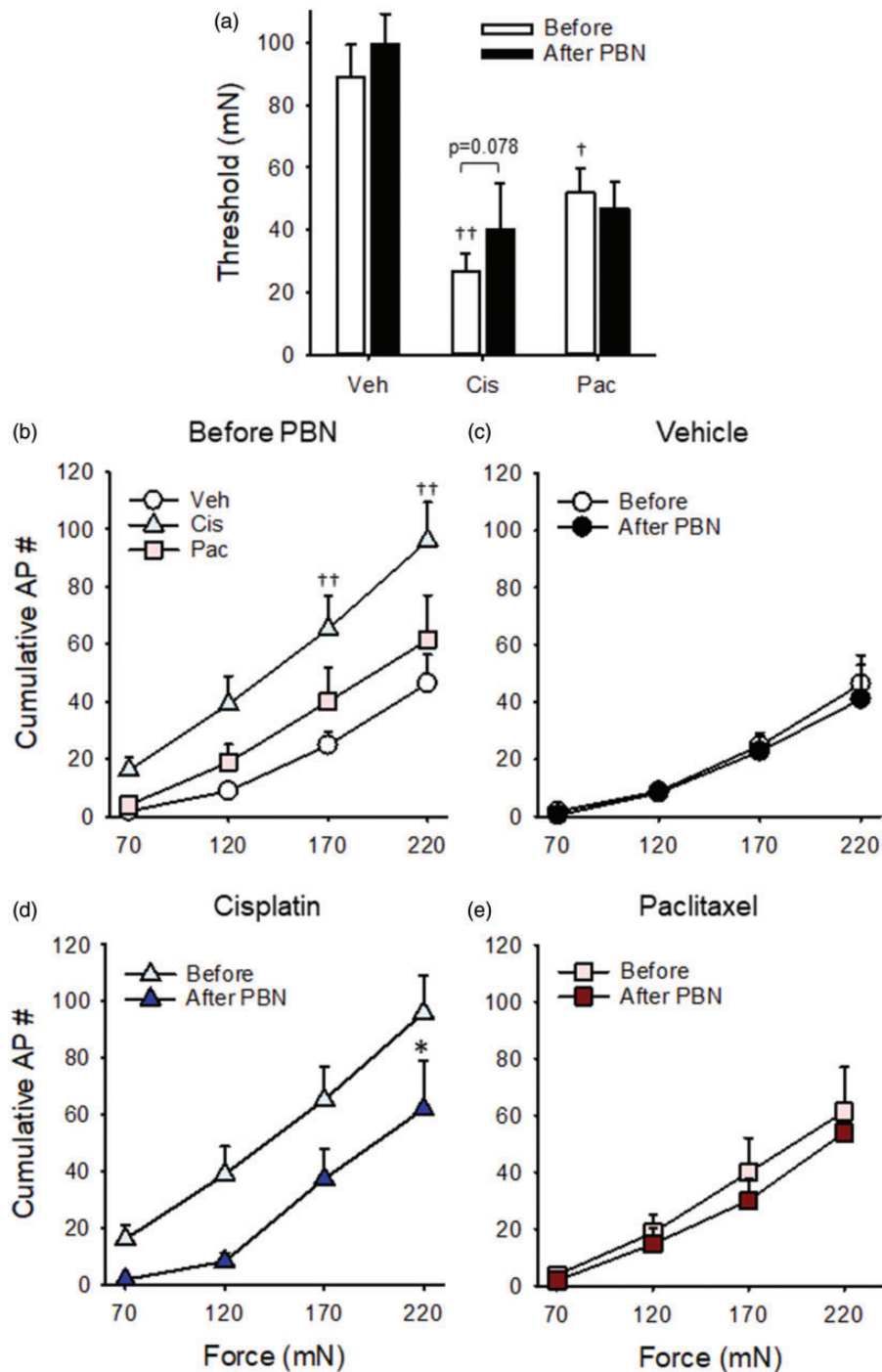
Figure 5. Mechanosensitivity of cutaneous C fibers in mouse models of chemotherapy-induced neuropathic pain. (a) Mechanical thresholds of C fibers before and after PBN application onto their receptive endings in vehicle (Veh, n = 6)-, cisplatin (Cis, n = 8)-, and paclitaxel (Pac, n = 5)-treated mice. (b) Stimulus intensity–response magnitude relationship of C fibers in the three mouse groups before PBN application. (c, d, and e) Effects of PBN on the relationship in the three mouse groups. $^{†}p < 0.05$, $^{††}p < 0.01$ versus "before" in Veh; $^{*}p < 0.05$ versus "before" in each mouse group by Holm–Sidak multiple comparison test following two-way RM ANOVA. PBN: phenyl-N-tert-butylnitrone; AP: action potential.

