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# Antinociceptive effectiveness of the inhibition of NCX reverse-mode action in rodent neuropathic pain model

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## Abstract

**Background:** Chronic neuropathic pain is a debilitating condition that remains difficult to treat. The $Na^+$-$Ca^{2+}$ exchanger (NCX) is a transporter that can exchange $Ca^{2+}$ with $Na^+$ in either direction to maintain intracellular $Ca^{2+}$ homeostasis. However, the effect of NCX on neuropathic pain remains unclear. Therefore, in this study, we aimed to clarify whether neuropathic pain is altered by NCX.

**Methods:** Adult Sprague–Dawley rats and mice (NCX2 knockout and wild type) were randomized to receive spinal nerve ligation surgery or intrathecal injection. Using behavioral testing to analyze the withdrawal thresholds and thermal withdrawal latency of rats after surgery or intrathecal injection. Immunohistochemistry and Western blotting were used to analyze the changes of NCX protein and downstream signaling pathways in rats dorsal root ganglion. We isolated the dorsal root ganglion neurons of adult rats using Fluo-4AM to detect the $Ca^{2+}$ imaging in neurons after drug treatment.

**Results:** NCX was expressed in the sensory neurons of rodent dorsal root ganglia. NCX expression was altered in ipsilateral L4–6 dorsal root ganglion neurons in spinal nerve ligation rats. Intrathecal injection of an inhibitor of reverse-mode NCX activity (KB-R7943 5∼20 μg) had an antinociceptive effect in spinal nerve ligation rats, and the effect lasted for 3 h. We measured the expression of signaling pathway molecules in dorsal root ganglion neurons, and only the p-extracellular signal-regulated kinase (ERK) 1/2 level was reduced after intrathecal injection in the spinal nerve ligation group compared to the control group. In cultured dorsal root ganglion neurons, inhibitors of reverse-mode NCX activity (KB-R7943 and ORM-10103) restrained $Ca^{2+}$ overload after tumor necrosis factor alpha (TNF-α) or lipopolysaccharide (LPS) treatment. NCX2 knockout mice presented an antinociceptive effect that lasted for more than 28 days after spinal nerve ligation surgery. The p-ERK1/2 level in NCX2 knockout mice ipsilateral L4–6 dorsal root ganglion neurons was lower than that in wild-type mice.

**Conclusions:** NCX proteins may mediate neuropathic pain progression via the $Ca^{2+}$ and ERK pathways. NCX represents a potential target for the treatment of neuropathic pain.

## Keywords

Neuropathic pain, sodium-calcium exchange, intrathecal, calcium, mitogen-activated protein kinases

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## Introduction

Neuropathic pain is a common complication of nerve injury, diabetes, and tumors. Neuropathic pain is one of the most intractable diseases and is observed as typical symptoms such as hyperalgesia and allodynia. The widely accepted theories for neuropathic pain include activation of spinal gliocytes, inflammatory cytokine release in the central nervous system, and ion

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($Na^+$, $K^+$ and $Ca^{2+}$) concentration changes in nerve cells.[1] However, the molecular mechanisms responsible for neuropathic pain are not fully known.

$Na^+$-$Ca^{2+}$ exchanger (NCX) is an important plasma membrane protein involved in intracellular $Ca^{2+}$ homeostasis[2] that is expressed in many organs within the body.[3] NCX is a bi-directional ion transporter that catalyzes the exchange of $Na^+$ with $Ca^{2+}$, depending on the electrochemical gradient of each ion.[4] Under physiological conditions, the primary role of NCX is to extrude $Ca^{2+}$ from cells using the $Na^+$ gradient across the cell membrane (forward mode of operation).[5] However, in some cases, NCX can contribute to $Ca^{2+}$ influx into cells by operating in the reverse mode (coupling $Ca^{2+}$ influx with $Na^+$ efflux).[6,7] Thus, NCX operates in two modes. In the forward mode, NCX generates an inward current by transferring 3 $Na^+$ ions into myocytes and removing 1 $Ca^{2+}$ ion.[8] In the reverse mode, the direction of ion transfer is reversed, and consequently, NCX produces an outward current, which contributes to the repolarization of cardiac myocytes.[9] Currently, four isoforms of NCX (NCX1–4) have been identified, and NCX1 and NCX2 are the predominant isoforms in rat dorsal root ganglion (DRG) neurons.[10]

Peripheral nerve injury elicits a marked immune response distal to the axonal lesion site in the spinal cord and the DRG. Large and small neurons constitute the majority of cells in the DRG.[11–13] Both cell types contain fibers that transmit pselaphesia and algesia to the central nervous system (large neurons contain A fibers and small neurons contain C fibers). These neurons show abnormal function during neuropathic pain.[14] Neuropathic pain is mediated by many factors, such as the abnormal release of inflammatory factors, alterations in channel expression in the nerve cells, and activation of glial cells.[15–17]

Recent studies have shown that $Ca^{2+}$ plays a very important role in the progression of neuropathic pain.[18,19] The level of $Ca^{2+}$ in neurons increases mainly from extracellular and intracellular $Ca^{2+}$, with extracellular calcium ions entering the neurons via activation of N-methyl-D-aspartate (NMDA) receptors, T-type calcium ion channels, and some subtypes of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionicacid (AMPA) receptors.A significant rise in the intracellular $Ca^{2+}$ concentration activates downstream signaling pathways, including mitogen-activated protein kinases (MAPKs), caspases, protein kinase C (PKC), protein kinase A (PKA), and calmodulin kinase II (CAMK II). These pathways may cause a series of biochemical reactions that induce changes in the synaptic membrane, eventually leading to increased sensitivity of neurons and producing hyperalgesia and pain hypersensitivity.

MAPKs have been implicated as key intracellular components in the transduction of biochemical and metabolic changes induced by hyperglycemia. Three distinct MAPK families have been identified: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Three MAPK members have been implicated in numerous diabetes-related pathologies.[20] In cultures of sensory neurons, ERK, JNK, and p38 are activated by osmotic perturbations induced by glucose stress and advanced glycation end products.[21] Apoptosis is also similar to neuronal plasticity under pathologic conditions, which can explain some problems that are not fully explained through paintheory.[22] Apoptosis is often mediated by caspase-mediated protein cleavage in which caspase-3 and caspase-9 play important roles.[23] The efficacy of neuropathic pain therapies is still unsatisfactory due to its complicated pathogenesis. In this study, we investigated the role of NCX in neuropathic pain.

