Contribution of the Chemokine (C-C Motif) Ligand 2 (CCL2) to Mechanical Hypersensitivity after Surgical Incision in Rats

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ABSTRACT

Background: Neural glial signaling in the spinal cord may underlie pain and sensitization after peripheral injury. The authors test the role of a glial activator, the chemokine (C-C motif) ligand 2 (CCL2), on mechanical hypersensitivity after plantar incision in a rat model of postoperative pain.

Methods: Twenty-four hours after hind paw incision, rats were intrathecally administered an anti-CCL2 neutralizing antibody (3 and 10 μg) or control immunoglobulin G (10 μg). Mechanical hypersensitivity was assessed acutely and for several days after administration of anti-CCL2 antibody using von Frey filaments. Immunohistochemical analysis was conducted on spinal cord sections to examine the effects of treatment on measures of microglial activation, including levels of ionized calcium-binding adaptor molecule 1 and phosphorylated p38 mitogen-activated protein kinase.

Results: Neutralization of spinal CCL2 acutely reversed mechanical hypersensitivity within 30 min in a dose-dependent manner. A single administration also produced a sustained decrease in mechanical hypersensitivity 48 and 72 h after incision. Anti-CCL2 antibody reduced microglial activation as measured by the levels of ionized calcium-binding adaptor molecule 1 immunoreactivity and the number of microglia containing phosphorylated p38 mitogen-activated protein kinase 48 h after incision but not within 30 min of administration.

Conclusions: These results provide evidence that CCL2 contributes to the maintenance of mechanical hypersensitivity after plantar incision and establish a role for neural glial signaling in postoperative pain. The long-term effects of anti-CCL2 treatment correlate with reduced microglial activation. Spinal blockade of CCL2 may serve as a useful therapy for the treatment of certain aspects of postoperative pain.

What We Already Know about This Topic

- ❖ Microglia in the spinal cord are activated after surgery and contribute to postoperative central sensitization and pain
- ❖ Factors that activate spinal microglia during surgery are not known

What This Article Tells Us That Is New

- ❖ In rats, surgery on the paw resulted in hypersensitivity to light touch and activation of spinal cord microglia, both of which were reduced by removing the chemokine CCL2 using an antibody administered spinally
- ❖ Spinal microglia are activated in part by CCL2 after surgery

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AN estimated 23 million people in the United States and 234 million worldwide undergo surgical procedures each year.[1] Despite increased preclinical and clinical research on pathologic mechanisms of postoperative pain and recent advances in analgesic therapies, many patients do not receive adequate analgesic support during the postoperative setting. Clinical reports indicate that 50–70% of patients experience moderate to severe pain after surgery.[2–4]

Preclinical models of acute postoperative pain have been developed involving surgical incision of the skin, muscle, and fascia, which leads to evoked and nonevoked pain-related behaviors that mirror the symptoms observed in patients undergoing surgery.[5–7] It is now widely recognized that activation of spinal glial cells, including microglia and astrocytes, is involved in central sensitization and mechanical hypersensitivity in acute and persistent pain states.[8–11] However, the contribution of glial cells to postoperative pain states has just begun to be investigated. After surgical incision, spinal microglia up-regulate the cell surface molecules CD11b (recognized by OX42 antibody) and the ionized calcium-binding adaptor molecule 1 (IBA1) in lumbar dermatomes ipsilateral to incision.[9,12,13] Furthermore, phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK)[14] and cyclooxygenase 1[15,16] are up-regulated in mi-

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croglia within hours of surgical incision. These data suggest that microglia serve as sources of proinflammatory cytokines and prostaglandins which could maintain central sensitization after incision. In support of this idea, intrathecal injection of the glial metabolic inhibitor fluorocitrate dose dependently reversed mechanical hypersensitivity 24 h after incision,[9] and spinal administration of agents that target microglial-mediated signaling, including p38 MAPK inhibitors[14] and the cyclooxygenase-1-preferring inhibitor ketorolac,[16,17] also partially reverse or prevent the development of mechanical hypersensitivity after incision. Recently, it has been shown that the selective cannabinoid receptor type 2 agonist JWH015 reduced microglial activation and mechanical hypersensitivity after surgical incision in rats.[18]

What has not been determined is what factors activate glial cells in the postoperative setting. Several factors that activate glial cells in the spinal cord have been identified in neuropathic pain states, including adenosine triphosphate, tumor necrosis factor-α, chemokine (C-C motif) ligand 2 (CCL2), fractalkine (CX3CL1), and toll-like receptor agonists.[19] As part of this study, we investigated the effects of spinal blockade of the chemokine CCL2 on mechanical hypersensitivity and spinal markers of microglial activation, including IBA1 and p-p38 MAPK after surgical incision.

Materials and Methods

Animal Preparation and Surgery

Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN), weighing 250–275 g, were used for experiments. All studies conformed to the Wake Forest University Guidelines on the ethical use of animals, and studies were performed after approval from the Animal Care and Use Committee (Winston-Salem, North Carolina). Animals were housed under a 12-h light–dark cycle, with food and water *ad libitum*.

Plantar incision was performed as previously described.[5] In brief, animals were anesthetized with inhalational isoflurane (2%) in oxygen, and the plantar aspect of the left hind paw was prepared in a sterile manner with a 10% povidone–iodine solution. A midline incision (1 cm) was made using a no. 11 blade on the left hind paw starting 0.5 cm from the heel. The plantaris muscle was elevated and incised longitudinally. The wound was closed with two 5.0 nylon mattress sutures. For sham procedures, animals were anesthetized with inhalational isoflurane (2%) in oxygen, and the plantar aspects of the left hind paw were prepared in a sterile manner with 10% povidone–iodine solution.

