

# Shifts in Cell-type Expression Accompany a Diminishing Role of Spinal P38-Mapkinase Activation over Time during Prolonged Postoperative Pain

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## ABSTRACT

**Background:** Surgery often causes prolonged postoperative pain, the mechanisms of which are unknown. The authors investigated the role of p38, a pain-associated mitogen-activated protein kinase, in induction and maintenance of such pain.

**Methods:** Male rats were subjected to the skin–muscle incision retraction procedure at the saphenous region; the procedure causes ~4 weeks of secondary tactile hyperalgesia in the ipsilateral plantar region, indicating central sensitization. The spinal cord was sectioned from L3 and L4 + L5 vertebral segments and stained for activated p38 (P-p38) at postoperative day 3 (POD 3), just as secondary hyperalgesia develops; at PODs 10–12, the time of maximum hyperalgesia; and at POD 35, after the resolution of hyperalgesia. Some sections were costained for microglia, astrocytes, and neurons. Intrathecal injections of a P-p38 inhibitor were performed at POD 2 or POD 9, and subsequent changes in pain were monitored.

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## What We Already Know about This Topic

- Skin incision causes activation of p38 mitogen activated protein kinase (P-p38 MAPK) in spinal cord microglia, but the response in prolonged postoperative pain is unknown

## What This Article Tells Us That Is New

- In rats, skin–muscle incision retraction resulted in prolonged pain and elevation of P-p38 MAPK, initially in microglia and later in neurons
- Inhibition of P-p38 MAPK early after surgery prevented prolonged hypersensitivity, whereas later treatment did not

**Results:** Skin–muscle incision retraction increased the numbers of dorsal horn P-p38 positive cells in L3 by ~3-fold and in L4 + L5 by ~7-fold from POD 3 to PODs 11–12. This increase was accompanied by a shift from microglia to neurons, resulting in a ~20-fold increase in P-p38–positive neurons in L4–L5 over this time. No P-p38 was detected in astrocytes. A P-p38 inhibitor given at POD 2 prevented development of secondary hypersensitivity, but when given at POD 9 the same dose gave weak relief of pain for less than 3 h.

**Conclusions:** Spinal P-p38 mitogen-activated protein kinase, activated after incision retraction, is important for the induction of prolonged pain, but despite increased pain near the time of maximum pain, its functional importance for the maintenance of pain is not great.

**P**OSTOPERATIVE pain of various durations can occur after different surgical procedures. Minor plastic surgery causes resting pain for 48–72 h, whereas thoracotomies and herniorrhaphies often result in pain that lasts 6 months or longer.<sup>1,2</sup> Prolonged pain slows recovery, causing personal discomfort and social withdrawal.

The reasons for these different pain durations are unknown. Both primary hyperalgesia, at the site of injury, and secondary hyperalgesia, at regions not directly affected by the surgical procedure, contribute to the course of postoperative pain. Secondary hyperalgesia results from “central sensitization” in the spinal cord and brain that accounts for the longer duration of pain that follows peripheral injury. Surgical incision through the skin and muscles of the foot<sup>3</sup> or back<sup>4</sup> leads to 3–5 days of acute postincisional pain. Animal models show the involvement in the spinal cord of increased gluta-

mate,<sup>5</sup> alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainate,<sup>6,7</sup> and metabotropic glutamate (mGluR5) receptors<sup>8</sup> and of activated p38 (P-p38) mitogen-activated protein kinase (MAPK)<sup>9–11</sup> and chemokine CCL-2<sup>12</sup> in this acute pain model. Peripheral treatments that cause more prolonged pain, such as nerve injury or inflammation, involve some but not all of these factors (*i.e.*, *N*-methyl-D-aspartate type glutamate receptors are involved in inflammatory and some nerve-injury pain, as are chemokines other than CCL-2).<sup>13</sup> However, surgical procedures resulting in prolonged postoperative pain have not been examined to identify the factors responsible for the chronic and acute phases.

In the current study, we used the skin-muscle incision retraction (SMIR) model that causes 4–5 weeks of postoperative secondary hyperalgesia and allodynia to examine the role of activated p38 in prolonged postoperative pain.<sup>14</sup> Incision and retraction of the skin and muscle, entrapping the saphenous nerve for 1 hr, results in mechanical hypersensitivity detected on the plantar surface of the ipsilateral paw, far from the site of incision or retraction. Importantly, there are no indications of any peripheral nerve injury in this model.<sup>15,16</sup> Systemic morphine and gabapentin can reverse the mechanosensitivity caused by SMIR.<sup>17</sup>

The saphenous nerve enters the spinal cord primarily at L3, with a lesser component entering at L4.<sup>14</sup> The L4 spinal segment is predominantly innervated by the sciatic nerve, whereas L5 exclusively receives input from the sciatic nerve,<sup>18</sup> which terminates in the plantar paw, the tested area for tactile hypersensitivity after SMIR. Thus, tests of altered plantar sensitivity after saphenous manipulations reveal coupling between the sciatic and saphenous nerves resulting from neuroplasticity caused by central sensitization.

We hypothesized that the course of mechanohyperalgesia from the SMIR procedure would be paralleled by a change in P-p38 expression in microglia, where it was known to be increased after paw incision. By examining spinal cord sections from the L3 and L4 + L5 segments taken from rats at different postoperative pain stages, we can correlate P-p38 expression with secondary hypersensitivity. Costaining for glia and neurons in the dorsal horn (DH) allows the identification of the cell types expressing P-p38. Intrathecal delivery of an inhibitor of P-p38 near these spinal segments at different pain stages indicates its functional importance in postoperative pain induction and maintenance.

## Materials and Methods

### Animals

All animal experimentation was approved by the Harvard Medical Area Standing Committee on Animals, Boston, Massachusetts. Male Sprague-Dawley rats were purchased from Charles River Laboratory (Wilmington, MA) and kept in the supervised animal housing facilities with controlled humidity (20–30% relative humidity), room temperature (24°C), a 12-h (6:00 AM–6:00 PM) light–dark cycle, and unlimited access to food and water. Before all behavioral

experiments, the animals were handled to familiarize them with the behavioral investigator, experimental environment, and specific experimental procedures for reduction of stress during experiments. At the time of surgery, rats weighed approximately 250–300g, and after the longest postoperative period, 13 days, they weighed 315–345 g.