## Methods and materials

### Animals

All experiments were approved by the Ethics Committee for Animal Use of Sun Yat-sen University and performed according to the European Commission guidelines for the care and use of animals. Specific pathogen-free adult male Sprague–Dawley rats (200–250 g) and NCX2 knockout (KO) or wild-type (WT) mice (25–35 g, age 12–13 weeks) were housed in individual cages with free access to food and water. The animals were maintained on a 12-h light–dark cycle at an ambient temperature of 21°C ± 1°C and a relative humidity of 50%–60%. All experiments were conducted in accordance with the guidelines defined by the Sun Yat-sen University Cancer Center, and the study was approved by the Animal Care and Ethics Committee of Sun Yat-sen University Cancer Center. All KO mice were provided by Cyagen Biosciences, Inc. They used the CRISPR/Cas9 method to KO the NCX2 gene "GeneSymbol Slc8a2" in oosperm.

### Intrathecal injection and drugs

Under isoflurane anesthesia, apolyethylene (PE-10) catheter was inserted through the foramen magnum and advanced 8 cm caudally to the intumescentia lumbalis, as previously described.[24] Rats that appeared to have hind limb paralysis or paresis after surgery were excluded from this study. Intrathecal injections were performed four to five days after catheter implantation. We administered KB-R7943 (Tocris, UK), which was dissolved in 1% dimethyl sulfoxide (DMSO). The KB-R7943 was intrathecally injected in a 10 μL volume, followed by a 10 μL saline flush to assure drug delivery to the

subarachnoid space. The control group received 1% DMSO.

### Neuropathic pain model

Spinal nerve ligation (SNL) surgery was performed as previously described.[25] Under isoflurane anesthesia, rats underwent surgery to ligate and transect the left L5 spinal nerves while maintaining 2 mm of nerve distal to the ligation site to prevent nerve regeneration. Sham animals underwent anesthesia and skin and muscle incisions identical to the SNL animals without ligation or injury of the spinal nerves.

### Immunohistochemistry

The DRGs in segments L4–6 of the spinal cord were dissected from anesthetized rats, fixed in formalin for 2 h, dehydrated overnight in 20% sucrose, and embedded in Tissue-Tek OCT Compound (Sakura Finetek, USA). Transverse sections through the DRGs were cut at 8-μm thickness on a cryostat microtome. We blocked nonspecific protein-binding sites by incubating the slices for 1 h in phosphate-buffered saline containing 0.5% donkey serum albumin and 0.1% Triton X-100. The slices were immunolabeled by overnight incubation at 4°C with primary antibodies. Then, the slices were incubated for 1 h with Cy3- or fluorescein isothiocyanate-labeled secondary antibodies. An inverted laser scanning microscope was used to analyze the protein expression. Anti-NCX 1–2 antibodies were purchased from Santa Cruz.Antibodies against neurofilament-200 (NF-200), isolectin B4 (IB-4), glial fibrillary acidic protein (GFAP), ERK, JNK, p38, and caspases were purchased from CST.

### Western blotting

We collected the ipsilateral L4–6 DRGs from anesthetized rats and homogenized the tissue by sonication in protein extraction buffer (pH 7.5). We employed NuPAGE Novex 4%–12% Bis-Tris gels for electrophoresis (Life Technologies) and transferred the protein to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking for 1 h at room temperature, we incubated the membranes with a primary antibody overnight at 4°C. Antibody–protein complexes were labeled with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, followed by incubation with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA). Chemiluminescent bands were revealed by exposing Hyperfilm electrochemiluminescence (ECL) (GE Healthcare, USA) to the membranes. GAPDH was used as a loading control and for normalization of the signal intensity of the immunoreactive bands.

### Behavioral testing

After habituating the animals to the testing environment, we obtained two baseline measures during the week prior to SNL surgery. All animals were examined 3 h after drug or vehicle injection. They were placed on an elevated wire grid and stimulated on the plantar surface of the hind paw in the territory of the "spared" sural nerve. We used calibrated von Frey monofilaments to determine the withdrawal threshold for punctate mechanical stimulation. Touch perception was measured with the up-down method to obtain the 50% threshold using von Frey filaments as described previously.[11,26] Thermal nociceptive thresholds were determined by measuring paw withdrawal latency using a Hargreaves apparatus.[27] Thermal place preference was used to assess cold avoidance. Rats were placed in a plexiglass chamber with two adjacent thermal surfaces, both with an accuracy of $\pm 0.1$°C.

### DRG culture

Experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals; all animal protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Connecticut Health care System, West Haven, Connecticut. Adult Sprague–Dawley rats (four- to six-week old) were deeply anesthetized by $CO_2$ narcosis and decapitated. DRGs were isolated and dissociated after anesthesia. In brief, dissected ganglia were placed in ice-cold oxygenated complete saline solution containing the following (in mM): 137 NaCl, 5.3 KCl, 1 $MgCl_2$, 25 sorbitol, 3 $CaCl_2$, and 10 HEPES, pH 7.2. DRGs were digested for 20 min at 37°C in complete saline solution containing collagenase D (1.5 mg/ml) and papain (30 U/ml). DRGs were centrifuged and resuspended in DRG media (Dulbecco's Modified Eagle Medium/F12 medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 1.5 mg/ml bovine serum albumin and 1.5 mg/ml trypsin inhibitor (Sigma)). DRGs were triturated in DRG media and centrifuged. The cell pellet was resuspended in DRG media, placed on top of a layer of 15% bovine serum albumin in DRG media and centrifuged at 200 relative centrifugal force for 10 min to remove non-neuronal cells.