Drug Administration

Rats that received plantar incision were intrathecally administered CCL2 neutralizing antibody (goat anti-mouse CCL2/MCP-1/LE 15 μl at 200 ng/ml or 15 μl at 667 ng/ml for 3 and 10 ng total, respectively) or the same amount of goat anti-mouse immunoglobulin G (IgG) (both antibodies from R&D Systems, Minneapolis, MN). Anti-CCL2 antibody and control IgG were prepared in 0.9% saline solution

as the vehicle. The goat anti-mouse CCL2/MCP-1/LE neutralizing antibody used for this study also recognizes rat CCL2.[20] Percutaneous intrathecal injections were performed between the L5 and L6 vertebrae of the spine using a 30-gauge ½-inch needle under 2% isoflurane or O_2 anesthesia. Successful puncture of the dura mater was assumed by the presence of a tail flick.

Behavioral Analysis

Paw withdrawal thresholds to mechanical stimuli were determined using von Frey filaments as previously described.[21] In brief, rats were placed in individual Plexiglas chambers with a plastic mesh floor and allowed to acclimate to the test apparatus at least 30 min before testing. Filaments were applied to the bending point for 6 s, and a brisk paw withdrawal was considered a positive response. Withdrawal threshold was determined using an up–down statistical method.[21] Individuals conducting behavioral assays were blinded to the treatment.

Tissue Preparation for Immunohistochemistry

Rats were anesthetized with sodium pentobarbital (intraperitoneal injection; 100 mg/kg), the thorax was opened, and 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4) was perfused through the left ventricle with a peristaltic pump (20 ml/min). The spinal cord was removed, immersed in fixative for 12 h at 4°C followed by immersion in 30% sucrose at 4°C for cryoprotection until ready to be sectioned. Spinal cord cross-sections (40 μm) were cut on a cryostat, and every fourth section was processed for immunohistochemistry for a given marker. For single labeling of microglia, an antibody against IBA1 (rabbit anti-rat IBA1, Wako Chemicals, Richmond, VA) was used. Spinal cord sections were processed free floating and incubated overnight at 4°C with primary antibody. Antibodies were diluted in a solution of PBS containing 1% normal donkey serum and 0.1% Triton X-100. Sections were washed in 0.1 M PBS solution and incubated in rabbit biotinylated secondary antiserum (1:500, Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. The Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used to link the antigen–antibody complex to horseradish peroxidase, which was then visualized with 3,3-diaminobenzidinetetrahydrochloride histochemistry. Finally, the sections were washed thoroughly in PBS, mounted on plus-slides, air dried, dehydrated in ethanol, cleared in xylene, and cover slipped with DPX mounting media (Sigma-Aldrich, St. Louis, MO).

For double immunofluorescence labeling, sections were incubated with a mixture of primary antibodies, rabbit anti–p-p38 MAPK (1:500, Cell Signaling Technologies, Danvers, MA) antibody and mouse monoclonal antibody, against the microglial-specific cell surface receptor CD11b (clone OX-42,1:250, Serotec Ltd, Raleigh, NC) overnight at 4°C. The sections were washed in 0.1 M PBS and incubated in a mixture of CY3-conjugated donkey anti-rabbit IgG (1:600,

Jackson Immunoresearch) and CY2-conjugated donkey anti-mouse IgG (1:200, Jackson Immunoresearch) for 2 h at room temperature. Then, sections were washed in PBS mounted on plus-slides, air-dried, dehydrated in ethanol, cleared in xylene, and cover slipped with DPX.

Image Analysis and Quantification

Sections were examined with brightfield illumination, and images were captured with a charge coupled device digital camera attached to the microscope using a 10× objective at a resolution of 1,600 × 1,200 pixels. Images of ipsilateral and contralateral L4–5 dorsal horn of sham operated and incision rats were captured. A square with a fixed area (250 × 250 μm^2) covering the region of laminae I-II was randomly positioned in the middle one third of the mediolateral extent of the spinal cord dorsal horn. The number of pixels occupied by IBA1 immunoreactive cells within a defined threshold was measured using image analysis software (Image J; NIH Image, National Institutes of Health, Bethesda, MD). Immunodensity measurements were obtained from a minimum of five spinal cord sections per rat and averaged.

For assessment of spinal levels of p-p38 MAPK, images of p-p38 MAPK immunoreactivity and OX42 immunoreactivity within ipsilateral and contralateral L4–5 dorsal horn of sham-operated and incision rats were sequentially captured using a 20× objective for each section by alternating between filter sets to capture in separate overlapping images CY3 and CY2 labeling, respectively. A square with a fixed area (250 × 250 μm^2) covering the dorsal medial aspects of the spinal cord was randomly positioned in the dorsal horn of the spinal cord images. The number of p-p38 MAPK immunoreactive cells was counted on six randomly chosen sections from each animal and averaged for each animal. The microglial phenotype of p-p38 MAPK immunoreactive cells in each section was confirmed by overlaying the p-p38 MAPK images with the OX42 image. The observer quantifying the sections was blinded to the condition of the sample being quantified.

Statistical Analysis

Statistical analysis was conducted using Sigma Plot (Version 11.0; Systat Software Inc., San Jose, CA) software. The effects of treatment on withdrawal thresholds were examined at each time point using the Kruskal-Wallis test. Significant effects were followed by pairwise comparisons of the mean ranks of the treatment groups at each time point using Student Newman-Keuls *post hoc* test. The effects of treatment over time on withdrawal thresholds were examined using Friedman repeated-measures ANOVA on ranks test. If significant effects were found pairwise, comparisons of the mean ranks at each time point within a treatment group were conducted using Student Newman-Keuls *post hoc* test. If group sizes were not equal, Dunn test was conducted for *post hoc* analysis. Immunocytochemical data were analyzed using a two-way ANOVA, assessing for effects of treatment and side followed by Bonferroni *post hoc* tests for pairwise comparisons. To examine whether markers of microglial activa-

tion (p-p38 MAPK immunoreactivity and IBA1 immunoreactivity) were associated with measures of mechanical hypersensitivity after sham procedure or plantar incision, linear regression analysis was conducted using a nonparametric Spearman ρ test. The criterion for statistical significance for all analysis was $P < 0.05$.

