

Upregulation of Nuclear Factor of Activated T-Cells by Nerve Injury Contributes to Development of Neuropathic Pain

You-Qing Cai, Shao-Rui Chen, and Hui-Lin Pan

Center for Neuroscience and Pain Research, Department of Anesthesiology and Perioperative Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas (Y.Q.C., S.R.C., H.L.P.); and Graduate programs in Neuroscience and Experimental Therapeutics, The University of Texas Graduate School of Biomedical Sciences, Houston, Texas (H.L.P.)

Received November 27, 2012; accepted February 4, 2013

ABSTRACT

Nerve injury induces long-term changes in gene expression in the nociceptive circuitry and can lead to chronic neuropathic pain. However, the transcriptional mechanism involved in neuropathic pain is poorly understood. Nuclear factor of activated T-cells (NFATc) is a transcriptional factor regulated by the Ca^{2+} -dependent protein phosphatase calcineurin. In this study, we determined nerve injury-induced changes in the expression of NFATc1–c4 in the dorsal root ganglia (DRG) and spinal cords and their role in the development of neuropathic pain. The mRNA of NFATc1–c4 was detected in the rat DRG and dorsal spinal cord. Nerve injury transiently elevated NFATc1–c3 mRNA levels and persistently increased NFATc4 and C-C chemokine receptor type 2 (CCR2) mRNA levels in the DRG. However, NFATc1–c4 mRNA levels in the spinal cord were not

altered significantly by nerve injury. Nerve injury also significantly increased the protein level of dephosphorylated NFATc4 in the DRG. Intrathecal injection of the specific NFATc inhibitor 11R-VIVIT or the calcineurin inhibitor FK-506 (tacrolimus) early after nerve injury significantly attenuated the development of tactile allodynia. In addition, treatment with FK-506 or 11R-VIVIT significantly reduced the mRNA levels of NFATc4 and CCR2 but not large-conductance Ca^{2+} -activated K^+ channels, in the DRG after nerve injury. Our findings suggest that peripheral nerve injury causes a time-dependent change in NFATc1–c4 expression in the DRG. Calcineurin-NFATc-mediated expression of pronociceptive cytokines contributes to the transition from acute to chronic pain after nerve injury.

Introduction

Chronic pain, such as neuropathic pain induced by peripheral nerve injury, leads to prolonged suffering and a lower quality of life. A major unresolved question in neuropathic pain research is the identity of the mechanism underlying the transition from acute to chronic pain after the initial nerve damage. Nerve injury can induce a differential change in the expression of pronociceptive and antinociceptive genes in the dorsal root ganglion (DRG) (Xiao et al., 2002; Kim et al., 2009). Differential changes in gene expression in the DRG may be involved in the induction or maintenance of chronic neuropathic pain. However, the transcriptional mechanisms involved in the sustained alterations in gene expression found in primary sensory neurons and their role in the transition from acute to chronic pain are still poorly understood.

Nuclear factor of activated T-cells (NFATc) represents a transcriptional factor family regulated by the Ca^{2+} -activated protein phosphatase calcineurin (Crabtree and Olson,

2002; Hogan et al., 2003). The NFATc family consists of at least five members: NFATc1–c4 and NFAT5. Unlike NFATc1–c4, which are substrates of calcineurin (Rao et al., 1997), NFAT5 is not regulated by Ca^{2+} /calmodulin but rather works as a tonicity-responsive transcriptional factor required for certain aspects of T-cell function and kidney homeostasis (Lopez-Rodriguez et al., 2001, 2004). NFATc1–c4 proteins are regulated by Ca^{2+} and calmodulin-dependent signaling, and a rise in intracellular Ca^{2+} activates the serine/threonine phosphatase calcineurin (Klee et al., 1979; Wu et al., 2005, 2006). Activated calcineurin rapidly dephosphorylates the serine-rich region in the amino termini of NFATc1–c4 proteins to expose nuclear localization sequences, leading to their rapid nuclear import or translocation. This unique feature of NFATc enables the integration and coincident detection of Ca^{2+} signals to regulate gene expression. However, the role of NFATc in the development of neuropathic pain has not been determined.

Nerve injury can increase the excitability of DRG neurons and increase Ca^{2+} influx and Ca^{2+} -mediated signaling to increase calcineurin activity in DRG neurons (Ma and LaMotte, 2005; Wu et al., 2005, 2006; Li et al., 2012). In the DRG and spinal cord, NFATc regulates the expression of several pronociceptive and proinflammatory genes, including cyclooxygenase-2, nerve growth factor, interleukin-1, and C-C

This study was supported by the National Institutes of Health [Grants DE022015 and NS073935]; and by N.G. and Helen T. Hawkins Endowment (to H.L.P.).

dx.doi.org/10.1124/jpet.112.202192.

ABBREVIATIONS: BK, large-conductance Ca^{2+} -activated K^+ ; CCR2, C-C chemokine receptor type 2; DRG, dorsal root ganglia; FK-506, tacrolimus; NFATc, nuclear factor of activated T-cells; PCR, polymerase chain reaction.

chemokine receptor type 2 (CCR2) (Iniguez et al., 2000; Marchand et al., 2005; Groth et al., 2007; White et al., 2007; Flockhart et al., 2008). Therefore, we reasoned that increased NFATc activation in the DRG after peripheral nerve injury could contribute to the development of neuropathic pain. In the present study, we used a rat model of neuropathic pain to determine 1) time-dependent changes in the expression of NFATc1–c4 in the DRG and spinal cord after nerve injury, and 2) the role of calcineurin-NFATc-mediated gene expression in the development of neuropathic pain after nerve injury. Our results suggest that upregulation of NFATc in the DRG contributes to the transition from acute to chronic pain after peripheral nerve injury.

Materials and Methods

Animal Model of Neuropathic Pain and Intrathecal Cannulation. Ligation of the left L5 and L6 spinal nerves in rats was used in this study as a model of neuropathic pain. First, male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing about 220 g were anesthetized with 2–3% isoflurane before surgical implantation of an intrathecal catheter. The catheter was inserted through an incision made in the cisternal membrane and advanced about 8 cm caudal so that the tip of each catheter was positioned at the lumbar spinal level (Chen et al., 2009; Zhou et al., 2012). These animals were allowed to recover for 1 week before spinal nerve ligation was performed. Under isoflurane anesthesia, the left L5 and L6 spinal nerves were isolated and ligated tightly with 4.0 silk sutures, as described previously (Kim and Chung, 1992; Chen et al., 2000). Sham surgery was performed on the right side to expose the L5 and L6 spinal nerves but without nerve ligation. The surgical preparation and experimental protocols were approved by the Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and conformed to the National Institutes of Health guidelines on the ethical care and use of animals.

