Pannexin-1 Up-regulation in the Dorsal Root Ganglion Contributes to Neuropathic Pain Development*

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Background: Pannexin-1 can release many signaling molecules, and blocking pannexin-1 at the spinal cord level reduces chronic pain.
Results: Nerve injury increases pannexin-1 expression in primary sensory neurons via histone modifications. Pannexin-1 knockdown reduces pain hypersensitivity.
Conclusion: Pannexin-1 up-regulation in primary sensory neurons contributes to neuropathic pain.
Significance: Understanding the molecular mechanism of neuronal plasticity will improve treatments for neuropathic pain.

Pannexin-1 (Panx1) is a large-pore membrane channel involved in the release of ATP and other signaling mediators. Little is known about the expression and functional role of Panx1 in the dorsal root ganglion (DRG) in the development of chronic neuropathic pain. In this study, we determined the epigenetic mechanism involved in increased Panx1 expression in the DRG after nerve injury. Spinal nerve ligation in rats significantly increased the mRNA and protein levels of Panx1 in the DRG but not in the spinal cord. Immunocytochemical labeling showed that Panx1 was primarily expressed in a subset of medium and large DRG neurons in control rats and that nerve injury markedly increased the number of Panx1-immunoreactive DRG neurons. Nerve injury significantly increased the enrichment of two activating histone marks (H3K4me2 and H3K9ac) and decreased the occupancy of two repressive histone marks (H3K9me2 and H3K27me3) around the promoter region of Panx1 in the DRG. However, nerve injury had no effect on the DNA methylation level around the Panx1 promoter in the DRG. Furthermore, intrathecal injection of the Panx1 blockers or Panx1-specific siRNA significantly reduced pain hypersensitivity induced by nerve injury. In addition, siRNA knockdown of Panx1 expression in a DRG cell line significantly reduced caspase-1 release induced by neuronal depolarization. Our findings suggest that nerve injury increases Panx1 expression levels in the DRG through altered histone modifications. Panx1 up-regulation contributes to the development of neuropathic pain and stimulation of inflammasome signaling.

Chronic neuropathic pain causes excruciating suffering and remains difficult to treat. Despite intense research in this area, the mechanisms underlying neuropathic pain development are not well understood. Nerve injury increases the excitability of primary sensory nerves and neurons (1, 2) and alters the expression level of many pro- and antinociceptive genes in the dorsal root ganglion (DRG)[2] (3, 4). Also, increased release of proinflammatory cytokines, including high-mobility group box 1 (5) and interleukin-1β (6), and inflammasomes (7) in primary sensory neurons and nerves can lead to pain hypersensitivity. However, it remains unclear exactly how injured primary sensory neurons are involved in neuropathic pain development.

Pannexin 1 (Panx1) is a large-pore membrane channel, and Panx1 activation can release ATP (8) and cytokines, including high-mobility group box 1 (9) and interleukin-1β (10). Panx1 channels can be opened by membrane depolarization (11), cytoplasmic calcium (12), and NMDA receptor activation (13, 14). Unlike connexins, which form intercellular gap junction channels, there is no *in vivo* evidence for the existence of Panx1-based intercellular channels (15, 16). In the central nervous system, Panx1 is expressed in many neuronal populations, including the hippocampus, olfactory bulb, cortex, and cerebellum (11, 17). It has been reported that Panx1 activation in the brain plays a role in the development of epilepsy (18) and headache (9). However, we know little about the distribution of Panx1 in DRG neurons and how nerve injury affects Panx1 expression in the DRG in neuropathic pain.

In this study, we determined the expression level and functional role of Panx1 in the DRG in a rat model of neuropathic pain. We showed that nerve injury significantly increased the expression level of Panx1 in the DRG. DNA methylation and histone modifications are two major components of epigenetic regulation of gene expression. We found that nerve injury-induced increases in Panx1 expression are associated with changes in histone modifications, but not DNA methylation, in the promoter region of Panx1. In addition, Panx1 inhibition reduces nerve injury-induced hypersensitivity and caspase-1 release. Our study provides important new evidence about the epigenetic mechanism regulating Panx1 expression in the DRG and the role of Panx1 in the development of neuropathic pain.

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[2] The abbreviations used are: DRG, dorsal root ganglion/ganglia; SNL, spinal nerve ligation; CBX, carbenoxolone.

Experimental Procedures

Rat Model of Neuropathic Pain—Male Sprague-Dawley rats (7–8 weeks old, Harlan, Indianapolis, IN) were used for the entire study. All surgical preparations 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 for the ethical use of animals. L5/L6 spinal nerve ligation (SNL) was used as an experimental model of neuropathic pain in our study. In brief, we induced anesthesia with 2–3% isoflurane, isolated the left L5 and L6 spinal nerves under a surgical microscope, and ligated them with a 6-0 silk suture. Control rats underwent a sham surgical procedure without nerve ligation. In this SNL model, stable pain hypersensitivity is typically developed within 10–14 days after SNL and lasts for at least 8 weeks (19, 20).

Intrathecal Catheter Cannulation and Injection—Intrathecal catheters were implanted in rats during isoflurane-induced anesthesia 2 weeks after SNL surgery. Briefly, we made a small incision at the back of the neck of the animal. Next, we made a small opening in the atlanto-occipital membrane of the cisterna magna and inserted a PE-10 catheter (~8.0 cm) so that its caudal tip reached the lumbar spinal region (19, 21). The rostral end of the catheter was exteriorized, and the wound was closed with sutures. The animals were allowed to recover for 4–5 days after the surgery. Animals displaying signs of motor or neurological dysfunction were excluded from the study.

For intrathecal injections, we used two structurally dissimilar Panx1 blockers, [10]Panx (Tocris Bioscience, Bristol, UK) and carbenoxolone (CBX; Sigma-Aldrich) (9), as well as universal negative control siRNA (SIC001, Sigma-Aldrich) and Panx1-specific siRNA (GAACAUAAGUGAGCUCAAA, Sigma-Aldrich). [10]Panx and CBX were freshly dissolved in saline and injected in a 5-μl volume followed by a 5-μl flush with normal saline. Control siRNA and Panx1-specific siRNA were mixed with i-FECT for intrathecal delivery (22). Intrathecal injections started at 3 weeks after SNL when the chronic pain was fully developed.

Behavioral Assessment of Tactile Allodynia and Hyperalgesia in Rats—To quantify tactile allodynia, rats were placed in individual plastic boxes on a mesh floor. After 30–45 min of acclimation, 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 s. Brisk paw withdrawals or flinching was considered a positive response. In the absence of a response, the filament of next greater force was applied. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the "up-down" calculating method.

Hyperalgesia was measured by testing the paw withdrawal threshold in response to a noxious pressure stimulus (Randall-Selitto test) using the paw pressure analgesy meter (Ugo Basile Biological Research, Comerio, Italy). When the rat displayed pain by either withdrawing the paw or vocalizing, the pedal was released immediately to record the nociceptive withdrawal threshold (19, 21).