Results

Acute Intrathecal Administration of CCL2 Sequestering Antibody 24 h after Incision Partially Reverses Mechanical Hypersensitivity

To examine the contribution of endogenous CCL2 to mechanical hypersensitivity after surgical incision, we measured the acute behavioral effect of intrathecal anti-CCL2 neutralizing antibody (10 and 3 ng) on mechanical withdrawal thresholds when administered 24 h after plantar incision. Withdrawal thresholds were significantly reduced 24 h after incision compared with baseline values ($P < 0.05$) in all groups examined (fig. 1A). Both doses of anti-CCL2 antibody significantly attenuated mechanical hypersensitivity compared with control IgG-treated rats at 30, 60, 90, and 120 min after administration. At 30 min after administration, the higher dose of anti-CCL2 antibody (10 ng) increased paw withdrawal thresholds significantly greater than the 3-ng dose, indicating a dose-dependent attenuation of mechanical hypersensitivity at this time point (fig. 1A, $P < 0.05$).

Acute Intrathecal Administration of Anti-CCL2 IgG after Plantar Incision Reduces Mechanical Hypersensitivity for Several Days

Because single spinal administration of exogenous CCL2 has been shown to induce long-term persistent changes in microglial activation and pain facilitation,[20,22] we examined the ability of anti-CCL2 neutralizing antibody to reduce mechanical hypersensitivity at later time points after administration. In a separate group of rats, a single administration of 3 or 10 ng anti-CCL2 IgG or control IgG was administered intrathecally 1 day after incision, and rats were assessed for mechanical hypersensitivity on days 2, 3, and 7 after incision (fig. 1B). Both doses of anti-CCL2 IgG significantly reduced mechanical hypersensitivity on days 2 and 3 after incision (1 day and 2 days after administration) compared with control IgG ($P < 0.05$, Kruskal-Wallis followed by Dunn test). The difference in the median values of the treatment groups 7 days after incision was not significantly different compared with values from control IgG-treated rats ($P = 0.264$, Kruskal-Wallis).

Effects of Spinal Blockade of CCL2 on Microglial Activation after Plantar Incision

Spinal microglia are activated after plantar incision as indicated by increases in the cell surface protein CD11b (OX42), up-regulation of the microglial-specific protein IBA1, and increased levels of phosphorylated p38 MAPK. Immunohis-
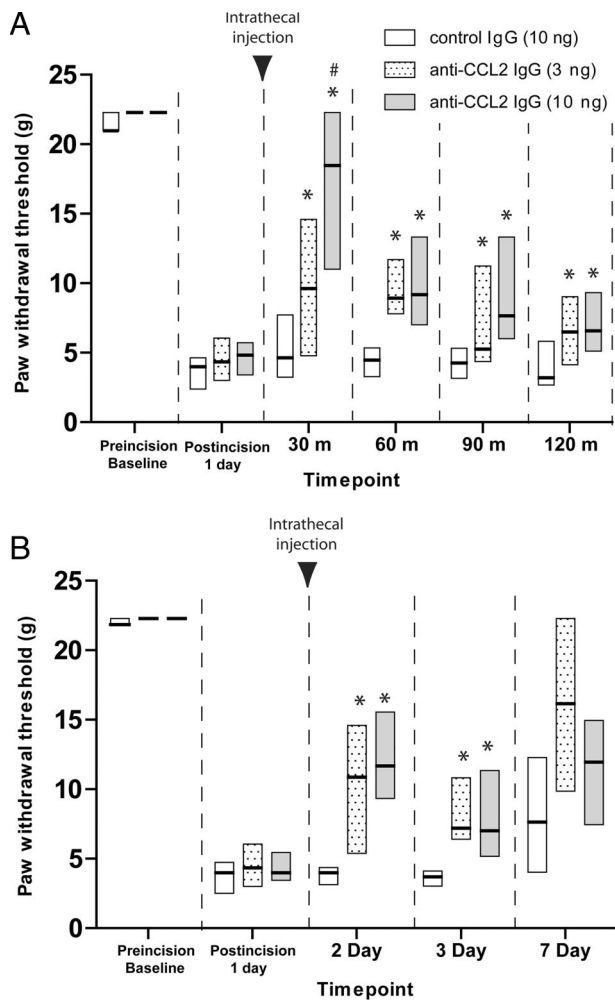
A

B

Fig. 1. Time course of mechanical hypersensitivity induced by paw incision after intrathecal treatment with anti-chemokine (C-C motif) ligand 2 (CCL2) immunoglobulin G (IgG) (3 ng, *dotted bars*; 10 ng, *gray bars*) or control IgG (10 ng, *open bars*) was examined acutely for several minutes after treatment beginning 1 day after surgical incision (*A*) and for several days after single treatment beginning 1 day after surgical incision (*B*). Withdrawal thresholds to von Frey filaments values are expressed as the median (*solid line*) and 25th and 75th percentiles of 8–14 rats per group. * $P < 0.05$ for comparisons to control IgG values at each time point or # $P < 0.05$ for comparison between 3 ng anti-CCL2– and 10 ng anti-CCL2–treated values at each time point by Kruskal-Wallis analysis of variance on ranks followed by Dunn *post hoc* test. *Black arrowhead* indicates time point of intrathecal injection.