an increase in mEPSC frequency in spinal dorsal horn neurons of cisplatin- and paclitaxel-treated mice. Notably, the increased excitatory synaptic strength was reduced by a ROS scavenger in paclitaxel-treated mice

but not in cisplatin-treated mice. This observation provides an explanation for the behavioral results that i.t. PBN was effective only on paclitaxel-induced mechanical hypersensitivity. Combined, the results of this study
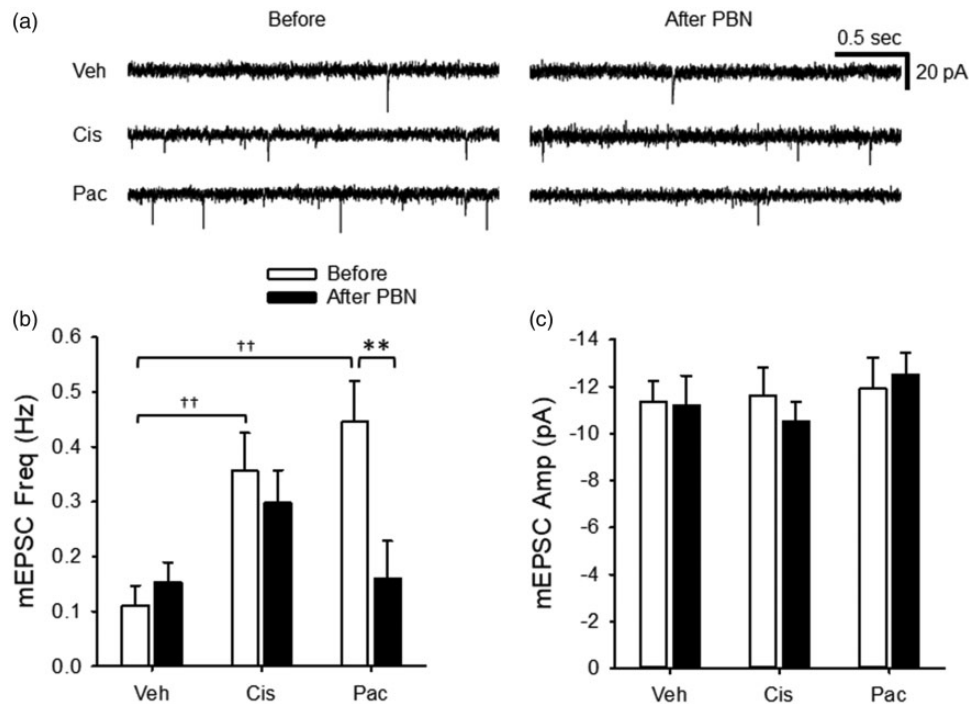
Figure 6. Involvement of spinal oxidative stress in increased excitatory synaptic strength in dorsal horn neurons. Ex vivo spinal cord slices were prepared from vehicle (Veh, n = 8)-, cisplatin (Cis, n = 8)-, and paclitaxel (Pac, n = 7)-treated mice when mechanical hypersensitivity fully developed in the latter two mouse groups. (a) Representative traces of miniature excitatory postsynaptic currents (mEPSC) in the three mouse groups before and after phenyl-N-tert-butylnitrone (PBN), a free radical scavenger, application into the recording chamber. (b) The frequency of mEPSC was significantly increased in dorsal horn neurons of Cis- and Pac-treated mice. However, PBN normalized it only in Pac-treated mice. (c) The amplitude of mEPSC did not differ between groups. $^{\dagger\dagger}p < 0.01$ versus before in Veh; $^{**}p < 0.01$ versus before in Pac by Holm–Sidak multiple comparison test following two-way RM ANOVA. PBN: phenyl-N-tert-butylnitrone; mEPSC: miniature excitatory postsynaptic currents.