Quantitative RT-PCR—The left DRG and dorsal spinal cord tissues at the L5 and L6 levels were removed from rats during isoflurane-induced anesthesia. Total RNA was extracted from the tissue using TRIzol/chloroform and treated with DNase I (Invitrogen). cDNA was prepared by using the Superscript III first-strand synthesis kit and treated with RNase H (Invitrogen). Quantitative RT-PCR was performed using the iQ5 real-time PCR detection system and SYBR Green. The thermal cycle condition used was as follows: 95 °C for 1 min, 40 cycles at 95 °C for 15 s, and 60 °C for 15 s. Primers used for quantitative PCR were as follows: Panx1 (forward), CTTCGCTCAGGAGATCTCCA; Panx1 (reverse), GGTTTCCAGACTCACTCTGC; Panx2 (forward), TTTTGACAAGCTGCACAAGG; Panx2 (reverse), CCGGTTAAGCGACTTGATGT; GAPDH (forward), TGCCACTCAGAAGACTGTGG; and GAPDH (reverse), TTCAGCTCTGGGATGACCTT. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western Immunoblotting—The DRG at the L5 and L6 levels were homogenized using a sonicator in ice-cold radioimmune precipitation assay buffer containing protease mixture inhibitors (Sigma-Aldrich). Proteins were separated by 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The following primary antibodies were used: rabbit anti-Panx1 (1:1000, catalog no. 488000, Invitrogen) and rabbit anti-GAPDH (1:5000, Cell Signaling Technology, Beverly, MA). ECL plus Western blotting substrate was used for visualizing immunoreactive protein bands. The Panx1 protein bands were normalized by GAPDH (used as a loading control) on the same gel.

Double Immunofluorescence Labeling—Under deep anesthesia with sodium pentobarbital (60 mg/kg intraperitoneally), rats were perfused intracardially with 4% paraformaldehyde in 0.1 M PBS and 10% sucrose in PBS. The L5/L6 DRG were removed, fixed in the fixative solution for 2 h, and cryoprotected in PBS containing 30% sucrose for 48 h at 4 °C. The tissues were cut to 30 μm in thickness in 0.1 M Tris-NaCl-Tween (TNT) buffer, rinsed in 0.1 M TBS, and blocked in 4% normal goat serum in TBS for 1 h. The floating sections were then incubated with rabbit anti-Panx1 and mouse anti-NeuN (a neuronal marker, Millipore, Billerica, MA) antibodies diluted in TBS (1:200) overnight at 4 °C. The sections were rinsed in TNT buffer three times and then incubated in secondary antibodies for 1 h. The sections were rinsed in TNT and PBS buffers and then mounted on slides, dried, and coverslipped. The sections were examined on a laser-scanning confocal microscope (Zeiss). Omission of the above primary antibodies resulted in negative labeling in all DRG sections examined. Also, the Panx1 antibody produced a single protein band at the predicted size (~45 kDa) of Panx1 proteins in the Western blotting experiments (Fig. 1C). To examine the number of Panx1-expressing DRG neurons in the control and SNL rats, five sections from the L5 DRG (1 DRG/rat, $n = 4$ rats per group) were sampled.

DNA Methylation Analysis—Genomic DNA from the DRG was isolated using the PureLink genomic DNA mini kit (Invitrogen). Bisulfite treatment of DNA was carried out using the Epitek bisulfite kit (Qiagen, Valencia, CA). Bisulfite sequencing PCR was carried out using the following primers: Panx1-1 (forward), TAAGGATTTTTTGATTATTGTTTGG; Panx1-1 (reverse),

AACTAAAAATAACCCCACCCTATCT; Panx1-2 (forward), TGGGGTTATTTTTAGTTAGTT; and Panx1-2 (reverse), AAAACAAAACTAACTCTC. The PCR condition was as follows: 94 °C for 2 min, 36 cycles at 95 °C for 20 s, 58 °C for 20 s, 72 °C for 20 s, and 7 min at 72 °C. PCR products were purified using the Gene JET gel extraction kit (Thermo Fisher Scientific, Waltham, MA) and then inserted into the PMD-19 vector (Takara, Mountain View, CA) according to the instructions of the manufacturer. Constructed plasmids were mixed with competent cells (Bioline, Taunton, MA) and placed on ice for 30 min. The mixture was heat-shocked in a water bath at 42 °C for 90 s, followed by adding 500 μl of lysogeny broth to the mixture at 37 °C for 40 min. The competent cells were then placed onto a lysogeny broth agar plate and incubated at 37 °C overnight. Thirty clones were randomly selected for sequencing.

ChIP—The ChIP assay was carried out using the Magna ChIP G tissue kit (Millipore) according to the instructions of the manufacturer. Briefly, fresh DRG tissues were stabilized for 3 min in the stabilization buffer. Then the DRG were incubated in 2% formaldehyde for 20 min at 25 °C. After washing three times with PBS, the DRG were incubated in lysis buffer for 15 min on ice. Finally, the DRG were sonicated in dilution buffer using a sonicator with a microprobe (Qsonica, Newtown, CT) at 4 °C. Chromatin was pulled down using the following antibodies: IgG (catalog no. ab124055, Abcam, Cambridge, MA), total H3 (catalog no. 2650s, Cell Signaling Technology), H3K4me3 (catalog no. 9751s, Cell Signaling Technology), H3K9ac (catalog no. 39917, Active Motif, Carlsbad, CA), H3K9me2 (catalog no. ab1220, Abcam), and H3K27me3 (catalog no. 9773s, Cell Signaling Technology). Real-time PCR was performed after chromatin precipitation. The real-time PCR condition was as follows: 95 °C for 1 min, 40 cycles at 95 °C for 15 s, and 60 °C for 15 s. Primers used in the ChIP-PCR were as follows: Panx1-1 (forward), AGGACCCCCTGACTACTGCT; Panx1-1 (reverse), GCTGCACCTTTCCAGAGTTC; Panx1-2 (forward), CGCTTCCGGAAACTGCTC; Panx1-2 (reverse), CTCTCCGCTACAGCTGCTC; Panx1-3 (forward), CTCGGACTTCTTGCTGAAGG; and Panx1-3 (reverse), GATGAGCAGCAGAGGCAGAC.

F11 Cell Culture and Caspase-1 Release—F11 cells, a DRG cell line, were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum in 5% CO_2 at 37 °C. About 1×10^6 cells were planted in a 10-cm dish, and some F11 cells were transfected with negative control siRNA or Panx1-specific siRNA using Lipofectamine 2000. Approximately 48 h after transfection, the culture medium was replaced with DMEM containing 20 mM KCl for 24 h. The supernatant and F11 cells were then collected separately. Caspase-1 proteins in the supernatant were isolated using trichloroacetic acid and quantified using Western immunoblotting with the use of rabbit anti-caspase-1 antibody (1:1000; Millipore). Also, total proteins from F11 cells used in each experiment were collected to measure the GAPDH protein level as a control.

Statistical Analysis—All data were expressed as the mean ± S.E. Because DNA samples were derived from mixtures of cell populations with heterogeneous DNA methylation profiles, this can cause the difference in methylated base positions between the samples. Chi-squared test was used to determine the difference in the ratio of methylated to unmethylated CpG

sites in the Panx1 promoter between the sham and SNL groups. When data (mRNA and protein levels) from two groups were compared, paired or unpaired Student's *t* test was used. For comparisons of biochemical and behavioral data from more than two groups, we used one-way analysis of variance followed by Tukey's or Dunnett's post hoc test or two-way analysis of variance followed by Bonferroni's post hoc test. $p < 0.05$ was considered statistically significant.