tochemical staining of IBA1 was significantly greater in the spinal cord dorsal horn ipsilateral to plantar incision compared with the ipsilateral dorsal horn of rats with sham surgery 24 and 48 h after surgery (figs. 2 and 3, $P < 0.05$). Rats with plantar incision had significantly greater number of IBA1 immunoreactive pixels in the ipsilateral spinal cord dorsal horn 1 day and 2 days postoperatively compared with contralateral values (fig. 3, $P < 0.05$). Microglia in the spinal cord of incised rats exhibited characteristic morphology of activation, including larger cell bodies and thicker processes, compared with the thin and highly ramified processes of

microglia within the spinal cord of rats that underwent sham procedure (fig. 2). We examined the short- and long-term effects of blocking spinal CCL2 on the number of IBA1 immunoreactive pixels beginning 24 h postoperatively after plantar incision. Rats treated with anti-CCL2 IgG *via* percutaneous lumbar intrathecal injections had similar number of IBA1 immunoreactive pixels in the ipsilateral dorsal horn as in control IgG-treated incision rats 30 min after administration (fig. 3A). Conversely, when IBA1 was examined 24 h after spinal anti-CCL2 treatment, the number of pixels was significantly reduced in the ipsilateral dorsal horn compared with incision vehicle-treated rats (fig. 3B). At this later time point, the IBA1 immunoreactive pixel values from ipsilateral dorsal horn of CCL2-treated rats were not significantly different compared with contralateral CCL2-treated values ($P = 0.832$) or values from rats that underwent sham procedures ($P = 0.226$).

We also examined the number of p-p38 MAPK immunoreactive microglia. Spinal cord sections were labeled with antibodies against p-p38 MAPK and the microglial cell surface antigen CD11b (OX42, fig. 4). Immunohistochemistry for both markers was performed in the same sections to confirm colabeling of p-p38 MAPK in microglia (fig. 4, C, F, and I) and allow for cell type-specific quantification. Incision increased the number of p-p38 MAPK microglia ipsilateral to surgery compared with sham-operated rats and compared with the contralateral side 24 and 48 h after incision (fig. 5; $P < 0.05$). Anti-CCL2 IgG did not alter the number of p-p38 MAPK microglia in the ipsilateral dorsal horn 30 min after administration on the first postoperative day compared with vehicle (fig. 5A). Similarly, the number of p-p38 MAPK microglia was significantly greater in ipsilateral *versus* contralateral spinal cord sections of anti-CCL2–treated rats 30 min after treatment (fig. 5A). Twenty-four hours after treatment, the number of p-p38 MAPK immunoreactive microglia was significantly reduced in the ipsilateral dorsal horn of anti-CCL2–treated incision rats compared with control IgG-treated incision rats, although not to the levels in rats that underwent sham procedures treated with control IgG (figs. 4, D and G and 5B).

Correlation of Markers of Microglial Activation to Mechanical Hypersensitivity after Plantar Incision
In a separate group of rats, we conducted behavioral analysis and collected spinal cord tissue 48 h after plantar incision or sham operation and 24 h after spinal treatment with anti-CCL2 antibodies or control IgG. We observed a strong correlation between the number of p-p38 MAPK immunoreactive microglia in the ipsilateral spinal cord and the degree of mechanical hypersensitivity in rats with plantar incision or sham procedure based on linear regression analysis (fig. 6A). There was also a strong correlation between number of IBA1 immunoreactive pixels in the spinal cord and mechanical hypersensitivity at this time point (fig. 6B).
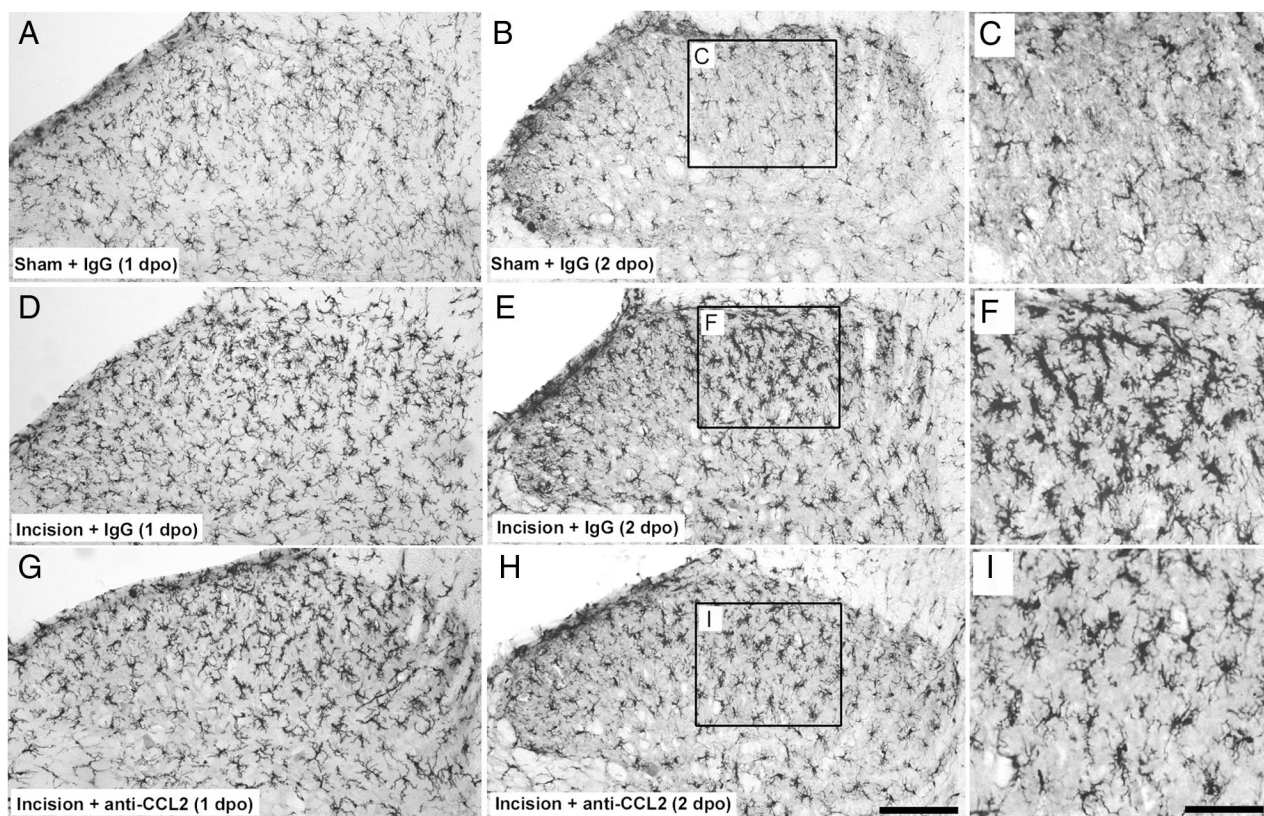
Fig. 2. Representative images of microglial staining with an antibody against ionized calcium-binding adapter molecule 1 (IBA1). Ipsilateral lumbar dorsal spinal cord of rats that underwent sham procedures administered control immunoglobulin G (IgG) (10 ng; *A*, *B*, and *C*) or ipsilateral spinal cord of rats with plantar incision administered 10 ng anti-chemokine (C-C motif) ligand 2 (CCL2) IgG (*G*, *H*, and *I*) or 10 ng control IgG (*D*, *E*, and *F*). Microglial IBA1 immunostaining was increased in the ipsilateral dorsal spinal cord of rats 1 day and 2 days postoperatively (1 dpo and 2 dpo) after plantar incision compared with the levels in rats that underwent sham procedure. A single intrathecal injection of anti-CCL2 IgG (10 ng) administered 1 day after plantar incision reduced the levels of ionized calcium-binding adaptor molecule 1 (IBA1)-immunoreactivity (IR) in the spinal cord of incision rats 24 h after administration (*H* compared with *E*) but not 30 min after administration (*G* compared with *D*) compared with incision rats that received intrathecal control IgG. Insets depict the morphologic differences in microglia 48 h after incision. Not increased size of microglia in control IgG-treated rats *versus* anti-CCL2–treated rats (*F* compared with *I*). Scale bar in *H* = 150 μm and scale bar in *I* = 120 μm.