suggest that four to five weeks after the initiation of chemotherapy, cisplatin-induced mechanical hypersensitivity is attributed to mechanical nociceptor sensitization maintained by peripheral oxidative stress, whereas paclitaxel-induced mechanical hypersensitivity is due to spinal oxidative stress that maintains central sensitization abnormally processing Aβ fiber inputs as nociceptive. With respect to the latter, compared to our experimental condition, the same (2 mg/kg) and a higher dose (4 mg/kg) of paclitaxel in rats[30] and mice,[31] respectively, are shown to cause spontaneous firing and hyperexcitability in small dorsal root ganglia neurons one to two weeks after the first paclitaxel treatment. Therefore, it may be important to consider potential species-, chemotherapeutic dose-, and the disease stage-dependent differences in the role of nociceptor sensitization in paclitaxel-induced mechanical hypersensitivity.

Since the mEPSC frequency but not amplitude was found to be increased in cisplatin- and paclitaxel-treated mice, the increased excitatory synaptic strength in the spinal dorsal horn may involve potentiation of presynaptic function. Identification of the potentiated presynaptic terminals is beyond the scope of present work; it will be necessary in future study to examine whether the central terminals of primary afferents, especially the ones undergoing CIPN in the periphery, are potentiated. Since Aδ fibers commonly showed a sign of neuropathy by their decreased CV in cisplatin- and paclitaxel-treated mice, we may obtain an insight into the potentiation of neuropathic primary afferent's central terminals by determining the paired pulse ratio and magnitude of Aδ fiber-mediated monosynaptic responses in our experimental CIPN setting.

Our results indicate that cisplatin-potentiated presynaptic function does not require ongoing oxidative stress to maintain the potentiated state, unlike the paclitaxel counterpart. Therefore, it would be interesting to further study the mechanistic differences in the increased excitatory presynaptic function between cisplatin- and paclitaxel-treated mice. It is noteworthy that selective activation of either microglia or astrocytes commonly increases mEPSC frequency without altering mEPSC amplitude.[32,33] With respect to this, cisplatin was shown to activate spinal microglia, not astrocytes, and upregulate the gene expression of inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α),[34] whereas paclitaxel activates astrocytes, not microglia, without changing the levels of

IL-1β and TNF-α in the spinal cord.[35] It could be that such differential glial activation in the spinal cord after cisplatin and paclitaxel treatments underlies/contributes to their mechanistic differences in the potentiation of excitatory presynaptic strength.

While demonstrating the differential involvement of "ongoing" peripheral and central oxidative stress in cisplatin- and paclitaxel-induced mechanical hypersensitivity, our results do not rule out the possibility that some "irreversible" changes may have already occurred in the nociceptive sensory pathway by oxidative stress after the chemotherapies, and the "irreversible" changes, maintenance of which being no longer dependent on oxidative stress, underlie the PBN-resistant part of mechanical hypersensitivity. Supporting this notion, previous studies showed that early intervention with ROS scavengers or a mitochondria protectant completely prevented cisplatin- and paclitaxel-induced mechanical hypersensitivity,[7,9,36] suggesting that there is a critical period of time during/after chemotherapy when oxidative stress irreversibly changes the sensory nervous system and establishes the perpetuation of oxidative stress.

In conclusion, we found that cisplatin- and paclitaxel-induced mechanical hypersensitivities are mediated in part by ongoing peripheral and central (spinal) oxidative stress, respectively. Oxidative stress at the two sites appears to maintain an increase in the mechanosensitivity of Aδ/C fibers in cisplatin-treated mice and a potentiation of excitatory synaptic strength in the dorsal horn in paclitaxel-treated mice. This provides mechanistic accounts for the site-specific inhibitory effects of a ROS scavenger on mechanical hypersensitivity caused by the two chemotherapeutics. From a therapeutic standpoint, because ROS is also required for physiological functions,[11] a "site-specific" ROS scavenging strategy may be useful for selectively targeting oxidative stress associated with chemotherapy-induced mechanical hypersensitivity. In this regard, it should be mentioned that spontaneous pain is another important manifestation of CIPN pain, and thus, it warrants further investigation of whether ongoing oxidative stress also maintains such spontaneous pain, and if so, whether the sites of oxidative stress correspond to those mediating mechanical hypersensitivity.