### SMIR Surgery

Skin-muscle incision retraction surgery was performed as developed by Flatters.<sup>14</sup> Rats (250–300 g) were anesthetized with intraperitoneal sodium pentobarbital (Nembutal®; Sigma-Aldrich Chemical Co., St. Louis, MO; 50 mg/ml) at doses of 65–75 mg/kg, laid supine, and the medial side of the left lower limb shaved. The shaved skin was sterilized with  $\beta$ -iodine and then alcohol to enable visualization through the skin of the saphenous vein. A 1.2–1.5-cm skin incision was made approximately 4 mm medial to the saphenous vein to reveal the muscle of the leg. An incision (7–10 mm long) was then made in the superficial muscle layer, approximately 4 mm medial to the saphenous nerve. The superficial muscle was then parted further by spreading it with blunt scissors within the muscle incision site to allow the insertion of a microdissecting retractor with four prongs, spaced over an 8-mm distance, each prong being 4 mm deep (Cat. No. 13-1090, Biomedical Research Instruments Inc., Silver Spring, MD). The retractor was inserted into the incision site on the thigh so as to position all prongs underneath the superficial gracilis muscle and above the adductor magnus muscle. The skin and this muscle were then retracted 2 cm, revealing the fascia of the underlying muscles. Covered by a sterile phosphate-buffered-saline-soaked dressing over the open wound, this retraction was maintained for 1 h. The animals were closely monitored during the retraction period; if a rat began to stir or blink its eyes, additional anesthesia was provided by muzzle inhalation of the volatile general anesthetic sevoflurane (Ultane; Abbott Labs, Abbott Park, IL). During this period the animals were completely covered (apart from the head) with a large absorbent bench pad (Cat. No. 56616-031; VWR International, West Chester, PA) to minimize heat loss. After the retractor was removed, muscle and skin wounds were closed with 4–0 Vicryl® (Myco Medical, Cary, NC) and 3–0 silk sutures (Angiotech; Surgical Specialties Corp., Reading PA), respectively. Sham-operated rats underwent the same incisional procedure, with the same general anesthetic, but without the retraction. After recovery from anesthesia, which occurred in 1–2 h, all animals could ambulate normally and rise up on their hind limbs to reach food and water.

### Immunocytochemistry

At 3, 11–12, and 35 days after surgery, animals were terminally anesthetized with pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with phosphate buffered saline at room temperature, followed by cold paraformaldehyde (4%) in 0.1 M phosphate buffer (pH 7.4, 4°C). The L3,

L4, and L5 spinal cord segments were dissected, postfixed overnight in the same perfusion fixative, and transferred to 20% sucrose-phosphate buffered saline for cryoprotection, and incubated overnight at 4°C.

For the different postoperative days (PODs) and conditions, the numbers of rats used for P-p38 staining alone were as follows: POD 3: SMIR = 6, sham = 5; PODs 11–12: SMIR = 8, sham = 6; POD 35: SMIR = 4, sham = 4; naive = 4. For each rat, 6–10 sections usually were counted to give an average number for that rat, and these averages were then averaged to give a mean value for that condition.

For the each of the colocalization experiments, with microglia, astrocytes, or neurons, three sections each from four rats were taken at POD 3, and seven sections from a total of four rats were taken at PODs 10–12.

Spinal cord segments were then frozen at  $-20^{\circ}\text{C}$  in a cryostat, and transverse free-floating sections were cut to 30- $\mu\text{m}$  thickness and collected in 0.1 M phosphate buffer. After the sections were blocked with 2% normal goat serum containing Triton X-100 (0.3%) for 1 h at room temperature, all were incubated with either a primary rabbit antibody to P-p38, Iba (or occasionally to OX-42, for microglial staining), glial fibrillary acid protein (GFAP; for astrocyte staining), neuron-specific nuclear protein (NeuN; for neuronal staining), or with no primary antibody. (Sections that were incubated in no primary antibody, but the usual titer of secondary antibody, showed no significant staining of individual cells, documenting the specificity of the secondary antibody.) After the sections were washed thoroughly to remove excess primary antibody, they were incubated with secondary antibodies for 1 h at 20–24°C. Sections were washed free of secondary antibody, placed on glass slides, dried, one drop of Vectashield™ (Vector Laboratories, Burlingame, CA) was placed over them, and they were covered with glass cover slips. Antibodies, and their respective titers, were as follows: P-p38 primary antibody (Cell Signaling, phospho-p38 MAPK [Thr180/Tyr182 rabbit monoclonal antibody, no. 9215; Thr180/Tyr182 mouse monoclonal antibody, no. 9216]), 1:400. P-p38 secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:800. CD11b primary (clone OX-42 [AbD monoclonal antibody; Serotec, Raleigh, NC]), 1:250. CD11b secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:200. Iba-1 primary antibody (Wako Chemicals USA, Richmond, VA), 1:5000; Iba-1 secondary antibody (Jackson ImmunoResearch Laboratories, Bar Harbor, ME), 1:400. GFAP primary monoclonal antibody (Millipore Bioscience Research Reagents; Millipore Corp., Woburn, MA), 1:4,000. GFAP secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:400. Neuronal-specific nuclear protein primary antibody (Millipore Bioscience Research Reagents), 1:3,000. Neuron Nuclear protein secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:400. For double immunofluorescence, sections were incubated with a mixture of pri-

mary antibodies: rabbit polyclonal antibody against phosphorylated p38 (P-p38), mouse monoclonal antibody against P-p38, GFAP, OX-42, or Iba-1 for 36–48 h at 4°C, followed by a mixture of goat antimouse fluorescein isothiocyanate-conjugated and goat antirabbit indocarbocyanine-conjugated secondary antibodies for 1 h at room temperature. The staining pattern with the rabbit polyclonal antibody against P-p38 was comparable with that obtained with the mouse monoclonal antibody, although a quantitative comparison was not conducted.

Fluorescence was viewed with an Olympus BX50 fluorescence microscope (Olympus America, Inc., Center Valley, PA), and the computer images captured and manipulated by Image-J software (NIH Image, National Institutes of Health, Bethesda MD). Positive cells were identified by bright spots at more than  $\times 5$  background intensity, and those in the DH were counted by hand. Cell bodies were selected by counting only “circular” bright areas and avoiding more diffuse or undefined bright spots. Similar instrument and software settings, adjusted slightly to give virtually equal cell luminosity, were applied to determine the number of P-p38–positive cells in the DH (lamina I–III) of L3 and L4 + L5 segments. These latter segments were merged for analysis because we lacked a clear demarcation between them during sectioning.

