

# Chemokine (C-C motif) Receptor 5 Is an Important Pathological Regulator in the Development and Maintenance of Neuropathic Pain

Katsuyuki Matsushita, M.D., Hidetoshi Tozaki-Saitoh, Ph.D., Chinami Kojima, B.S., Takahiro Masuda, Ph.D., Makoto Tsuda, Ph.D., Kazuhide Inoue, Ph.D., Sumio Hoka, M.D., Ph.D.

## ABSTRACT

**Background:** The chemokine family has been revealed to be involved in the pathogenesis of neuropathic pain. In this study, the authors investigated the role of chemokine (C-C motif) ligand 3 and its receptors chemokine (C-C motif) receptor 1 and chemokine (C-C motif) receptor (CCR) 5 in neuropathic pain.

**Methods:** A spinal nerve injury model was established in adult male Wistar rats. The von Frey test and hot plate test were performed to evaluate neuropathic pain behavior, and real-time quantitative reverse transcription polymerase chain reaction, *in situ* hybridization, and immunohistochemistry were performed to understand the molecular mechanisms.

**Results:** The expression levels of chemokine (C-C motif) ligand 3 and CCR5 messenger RNA in the spinal cord were up-regulated after nerve injury, which was possibly due to CD11b-positive microglia. Single intrathecal administration of recombinant chemokine (C-C motif) ligand 3 produced biphasic tactile allodynia; each phase of pain behavior was induced by different receptors. Intrathecal injection of CCR5 antagonist suppressed the development of tactile allodynia ( $12.81 \pm 1.33$  g *vs.*  $3.52 \pm 0.41$  g [mean  $\pm$  SEM, drug *vs.* control in paw-withdrawal threshold];  $P < 0.05$ ,  $n = 6$  each) and could reverse established tactile allodynia ( $10.87 \pm 0.91$  g *vs.*  $3.43 \pm 0.28$  g;  $P < 0.05$ ,  $n = 8$  and 7). Furthermore, Oral administration of CCR5 antagonist could reverse established tactile allodynia ( $8.20 \pm 1.27$  g *vs.*  $3.18 \pm 0.46$  g;  $P < 0.05$ ,  $n = 4$  each).

**Conclusions:** Pharmacological blockade of CCR5 was effective in the treatment of the development and maintenance phases of neuropathic pain. Thus, CCR5 antagonists may be potential new drugs for the treatment of neuropathic pain. (ANESTHESIOLOGY 2014; 120:1491-503)

NEUROPATHIC pain is a debilitating pain state, which is often caused by injury to the nervous system arising from bone compression in cancer, diabetes mellitus, infection, autoimmune disease, or physical injury.<sup>1</sup> From general population studies, neuropathic pain affects up to 5% of the population.<sup>2,3</sup> Hallmark symptoms of neuropathic pain include tactile allodynia and thermal hyperalgesia, which are refractory to currently available treatments such as nonsteroidal antiinflammatory drugs and opioids.<sup>4,5</sup> The mechanism underlying the development of neuropathic pain remains largely unknown. Therefore, unraveling the molecular and cellular basis of the development and maintenance of neuropathic pain is essential for the discovery of new treatments.

Accumulating evidence from studies using animal models of neuropathic pain indicates that neuropathic pain is a reflection of aberrant excitability of dorsal horn neurons evoked by peripheral sensory input.<sup>6,7</sup> This hyperexcitability might result from multiple cellular and molecular alternations in the dorsal horn after nerve injury. It has been suggested that there are relevant changes in neurons, but recent

### What We Already Know about This Topic

- Chemokines, small heparin-binding proteins, are up-regulated in the spinal cord after peripheral nerve injury
- Their role in the development of nerve injury-induced neuropathic pain is incompletely understood

### What This Article Tells Us That Is New

- In a spinal nerve ligation injury model, neutralization of the chemokine (C-C motif) ligand 3 or administration of an antagonist of its receptor chemokine (C-C motif) receptor 5 attenuated development of early- and late-phase tactile allodynia
- Antagonists of chemokine (C-C motif) receptor 5 may be a novel therapeutic approach to the treatment of neuropathic pain

studies provide convincing evidence that spinal microglia, immune-like glial cells in the central nervous system (CNS), respond to peripheral nerve injury (PNI). These cells have been suggested to become morphologically activated, increase their number, and express a variety of genes required for inflammatory responses.<sup>8-12</sup>

Chemokines are small heparin-binding proteins with conserved cysteine residues in their amino acid sequences. The

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site ([www.anesthesiology.org](http://www.anesthesiology.org)). The first two authors contributed equally to this work (K.M. and H.T.-S.).

Submitted for publication July 17, 2013. Accepted for publication January 28, 2014. From the Department of Anesthesiology and Critical Care Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan (K.M., S.H.); and Department of Molecular and System Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan (H.T.-S., C.K., T.M., M.T., K.I.).

Copyright © 2014, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2014; 120:1491-503

approximately 50 human chemokines are classified into four families (CC, CXC, CX3C, and XC family) on the basis of the presence and position of the first cysteine residue. Animal models of pain studies have revealed that several molecules of the chemokine family are involved in the pathogenesis of neuropathic and inflammatory pain.<sup>13–20</sup> Chemokine (C-C motif) ligand (CCL) 2 was detected in the soma and processes of dorsal root ganglion neurons, and intrathecal administration of exogenous CCL2 induced rapid and sustained thermal hyperalgesia and mechanical allodynia through chemokine (C-C motif) receptor (CCR) 2.<sup>20</sup> CCL21 was also detected in the soma and processes of injured dorsal root ganglion neurons and was transported into the dorsal horn, which participates in the up-regulation of P2X4 receptors in spinal microglia.<sup>21</sup> Chemokine (C-X3-C motif) ligand 1, which is liberated by the microglia-derived protease cathepsin S from neuronal cell surface activated chemokine (C-X3-C motif) receptor 1 on microglia, leads to further release of microglial mediators to establish the chronic pain state.<sup>14</sup> In the peripheral site, CCL4 correlates with neuropathic pain behavior after sciatic nerve injury in mice. CCL4 messenger RNA (mRNA) was significantly up-regulated in the injured nerve and neutralizing CCL4 in the injured nerve attenuated mechanical allodynia (not thermal hyperalgesia).<sup>17</sup> CCL3 was also found to be up-regulated in the peripheral and CNS after PNI and contributed to chronic pain.<sup>22,23</sup> CCL3 and CCL4 share the receptors CCR1 and CCR5. These studies propose that the chemokine receptors might be the therapeutic targets for neuropathic pain in the periphery. However, the roles of these ligands and receptors in the spinal cord remain unknown. In the current study, we sought to find the detailed role of CCL3 and its interaction with the receptors CCR1 and CCR5 in neuropathic pain and to determine their therapeutic potential in disturbing CCL3 signaling in the mechanism of tactile allodynia and thermal hyperalgesia.

## Materials and Methods

### Animals

Male Wistar rats (weighing 250 to 270 g; Japan SLC, Shizuoka, Japan) were housed in individual cages at a temperature of 22° ± 1°C with a 12-h light–dark cycle (light on 8:00 to 20:00) and fed food and water *ad libitum*. All animal experiments were conducted according to the relevant national and international guidelines and the “Act on Welfare and Management of Animals” (Ministry of Environment of Japan, Chiyoda-ku, Tokyo, Japan), “Regulation of Laboratory Animals” (Kyushu University, Higashi-ku, Fukuoka, Japan), and “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health, Bethesda, Maryland). All protocols were approved by the Institutional Animal Care and Use committee review panels at Kyushu University (Fukuoka, Fukuoka, Japan).

### Neuropathic Pain Model

We used the spinal nerve injury model<sup>24</sup> with some modifications.<sup>11,25</sup> In brief, a small left-side incision at L3–S1 was made with isoflurane (2% [v/v]) anesthesia. The paraspinal muscle and fat were removed from the L6 traverse process, and part of this traverse process was removed to expose the parallel-lying L4 and L5 spinal nerves. The left L5 spinal nerve was tightly ligated with 5-0 silk and cut just distal to the ligature. The wound and the surrounding skin were sutured with 3-0 silk.

### Real-time Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

Rats were deeply anesthetized with pentobarbital (100 mg/kg, intraperitoneally), perfused transcardially with phosphate buffered saline (PBS), and the L5 spinal cord was immediately removed and separated half at middle of the spinal cord, so that a sample contained the dorsal and ventral horn of the ipsilateral or contralateral side to the PNI. Total RNA from the L5 spinal cord was extracted using Trisure (Bioline, Danwon-Gu, Korea) according to the manufacturer’s protocol. For sorting CD11b-positive cells, the removed half of the L5 spinal cord was digested with Liberase DL enzyme mix (2 Wünsch units/ml in hank balanced salt solution; Roche, Basel, Switzerland) for 20 min at 37°C, triturated gently, incubated with 1 mg/ml DNase I (Roche) for 5 min at 37°C, triturated, filtered through a 70-µm cell strainer, and centrifuged (600g, 4°C) for 5 min. Myelin debris was removed by magnetic separation using Myelin Removal Beads II and MACS LS column (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s protocol. After cells were washed and resuspended in PBS with 1% (v/v) fetal bovine serum, cells were incubated with anti-rat CD11b/c antibody conjugated to allophycocyanin (1:100; BioLegend, San Diego, CA) on ice. Six thousand CD11b/c-positive single cells, which is approximately 70 to 100% of total CD11b/c-positive cells isolatable from our procedure, were counted and isolated in each samples by FACS Aria III (BD Biosciences, San Jose, CA). Total RNA from sorted cells were extracted and purified with the NucleoSpin RNA XS kit (Takara, Otsu, Japan). First-strand complementary DNA was reverse transcribed with Prime Script reverse transcriptase (Takara). Quantitative reverse transcription polymerase chain reaction was carried out using a 7500 real-time polymerase chain reaction analysis system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol, and the data were analyzed using the 7500 System SDS Software 1.3.1 (Applied Biosystems). All values except for the sorted samples were normalized to glyceraldehyde-3-phosphate dehydrogenase. Expression values from sorted cells were normalized to the total cell number in the sample (6,000 cells). Results of reverse transcription polymerase chain reaction analysis were shown in figure 1, and sequences for the TaqMan probe and forward and reverse primers used in this study were designed as indicated in table 1.