The selective calcineurin inhibitor FK-506 (tacrolimus; Tocris Bioscience, Bristol, UK) was dissolved in dimethylsulfoxide to 4 mg/ml. The NFATc inhibitor 11R-VIVIT (Calbiochem, Darmstadt, Germany) was dissolved in sterile saline to 4 mg/ml. We showed previously that agents can directly access the DRG after intrathecal injection (Cai et al., 2009). These drugs were injected intrathecally in a volume of 10 μ l, followed by a 10- μ l flush with normal saline. The rats were treated with FK-506 (20 μ g) or 11R-VIVIT (20 μ g) twice daily via

intrathecal injection for the first 5 consecutive days. These doses of FK-506 and 11R-VIVIT were selected on the basis of preliminary data showing their effects on the NFATc4 and CCR2 mRNA levels. For vehicle control groups, rats were injected with the same volume of dimethylsulfoxide or saline.

Behavioral Assessment of Allodynia in Rats. To quantify tactile allodynia, rats were placed in individual plastic boxes on a mesh floor and allowed to acclimate for 30–45 minutes. A series of calibrated von Frey filaments was applied perpendicularly to the plantar surface of the hind paw with sufficient force to bend the filaments for 6 seconds. Brisk paw withdrawal or flinching was considered a positive response. In the absence of a response, the filament of next greater force was applied. If a response occurred, the filament of next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the “up-down” calculating method (Chaplan et al., 1994; Chen et al., 2000).

Rotarod Performance Test. This test was conducted using a rotarod accelerator treadmill (Med Associates Inc., St. Albans, VT) to determine whether FK-506 and 11R-VIVIT affect motor function. Briefly, rats were first habituated and trained for 2 days at a fixed speed of 8 rpm. After this conditioning period, rats were tested 30 minutes after intrathecal delivery of FK-506 or 11R-VIVIT on an accelerating mode (4–40 rpm in 5 minutes). The time (latency) was measured from the start of the acceleration period until the rat fell off the drum (Fairbanks et al., 2000).

Detection and Quantification of NFATc1–c4 mRNA. Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.), and then the DRG and dorsal spinal cord tissues at the L5 and L6 levels were rapidly removed. Total RNA was extracted from the spinal cord and DRGs using the Purelink total RNA purification system (Invitrogen, Carlsbad, CA) with on-column DNase I digestion according to the manufacturer's instructions. cDNA was prepared by using the Superscript III first-strand synthesis kit (Invitrogen). The mRNA of NFATc1–c4 in the spinal cord and DRG were detected using agarose gel (Li et al., 2012). Quantitative polymerase chain reaction (PCR) was performed using the iQ5 real-time PCR detection system with the SYBR green PCR kit (Bio-Rad, Hercules, CA). All samples were analyzed in duplicate using an annealing temperature of 60°C. The primer pairs used for NFATc1–c4, CCR2, and BK α 1 are listed in Table 1. To calculate the relative mRNA levels of different genes, standard curves were generated using a 2-fold dilution of the cDNA from the DRGs as the PCR template (Cai et al., 2009; Chen et al., 2009). The relative amount of target genes in each sample was first

TABLE 1
List of primers used for real-time polymerase chain reaction analysis

| Gene Name | Primer | Sequence (5' to 3') | Location |
|-------------------------------------|---------|---------------------------------------------|-----------|
| Rat NFATc1 (XM_001058445) | c1-p1 | 5'-CCC AGC GGT CGG CCC AGG AAC T-3' | 1779–1800 |
| | c1-p2 | 5'-GCG TAG GCG GGA GAG GAA AGG TCG TG-3' | 2346–2321 |
| | c1-p3 | 5'-CAG CTG CCG TCG CAC TCT GGT C-3' | 1268–1289 |
| | c1-p4 | 5'-CCC GGC TGC CTT CCG TCT CAT A-3' | 1361–1340 |
| Rat NFATc2 (XM_001072834) | c2-p1 | 5'-CAC GGC CGG CTC TGC TGT TCT CA-3' | 953–975 |
| | c2-p2 | 5'-TGG TGG CCC GCA TGT TGT TCT TTG-3' | 1602–1579 |
| | c2-p3 | 5'-TGG GGC CGT GTG AGC AGG AGG AGA G-3' | 1120–1144 |
| | c2-p4 | 5'-AGT CAC GGG GAT GCT GCA GAT GGG AAT G-3' | 1238–1211 |
| Rat NFATc3 (XM_001074460) | c3-p1 | 5'-GGA ACA GCC GAT GAT AGA TAT TTA CG AC-3' | 1707–1734 |
| | c3-p2 | 5'-GAC CCC CTT GAC ATT TTT CTC TGA TTA T-3' | 2235–2208 |
| | c3-p3 | 5'-CCC CAT CCC GGG AGA CAT CAG TAG-3' | 1357–1380 |
| | c3-p4 | 5'-TAT AGG AGT GTG GCC AGG CTT TGG TTT G-3' | 1493–1466 |
| Rat NFATc4 (NM_001107264) | c4-p1 | 5'-CAG GGC GGC GGG AAG GTC GTC-3' | 1918–1938 |
| | c4-p2 | 5'-AGG AAA GGG GCG CGG AAG AAA GGA GA-3' | 2604–2579 |
| | c4-p3 | 5'-GGG AGG AAG AGG CCG CAG TGA AC-3' | 2129–2151 |
| | c4-p4 | 5'-CCG CCC ATT GGA GAC ATA AAA GTA GAC C-3' | 2256–2229 |
| Rat CCR2 (NM_021866) | CCR2-p1 | 5'-AGG GGG CCA CCA CAC CGT ATG A-3' | 92–113 |
| | CCR2-p2 | 5'-CAT GTT GCC CAC AAA ACC AAA GAT GAA-3' | 222–196 |
| Rat S18 (NM_213557) | S18-p1 | 5'-ACT TTT GGG GCC TTC GTG TC-3' | 419–438 |
| | S18-p2 | 5'-GCC CAA AGA CTC ATT TCT TCT TG-3' | 514–492 |

normalized to the level of the housekeeping gene S18 and then normalized to its expression level in sham control or vehicle-treated rats. The PCR product specificity was verified by melting-curve analysis and agarose gel electrophoresis.