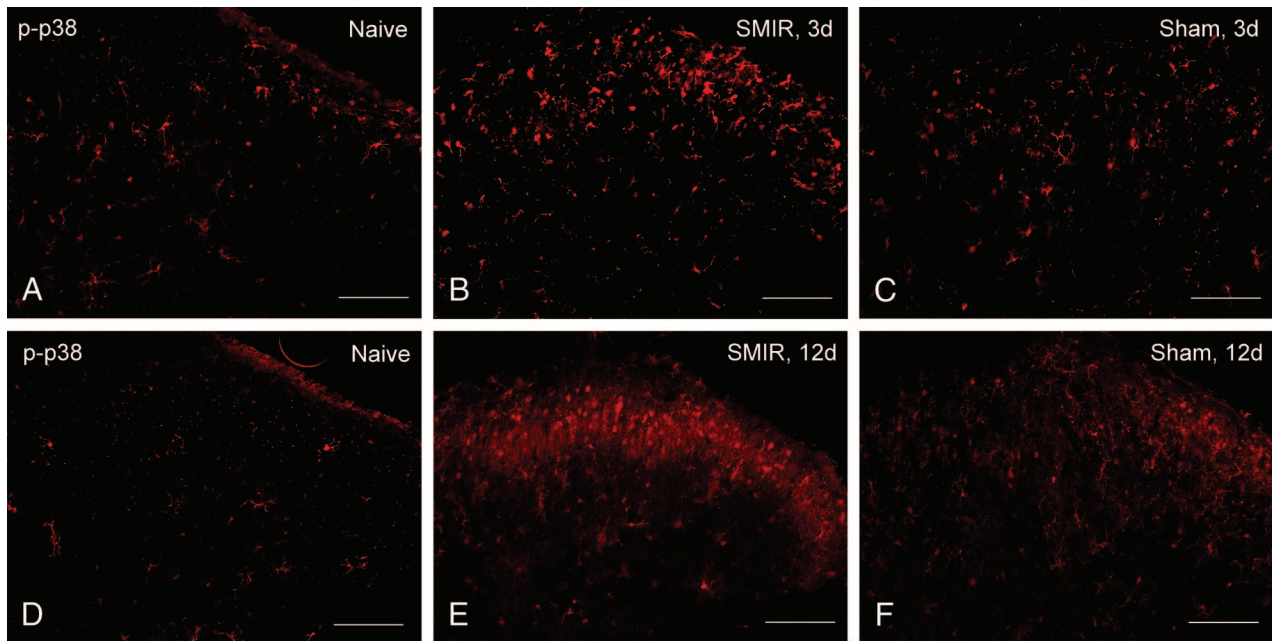
### **Behavioral Testing of Mechanical Sensitivity**

All animals to be tested behaviorally were habituated three times to the testing environment on three sequential days just before testing. Throughout the behavioral testing time courses, the experimenter was blind to the injected agents (P-p38 inhibitor or vehicle solution).

Animals were placed on an increased wire mesh floor and confined underneath individual overturned plastic boxes. Mechanical allodynia or hyperalgesia was assessed using four von Frey filaments (von Frey hair) with bending forces of 4, 6, 10, and 15 g (Touch-Test™ Sensory Evaluators; Stoelting Co., Wood Dale, IL). In ascending order of force, each von Frey hair filament was applied 10 times, to the midplantar-central area of the hind paw encircled by tori (footpads). Withdrawal responses to each of the von Frey hair filaments from both hind paws were counted and recorded. Although significant increases in withdrawal response frequency occurred for tests using the 6-, 10-, and 15-g filaments, only the hyperalgesic response to the 15-g von Frey hair is reported here. The three baseline measurements of mechanical sensitivity were taken on separate days and averaged to provide the preoperative baseline response, denoted by the value graphed on POD 0.

For each time of testing and treatment group, the following numbers of rats were used: Early injection of SB203580 (on POD 2): SB-treated rats,  $n = 8$ ; vehicle-treated rats,  $n = 6$ . Late injection of SB203580 (on POD 9): SB-injected rats,  $n = 7$ ; vehicle-injected rats,  $n = 4$ .





**Fig. 1.** Immunofluorescence of dorsal horn (DH) from an ipsilateral L3 spinal segment taken from a naive rat (A) (of the same age and weight as for B and C), at postoperative day (POD) 3 after skin–muscle incision retraction (SMIR) treatment (B), and 3 days after sham treatment (C). By comparison, segments from L3 were also taken from SMIR-treated rats at POD 12 (E), and 12 days after sham treatment (F). The naive control for the second group (D) shows a similar low density of positive cells. Scale bar = 100  $\mu$ m.

### Statistics

Data are presented as means  $\pm$  SD. For immunocytochemistry, numbers of P-p38–positive cells, averaged for each individual rat (see Materials and Methods) were further averaged to give grand mean  $\pm$  SD. The density of these cells, counted in lamina I–III of the ipsilateral DH, were compared between SMIR and sham rats using a generalized linear model (PROC GLM), with simultaneous comparisons between treatment groups (SMIR *vs.* sham), among the three PODs (3 *vs.* 11 *vs.* 35), and between spinal segments (L3 *vs.* L4 + L5). This was followed by pairwise comparisons when allowed, using Tukey multiple comparison procedure (SAS Software, Cary, NC).

Response values from the behavioral experiments were compared between drug (SB203580)-injected and vehicle-injected rats using one-way ANOVA.

For all analyses, the significance minimum is reported for all comparison, which were two-tailed with significance assigned at  $P < 0.05$  for pairwise comparisons, and at adjusted values for comparison among larger groups.

## Results

### Immunocytochemistry

**Numbers of P-p38–positive Cells Increase with Hyperalgesia.** The SMIR procedure results in a tactile hyperalgesia of the ipsilateral hind paw that first develops at PODs 3–4, reaches a maximum at PODs 10–14, and resolves to the preoperative level by POD 35.<sup>14</sup> A direct test of the primary incision area is not possible in awake rats, but a sham procedure similar to this one, which included incision but omitted the retraction step, on the rat's back showed an acute elevation of the primary response over only the first 5–7 days.<sup>4</sup>

The experiments in this article are directed toward understanding the secondary hypersensitivity that indicates central sensitization after surgical procedures.