Results

Nerve Injury Increases the Expression Level of Panx1 in the DRG—We first used quantitative RT-PCR to determine the mRNA level of Panx1 in L5 and L6 DRG 5, 10, and 21 days after surgery. The mRNA level of Panx1 in the DRG increased significantly at all three time points (Fig. 1A). However, the Panx1 mRNA level in the dorsal spinal cord did not differ significantly between the sham control and SNL rats (Fig. 1B). Western immunoblotting detected a single Panx1 protein band in the DRG tissue, and the Panx1 protein level in the DRG was also increased significantly 5, 10, and 21 days after SNL (Fig. 1C).

The pannexin family consists of three members: Panx1, Panx2, and Panx3 (16, 23). We therefore examined the mRNA levels of Panx2 and Panx3 in L5 and L6 DRG and dorsal spinal cords 21 days after nerve injury. SNL significantly decreased the Panx2 mRNA level in the DRG, but it had no significant effect on the Panx2 mRNA level in the spinal cord (Fig. 1D). However, the mRNA levels of Panx3 in the DRGs and dorsal spinal cords were not detected in sham control or SNL rats.

Nerve Injury Increases the Number of Panx1-expressing DRG Neurons—We next used double immunofluorescence labeling to determine the distribution of Panx1 in DRG neurons. Confocal imaging analysis revealed that Panx1 immunoreactivity was evenly present in the cytoplasm and plasma membrane of a subset of medium- and large-diameter DRG neurons in sham control rats (Fig. 2). Approximately 44.5% of NeuN-positive DRG neurons showed Panx1 immunoreactivity in control rats. By contrast, in nerve-injured rats, Panx1 immunoreactivity was distributed in almost all NeuN-positive DRG neurons (97.1%). Also, in most DRG neurons from SNL rats, Panx1 immunoreactivity was clustered around the plasma membrane (Fig. 2). Notably, some Panx1 immunoreactivities surrounded DRG neurons and were not colocalized with NeuN. The above data suggest that nerve injury causes up-regulation of Panx1 in DRG neurons and, possibly, satellite glial cells.

Nerve Injury Has No Effect on the DNA Methylation Level of the Panx1 Promoter in the DRG—DNA methylation and histone tail modifications are the two predominant epigenetic mechanisms involved in regulating long-lasting changes in gene expression in post-mitotic neurons (24, 25). The DNA methylation level in the promoter region controls gene expression. We measured the DNA methylation pattern in the Panx1 promoter region in DRG 21 days after surgery. A total of 32 CpG sites at the Panx1 promoter region (from −345 bp to +98 bp) were selected for analysis. The methylation level in the Panx1 promoter in the control DRG was very low (Fig. 3). There was no significant difference in DNA methylation level at the Panx1 promoter region in the DRG between sham control and SNL rats (Fig. 3). These results suggest that nerve injury-in-
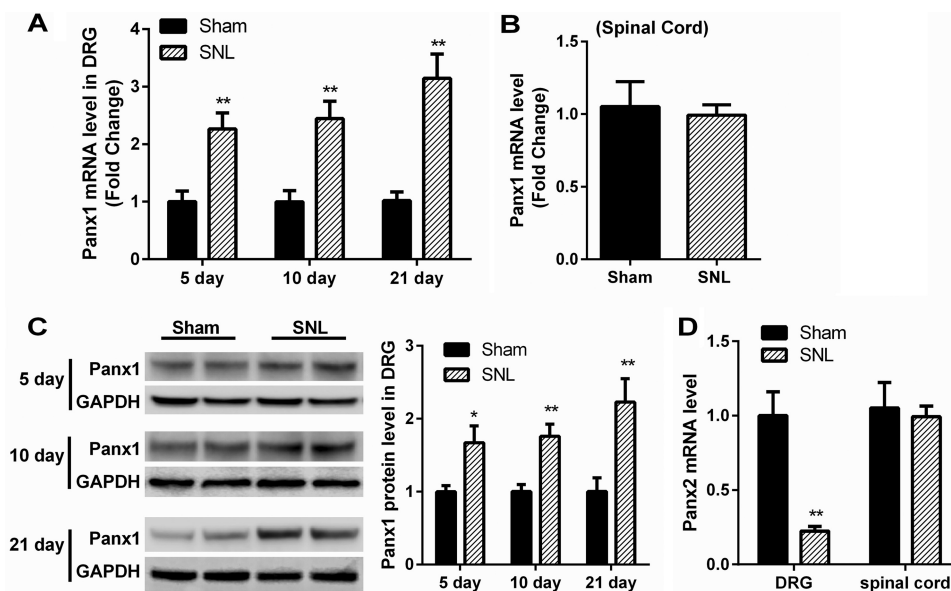
FIGURE 1. **Nerve injury increases the Panx1 expression level in the DRG.** *A*, the mRNA levels of Panx1 in the DRG at 5, 10, and 21 days after sham and SNL surgery ($n = 6$ rats/group). *B*, the mRNA level of Panx1 in the dorsal spinal cord 21 days after sham and SNL surgery ($n = 6$ rats/group). *C*, original gel images and mean data ($n = 6$ rats/group) showing the Panx1 protein (~45 kDa) level in the DRG 21 days after sham and SNL surgery. *D*, the mRNA levels of Panx2 in the DRG and dorsal spinal cord 21 days after sham and SNL surgery ($n = 6$ rats/group). The mRNA level was measured by quantitative RT-PCR and normalized to GAPDH. For Western immunoblotting, GAPDH (37 kDa) was used as a loading control. Data are mean $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* the sham group.
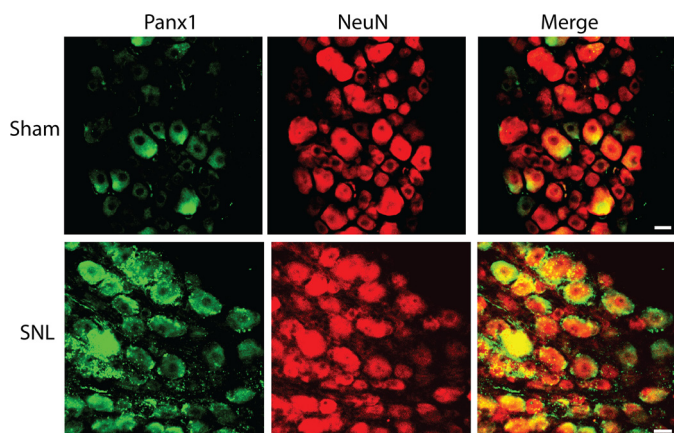

FIGURE 2. **Cellular distribution of Panx1 in control and injured DRG.** Double immunostaining and confocal images show the colocalization of Panx1 with a neuronal marker (*NeuN, red*) in L5 and L6 DRG sections from sham and SNL rats 21 days after surgery. Digitally merged images are shown at the *right*. *Scale bar* = 20 μm.

FIGURE 3. **Nerve injury has no effect on the DNA methylation level at the Panx1 promoter in DRG.** Bisulfite sequencing PCR analysis of Panx1 in DRG from sham and SNL rats 21 days after surgery ($n = 4$ independent experiments/group). The DNA methylation pattern of CpG sites near the transcriptional start site ($-345/+98$ bp) was analyzed. Each row represents the methylation pattern of a single clone (*i.e.* one allele). *Open circles* represent unmethylated CpG sites, and *closed circles* represent methylated CpG sites at the transcriptional start site.

duced Panx1 up-regulation in the DRG is not associated with changes in DNA methylation status.

