

Nerve Conduction Blockade in the Sciatic Nerve Prevents but Does Not Reverse the Activation of p38 Mitogen-activated Protein Kinase in Spinal Microglia in the Rat Spared Nerve Injury Model

Yeong-Ray Wen, M.D.,* Marc R. Suter, M.D.,† Yasuhiko Kawasaki, Ph.D.,‡ Jin Huang,§ Marie Pertin, B.Sc.,|| Tatsuro Kohno, M.D.,# Charles B. Berde, M.D.,** Isabelle Decosterd, M.D.,†† Ru-Rong Ji, Ph.D.‡‡

Background: Current evidence indicates that p38 mitogen-activated protein kinase activation in spinal microglia contributes to the development of neuropathic pain. However, how nerve injury activates p38 in spinal microglia is incompletely unknown. Nerve injury–induced **ectopic spontaneous activity** is essential for the generation of neuropathic pain. The authors examined whether peripheral neural activity is necessary for p38 activation in spinal microglia.

Methods: To examine whether spinal microglia activation depends on peripheral activity in the rat spared nerve injury (SNI) model, the authors blocked conduction in the sciatic nerve **before or 2 days after SNI**. The block was produced by applying **bupivacaine-loaded microspheres** above the nerve injury site. The p38 activation was examined by p38 phosphorylation using a phosphorylated p38 antibody, and neuropathic pain-related behavior was evaluated before and after intrathecal infusion of a p38 inhibitor.

Results: Three days after SNI, there was a marked p38 activation in the medial two thirds of the dorsal horn, where the injured tibial and peroneal nerves terminated and where isolectin B4 staining was lost. Phosphorylated **p38 was only colocalized with the microglial surface marker OX-42**, indicating a microglial localization of phosphorylated p38 in the SNI model. **Bupivacaine microspheres** produced persistent block (loss of sensory and motor function) of the sciatic nerve for the whole period of the study (3 days). This blockade **prevented but did not reverse p38 activation in spinal microglia**. **Intrathecal infusion of the p38 inhibitor FR167653 prevented and reversed mechanical allodynia on post-SNI day 3.**

Conclusions: After nerve injury, activity in the peripheral nerve is required for the induction but not the maintenance of p38 activation in spinal microglia.

MECHANISMS of nerve injury–induced neuropathic pain are incompletely known, which hinders effective treatment of this disabled pain condition. Increasing evidence suggests that spinal microglia play an important role in neuropathic pain sensitization.¹⁻⁴ One of the most robust changes in the spinal cord after peripheral nerve injury is the **up-regulation of the microglial surface marker CD11b (OX-42)**.^{5,6} Nerve injury also increases the expression of multiple receptors in spinal microglia, such as the **chemokine receptor CX3CR1** and the **adenosine triphosphate receptor P2X4**. Nerve injury may also induce expression of the **chemokine receptor CCR2** and **Toll-like receptor 4** in spinal microglia. Importantly, blocking or deleting these receptors can reduce neuropathic pain.⁷⁻¹⁰ Further, injection of **adenosine triphosphate-activated microglia** into the spinal cord is sufficient to induce mechanical allodynia.^{9,11}

Nerve injury also activates p38 mitogen-activated protein kinase signaling pathway in spinal microglia.^{6,12,13} Intrathecal inhibition of p38 has been shown to attenuate neuropathic pain in different animal models.^{6,12-17} The upstream mechanisms causing p38 activation in spinal microglia began to be revealed. For example, the cytokine **tumor necrosis factor α** and the chemokine **fractalkine** are necessary for p38 activation after nerve injury.^{12,18}

However, it still remains largely unknown how nerve damage activates spinal microglia. Peripheral nerve injury induces spontaneous activity in the axons and soma of primary sensory neurons. Injury discharge is followed by the onset of **spontaneous activity that matches the onset of mechanical allodynia, and this activity is strongly implicated in the generation of neuropathic pain**.¹⁹⁻²⁵ We examined whether spontaneous activity is critical for the activation of p38 in spinal microglia. To persistently block injury discharge and the spontaneous discharge originating from the injury site, we used **bio-degradable polymeric microspheres loaded with bupivacaine**, allowing continuous drug delivery and long-term nerve blockade for more than 3 days.²⁶ We showed that the **induction but not maintenance of p38 activation in spinal microglia requires spontaneous activity in the rat spared nerve injury (SNI) model**.

* Visiting Research Fellow, Pain Research Center, Department of Anesthesiology, Brigham and Women's Hospital and Harvard Medical School; Department of Anesthesiology, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan. † Clinical Fellow, || Research Assistant, ‡‡ Assistant Professor, Anesthesiology Pain Research Group, Anesthesiology Department, University Hospital Center and University of Lausanne, Lausanne, Switzerland; Department of Cell Biology and Morphology, Faculty of Biology and Medicine, Lausanne University, Lausanne, Switzerland. ‡ Postdoctoral Fellow, § Summer College Student, ‡‡ Associate Professor, Pain Research Center, Department of Anesthesiology, Brigham and Women's Hospital and Harvard Medical School. # Associate Professor, Department of Anesthesiology, Medical School of Niigata University, Japan. ** Professor, Department of Anesthesiology, Children's Hospital and Harvard Medical School, Boston, Massachusetts.

Received from the Department of Anesthesiology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts. Submitted for publication January 19, 2007. Accepted for publication April 25, 2007. Supported by R01 grant Nos. DE17794 and NS54932, and R03 grant No. TW7180 from the National Institutes of Health, Bethesda, Maryland (Dr. Ji); a Collaboration Grant from the International Association for the Study of Pain, Seattle, Washington (Drs. Ji and Decosterd); and grants from the Swiss National Science Foundation (31000-112577), Bern, Switzerland (Dr. Decosterd), and the P. Mercier Science Foundation, Pully, Switzerland (Dr. Decosterd). Tissucol was provided by Baxter AG, Switzerland. Drs. Wen and Suter contributed equally to this study.

Address correspondence to Dr. Ji: Department of Anesthesiology, Brigham and Women's Hospital, 75 Francis Street, Medical Research Building, Room 604, Boston, Massachusetts 02115. rjji@zeus.bwh.harvard.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

Materials and Methods

Animals

Male adult Sprague-Dawley rats (240–300 g) were housed (two per cage) with free access to water and rat chow. The animal room was artificially illuminated on a 12-h day-night cycle and maintained at ambient temperature (22°–23°C). All the animals were used under animal care institutional guidelines for Harvard Medical School, Boston, Massachusetts, and Lausanne University, Lausanne, Switzerland.