Spinal cord sections were taken from rats undergoing SMIR or sham procedures at these respective postoperative time periods and stained for P-p38 using phospho-specific antibodies (fig. 1, A, B, and C). Positively stained cells were apparent in the ipsilateral DH from L3 segments at POD 3 (fig. 1B), primarily in lamina I–III, and were increased over the numbers in the L3 DH taken at POD 3 from naive (fig. 1A) and sham rats (fig. 1C). The L3 spinal segment receives the major input ( $\sim 75\%$ ) of the saphenous nerve, with the remaining 25% entering at L4, which also receives afferents from the sciatic nerve.<sup>14,18</sup> No staining above the naive level was observed in the contralateral L3 DH (data not shown).

Stained segments taken at the time of maximum pain are shown in figure 1, D, E, and F. P-p38–positive cells were more abundant in the L3 DH from SMIR rats at the time of maximum pain, PODs 11–12 (fig. 1E, table 1). Segments from L3 of sham-treated rats were less densely stained (fig. 1F) but still greater than those from naive rats of the same weight and age (fig. 1, A and D). In addition to the cell bodies, which are distinguished by bright spots, axons passing through the DH, shown by a more diffuse fluorescence, were positive for P-p38 at POD 12, for both SMIR and sham

**Table 1.** Numbers of P-p38–positive Cells in Lamina I–III of Dorsal Horn of Lumbar Segments from SMIR and Sham-operated Rats

Spinal Cord Segment	(Mean $\pm$ SD)					
	Postoperative Day 3		Postoperative Day 11		Postoperative Day 35	
	L3	L4 + L5	L3	L4 + L5	L3	L4 + L5
Procedure						
SMIR	27 $\pm$ 4.8 (4)*	7.4 $\pm$ 4.7 (7)*	74.3 $\pm$ 9.8 (7)*†‡	52.5 $\pm$ 5 (4)*†‡	40.5 $\pm$ 7.1 (4)*	32.2 $\pm$ 3.4 (8)*
Sham	12 $\pm$ 2.2 (4)*	9.3 $\pm$ 2.4 (7)*	35 $\pm$ 5 (5)*‡	27.5 $\pm$ 5.2 (6)*‡	31.7 $\pm$ 3.5 (3)	28 $\pm$ 2.4 (6)

Significant differences, by the three-way procedure described in Materials and Methods: \* Day 3 vs. day 11 vs. day 35,  $P < 0.05$  for all SMIR in L3 and also in L4 + L5, and for sham between day 3 and day 11 in L3 and in L4 + L5. † L3 vs. L4 + L5, for SMIR on day 11,  $P = 0.031$ . ‡ SMIR versus sham in L3 ( $P = 0.017$ ) and in L4 + L5 ( $P = 0.034$ ) on day 11.

L3 = lumbar spinal segment 3; L4 + L5 = lumbar spinal segments 4 and 5; SMIR = skin–muscle incision retraction.

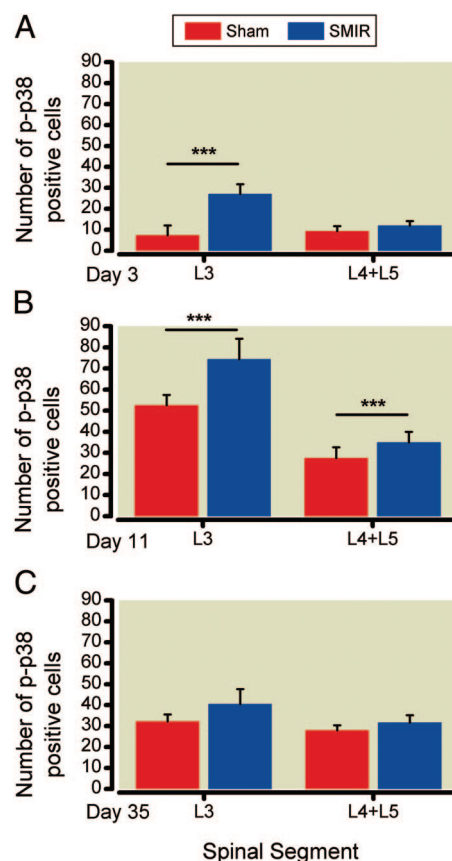
rats (fig. 1, E and F). Contralateral segments of SMIR and sham rats taken at POD 12 remained stained only to the level of the naive DH (data not shown).

Cells positive for P-p38 in lamina I–III of spinal cord segments from L3 and L4 + L5 were counted separately for both SMIR and sham rats. (Segments from naive rats were also examined, but so few positive cells were detected that those numbers are not reported here.) Average values for numbers of P-p38–positive cells for the separate segments and conditions are graphed in figure 2 and collected, with significance parameters, in table 1. At POD 3 the number of P-p38–positive cells in the L3 segment from SMIR rats exceeds that in sham rats but is equal between the two procedures in L4 + L5. At POD 11, in both L3 and L4 + L5 segments, the number of positive cells is greater in SMIR than in sham segments. The number of these cells is also greater in L3 than in L4 + L5 at POD 11 in SMIR animals. Finally, there is a significant difference in the number of P-p38–positive cells among POD 3, POD 11, and POD 35 for both L3 and L4 + L5 in SMIR rats and in L3 of sham rats, and between POD 3 and POD 11 in L4 + L5 of sham rats (table 1).

By POD 11, at the time of maximum postoperative mechanohyperalgesia, the number of positive cells in L3 of SMIR rats had approximately tripled compared with that of POD 3. A similar increase in positive cells in sham rats had occurred, although the numbers remain lower than in SMIR rats (fig. 2, table 1). Segments from L4 + L5 taken at these times showed a 7-fold increase in positive cells from SMIR rats, and a 3-fold increase in those from sham rats (table 1). By POD 35, when allodynia had disappeared, the numbers of positive cells in L3 from SMIR rats was approximately half that PODs 11–12 and similarly reduced in L4 + L5 segments, so that these densities were now equal (fig. 2). In contrast, the number of positive cells in L3 of sham-treated rats did not change between PODs 11–12 and POD 35, and the same was true for those in L4 + L5 (table 1). Consequently, by POD 35 the numbers of P-p38–positive cells was the same in both segments for both procedures (fig. 2C).