Discussion

In this study, we provide behavioral evidence that endogenous spinal CCL2 contributes to mechanical hypersensitivity after surgical incision. First, we demonstrate that acute blockade of spinal CCL2 with a neutralizing antibody partially reverses mechanical hypersensitivity within minutes of administration independent of effects on microglial activation. Second, we demonstrate that a single spinal administration of anti-CCL2 antibody attenuates mechanical hypersensitivity for days after incision by inhibiting microglial activation and p38 MAPK signaling. These results carry implications for the mechanisms of neural glial signaling in pain facilitation after incision and for postoperative pain treatment.

CCL2 also known as monocyte chemotactic protein 1 is a small 14-kDa protein that signals through the G-protein coupled receptor CCR2. CCL2, similar to other chemokines, was initially identified as an immunomodulatory factor that regulates activation and migration of peripheral immune cells. Recently, it has been shown to have a neuromodulatory role in spinal nociceptive processing (for review see Ref. 23). CCL2 messenger RNA and protein are constitutively expressed in a population of predominantly small and medium-sized neurons within the dorsal root ganglia.[22] CCL2 immunoreactivity colocalizes with markers of peptidergic[20,22] and nonpeptidergic[20,24] primary afferents in the dorsal root ganglia and their central terminals within the dorsal horn of the spinal cord.[25] Under normal conditions, CCL2 is not present at detectable levels within nonneuronal cells, including microglia and astrocytes, of the rat spinal cord.[22,26,27] However, CCL2 may be up-regulated in sensory neurons of rats after peripheral nerve injury and inflammation[20,22,25–29] and has also been shown to be up-regulated within spinal cord astrocytes after chronic constriction injury of the sciatic nerve in mice.[30]

CCR2 receptors have been localized in the spinal cord based on autoradiographical binding studies that reported dense binding of radiolabeled CCL2 in the dorsal horn (laminae I–IV).[29] There is some debate regarding the cell type expression of CCR2 within the spinal cord, and immunohistochemical and transgenic approaches have provided evidence of CCR2 predominantly in microglia and macrophage,[31,32]
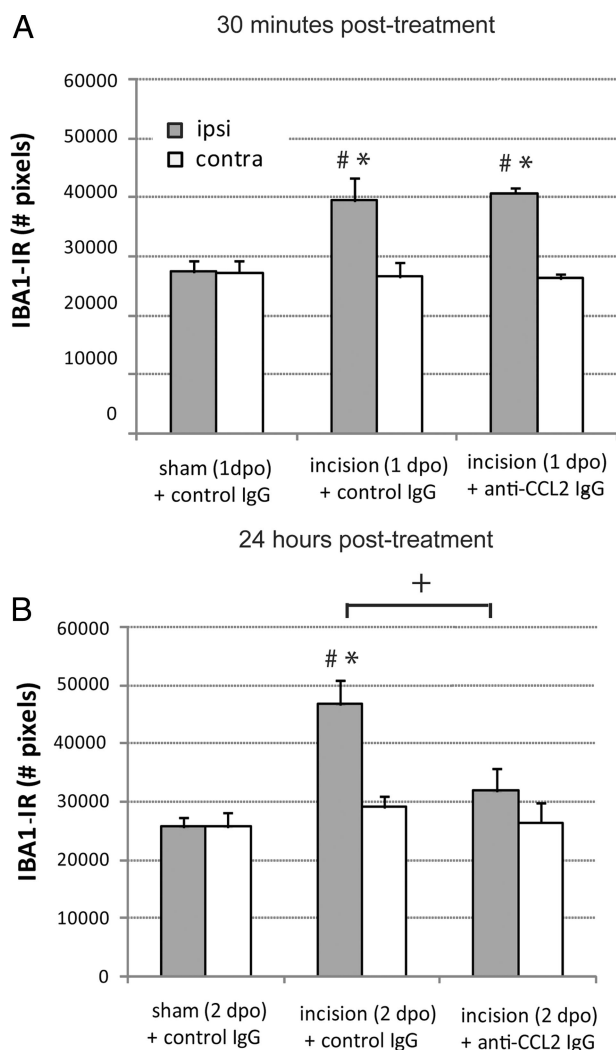
A
30 minutes post-treatment