Nerve Blockade and Sensory/Motor Function

Testing

As described in our previous study,²⁶ bupivacaine microspheres were used to produce a slow release of the local anesthetic and a long-term conduction blockade of the sciatic nerve. The sciatic nerve was exposed from the emergence of the musculocutaneous branch to the trifurcation of the sural, tibial, and peroneal branches. A silicone tube (11 mm long) was incised on its long axis and placed carefully around the sciatic nerve. Two 5-0 silk ligatures were used to close the longitudinal slit. The distal end of the tube was sealed with fibrin glue (Tissucol; Baxter, Volketswil, Switzerland). Bupivacaine microspheres containing 75% bupivacaine (wt/wt) (provided by Dr. Charles Berde) were prepared in a fibrin solution (300 mg/ml). This solution also contained low concentration of dexamethasone (0.05%), which might increase the penetration of bupivacaine to nerve fibers, therefore prolonging the duration of nerve block.²⁷ The mixture solution (bupivacaine microsphere in fibrin solution, 50 μ l) was then slowly poured inside the tube through the proximate end.²⁶ Special care was taken to avoid any stretch or damage to the sciatic nerve and its branches. A sham blockade procedure was performed by filling the tube with fibrin without bupivacaine microspheres, followed by SNI (vehicle control) or sham surgery (sham control). An additional control group was added by filling the perisciatic tube with a solution of fibrin containing dexamethasone (0.05%). All the animals received a subcutaneous injection of amoxicillin (150 mg/kg) before surgery. To block nerve injury-induced immediate discharge, SNI was performed 3 h after nerve blockade, because a complete nerve blockade can be achieved 3 h after treatment with bupivacaine microspheres.

To test the efficacy of the nerve blockade, rats were allowed to recover from anesthesia and then gently held with a cloth wrapped above their waist to restrain upper extremities, and sensitivity measurements were performed. Measurements were also performed daily for 3 days after nerve blockade.²⁶ The nociceptive sensory function was examined with noxious mechanical stimulation (pinch with forceps) and noxious heat stimulation (hot plate testing for the ipsilateral or contralateral hind paws, with 12 s as cutoff to avoid tissue injury). The

motor function was examined by the presence or absence of hopping response and tactile placing.²⁶

Spared Nerve Injury

Although we initially found p38 activation in the spinal nerve ligation model,^{6,28} we chose the SNI model²⁹ in this study because (1) nerve conduction blockade is easier to perform on the sciatic nerve than on the spinal nerve and (2) the role of p38 has not been examined in the SNI model. To produce SNI, two of the three sciatic nerves' terminal branches, the common peroneal and tibial nerves, were ligated by a 5-0 silk suture and cut, and a 3-mm portion of the nerves was removed. The third branch, the sural nerve, was untouched, and great care was taken not to stretch this nerve during the surgical procedure. All the animals were killed 3 days after SNI, because at this time point (1) mechanical allodynia is fully developed and (2) p38 is induced in spinal microglia.⁶

Spinal Drug Delivery

The p38 inhibitor FR167653 was provided by Fujisawa Pharmaceutical Company (Osaka, Japan). For the study of pretreatment (treatment given before and during SNI), an osmotic pump (0.5 μ l/h, 7-day capacity; Alzet, Cupertino, CA) was filled with FR167653 (30 μ g/ μ l, n = 5) or vehicle (30% dimethyl sulfoxide [DMSO], n = 8) to produce stable and sustained infusion of the inhibitor, starting 2 days before SNI. Laminectomy was performed under a microscope at the spinal level 2 cm below the lumbar enlargement. A polyethylene (PE5) catheter was implanted into the intrathecal space of the spinal cord (L4–L5). The other end of the catheter was connected to the pump. After filling, the pump was soaked in saline for 3 h before implantation and placed subcutaneously on the back.^{30,31} The concentrations of FR167653 (30 μ g/ μ l) and DMSO (30%) for pump infusion were chosen on the basis of previous studies.^{15,30}

For single intrathecal injection, a spinal cord puncture was made with 27-gauge needle between L5 and L6 level to deliver the drug and vehicle to the cerebral spinal fluid space around the lumbosacral spinal cord.^{31,32} Animals were briefly anesthetized with sevoflurane before the puncture, and 20 μ l liquid was injected with a microsyringe. Immediately after the needle entry into subarachnoid space (change in resistance), a brisk tail flick was observed after the needle puncture. For single injection, FR167653 was dissolved in 10% DMSO and intrathecally injected in 20 μ l at the doses of 20, 50, and 100 μ g (n = 8 for each dose). DMSO, 10% (20 μ l), was also injected as vehicle control (n = 5). The doses of FR167653 and the concentration of DMSO for bolus injection were chosen on the basis of previous studies^{15,30} and our pilot study.

Immunohistochemistry

Three days after SNI or sham surgery, animals ($n = 4$ for each group) were terminally anesthetized with isoflurane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde with 1.5% picric acid in 0.16 M phosphate buffer (pH 7.2–7.4, 4°C). After the perfusion, the L4 spinal cord segments were removed, postfixed in the same fixative for 24 h, and then replaced with 15% sucrose overnight. Transverse spinal sections (free-floating, 30 μm) were cut in a cryostat and processed for immunofluorescence.^{6,30} All of the sections were blocked with 2% goat serum in 0.3% Triton for 1 h at room temperature and incubated overnight at 4°C with anti-phospho-p38 (p-p38) antibody (rabbit, 1:500; Cell Signaling, Beverly, MA). The sections were then incubated for 1 h at room temperature with Cy3-conjugated secondary antibody (1:300; Jackson Immunolab, West Grove, PA). For double immunofluorescence, spinal sections were incubated with a mixture of polyclonal p-p38 and monoclonal OX-42 (for CD11b, a microglia marker, 1:5,000; Chemicon, Temecula, CA) antibodies overnight at 4°C, followed by a mixture of fluorescein isothiocyanate-conjugated and Cy3-conjugated secondary antibodies for 1 h at room temperature. The stained sections were examined with a Nikon fluorescence microscope (Tokyo, Japan), and images were captured with a Spot charge-coupled device microscope digital camera (Diagnostic Instruments, Sterling Heights, MI). The specificity of the staining and antibodies has been tested in previous studies.^{6,18}

IB4 Staining

The free-floating spinal sections prepared for immunostaining were also used for the staining of isolectin B4 (IB4). The spinal sections were incubated with IB4 (1:200; Sigma, St. Louis, MO) for 2 h in phosphate-buffered saline solution at room temperature.³³ The sections were then washed in phosphate-buffered saline and examined under a Nikon fluorescence microscope.

Western Blotting

Western blotting was performed as previously reported.^{6,31,32} Animals were rapidly killed 3 days after SNI or sham surgery ($n = 8$), and the L4–L5 spinal segments (dorsal part) were quickly removed and homogenized in a lysis buffer containing a cocktail of proteinase and phosphatase inhibitors (Sigma). Protein samples (40 μg) were separated on sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride blots (Millipore, Billerica, MA). The blots were blocked with 5% milk and incubated overnight at 4°C with antibody against p-p38 (anti-rabbit, 1:1,000; Cell Signaling), or p38 (anti-rabbit, 1:1,000; Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; anti-rabbit, 1:10,000; Abcam, Cambridge, England). These blots were further incubated with horseradish peroxidase-

conjugated secondary antibody (1:5,000; Amersham, Piscataway, NJ), developed in ECL solution (PerkinElmer, Boston, MA), and exposed onto hyperfilms (Amersham). The pixels of the specific bands were quantified with Image Quant TL software (Zurich, Switzerland), and the ratios of p-p38/p38, p38/GAPDH, and p-p38/GAPDH were determined.