Nerve Injury Alters Histone Modifications around the Panx1 Promoter in the DRG—Both H3K4me3 and H3K9ac are activating histone markers associated with transcriptional activation (26–28). In contrast, H3K9me2 and H3K27me3 are repressive histone markers associated with gene silencing (29, 30). We used a ChIP-PCR approach to determine whether nerve injury alters histone modifications at the promoter region of Panx1 in the DRG. ChIP-PCR experiments showed that SNL significantly increased the occupancy of H3K4me3 and H3K9ac at the Panx1 promoter around its transcriptional start site in the DRG (Fig. 4*A*). Also, SNL significantly reduced the H3K9me2 and H3K27me3 levels at the Panx1 promoter region around its transcriptional start site (Fig. 4*A*). Additional experiments showed significant increases in H3K4me3 and H3K9ac enrichment and
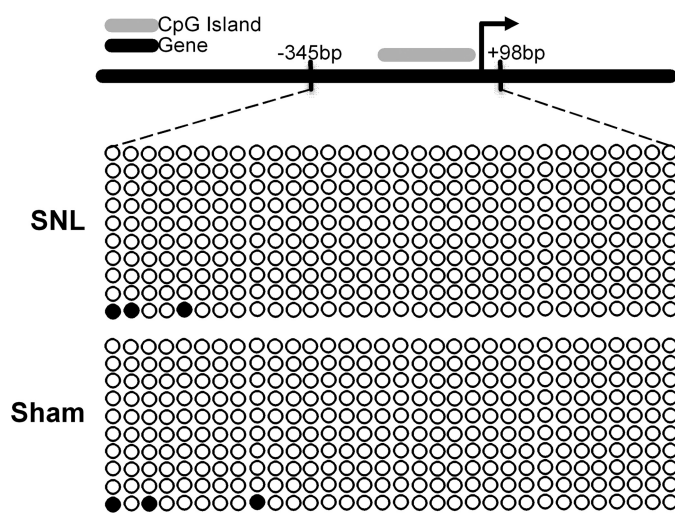
significant reductions in H3K9me2 and H3K27me3 occupancy in two other promoter regions of Panx1 in the injured DRG (Fig. 4, *B* and *C*). These data suggest that an open chromatin structure contributes to increased Panx1 expression in the injured DRG.

Panx1 Up-regulation Contributes to Pain Hypersensitivity Induced by Nerve Injury—Intrathecal injections allow agents direct access to DRG neurons (21, 22). To determine whether increased Panx1 expression in the DRG contributes to pain hypersensitivity induced by nerve injury, we first used two different Panx1 blockers, CBX and [10]Panx (9). Intrathecal injec-
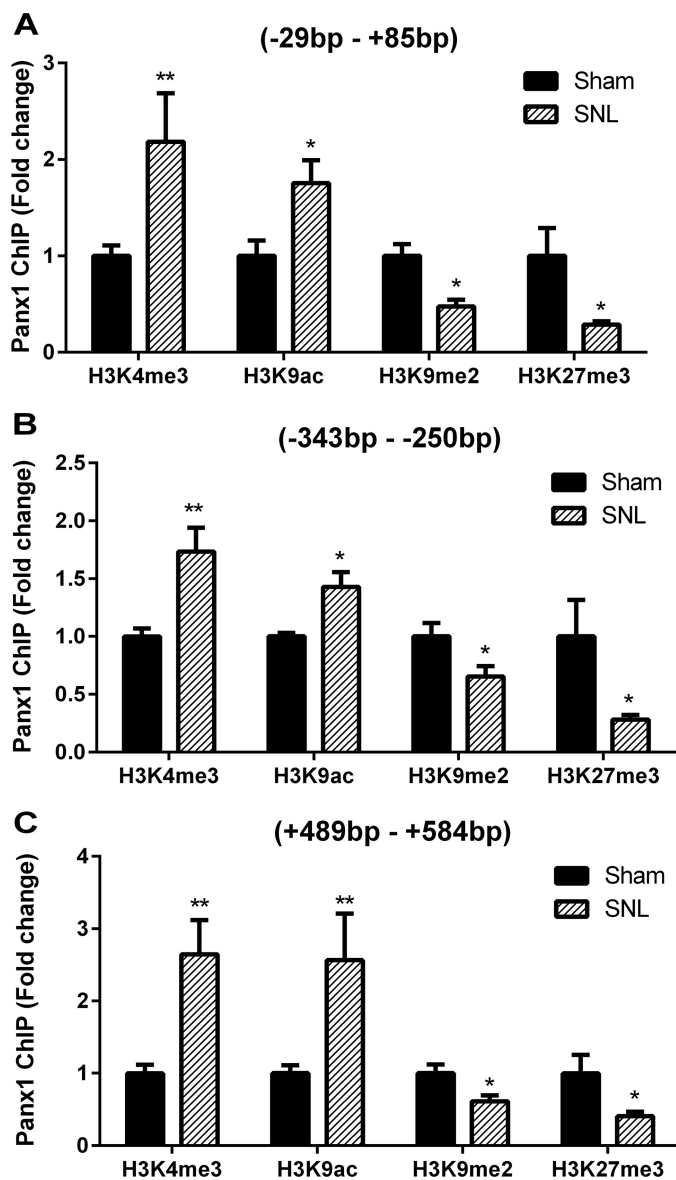
A

(-29bp - +85bp)

B

(-343bp - -250bp)

C

(+489bp - +584bp)

FIGURE 4. **Nerve injury alters histone modifications at the Panx1 promoter in the DRG.** *A–C*, ChIP-PCR quantification data showing the enrichment of histone H3K4me3, H3K9ac, H3K9me2, and H3K27me3 at the three different promoter regions of Panx1: $-29/+85$ bp (*A*), $-343/-250$ bp (*B*), and $+489/+584$ bp (*C*). L5 and L6 DRG were obtained from sham and SNL rats 21 days after surgery ($n = 6$ independent experiments/group). Data were normalized by total H3 values and are mean $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* the sham group.

tion of CBX (0.5–20 μg) dose-dependently increased the tactile threshold and the pressure threshold of SNL rats (Fig. 5, *A* and *B*). The CBX effect at 10–20 μg appeared within 1 h after injection and lasted for at least 3 h. Furthermore, intrathecal injection of ^{10}Panx (5–40 μg) also significantly increased the tactile threshold and the pressure threshold in a dose-dependent manner in separate SNL rats (Fig. 5, *C* and *D*).

Because the Panx1 blockers may have effects on targets other than Panx1, we next used Panx1-specific siRNA to knockdown the Panx1 expression level in the DRG of SNL rats. We initially screened three Panx1 siRNAs in F11 cells and selected the most effective one (GAACAUAAGUGAGCUCAAA) for intrathecal injection in SNL rats. Intrathecal injection of Panx1-specific

siRNA (5 μg) daily for 4 days caused a large reduction in the mRNA and protein levels of Panx1 in the DRG (Fig. 6, *A* and *B*). The Panx1-specific siRNA slightly reduced the Panx1 mRNA level in the dorsal spinal cord (Fig. 6*A*) and had no significant effect on the mRNA level of Panx2 in the DRG (Fig. 6*C*). Furthermore, intrathecal injection of Panx1-specific siRNA for 4 days gradually increased the withdrawal thresholds in response to application of von Frey filaments and a noxious stimulus (Fig. 6, *D* and *E*). Together, these findings suggest that Panx1 up-regulation in the DRG contributes to pain hypersensitivity induced by nerve injury.