B
24 hours post-treatment

Fig. 3. Quantification of ionized calcium-binding adapter molecule 1 (IBA1) immunostaining in ipsilateral (ipsi) (*gray bar*) and contralateral (contra) (*open bar*) dorsal spinal cord of sham-operated and incision rats 30 min after intrathecal administration of anti-chemokine (C-C motif) ligand 2 (CCL2) (10 ng) or control immunoglobulin G (IgG) (10 ng) on day 1 postoperatively (1 dpo; *A*) or 24 h after administration of anti-CCL2 (10 ng) or control IgG (10 ng) on day 2 postoperatively (2 dpo; *B*). * $P < 0.05$ compared with sham + control IgG value within side. # $P < 0.05$ compared with contralateral side within treatment group. + $P < 0.05$ compared with incision control IgG-treated values by two-way analysis of variance with Bonferroni *post hoc* test (n = 8 rats per group).

whereas other studies report it predominantly in second-order neurons and terminals of primary afferents.[30,33] Therefore, in the context of postoperative pain state, primary afferent-derived CCL2 may have actions on both neuronal and nonneuronal cell populations within the spinal cord.

In this study, we provide evidence that endogenous spinal CCL2 contributes, in part, to mechanical hypersensitivity after surgical incision in the rats. In support of our findings, there is substantial evidence that endogenous CCL2 and CCR2 are involved in the development of mechanical hypersensitivity in acute and persistent pain states. CCR2 antagonists attenuate mechanical hypersensitivity in neuropathic

pain models,[20,28,30] and transgenic mice lacking CCR2 do not develop mechanical hypersensitivity after partial sciatic nerve ligation.[32] In two recent studies, intrathecal administration of a CCL2-neutralizing antibody attenuated ipsilateral mechanical allodynia for several days after chronic constriction injury in rats[20] and transiently reversed mechanical hypersensitivity after spinal nerve ligation in mice.[30] In acute inflammatory pain states, CCR2 knockout mice exhibited decreased phase II formalin-induced nociceptive behavior and a modest attenuation of mechanical allodynia induced by hind paw injection of complete Freund's adjuvant. Spinal administration of exogenous CCL2 in naïve rats induces mechanical allodynia, which is sustained for several days after single injection,[20,22] suggesting long-term effects of CCL2 on spinal pain processing.