Behavioral Analysis

Animals were habituated to the testing environment daily for 3 days before baseline testing. The room temperature and humidity remained stable for all experiments. For testing mechanical sensitivity, animals were put in plastic boxes (11 \times 13 \times 24 cm) on an elevated metal mesh floor and allowed 30 min for habituation, and mechanical allodynia was tested using von Frey filaments. Mechanical paw withdrawal thresholds were determined using the up-down method described by Chaplan *et al.*³⁴ Hind paws were pressed with one of a series von Frey hairs with logarithmically incremental stiffness (0.6–26 g; Stoelting, Wood Dale, IL), presented perpendicular to the plantar surface for 3–5 s for each hair.³¹ The 50% withdrawal threshold was determined using the up-down method of Dixon.³⁵ The experimenter was not aware of drug treatment.

Quantification of p-p38 Immunoreactivity in Microglia

Five nonadjacent sections (30 μm) from the L4 spinal cord segments were randomly selected, and the numbers of p-p38-immunoreactive cell profiles with morphology of microglia were counted. The region of the dorsal horn sampled in all experiments is the medial two thirds of the dorsal horn (laminae I–III), where most p-p38-immunoreactive cells were found after SNI. This area was captured under 20 \times objective in a square box (450 \times 338 μm), with the top line of the square in parallel with the edge of the gray matter of the dorsal horn.^{18,36} The size of the box was kept the same in all conditions. The observer was unaware of the treatments during counting.

Statistical Analysis

To minimize the number of animals used in this study, we included four rats per group for quantification of immunohistochemistry and Western blotting^{18,30,33,36} and five to eight rats per group for behavioral studies.^{18,31} Because paw withdrawal thresholds determined by up-down method passed normality test for all the groups, we compared differences using parametric tests as previously shown.³⁷ Differences were compared (GraphPad InStat; GraphPad Software, Inc., San Diego, CA, and SPSS 10; SPSS Inc., Chicago, IL) using the Student *t* test (two groups) or one-way analysis of variance (multiple groups) followed by the Fisher protected least significant difference *post hoc* test.^{18,31} Data are pre-

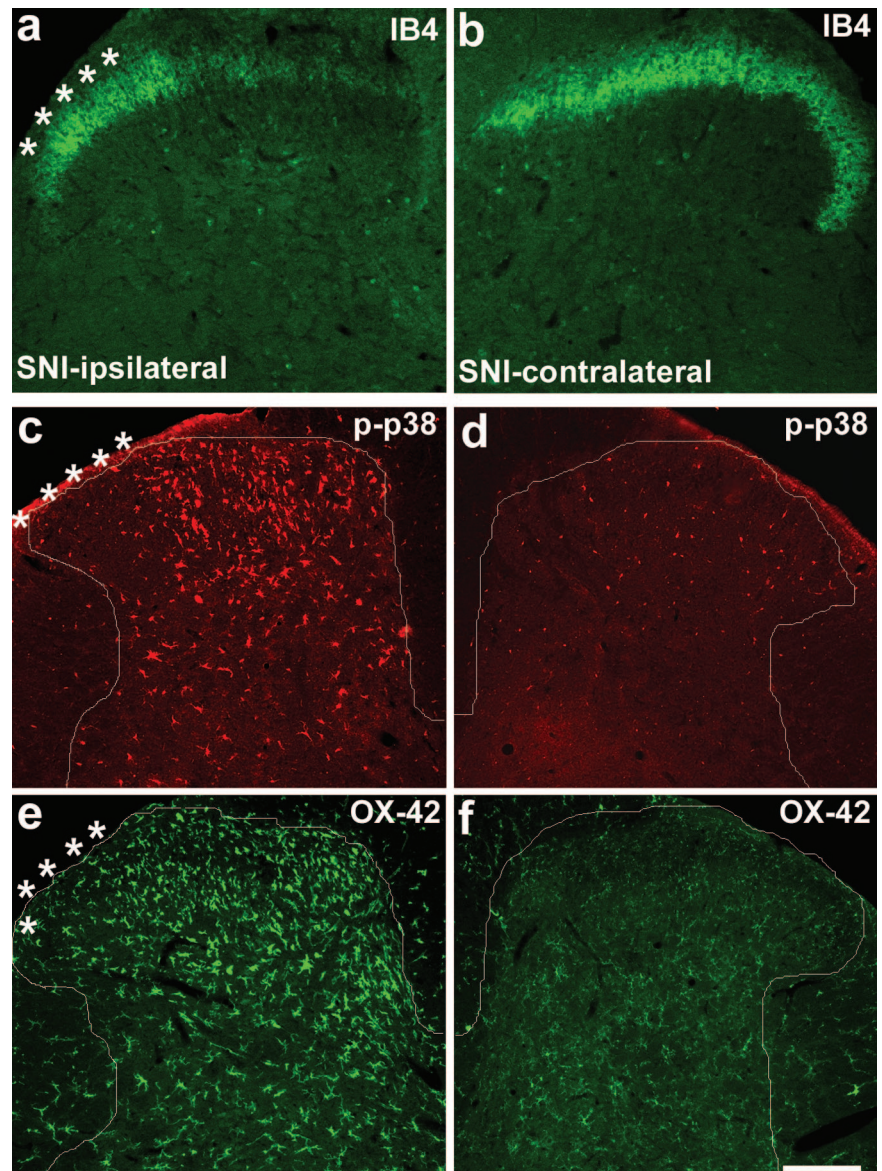


Fig. 1. Spared nerve injury (SNI) induces isolectin B4 (IB4) down-regulation, p38 activation, and CD11b (OX-42) up-regulation in the spinal cord dorsal horn 3 days after injury. (A and B) IB4 staining shows a reduction of the staining in the medial two thirds of the dorsal horn on the ipsilateral side after SNI. (C–F) Immunohistochemistry with phospho-p38 (p-p38) and OX-42 antibodies reveals an increase of p-p38-immunoreactive (C and D) and OX-42-immunoreactive (E and F) cells in the medial two thirds of the ipsilateral dorsal horn (L4) after SNI. White lines indicate the borders of the dorsal horn. White stars indicate the spinal area terminated by the intact sural nerve. Scale, 100 μ m.

sented as mean \pm SEM. The criterion for statistical significance was $P < 0.05$. The effect sizes were also estimated using Cohen's d values that are larger than 1.8 when $P < 0.05$.

Results

IB4 Staining in the Spinal Cord after SNI

Isolectin B4 has been widely used to label nonpeptidergic primary sensory neurons in the dorsal root ganglion. IB4 binding was also found in the central terminals of dorsal root ganglion neurons in lamina II of the spinal cord. After peripheral nerve injury, IB4 binding is rapidly lost in the dorsal root ganglion neurons and their central terminals.³⁸ Therefore, we used IB4 as a marker to characterize the projection of injured and intact nerve axons after SNI.

Three days after SNI, there was a significant reduction

of IB4 binding in the medial two thirds of the dorsal horn (figs. 1A and B), which is terminated by the injured tibial and peroneal nerve. However, IB4 binding in the lateral one third of the dorsal horn, which is terminated by the intact sural nerve,³⁹ remained unchanged (figs. 1A and B). This IB4 staining pattern validated the SNI model we produced.