Panx1 Activation Increases the Release of Caspase-1 in a DRG Cell Line—The inflammasome caspase-1 is a primary mechanism responsible for the maturation of interleukin-1β (31, 32). Caspase-1 and interleukin-1β in the peripheral nerve play an important role in the development of pain hypersensitivity (6, 32). Because it is very difficult to measure the caspase-1 release in the DRG *in vivo*, we used a DRG cell line, F11, to determine whether Panx1 activation is involved in caspase-1 release. Neuronal depolarization with 20 mM KCl significantly increased the caspase-1 protein level in the supernatant (Fig. 7*A*). Furthermore, treatment with Panx1-specific siRNA significantly attenuated the release of caspase-1 from F11 cells induced by 20 mM KCl (Fig. 7, *B* and *C*). These results suggest that activation of Panx1 is involved in caspase-1 release from DRG cells.

Discussion

Our study provides new information about the expression of Panx1 in primary sensory neurons in the DRG and their role in neuropathic pain. The mammalian pannexin family consists of three members: Panx1, Panx2, and Panx3. The opening of pannexin channels allows the release of signaling molecules such as ATP, glutamate, and cytokines (8–10, 16). Although Panx1 is expressed in many regions of the central nervous system, Panx2 appears to be more restricted to several areas of the human brain, including the cerebellum, cerebral cortex, medulla, occipital pole frontal lobe, temporal lobe, and putamen (23). In the brain, only low levels of Panx3 transcripts are detected in the hippocampus (23). We found, in this study, that nerve injury profoundly increased the mRNA and protein levels of Panx1 in the DRGs within 5 days and that this Panx1 up-regulation lasted for at least 3 weeks after surgery. In contrast, we showed that Panx3 was not expressed in the rat DRG and that the expression level of Panx2 in the DRG was reduced by nerve injury. We also found that Panx1 was present in a subset of medium- and large-diameter DRG neurons in control rats. Strikingly, Panx1 was abundantly expressed in almost all DRG neurons and, possibly, satellite glial cells after nerve injury. It has been reported that both Panx1 and Panx2 are expressed in DRG somata and the surrounding satellite glial cells (33), which may be involved in the communication between DRG neuronal somata and satellite glial cells via ATP release and P2X7 receptors (34). Also, inflammation increases Panx1 expression in the trigeminal ganglia, and orofacial pain hypersensitivity is reduced in P2X7R-null and Panx1-null mice (35). These data suggest that sustained increases in Panx1 expression in primary sensory neurons may be involved in neuropathic pain development.
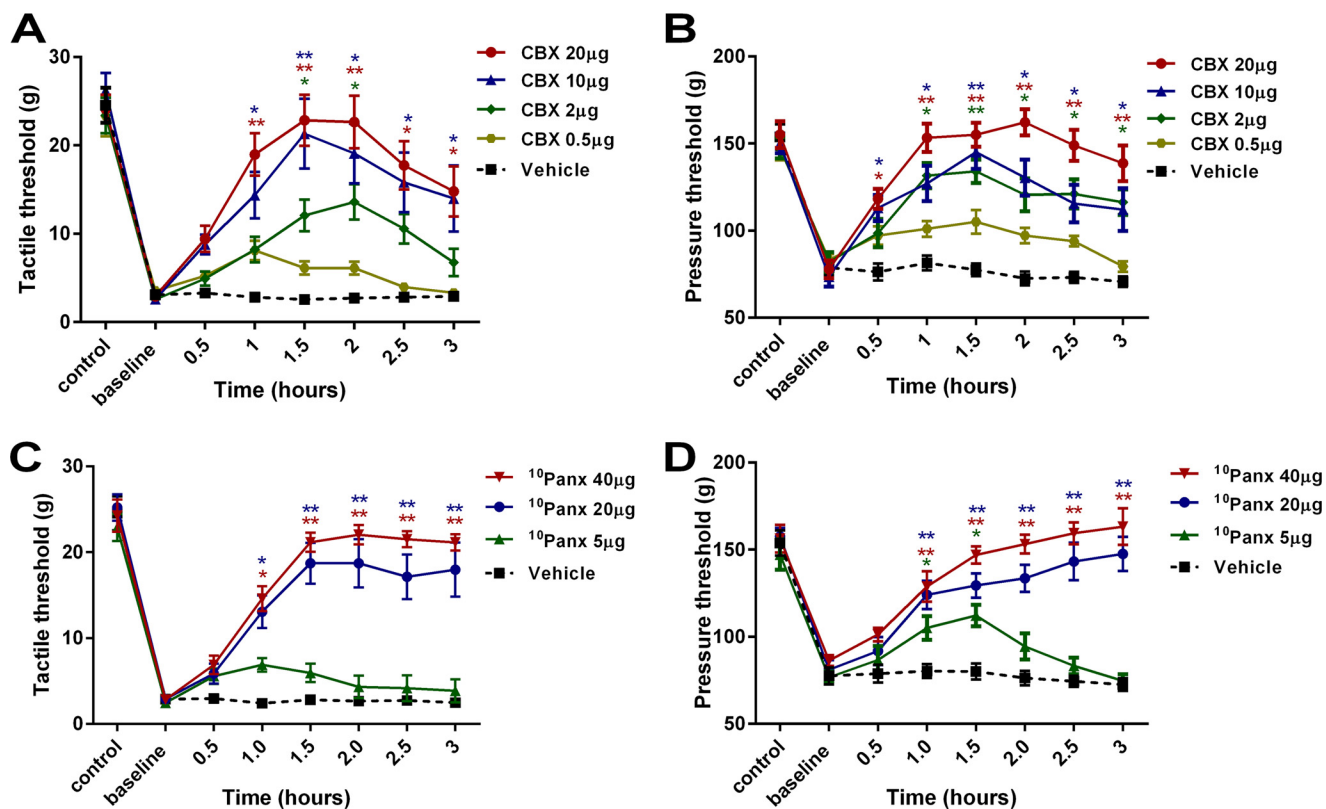
FIGURE 5. **Panx1 blockers reduce pain hypersensitivity induced by nerve injury.** *A* and *B*, time course of the effects of intrathecal injection of CBX on the tactile and pressure withdrawal threshold of SNL rats ($n = 8–9$ rats/group). *C* and *D*, time course of the effects of intrathecal administration of [10]Panx1 on the tactile and pressure withdrawal threshold of SNL rats ($n = 8–9$ rats/group). Data are mean $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$, *versus* the same color used for the baseline value. Withdrawal thresholds before SNL surgery were plotted as the control.
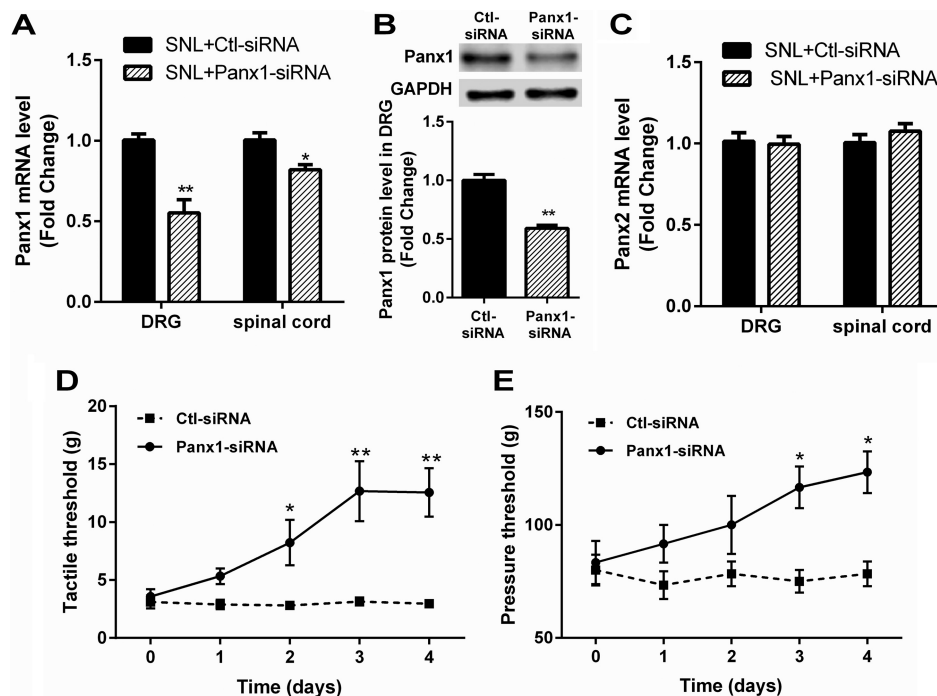

