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# Dysregulation of Cellular Calcium Homeostasis in Chemotherapy-Evoked Painful Peripheral Neuropathy

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## Abstract

Paclitaxel and vincristine are chemotherapeutic drugs that often evoke a long-lasting painful peripheral neuropathy. Using drugs that reduce intracellular or extracellular calcium ions ($Ca^{2+}$), we investigated the hypothesis that impaired $Ca^{2+}$ regulation contributes to the chemotherapy-evoked neuropathic pain syndrome. For comparison, we also tested rats with painful peripheral neuropathy caused by nerve trauma and to the anti-human immunodeficiency virus nucleoside analog 2′, 3′ dideoxycytidine (ddC). Normal naïve (without neuropathy), paclitaxel-treated, and vincristine-treated rats received the following intrathecal injections: TMB-8 (46 nmol), Quin-2 (1.8 nmol), EGTA (0.1 μmol), EGTA-AM (0.1μmol), and their vehicle controls. Chronic constriction injury (CCI) rats were examined after TMB-8 and Quin-2 injections, and ddC-treated rats were examined after receiving TMB-8. Mechano-allodynia and mechano-hyperalgesia were evaluated after each injection. Drug effects on heat hyperalgesia were also tested in CCI rats. All four $Ca^{2+}$-reducing drugs significantly inhibited mechano-allodynia and mechano-hyperalgesia in the rats treated with paclitaxel, vincristine, or ddC, but no effects were seen in the CCI or naïve rats. We conclude that a similar abnormality of cellular $Ca^{2+}$ homeostasis contributes to the pain caused by paclitaxel, vincristine, and ddC, but not posttraumatic painful peripheral neuropathy.

Paclitaxel and vincristine are chemotherapeutic drugs that are used widely for the treatment of solid tumors. Both evoke a dose-limiting peripheral neuropathy that often presents with a distal, symmetrical pain syndrome. Even with cessation of chemotherapy, the pain is variably reversible and may persist for months to years. The cause of this chemotherapy-evoked neuropathic pain is unknown. There are no confirmed prophylactic or therapeutic treatments (1).

Calcium is a key regulator of major cellular processes. Its cytosolic concentration is determined mainly by extracellular $Ca^{2+}$ influx, release of $Ca^{2+}$ from internal stores, and mitochondrial uptake. We have shown that ethosuximide, a relatively selective T-type $Ca^{2+}$-channel blocker, and gabapentin, an antagonist of $Ca^{2+}$ channels containing the $\alpha_2\delta$ subunit, significantly reduce paclitaxel- and vincristine-evoked neuropathic pain (2,3). Recently, we have obtained evidence that rats with paclitaxel-evoked neuropathic pain have an abnormality of mitochondria within myelinated and unmyelinated sensory axons (4). A mitochondrial abnormality can potentially lead to dysregulation of intracellular $Ca^{2+}$. Similarly, there is evidence that the painful peripheral neuropathy produced by anti-human immunodeficiency virus (HIV) nucleoside

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analog therapy is associated with disrupted $Ca^{2+}$ homeostasis secondary to mitochondrial dysfunction (5).

The experiments reported here explore the hypothesis that impaired $Ca^{2+}$ regulation is associated with paclitaxel- and vincristine-evoked neuropathic pain in rats. In addition, we compared the chemotherapy-evoked pain syndrome with those produced by traumatic nerve injury and anti-HIV nucleoside analog therapy.

## Methods

These experiments conformed to the ethics guidelines of the International Association for the Study of Pain (6), the National Institutes of Health, and the Canadian Institutes of Health Research. Experimental protocols were approved by the Facility Animal Care Committee of the Faculty of Medicine, McGill University.

Adult male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, IN; breeding colony, Frederick, MD), weighing 250–270 g at the start of the experiments, were housed with sawdust bedding under a 12-h dark/light cycle and had access to food and water *ad libitum*.

Paclitaxel (Taxol®; Bristol-Myers-Squibb, Montreal, Quebec, Canada) was injected intraperitoneally (IP; 2 mg/kg) every other day for 4 days (7); it was prepared from a stock solution of 6 mg/mL in Cremophor EL and ethanol (50:50) diluted to 2 mg/mL with normal saline. Vincristine (Novopharm Ltd, Toronto, Ontario, Canada) in saline vehicle was injected IP (50 μg/kg) for 10 consecutive days (3). 2′,3′-dideoxycytidine (ddC; saline vehicle; Sigma-Aldrich, Oakville, Ontario, Canada) was administered as a single IV bolus of 50 mg/kg via the tail vein (5). The chronic constriction injury (CCI) model was produced as described by Bennett and Xie (8). CCI rats received a contralateral sham operation (the nerve was similarly manipulated but not ligated).