Western Blotting. The DRGs and dorsal spinal cord tissues at the L5 and L6 levels were removed, dissected, and homogenized in 150 μ l of RIPA buffer: 50 mM Tris-HCl (pH 7.4), 1% NP-40 (nonyl phenoxypolyethoxyethanol), 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 , and 1 mM NaF (in the presence of proteinase inhibitor cocktail). Samples were then put on ice for 30 minutes with shaking. Lysates were centrifuged at 13,000g for 30 minutes at 4°C. The supernatant was carefully collected, and the protein concentration was measured using the DC protein assay kit (Bio-Rad). Thirty micrograms of total protein from each sample was loaded and separated on 6% SDS-PAGE using a standard protocol. The resolved proteins were transferred to nitrocellulose membranes that were treated with 5% nonfat milk in Tris buffer containing Tween 20 (Tris-buffered saline and Tween-20; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 2 hours and then incubated with rabbit anti-NFATc4 primary antibody (sc-13036, Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) overnight at 4°C. For the loading control, the same blot was incubated with a rabbit anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO; 1:2000). The membrane was washed several times and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA; 1:20,000) for 1 hour at room temperature. After three washes in TBST, the protein band was revealed using the ECL Plus detection kit (GE Healthcare Life Sciences, Pittsburgh, PA), and the protein band intensity was quantified using the ImageJ software program (NIH, Bethesda, MD).

Statistical Analysis. All values are presented as means \pm S.E.M. We used the *t* test to compare two groups and one-way analysis of variance to compare more than two groups. Two-way analysis of variance followed by Bonferroni's post hoc test was used to determine any significant differences in the effects of NFATc and calcineurin inhibitors on the rat paw withdrawal thresholds. *P* value less than 0.05 was considered statistically significant.

Results

Nerve Injury-Induced Changes in the Expression of NFATc1–c4 in the DRG and Spinal Cord. To determine which isoforms of NFATc are present in the DRG and spinal cord, we first used agarose gel to detect the mRNA of NFATc1–c4. The mRNA of all four subtypes of NFATc was detected in the dorsal spinal cord and DRG tissues (Fig. 1A).

We next determined time-dependent changes in the expression levels of NFATc1–c4 after nerve injury. Real-time PCR was used to quantify the mRNA levels of NFATc1–c4 in the dorsal spinal cord and DRG. The mRNA level of NFATc1 in the DRG significantly increased 3 and 7 days after nerve ligation (Fig. 1B). The mRNA levels of NFATc2 and NFATc3 significantly increased in the DRG only at day 7 after nerve injury. Notably, nerve injury caused a large increase in the mRNA level of NFATc4 in the DRG at day 3, and this increase persisted for at least 14 days after nerve injury (Fig. 1B). In contrast, the mRNA levels of NFATc1–c4 in the dorsal spinal cord were not significantly altered from days 3 to 14 after nerve injury (Fig. 1C).

Because nerve injury caused a large and persistent increase in the mRNA level of NFATc4, we further determined the protein level of NFATc4 in the DRG using Western blot analysis. Immunoblotting of DRG tissues showed two protein bands, indicative of dephosphorylated (~140 kDa) and phosphorylated (~160 kDa) forms of NFATc4, as reported

previously (Arron et al., 2006). In injured DRGs, the dephosphorylated NFATc4 protein level was significantly increased, whereas the phosphorylated NFATc4 protein level was diminished compared with those in DRGs from control rats (Fig. 2). However, the protein level of dephosphorylated NFATc4 in the dorsal spinal cord did not differ significantly between the nerve injury and sham surgery groups.

Effect of Intrathecal 11R-VIVIT or FK-506 on the Development of Neuropathic Pain. To determine the role of NFATc at the spinal level in the development of neuropathic pain, we used a selective and cell-permeable NFATc inhibitor, 11R-VIVIT, which specifically blocks the calcineurin-NFATc interaction (Aramburu et al., 1999; Noguchi et al., 2004). 11R-VIVIT (20 μ g, twice/day) was injected intrathecally for 5 days after nerve injury, and tactile allodynia was assessed for 14 days after nerve injury. Treatment with 11R-VIVIT significantly reduced the development of tactile allodynia in rats compared with vehicle only treatment (Fig. 3A).

Because NFATc1–c4 are the substrates of calcineurin (Crabtree and Olson, 2002; Hogan et al., 2003), we next determined whether inhibition of calcineurin-NFATc attenuates the development of pain hypersensitivity induced by nerve injury. We treated the rats with FK-506 (20 μ g, twice/day), a selective inhibitor of calcineurin (Liu et al., 1991), or vehicle via an intrathecal catheter for the first 5 days after spinal nerve ligation. Intrathecal treatment with FK-506 significantly reduced the development of tactile allodynia in rats compared with vehicle only treatment (Fig. 3B).

Intrathecal injection of 20 μ g of FK-506 or 20 μ g of 11R-VIVIT had no significant effect on the motor function, quantified with a rotarod test, in rats 3 weeks after spinal nerve ligation. The fall latency was 109.7 ± 5.8 , 121.2 ± 6.6 , and 112.8 ± 4.4 seconds ($P > 0.05$, $n = 6$ rats per group) for vehicle-, FK-506-, and 11R-VIVIT-treated rats, respectively.

Effect of Nerve Injury on the mRNA Level of CCR2 in the DRG and Spinal Cord. CCR2 is an important target gene of NFATc (Jung and Miller, 2008). We used quantitative PCR to examine whether nerve injury affects the expression of CCR2 in the DRG and spinal cord. The mRNA level of CCR2 in the DRG was significantly increased at day 3 and remained elevated at day 14 after nerve injury (Fig. 4). However, the mRNA level of CCR2 in the dorsal spinal cord did not differ significantly between the nerve injury and sham control groups (Fig. 4).

Effects of Intrathecal 11R-VIVIT on the mRNA Levels of NFATc4, CCR2, and BK Channels in the DRG. To validate the effect of intrathecal treatment with 11R-VIVIT on NFATc4, we used real-time PCR analysis to measure the mRNA level of NFATc4 in the DRG of nerve-injured rats. The DRG tissues were obtained from 11R-VIVIT- and vehicle-treated rats 14 days after nerve injury. Compared with the vehicle-treated group, treatment with 11R-VIVIT significantly reduced the increase in the mRNA level of NFATc4 in the DRG of nerve-injured rats (Fig. 5A).