Author Contributions

HSS was involved in data acquisition/analysis/interpretation and manuscript drafting. CB performed data acquisition/analysis/interpretation and manuscript revision. JW contributed to data acquisition/analysis. K-HL was involved in data acquisition/analysis. KMH was involved in data acquisition and manuscript revision. HKK contributed to study design and manuscript revision. JMC was involved in research funding acquisition, data interpretation, and manuscript revision. J-HL contributed to study design, data analysis/interpretation, and manuscript drafting.

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References

1. Burton AW, Fanciullo GJ, Beasley RD and Fisch MJ. Chronic pain in the cancer survivor: a new frontier. *Pain Med* 2007; 8: 189–198.
2. Miaskowski C, Mastick J, Paul SM, Topp K, Smoot B, Abrams G, Chen LM, Kober KM, Conley YP, Chesney M, Bolla K, Mausisa G, Mazor M, Wong M, Schumacher M and Levine JD. Chemotherapy-Induced Neuropathy in Cancer Survivors. *J Pain Symptom Manage* 2017; 54: 204. e2–218.e2.
3. Gutiérrez-Gutiérrez G, Sereno M, Miralles A, Casado-Sáenz E and Gutiérrez-Rivas E. Chemotherapy-induced peripheral neuropathy: clinical features, diagnosis, prevention and treatment strategies. *Clin Transl Oncol* 2010; 12: 81–91.
4. Quasthoff S and Hartung HP. Chemotherapy-induced peripheral neuropathy. *J Neurol* 2002; 249: 9–17.
5. Peltier AC and Russell JW. Recent advances in drug-induced neuropathies. *Curr Opin Neurol* 2002; 15: 633–638.
6. Areti A, Yerra VG, Naidu V and Kumar A. Oxidative stress and nerve damage: role in chemotherapy induced peripheral neuropathy. *Redox Biol* 2014; 2: 289–295.
7. Kim HK, Zhang YP, Gwak YS and Abdi S. Phenyl N-tert-butylnitrone, a free radical scavenger, reduces mechanical allodynia in chemotherapy-induced neuropathic pain in rats. *Anesthesiology* 2010; 112: 432–439.
8. Fidanboylu M, Griffiths LA and Flatters SJ. Global inhibition of reactive oxygen species (ROS) inhibits paclitaxel-induced painful peripheral neuropathy. *PLoS One* 2011; 6: e25212.
9. Kim HK, Hwang SH and Abdi S T. Ameliorates and prevents mechanical hyperalgesia in a rat model of chemotherapy-induced neuropathic pain. *Front Pharmacol* 2016; 7: 532.
10. Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol* 2011; 194: 7–15.
11. Sena LA and Chandel NS. Physiological roles of mitochondrial reactive oxygen species. *Mol Cell* 2012; 48: 158–167.

12. Authier N, Balayssac D, Marchand F, Ling B, Zangarelli A, Descoeur J, Coudore F, Bourinet E and Eschalier A. Animal models of chemotherapy-evoked painful peripheral neuropathies. *Neurotherapeutics* 2009; 6: 620–629.

13. Binshtok AM, Bean BP and Woolf CJ. Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* 2007; 449: 607–610.

14. Xu ZZ, Kim YH, Bang S, Zhang Y, Berta T, Wang F, Oh SB and Ji RR. Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fiber blockade. *Nat Med* 2015; 21: 1326–1331.

15. Kim HK, Park SK, Zhou JL, Taglialatela G, Chung K, Coggeshall RE and Chung JM. Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain* 2004; 111: 116–124.

16. Yowtak J, Lee KY, Kim HY, Wang J, Kim HK, Chung K and Chung JM. Reactive oxygen species contribute to neuropathic pain by reducing spinal GABA release. *Pain* 2011; 152: 844–852.

17. Ichikizaki K, Toya S and Hoshino T. A new procedure for lumbar puncture in the mouse (intrathecal injection) preliminary report. *Keio J Med* 1979; 28: 165–171.

18. De la Calle JL and Paíno CL. A procedure for direct lumbar puncture in rats. *Brain Res Bull* 2002; 59: 245–250.

19. Cain DM, Khasabov SG and Simone DA. Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an in vivo study. *J Neurophysiol* 2001; 85: 1561–1574.