### $Ca^{2+}$ imaging

$Ca^{2+}$ concentrations were assayed using the ratiometric intracellular calcium indicator Fluo-4AM (Dojindo). DRG neurons cultured for 18 days were loaded at room temperature for 30 min with 2 μM Fluo-4AM in Hank's balanced salt solution (HBSS) containing the

following (in mM): 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES, pH 7.3, along with 0.02% Pluronic (Sigma). Neuronal cultures were illuminated with ordinary light to reveal the neurons that were cultured with TNF-α and LPS overnight. After that, we use saline or 1.4 μM KB-R7943(Tocris, UK) or 960 nM ORM-10103 (Sigma, USA) to incubate these neurons for 2 h. Neuronal cell bodies and neurites identified from the visible light observation were selected for $Ca^{2+}$ imaging. Neurons were illuminated with 488 nm light using Laser confocal microscope (OLYMPUS FV1000).

### Statistics

All data are shown as the means ± S.E.M. We used one-way factorial analysis of variance to analyze the data, and the Student-Newman-Keuls test was used to analyze differences between groups. All the tests were performed using SPSS 11.0 (SPSS Inc.). Differences were considered significant when $p < 0.05$.

## Results

### Cellular localization and expression in the DRG after SNL surgery

To determine the regions of the DRG in which NCX1 and NCX2 expression was modulated after SNL surgery, we used immunofluorescence microscopy to examine the localization of NCX1 and NCX2 in DRGs. Large DRG neurons (NF200-labeled cells), but not small DRG neurons (IB-4-labeled cells) astrocytes (GFAP-labeled cells), showed NCX1 expression in DRGs of rats that received SNL surgery (Figure 1). Large DRG neurons (NF200-labeled cells) and small DRG neurons (IB-4-labeled cells), but not astrocytes (GFAP-labeled cells), showed NCX2 expression in the DRGs of rats that received SNL surgery (Figure 1).

At the third day post-SNL surgery, Western blot analysis showed no significant changes in the expression levels of NCX1 or NCX2 in the ipsilateral DRGs compared to the control DRGs (Figure 2). We also measured the NCX expression levels at the first, second, and fourth weeks after SNL. Interestingly, NCX1 expression at the second week after SNL was decreased to 38% ± 3.5% of that following the control treatment (Figure 2). At the second and fourth weeks after SNL, the expression of NCX2 in ipsilateral DRGs still showed a trend of an increase compared to the control levels (increase to 341% ± 29% of the control level in the second week and 303% ± 21% in the fourth week) (Figure 2). These data demonstrate that the development of neuropathic pain was associated with the alteration of NCX expression in DRG neurons and that the changes in NCX1 and NCX2 expression showed opposing trends.

### The antinociceptive effect of intrathecal KBR-7943 injection in SNL rats

We observed a reduction in allodynia and thermal withdrawal thresholds at the first week after SNL. No significant change in the withdrawal threshold was observed in the contralateral paws. The subarachnoid catheter placement neither changed the peripheral nerve injury-induced hypersensitivity nor modified the withdrawal thresholds in the paws after SNL (data not shown). Intrathecal injection of 1% DMSO had no significant effect on the withdrawal threshold. In contrast, intrathecal injection of KB-R7943 (5–20 μg) produced a significant reversal of the pain hypersensitivity and reduced thermal withdrawal latency induced by SNL. The effect of a single injection of 20 μg KB-R7943 lasted for more than 3 h (Figure 3). These antinociceptive effects of KB-R7943 suggest that reverse-mode NCX activity in the DRGs may be part of the mechanism of SNL-induced hyperpathia in neuropathic pain.

### MAPK phosphorylation level and cleaved caspase-3 and caspase-9 levels in DRGs after intrathecal KBR-7943 treatment

To investigate the possible mechanisms underlying the antinociceptive effects of KB-R7943, we used Western blot to measure MAPK and caspase signaling, the phosphorylated protein levels of ERK1/2, p38, and JNK and the levels of cleaved caspase-3 and caspase-9 in the ipsilateral L4–6 DRGs from the group treated with 20 μg KB-R7943. We found that intrathecal injection of KBR-7943 (20 μg) resulted in only the downregulation of MAPK-ERK1/2 phosphorylation (the phosphorylation level was reduced to 26% ± 2% of the control level at 1 h post-intrathecal injection and to 33% ± 3% at 2 h post-intrathecal injection). The cleavage of caspase-3 and caspase-9 and the phosphorylation of JNK and p38 showed no significant changes (Figure 4).

### Reverse-mode NCX activity contributes to $Ca^{2+}$ overload in neurons

We found that both neuronal cell bodies and neurites of DRG neurons exhibited low $Ca^{2+}$ concentrations in the treated group (Figure 5). To determine whether NCX contributes to the observed $Ca^{2+}$ overload in DRG neurons, DRG neurons were cultured for 18 days and then pretreated with LPS (1000 ng/ml) or TNF-α (4000 pg/ml) 24 h before treatment. The cell bodies of DRG neurons exhibited similar basal $Ca^{2+}$ levels and decreased transient peak $Ca^{2+}$ levels compared with those of untreated neurons (Figure 5). Quantitative analysis revealed that after KB-R7943 and ORM-10103 treatment for 1 h, the intracellular $Ca^{2+}$ concentration was not significantly different