We then determined the effect of intrathecal treatment with 11R-VIVIT on the CCR2 mRNA level in the DRG of nerve-injured rats. The DRG tissues were removed from vehicle- and inhibitor-treated rats at the end of the behavioral test (14 days after nerve injury). Compared with the vehicle group, chronic treatment with 11R-VIVIT largely diminished the increase in the mRNA level of CCR2 in the DRG of nerve-injured rats (Fig. 5B). We previously showed that nerve injury

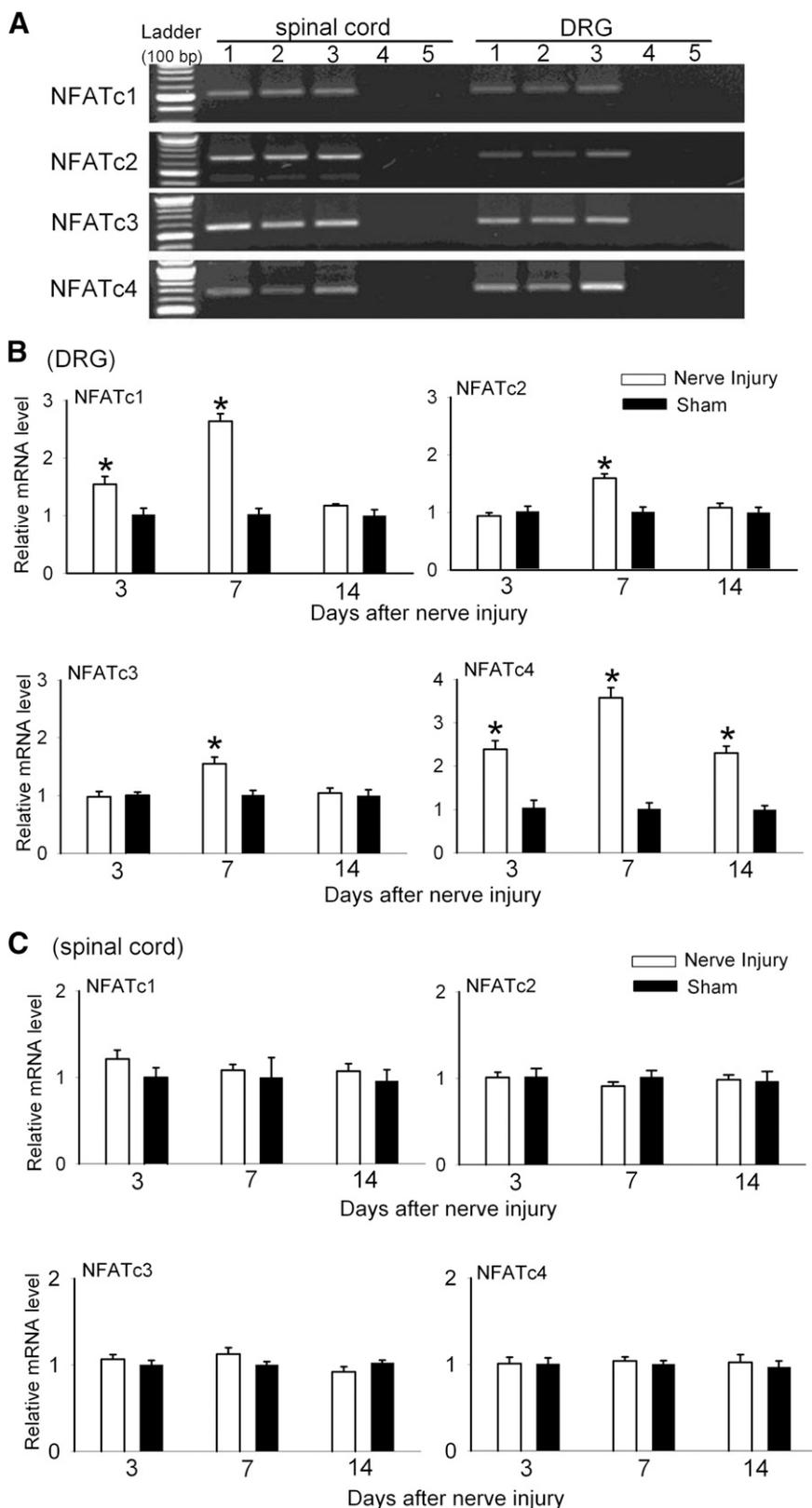


Fig. 1. Effects of nerve injury on the expression levels of NFATc1–c4 in the DRG and dorsal horn spinal cord. (A) Agarose gel electrophoresis showing the presence of the DNA products of NFATc1–c4 in the DRG and dorsal spinal cord. Lanes 1–3: cDNA samples from three different rats. Lane 4: Total RNA as PCR template. Lane 5: H₂O as PCR template. (B) Quantitative PCR analysis of mRNA levels of NFATc1–c4 in the DRG at days 3, 7, and 14 after nerve injury ($n = 5$ in each group). (C) mRNA levels of NFATc1–c4 in the dorsal spinal cord at days 3, 7, and 14 after nerve injury ($n = 5$ in each group). Note that the mRNA level in the sham group was normalized and considered 1 (100%). * $P < 0.05$ compared with the sham control group.

suppresses the expression of large-conductance Ca²⁺-activated K⁺ (BK) channels in the DRG (Chen et al., 2009). However, the mRNA level of the BK α 1 subunit in the DRG did not differ significantly between vehicle-treated and 11R-VIVIT-treated rats (Fig. 5C).

Effects of Intrathecal FK-506 on the mRNA Levels of NFATc4, CCR2, and BK Channels in the DRG. To confirm the effect of chronic FK-506 treatment on NFATc4 expression in the DRG, we analyzed the mRNA level of NFATc4 in the DRG using real-time PCR. Compared with the

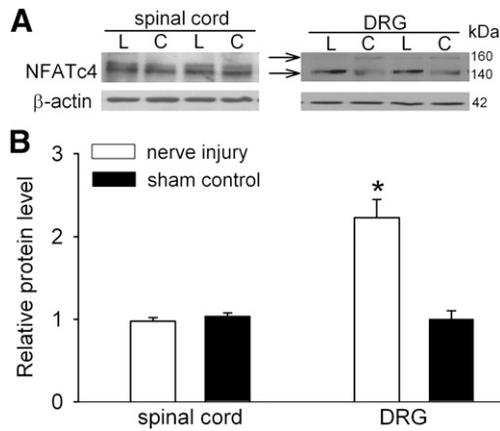


Fig. 2. Effect of nerve injury on the protein level of dephosphorylated NFATc4 in the DRG and dorsal spinal cord. Western blots (A) and quantification (B) of the protein amount of dephosphorylated NFATc4 (~140 kDa) in the DRG and dorsal spinal cord. The tissues were obtained 14 days after L5 and L6 spinal nerve ligation ($n = 4$ rats in each group). β -Actin was used as a protein loading control. * $P < 0.05$ compared with the sham control group. C, sham control; L, nerve ligation.

vehicle group, treatment with FK-506 largely prevented the increase in the mRNA level of NFATc4 in the DRG caused by nerve injury (Fig. 6A).