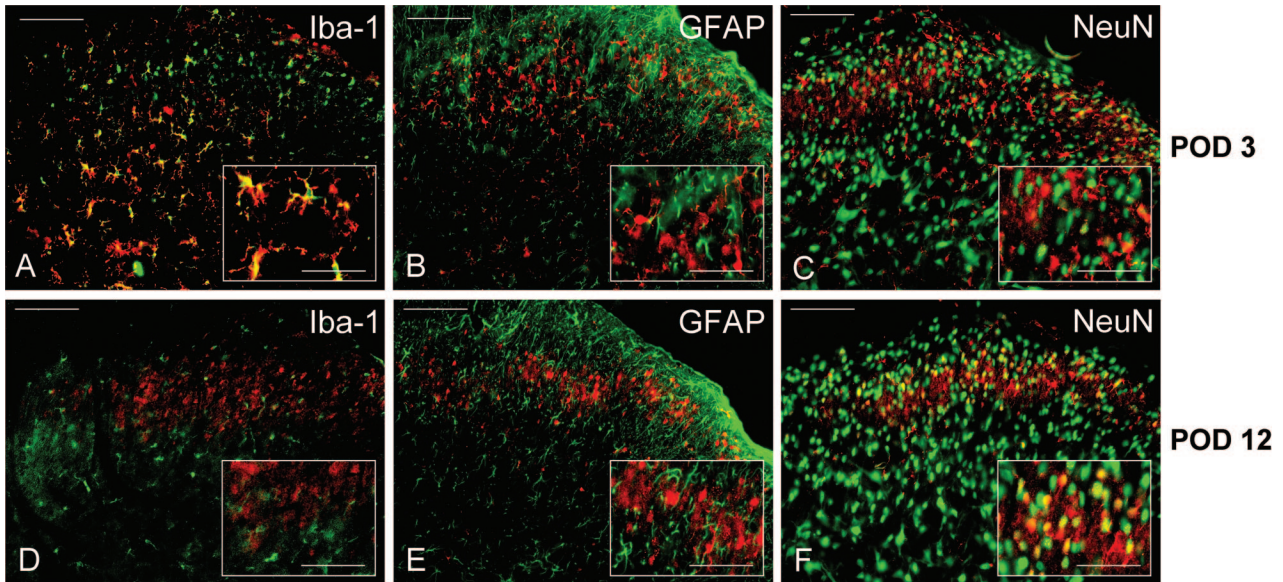
### P-p38 Distribution Changes in Microglia and Neurons over Time after Surgery

Counter-staining of P-p38 segments with antibodies that identify activated microglia, OX-42 (data not shown), all microglia (Iba), or activated astrocytes (GFAP) allowed the



**Fig. 2.** Average values ( $\pm$ SD) of numbers of P-p38–positive cells in lamina I–III of the dorsal horn of ipsilateral L3 and merged L4 + L5 segments taken from rats treated by the skin–muscle incision retraction (SMIR; blue bars) and sham (red bars) procedures. Postoperative day (POD) 3 (before pain is increased) (A), POD 11 (at the hyperalgesic maximum) (B), and POD 35 (when all hyperalgesia has resolved) (C). SMIR and sham values compared for the same time and segment by one-way ANOVA. \*\*\*  $P < 0.001$ .





**Fig. 3.** Immunofluorescence of P-p38–stained (red) ipsilateral dorsal horn L3 spinal segments taken from postoperative day (POD) 3 skin–muscle incision retraction (SMIR) rats (top row, A–C) or POD 12 SMIR rats (bottom row, D–F) and counter-stained (green) for the microbial marker Iba-1 (A, D), the astrocyte marker glial fibrillary acid protein (GFAP) (B, E), and the neuronal marker neuron-specific nuclear protein (NeuN) (C, F). Scale bar = 100  $\mu$ m for the large panels and 50  $\mu$ m for the magnified insets.

identification of p38 activation among these different cell types. At POD 3, the time of first detection of secondary hyperalgesia, there was strong colocalization of P-p38 and microglia in the L3 DH (fig. 3A). No P-p38 cells were identified as astrocytes, as shown by the absence of colocalization with GFAP (fig. 3B). Of the many neurons in the DH, identified by neuron-specific nuclear protein staining, a small but significant number were positive for P-p38 (fig. 3C, table 2). (Although we did not costain segments from naive rats, the morphology of the few P-p38–positive cells that were detected was like that of microglia and not of neurons.)

However, by the time that postoperative hyperalgesia was maximum, this pattern of cellular distribution had shifted. In segments taken at PODs 10–12 there was virtually no P-p38 detected in microglia (fig. 3D), and P-p38 continued to be absent from astrocytes (fig. 3E). Remarkably, neuronal expression of P-p38 had increased markedly by that time (fig.

3F), and a quantitative analysis showed that the fraction of P-p38–positive cells that were neurons had risen 2- to 3-fold from POD 3 to PODs 10–12 (table 2).

### Behavioral Pharmacology

The early intrathecal delivery of an inhibitor of P-p38 was able to prevent the development of SMIR-induced mechanosensitivity. Injection of 10  $\mu$ g SB203580 at the L4–L5 region on POD 2 prevented any significant increase of response to von Frey hair stimulation during the next 11 days (the duration of testing), to the time of maximum response (fig. 4A). (The significance of differences between treated and control responses at POD 8, POD 11, and POD 13 established in pairwise comparisons, disappears when adjusted for comparisons among all the measured days in both groups.) In contrast, when the same dose of this inhibitor was given at POD 9, after the mechanosensitivity was well developed, the pain-relieving effects were brief (fig. 4B). (This significant difference between the SB203580-treated and the vehicle-treated groups, analyzed pairwise, also disappeared when adjusted for comparison of all eight sets of data.) The response to tactile stimulation fell to preoperative levels, but only at 1 h after the injection; by 3 h, the postoperative hypersensitivity had returned to its predrug level.