20. Bae C, Wang J, Shim HS, Tang SJ, Chung JM and La JH. Mitochondrial superoxide increases excitatory synaptic strength in spinal dorsal horn neurons of neuropathic mice. *Mol Pain* 2018; 14: 174480691879703.

21. Lee KY, Chung K and Chung JM. Involvement of reactive oxygen species in long-term potentiation in the spinal cord dorsal horn. *J Neurophysiol* 2010; 103: 382–391.

22. Boehmerle W, Huehnchen P, Peruzzaro S, Balkaya M and Endres M. Electrophysiological, behavioral and histological characterization of paclitaxel, cisplatin, vincristine and bortezomib-induced neuropathy in C57Bl/6 mice. *Sci Rep* 2014; 4: 6370.

23. Lennertz RC, Kossyreva EA, Smith AK and Stucky CL. TRPA1 mediates mechanical sensitization in nociceptors during inflammation. *PLoS One*. 2012; 7: e43597.

24. Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, Hjerling-Leffler J, Haeggström J, Kharchenko O, Kharchenko PV, Linnarsson S and Ernfors P. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. *Nat Neurosci* 2015; 18: 145–153.

25. Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS and Levings MK. Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J Immunol* 2005; 175: 8051–8059.

26. Khalil M, Alliger K, Weidinger C, Yerinde C, Wirtz S, Becker C and Engel MA. Functional role of transient receptor potential channels in immune cells and epithelia. *Front Immunol* 2018; 9: 174.

27. Nassini R, Gees M, Harrison S, De Siena G, Materazzi S, Moretto N, Failli P, Preti D, Marchetti N, Cavazzini A, Mancini F, Pedretti P, Nilius B, Patacchini R and Geppetti P. Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation. *Pain* 2011; 152: 1621–1631.

28. Ta LE, Bieber AJ, Carlton SM, Loprinzi CL, Low PA and Windebank AJ. Transient receptor potential vanilloid 1 is essential for cisplatin-induced heat hyperalgesia in mice. *Mol Pain* 2010; 6: 15.

29. Latremoliere A and Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* 2009; 10: 895–926.

30. Li Y, Tatsui CE, Rhines LD, North RY, Harrison DS, Cassidy RM, Johansson CA, Kosturakis AK, Edwards DD, Zhang H and Dougherty PM. Dorsal root ganglion neurons become hyperexcitable and increase expression of voltage-gated T-type calcium channels (Cav3.2) in paclitaxel-induced peripheral neuropathy. *Pain* 2017; 158: 417–429.

31. Megat S, Ray PR, Moy JK, Lou TF, Barragán-Iglesias P, Li Y, Pradhan G, Wanghzou A, Ahmad A, Burton MD, North RY, Dougherty PM, Khoutorsky A, Sonenberg N, Webster KR, Dussor G, Campbell ZT and Price TJ. Nociceptor translational profiling reveals the ragulator-Rag GTPase complex as a critical generator of neuropathic pain. *J Neurosci* 2019; 39: 393–411.

32. Clark AK, Gruber-Schoffnegger D, Drdla-Schutting R, Gerhold KJ, Malcangio M and Sandkuhler J. Selective activation of microglia facilitates synaptic strength. *J Neurosci* 2015; 35: 4552–4570.

33. Perea G, Yang A, Boyden ES and Sur M. Optogenetic astrocyte activation modulates response selectivity of visual cortex neurons in vivo. *Nat Commun* 2014; 5: 3262.

34. Hu LY, Zhou Y, Cui WQ, Hu XM, Du LX, Mi WL, Chu YX, Wu GC, Wang YQ and Mao-Ying QL. Triggering receptor expressed on myeloid cells 2 (TREM2) dependent microglial activation promotes cisplatin-induced peripheral neuropathy in mice. *Brain Behav Immun* 2018; 68: 132–145.

35. Zhang H, Yoon S-Y, Zhang H and Dougherty P M. Evidence that spinal astrocytes but not microglia contribute to the pathogenesis of Paclitaxel-induced painful neuropathy. *J Pain* 2012; 13: 293–303.

36. Krukowski K, Nijboer CH, Huo X, Kavelaars A and Heijnen CJ. Prevention of chemotherapy-induced peripheral neuropathy by the small-molecule inhibitor pifithrin-μ. *Pain* 2015; 156: 2184–2192.