Activation of calcineurin-NFATc signaling mediates upregulation of CCR2 chemokine receptors in the DRG (Jung and Miller, 2008). We therefore determined the effect of chronic FK-506 treatment on the mRNA level of CCR2 in the DRG of nerve-injured rats. The CCR2 mRNA level in the DRG was significantly reduced by FK-506 treatment compared with

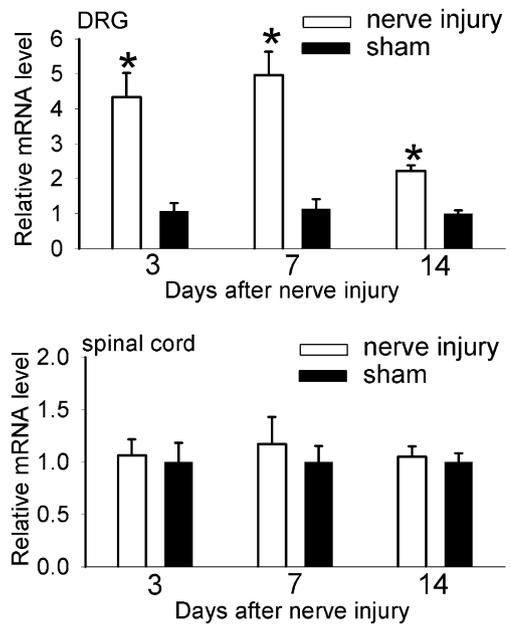


Fig. 4. Effect of nerve injury on the expression levels of CCR2 in the DRG and dorsal horn spinal cord. The mRNA level of CCR2 was measured using quantitative PCR analysis at days 3, 7, and 14 after nerve injury ($n = 6$ rats in each group) and sham surgery ($n = 5$ rats in each group). * $P < 0.05$ compared with the sham control group.

vehicle treatment (Fig. 6B). However, there was no significant difference in the mRNA level of the BK α 1 subunit in the DRG between FK-506-treated and vehicle-treated groups (Fig. 6C).

Discussion

Nerve injury may increase NFATc expression in the DRG and facilitate the development of chronic pain through NFATc-dependent expression of pronociceptive and proinflammatory genes. In this study, we found that nerve injury caused a transient increase in the mRNA levels of NFATc1–c3 and a sustained increase in the mRNA level of NFATc4 in the DRG. However, nerve injury did not significantly affect the mRNA level of NFATc1–c4 in the spinal cord. Inhibition of calcineurin-NFATc signaling early after nerve injury significantly reduced the mRNA level of CCR2 in the DRG and attenuated the development of pain hypersensitivity. Our findings suggest that NFATc-dependent expression of pronociceptive and proinflammatory genes in the DRG plays an important role in the development of neuropathic pain.

Chronic neuropathic pain is associated with changes in gene expression in the DRG (Xiao et al., 2002; Maratou et al., 2009). Calcineurin is a Ca²⁺/calmodulin-dependent serine/threonine protein phosphatase (Klee et al., 1979) that directly dephosphorylates NFATc1–c4, allowing NFATc to translocate into the nucleus (Rao et al., 1997). Combined with its transcriptional partners, such as activator protein-1 in the nucleus, NFATc regulates a large number of inducible genes, including cytokines and cell-surface receptors (Macian et al., 2001). In the central nervous system, NFATc is involved in axon guidance, cell survival, and synaptogenesis (Graef et al., 1999, 2003). Although NFATc1–c4 are expressed in both neurons and glia (Jones et al., 2003; Jung and Miller, 2008; Nagamoto-Combs and Combs, 2010; Serrano-Perez et al.,

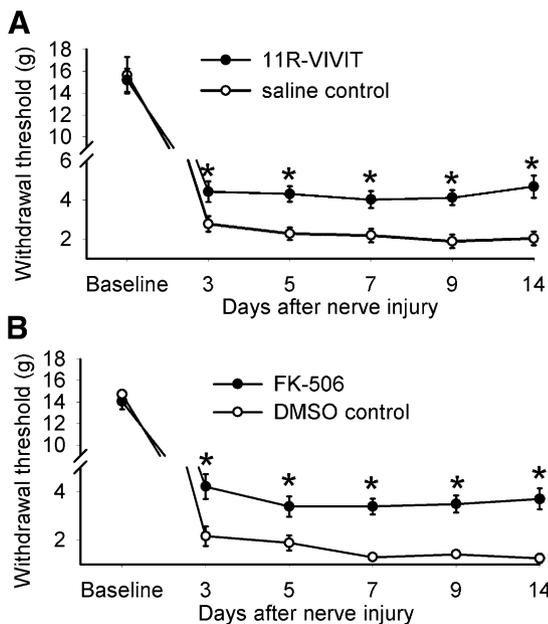


Fig. 3. Effect of intrathecal injection of 11R-VIVIT or FK-506 on the development of pain hypersensitivity after nerve injury. (A) Time course of the development of tactile allodynia in vehicle- and 11R-VIVIT-treated rats ($n = 8$ rats in each group). (B) Time course of the development of tactile allodynia in vehicle- and FK-506-treated rats ($n = 11$ rats in each group). Tactile allodynia was tested using von Frey filaments. Rats were treated with 11R-VIVIT or FK-506 for the first 5 days after L5 and L6 spinal nerve ligation. * $P < 0.05$ compared with the vehicle-treated rats. DMSO, dimethylsulfoxide.

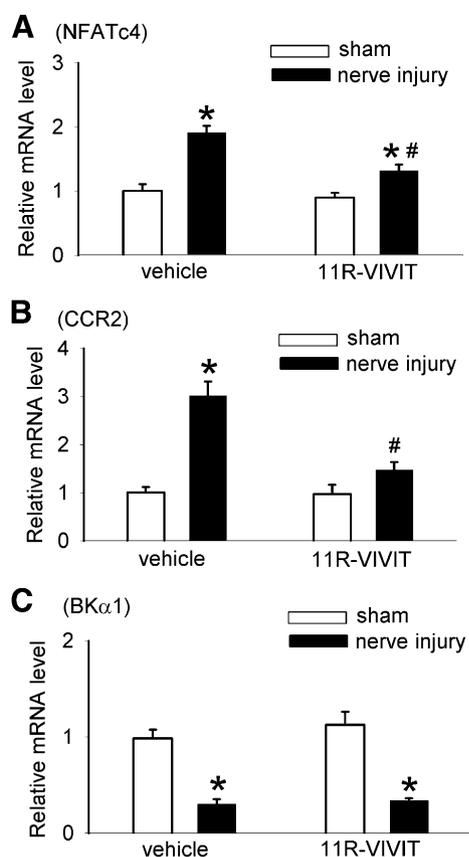


Fig. 5. Effects of intrathecal treatment with 11R-VIVIT on the expression levels of NFATc4, CCR2, and BK α 1 in the DRG of nerve-injured rats. 11R-VIVIT (20 μ g, twice/day, $n = 8$) or saline ($n = 8$) was injected for the first 5 days after nerve injury. The mRNA levels of NFATc4 (A), CCR2 (B), and BK α 1 (C) were quantified using real-time PCR from left L5 and L6 DRG tissues obtained 14 days after nerve injury ($n = 8$ in each group). * $P < 0.05$ compared with sham control group. # $P < 0.05$ compared with the vehicle-treated rats.