### Fluorescent In Situ Hybridization (ViewRNA Assay)

Rat CCL3 mRNA expression was detected using the QuantiGene® ViewRNA tissue assay (Affymetrix Panomics, Fremont, CA) according to the manufacturer's instructions with optimal conditions of boiling in Affymetrix pretreatment solution and protease digestion. Rat CCL3 probes were purchased from Affymetrix Panomics (cat. No. VC1-11302). In brief, rat lumbar spinal cord tissues were fixed in 4% (w/v) PBS-buffered paraformaldehyde and embedded in OCT Compound (SAKURA finetek, Tokyo, Japan) and sectioned into 12- $\mu$ m thick sections. The tissue slides were treated with protease at 40°C for 20 min. The RNA probe was used at a 1:40 dilution and was incubated with sample at 40°C for 120 min. After washing, the RNA/probe complex was hybridized with preamplifier, amplifier, and alkaline phosphatase-conjugated oligonucleotides at 40°C for 25, 15, and 15 min, respectively. After the removal of free alkaline phosphatase-conjugated oligonucleotide by washing in PBS, the slides were incubated with Fast Red substrate at room temperature for 30 min. The tissue images were acquired with a confocal microscope (LSM 5 Pascal; Carl Zeiss, Jena, Germany) and analyzed using the Zeiss LSM Image Browser (Carl Zeiss).

### Behavioral Tests

**Measurement of Mechanical Paw-withdrawal Threshold (von Frey Test).** Tactile allodynia was evaluated using the von Frey test. Rats were placed individually in a wire mesh cage and habituated for 30 to 60 min to allow acclimatization to the new environment.<sup>11,25</sup> Calibrated von Frey filaments (0.4 to 15 g; Stoelting, Wood Dale, IL) were applied to the plantar surface of the rat hind paw from below the mesh floor. The 50% paw-withdrawal threshold (PWT) was determined using the up-down method.<sup>26</sup>

### Measurement of Thermal Hyperalgesia (Hot Plate Test).

Thermal hyperalgesia was evaluated using the hot plate test. Rats were placed individually on a hot plate metallic surface maintained at 52°C, and the time between placement of the animal on the hot plate and the occurrence of either licking or shaking of each hind paw was recorded as the reaction time (s). The cutoff time was assessed at 60 s. The measurements were repeated three times at 30-min intervals, and the average of the measurements was used in the final analysis.

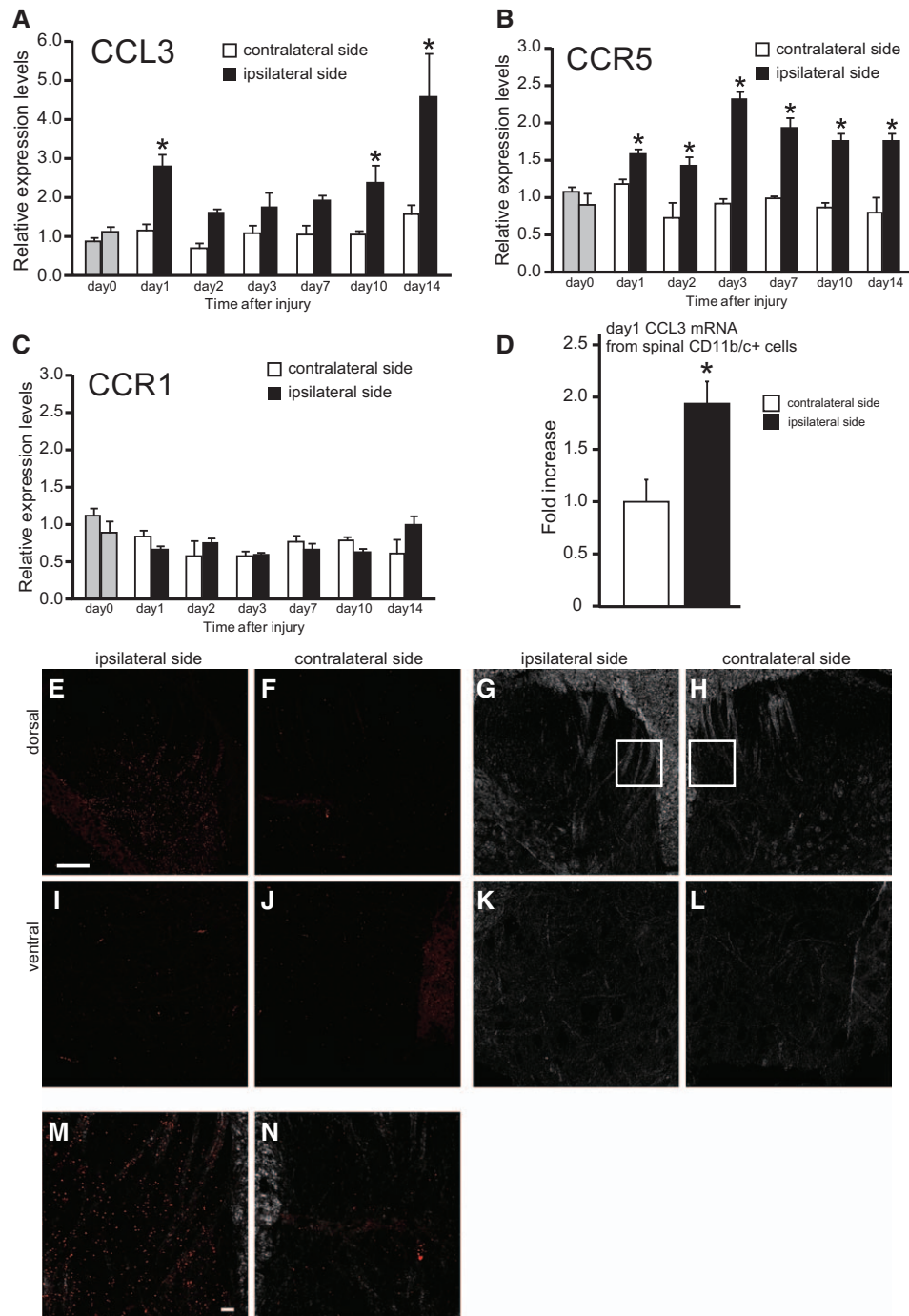
**Intrathecal Drug Administration.** For intrathecal drug administration, with isoflurane (2% [v/v]) anesthesia, a 32-gauge intrathecal catheter (ReCathCo, Allison Park, PA) was inserted through the atlanto-occipital membrane into the lumbar enlargement and externalized through the skin.<sup>25</sup> Four days after catheterization, the catheter placement was verified by the observation of hind limb paralysis after intrathecal injection of lidocaine (2% [v/v], 5  $\mu$ l). Animals that failed to display paralysis by lidocaine were not included in the experiments. The below-mentioned experiments were performed at least 2 to 3 days after the lidocaine test.

**Behavioral Test Procedure.** First, rats were injected with recombinant CCL3 (rCCL3, 0.1 and 10 fmol/10  $\mu$ l in PBS; Peprotech, Rocky Hill, NJ) once through the catheter, and behavioral measurements were carried out 30 min before, 30, 60, 120, 180, 240, 300 min, and 1, 3, 5, 7, 14 days after drug administration (fig. 2). For examining the effect of CCR1 or CCR5 antagonists on rCCL3-induced tactile allodynia, preinjection (30 min before intrathecal injection of rCCL3) of the selective CCR1 antagonist BX513 hydrochloride (0.01, 0.1, and 1 pmol/10  $\mu$ l; Tocris Bioscience, Bristol, United Kingdom) or the selective CCR5 antagonist maraviroc (Selzentry, Celsentri) (1, 10, and 100 pmol/10  $\mu$ l; Sellek, Houston, TX) was performed. In another experiment, BX513 hydrochloride was injected 1 day after CCL3 injection, and behavioral measurements were carried out as described above (fig. 3).

For examining the effect of CCL3-neutralizing antibody, CCR1, or CCR5 antagonists on development of neuropathic pain, the unilateral L5 spinal nerve of rats was injured as described above, and rats were intrathecally injected with CCL3-neutralizing antibody (20 ng in 10  $\mu$ l; Roche) once a day from 1 day before PNI to 3 days after PNI or either maraviroc or BX513 hydrochloride once a day from day 0 (just before nerve injury) up to day 6. The behavioral measurements were carried out before PNI and on days 1, 3, 5, and 7 (individual measurements carried out before daily injection of the drugs to prevent observing acute effects of the drug injection) (fig. 4). Furthermore, for examining the effect of CCL3-neutralizing antibody, BX513 hydrochloride or maraviroc on the established tactile allodynia, CCL3-neutralizing antibody, BX513 hydrochloride, or maraviroc were injected from day 7 to day 13 after PNI. Behavioral measurements were carried out 7 days after PNI, 30, 60, 120, 180, 240, 300 min, and 1, 3, 5, 7 days after first drug administration (fig. 5, A–C). Single maraviroc injections were also performed after 7 days after PNI, and behavioral measurements were carried out for 3 days after the injection (8, 9, and 10 days after PNI; fig. 5D). The hot plate test was carried out before PNI, 3 and 7 days after PNI, or 3 and 24 h after drug administration in animals that received PNI 7 days before the injection (fig. 6).