In this study, we provide evidence that microglial mechanisms may be relevant to CCL2-induced central sensitization in the postoperative pain setting. We observed a reduction in IBA1 immunoreactivity and a reduced number of p-p38 MAPK immunoreactive microglia in the ipsilateral spinal cord of rats with plantar incision 24 h after administration of neutralizing anti-CCL2 antibody. Consistent with our findings, there is substantial evidence in support of a microglial-mediated mechanism contributing to CCL2s pro-hypersensitivity effects. A causal relationship between microglial activation and CCR2-mediated signaling was initially suggested based on the observations that up-regulation of CCL2 in primary afferent nerve terminals correlates with the spatial and temporal profile of activated microglia within the spinal cord after peripheral nerve injury,[27] and exogenous administration of CCL2 in the spinal cord of naïve rats induces extensive spinal microglial activation at the site of injection[20] as indicated by increased OX42 immunoreactivity. In addition, spinal CCR2 antagonists attenuate microglial activation (reduce OX42 immunoreactivity) after peripheral nerve injury,[20] and CCR2 knockout mice have a reduced number of p-p38 MAPK-positive microglia after partial sciatic nerve injury compared with wild-type controls.[32] Similar to our results, Wen *et al.* observed a significant increase in the number of p-p38 MAPK immunoreactive microglia 1 day and 2 days after hind paw incision in rats. In their study, a single intrathecal injection of the p38 inhibitor ZR167653 before incision attenuated mechanical hypersensitivity and reduced the number of p-p38 MAPK microglia after incision. These results and our findings showing a strong correlation between measures of mechanical hypersensitivity and the number of p-p38 MAPK microglia in the ipsilateral spinal cord of treated rats (fig. 6) suggest that microglial signaling *via* p38 MAPK is important for the persistence of mechanical hypersensitivity after surgical incision. In contrast to our findings, Ito *et al.*[34] reported that single systemic administration of the microglial inhibitor minocycline only slightly attenuated mechanical hypersensitivity when administered acutely 1 day after plantar incision. In the same study, intrathecal administration of the p38 inhibitor SB203580 1 day and 3 days after incision was without effect. Chronic admin-
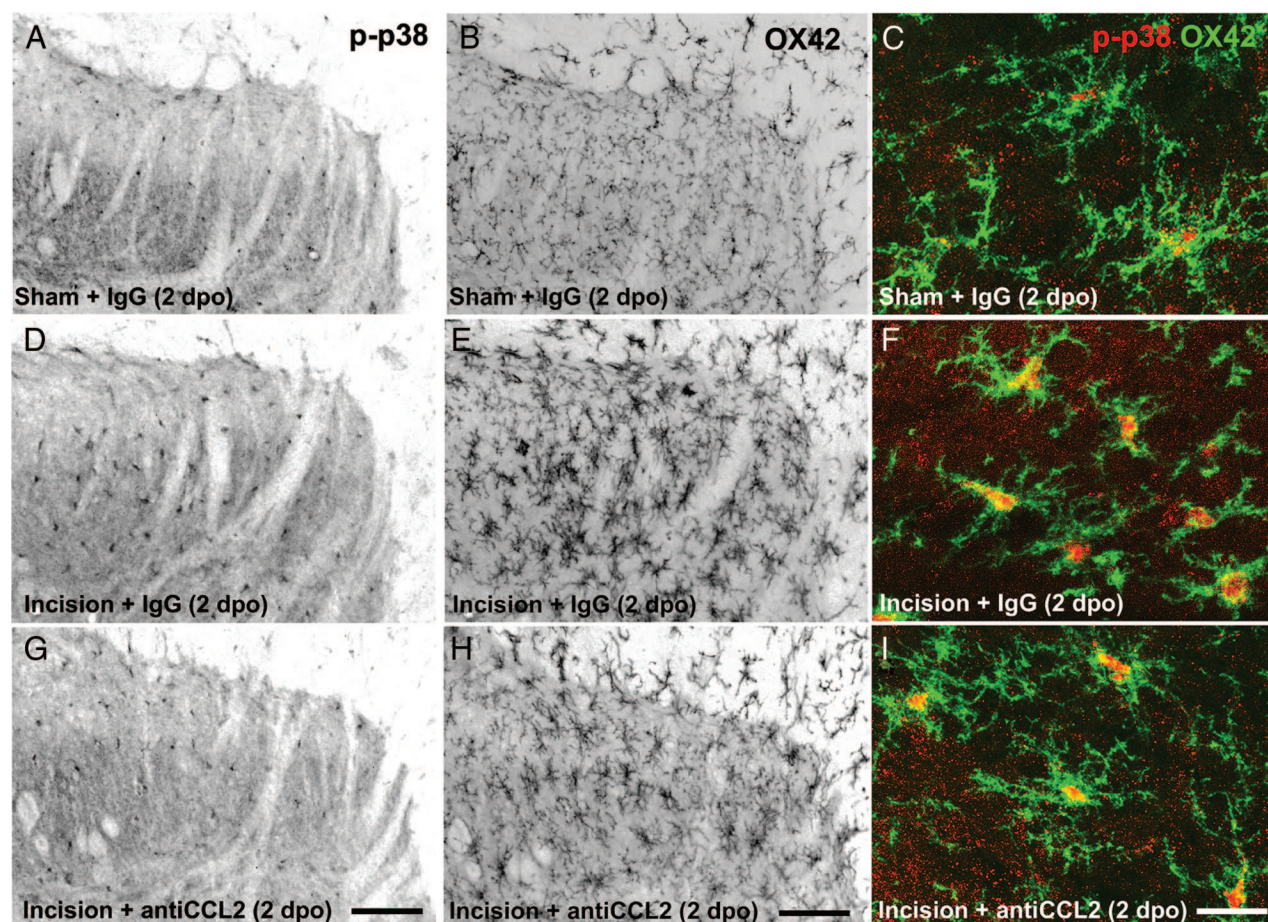
Fig. 4. Representative images of phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) labeling (*A*, *D*, and *G*) and Cd11b (OX42; *B*, *E*, and *H*) in the ipsilateral dorsal spinal cord of sham-operated (*A*, *B*, and *C*) and incision rats treated with 10 ng control immunoglobulin G (IgG) (*D*, *E*, and *F*) or 10 ng anti-chemokine (C-C motif) ligand 2 (CCL2) IgG (*G*, *H*, and *I*) rats 2 days postoperatively (2 dpo) after plantar incision. Rats received a single acute administration of anti-CCL2 IgG (10 ng) on day 1 after plantar incision. High-power confocal images show colocalization of p-p38 MAPK (*red*) and OX42 (*green*). Increased levels of cytoplasmic p-p38 MAPK in OX42 immunoreactive microglia of incision rats treated with control IgG compared with sham-operated rats (*F* compared with *C*) and incision rats treated with anti-CCL2 IgG (*F* compared with *I*). Fluorescent images in panels *A*, *B*, *D*, *E*, *G*, and *H* were inverted in Photoshop (Adobe Systems Inc., San Jose, CA) and converted to grayscale to enhance contrast. Scale bars in *G* and *H* = 75 μm and scale bar in *I* = 5 μm.

istration of minocycline twice daily for 3 days failed to reduce mechanical hypersensitivity despite producing a reduction in OX42 immunoreactive microglia by day 3 after incision. Although these data suggest that microglia may have a minor role in postoperative pain, it is also possible that the systemic doses of minocycline and p38 inhibitor used in this former study were insufficient to block phosphorylation of p38 MAPK, which seems to be essential for the development and maintenance of mechanical hypersensitivity in this postoperative pain model.[14] The effects of systemic minocycline on the levels of p-p38 MAPK were not examined in the former study. This may be an important distinction because OX42 immunoreactivity does not parallel the development of mechanical hypersensitivity after incision unlike the up-regulation of p-p38 MAPK and IBA1 in microglia, which has been observed as early as 1–24 h after plantar incision. Several studies suggest that microglial changes in IBA1 immunoreactivity or levels of p-p38 MAPK in microglia may be a more functionally relevant marker of microglial activation particularly in acute pain states.[10,35,36]