2011), NFATc4 seems to be the major isoform of NFATc in DRG neurons (Groth et al., 2007). Because calcineurin and NFATc can be activated by neuronal depolarization in a Ca²⁺-dependent manner (Rao et al., 1997; Graef et al., 1999), NFATc is likely activated by peripheral nerve injury and regulates the expression of many pronociceptive and proinflammatory genes in the DRG (Groth and Mermelstein, 2003; Groth et al., 2007; Jackson et al., 2007; Jung and Miller, 2008). In the present study, we found that NFATc1–c4 were detected in the DRG and spinal cord. Peripheral nerve injury caused a sustained increase in the mRNA levels of NFATc4 and CCR2 in the DRG. Because NFATc4 can upregulate itself via a positive feedback mechanism (Arron et al., 2006), the elevated mRNA level of NFATc4 likely reflects increased activation of NFATc4 in the DRG after nerve injury. Furthermore, nerve injury significantly increased the protein level of dephosphorylated NFATc4 (reflecting its nucleus location) and diminished the phosphorylated NFATc4 (reflecting its cytoplasmic level) protein level in the DRG, suggesting that the NFATc4 protein is translocated to the nucleus of DRG neurons after nerve injury. In contrast, the mRNA levels of NFATc1–c3 in the DRG were transiently increased for only 3 or 7 days after nerve injury. Interestingly, nerve injury had no evident effect on the mRNA levels of NFATc1–c4 in the

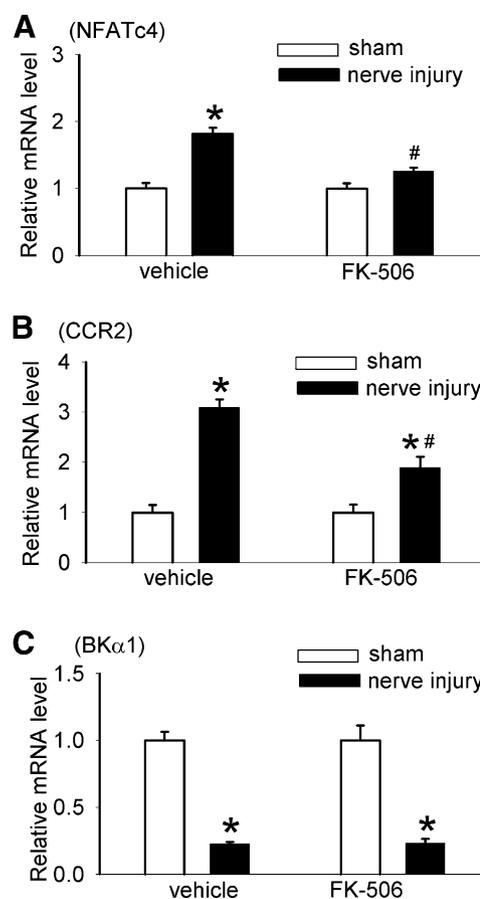


Fig. 6. Effects of intrathecal treatment with FK-506 on the expression levels of NFATc4, CCR2, and BK α 1 in the DRG of nerve-injured rats. FK-506 (20 μ g, twice/day, $n = 8$) or dimethylsulfoxide (DMSO) ($n = 7$) was intrathecally injected for the first 5 days after nerve injury. The mRNA levels of NFATc4 (A), CCR2 (B), and BK α 1 (C) were quantified using real-time PCR from left L5 and L6 DRG tissues obtained 14 days after nerve injury. * $P < 0.05$ compared with sham control group. # $P < 0.05$ compared with the vehicle-treated rats.

dorsal spinal cord, indicating that upregulation of NFATc-mediated gene expression primarily occurs in the DRG after nerve injury. This finding is consistent with our previous studies showing that nerve injury-induced changes in the expression level of calcium-activated potassium channel and voltage-activated calcium channel subunits take place in the DRG but not in the spinal cord (Chen et al., 2009; Li et al., 2012).

The most salient finding of our study is that inhibition of calcineurin-NFATc with FK-506 or 11R-VIVIT significantly reduced the development of tactile allodynia induced by nerve injury. Our data suggest that NFATc in the DRG is an important transcriptional factor involved in the development of neuropathic pain after nerve injury. We also found that nerve injury caused a large increase in the CCR2 mRNA level in the DRG, which lasted for at least 14 days after nerve injury. Because the time course of changes in the mRNA level of NFATc4 was similar to that of CCR2 in the DRG after nerve injury, it is possible that sustained upregulation of CCR2 is induced by increased NFATc4 activity. Treatment with FK-506 or 11R-VIVIT largely attenuated the increase in the mRNA level of CCR2, but not BK channel expression, in the DRG. This finding indicates that calcineurin-NFATc serves as

an important transcriptional mechanism for increased CCR2 expression induced by nerve injury. Because the effect of FK-506 and 11R-VIVIT on tactile allodynia was small relative to their effect on mRNA levels of CCR2 and NFATc4 in the DRG, it should be acknowledged that the smaller effects may represent smaller changes in protein levels of CCR2 and NFATc4. It has been shown that activation of NFATc can upregulate CCR2 in cultured DRG neurons (Jung and Miller, 2008). Although NFATc1–c4 are the best characterized substrates of calcineurin, calcineurin may affect other substrates to attenuate chronic pain development after nerve injury. It has been shown that FK-506 can block the calcineurin-NF- κ B and MEF2 signaling pathways (Martinez-Martinez and Redondo, 2004). We noted that FK-506 or 11R-VIVIT did not fully block nerve injury-induced increases in the NFATc4 and CCR2 expression in the DRG. It is possible that other signaling pathways also regulate CCR2 expression induced by nerve injury. Also, we performed FK-506 or 11R-VIVIT treatment of only 5 days after nerve injury but measured NFATc4 and CCR2 expression at the end of behavioral testing (i.e., 14 days after nerve injury). It is likely that the mRNA levels of NFATc4 and CCR2 in the DRG would have been much lower if the assay had been done shortly after FK-506 or 11R-VIVIT injection.