For examination of oral administration of maraviroc on existing tactile allodynia, maraviroc (100 mg/kg, suspended in 0.5% [w/v] carboxymethyl cellulose) was orally administered (by gavage) 7 days after PNI, and behavioral measurements were carried out 30, 60, 120, 180, 240, 300, 360 min, and 1, 2, 3, 5 days after drug administration (fig. 7).

**Immunohistochemistry.** Rats were deeply anesthetized with pentobarbital (100 mg/kg, intraperitoneally) and perfused transcardially with 100 ml of PBS, followed by 150 ml of ice-cold 4% (w/v) paraformaldehyde/PBS. The L5 segment of the spinal cord was removed, postfixed in the same fixative for 3 h at 4°C, and placed in 30% (w/v) sucrose solution for 24 h at 4°C. The transverse spinal cord sections (30  $\mu$ m)



**Fig. 1.** Expression levels of chemokine (C-C motif) ligand 3 (CCL3), chemokine (C-C motif) receptor 1 (CCR1), and chemokine (C-C motif) receptor 5 (CCR5) messenger RNA (mRNA) in the spinal cord after injury to the L5 spinal nerve. Total RNA extracted from the rat spinal cord was subjected to real-time quantitative reverse transcription polymerase chain reaction analysis for CCL3 (A), CCR5 (B), and CCR1 (C) mRNA expression after peripheral nerve injury. *Bar graphs* show the average fold increase in the level of mRNA expression in spinal cord sagittal hemisections compared with the mean expression level of mRNA in naive (day 0) animals. Each measurement was normalized to glyceraldehyde-3-phosphate dehydrogenase content. Data are the means  $\pm$  SEM of five individual animals ( $*P < 0.05$ ; vs. the naive spinal cord, one-way ANOVA *post hoc* Tukey test). CD11b/c-positive cells sorted from day 1 spinal cord (6,000 cells from each sample) were also subjected to real-time quantitative reverse transcription polymerase chain reaction analysis of CCL3 transcript (D). Data are the means  $\pm$  SEM of nine individual animals ( $*P < 0.05$ , paired *t* test). Fluorescent *in situ* hybridization for CCL3 mRNA was performed using ViewRNA assay to visualize the transcript localization in day 1 spinal cord tissue (E–N). Spotted fluorescent signal of Fast Red represents CCL3 mRNA localization (E, F, I, J) and corresponding transmitted light images (G, H, K, L) are shown. High-magnification images of E with G and F with H indicated by the *white square region* in G and H are represented in M and N. *Scale bars* indicate 100  $\mu$ m (E–L) and 20  $\mu$ m (M and N).

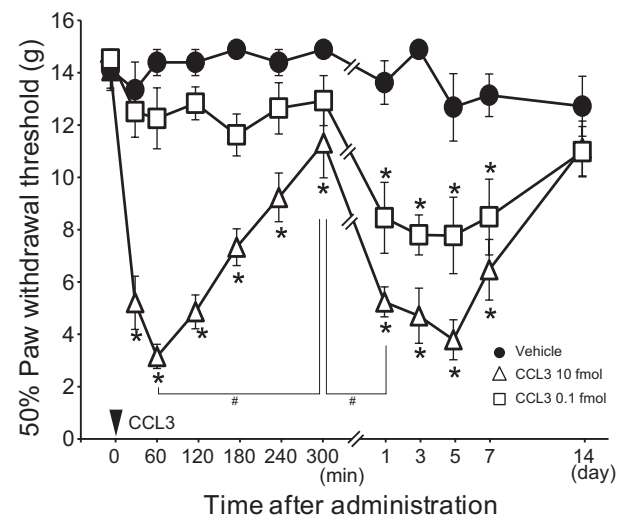
**Table 1.** Sequences of Probes and Primers for Real-time Quantitative Reverse Transcription Polymerase Chain Reaction

| Gene Name     | Probe or Primer | Sequence                                     |
|---------------|-----------------|--|
| <i>ccl3</i>   | Probe           | 5'-FAM-CGCCATATGGAGCTGACACCCCG-TAMRA-3'      |
|               | Forward primer  | 5'-CCACTGCCCTTGCTGTTCTT-3'                   |
|               | Reverse primer  | 5'-GCAAAGGCTGCTGGTTTCAA-3'                   |
| <i>ccr1</i>   | Probe           | 5'-FAM-CCTGGGCTTATACAGTGAGATCTTC-TAMRA-3'    |
|               | Forward primer  | 5'-CTAAGATGGCTAGGGCCCAAATA-3'                |
|               | Reverse primer  | 5'-TCCCTGAGGGCCCGAACTGTCA-3'                 |
| <i>ccr5</i>   | Probe           | 5'-FAM-CAGGCAATGCAGGTGACAGA-TAMRA-3'         |
|               | Forward primer  | 5'-GACCGGTATAGACTGAGCTTACAC-3'               |
|               | Reverse primer  | 5'-ACTCTTGGGATGACACACTGCTGCCTC-3'            |
| <i>gapdh</i>  | Probe           | 5'-FAM-ACCACCACTGCTTAGCCCCCTG-TAMRA-3'       |
|               | Forward primer  | 5'-TGCCCCCATGTTTGTGATG-3'                    |
|               | Reverse primer  | 5'-GGCATGGACTGTGGTCATGA-3'                   |
| $\beta$ actin | Probe           | 5'-FAM-CCTGGCCTCACTGTCCACCTTCCA-TAMRA-3'     |
|               | Forward primer  | 5'-CCTGAGCGCAAGTACTCTGTGT-3'                 |
|               | Reverse primer  | 5'-CTGCTTGTCTGATCCACATCTG-3'                 |
| <i>il-6</i>   | Probe           | 5'-FAM-CACAGAGGATACCACCACAACAGACCAG-TAMRA-3' |
|               | Forward primer  | 5'-CTTCTTGGGACTGATGTTTGTGAC-3'               |
|               | Reverse primer  | 5'-TTGCCATTGCACAACCTTTTC-3'                  |
| <i>Tnfa</i>   | Probe           | 5'-FAM-CGTAGCCCAOGTCGA-TAMRA-3'              |
|               | Forward primer  | 5'-GACCCTCACACTCAGATCATCTTCT-3'              |
|               | Reverse primer  | 5'-GGTACAGCCCATCTGCTGGTA-3'                  |

Sequences of TaqMan probes, forward primers, and reverse primers for real-time quantitative reverse transcription polymerase chain reaction.

were cut. The sections were incubated for 48 h at 4°C with the primary antibody OX-42 (rat monoclonal 5C6, 1:1,000; Serotec, Oxford, United Kingdom). After incubation, the

sections were washed and incubated with the appropriate secondary antibody (Alexa Fluor 543 conjugated with goat anti-rat/rabbit IgG, 1:1,000; Life Technologies Japan Ltd., Tokyo, Japan). Fluorescent images were obtained with a confocal microscope (LSM 5 Pascal; Carl Zeiss) and analyzed using the Zeiss LSM Image Browser (Carl Zeiss).



**Fig. 2.** Intrathecal injection of recombinant chemokine (C-C motif) ligand 3 (CCL3) induced biphasic tactile allodynia behavior. The paw-withdrawal thresholds of the hind paw in response to tactile stimulation in rats were examined at the indicated time points after intrathecal injection of recombinant CCL3 or vehicle (0.1 and 10 fmol in 10  $\mu$ l phosphate buffered saline,  $n = 8$  and 8,  $n$  of vehicle is 6). Each data point represents the mean  $\pm$  SEM of paw-withdrawal thresholds (\* $P < 0.05$ ; vs. vehicle group at each time point, # $P < 0.05$ ; significance between two time points indicated in figure. Multiple comparison by Bonferroni test after repeated-measure two-way ANOVA was used).