FIGURE 6. **siRNA knockdown of Panx1 attenuates nerve injury-induced pain hypersensitivity.** *A*, mRNA levels of Panx1 in the DRG and dorsal spinal cord from control (*Ctl*) siRNA- and Panx1-specific siRNA-treated SNL rats ($n = 6$ rats/group). *B*, original gel images and mean data showing the protein levels of Panx1 (45 kDa) in the DRG obtained from control siRNA- and Panx1-specific siRNA-treated rats ($n = 6$ rats/group). *C*, mRNA levels of Panx2 in the DRG and dorsal spinal cord from control siRNA- and Panx1-specific siRNA-treated SNL rats ($n = 6$ rats/group). *D* and *E*, time course of the effects of daily intrathecal injection of control siRNA- and Panx1-specific siRNA on tactile and pressure withdrawal thresholds of SNL rats 3 weeks after surgery ($n = 6$ rats/group). Data are mean $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* the control siRNA group.
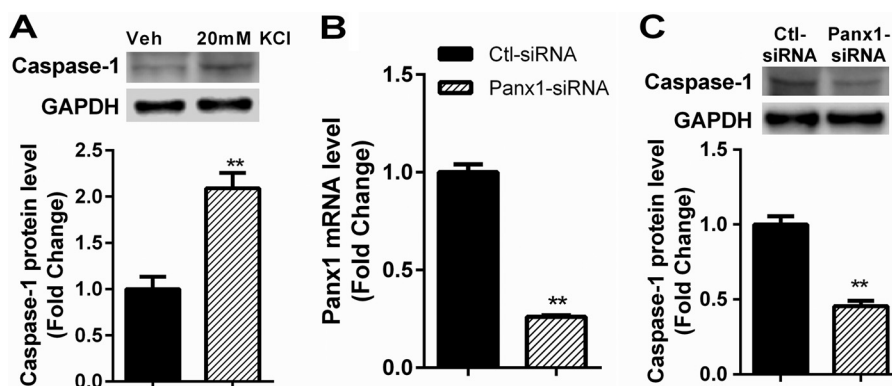
FIGURE 7. **siRNA knockdown of Panx1 reduces caspase-1 release in F11 cells.** *A*, mRNA levels of Panx1 in F11 cells treated with control siRNA and Panx1-specific siRNA ($n = 5$ independent experiments/group). *Veh*, vehicle. *B*, caspase-1 protein (45 kDa) levels in the supernatant of F11 cells treated with 20 mM KCl or vehicle ($n = 5$ independent experiments/group). *Ctl*, control. *C*, caspase-1 protein levels in the supernatant of F11 cells stimulated with 20 mM KCl. Cells were pretreated with control siRNA or Panx1-specific siRNA ($n = 5$ independent experiments/group). Data are mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ *versus* the control siRNA or vehicle group.

A salient finding of our study is that blocking Panx1 channels with CBX or [10]Panx and siRNA knockdown of Panx1 expression in the DRG significantly attenuated tactile allodynia and mechanical hyperalgesia induced by nerve injury. A recent study has reported that intrathecal injection of Panx1 blockers reduces pain hypersensitivity caused by sural nerve transection in rats (36). However, the conclusion of the authors is entirely on the basis of the use of pharmacological agents that may not specifically block Panx1 channels (37). Also, the authors assume that the analgesic effect produced by intrathecal administration of Panx1 blockers is through inhibition of Panx1 in the spinal cord (36). However, neither spinal nerve ligation (used in our study) nor sural nerve transection (36) has any effect on Panx1 expression level in the rat spinal cord. We found that intrathecal injection of Panx1-siRNA primarily reduced Panx1 expression level in the DRG. Therefore, intrathecally injected Panx1 blockers and Panx1 siRNA reduce neuropathic pain primarily by targeting up-regulated Panx1 in injured DRG neurons.

Nerve injury-induced releases of proinflammatory cytokines contribute to the development of neuropathic pain (38–40). Caspase-1 is the major component of inflammasomes (41, 42) and is involved in inflammatory responses by cleaving the precursors of interleukin-1β and interleukin-18 (43). Because the sensitivity of antibody-based caspase-1 assays is limited, we had to use the DRG F11 cell line, which allowed us reproducibly detect caspase-1 release when a large number of cells ($\sim 10^6$ F11 cells) were used. However, each DRG only has a few hundred neurons. Also, DRG neurons in prolonged culture often change their phenotypes, and it is not feasible to compare caspase-1 release under two conditions (sham and injury DRGs). Our study using a DRG cell line showed that siRNA knockdown of Panx1 significantly attenuated caspase-1 release induced by neuronal depolarization. Increased caspase-1 release could result in increased interleukin-1β maturation and activation to induce pain hypersensitivity (6). Therefore, nerve injury-induced Panx1 up-regulation in the DRG may contribute to neuropathic pain development by promoting caspase-1 release and stimulation of the inflammasome-cytokine signaling cascade.