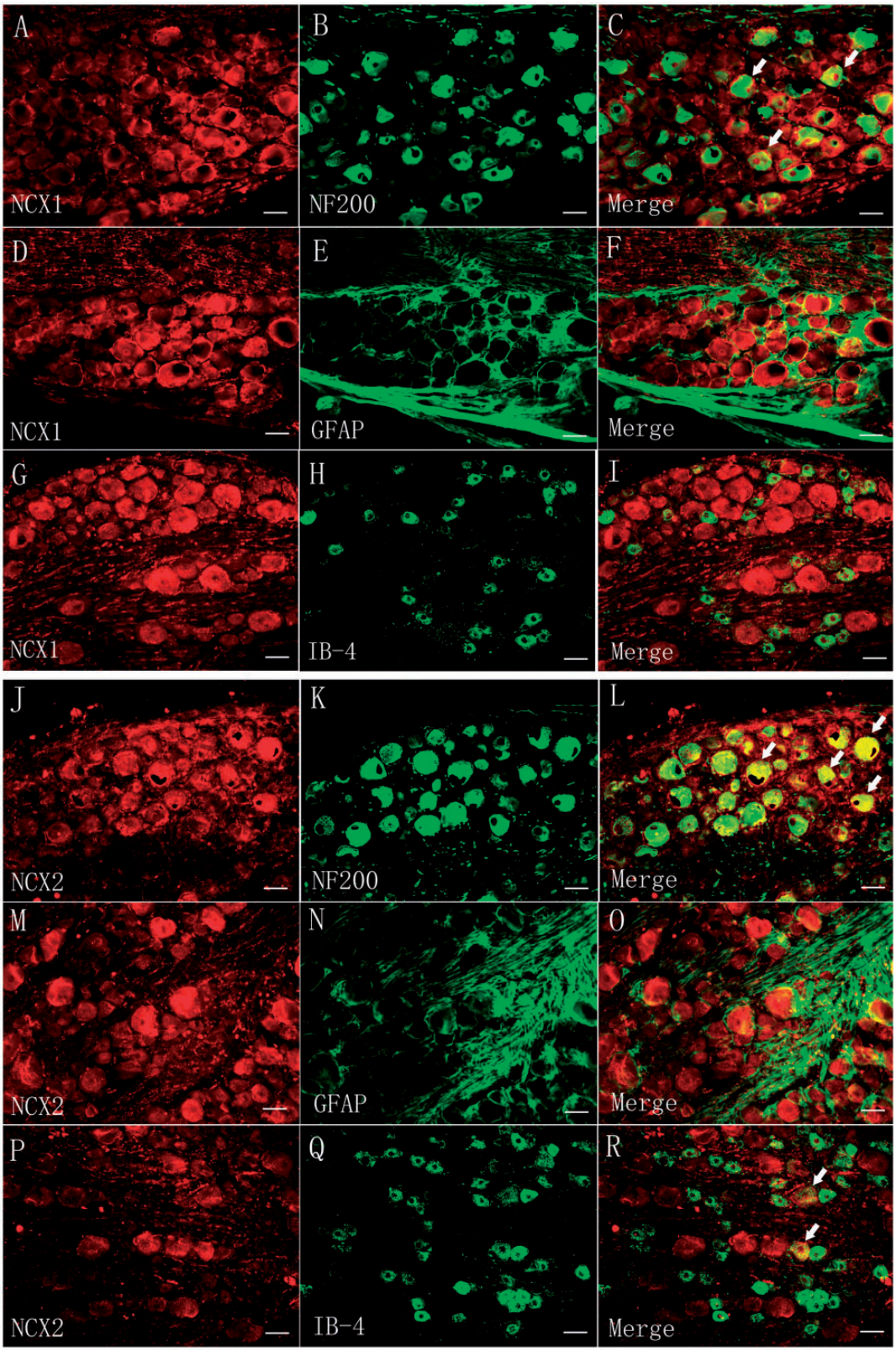


**Figure 1.** Immunofluorescence photomicrographs of NCX and p-ERK expression in the ipsilateral L4–6 DRGs of rats on the seventh day after SNL surgery. Double labeling immunostaining of GFAP, NF200, and IB-4 was performed. We found colocalization between NCX1 (red) and NF200 (green), but NCX1 (red) did not colocalize with GFAP (green) or IB-4 (green), which suggests that NCX1 is located in the large neurons of the DRGs ((a) to (i)). NCX2 (red) colocalized with NF200 (green) and IB-4 (green) but not GFAP (green), which suggests that both NCX2 and p-ERK are located in the large and small neurons of the DRGs ((j) to (r)). Arrows show colocalization. Scale bar: 50 μm. NCX: $Na^{+}$-$Ca^{2+}$ exchanger; GFAP: glial fibrillary acidic protein; NF-200: neurofilament-200; IB-4: isolectin B4.

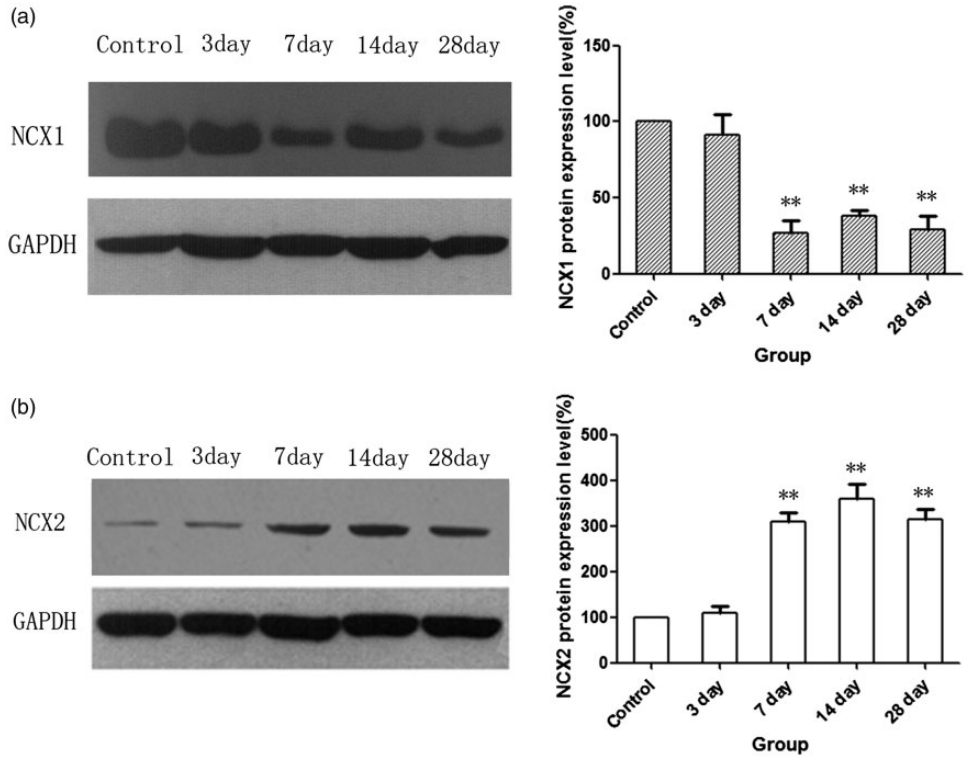


**Figure 2.** The expression of NCX1 and NCX2 in the ipsilateral L4–6 DRGs of SNL model rats. The ipsilateral L4–6 DRGs of SNL model rats were harvested on the seventh day after surgery under anesthesia, and NCX1–2 expression was examined using Western blot analysis. Naive rats were used as the control group. (a) NCX1 was significantly downregulated after the seventh day in the ipsilateral L4–6 DRGs of SNL model rats. $*p < 0.01$ compared to the control group, n = 3. (b) NCX2 was significantly upregulated on the seventh day after surgery in the ipsilateral L4–6 DRGs of SNL model rats. $**p < 0.01$ compared to the control group, n = 3. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NCX: $Na^+$-$Ca^{2+}$ exchanger.

from that following the control treatment ($p < 0.05$). The intracellular $Ca^{2+}$ concentration of neurons incubated with LPS or TNF-α was higher than that of untreated neurons ($p < 0.05$). However, after KB-R7943 and ORM-10103 treatment for 1 h, the intracellular $Ca^{2+}$ concentration showed no significant difference from that following the control treatment ($p < 0.05$).