Increased cytokine levels have been shown to influence neuronal activity through several mechanisms, including increases in the neurotransmitter release through Ca^{2+} -dependent mechanisms and upregulation of transient receptor potential cation channel, subfamily V, member 1 and Nav1.8 sodium channels in DRG neurons (White et al., 2007; Kao et al., 2012). Many cytokines and their receptors, such as the C-C chemokine ligand (CCL2, also known as monocyte chemoattractant protein 1) and its receptor CCR2, are critically involved in neuropathic pain induction. CCL2 is constitutively expressed in primary afferent neurons and their central terminals in the spinal dorsal horn (Dansereau et al., 2008). Nerve injury can lead to increased activity of CCR2 in the DRG in the neuropathic pain model (Jung et al., 2009). CCR2 knockout mice fail to display mechanical pain hypersensitivity after partial ligation of the sciatic nerve (Abbadie et al., 2003). Also, mice overexpressing CCL2 in astrocytes exhibit enhanced nociceptive responses (Menetski et al., 2007). Although our data suggest that calcineurin-NFATc signaling contributes to the development of neuropathic pain through increased CCR2 expression, calcineurin and NFATc are likely involved in the regulation of other target genes in neuropathic pain. For example, it has been reported that COX-2 is another target gene of NFATc (Iniguez et al., 2000; Flockhart et al., 2008). We have shown that COX-2 is involved in the induction, but not the maintenance, of neuropathic pain in rats subjected to spinal nerve injury (Zhao et al., 2000). Thus, increased expression of cytokines and cyclooxygenase-2 products (e.g., prostaglandins) may result from NFATc upregulation and play an important role in the development of neuropathic pain after nerve injury. Altered gene expression in primary sensory neurons caused by nerve injury is regulated by many transcriptional factors, such as NF- κ B, cyclic AMP response element binding protein, and NFATc. It has been shown that the expression of BK channels is regulated by the cyclic AMP response element binding protein (Wang et al., 2009). We found that treatment with FK-506 or 11R-VIVIT had no effect on the mRNA level of BK α 1 in the DRG, suggesting

that calcineurin-NFATc signaling does not regulate BK α 1 expression altered by nerve injury.

In summary, we found that NFATc1-c4 expression in the DRG significantly increased soon after nerve injury. Early inhibition of calcineurin-NFATc significantly attenuated CCR2 expression in the DRG and the development of pain hypersensitivity after nerve injury. Our findings suggest that calcineurin-NFATc-mediated nociceptive gene expression in the DRG contributes to the development of chronic neuropathic pain. This important new information greatly improves our understanding of the transcriptional mechanism involved in the transition from acute to chronic pain after nerve injury.

Authorship Contributions

Participated in research design: Cai, Chen, Pan.

Conducted experiments: Cai, Chen.

Performed data analysis: Cai, Chen, Pan.

Wrote or contributed to the writing of the manuscript: Cai, Chen, Pan.

References

- Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, and Forrest MJ (2003) Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci USA* **100**:7947–7952.
- Aramburu J, Yaffe MB, López-Rodríguez C, Cantley LC, Hogan PG, and Rao A (1999) Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* **285**:2129–2133.
- Arron JR, Winslow MM, Polleri A, Chang CP, Wu H, Gao X, Neilson JR, Chen L, Heit JJ, and Kim SK, et al. (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature* **441**:595–600.
- Cai YQ, Chen SR, Han HD, Sood AK, Lopez-Berestein G, and Pan HL (2009) Role of M2, M3, and M4 muscarinic receptor subtypes in the spinal cholinergic control of nociception revealed using siRNA in rats. *J Neurochem* **111**:1000–1010.
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, and Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* **53**:55–63.
- Chen SR, Cai YQ, and Pan HL (2009) Plasticity and emerging role of BKCa channels in nociceptive control in neuropathic pain. *J Neurochem* **110**:352–362.
- Chen SR, Eisenach JC, McCaslin PP, and Pan HL (2000) Synergistic effect between intrathecal non-NMDA antagonist and gabapentin on allodynia induced by spinal nerve ligation in rats. *Anesthesiology* **92**:500–506.
- Crabtree GR and Olson EN (2002) NFAT signaling: choreographing the social lives of cells. *Cell* **109** (Suppl):S67–S79.
- Dansereau MA, Gosselin RD, Pohl M, Pommier B, Mechighel P, Mauborgne A, Rostene W, Kitabgi P, Beaudet N, and Sarret P, et al. (2008) Spinal CCL2 pro-nociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J Neurochem* **106**:757–769.
- Fairbanks CA, Schreiber KL, Brewer KL, Yu CG, Stone LS, Kitto KF, Nguyen HO, Grocholski BM, Shoeman DW, and Kehl LJ, et al. (2000) Agmatine reverses pain induced by inflammation, neuropathy, and spinal cord injury. *Proc Natl Acad Sci USA* **97**:10584–10589.
- Flockhart RJ, Diffey BL, Farr PM, Lloyd J, and Reynolds NJ (2008) NFAT regulates induction of COX-2 and apoptosis of keratinocytes in response to ultraviolet radiation exposure. *FASEB J* **22**:4218–4227.
- Graef IA, Mermelstein PG, Stankunas K, Neilson JR, Deisseroth K, Tsien RW, and Crabtree GR (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. *Nature* **401**:703–708.
- Graef IA, Wang F, Charron F, Chen L, Neilson J, Tessier-Lavigne M, and Crabtree GR (2003) Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* **113**:657–670.
- Groth RD, Coicou LG, Mermelstein PG, and Seybold VS (2007) Neurotrophin activation of NFAT-dependent transcription contributes to the regulation of pro-nociceptive genes. *J Neurochem* **102**:1162–1174.
- Groth RD and Mermelstein PG (2003) Brain-derived neurotrophic factor activation of NFAT (nuclear factor of activated T-cells)-dependent transcription: a role for the transcription factor NFATc4 in neurotrophin-mediated gene expression. *J Neurosci* **23**:8125–8134.
- Hogan PG, Chen L, Nardone J, and Rao A (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* **17**:2205–2232.
- Iniguez MA, Martinez-Martinez S, Punzón C, Redondo JM, and Fresno M (2000) An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. *J Biol Chem* **275**:23627–23635.
- Jackson JG, Usachev YM, and Thayer SA (2007) Bradykinin-induced nuclear factor of activated T-cells-dependent transcription in rat dorsal root ganglion neurons. *Mol Pharmacol* **72**:303–310.
- Jones EA, Sun D, Kobierski L, and Symes AJ (2003) NFAT4 is expressed in primary astrocytes and activated by glutamate. *J Neurosci Res* **72**:191–197.
- Jung H, Bhargoo S, Banisadr G, Freitag C, Ren D, White FA, and Miller RJ (2009) Visualization of chemokine receptor activation in transgenic mice reveals peripheral activation of CCR2 receptors in states of neuropathic pain. *J Neurosci* **29**:8051–8062.