### Discussion

The results of this study show that the activated form of p38 MAPK, P-p38, appears in an increased number of cells in the DH after skin–muscle incision retraction surgery. The patterns of distribution of P-p38 change after surgery, among the different cell types in the DH, and between segments

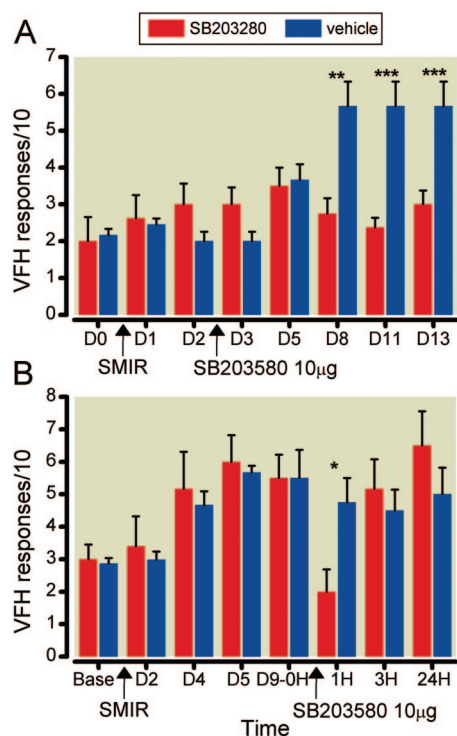
**Table 2.** Percent of P-p38–positive Neurons\* after the SMIR Procedure

	Postoperative Day 3	Postoperative Days 10–12
% NeuN-positive cells	14.7 $\pm$ 0.8%	32.4 $\pm$ 0.5%

Mean  $\pm$  SD for numbers of colocalized cells from three sections from each of four animals for post-SMIR day 3 and seven sections from four animals at post-SMIR days 10–12.

\* Stained by NeuN.

NeuN = neuron-specific nuclear protein; SMIR = skin–muscle incision retraction.



**Fig. 4.** Effect of P-p38 inhibitor on behavioral responses after skin-muscle incision retraction (SMIR). Rats were stimulated by a 15-g von Frey filament (VFH) pressed against the mid-plantar paw surface of the leg ipsilateral to the surgical procedure; the vertical axis plots the number of withdrawal responses per 10 VFH stimulations (mean  $\pm$  SD). Intrathecal injection of 10  $\mu$ g SB203580 at postoperative day (POD) 2 keeps the response level at the preoperative baseline (denoted at day 0 [D0]) and at PODs 8–13 significantly below that for the control, hyperalgesic rats that received only a vehicle injection (A). Intrathecal injection of 10  $\mu$ g SB203580 at POD 9, after hyperalgesia has developed, results in only a brief reduction of the increased response. Comparison of vehicle- and SB203580-injected rats on the same days (B). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  (one-way ANOVA).

receiving input exclusively from the saphenous nerve (L3) or from the saphenous and the sciatic nerve (L4) or the sciatic nerve alone (L5). At the earliest time of observation, POD 3, when secondary hypersensitivity may be detected but when primary postincisional pain is almost certainly present (but not testable in this model), P-p38 appears mostly in microglia. During peak secondary hyperalgesia, at PODs 11–12, a time when primary postincisional pain in other models has disappeared,<sup>3,4,11,12</sup> P-p38-positive cells increase markedly in L3 and even more in L4 + L5. In addition to this increase there is a shift in the distribution of P-p38 from microglia to neurons; this 2- to 3-fold increase in the proportion of positive cells that are neurons, coupled with the 7-fold increase in total P-p38-positive cells in L4 + L5, results in an approximately 20-fold increase in the number of P-p38-positive neurons in L4 + L5, from the very beginning of secondary hypersensitivity to its maximum. Because L4 + L5 segments contain the inputs of the sciatic nerve from the

behavioral test area of the plantar hind paw, it is noteworthy that such a large increase in neuronal P-p38 occurs over the time when secondary hyperalgesia develops in an area innervated by the sciatic nerve, far removed from the locus of saphenous nerve manipulation. The L3 spinal segments, exclusively innervated by the saphenous nerve, show the same numbers of P-p38-positive cells at POD 3 after SMIR and POD 11 after sham, both conditions when secondary hyperalgesia is absent. These same, lower numbers of P-p38-positive cells are present in L4 + L5 in both SMIR and sham rats at POD 35, a time when secondary hyperalgesia (and probably primary hypersensitivity<sup>3,4</sup>) has fully resolved. Although these numbers are above the baseline values seen in naive rats, they may reflect changes in spinal circuitry that are inadequate to drive the secondary hypersensitivity from central sensitization. Thus, the rising numbers of P-p38-positive cells in L3 and in L4 + L5 during the first 2 weeks or so appear to correlate with the induction of functional central sensitization after retraction, but not after incision alone, and the retention of P-p38 levels above the baseline at a time beyond the resolution of pain implies that p38 activation *per se* is not tightly coupled to the maintenance of hyperalgesia.

The importance of P-p38 for pain induction is supported by the functional effects of an inhibitor of this MAPK. Injected intrathecally a few days after the SMIR procedure, this agent is able to virtually prevent secondary hypersensitivity. Injected a week later, at a time near the peak of the induced secondary pain, it is marginally and only transiently effective.

How do these results compare with other studies about P-p38 involvement in injury- or inflammation-induced pain hypersensitivity and with studies on the role of P-p38 in the acute responses to (paw) incision-induced pain? How can we explain these findings in terms of a model for the induction and resolution of prolonged postoperative pain?

### Role of P-p38 in Models of Nerve Injury and Inflammation

P-p38 is increased in response to peripheral nerve injury and inflammation.<sup>19</sup> Chronic constriction injury<sup>20</sup> and spinal nerve ligation<sup>21,22</sup> increase the activated P-p38 in spinal cord microglia for at least 2 weeks. Activation of p38 occurs in spinal cord microglia after inflammation, with significant increases after the injection of formalin,<sup>19,23</sup> carrageenan,<sup>23</sup> or Freund's complete adjuvant<sup>24</sup> into the rat's foot pad. Such activation is quite rapid, appearing within minutes, and quite transient.<sup>25,26</sup> Plantar injection of Freund's complete adjuvant activates p38 only in peripheral neurons, in the dorsal root ganglia, and not in spinal neurons,<sup>27,28</sup> cells where the extracellular signal-regulated MAPK is activated early after inflammation<sup>29</sup> and incision.<sup>30</sup> These findings suggest a temporal sequence of activation of different MAPK, as well as other kinases, first in peripheral and then in central structures, and moving from sensory neurons to spinal glia.<sup>31–33</sup> In turn, activated p38, is known to increase peripheral neuronal transient receptor potential vanilloid 1 expression and

facilitate the release of cytokines and growth factors, which in turn enhance release of excitatory amino acid transmitters essential for pain transmission in the spinal cord, and sensitize their receptors on postsynaptic neurons.<sup>34</sup>