Drugs that modify cellular $Ca^{2+}$ levels were injected intrathecally while the rats were anesthetized with 2% isoflurane in oxygen. The rat was placed prone with its spinal column arched over a support. Lumbar puncture was performed at the level of L5-6 intervertebral space using a 30-gauge needle. We verified the accuracy of these injections in pilot experiments in which the injection of 2% lidocaine produced a brief bilateral paralysis of the hindlimbs. A transient lateral flick of the rat's tail confirmed intrathecal needle placement. The test solution was then injected over 5 s via a 50-μL Hamilton syringe. For rats with neuropathic pain, the effects of all $Ca^{2+}$-decreasing drugs were tested at the approximate time of the models' peak symptom severity (22–28 days after paclitaxel injection, 13–19 days after vincristine injection, 8–12 days after CCI, and 8 days after ddC) in rats with confirmed pain hypersensitivity.

The following compounds, all delivered in a volume of 20 μL, were used to manipulate neuronal $Ca^{2+}$ levels: (a) TMB-8 (8-(dimethylamino) octyl 3,4,5-trimethoxybenzoate (46 nmol; Sigma-Aldrich, Oakville, Ontario, Canada). Intrathecal TMB-8 (up to 46 nmol) reduced anti-HIV nucleoside analog-evoked neuropathic pain in rats (5). (b) Quin-2 (2-([2-bis (carboxymethyl)amino-5methylphenoxy] methyl)-6-methoxy-8-bis(carboxymethyl) aminoquinoline potassium (1.8 nmol; EMD Biosciences [Calbiochem], San Diego, CA). This intrathecal dose of Quin-2 has been used to investigate the role of $Ca^{2+}$channels in formalin-evoked pain (9). (c) EGTA (ethylene glycolbis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt (0.1 μmol; Sigma-Aldrich). Intracerebroventricular EGTA (up to 1 μmol) has been used in experiments on opioid analgesia in mice (10). In pilot experiments, we investigated the intrathecal dose of EGTA at 0.01, 0.1, and 1 μmol to determine a dose that did not elicit ataxia in rats. The largest tolerated dose was 0.1 μmol. (d) EGTA-$_{AM}$ (ethylene glycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester (0.1 μmol; EMD Biosciences [Calbiochem]). EGTA-$_{AM}$ is the acetoxymethyl ester of EGTA. EGTA-$_{AM}$ and TMB-8 act

intracellularly, whereas Quin-2 and EGTA act extracellularly. Their effects were examined 30 min after the intrathecal injection.

TMB-8, Quin-2, and EGTA were freshly prepared in normal saline; EGTA-$_{AM}$ was freshly prepared in 20% Pluronic F-127 in dimethyl sulfoxide (Molecular Probes, Eugene, OR). For each drug, a group of control rats received an intrathecal injection of the same volume of the relevant vehicle.

The effects of intradermal injections of TMB-8 (116 nmol/10 μL) or an equal volume of saline were examined in ddC- and paclitaxel-treated rats. These injections were made while the rats were under isoflurane anesthesia into the dorsal skin of one hindpaw using a 30-gauge needle, and the rats were tested 30 min later.

The rats were assessed for mechano-allodynia and mechano-hyperalgesia. In CCI rats, we also examined heat hyperalgesia. Paclitaxel-treated rats have little or no heat hyperalgesia (7). Rats were habituated to the testing environment for 15 min on three separate occasions before data collection. Naïve baselines were noted before the establishment of the pain models.

Mechanical hypersensitivity was evaluated using von Frey hairs (VFH; Stoelting, Wood Dale, IL). Mechano-allodynia was assessed as an increased incidence of response to stimulation with a 4-g VFH; naïve rats almost never withdraw from this stimulus. Subsequently, mechano-hyperalgesia was assessed with a 15-g VFH, which evokes a relatively infrequent incidence of withdrawals in naïve rats. The rats were confined under inverted plastic cages and placed on an elevated wire mesh platform, which allowed access to the plantar surface of the hindpaws. Each VFH was applied to the mid-plantar hindpaw (the region in the center of the ring formed by the tori at the base of each of the digits) and held for 5 s. This was repeated five times for each paw, and the sum of withdrawal responses from both paws to each VFH was expressed as a percentage response (number of hindpaw withdrawals/10 × 100). For the CCI rats, the response frequencies to each VFH for the ipsilateral and contralateral paws were calculated separately.