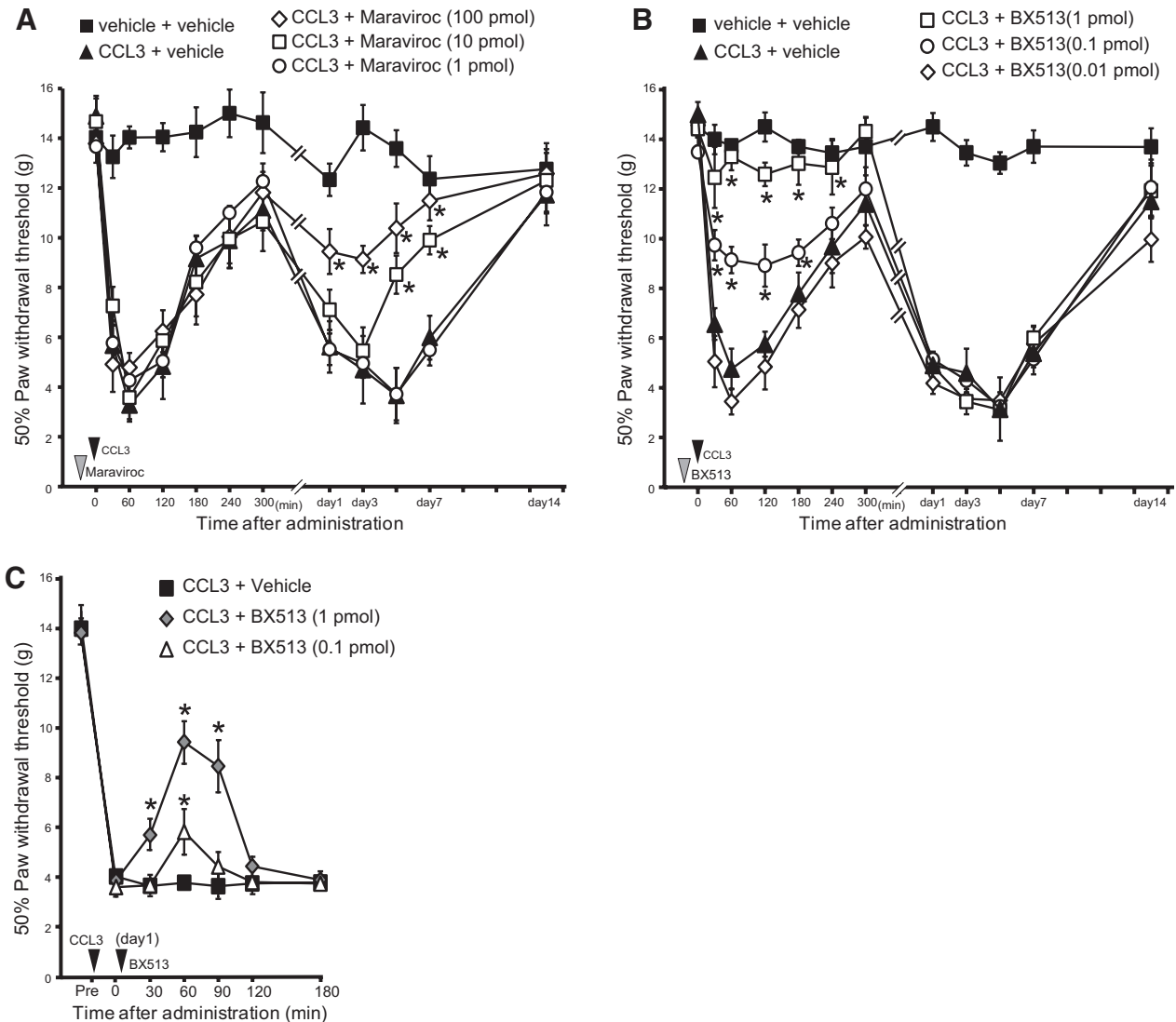
### Statistical Analysis

Data are presented as the mean  $\pm$  SEM. Statistical analyses were computed using the software SigmaPlot (build 11.2.0.5; Systat Software, Inc., Chicago, IL) using one-way ANOVA *post hoc* Tukey multiple comparisons, two-way ANOVA followed by Bonferroni multiple comparisons test, or paired  $t$  tests. The values were considered significantly different at  $P$  value less than 0.05.

## Results

### Up-regulation of CCL3 and CCR5 mRNA Expression in the Spinal Cord after PNI

Using real-time quantitative reverse transcription polymerase chain reaction, we examined the levels of CCL3, CCR1, and CCR5 mRNA in total RNA extracts from the spinal cord ipsilateral and contralateral to injury at the L5 spinal nerve (fig. 1). A significant increase in CCL3 mRNA expression in the ipsilateral spinal cord was observed 1, 10, and 14 days after PNI ( $P < 0.01$ ,  $P < 0.05$ ,  $P < 0.001$ ) (fig. 1A). The expression of CCR5 mRNA in the ipsilateral spinal cord significantly increased from 1 to 14 days after PNI (fig. 1B), and an increase in protein expression at day 7 after PNI was suggested by Western blotting (see fig. 1, Supplemental Digital Content 1, <http://links.lww.com/ALN/B36>, and Supplemental

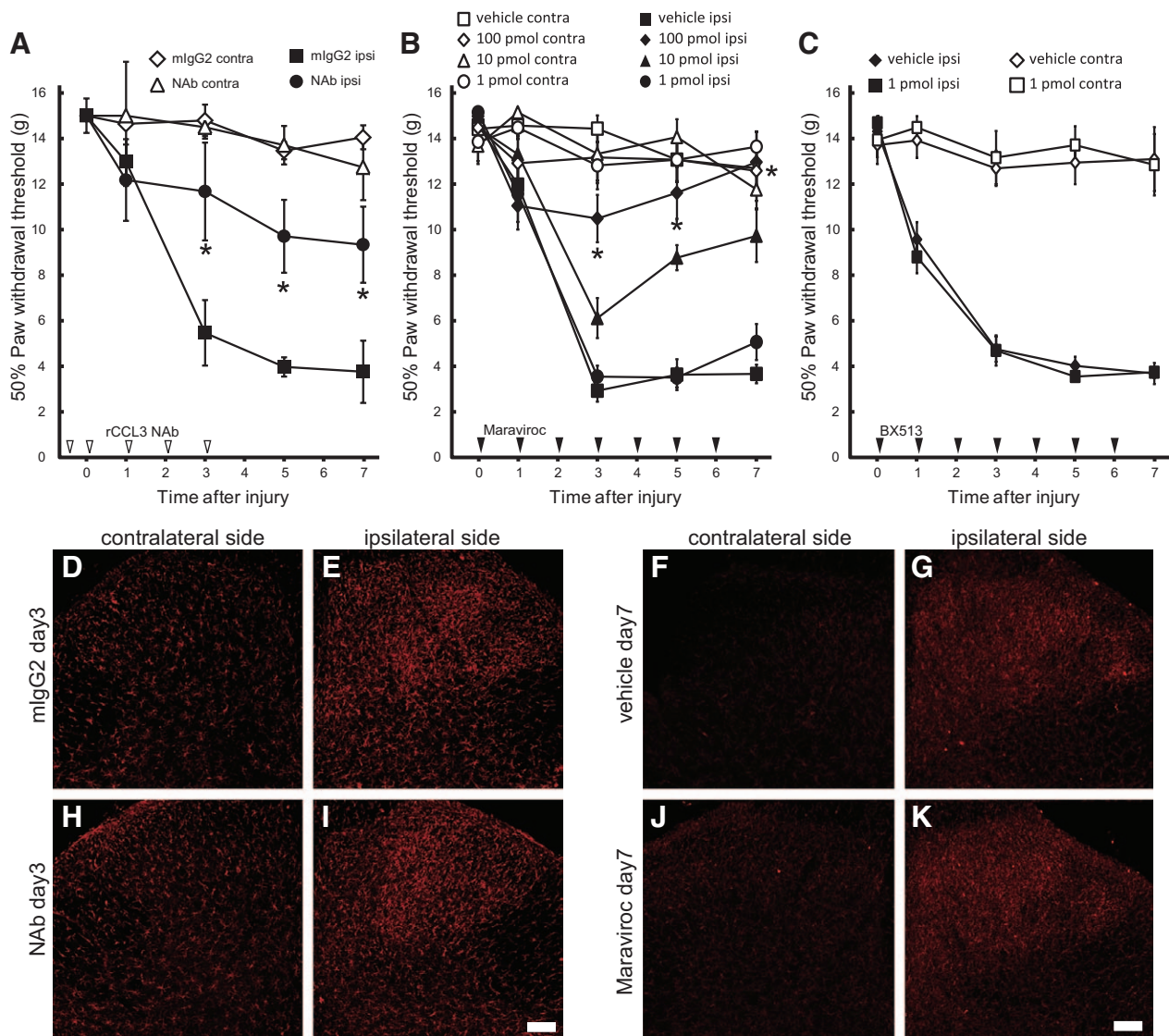


**Fig. 3.** Effect of chemokine (C-C motif) receptor 1 (CCR1) and chemokine (C-C motif) receptor 5 (CCR5) antagonists on chemokine (C-C motif) ligand 3 (CCL3)-induced tactile allodynia. The CCR5 antagonist maraviroc (A, 1, 10, and 100 pmol in 10  $\mu$ l phosphate buffered saline [PBS],  $n = 6, 6,$  and  $7$ ), the CCR1 antagonist BX513 hydrochloride (BX513) (B, 0.01, 0.1, and 1 pmol in 10  $\mu$ l PBS,  $n = 6, 6,$  and  $7$ ), or vehicle ( $n = 7$  for each A and B) were pretreated 30 min before CCL3 injection (10 fmol in 10  $\mu$ l PBS, indicated *gray inverted triangle*). BX513 hydrochloride (C, 0.1 and 1 pmol in 10  $\mu$ l PBS,  $n = 6$  and  $7$ ,  $n$  of vehicle is  $6$ ) was administered to animals with CCL3-induced allodynia at a day after CCL3 injection (10 fmol in 10  $\mu$ l PBS). The paw-withdrawal thresholds of the hind paw in response to tactile stimulation in rats were examined at the indicated time points after intrathecal injection of CCL3. Each data point represents the mean  $\pm$  SEM of the paw-withdrawal thresholds ( $*P < 0.05$ ; vs. CCL3 + vehicle group [ipsilateral side] at each time point by multiple comparison by Bonferroni test after repeated-measure two-way ANOVA).

Digital Content 2, <http://links.lww.com/ALN/B37>). In contrast, CCR1 mRNA expression in the spinal cord remained unchanged statistically (fig. 1C). It is widely accepted that spinal microglia are activated after PNI and that microglia have the potential to be a source of inflammatory mediators including chemokines. Thus, we collected CD11b/c-positive cells from the rat spinal cord 20 h after PNI using flow cytometric cell sorting. Detected CCL3 mRNA expression levels were significantly higher on the ipsilateral side to PNI (fig. 1D). Localization of CCL3 mRNA detected by fluorescent *in situ* hybridization indicated that mRNA expression indicated by red spots was apparently increased in the dorsal horn

(fig. 1, E–N). Signals in the dorsal horn of the spinal cord ipsilateral side to the PNI existed more densely around innervating nerve bundles recognized by scattering structures in the transmitted light image (fig. 1M). However, we failed to detect protein expression of CCL3 by immunohistochemistry or Western blotting (data not shown). These results represent that at least in part spinal microglia in the dorsal horn may enhance the expression of CCL3 mRNA after PNI.