### *Nociceptive function in KO and WT mice after SNL*

Four weeks after SNL, we observed a reduction in the mechanical withdrawal threshold in the ipsilateral paws of WT mice. However, NCX2 KO mice presented a higher mechanical stimulation threshold in the ipsilateral hind paws than WT mice ($*p < 0.01$ compared to the WT mice) (Figure 6(a)). This result suggests that mice lacking NCX2 expression show greater adaptation to pain after nerve injury.

### *The expression of p-ERK in WT and NCX2 KO mice after SNL surgery*

To better understand the MAPK signaling changes that occur in DRG neurons, we investigated the phosphorylated protein levels of ERK. At the first week after SNL, p-ERK expression in WT mice was increased by almost 2.5-fold compared to sham mice, whereas p-ERK expression in the ipsilateral DRGs of NCX2 KO mice in the SNL group was decreased relative to that of WT mice (Figure 6(b)). This result suggests that the alleviation of pain behavior in NCX2 KO mice may be related to their reduced p-ERK expression in DRGs after nerve injury.

## Discussion

Neuropathic pain is a disease with complex causes. In present study, it is considered that the neuropathic pain is mainly caused by the central sensitization and peripheral sensitization after nerve injury. The peripheral sensitization is mainly related to the changes of peripheral nerve and DRG, which caused by the changes of ion channels (calcium channels, sodium channels, etc.) and the role of inflammatory mediators.[28] In recent years, many drugs have been developed to target the calcium channels (T-type, L-type etc.) for treating neuropathic pain.[29] And some researchers considered that the

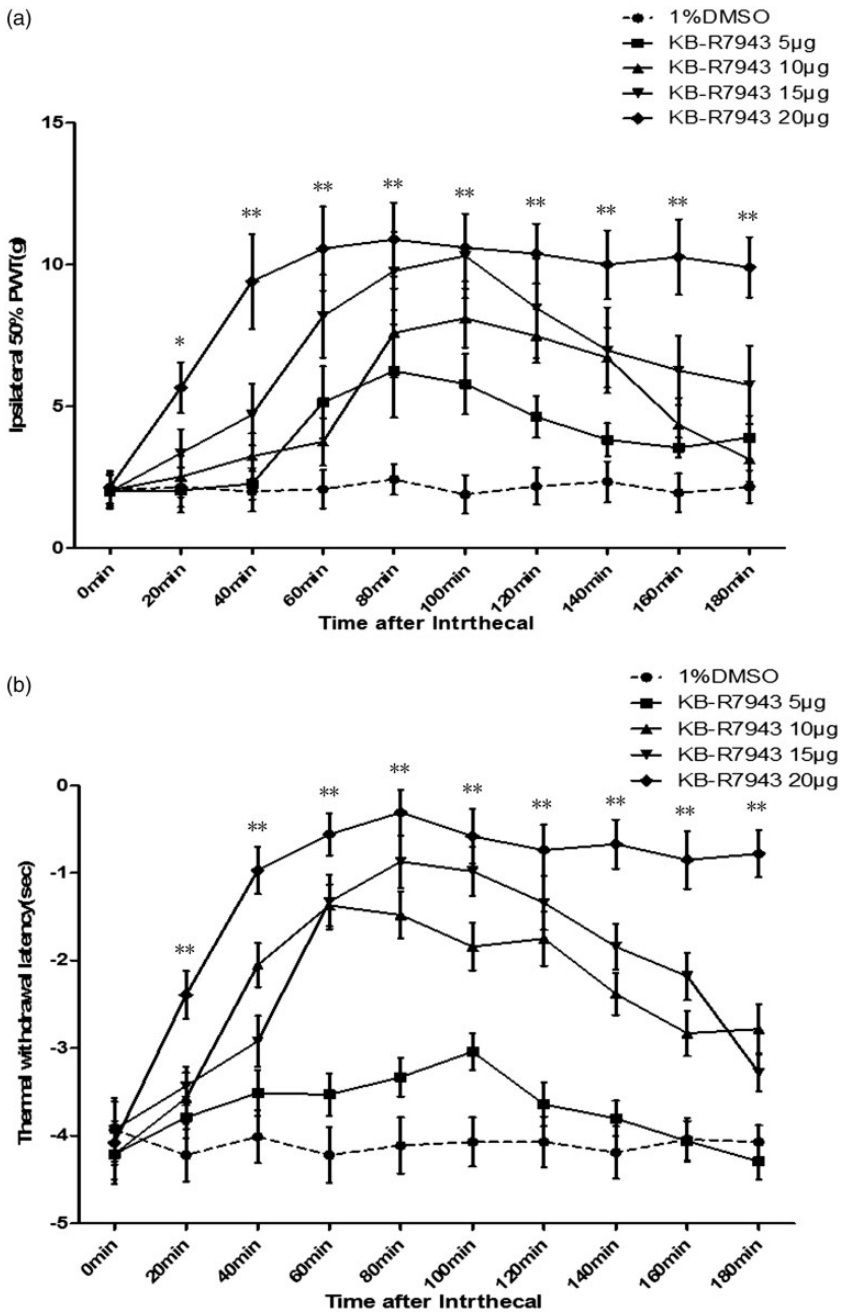


**Figure 3.** The time course of the mechanical withdrawal threshold after intrathecal injection of KB-R7943. SNL model rats developed persistent mechanical allodynia of the ipsilateral hind paw. The results of intrathecal KB-R7943 administration on the seventh day after SNL surgery are shown. (a) Intrathecal KB-R7943 application produced a dose- and time-dependent antinociceptive effect in the ipsilateral hind paw. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 1% DMSO treatment, n = 6. (b) Intrathecal KB-R7943 application produced a greater thermal withdrawal latency on the thermal paw withdrawal test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 1% DMSO treatment, n = 6. DMSO: dimethyl sulfoxide; PWT: paw withdrawal threshold.