Pain hypersensitivity to mechanical stimuli was also assessed with the Randall-Selitto paw-pressure test using an Analgesy-meter® (Ugo Basile, ComerioVarese, Italy). Testing began approximately 5 min after VFH testing. Rats were wrapped loosely in a fabric towel with the hindpaw placed between the Analgesy-meter platform and stylus. A linearly increasing mechanical force was then applied until the paw was withdrawn, and the corresponding force was recorded as the withdrawal threshold. Three readings for each paw were taken at 5-min intervals and averaged. With the pain models used here, there is a decrease in the paw-pressure threshold of approximately 25%; we classify this decrease as mechano-hyperalgesia. However, we note that the paw-pressure stimulus may activate both cutaneous and deep-tissue sensory afferents, whereas the 15-g VFH stimulus delivers most of its energy to the cutaneous innervation.

Heat hypersensitivity was assessed using the method of Hargreaves et al. (11). Rats were confined in a plastic enclosure on the glass surface of a thermal stimulator (UCSD, San Diego, CA). A focused beam of radiant heat under the glass floor was aimed at the fat part of the heel. The latency to paw withdrawal was taken as an index of the heat-pain threshold. The heat intensity was standardized such that mean naïve baseline latencies were between 8 and12 s and remained constant throughout the experiment. A cutoff latency of 20 s was imposed to avoid possible tissue damage. Testing was repeated 3 times for each paw at 10-min intervals, and the scores were averaged.

All data are expressed as mean ± sem. The effects of intrathecal or intradermal drug versus vehicle were analyzed with unpaired *t*-tests. Differences were considered significant at $P < 0.05$.

## Results

Treatment with paclitaxel, vincristine, or ddC produced the expected bilateral neuropathic pain, with statistically significant increases in pain sensitivity as measured with the 4-g and 15-g VFH tests and the paw-pressure test. The CCI procedure produced the expected statistically significant increases in pain sensitivity to VFH, paw pressure, and heat on the nerve-injured side; no significant changes were found on the sham-operated side with any of the four tests.

In paclitaxel- and vincristine-treated rats tested with VFH (Fig. 1), intrathecal TMB-8, Quin-2, EGTA, and EGTA-AM all produced statistically significant reductions in mechano-allodynia and mechano-hyperalgesia, whereas intrathecal injections of their respective vehicles had no effect. The degree of pain reduction was similar for each of the four drugs. In addition, all four drugs produced statistically significant reduction of mechano-hyperalgesia, as assessed in the paw-pressure test (Fig. 2). None of the four drugs had any effect on responses of naïve animals tested with 4-g or 15-g VFH and the paw-pressure test using the same doses ($n = 6$ per group; data not shown).

In CCI rats, neither TMB-8 nor Quin-2 had any effect on the mechano-allodynia and mechano-hyperalgesia assessed with VFH, mechano-hyperalgesia assessed with the paw-pressure test, or on heat hyperalgesia (Fig. 3). Neither drug had any effect in any of the four tests on the response sensitivity of the contralateral hindpaw (Fig. 3).

As shown in Figure 4, both intradermal and intrathecal injections of TMB-8 reduced mechano-hyperalgesia (paw-pressure test) in ddC-treated rats, as previously reported (5). Although intrathecal TMB-8 was effective in paclitaxel-treated rats, the intradermal TMB-8 dose was without effect.

## Discussion

An increase in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) mediates a wide range of neuronal functions including membrane excitability, neurotransmitter release, synaptic plasticity, gene expression, and excitotoxicity (12). It is probable that $[Ca^{2+}]_c$ also plays a critical role in glial function. Altered $[Ca^{2+}]_c$ has been demonstrated after formalin-evoked pain (9), posttraumatic painful neuropathy (13), and anti-HIV nucleoside analog-evoked painful peripheral neuropathy (5).