We could not identify the responsible cell types for CCR1 and CCR5 mRNA expression, but it was expected that different cell types in the spinal cord express CCR1 or CCR5, because chromogenic *in situ* hybridization assays revealed distinct

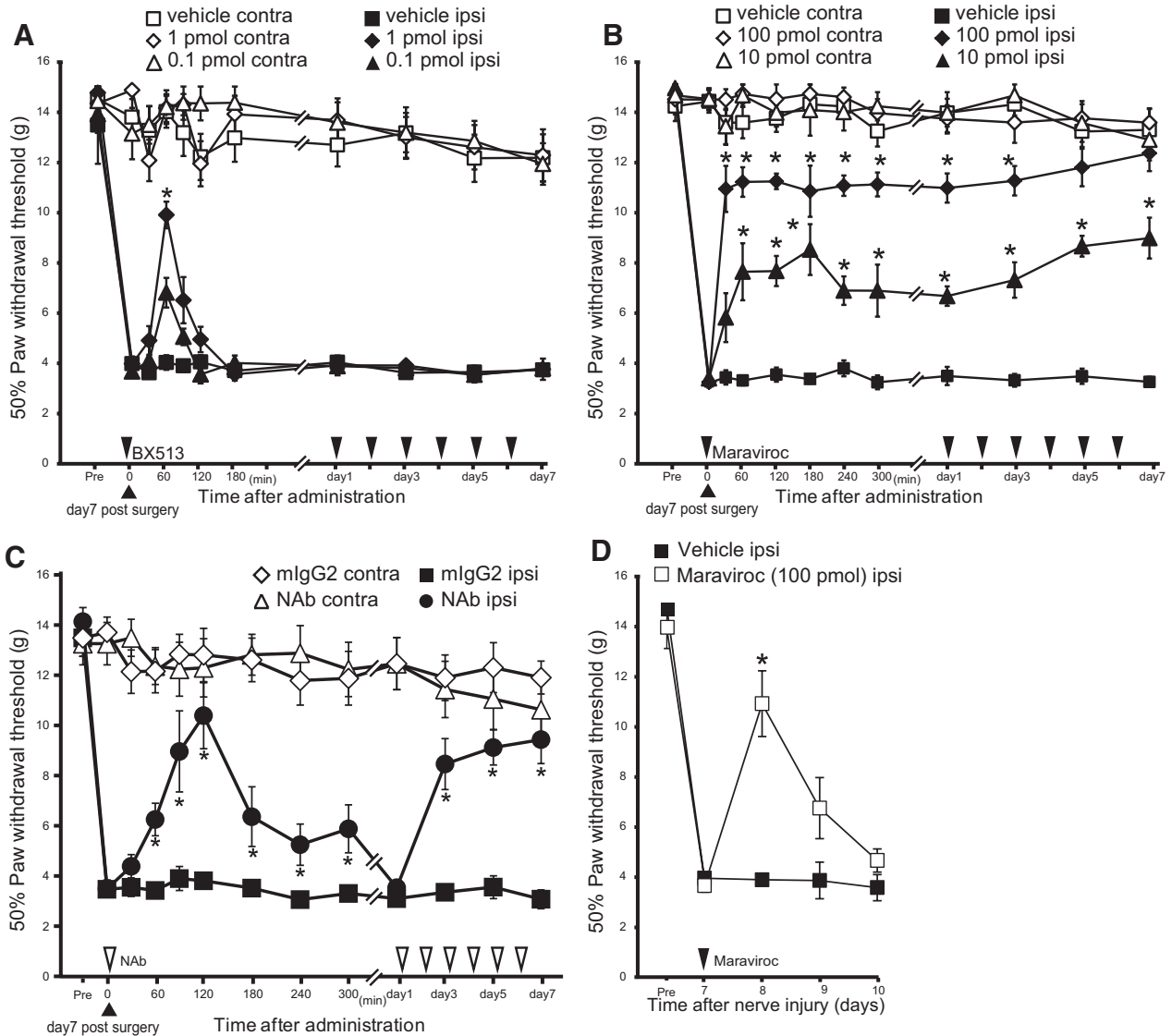


**Fig. 4.** Effect of chemokine (C-C motif) ligand 3 (CCL3)-neutralizing antibody, chemokine (C-C motif) receptor 1 (CCR1), and chemokine (C-C motif) receptor 5 (CCR5) antagonists on peripheral nerve injury-induced tactile allodynia. The CCL3-neutralizing antibody (NAb) or mouse immunoglobulin G 2 for isotype control (mIgG2, 20 ng in 10  $\mu$ l in an injection,  $n = 6$  for each group) was intrathecally injected once daily during the indicated time to rats with L5 spinal nerve transection (A). The CCR5 antagonist maraviroc (B, vehicle, 1, 10, and 100 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 6$  for each) and the CCR1 antagonist BX513 hydrochloride (C, vehicle and 1 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 6$  and 7) were intrathecally injected once daily at the indicated time to rats with L5 spinal nerve transection. The paw-withdrawal thresholds of the hind paw in response to tactile stimulation in rats were examined at the indicated time points after peripheral nerve injury. Each data point represents the mean  $\pm$  SEM of the paw-withdrawal thresholds ( $*P < 0.05$ ; vs. mIgG2 or vehicle group [ipsilateral side] at each time point by multiple comparison by Bonferroni test after a repeated-measure two-way ANOVA). L5 spinal cord segments from 3-day postoperative rats with intrathecal administration of neutralizing antibody or isotype IgG (D, E, H, and I), and 7-day postoperative rats with maraviroc or vehicle were subjected to immunohistochemistry using OX-42 antibody (F, G, J, and K). Scale bar indicates 100  $\mu$ m.

staining patterns for each receptor mRNA (see figs. 2 and 3, Supplemental Digital Content 1, <http://links.lww.com/ALN/B36>, and Supplemental Digital Content 2, <http://links.lww.com/ALN/B37>). Flow cytometric analysis showed that spinal CD11b/c-positive cells had surface expression of CCR5 receptor protein, but expression levels did not change between the ipsilateral and contralateral side at day 3 (unpublished result, October 14, 2013, Katsuyuki Matsushita, M.D., Fukuoka, Fukuoka, Japan). The increased number of CCR5 mRNA-positive cells

was obvious on the ipsilateral side of the dorsal horn. Thus, it was suggested that CCR5 was expressed basally in the spinal microglia, but the expression levels were not altered at a single-cell level. The detected increase in CCR5 mRNA expression in total RNA from whole spinal tissue samples may be due to the increases in the number of spinal microglia on the ipsilateral side to the PNI.

We also examined the expression of interleukin 6 and tumor necrosis factor- $\alpha$  mRNA at 16 and 20 h after PNI. The transcripts of these genes were significantly increased at



**Fig. 5.** Effect of chemokine (C-C motif) receptor 1 (CCR1), chemokine (C-C motif) receptor 5 (CCR5) antagonists, and chemokine (C-C motif) ligand 3 (CCL3)-neutralizing antibody on existing peripheral nerve injury-induced tactile allodynia. The CCR1 antagonist BX513 hydrochloride (A, vehicle, 0.1 and 1 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 6, 7,$  and  $7$ ), the CCR5 antagonist maraviroc (B, vehicle, 10 and 100 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 9, 8,$  and  $7$ ), or CCL3-neutralizing antibody (NAb) or isotype mouse immunoglobulin G 2 (mIgG2) (C, 20 ng in 10  $\mu$ l phosphate buffered saline,  $n = 7$  for each) were intrathecally injected once daily during the indicated time to rats with tactile allodynia 7 days after L5 spinal nerve transection. Maraviroc (vehicle and 100 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 5$  for each group) was intrathecally injected once to rats with tactile allodynia 7 days after L5 spinal nerve transection (D). The paw-withdrawal thresholds of the hind paw in response to tactile stimulation in rats were examined at the indicated time points after drug administrations. Each data point represents the mean  $\pm$  SEM of the paw-withdrawal thresholds ( $*P < 0.05$ ; vs. vehicle or mIgG2 group (ipsilateral side) at each time point by multiple comparison by Bonferroni test after a repeated-measure two-way ANOVA).

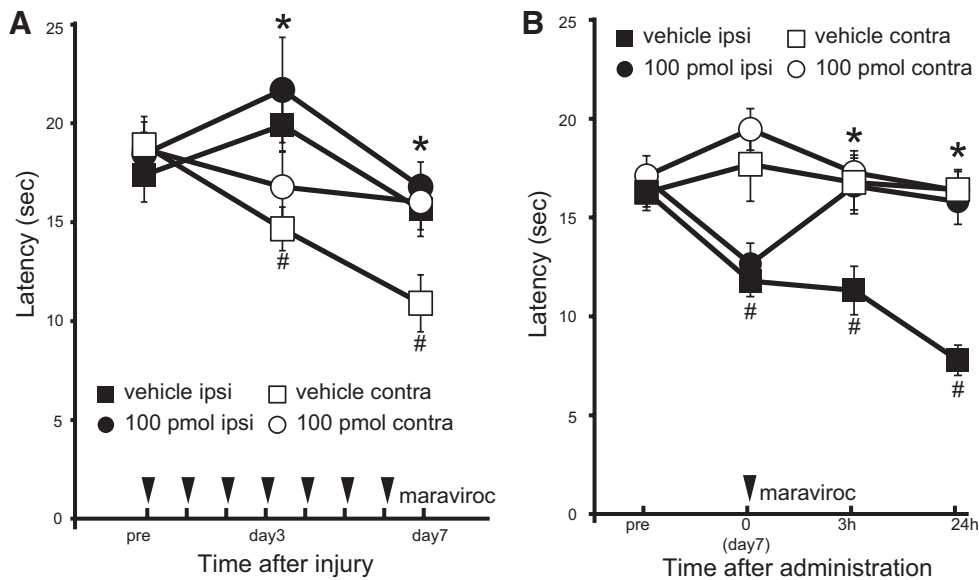
both time points. Thus, it is suggested that some inflammatory responses were induced as early as 16 h after PNI (unpublished result, September 25, 2012, Katsuyuki Matsushita, M.D., Fukuoka, Fukuoka, Japan).