Inhibitors of P-p38 generally are effective in the prevention and the reversal of thermal and mechanical hypersensitivity after nerve injury<sup>15,20,35,36</sup> but are selectively effective on the thermal but not the mechanohypersensitivity after inflammation.<sup>23,25,36</sup> An inhibitor of P-p38 (SB203580, as used here) given daily intrathecally starting just before spinal nerve ligation, delays development of mechanical allodynia, with the strongest effect occurring 1 day after nerve ligation and a much weaker effect occurring 10 days later.<sup>21</sup> Later bolus administration of the inhibitor gives only transient relief, which is longer lasting for injections at day 1 then at day 10 after spinal nerve ligation.<sup>21</sup> This time-dependent effectiveness parallels the behavior observed with the SMIR-induced pain reported in the current study, despite the absence of nerve injury in the SMIR model.<sup>14</sup>

### Postincision Pain and P-p38

Incision of the plantar paw leads to activation of spinal microglia and astrocytes<sup>10,11</sup> and to elevation of P-p38 that is coupled to the relatively short-lasting postincisional mechanical allodynia.<sup>9,11</sup> The density of P-p38–positive cells in the ipsilateral DH is maximum at 1 and 2 days after paw incision,<sup>9,11</sup> declining by ~ 20% at POD 3, and to an insignificant increase compared with preoperative levels at POD 7.<sup>9</sup> This time course roughly parallels the time course of mechanoallodynia and thermal hyperalgesia,<sup>3,8,9</sup> although the resolution of this pain is quite variable<sup>11,36,37</sup> and appears to depend, among other factors, on the anesthetic used during the surgical procedure.<sup>38,39</sup>

Postincisional P-p38 has been localized during the acute period of hypersensitivity primarily in microglia, but this activated MAPK is also seen in some spinal neurons<sup>10</sup> and in astrocytes.<sup>11</sup> Blocking the activation of p38 by preoperative intrathecal injection of an inhibitor of the upstream, activating kinase MAPK significantly suppressed mechanoallodynia from paw incision through POD 2 but had a much smaller effect, and only at 1 h after surgery, on thermal hyperalgesia.<sup>10</sup> Intrathecal administration of the glial inhibitor fluorocitrate at POD 1 resulted in a partial but long-lasting (up to POD 5) reversal of established mechanoallodynia.<sup>11</sup> In contrast to this result, the intraperitoneal injection of antagonists or inverse agonists of cannabinoid receptors to rats after paw incision enhanced the expression of P-p38 in astrocytes, increased GFAP, and concomitantly delayed the resolution of postincisional pain.<sup>9</sup> Perhaps this treatment results in a shift of the cause of postincisional pain from microglia to astrocytes, akin to the progression of pain-associated cell loci during nerve injury-induced pain.<sup>31</sup>

The current report is the first to use a model of prolonged postoperative pain to study the role of MAPK. The current

results extend the previous work on P-p38 and paw incision, showing that the continuously activated form of this MAPK is not essential for maintaining increased pain. In the SMIR model, secondary tactile allodynia and hyperalgesia that typically involve central sensitization<sup>40,41</sup> appear after the initial increase of P-p38 in the spinal cord, an early event that also occurs after sham incision without retraction. This suggests that P-p38 is increased by both procedures during the initial insult, the incision, and is further increased at later times when retraction is used during surgery.

The spread of P-p38 activation from L3 into L4 + L5, and the approximately 20-fold increase in P-p38–expressing DH neurons in L4 + L5 correlates with the development of secondary hypersensitivity. Early activation of p38 in microglia may be necessary for hypersensitivity to appear, judging by the effectiveness of the inhibitor given at POD 2 in preventing the elevation of the pain response. However, the increase in P-p38 in neurons, if it does contribute to later hypersensitivity, by itself does not appear to be sufficient to sustain the sensitivity, as shown by the relatively weak effectiveness of P-p38 inhibitors to reverse this sensitivity once it is established.

Thus, in SMIR-induced tactile hypersensitivity, even in the absence of peripheral nerve injury, there appears to be adequate afferent input to cause long-term sensitization of central pathways. Accordingly, intrathecal inhibitors of P-p38 may have time-sensitive effectiveness for two reasons: first, P-p38 *per se* may be more important for the induction of hypersensitivity than for its maintenance, or second, the locus of activated P-p38 essential for hypersensitivity may shift over days from spinal cord to brain. In this second explanation, inhibition of spinal P-p38 may interrupt temporarily the pain facilitating afferent throughput to the brain but does not reverse the essential, altered brain state. Once the inhibitor has been removed or degraded, the conditions necessary for hypersensitivity are restored, and increased postoperative pain reemerges. If this second hypothesis is true, studies of glial and neuronal changes in spinal cord alone are not adequate for a complete understanding of the mechanisms underlying prolonged postoperative pain. Persistent or chronic pain's maturation may require a temporary stage of neuroplasticity in which spinal processes are critical, but the stable phenotypic changes in the brain are likely to be the essential targets for treatment of chronic pain that occurs after surgery.