SNI Activates p38 in Spinal Microglia

A specific anti-p-p38 antibody was used to study changes in p38 phosphorylation. The basal expression of p-p38 was low in the noninjured (contralateral) side of the spinal cord after nerve injury, as previously shown.⁶ SNI induced a marked increase in p-p38 expression in the spinal cord 3 days after nerve injury (figs. 1C and D). Numerous p-p38-immunoreactive cells were found in the ipsilateral spinal cord, especially in the medial two thirds of superficial dorsal horn (laminae I–III; figs. 1C

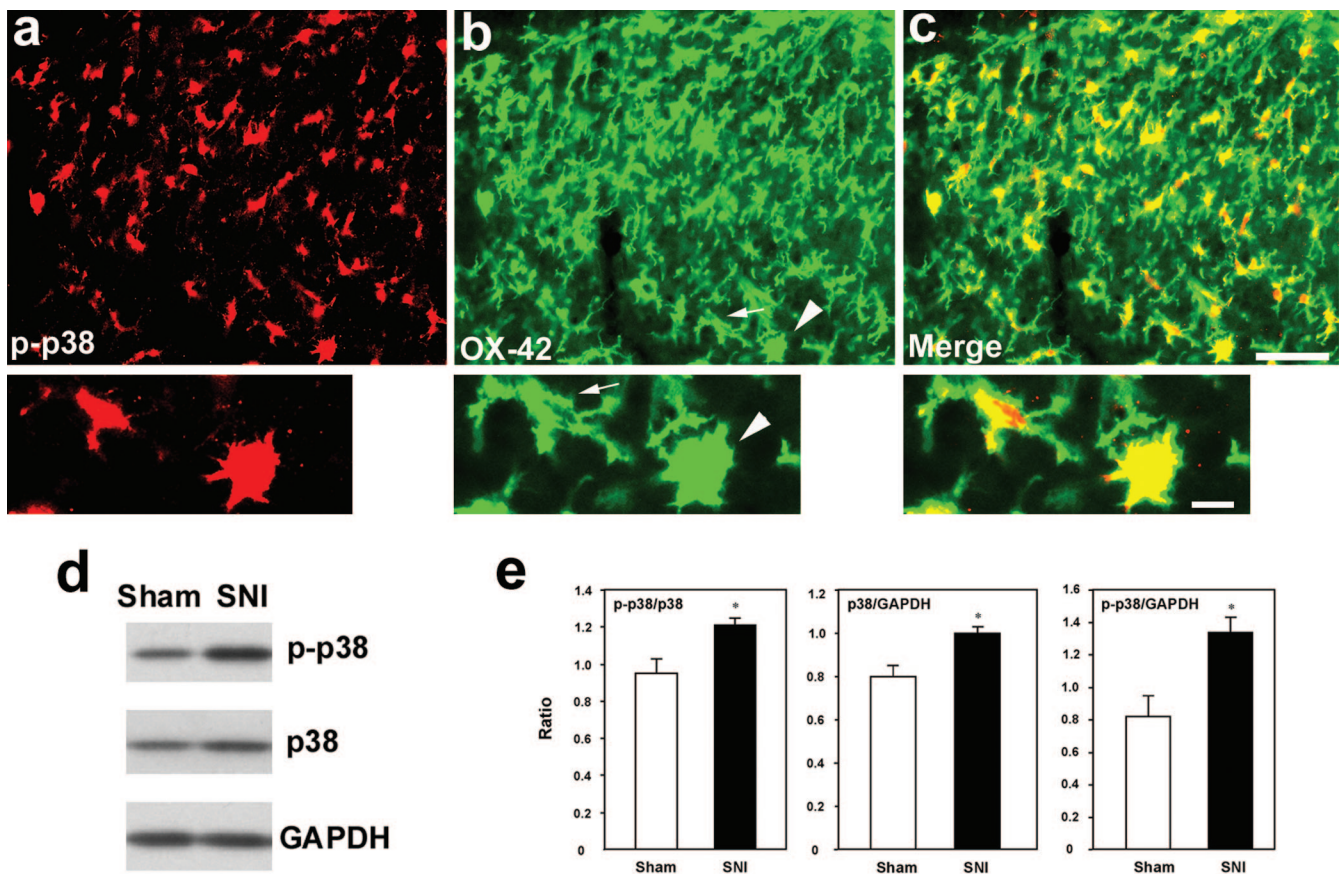


Fig. 2. p38 is activated in spinal microglia after spared nerve injury (SNI). (A–C) Double immunofluorescence shows phospho-p38 (p-p38)/OX-42 colocalization in the medial superficial dorsal horn 3 days after SNI. C is an overlay of A and B. The lower panel is high magnification of the upper panel showing two microglia cells that are double-stained for p-p38 and OX-42. Arrow and arrowhead show ramified (resting) and hypertrophy (active) states of microglia, respectively. Scales, 50 μ m (upper panel) and 10 μ m (lower panel). (D and E) Western blotting reveals an increase of p-p38 levels in the spinal dorsal horn 3 days after SNI. The p-p38 or p38 levels (pixels of specific Western bands) were normalized to corresponding p38 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. * $P < 0.05$ (t test).

and D). This result indicates that p38 is mainly activated in the spinal region innervated by the injured axons after SNI.

To further examine the SNI-induced changes in spinal microglia, we also performed immunohistochemistry using OX-42 antibody, which labels the microglial surface marker CD11b (also called complement receptor 3 or Mac-1). SNI induced robust increase in OX-42 immunoreactivity also in the medial two thirds of the superficial dorsal horn (laminae I–III), 3 days after injury (figs. 1E and F). This increase is more likely to reflect an increase in OX-42 expression or activation of microglia rather than a proliferation of microglia. Double immunofluorescence showed that p-p38 was almost completely colocalized with OX-42 (figs. 2A–C), indicating that p38 is exclusively activated in spinal microglia on post-SNI day 3.

To confirm p-p38 increase after SNI, we also performed Western analysis using dorsal horn tissues from rats 3 days after SNI or sham surgery. When normalized to total p38 levels, SNI only induced a 26% increase in p-p38 levels (unpaired t test, $P = 0.0114$, $t = 3.031$, df

$= 11$; figs. 2D and E). When normalized to GAPDH, SNI also induced a 25% increase in p38 levels (unpaired t test, $P = 0.0196$, $t = 3.158$, $df = 6$). Thus, both phosphorylated and total p38 levels were increased after SNI, and the actual increase of dorsal horn p-p38 levels is 63% when normalized to GAPDH (fig. 2E). However, SNI only induced p-p38 increase in a very restricted area of the dorsal horn, *i.e.*, in the medial superficial dorsal horn of the gray matter. Therefore, collecting the whole dorsal horn (a quarter of spinal cord including both white and gray matter) for Western analysis largely masked the p-p38 increase. In the remaining studies, we measured p-p38 immunoreactivity in microglia of the medial superficial dorsal horn to quantify p-p38 levels, which showed a fivefold increase after SNI (see next section).