#### Development of Tactile Allodynia by Intrathecal Injection of rCCL3

To investigate the effect of CCL3 up-regulation on the spinal cord during neuropathic pain, we performed a single

intrathecal injection of rCCL3 to naive rats. Single intrathecal injection of rCCL3 produced two phases of tactile allodynia behavior (fig. 2). In the first phase, the PWT to mechanical stimulation decreased during the first 60 min ( $3.18 \pm 0.46$  g in 10-fmol treated rats and  $14.49 \pm 0.50$  g in vehicle-treated rats;  $P < 0.001$ ) and gradually recovered. At 5 h after injection, significant recovery of PWT from a peak decrease was observed. In the second phase, the decreased PWT was again observed at 24 h after rCCL3





**Fig. 6.** Effect of chemokine (C-C motif) receptor 5 (CCR5) antagonist on peripheral nerve injury-induced thermal hyperalgesia. The duration until licking or shaking of each hind paw on the hot plate maintained at 52°C was measured as the Latency (s). The CCR5 antagonist maraviroc or vehicle was intrathecally injected once daily during the indicated time (A, 100 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 8$  and 7) or injected once (B, 100 pmol in 10  $\mu$ l phosphate buffered saline,  $n = 6$  each) to rats 7 days after L5 spinal nerve transection. Each data point represents the mean  $\pm$  SEM of the paw-withdrawal thresholds ( $*P < 0.05$ ; vs. vehicle group [ipsilateral side] in each time point,  $\#P < 0.05$ ; vs. premeasurement at each treatment. Multiple comparison by Bonferroni test after a repeated-measure two-way ANOVA was used).

injection ( $P < 0.001$ ;  $5.28 \pm 0.58$  g in 10-fmol treated rats and  $13.72 \pm 0.83$  g in vehicle-treated rats) and persisted to day 7 ( $P < 0.01$ ;  $6.52 \pm 1.16$  g in 10-fmol treated rats and  $13.24 \pm 0.82$  g in vehicle-treated rats) after injection. PWT recovered 14 days after intrathecal injection. It should be noted that a lower dose of rCCL3 produced only a later phase of PWT decrease. Thus, each phase of pain behavior may not be a sequential incident, but different mechanisms induced each behavior.

### CCR1 and CCR5 Antagonists Inhibited rCCL3-induced Tactile Allodynia

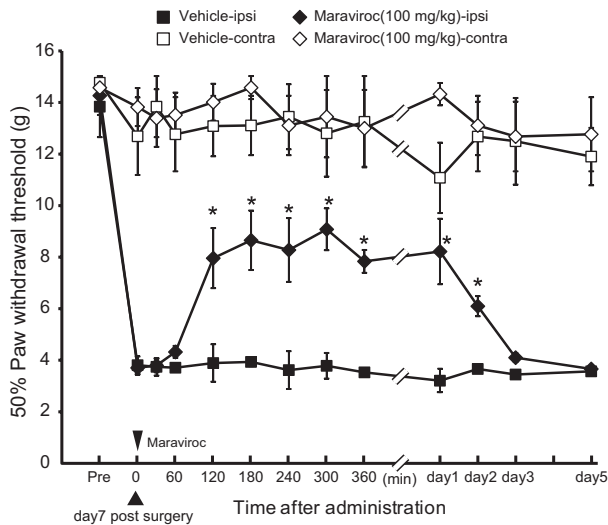
CCL3 is known to be the ligand for CCR1 and CCR5, and mRNAs of these receptors were differentially expressed in the spinal cord. Thus, to investigate the detailed role of CCR1 and CCR5 in rCCL3-induced tactile allodynia, the CCR1 antagonist BX513 hydrochloride or the CCR5 antagonist maraviroc were intrathecally injected 30 min before intrathecal injection of rCCL3. In maraviroc pretreated rats, the first phase of rCCL3-induced tactile allodynia was not suppressed. However, in the second phase of rCCL3-induced tactile allodynia, maraviroc dose-dependently suppressed the decrease of PWT from day 1 ( $P < 0.05$ ;  $9.45 \pm 1.04$  g in 100-pmol treated rats and  $5.61 \pm 0.65$  g in vehicle-treated rats) up to day 7 after injection ( $P < 0.001$ ;  $11.49 \pm 0.88$  g in 100-pmol treated rats and  $5.99 \pm 0.93$  g in vehicle-treated rats) (fig. 3A). In contrast, in BX513 hydrochloride pretreated rats, the first phase of rCCL3-induced tactile allodynia was clearly suppressed in a dose-dependent manner. However, the second phase was not

affected at all (fig. 3B). It should be noted that this transient effect of BX513 hydrochloride was also observed at the second phase of rCCL3-induced tactile allodynia. When BX513 hydrochloride was intrathecally injected 1 day after intrathecal injection of rCCL3, the rCCL3-induced decreased PWT transiently recovered (fig. 3C) ( $P < 0.001$ ;  $9.45 \pm 0.88$  g in 1-pmol BX513 hydrochloride-treated rats and  $3.77 \pm 0.24$  g in vehicle-treated rats). The PWT decreased again 120 min after BX513 hydrochloride injection. These results suggest that CCL3 has dual effects on pain induction: acute and transient effects through CCR1 and slowly develops long-lasting effects through CCR1 and CCR5.

### The CCR5 Antagonist and CCL3-neutralizing Antibody Attenuated the Development of PNI-induced Tactile Allodynia

To investigate whether endogenous CCL3 protein is involved in the mechanism of developing neuropathic pain, we intrathecally injected CCL3-neutralizing antibody to rats with developing neuropathic pain. A significant suppression of the PWT decrease was observed during the period of antibody injection, and this effect lasted at least 4 days after discontinuing the injection (the PWT on the ipsilateral side 7 days after PNI,  $P < 0.001$ ;  $9.34 \pm 1.67$  g in CCL3-neutralizing antibody-treated rats and  $2.60 \pm 0.53$  g in vehicle-treated rats) (fig. 4A).

To investigate whether endogenous CCR1 or CCR5 is involved in the mechanism of developing neuropathic pain, we intrathecally injected BX513 hydrochloride or maraviroc



**Fig. 7.** Effect of oral administration of chemokine (C-C motif) receptor 5 (CCR5) antagonist on existing peripheral nerve injury-induced tactile allodynia. The CCR5 antagonist maraviroc or vehicle (100 mg/kg,  $n = 4$  for each group) was orally administered once to rats with tactile allodynia 7 days after L5 spinal nerve transection. The paw-withdrawal thresholds of the hind paw in response to tactile stimulation in rats were examined at the indicated time points after drug administrations. Each data point represents the mean  $\pm$  SEM of the paw-withdrawal thresholds ( $*P < 0.05$ ; vs. vehicle group [ipsilateral side] at each time point by multiple comparison by Bonferroni test after a repeated-measure two-way ANOVA).

in rats with developing neuropathic pain. In animals subjected to BX513 hydrochloride or maraviroc injection, reagents were intrathecally injected once a day from just before PNI to 6 days after PNI. Behavioral measurements were scheduled before daily injection to exclude the acute effects of reagents from the measurements. In maraviroc-treated rats, a significant suppression of PWT decrease was observed from 3 days to 7 days in a dose-dependent manner (fig. 4B). On day 7 after PNI, a significant decrease in PWT was observed on the ipsilateral side in vehicle-treated rats ( $P < 0.001$ ;  $3.52 \pm 0.41$  g on ipsilateral side and  $12.54 \pm 0.90$  g on contralateral side, not shown in fig. 4, A–C). In contrast, the decreased PWT recovered in maraviroc-treated rats ( $12.81 \pm 1.33$  g on the ipsilateral side of 100-pmol maraviroc-treated rats, whereas  $12.43 \pm 1.32$  g on the contralateral side) after PNI. In BX513 hydrochloride-treated rats, development of PNI-induced tactile allodynia was not suppressed at all (fig. 4C). To examine whether CCL3-neutralizing antibody or maraviroc affects activation of spinal microglia, we performed immunolabeling of spinal sections from antibody-treated (day 3) and maraviroc-treated (day 7) nerve-injured rats with the microglial marker OX-42. As reported previously,<sup>11</sup> a marked increase in OX-42 immunoreactivity was observed in the ipsilateral dorsal spinal cord of vehicle-treated rats 3 and 7 days after PNI (fig. 4, D–G). In contrast to the effect of tactile allodynia, repeated intrathecal administration of either the CCL3-neutralizing antibody or

maraviroc did not suppress this increase in OX-42 labeling (fig. 4, H–K).