change of intracellular calcium concentration which caused by voltage-gated calcium channels was an important cause of peripheral sensitivity.[30,31] NCXs widely participate in the pathogenesis of nerve disorders.[32,33] NCX is also an important element that regulates the intracellular $Ca^{2+}$ concentration in various excitable cells, and it is primarily involved in the maintenance of intracellular $Ca^{2+}$ homeostasis.[3,5] In our preliminary work, we found that clonidine and ouabain had analgesic effects in a rodent pain model.[34] Intrathecal injection of ouabain in SNL rats alters the ion concentrations in spinal dorsal horn neurons, thereby modulating the

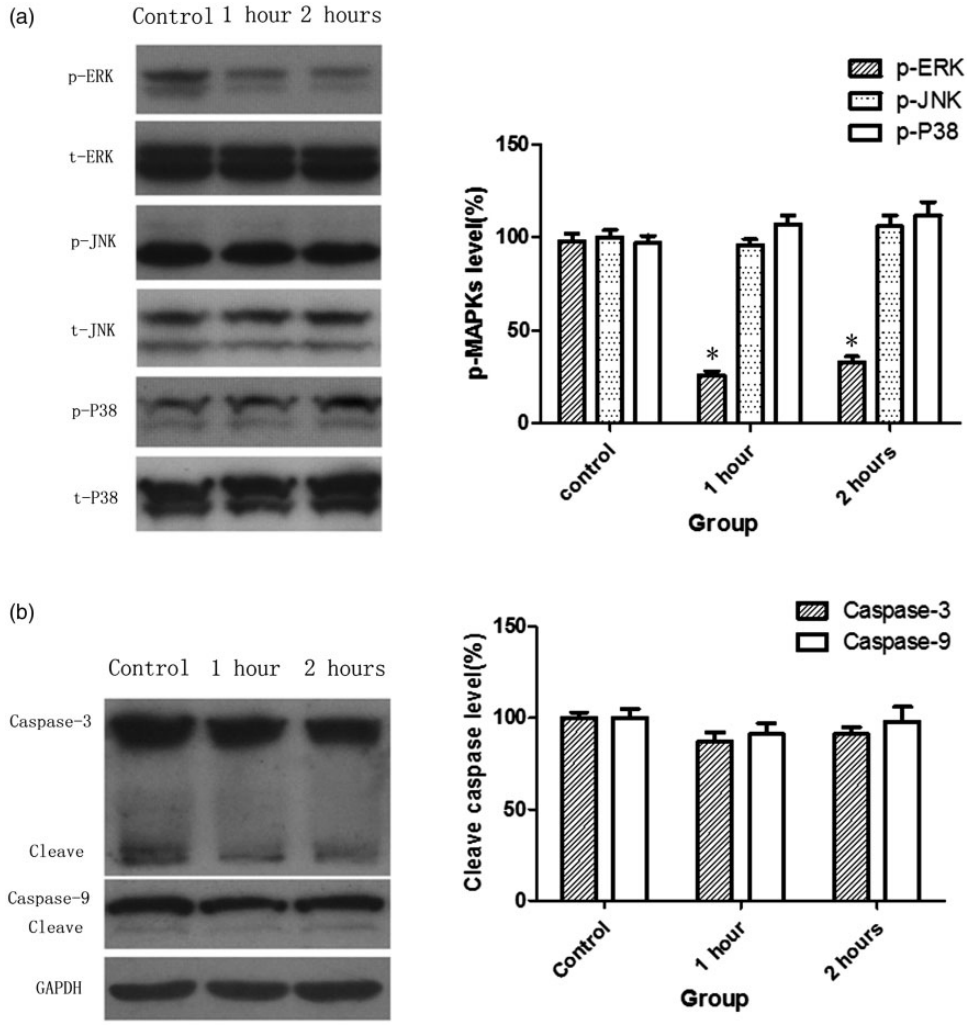


**Figure 4.** The expression of MAPKs in the ipsilateral L4–6 DRGs of SNL model rats after intrathecal injection of KB-R7943 based on Western blot analysis. (a) One and 2 h after intrathecal KB-R7943 administration, ipsilateral L4–6 DRGs were harvested under anesthesia, and the expression of three types of MAPKs were examined using Western blot analysis. Only p-ERK was significantly downregulated in the ipsilateral L4–6 DRGs. $*p < 0.01$ compared to the control DRGs, $n = 3$, Student's t test. (b) One and 2 h after intrathecal KB-R7943 administration, ipsilateral L4–6 DRGs were harvested under anesthesia, and caspase-3 and caspase-9 expression were examined using Western blot analysis. There was no significant change in the cleaved caspase-3 or caspase-9 levels in the ipsilateral L4–6 DRGs, $n = 3$. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase.

activity of kinases (such as MAPKs) and increasing the pain threshold. p38 (one of the MAPKs) is expressed in the gliocytes of the spinal dorsal horn. Ouabain reduces the phosphorylation level of p38, thereby inhibiting microglia. Moreover, we found that ouabain inhibits not only the $Na^+$-$K^+$ exchanger but also the NCX and $K^+$ channels. Ouabain mainly inhibits the NCX and the $Na^+$-$K^+$ exchanger at the dose used for intrathecal injection. From those studies, we could not distinguish the antinociceptive effect produced by inhibition of the $Na^+$-$K^+$ exchanger or the NCX. Recent studies have found that NCX play an important role in chemotherapy-induced peripheral neuropathy.[35] Chemotherapy-induced peripheral neuropathy has a similar mechanism with neuropathic pain. So we selected the NCX as a separate object of interest in this study. This may be the first time that the constitutive expression of NCX in DRG neurons was found to play an important role in neuropathic pain.