Our data show that the neuropathic pain (mechano-allodynia and mechano-hyperalgesia) produced by paclitaxel and vincristine is significantly ameliorated by drugs that decrease the extracellular and intracellular availability of $Ca^{2+}$. We confirmed this effect with 4 different drugs (TMB-8, Quin-2, EGTA, and EGTA-AM). Three of the drugs (TMB-8, Quin-2, and EGTA) are chemically distinct, and 3 have distinct mechanisms or sites of action. Quin-2 is a highly selective $Ca^{2+}$ chelator (14), as is EGTA. Both are membrane impermeable and will preferentially chelate extracellular $Ca^{2+}$, reducing $Ca^{2+}$ influx. We used intracellular $Ca^{2+}$-decreasing drugs to further explore the regulation of $Ca^{2+}$ downstream to extracellular $Ca^{2+}$ influx. TMB-8 is membrane permeable and works intracellularly to prevent $Ca^{2+}$-induced $Ca^{2+}$ release from the endoplasmic reticulum via binding to ryanodine receptors (15). EGTA-AM is a nonpolar ester of EGTA, making it membrane permeable. It is inactive until de-esterification by intracellular esterases and, thus, preferentially chelates, free intracellular $Ca^{2+}$. None of these drugs had any effect on the responsiveness of naïve rats, showing that

their effects were not caused by a general analgesic action or by an impairment of normal $Ca^{2+}$ signaling.

Our results are consistent with a previous report (5) that intrathecal TMB-8 is also effective against the neuropathic pain produced by anti-HIV nucleoside analog therapy. We confirm that there is a peripheral site of action for TMB-8 in the ddC syndrome. However, using the same dose of TMB-8, we found no evidence for a peripheral site of action for paclitaxel-evoked pain.

Although the intrathecal dosages of TMB-8 and Quin-2 used were effective in paclitaxel-, vincristine-, and ddC-evoked pain, they were ineffective against the mechano-allodynia, mechano-hyperalgesia, and heat hyperalgesia seen with posttraumatic painful peripheral neuropathy. We suspect that the absence of an effect in the CCI rats may reflect a true difference in underlying pathogenesis. Activation of the *N*-methyl-D-aspartate receptor (NMDAR) leads to an influx of extracellular $Ca^{2+}$, which increases $[Ca^{2+}]_c$ and initiates neuronal events that contribute to central sensitization (9). Increased intracellular $Ca^{2+}$ from NMDAR activation has been demonstrated in the ipsilateral dorsal horn of spinal cords of CCI rats (16). Thus, a larger dose of the $Ca^{2+}$-decreasing drugs may be required to show an effect in CCI rats.

Paclitaxel and vincristine are thought to exert their antitumor activity largely by binding to β-tubulin and disrupting mitotic spindle formation in actively dividing cells. Paclitaxel stabilizes β-tubulin polymerization, whereas vincristine inhibits spindle assembly (17). Axonal microtubules are composed largely of β-tubulin, and it has generally been accepted that the neurotoxicity caused by paclitaxel and vincristine is caused by disruption of microtubule structure that impairs axoplasmic transport and leads to a dying-back neuropathy. Although this hypothesis may be germane to the axonal degeneration caused by large doses of paclitaxel and vincristine, there are reasons to question that it can account for the neuropathic pain produced by small doses of paclitaxel and vincristine. Importantly, electron microscopy studies of sensory nerves from rats with paclitaxel- and vincristine-evoked neuropathic pain have found no evidence of dying-back and only subtle changes in the organization of axonal microtubules (4,7,18).

Vincristine has been reported to affect $Ca^{2+}$ movement through the mitochondrial membrane, reducing both the amount and rate of $Ca^{2+}$ uptake and decreasing $Ca^{2+}$ efflux (19). Paclitaxel has been shown to abolish the normal oscillations in $[Ca^{2+}]_c$ and to evoke a spike increase in $[Ca^{2+}]_c$ that is independent of extracellular $Ca^{2+}$ levels and intracellular inositol triphosphate-sensitive $Ca^{2+}$ stores (20). The same study also demonstrated that paclitaxel causes a rapid decline in mitochondrial membrane potential and, notably, a loss of mitochondrial $Ca^{2+}$ via the mitochondrial permeability transition pore. It is associated with β-tubulin (21) and is a likely binding site for paclitaxel and vincristine on mitochondria. In support of this alternative hypothesis of impaired mitochondrial function in paclitaxel- and vincristine-evoked neuropathic pain, we have reported that sensory primary afferent axons from rats with paclitaxel-evoked neuropathic pain have a greatly increased incidence of mitochondria that are swollen, vacuolated, and have severely disrupted cristae, which are seen to have collapsed, fragmented, and puddled at the periphery of the organelle (4). Mitochondrial function is critically dependent on molecular exchange between its outer and inner membranes; thus, disrupted cristae indicate impaired function. These structural abnormalities suggest that there would be pathological changes in mitochondrial function.