### **The CCL3-neutralizing Antibody, CCR1 Antagonist, and CCR5 Antagonist Relieved Existing Tactile Allodynia**

To examine whether the CCR1 antagonist or CCR5 antagonist is effective in treating tactile allodynia, we intrathecally injected BX513 hydrochloride or maraviroc once a day from day 7 to day 13 after PNI. Under these conditions, short-term behavioral tests were performed immediately after the first injection to clarify the acute effect of drugs. In BX513 hydrochloride-treated rats, the decreased PWT on the ipsilateral side increased at 60 min ( $P < 0.001$ ;  $9.89 \pm 0.53$  g in 1-pmol treated rats and  $4.02 \pm 0.29$  g in vehicle-treated rats) after injection (fig. 5A). This effect was not observed 120 min after BX513 hydrochloride injection, and the decreased PWT was unchanged by daily administration of BX513 hydrochloride. In maraviroc-treated rats, the decreased PWT on the ipsilateral side increased 30 min after injection ( $P < 0.001$ ;  $10.87 \pm 0.91$  g in 100-pmol treated rats and  $3.43 \pm 0.28$  g in vehicle-treated rats). Contrary to BX513 hydrochloride-treated rats, the increase in PWT persisted for 24 h after maraviroc injection dose-dependently ( $P < 0.001$ ;  $11.00 \pm 0.42$  g in 100-pmol treated rats and  $3.79 \pm 0.31$  g in vehicle-treated rats) (fig. 5B). Daily injection of maraviroc reversed the decreased PWT on the ipsilateral side continuously in a dose-dependent manner. Single injection of maraviroc recovered the decrease in PWT on the ipsilateral side up to 1 day after injection (fig. 5D). CCL3-neutralizing antibody at the same dose that suppressed the development of tactile allodynia also reversed PWT transiently and partially. However, repeated administration showed enhanced and longer recovery effects (fig. 5C).

### **The CCR5 Antagonist Relieved Thermal Hyperalgesia after PNI**

To investigate whether maraviroc was effective in thermal hyperalgesia, we intrathecally injected maraviroc once a day from just before PNI to 6 days after PNI. Similar to the effect on tactile allodynia, repetitive administration of maraviroc suppressed the development of thermal hyperalgesia (fig. 6A). Single administration of maraviroc to PNI rats also resulted in a significant recovery of thermal hyperalgesia (fig. 6B).

### **Oral Administration of the CCR5 Antagonist Relieved Established Tactile Allodynia**

To examine the effect of oral administration of maraviroc on PNI-induced tactile allodynia, we performed single oral administration of maraviroc (by gavage) 7 days after PNI. The decreased PWT of rats orally administered maraviroc increased from 120 min ( $P < 0.001$ ;  $7.93 \pm 1.16$  g in maraviroc-treated rats and  $3.39 \pm 0.13$  g in vehicle-treated rats) up to day 2 ( $P < 0.001$ ;  $6.07 \pm 0.39$  g in maraviroc-treated rats and  $3.63 \pm 0.12$  g in vehicle-treated rats) after administration (fig. 7).

## Discussion

In the current study, we provide evidence that the CCL3 transcript is up-regulated in the ipsilateral dorsal spinal cord after PNI, and that CCL3 participates in the development and maintenance of neuropathic pain. Consistent with a previous study,<sup>22</sup> neutralizing endogenous CCL3 in the spinal cord with a specific antibody prevented development of tactile allodynia after PNI. In addition, we found that established tactile allodynia recovered after repetitive intrathecal injection of CCL3-neutralizing antibody. In a pain model of partial sciatic nerve ligation, it was demonstrated that CCL2, CCL3, CCL4, and chemokine (C-X-C motif) ligand 2 were up-regulated in the injured site of sciatic nerves. Using neutralizing antibodies, it was shown that CCL3 and chemokine (C-X-C motif) ligand 2 contribute to the development of tactile allodynia and thermal hyperalgesia, but CCL4 contributes to only tactile allodynia.<sup>17,19,23,27</sup> Several studies indicate that infiltrating immune cells produce chemokines around sciatic nerves.<sup>19,22,23</sup> In this study, we found that CCL3 mRNA was also up-regulated in the injured side of the spinal cord, and similar up-regulation was observed in sorted CD11b/c-positive cells from the spinal cord that are considered spinal microglia. Thus, in the spinal cord, resident microglia may be one of the CCL3-producing cells that are involved in establishing tactile allodynia and thermal hyperalgesia. Because of technical limitations, we could not obtain reliable evidence for the presence and up-regulation of CCL3 protein. However, the inhibitory effect of the neutralizing antibody on pain hypersensitivity suggests the presence of endogenous CCL3 protein in the spinal cord after PNI. In parallel, we found that intrathecal administration of rCCL3 protein could produce tactile allodynia. Similar observations were demonstrated in a previous study,<sup>22</sup> but we carried out behavioral assays to see the acute effect of rCCL3 injection and found that the rCCL3-induced tactile allodynia was a biphasic phenomenon. Single injection induces an early phase and late phase of tactile allodynia. Differences between phases have been more clearly understood from results showing that low doses of rCCL3 (0.1 fmol) do not induce early phase but a significant decrease in PWT in later phase. Indeed, we found that pretreatment with a CCR1 antagonist suppressed tactile allodynia at early phase and that CCR5 antagonist reduced allodynia in later phase. Difference in sensitivity to CCL3, which were observed in the case of low-dose rCCL3 administration, may be due to differences in cell types expressing CCR1 or CCR5. Localization of CCR1 mRNA was observed in neuron-like cells (large round cell body) at inner parts of the spinal cord (see fig. 2, Supplemental Digital Content 1, <http://links.lww.com/ALN/B36>), whereas CCR5 mRNA was observed in small cells that increased in number on the injured side of the spinal cord and surrounded large motor neuron-like cells in the ventral horn (see fig. 3, Supplemental Digital Content 1, <http://links.lww.com/ALN/B36>). In addition, we observed that mRNA of CCR5 was up-regulated on the ipsilateral spinal

cord after PNI, but CCR1 was unchanged (fig. 1). Thus, it was considered that each receptor was differentially expressed and regulated in the spinal cord because of the different cell types expressing each receptor or because of the difference in mechanism regulating receptor transcription. Together with these observations, it is suggested that CCR1 stimulates CCR1-mediated transient pain signaling and CCR5 triggers the development of relatively long-lasting tactile allodynia. It should be noted that the CCR1 antagonist showed transient relief to later phase of allodynia, but this effect was reversed within a few hours (fig. 3C). It seems unlikely that intrathecally injected rCCL3 maintains an effective concentration in the spinal cord a day after injection. Thus, it may be implicated that CCR5-triggered later-phase tactile allodynia is mediated by CCR1-induced pain signaling or that basal CCR1 activity is required for pain sensation.

Blockade of CCR5 successfully suppressed the development of tactile allodynia. Several previous studies also demonstrated the availability of CCR5 inhibition for treating tactile allodynia after PNI or inflammation.<sup>17,18,28,29</sup> We found that both the CCR1 antagonist and CCR5 antagonist immediately removed established tactile allodynia. However, the effect of the CCR1 antagonist was transient, and daily administration of the CCR1 antagonist was not effective to obtain successive relief from tactile allodynia after PNI. In contrast, the effect of the CCR5 antagonist was long lasting. A previous study suggested that CCR5 was expressed in spinal microglia, with CCR5 mRNA being expressed in Iba-1-positive activated microglia after partial sciatic nerve ligation.<sup>29</sup> Our results seem to support this suggestion. Similarly, in this study, the expression of CCR5 mRNA increased in a similar manner to the increase in microglial number after PNI,<sup>30</sup> with surface expression of CCR5 protein being detected by flow cytometry in CD11b/c-positive cells (unpublished result, October 14, 2013, Katsuyuki Matsushita, M.D., Fukuoka, Fukuoka, Japan). Although mRNA expression levels in the spinal cord were significantly increased at day 3 after PNI, we did not detect enhanced surface expression of CCR5 in CD11b/c-positive cells. However, the number of CD11b/c-positive cells increased (unpublished result, October 14, 2013, Katsuyuki Matsushita, M.D., Fukuoka, Fukuoka, Japan). These two observations suggest that up-regulation of CCR5 mRNA expression levels is due to the increase in the number of spinal microglia in response to PNI. Together, it is suggested that CCR5 in spinal cord microglia is a crucial factor for maintenance of tactile allodynia after PNI. It was reported that inhibition of microglial activation with minocycline attenuates the development but not existing hypersensitivity in a rat model of neuropathy.<sup>31</sup> However, in our case, the CCR5 antagonist recovered established tactile allodynia and thermal hyperalgesia. We have observed similar results using an antagonist of the P2Y12 receptor which is specifically expressed in microglia<sup>32,33</sup> and suppresses gene expression of interferon regulatory factor 8, which regulates

the activation state of microglia.<sup>34</sup> Furthermore, morphological signs of microglial activation were suppressed by minocycline, but the P2Y<sub>12</sub> receptor antagonist did not affect the morphology of cells and increased cell number, as was observed in our current study using the CCL3-neutralizing antibody and the CCR5 antagonist (fig. 4, D–K). Therefore, it is suggested that at least some part of the cellular signaling in microglia that includes CCR5 but not the target of minocycline contributes to the maintenance of tactile allodynia after PNI. The mechanism by which CCR5 develops and maintains tactile allodynia and thermal hyperalgesia is still unknown and may include beneficial therapeutic targets for the treatment of neuropathic pain. It is also important to study the molecular mechanisms regulating CCR5 function in the spinal cord after PNI, as it was indicated in a previous study that posttranslational modification of the N-terminal of CCR5 modulates binding affinity of chemokines to CCR5.<sup>35</sup>

One intriguing point of our experiments is that maraviroc, an antagonist for CCR5, is an approved drug for the treatment of human immunodeficiency virus (HIV) infection. Maraviroc is a selective, reversible, small-molecule CCR5 antagonist for the treatment of HIV-1 infection. Maraviroc is the first member of a new class of antiretroviral medications and has been approved by the U.S. Food and Drug Administration for the use in combination with other antiretroviral agents for treatment-experienced patients infected with multidrug-resistant CCR5-tropic HIV-1. The most frequently reported (>10%) adverse effects of maraviroc are upper respiratory tract infection (20.0%), cough (12.7%), and pyrexia (12.0%).<sup>36</sup> Tricyclic antidepressants,  $\alpha$ 2- $\delta$  anticonvulsants, serotonin, and norepinephrine reuptake inhibitors are used as a treatment of neuropathic pain,<sup>37</sup> but these drugs are associated with several CNS-related side effects including sedation and dizziness.<sup>37,38</sup> Dizziness has been reported as one of the side effects of maraviroc, but in comparison with currently available treatments, oral administration of maraviroc is associated with fewer CNS-related side effects. However, in our study, we have been unable to address this problem given that the dose of oral maraviroc administration was higher than that being currently used for the treatment of HIV infection, because only 10% of maraviroc in the plasma is distributed to the CNS in rats.<sup>39,40</sup> Thus, although it is necessary to take proper account of the dose and route of administration for clinical use or new drug design, we consider that the CCL3–CCR5 signaling pathway may be a new therapeutic target for the treatment of neuropathic pain.