Nerve Blockade Prevents p38 Activation in Spinal Microglia

As we previously reported,²⁶ all animals demonstrated complete sensory and motor blockade 3 h after application of bupivacaine microspheres to the sciatic nerve

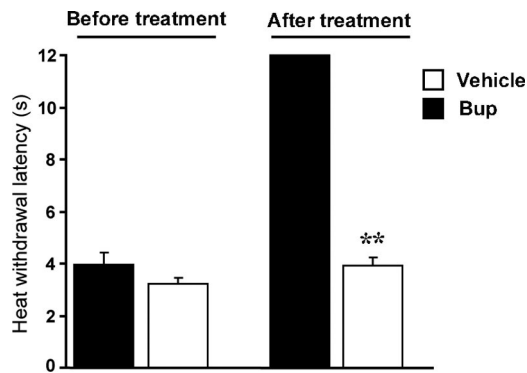


Fig. 3. Application of bupivacaine (Bup) microspheres to the sciatic nerve blocks heat responses. No rats respond to heat stimulation in hot plate testing (56°C), as measured by paw withdrawal latency. A 12-s cutoff was set to avoid tissue damage. Paw withdrawal latency of sham control rats receiving vehicle treatment did not change. ** $P < 0.01$, t test, compared with corresponding bupivacaine group.

that was checked before SNI. The block maintained for the whole period of study (3 days). Therefore, none of the rats receiving bupivacaine microspheres responded to noxious heat stimulus in hot plate testing ($P < 0.0001$, $t = 20.49$, $df = 13$; fig. 3). Nor did they respond to skin pinch in the territory of the sciatic nerve. In addition, paw positioning and hopping responses were abolished. In contrast, all sensory and motor functions were preserved in rats receiving a sham procedure for nerve blockade (vehicle). A complete sensory blockade lasted more than a week, without signs of nerve damage, because all of the animals recovered to full responsiveness in sensory and motor tests when the block wore off.²⁶

To investigate whether injury-induced spontaneous activity generated in axons is essential for the induction of p38 activation, we applied bupivacaine microspheres to the sciatic nerve (proximal to the surgery site) before

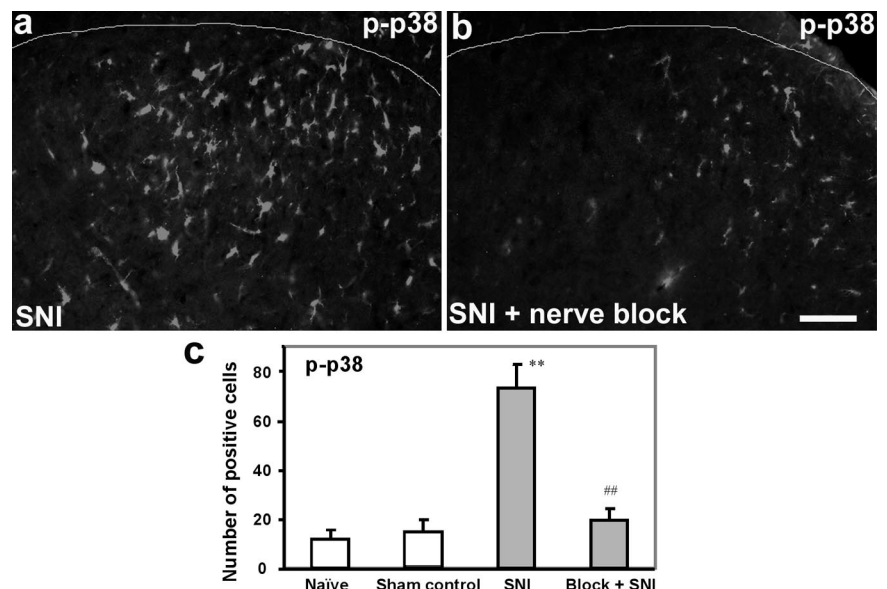
SNI and examined spinal p38 activation 3 days after SNI. A comparison among four groups (naive, sham, SNI, and SNI plus nerve block) was statistically significant in the number of p-p38-immunoreactive cells (analysis of variance, $F_{3,12} = 22.318$, $P < 0.001$). A *post hoc* comparison showed no difference between naive and sham control rats, but SNI induced a fivefold increase in p-p38 immunoreactivity ($P < 0.001$, compared with sham control). The nerve blockade completely prevented SNI-induced increase in spinal p-p38 immunoreactivity (figs. 4A–C) compared with the SNI group (*post hoc* comparison, $P < 0.001$).

To examine whether the low concentration of dexamethasone ($20 \mu\text{g}$, 0.05%) contained in the blocking solution has any effect on SNI-induced p38 activation, we included an additional control group receiving treatment, before SNI, of a fibrin solution that contains dexamethasone but not bupivacaine. Dexamethasone did not inhibit SNI-induced p38 activation: The numbers of p-p38-immunoreactive cells/spinal section in the dexamethasone-treated and vehicle (fibrin solution only)-treated groups were 76 ± 3 and 73 ± 9 , respectively ($t = 0.2338$, $P = 0.8244$, $df = 5$). Therefore, suppression of p38 activation by blockade solution is not caused by low concentration of dexamethasone.

Nerve Blockade Does Not Reverse p38 Activation in Spinal Microglia

To investigate whether injury-induced spontaneous activity generated in axons is essential for the maintenance of p38 activation, we also applied bupivacaine microspheres to the sciatic nerve 2 days after SNI and examined spinal p38 activation 24 h later on post-SNI day 3. This delayed nerve block did not reverse SNI-induced p38 activation ($t = 1.774$, $P = 0.1265$, $df = 6$; fig. 5).

Fig. 4. Nerve conduction blockade prevents p38 activation in spinal microglia. Immunofluorescence shows phospho-p38 (p-p38) immunoreactivity in the spared nerve injury (SNI) rats (A) and SNI rats receiving nerve blockade (B). Scale, $20 \mu\text{m}$. (C) Number of p-p38-immunoreactive cells in the medial superficial (laminae I–III) spinal cord with pretreatment of bupivacaine microspheres. ** $P < 0.01$, compared with sham controls; ## $P < 0.01$, compared with SNI, analysis of variance; $n = 4$. Sham control rats were given vehicle (fibrin solution of microspheres). SNI rats were given vehicle before SNI. Bupivacaine microspheres were applied before SNI.



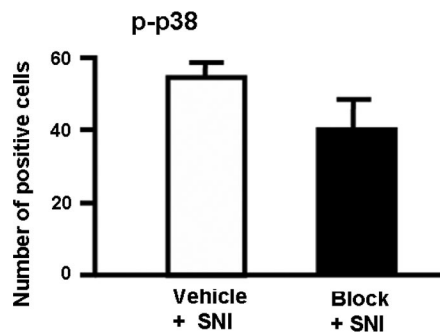


Fig. 5. Nerve conduction blockade does not reverse p38 activation in spinal microglia. Number of phospho-p38 (p-p38)-immunoreactive cells in the medial superficial (laminae I–III) spinal cord with posttreatment of bupivacaine microspheres. Bupivacaine microspheres were applied to the sciatic nerve 2 days after spared nerve injury (SNI). $P > 0.05$, t test ($n = 4$). All of the rats were transcardially perfused 3 days after surgery.

Effects of Contralateral Nerve Blockade on Spinal p38 Activation

To examine whether this treatment of bupivacaine microspheres would produce systemic effects, we applied bupivacaine microspheres to the contralateral (noninjured) sciatic nerve before the ipsilateral nerve injury and examined p-p38 expression on post-SNI day 3. This contralateral blockade did not affect the activation of p38 in the ipsilateral spinal cord ($t = 0.7470$, $P = 0.4833$, $df = 6$; fig. 6A).