Mitochondria play a key role in intracellular $Ca^{2+}$ homeostasis. The influx of extracellular $Ca^{2+}$ via activated voltage- and ligand-gated $Ca^{2+}$ channels on the cell membrane and the release of $Ca^{2+}$ from endoplasmic reticulum can generate large, localized increases in $[Ca^{2+}]_c$. Mitochondria, with their large buffering capacity and close proximity to these structures, can rapidly sequester this free $Ca^{2+}$ (12,22). Impaired mitochondrial $Ca^{2+}$ uptake

(or increased leakage of mitochondrial $Ca^{2+}$) would thus alter the spatio-temporal changes of $[Ca^{2+}]_c$, increase propagation of $[Ca^{2+}]_c$ signals (23), and modulate $Ca^{2+}$-dependent processes such as increased exocytosis of neurotransmitters (22). All these changes may result in heightened neuronal excitability and impaired glial function. Reducing the availability of extracellular $Ca^{2+}$, blocking $Ca^{2+}$ release from intracellular stores, or chelating cytoplasmic-free $Ca^{2+}$ would thus be expected to reverse some of the adverse consequences of impaired mitochondrial $Ca^{2+}$ regulation.

The increase in neuronal $[Ca^{2+}]_c$ after NMDAR activation is usually buffered by mitochondrial uptake (24,25). When mitochondrial $Ca^{2+}$ uptake is impaired, an abnormal accumulation of cytoplasmic $Ca^{2+}$ would occur in the vicinity of the NMDAR. This engages regulatory proteins such as $Ca^{2+}$-dependent phosphatase, which inactivate the NMDAR and shorten the duration of $Ca^{2+}$ influx (26). Experiments using cortical brain-slice preparation have shown a reduction of $Ca^{2+}$ influx and intracellular $Ca^{2+}$ load caused by NMDAR activation in the presence of impaired mitochondrial $Ca^{2+}$ uptake (25). This mechanism would reduce the contribution of NMDAR to central sensitization in chemotherapy-evoked pain. This could (further) account for the effectiveness of the $Ca^{2+}$ decreasing drugs in reducing paclitaxel-, vincristine-, and ddC-evoked pain, but not trauma-evoked pain, in CCI rats. Additionally, it would also explain the lack of effect of MK801 in paclitaxel-treated animals (2) when NMDAR antagonists are invariably effective in models of posttraumatic painful peripheral neuropathy.

It is possible that the $Ca^{2+}$-decreasing drugs used in this experiment exerted their effects on altered $Ca^{2+}$-mediated neuronal and glial functions other than mitochondrial buffering. Of course, mitochondria also perform other cellular functions, including energy production and the regulation of free radicals. Our results do not exclude these additional possible mechanisms for the generation of chemotherapy-evoked neuropathic pain. Moreover, our results do not resolve the locus of the observed effect. Intrathecally administered drugs might act in the spinal cord dorsal horn, the dorsal nerve root, or the dorsal root ganglion. Further evaluation of the role of mitochondria and $Ca^{2+}$ dysregulation in chemotherapy-evoked pain may have a significant impact to developing successful clinical strategies for the management of this painful complication of cancer treatment.

In summary, our findings suggest that chemotherapy-evoked neuropathic pain is, at least in part, mediated by altered cellular $Ca^{2+}$ homeostasis. Given the critical role mitochondria play in modulating $[Ca^{2+}]_c$ signals and the effects of paclitaxel and vincristine on mitochondria, this dysregulation of $Ca^{2+}$ could possibly be secondary to impaired mitochondria function.

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**Figure 1.**

Effect of intrathecal (i.t.) 20 μL of TMB-8 (46 nmol), Quin-2 (1.8 nmol), EGTA-$_{AM}$ (0.1 μmol), and EGTA (0.1 μmol) versus the respective vehicles on the responses of paclitaxel- and vincristine-treated rats to von Frey hairs (VFH) exerting 4 g (mechano-allodynia) and 15 g (mechano-hyperalgesia) of force. Data from paclitaxel- and vincristine-treated rats were analyzed separately. The dotted lines indicate baseline values before chemotherapy treatment (mean of all groups combined). There was no difference in response to 4-g and 15-g VFH for both chemotherapy-treated groups after i.t. N/S. For the purpose of graphic presentation, results from paclitaxel- and vincristine-treated rats given i.t. N/S were combined and labeled as "Control". N/S = normal saline; DMSO+P = 20% Pluronic F-127 in dimethyl sulfoxide (the vehicle for EGTA-$_{AM}$). *$P < 0.05$; **$P < 0.01$; $n = 10$ per group.