Interestingly, in this study, spinal neutralization of CCL2 rapidly attenuated mechanical hypersensitivity within 30 min of administration but failed to reduce the number of p-p38 MAPK immunoreactive microglia or IBA1 immunoreactivity at this acute time point. There are several explanations for the lack of acute effect on microglial activation. First, it is possible that the immunohistochemical approaches used in this study are not sensitive enough to detect acute effects of treatment. However, several studies have observed acute effects of pharmacologic agents on p-p38 MAPK immunoreactivity in the peripheral and central nervous system.[37] Another possibility is that CCL2 contributes to mechanical hypersensitivity at early time points by direct actions on spinal cord neurons in addition to the delayed effects on microglia that we observed at 48 h after incision. There is increasing *in vitro* and *in vivo* evidence that CCL2 directly excites or sensitizes neurons in the superficial laminae of the spinal cord and dorsal root ganglia. Spinal administration of exogenous CCL2 *in vivo* induces phosphoryla-
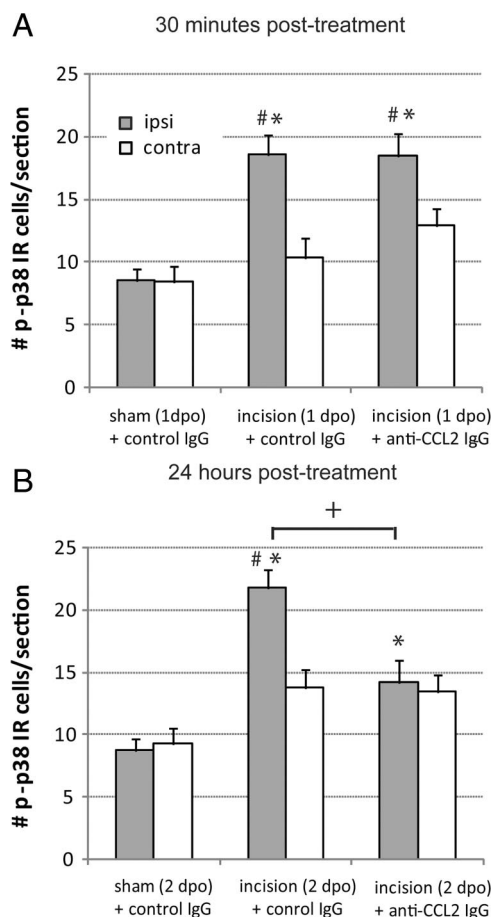
Fig. 5. Quantification of microglial phosphorylated p38 mitogen-activated protein kinase immunostaining in ipsilateral (ipsi) (*gray bar*) and contralateral (contra) (*open bar*) dorsal spinal cord of sham-operated and incision rats 30 min after intrathecal administration of anti-chemokine (C-C motif) ligand 2 (CCL2) (10 ng) or control immunoglobulin G (IgG) (10 ng) on day 1 postoperatively (1 dpo; *A*) or 24 h after administration of anti-CCL2 (10 ng) or control IgG (10 ng) on day 2 postoperatively (2 dpo; *B*). * $P < 0.05$ compared with sham + control IgG value within side. # $P < 0.05$ compared with contralateral side within the treatment group. + $P < 0.05$ compared with incision control IgG-treated values by two-way analysis of variance with Bonferroni *post hoc* test (n = 8 rats per group).

tion of extracellular signal regulated kinase in neurons within the superficial dorsal horn and *in vitro* after application to spinal cord slices.[30] Patch clamp recordings of lamina II spinal cord neurons have been conducted in slice preparations to examine direct functional effects of CCL2. Bath application of CCL2 increased excitatory synaptic transmission as indicated by an increased frequency and amplitude of spontaneous excitatory postsynaptic currents.[30] Whole cell patch clamp recordings of cultured spinal neurons showed that CCL2 inhibits γ-amino butyric acid–induced currents, suggesting a neuromodulatory role for this chemokine.[29] The presence of CCL2 in primary afferents fibers and localization of CCR2 on sensory neurons suggest that CCL2 may act in an autocrine or paracrine manner on the central terminals of primary afferent fibers to promote the release of neurotransmitters into the spinal cord. In support of this, CCL2 has
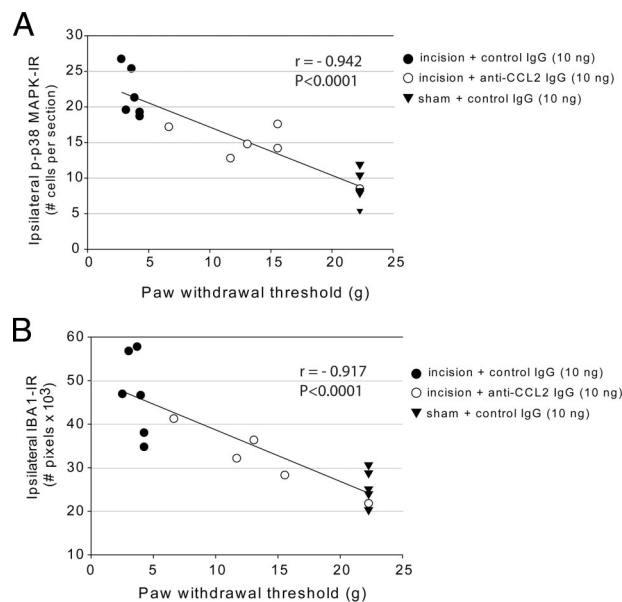

Fig. 6. Correlation between spinal markers of microglial activation and behavioral measures of mechanical hypersensitivity 2 days after surgical incision or sham procedure. The association between number of microglial ionized calcium-binding adaptor molecule 1 immunoreactive (IBA1-IR) pixels (*A*) or number of microglia positive for phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) (*B*) and mechanical paw withdrawal thresholds (g) were examined using Spearman rho nonparametric linear regression analysis. CCL2 = chemokine (C-C motif) ligand 2; IgG = immunoglobulin G.

been shown to trigger the release of calcitonin gene-related peptide from cultured dorsal root ganglia neurons.[38] It is not known whether similar mechanisms occur after surgical incision. Clearly, additional research is warranted to more precisely define the mechanisms by which CCL2 modulates nociceptive processing in the context of postoperative pain.

In summary, we administered a CCL2-neutralizing antibody beginning 1 day after plantar incision in the rat after the postoperative pain state was established. By using this posttreatment paradigm, we observed that spinal blockade of CCL2 attenuated mechanical hypersensitivity within minutes of administration, an effect that was sustained for several days. These findings establish a role for spinal CCL2-mediated signaling in the maintenance of mechanical hypersensitivity after surgical incision and suggest that spinal CCL2 antagonists may have use for treating postoperative pain.

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