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## References

- Perkins FM, Kehlet H: Chronic pain as an outcome of surgery: A review of predictive factors. *ANESTHESIOLOGY* 2000; 93:1123-33
- Kehlet H, Jensen TS, Woolf CJ: Persistent postsurgical pain: Risk factors and prevention. *Lancet* 2006; 367:1618-25
- Brennan TJ, Vandermeulen EP, Gebhart GF: Characterization of a rat model of incisional pain. *Pain* 1996; 64:493-501
- Duarte AM, Pospisilova E, Reilly E, Mujenda F, Hamaya Y, Strichartz GR: Reduction of postincisional allodynia by subcutaneous bupivacaine: Findings with a new model in the hairy skin of the rat. *ANESTHESIOLOGY* 2005; 103:113-25
- Zahn PK, Sluka KA, Brennan TJ: Excitatory amino acid release in the spinal cord caused by plantar incision in the rat. *Pain* 2002; 100:65-76
- Lee HJ, Pogatzki-Zahn EM, Brennan TJ: The effect of the AMPA/kainate receptor antagonist LY293558 in a rat model of postoperative pain. *J Pain* 2006; 7:768-77
- Pogatzki EM, Niemeier JS, Sorkin LS, Brennan TJ: Spinal glutamate receptor antagonists differentiate primary and secondary mechanical hyperalgesia caused by incision. *Pain* 2003; 105:97-107
- Zhu CZ, Hsieh G, El-Kouhen O, Wilson SG, Mikusa JP, Hollingsworth PR, Chang R, Moreland RB, Brioni J, Decker MW, Honore P: Role of central and peripheral mGluR5 receptors in post-operative pain in rats. *Pain* 2005; 114:195-202
- Alkatis MS, Solorzano C, Landry RP, Piomelli D, DeLeo JA, Romero-Sandoval EA: Evidence for a role of endocannabinoids, astrocytes and p38 phosphorylation in the resolution of postoperative pain. *PLoS One* 2010; 5:e10891
- Wen YR, Suter MR, Ji RR, Yeh GC, Wu YS, Wang KC, Kohno T, Sun WZ, Wang CC: Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia. *ANESTHESIOLOGY* 2009; 110:155-65
- Obata H, Eisenach JC, Hussain H, Bynum T, Vincler M: Spinal glial activation contributes to postoperative mechanical hypersensitivity in the rat. *J Pain* 2006; 7:816-22
- Peters CM, Eisenach JC: Contribution of the chemokine (C-C motif) ligand 2 (CCL2) to mechanical hypersensitivity after surgical incision in rats. *ANESTHESIOLOGY* 2010; 112:1250-8
- Ji R-R, Woolf CJ: Neuronal plasticity and signal transduction in nociceptive neurons: Implications for the initiation and maintenance of pathological pain. *Neurobiol Disease* 2001; 8:1-10
- Flatters SJ: Characterization of a model of persistent postoperative pain evoked by skin/muscle incision and retraction (SMIR). *Pain* 2008; 135:119-30
- 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
- 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
- Flatters SJ: Effect of analgesic standards on persistent postoperative pain evoked by skin/muscle incision and retraction (SMIR). *Neurosci Lett* 2010; 477:43-7
- 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 Neur* 1985; 231:66-77
- Svensson CI, Yaksh TL, Sorkin LS: The role of p38 in microglial regulation of spinal pain processing, *Immune and Glial Regulation of Pain*. Edited by DeLeo JA, Sorkin LS, Watkins LR. Seattle, Washington, IASP Press, 2007, pp 297-317
- Kim SY, Bae JC, Kim JY, Lee HL, Lee KM, Kim DS, Cho HJ: Activation of p38 MAP kinase in the rat dorsal root ganglia and spinal cord following peripheral inflammation and nerve injury. *Neuroreport* 2002; 13:2483-6
- 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
- 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
- Hua XY, Svensson CI, Matsui T, Fitzsimmons B, Yaksh TL, Webb M: Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38 MAPK in spinal microglia. *Eur J Neurosci* 2005; 22:2431-40
- Boyle DL, Jones TL, Hammaker D, Svensson CI, Rosengren S, Albani S, Sorkin L, Firestein GS: Regulation of peripheral inflammation by spinal p38 MAP kinase in rats. *PLoS Med* 2006; 3:e338
- Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, Catalano R, Feng Y, Protter AA, Scott B, Yaksh TL: Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem* 2003; 86:1534-44
- Svensson CI, Fitzsimmons B, Azizi S, Powell HC, Hua XY, Yaksh TL: Spinal p38beta isoform mediates tissue injury-induced hyperalgesia and spinal sensitization. *J Neurochem* 2005; 92:1508-20
- Beloeil H, Ji RR, Berde CB: Effects of bupivacaine and tetrodotoxin on carrageenan-induced hind paw inflammation in rats (Part 2): Cytokines and p38 mitogen-activated protein kinases in dorsal root ganglia and spinal cord. *ANESTHESIOLOGY* 2006; 105:139-45
- Ji RR, Samad TA, Jin SX, Schmol 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
- Ji RR, Baba H, Brenner GJ, Woolf CJ: Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci* 1999; 2:1114-9
- Campillo A, González-Cuello A, Cabañero D, Garcia-Nogales P, Romero A, Milanés MV, Laorden ML, Puig MM: Increased spinal dynorphin levels and phospho-extracellular signal-regulated kinases 1 and 2 and c-Fos immunoreactivity after surgery under remifentanyl anesthesia in mice. *Mol Pharmacol* 2010; 77:185-94
- Ji RR, Gereau RW 4th, Malcangio M, Strichartz GR: MAP kinase and pain. *Brain Res Rev* 2009; 60:135-48
- Ji RR, Kawasaki Y, Zhuang ZY, Wen YR, Zhang YQ: Protein kinases as potential targets for the treatment of pathological pain. *Handb Exp Pharmacol* 2007; 177:359-89
- Gao YJ, Ji RR: Chemokines, neuronal-glial interactions, and central processing of neuropathic pain. *Pharmacol Ther* 2010; 126:56-68
- 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
- Zhang FE, Cao JL, Zhang LC, Zeng YM: Activation of p38 mitogen-activated protein kinase in spinal cord contributes to chronic constriction injury-induced neuropathic pain. *Sheng Li Xue Bao* 2005; 57:545-51
- 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
- Romero-Sandoval A, Chai N, Natile-McMenemy N, DeLeo JA: A

- comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Res* 2008; 1219:116–26
38. Horvath RJ, Landry RP, Romero-Sandoval EA, DeLeo JA: Morphine tolerance attenuates the resolution of postoperative pain and enhances spinal microglial p38 and extracellular receptor kinase phosphorylation. *Neuroscience* 2010; 169: 843–54
  39. Cabañero D, Célérier E, García-Nogales P, Mata M, Roques BP, Maldonado R, Puig MM: The pro-nociceptive effects of remifentanyl or surgical injury in mice are associated with a decrease in delta-opioid receptor mRNA levels: Prevention of the nociceptive response by on-site delivery of enkephalins. *Pain* 2009; 141:88–96
  40. Dirks J, Møiniche S, Hilsted KL, Dahl JB: Mechanisms of postoperative pain: Clinical indications for a contribution of central neuronal sensitization. *ANESTHESIOLOGY* 2002; 97: 1591–6
  41. Kawamata M, Koshizaki M, Shimada SG, Narimatsu E, Kozuka Y, Takahashi T, Namiki A, Collins JG: Changes in response properties and receptive fields of spinal dorsal horn neurons in rats after surgical incision in hairy skin. *ANESTHESIOLOGY* 2005; 102:141–51