DRG neurons consist of large neurons and small neurons.[10] These two types of neurons project A fibers and C fibers to the spinal dorsal horn, which participate in sensory conduction. The main function of the A fibers and C fibers is to transmit pain messages to the spinal dorsal horn. These fibers form synapses with the primary sensory neurons and secondary sensory neurons in the spinal dorsal horn, which play an important role in the process of nociceptive information transfer and

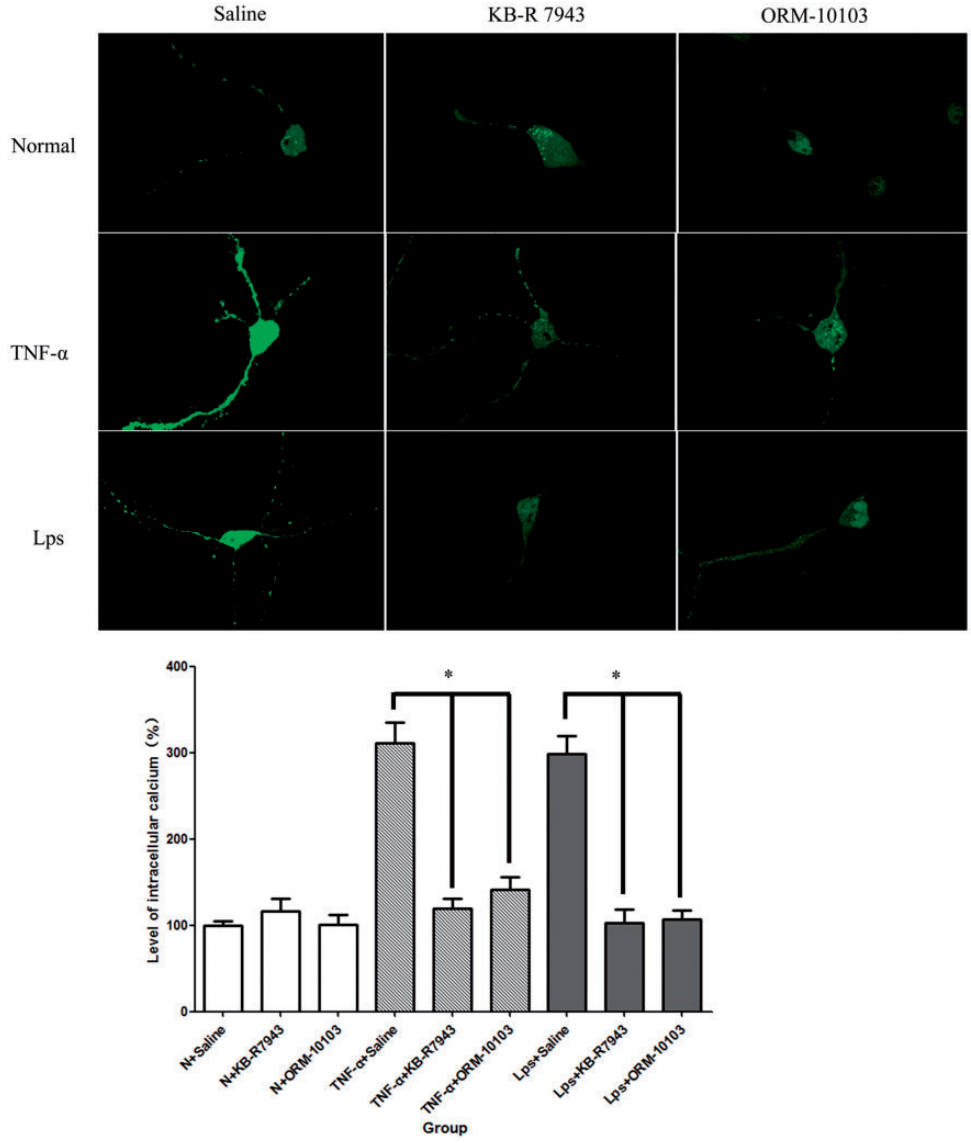


**Figure 5.** Primary cultures of DRG neurons incubated with LPS (1000 ng/ml) or TNF-α (4000 pg/ml) overnight were subjected to three different processes (saline treatment, KB-R7943 treatment or ORM-10103 treatment). Using the same exposure conditions and excitation light source, we performed laser confocal microscopy (1000×) to measure the fluorescence intensity of Flou-4AM in DRG neurons after intracellular staining with the fluorescent calcium dye Fluo-4 AM. The Normal+Saline group was used as the control; we found that in normal neurons, the $Ca^{2+}$ concentration was lower than that in neurons incubated with LPS or TNF-α. After KB-R7943 and ORM-10103 treatment for 1 h, the intracellular $Ca^{2+}$ concentration was not significantly different from that after the control treatment (*$p < 0.05$). The neurons incubated with LPS or TNF-α had higher intracellular $Ca^{2+}$ concentrations than normal neurons ($p < 0.05$). In addition, after KB-R7943 and ORM-10103 treatment for 1 h, the intracellular $Ca^{2+}$ concentration had no significant difference from that following the control treatment (*$p < 0.05$) (n = 6 in each group). TNF: tumor necrosis factor; LPS: lipopolysaccharide.

integration. Therefore, the enhanced efficiency of synaptic transmission between the A/C fibers and spinal dorsal horn neurons could translate into enhancement of pain signals in the spinal region, which may be related to the mental experience of neuropathic pain. DRG neurons participate in not only peripheral sensitization but also central sensitization.[1] These results suggest that NCX may be an important factor in the development of peripheral hypersensitivity.