### Acknowledgments

This work was partly supported by grants-in-aid for Scientific Research (KAKEN S-23229008 and KAKEN C-25460721) from Japan Society of the Promotion of Science (Chiyoda-ku, Tokyo, Japan; to Drs. Inoue and Tozaki-Saitoh), the Core Research for Evolutional Science and Technology (CREST)

program of the Japan Science and Technology (JST) Corporation (Kawaguchi, Saitama, Japan), and Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (Chiyoda-ku, Tokyo, Japan).

### Competing Interests

The authors declare no competing interests.

### Correspondence

Address correspondence to Dr. Inoue: Department of Molecular and System Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan. inoue@phar.kyushu-u.ac.jp. Information on purchasing reprints may be found at [www.anesthesiology.org](http://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.

### References

1. Baron R: Mechanisms of disease: Neuropathic pain—A clinical perspective. *Nat Clin Pract Neurol* 2006; 2:95–106
2. Bouhassira D, Lantéri-Minet M, Attal N, Laurent B, Touboul C: Prevalence of chronic pain with neuropathic characteristics in the general population. *Pain* 2008; 136:380–7
3. Torrance N, Smith BH, Bennett MI, Lee AJ: The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. *J Pain* 2006; 7:281–9
4. Scholz J, Woolf CJ: Can we conquer pain? *Nat Neurosci* 2002; 5(suppl):1062–7
5. Woolf CJ, Mannion RJ: Neuropathic pain: Aetiology, symptoms, mechanisms, and management. *Lancet* 1999; 353:1959–64
6. Costigan M, Scholz J, Woolf CJ: Neuropathic pain: A maladaptive response of the nervous system to damage. *Annu Rev Neurosci* 2009; 32:1–32
7. Woolf CJ, Salter MW: Neuronal plasticity: Increasing the gain in pain. *Science* 2000; 288:1765–9
8. McMahon SB, Malcangio M: Current challenges in glia-pain biology. *Neuron* 2009; 64:46–54
9. Scholz J, Woolf CJ: The neuropathic pain triad: Neurons, immune cells and glia. *Nat Neurosci* 2007; 10:1361–8
10. 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
11. Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K: P2X<sub>4</sub> receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 2003; 424:778–83
12. Watkins LR, Milligan ED, Maier SF: Glial activation: A driving force for pathological pain. *Trends Neurosci* 2001; 24:450–5
13. Abbadie C: Chemokines, chemokine receptors and pain. *Trends Immunol* 2005; 26:529–34
14. Abbadie C, Bhangoo S, De Koninck Y, Malcangio M, Melik-Parsadaniantz S, White FA: Chemokines and pain mechanisms. *Brain Res Rev* 2009; 60:125–34
15. Charo IF, Ransohoff RM: The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006; 354:610–21
16. Ransohoff RM: Chemokines and chemokine receptors: Standing at the crossroads of immunobiology and neurobiology. *Immunity* 2009; 31:711–21

17. Saika F, Kiguchi N, Kobayashi Y, Fukazawa Y, Kishioka S: CC-chemokine ligand 4/macrophage inflammatory protein-1 $\beta$  participates in the induction of neuropathic pain after peripheral nerve injury. *Eur J Pain* 2012; 16:1271–80
18. Lee YK, Choi DY, Jung YY, Yun YW, Lee BJ, Han SB, Hong JT: Decreased pain responses of C-C chemokine receptor 5 knockout mice to chemical or inflammatory stimuli. *Neuropharmacology* 2013; 67:57–65
19. Kiguchi N, Kobayashi Y, Saika F, Kishioka S: Epigenetic upregulation of CCL2 and CCL3 *via* histone modifications in infiltrating macrophages after peripheral nerve injury. *Cytokine* 2013; 64:666–72
20. Dansereau MA, Gosselin RD, Pohl M, Pommier B, Mechighel P, Mauborgne A, Rostene W, Kitabgi P, Beaudet N, Sarret P, Melik-Parsadaniantz S: Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J Neurochem* 2008; 106:757–69
21. Biber K, Tsuda M, Tozaki-Saitoh H, Tsukamoto K, Toyomitsu E, Masuda T, Boddeke H, Inoue K: Neuronal CCL21 up-regulates microglia P2X4 expression and initiates neuropathic pain development. *EMBO J* 2011; 30:1864–73
22. Kiguchi N, Kobayashi Y, Maeda T, Saika F, Kishioka S: CC-chemokine MIP-1 $\alpha$  in the spinal cord contributes to nerve injury-induced neuropathic pain. *Neurosci Lett* 2010; 484:17–21
23. Kiguchi N, Maeda T, Kobayashi Y, Fukazawa Y, Kishioka S: Macrophage inflammatory protein-1 $\alpha$  mediates the development of neuropathic pain following peripheral nerve injury through interleukin-1 $\beta$  up-regulation. *Pain* 2010; 149:305–15
24. Kim SH, Chung JM: An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 1992; 50:355–63
25. Tsuda M, Masuda T, Kitano J, Shimoyama H, Tozaki-Saitoh H, Inoue K: IFN- $\gamma$  receptor signaling mediates spinal microglia activation driving neuropathic pain. *Proc Natl Acad Sci U S A* 2009; 106:8032–7
26. 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
27. Kiguchi N, Kobayashi Y, Maeda T, Fukazawa Y, Tohya K, Kimura M, Kishioka S: Epigenetic augmentation of the macrophage inflammatory protein 2/C-X-C chemokine receptor type 2 axis through histone H3 acetylation in injured peripheral nerves elicits neuropathic pain. *J Pharmacol Exp Ther* 2012; 340:577–87
28. Okamoto M, Suzuki T, Watanabe N: Modulation of inflammatory pain in response to a CCR2/CCR5 antagonist in rodent model. *J Pharmacol Pharmacother* 2013; 4:208–10
29. Padi SS, Shi XQ, Zhao YQ, Ruff MR, Baichoo N, Pert CB, Zhang J: Attenuation of rodent neuropathic pain by an orally active peptide, RAP-103, which potently blocks CCR2- and CCR5-mediated monocyte chemotaxis and inflammation. *Pain* 2012; 153:95–106
30. Echeverry S, Shi XQ, Zhang J: Characterization of cell proliferation in rat spinal cord following peripheral nerve injury and the relationship with neuropathic pain. *Pain* 2008; 135:37–47
31. Raghavendra V, Tanga F, DeLeo JA: Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther* 2003; 306:624–30
32. Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K: P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J Neurosci* 2008; 28:4949–56
33. Hickman SE, Kingery ND, Ohsumi TK, Borowsky ML, Wang LC, Means TK, El Khoury J: The microglial sensome revealed by direct RNA sequencing. *Nat Neurosci* 2013; 16:1896–905
34. Masuda T, Tsuda M, Yoshinaga R, Tozaki-Saitoh H, Ozato K, Tamura T, Inoue K: IRF8 is a critical transcription factor for transforming microglia into a reactive phenotype. *Cell Rep* 2012; 1:334–40
35. Bannert N, Craig S, Farzan M, Sogah D, Santo NV, Choe H, Sodroski J: Sialylated O-glycans and sulfated tyrosines in the NH<sub>2</sub>-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J Exp Med* 2001; 194:1661–73
36. Lieberman-Blum SS, Fung HB, Bandres JC: Maraviroc: A CCR5-receptor antagonist for the treatment of HIV-1 infection. *Clin Ther* 2008; 30:1228–50
37. Freynhagen R, Bennett MI: Diagnosis and management of neuropathic pain. *BMJ* 2009; 339:b3002
38. Nightingale S: The neuropathic pain market. *Nat Rev Drug Discov* 2012; 11:101–2
39. Walker DK, Abel S, Comby P, Muirhead GJ, Nedderman AN, Smith DA: Species differences in the disposition of the CCR5 antagonist, UK-427,857, a new potential treatment for HIV. *Drug Metab Dispos* 2005; 33:587–95
40. Walker DK, Bowers SJ, Mitchell RJ, Potchoiba MJ, Schroeder CM, Small HF: Preclinical assessment of the distribution of maraviroc to potential human immunodeficiency virus (HIV) sanctuary sites in the central nervous system (CNS) and gut-associated lymphoid tissue (GALT). *Xenobiotica* 2008; 38:1330–9