To determine whether this treatment would affect basal activation of p38 on the noninjured side, we also examined p-p38 expression on the contralateral spinal cord after block. This contralateral nerve blockade did not affect p-p38 levels on the contralateral (noninjured) side ($t = 0.4368$, $P = 0.6775$, $df = 6$), suggesting that basal activation of p38 does not require nerve activity (fig. 6B).

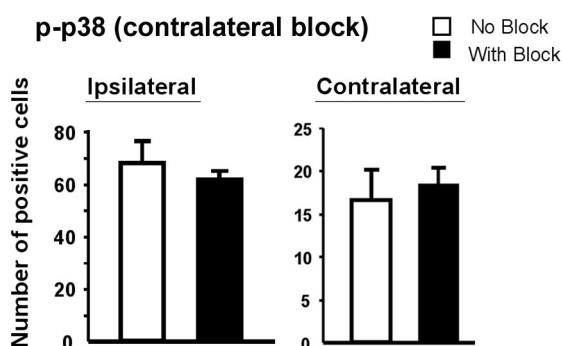


Fig. 6. Lack of effects of contralateral nerve blockade on p38 activation on the ipsilateral and contralateral spinal cord. The number of phospho-p38 (p-p38)-immunoreactive cells in the medial superficial (laminae I–III) spinal cord was quantified. Rats were given vehicle ($n = 4$, left and ipsilateral side) or bupivacaine microspheres ($n = 4$, right and contralateral side) before spared nerve injury and transcardially perfused 3 days after spared nerve injury. $P > 0.05$, t test ($n = 4$).

Intrathecal p38 Inhibitor Prevents and Reverses SNI-induced Mechanical Allodynia

To determine whether p38 activation is required for the development of neuropathic pain in the SNI model that may have different sensitivity to drug treatment compared with other neuropathic pain models,⁴⁰ we examined whether intrathecal infusion of the p38 inhibitor FR167653 would prevent SNI-induced neuropathic pain. As previously shown,²⁹ SNI induced rapid mechanical allodynia that was robust on day 2 (fig. 7A). Intrathecal infusion of FR167653 (30 $\mu\text{g}/\mu\text{l}$) via an osmotic pump, starting 2 days before SNI and continuing to post-SNI day 3, did not affect the basal mechanical pain threshold, but completely prevented SNI-induced mechanical allodynia on SNI day 2 ($t = 5.514$, $df = 11$, $P = 0.0002$) and day 3 ($t = 6.868$, $df = 11$, $P < 0.0001$; fig. 7A).

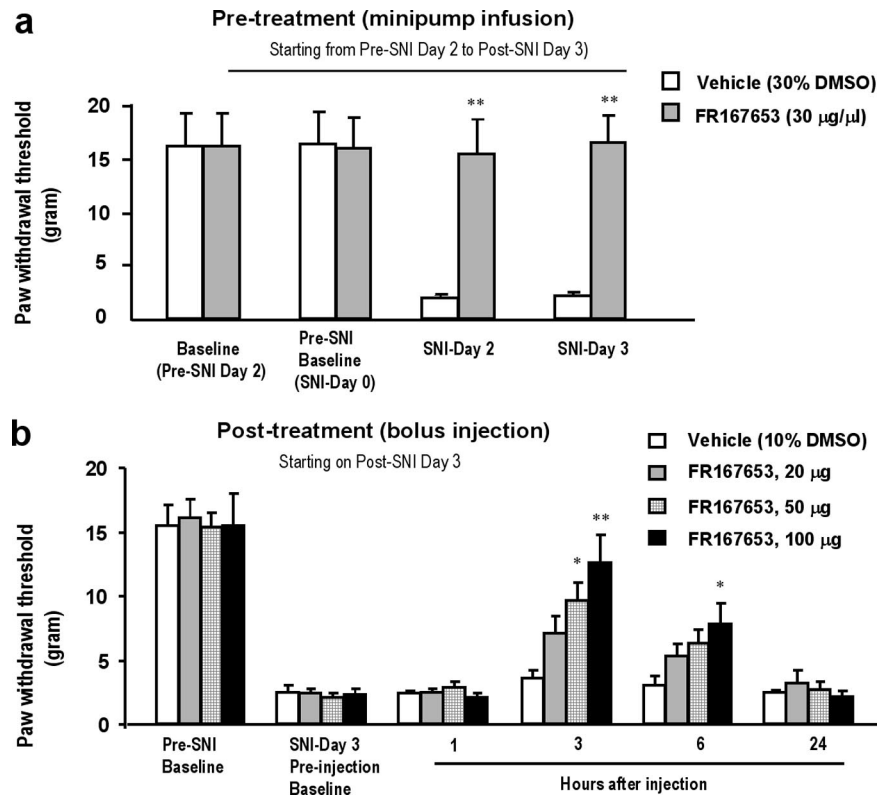
To further determine whether p38 activation is also essential for the maintenance of neuropathic pain, we gave a bolus injection of FR167653 (20, 50, or 100 μg) into the spinal cerebrospinal fluid space by lumbar puncture on post-SNI day 3. FR167653 produced a dose-dependent reversal of mechanical allodynia at 3 h after the injection (analysis of variance, $F_{3,25} = 6.728$, $P = 0.0018$; fig. 7B). The antiallodynic effect of FR167653 was maintained at 6 h after the injection (analysis of variance, $F_{3,25} = 3.050$, $P = 0.0471$; fig. 7B) but completely recovered 24 h later. Vehicle (10% DMSO) had no effect on SNI-induced mechanical allodynia (fig. 7B).

Discussion

We have made several interesting observations in this study. First, SNI activated p38 mitogen-activated protein kinase in microglia in the medial two thirds of dorsal horn, which is terminated by the injured tibial and peroneal nerve. Second, intrathecal administration of the p38 inhibitor FR167653 both prevented and reversed SNI-induced mechanical allodynia on day 3. Third, nerve conduction block in the sciatic nerve with bupivacaine microspheres prevented but did not reverse spinal p38 activation in spinal microglia. Fourth, application of bupivacaine microspheres to the contralateral nerve did not prevent SNI-induced p38 activation.