KBR-7943 inhibits the reverse mode of NCX-mediated ion transport, which reduces $Ca^{2+}$ influx into the cells, and it also slightly inhibits NMDA receptors, $Na^{+}$-$K^{+}$ exchangers and $K^{+}$ channels. However, at the working concentration that we used, KB-R7943 inhibits only the reverse mode of NCX-mediated ion transport. KB-R7943 has been used as a powerful anti-calcium overload drug for the treatment of heart failure through inhibiting the overexpression of NCX protein and

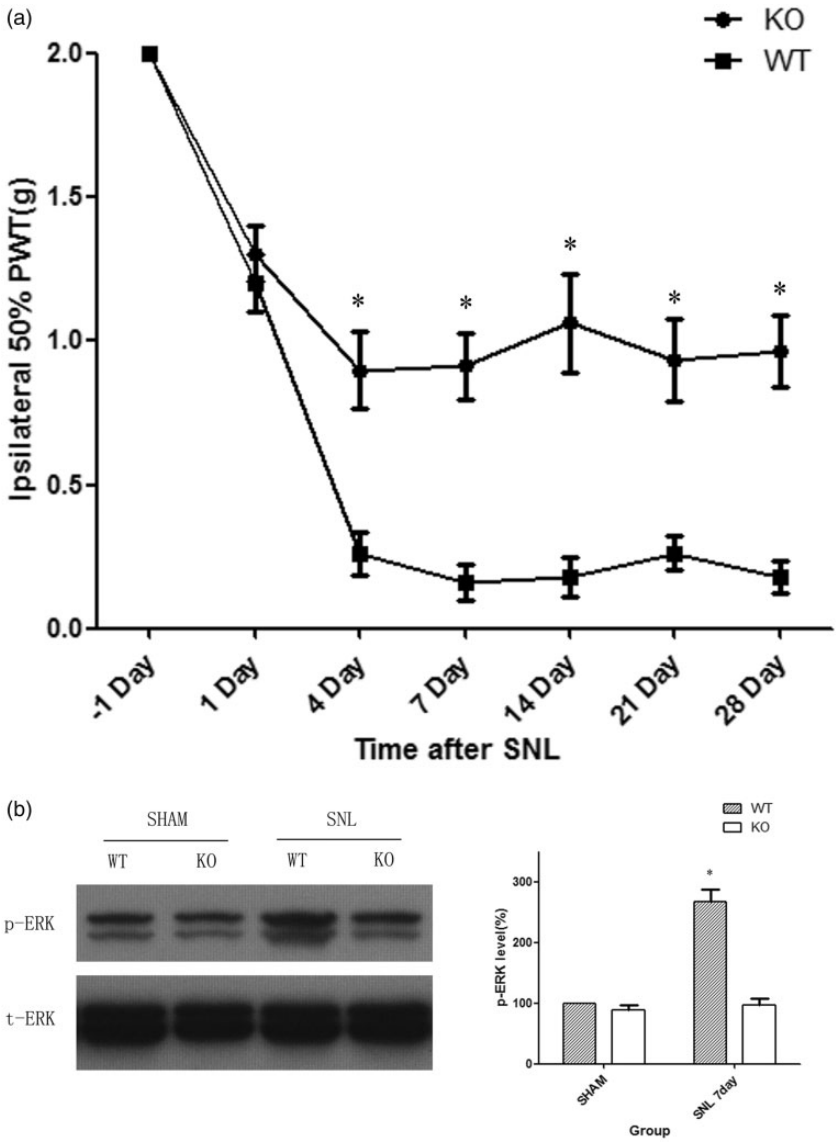


**Figure 6.** (a) Four weeks after SNL surgery, we observed a reduction in the mechanical withdrawal threshold in the ipsilateral paws of the WT mice. However, NCX2 KO mice presented a higher mechanical withdrawal threshold in the ipsilateral hind paws than WT mice (*$p < 0.01$ compared to the WT mice, n = 6). (b) At one-week post-surgery, the sham group of NCX2 KO mice showed no significant change in p-ERK expression in the ipsilateral DRGs relative to the WT group, but at one-week post-surgery, the SNL group of NCX2 KO mice showed decreased p-ERK expression in the ipsilateral DRG (by 68%) (*$p < 0.01$) relative to the WT group (n = 3). KO: knockout; WT: wild type.

preventing the reverse-mode activity of NCX.[36] ORM-10103 is also a potent, specific inhibitor of the $Na^+/Ca^{2+}$ exchanger. But ORM-10103 was different from KB-R7943 which could inhibit both inward and outward NCX currents.

The downstream signal transmission pathways of reactive oxygen species (ROS) and the MAPK family are believed to function as regulators of cell growth, function, survival, and death. In general, the p38 kinase cascade mediates cell apoptosis and cytokine reactions; the JNK kinase cascade mediates cell inflammation, differentiation, and apoptosis; and the ERK kinase cascade regulates cell differentiation and growth.[37] They also play critical roles in the inflammatory response and the regulation of neuroplasticity.[38] LysoPC-induced calcium overload induced ROS production and activated MAPK signaling pathways.[39] Increasing evidence has shown that p38 regulates inflammation-induced pain, and p38 activation in microglia plays a key role in central sensitization processing.[40] Increasing p38 expression in spinal microglia and administering antisense oligonucleotides that inhibit p-p38 expression reliably attenuate hyperalgesia.[41] Therefore, p-p38 MAPK expression is an appropriate marker for

the study of inflammatory pain and the assessment of the efficacy of anti-analgesic compounds.[42] Some studies have reported that KB-R7943 decreases the expression of protein kinase C and p38 MAPK and induces the apoptosis of endothelial cells and smooth muscle cells.[43] Therefore, we hypothesized that the antinociceptive action of KB-R7943 may involve a similar mechanism in DRG neurons. KBR-7943 markedly inhibited the surgery-evoked expression of p-ERK1/2 in the neurons of the ipsilateral DRGs, and we found the same manifestation in NCX2 KO mice. These results demonstrate that NCX exerted antinociceptive effects on the neural processes of noxious stimulus transmission and spinal sensitization. NCX2 levels increased rapidly after surgery, and the $Ca^{2+}$ ion concentration increased in DRG neurons to induce the phosphorylation of ERK1/2. p-ERK1/2 can regulate transcription in DRG neurons and possibly increase the density of sodium or calcium channels on the cytomembrane and nerve endings of DRG neurons. Finally, p-ERK1/2 induces the sensitivity and spontaneous activity of neurons of the sensory nerve in DRGs. Although not all the mechanisms for the antinociceptive effect of KB-R7943 are completely clear, we conclude that the observed reduction in pain sensitivity was due to the pharmacological activities of KB-R7943.

In summary, the data presented here suggest that reverse-mode NCX activity induces peripheral sensitization in rodents. Thus, the interplay between the effects of inflammatory factors and peripheral nerve sensitization appears to underlie NCX function in DRG neurons. Therefore, NCX may be a potential clinical therapeutic target in neuropathic pain. Experiments on human beings and further studies of the detailed mechanisms for these effects are needed.

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