- Jung H and Miller RJ (2008) Activation of the nuclear factor of activated T-cells (NFAT) mediates upregulation of CCR2 chemokine receptors in dorsal root ganglion (DRG) neurons: a possible mechanism for activity-dependent transcription in DRG neurons in association with neuropathic pain. *Mol Cell Neurosci* **37**:170–177.
- Kao DJ, Li AH, Chen JC, Luo RS, Chen YL, Lu JC, and Wang HL (2012) CC chemokine ligand 2 upregulates the current density and expression of TRPV1 channels and Na^v1.8 sodium channels in dorsal root ganglion neurons (Abstract). *J Neuroinflammation* **9**:189.
- Kim DS, Figueroa KW, Li KW, Boroujerdi A, Yolo T, and Luo ZD (2009) Profiling of dynamically changed gene expression in dorsal root ganglia post peripheral nerve injury and a critical role of injury-induced glial fibrillary acidic protein in maintenance of pain behaviors [corrected]. *Pain* **143**:114–122.
- Kim SH and Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50**:355–363.
- Klee CB, Crouch TH, and Krinks MH (1979) Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. *Proc Natl Acad Sci USA* **76**:6270–6273.
- Li L, Cao XH, Chen SR, Han HD, Lopez-Berestein G, Sood AK, and Pan HL (2012) Up-regulation of Cavβ3 subunit in primary sensory neurons increases voltage-activated Ca²⁺ channel activity and nociceptive input in neuropathic pain. *J Biol Chem* **287**:6002–6013.
- Liu J, Farmer JD, Jr, Lane WS, Friedman J, Weissman I, and Schreiber SL (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**:807–815.
- López-Rodríguez C, Antos CL, Shelton JM, Richardson JA, Lin F, Novobrantseva TI, Bronson RT, Igarashi P, Rao A, and Olson EN (2004) Loss of NFAT5 results in renal atrophy and lack of tonicity-responsive gene expression. *Proc Natl Acad Sci USA* **101**:2392–2397.
- López-Rodríguez C, Aramburu J, Jin L, Rakeman AS, Michino M, and Rao A (2001) Bridging the NFAT and NF-κB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. *Immunity* **15**:47–58.
- Ma C and LaMotte RH (2005) Enhanced excitability of dissociated primary sensory neurons after chronic compression of the dorsal root ganglion in the rat. *Pain* **113**:106–112.
- Macián F, López-Rodríguez C, and Rao A (2001) Partners in transcription: NFAT and AP-1. *Oncogene* **20**:2476–2489.
- Maratou K, Wallace VC, Hasnie FS, Okuse K, Hosseini R, Jina N, Blackbeard J, Pheby T, Orengo C, and Dickenson AH, et al. (2009) Comparison of dorsal root ganglion gene expression in rat models of traumatic and HIV-associated neuropathic pain. *Eur J Pain* **13**:387–398.
- Marchand F, Perretti M, and McMahon SB (2005) Role of the immune system in chronic pain. *Nat Rev Neurosci* **6**:521–532.
- Martínez-Martínez S and Redondo JM (2004) Inhibitors of the calcineurin/NFAT pathway. *Curr Med Chem* **11**:997–1007.
- Menetski J, Mistry S, Lu M, Mudgett JS, Ransohoff RM, Demartino JA, Macintyre DE, and Abbadie C (2007) Mice overexpressing chemokine ligand 2 (CCL2) in astrocytes display enhanced nociceptive responses. *Neuroscience* **149**:706–714.
- Nagamoto-Combs K and Combs CK (2010) Microglial phenotype is regulated by activity of the transcription factor, NFAT (nuclear factor of activated T cells). *J Neurosci* **30**:9641–9646.
- Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, Li ST, Kobayashi N, Matsumoto S, and Tanaka K, et al. (2004) A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* **10**:305–309.
- Rao A, Luo C, and Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* **15**:707–747.
- Serrano-Pérez MC, Martín ED, Vaquero CF, Azcoitia I, Calvo S, Cano E, and Tranque P (2011) Response of transcription factor NFATc3 to excitotoxic and traumatic brain insults: identification of a subpopulation of reactive astrocytes. *Glia* **59**:94–107.
- Wang Y, Ghezzi A, Yin JC, and Atkinson NS (2009) CREB regulation of BK channel gene expression underlies rapid drug tolerance. *Genes Brain Behav* **8**:369–376.
- White FA, Jung H, and Miller RJ (2007) Chemokines and the pathophysiology of neuropathic pain. *Proc Natl Acad Sci USA* **104**:20151–20158.
- Wu ZZ, Chen SR, and Pan HL (2005) Transient receptor potential vanilloid type 1 activation down-regulates voltage-gated calcium channels through calcium-dependent calcineurin in sensory neurons. *J Biol Chem* **280**:18142–18151.
- Wu ZZ, Chen SR, and Pan HL (2006) Signaling mechanisms of down-regulation of voltage-activated Ca²⁺ channels by transient receptor potential vanilloid type 1 stimulation with olvanil in primary sensory neurons. *Neuroscience* **141**:407–419.
- Xiao HS, Huang QH, Zhang FX, Bao L, Lu YJ, Guo C, Yang L, Huang WJ, Fu G, and Xu SH, et al. (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc Natl Acad Sci USA* **99**:8360–8365.
- Zhao Z, Chen SR, Eisenach JC, Busija DW, and Pan HL (2000) Spinal cyclooxygenase-2 is involved in development of allodynia after nerve injury in rats. *Neuroscience* **97**:743–748.
- Zhou HY, Chen SR, Byun HS, Chen H, Li L, Han HD, Lopez-Berestein G, Sood AK, and Pan HL (2012) N-methyl-D-aspartate receptor- and calpain-mediated proteolytic cleavage of K⁺-Cl⁻ cotransporter-2 impairs spinal chloride homeostasis in neuropathic pain. *J Biol Chem* **287**:33853–33864.

Address correspondence to: Dr. Hui-Lin Pan, Department of Anesthesiology and Perioperative Medicine, Unit 110, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas. E-mail: huilpan@mdanderson.org