Accumulating evidence suggests a glial activation of p38 in the spinal cord. We initially found that p-p38 is present in spinal glial cells and that chronic inflammation by complete Freund adjuvant induces a modest increase in spinal p-p38 levels.³³ Inflammation by formalin, capsaicin, or complete Freund adjuvant was also shown to activate p38 in spinal microglia.^{41–44} In these inflammatory pain models, activation of peripheral nociceptors by inflammatory mediators may produce spontaneous activity in the axons and cell bodies of the primary sensory neurons.⁴² Therefore, p38 activation in

Fig. 7. Spared nerve injury (SNI)-induced mechanical allodynia is both prevented and reversed by p38 inhibitor FR167653. (A) Prevention of mechanical allodynia. FR167653 (30 $\mu\text{g}/\mu\text{l}$, $n = 5$) was infused into intrathecal space *via* an osmotic pump (0.5 $\mu\text{l}/\text{h}$ for 5 days) starting 2 days before SNI. Note that the basal mechanical sensitivity does not change after FR167653. $** P < 0.01$, t test, compared with corresponding vehicle controls (30% dimethyl sulfoxide [DMSO], $n = 8$). **(B) Reversal of mechanical allodynia.** A bolus injection of FR167653 (20, 50, and 100 μg , 20 μl , $n = 8$ for each dose) into lumbar cerebral spinal fluid space, starting 3 days after SNI, reduces SNI-induced mechanical allodynia in a dose-dependent manner. The paw withdrawal thresholds were tested before the injection and at 1, 3, 6, and 24 h after the injection. $* P < 0.05$, $** P < 0.01$, compared with corresponding vehicle control (10% DMSO, $n = 5$).



spinal microglia under inflammatory pain conditions could be activity dependent. However, in a recent study, application of bupivacaine (repeated 6 h later) to the sciatic nerve did not block spinal p38 activation examined 15 h after hind paw carrageenan injection.⁴³ This failure may be caused by incomplete conduction blockade due to afferent input *via* saphenous nerves.⁴³

Compared with inflammation, peripheral nerve injury produces a more robust activation of p38 in spinal microglia.^{6,13} p38 activation was also found in spinal microglia after spinal cord injury, a central neuropathic pain condition.^{44,45} Growing evidence from different laboratories demonstrates that p38 inhibitors can attenuate neuropathic pain in different animal models.^{6,12-17} In the current study, we further confirmed microglial activation of p38 in another neuropathic pain condition after SNI. We have also shown that a different p38 inhibitor, FR167653, which can inhibit p38 activation,⁴⁶ prevents and reverses mechanical allodynia by SNI.

A major finding of this study is that peripheral nerve blockade can prevent p38 activation in the spinal cord, suggesting that spontaneous activity, especially in the neuroma, is indispensable for initial p38 activation in spinal microglia. Spontaneous activity may release neurotransmitters (glutamate, adenosine triphosphate, substance P) from central terminals of the primary afferents to activate p38 in spinal microglia. Recently, we have shown that nerve injury may cause the release of the chemokine fractalkine, which is sufficient and required to produce spinal p38 activation by activating the

CX3CR1 receptor expressed by microglia.¹⁸ However, nerve conduction blockade in the sciatic nerve 2 days after SNI cannot reverse p38 activation in spinal microglia, indicating that maintenance of p38 activation may not require spontaneous activity. After activation, microglia produce multiple inflammatory mediators, such as the cytokines interleukin 1 β , interleukin 6, and tumor necrosis factor α ,⁴⁷ which could maintain p38 activation in spinal microglia, even in the absence of primary afferent input.

It is generally believed that spontaneous activity or ectopic discharge induced by peripheral nerve injury is responsible for the generation of neuropathic pain.¹⁹⁻²⁵ Local anesthetics are generally used to block nerve conduction and suppress spontaneous activity. The concentrations of local anesthetics required for blocking spontaneous activity are often lower than that required for blocking nerve conduction.^{48,49} Local anesthetics may produce nonspecific effects, such as neurotoxicity and inhibition of fast axonal transport.^{2,50} Because cytokines such as tumor necrosis factor α have been implicated in spinal p38 activation,^{12,16} we cannot exclude the possibilities that bupivacaine may inhibit spinal p38 activation by suppressing (1) the local production of tumor necrosis factor α in the sciatic nerve (e.g., in Schwann cells) and (2) axonal transport of tumor necrosis factor α to central axons in the spinal cord. However, severe disturbance of axonal transport would cause nerve damage, but we did not find obvious damage in the sciatic nerve treated with bupivacaine microspheres; all of the treated

animals recovered to full responsiveness in sensory and motor tests when the block wore off.²⁶ Because **low concentration of bupivacaine was shown to suppress inflammatory responses of immune cells and extracellular signal-regulated kinase activation in spinal neurons,**^{51,52} we tried to avoid the systemic effects of bupivacaine by using microspheres. The lack of systemic effects is supported by our data showing that application of bupivacaine microspheres to the contralateral side of the sciatic nerve does not change spinal p38 activation on the other side (fig. 6).

Abnormal peripheral activity is elicited by immediate injury discharge during axonal damage and sustained by ectopic activity in both injured and noninjured fibers.¹⁹⁻²⁵ Application of bupivacaine to the sciatic nerve in the SNI model should block spontaneous activity originated from both injured fibers in common peroneal and tibial nerves and intact fibers in sural nerves. However, **p38 activation was mainly found in the medical dorsal horn, which is innervated by the injured tibial and peroneal nerve** (fig. 1). Therefore, spontaneous activity in the injured axons should play a major role in spinal p38 activation. Although application of bupivacaine microspheres at the sciatic nerve should block spontaneous activity originated at the distal site (e.g., neuroma) of the block, we do not know whether this block can also suppress spontaneous activity at the proximal site (e.g., dorsal root ganglion soma and dorsal root).²² Therefore, the role of proximal activity in p38 and microglia activation remains to be investigated.

In summary, this study has provided further evidence that p38 activation (p-p38 induction) in spinal microglia is a useful marker for microglia activation in the spinal cord. Importantly, this marker is directly correlated with chronic pain sensitization. In contrast, **pain states may not be associated with the changes of the commonly used microglial surface marker CD11b (OX-42).**^{5,9,53} Our data also suggest that peripheral spontaneous activity is important for the induction but not the maintenance of p38 activation in spinal microglia after nerve injury. Therefore, afferent barrage after the nerve injury may trigger neuropathic pain by activating p38 in spinal microglia.

References

- DeLeo JA, Zezierski RP: The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain* 2001; 90:1-6
- Ji RR, Strichartz G: Cell signaling and the genesis of neuropathic pain. *Sci STKE* 2004; 2004:reE14
- Tsuda M, Inoue K, Salter MW: Neuropathic pain and spinal microglia: A big problem from molecules in "small" glia. *Trends Neurosci* 2005; 28:101-7
- Watkins LR, Milligan ED, Maier SF: Glial activation: A driving force for pathological pain. *Trends Neurosci* 2001; 24:450-5
- Colburn RW, DeLeo JA, Rickman AJ, Yeager MP, Kwon P, Hickey WF: Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. *J Neuroimmunol* 1997; 79:163-75
- Jin SX, Zhuang ZY, Woolf CJ, Ji RR: p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root

ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* 2003; 23:4017-22

- Abbadie C, LINDIA JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ: Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci U S A* 2003; 100:7947-52
- Tanga FY, Nutile-McMenemy N, DeLeo JA: The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 2005; 102:5856-61
- Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K: P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 2003; 424:778-83
- Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS, Foster AC: Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. *Eur J Neurosci* 2004; 20:1150-60
- Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y: BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 2005; 438:1017-21
- Svensson CI, Schafers M, Jones TL, Powell H, Sorkin LS: Spinal blockade of TNF blocks spinal nerve ligation-induced increases in spinal p-p38. *Neurosci Lett* 2005; 379:209-13
- Tsuda M, Mizokoshi A, Shigemoto-Mogami Y, Koizumi S, Inoue K: Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia* 2004; 45:89-95
- Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, Tracey K, Martin D, Maier SF, Watkins LR: Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J Neurosci* 2003; 23:1026-40
- Obata K, Yamanaka H, Kobayashi K, Dai Y, Mizushima T, Katsura H, Fukuoka T, Tokunaga A, Noguchi K: Role of mitogen-activated protein kinase activation in injured and intact primary afferent neurons for mechanical and heat hypersensitivity after spinal nerve ligation. *J Neurosci* 2004; 24:10211-22
- Schafers M, Svensson CI, Sommer C, Sorkin LS: Tumor necrosis factor- α induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J Neurosci* 2003; 23:2517-21
- Sweitzer SM, Medicherla S, Almiraz R, Dugar S, Chakravarty S, Shumilla JA, Yeomans DC, Trotter AA: Antinociceptive action of a p38 α MAPK inhibitor, SD-282, in a diabetic neuropathy model. *Pain* 2004; 109:409-19
- Zhuang ZY, Kawasaki Y, Tan PH, Wen YR, Huang J, Ji RR: Role of the CX3CR1/p38 MAPK pathway in spinal microglia for the development of neuropathic pain following nerve injury-induced cleavage of fractalkine. *Brain Behav Immun* 2007; 21:642-51
- Devor M, Seltzer Z: Pathophysiology of damaged peripheral nerves in relation to chronic pain, Wall and Melzack's Textbook of Pain, Fourth edition. Edinburgh, Churchill Livingstone, 1999, pp 129-64
- Liu CN, Wall PD, Ben Dor, E Michaelis M, Amir R, Devor M: Tactile allodynia in the absence of C-fiber activation: Altered firing properties of DRG neurons following spinal nerve injury. *Pain* 2000; 85:503-21
- Liu X, Chung K, Chung JM: Ectopic discharges and adrenergic sensitivity of sensory neurons after spinal nerve injury. *Brain Res* 1999; 849:244-7
- Ma C, Shu Y, Zheng Z, Chen Y, Yao H, Greenquist KW, White FA, LaMotte RH: Similar electrophysiological changes in axotomized and neighboring intact dorsal root ganglion neurons. *J Neurophysiol* 2003; 89:1588-602
- Sun Q, Tu H, Xing GG, Han JS, Wan Y: Ectopic discharges from injured nerve fibers are highly correlated with tactile allodynia only in early, but not late, stage in rats with spinal nerve ligation. *Exp Neurol* 2005; 191:128-36
- Wu G, Ringkamp M, Hartke TV, Murinson BB, Campbell JN, Griffin JW, Meyer RA: Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. *J Neurosci* 2001; 21:RC140
- Xie W, Strong JA, Meij JT, Zhang JM, Yu L: Neuropathic pain: Early spontaneous afferent activity is the trigger. *Pain* 2005; 116:243-56
- Suter MR, Papaloizos M, Berde CB, Woolf CJ, Gilliard N, Spahn DR, Decosterd I: **Development of neuropathic pain in the rat spared nerve injury model is not prevented by a peripheral nerve block.** *ANESTHESIOLOGY* 2003; 99:1402-8
- Drager C, Benziger D, Gao F, Berde CB: Prolonged intercostal nerve blockade in sheep using controlled-release of bupivacaine and dexamethasone from polymer microspheres. *ANESTHESIOLOGY* 1998; 89:969-79
- Kim SH, Chung JM: An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 1992; 50:355-63
- Decosterd I, Woolf CJ: Spared nerve injury: An animal model of persistent peripheral neuropathic pain. *Pain* 2000; 87:149-58
- Ji RR, Befort K, Brenner GJ, Woolf CJ: ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. *J Neurosci* 2002; 22:478-85
- Zhuang ZY, Wen YR, Zhang DR, Borsello T, Bonny C, Strichartz GR, Decosterd I, Ji RR: A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *J Neurosci* 2006; 26:3551-60

32. Zhuang ZY, Gerner P, Woolf CJ, Ji RR: ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. *Pain* 2005; 114:149-59
33. Ji RR, Samad TA, Jin SX, Schmolz R, Woolf CJ: p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 2002; 36:57-68
34. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53:55-63
35. Dixon WJ: Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 1980; 20:441-62
36. Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der, MC Befort K, Woolf CJ, Ji RR: Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J Neurosci* 2004; 24:8310-21
37. Romero-Sandoval A, Eisenach JC: Spinal cannabinoid receptor type 2 activation reduces hypersensitivity and spinal cord glial activation after paw incision. *ANESTHESIOLOGY* 2007; 106:787-94
38. Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV: A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci* 1998; 18:3059-72
39. Swett JE, Woolf CJ: The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. *J Comp Neurol* 1985; 231:66-77
40. Decosterd I, Allchorne A, Woolf CJ: Differential analgesic sensitivity of two distinct neuropathic pain models. *Anesth Analg* 2004; 99:457-63
41. Boyle DL, Jones TL, Hammaker D, Svensson CI, Rosengren S, Albani S, Sorokin L, Firestein GS: Regulation of peripheral inflammation by spinal p38 MAP kinase in rats. *PLoS Med* 2006; 3:e338
42. Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN: Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. *J Neurosci* 2006; 26:1281-92
43. Beloeil H, Ji RR, Berde CB: Effects of bupivacaine and tetrodotoxin on carrageenan-induced hind paw inflammation in rats: II. Cytokines and p38 mitogen-activated protein kinases in dorsal root ganglia and spinal cord. *ANESTHESIOLOGY* 2006; 105:139-45
44. Crown ED, Ye Z, Johnson KM, Xu GY, McAdoo DJ, Hulsebosch CE: Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. *Exp Neurol* 2006; 199:397-407
45. Hains BC, Waxman SG: Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci* 2006; 26:4308-17
46. Mizushima T, Obata K, Yamanaka H, Dai Y, Fukuoka T, Tokunaga A, Mashimo T, Noguchi K: Activation of p38 MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. *Pain* 2005; 113:51-60
47. Hanisch UK: Microglia as a source and target of cytokines. *Glia* 2002; 40:140-55
48. Devor M, Wall PD, Catalan N: Systemic lidocaine silences ectopic neuroma and DRG discharge without blocking nerve conduction. *Pain* 1992; 48:261-8
49. Khodorova A, Meissner K, Leeson S, Strichartz GR: Lidocaine selectively blocks abnormal impulses arising from noninactivating Na channels. *Muscle Nerve* 2001; 24:634-47
50. Kanai A, Hiruma H, Katakura T, Sase S, Kawakami T, Hoka S: Low-concentration lidocaine rapidly inhibits axonal transport in cultured mouse dorsal root ganglion neurons. *ANESTHESIOLOGY* 2001; 95:675-80
51. Fischer LG, Bremer M, Coleman EJ, Conrad B, Krumm B, Gross A, Hollmann MW, Mandell G, Durieux ME: Local anesthetics attenuate lysophosphatidic acid-induced priming in human neutrophils. *Anesth Analg* 2001; 92:1041-7
52. Yanagidate F, Strichartz GR: Bupivacaine inhibits activation of neuronal spinal extracellular receptor-activated kinase through selective effects on ionotropic receptors. *ANESTHESIOLOGY* 2006; 104:805-14
53. Winkelstein BA, DeLeo JA: Nerve root injury severity differentially modulates spinal glial activation in a rat lumbar radiculopathy model: Considerations for persistent pain. *Brain Res* 2002; 956:294-301