**Figure 2.**

Effect of intrathecal (i.t.) 20 μL of TMB-8 (46 nmol), Quin-2 (1.8 nmol), EGTA-$_{AM}$ (0.1μmol), and EGTA (0.1μmol) and their respective vehicle controls on the responses of paclitaxel- and vincristine-treated rats in the paw-pressure test of mechano-hyperalgesia. The dotted lines indicate baseline values before chemotherapy treatment (mean of all groups combined). N/S = normal saline; DMSO+P = 20% Pluronic F-127 in dimethyl sulfoxide (the vehicle for EGTA-$_{AM}$). $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 10–15$ per group.

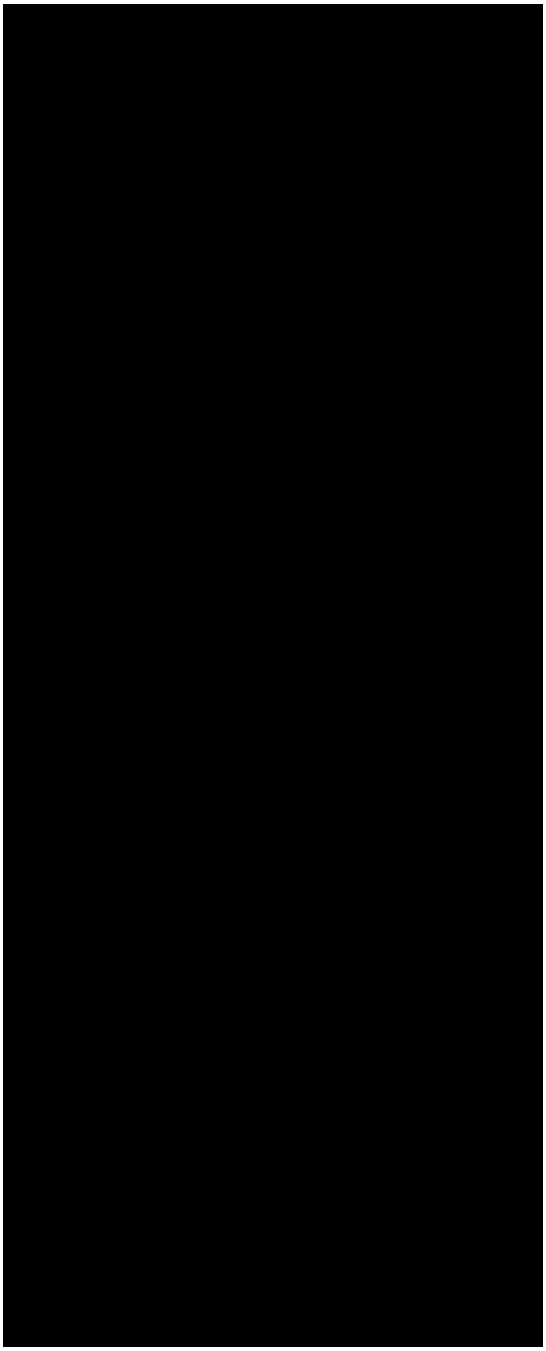

**Figure 3.**
Effect of intrathecal (i.t.) 20 μL of TMB-8 (46 nmol), Quin-2 (1.8 nmol), and the vehicle control (normal saline [N/S]) on responses of the ipsilateral and contralateral hindpaws of chronic constriction injury (CCI) rats to 4-g von Frey hairs (VFH; mechano-allodynia), 15-g VFH (mechano-hyperalgesia), paw-pressure test (mechano-hyperalgesia), and noxious heat (heat hyperalgesia). Dotted lines indicate the baseline values obtained before CCI surgery. N/S group represents the combined result from the controls for TMB-8– and Quin-2–injected groups. There are no statistically significant effects. $n = 8$ per group.

**Figure 4.**
(ID) Effect of intradermal (ID) 10 μL of TMB-8 (116 nmol) versus normal saline (N/S) on the paw-withdrawal thresholds of ddC- and paclitaxel-treated rats. (IT) Effect of intrathecal (IT) 20 μl of TMB-8 (46 nmol) on ddC- and paclitaxel-treated rats (the paclitaxel data are repeated from Fig. 2 for comparison). Both ID and IT TMB-8 reverse ddC pain, but only IT administration is effective for paclitaxel pain. The dotted lines indicate baseline values before ddC or paclitaxel treatment (mean of all groups combined). ***$P < 0.001$; $n = 12–15$ per group.