DNA methylation and histone modification are two major components of epigenetic mechanisms involved in gene expression (24, 25). We showed that nerve injury significantly increased the enrichment of two activating histone marks, H3K4me3 and H3K9ac, around the promoter region of Panx1 in the DRG. Also, nerve injury significantly decreased the occupancy of two repressive histone marks, H3K4me2 and H3K27me3, at the Panx1 promoter. However, nerve injury had no significant effect on the DNA methylation level in the promoter region of the Panx1 gene in the DRG. H3K4 methylation is catalyzed by trithorax group proteins (TrxG), whereas H3K27 trimethylation is mediated through polycomb group proteins (30). Polycomb repressive complex 2 (PRC2) of polycomb group proteins is typically found in the regions that are trimethylated at H3K27 of silenced genes (44), and the H3K4me2 is present at most active promoters in the genome (45). Because PRC2 not only has signature activity to trimethylate H3K27 (46) but can also target retinol binding protein 2, an H3K4 demethylase (47), the ratio of H3K27me3 and H3K4me2 in the gene promoter can greatly affect gene transcription. When H3K27me3 is reduced, TrxG complexes can bind to the H3K4 position to induce H3K4me3 and gene activation (48).

Increased H3K9ac enrichment (regulated by histone acetyltransferases and deacetylases) in gene promoters is associated with transcriptional activation and increased gene expression (28). On the other hand, increased H3K9me2 occupancy, mediated through the G9a/GLP histone lysine dimethyltransferase, in gene promoters is generally involved in transcriptional repression and gene silencing (49). The balance of acetylation and methylation levels at the H3K9 position could also be critical for regulating gene transcription (50). Therefore, the combined changes in histone modifications (*i.e.* increased enrichment of H3K4me3 and H3K9ac and decreased levels of H3K9me2 and H3K27me3) at the Panx1 promoter region are likely involved in the increased Panx1 expression level in the injured DRG. The upstream molecules that contribute to the histone methylation pathway include histone methyltransferases (*e.g.* trithorax group proteins, polycomb group proteins, and G9a/GLP) and histone demethylases (*e.g.* LSD1, JMJD1A, and JMJD3). The downstream signals engaged in histone methylation may include methyllysine reader proteins such as PHD, MBT, and WD40 domains. Further studies are needed to identify specific upstream and downstream signaling molecules

(including transcriptional factors) that participate in altered histone modifications leading to increased Panx1 expression in the injured DRG.

In conclusion, we found, in this study, that nerve injury caused a large and persistent Panx1 up-regulation in DRG. Increased Panx1 expression was associated with altered histone modifications around the Panx1 promoter region: increased enrichment of H3K4me3 and H3K9ac and decreased occupancy of H3K9me2 and H3K27me3. Furthermore, inhibition of Panx1 significantly reduced pain hypersensitivity and caspase-1 release. Therefore, our findings suggest that peripheral nerve injury increases Panx1 expression through histone modifications in the DRG. Because Panx1 up-regulation in the DRG contributes to neuropathic pain development, Panx1 may be a new therapeutic target for treating chronic neuropathic pain.

References

1. Ma, C., and LaMotte, R. H. (2005) Enhanced excitability of dissociated primary sensory neurons after chronic compression of the dorsal root ganglion in the rat. *Pain* **113,** 106–112
2. Pan, H. L., Eisenach, J. C., and Chen, S. R. (1999) Gabapentin suppresses ectopic nerve discharges and reverses allodynia in neuropathic rats. *J. Pharmacol. Exp. Ther.* **288,** 1026–1030
3. Wang, H., Sun, H., Della Penna, K., Benz, R. J., Xu, J., Gerhold, D. L., Holder, D. J., and Koblan, K. S. (2002) Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. *Neuroscience* **114,** 529–546
4. Xiao, H. S., Huang, Q. H., Zhang, F. X., Bao, L., Lu, Y. J., Guo, C., Yang, L., Huang, W. J., Fu, G., Xu, S. H., Cheng, X. P., Yan, Q., Zhu, Z. D., Zhang, X., Chen, Z., Han, Z. G., and Zhang, X. (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 8360–8365
5. Shibasaki, M., Sasaki, M., Miura, M., Mizukoshi, K., Ueno, H., Hashimoto, S., Tanaka, Y., and Amaya, F. (2010) Induction of high mobility group box-1 in dorsal root ganglion contributes to pain hypersensitivity after peripheral nerve injury. *Pain* **149,** 514–521
6. Zelenka, M., Schäfers, M., and Sommer, C. (2005) Intraneural injection of interleukin-1beta and tumor necrosis factor-α into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain* **116,** 257–263
7. Li, Q., Tian, Y., Wang, Z. F., Liu, S. B., Mi, W. L., Ma, H. J., Wu, G. C., Wang, J., Yu, J., and Wang, Y. Q. (2013) Involvement of the spinal NALP1 inflammasome in neuropathic pain and aspirin-triggered-15-epi-lipoxin A4 induced analgesia. *Neuroscience* **254,** 230–240
8. Sandilos, J. K., Chiu, Y. H., Chekeni, F. B., Armstrong, A. J., Walk, S. F., Ravichandran, K. S., and Bayliss, D. A. (2012) Pannexin 1, an ATP release channel, is activated by caspase cleavage of its pore-associated C-terminal autoinhibitory region. *J. Biol. Chem.* **287,** 11303–11311
9. Karatas, H., Erdener, S. E., Gursoy-Ozdemir, Y., Lule, S., Eren-Koçak, E., Sen, Z. D., and Dalkara, T. (2013) Spreading depression triggers headache by activating neuronal Panx1 channels. *Science* **339,** 1092–1095
10. Pelegrin, P., and Surprenant, A. (2006) Pannexin-1 mediates large pore formation and interleukin-1β release by the ATP-gated P2X7 receptor. *EMBO J.* **25,** 5071–5082
11. Bruzzone, R., Hormuzdi, S. G., Barbe, M. T., Herb, A., and Monyer, H. (2003) Pannexins, a family of gap junction proteins expressed in brain. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 13644–13649
12. Locovei, S., Wang, J., and Dahl, G. (2006) Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium. *FEBS Lett.* **580,** 239–244
13. Weilinger, N. L., Tang, P. L., and Thompson, R. J. (2012) Anoxia-induced NMDA receptor activation opens pannexin channels via Src family kinases. *J. Neurosci.* **32,** 12579–12588
14. Thompson, R. J., Jackson, M. F., Olah, M. E., Rungta, R. L., Hines, D. J., Beazely, M. A., MacDonald, J. F., and MacVicar, B. A. (2008) Activation of pannexin-1 hemichannels augments aberrant bursting in the hippocampus. *Science* **322,** 1555–1559
15. Sosinsky, G. E., Boassa, D., Dermietzel, R., Duffy, H. S., Laird, D. W., MacVicar, B., Naus, C. C., Penuela, S., Scemes, E., Spray, D. C., Thompson, R. J., Zhao, H. B., and Dahl, G. (2011) Pannexin channels are not gap junction hemichannels. *Channels* **5,** 193–197
16. Penuela, S., Gehi, R., and Laird, D. W. (2013) The biochemistry and function of pannexin channels. *Biochim. Biophys. Acta* **1828,** 15–22
17. Zappalà, A., Cicero, D., Serapide, M. F., Paz, C., Catania, M. V., Falchi, M., Parenti, R., Pantò, M. R., La Delia, F., and Cicirata, F. (2006) Expression of pannexin1 in the CNS of adult mouse: cellular localization and effect of 4-aminopyridine-induced seizures. *Neuroscience* **141,** 167–178
18. Santiago, M. F., Veliskova, J., Patel, N. K., Lutz, S. E., Caille, D., Charollais, A., Meda, P., and Scemes, E. (2011) Targeting pannexin1 improves seizure outcome. *PLoS ONE* **6,** e25178
19. Chen, S. R., Zhou, H. Y., Byun, H. S., Chen, H., and Pan, H. L. (2014) Casein kinase II regulates *N*-methyl-D-aspartate receptor activity in spinal cords and pain hypersensitivity induced by nerve injury. *J. Pharmacol. Exp. Ther.* **350,** 301–312
20. Chen, S. R., Cai, Y. Q., and Pan, H. L. (2009) Plasticity and emerging role of BKCa channels in nociceptive control in neuropathic pain. *J. Neurochem.* **110,** 352–362
21. Cai, Y. Q., Chen, S. R., Han, H. D., Sood, A. K., Lopez-Berestein, G., and Pan, H. L. (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
22. Luo, M. C., Zhang, D. Q., Ma, S. W., Huang, Y. Y., Shuster, S. J., Porreca, F., and Lai, J. (2005) An efficient intrathecal delivery of small interfering RNA to the spinal cord and peripheral neurons. *Mol. Pain* **1,** 29
23. Baranova, A., Ivanov, D., Petrash, N., Pestova, A., Skoblov, M., Kelmanson, I., Shagin, D., Nazarenko, S., Geraymovych, E., Litvin, O., Tiunova, A., Born, T. L., Usman, N., Staroverov, D., Lukyanov, S., and Panchin, Y. (2004) The mammalian pannexin family is homologous to the invertebrate innexin gap junction proteins. *Genomics* **83,** 706–716
24. Riccio, A. (2010) Dynamic epigenetic regulation in neurons: enzymes, stimuli and signaling pathways. *Nat. Neurosci.* **13,** 1330–1337
25. Ronan, J. L., Wu, W., and Crabtree, G. R. (2013) From neural development to cognition: unexpected roles for chromatin. *Nat. Rev. Genet.* **14,** 347–359
26. Ng, H. H., Robert, F., Young, R. A., and Struhl, K. (2003) Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* **11,** 709–719
27. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature* **419,** 407–411
28. Jenuwein, T., and Allis, C. D. (2001) Translating the histone code. *Science* **293,** 1074–1080
29. Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292,** 110–113
30. Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007) Genome regulation by polycomb and trithorax proteins. *Cell* **128,** 735–745
31. Martinon, F., Burns, K., and Tschopp, J. (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol. Cell* **10,** 417–426
32. Cunha, T. M., Talbot, J., Pinto, L. G., Vieira, S. M., Souza, G. R., Guerrero, A. T., Sonego, F., Verri, W. A., Jr., Zamboni, D. S., Ferreira, S. H., and Cunha, F. Q. (2010) Caspase-1 is involved in the genesis of inflammatory hypernociception by contributing to peripheral IL-1β maturation. *Mol. Pain* **6,** 63
33. Huang, L. Y., and Gu, Y. (2012) Purinergic P2X7 receptor-activated and pannexin-mediated responses in satellite glial cells in rat dorsal root ganglia. *Abstracts of the 2012 Neuroscience Meeting, New Orleans, LA, October 13–17, 2012*, Abstract 180.06/II4, Society for Neuroscience, Washington, D. C.
34. Huang, L. Y., Gu, Y., and Chen, Y. (2013) Communication between neuronal somata and satellite glial cells in sensory ganglia. *Glia* **61,** 1571–1581

35. Hanstein, R., Zhao, J. B., Gulinello, M., Hanani, M., and Spray, D. C. (2012) Transient inflammation causes chronic tactile hypersensitivity: a new role for pannexin1 channels in pain. *Abstracts of the 2012 Neuroscience Meeting, New Orleans, LA, October 13–17, 2012*, Abstract 180.02/HH20, Society for Neuroscience, Washington, D. C.

36. Bravo, D., Ibarra, P., Retamal, J., Pelissier, T., Laurido, C., Hernandez, A., and Constandil, L. (2014) Pannexin 1: a novel participant in neuropathic pain signaling in the rat spinal cord. *Pain* **155,** 2108 –2115

37. Vessey, J. P., Lalonde, M. R., Mizan, H. A., Welch, N. C., Kelly, M. E., and Barnes, S. (2004) Carbenoxolone inhibition of voltage-gated Ca channels and synaptic transmission in the retina. *J. Neurophysiol.* **92,** 1252–1256

38. Wolf, G., Gabay, E., Tal, M., Yirmiya, R., and Shavit, Y. (2006) Genetic impairment of interleukin-1 signaling attenuates neuropathic pain, autotomy, and spontaneous ectopic neuronal activity, following nerve injury in mice. *Pain* **120,** 315–324

39. Martucci, C., Trovato, A. E., Costa, B., Borsani, E., Franchi, S., Magnaghi, V., Panerai, A. E., Rodella, L. F., Valsecchi, A. E., Sacerdote, P., and Colleoni, M. (2008) The purinergic antagonist PPADS reduces pain related behaviours and interleukin-1 β, interleukin-6, iNOS and nNOS overproduction in central and peripheral nervous system after peripheral neuropathy in mice. *Pain* **137,** 81–95

40. Dansereau, M. A., Gosselin, R. D., Pohl, M., Pommier, B., Mechighel, P., Mauborgne, A., Rostene, W., Kitabgi, P., Beaudet, N., Sarret, P., and Melik-Parsadaniantz, S. (2008) Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J. Neurochem.* **106,** 757–769

41. Chen, M. C., and Meckfessel, M. H. (2013) Autoinflammatory disorders, pain, and neural regulation of inflammation. *Dermatol. Clin.* **31,** 461–470

42. Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., and Nuñez, G. (2009) The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* **10,** 241–247

43. Raupach, B., Peuschel, S. K., Monack, D. M., and Zychlinsky, A. (2006) Caspase-1-mediated activation of interleukin-1β (IL-1β) and IL-18 contributes to innate immune defenses against *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.* **74,** 4922– 4926

44. Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K., Bell, G. W., Otte, A. P., Vidal, M., Gifford, D. K., Young, R. A., and Jaenisch, R. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441,** 349 –353

45. Kim, T. H., Barrera, L. O., Zheng, M., Qu, C., Singer, M. A., Richmond, T. A., Wu, Y., Green, R. D., and Ren, B. (2005) A high-resolution map of active promoters in the human genome. *Nature* **436,** 876 – 880

46. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298,** 1039 –1043

47. Pasini, D., Hansen, K. H., Christensen, J., Agger, K., Cloos, P. A., and Helin, K. (2008) Coordinated regulation of transcriptional repression by the RBP2 H3K4 demethylase and Polycomb-repressive complex 2. *Genes Dev.* **22,** 1345–1355

48. Papp, B., and Müller, J. (2006) Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. *Genes Dev.* **20,** 2041–2054

49. Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. (2007) High-resolution profiling of histone methylations in the human genome. *Cell* **129,** 823– 837

50. Gupta-Agarwal, S., Franklin, A. V., Deramus, T., Wheelock, M., Davis, R. L., McMahon, L. L., and Lubin, F. D. (2012) G9a/GLP histone lysine dimethyltransferase complex activity in the hippocampus and the entorhinal cortex is required for gene activation and silencing during memory consolidation. *J. Neurosci.* **32,** 5440 –5453