Epigenetic transcriptional activation of monocyte chemotactic protein 3 contributes to long-lasting neuropathic pain

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A multiplex analysis for profiling the expression of candidate genes along with epigenetic modification may lead to a better understanding of the complex machinery of neuropathic pain. In the present study, we found that partial sciatic nerve ligation most remarkably increased the expression of monocyte chemotactic protein 3 (MCP-3, known as CCL7) a total of 33,541 genes in the spinal cord, which lasted for 4 weeks. This increase in MCP-3 gene transcription was accompanied by the decreased trimethylation of histone H3 at Lys27 at the MCP-3 promoter. The increased MCP-3 expression associated with its epigenetic modification observed in the spinal cord was almost abolished in interleukin 6 knockout mice with partial sciatic nerve ligation. Consistent with these findings, a single intrathecal injection of recombinant proteins of interleukin 6 significantly increased MCP-3 messenger RNA with a decrease in the level of Lys27 trimethylation of histone H3 at the MCP-3 promoter in the spinal cord of mice. Furthermore, deletion of the C–C chemokine receptor type 2 (CCR2) gene, which encodes a receptor for MCP-3, failed to affect the acceleration of MCP-3 expression in the spinal cord after partial sciatic nerve ligation. A robust increase in MCP-3 protein, which lasted for up to 2 weeks after surgery, in the dorsal horn of the spinal cord of mice with partial sciatic nerve ligation was seen mostly in astrocytes, but not microglia or neurons. On the other hand, the increases in both microglia and astrocytes in the spinal cord by partial sciatic nerve ligation were mostly abolished in interleukin 6 knockout mice. Moreover, this increase in microglia was almost abolished by CCR2 gene deletion, whereas the increase in astrocytes was...
not affected in nerve-ligated mice that lacked the CCR2 gene. We also found that either in vivo or in vitro treatment with MCP-3 caused robust microglia activation. Under these conditions, intrathecal administration of MCP-3 antibody suppressed the increase in microglia within the mouse spinal cord and neuropathic pain-like behaviours after nerve injury. With the use of a functional magnetic resonance imaging analysis, we demonstrated that a single intrathecal injection of MCP-3 induced dramatic increases in signal intensity in pain-related brain regions. These findings suggest that increased MCP-3 expression associated with interleukin 6 dependent epigenetic modification at the MCP-3 promoter after nerve injury, mostly in spinal astrocytes, may serve to facilitate astrocyte–microglia interaction in the spinal cord and could play a critical role in the neuropathic pain-like state.

**Keywords**: microglia; neuropathic pain; chemokines; spinal cord plasticity; nerve injury

**Abbreviations**: CCL = C-C Chemokine ligand; CCR2 = C-C chemokine receptor type 2; FEAT = FMRI expert analysis tool; IL6 = interleukin 6; JAK = Janus kinase signal; MCP-3 = monocyte chemotactic protein 3; PBS = phosphate buffered saline; RANTES = regulated upon activation normal T expressed and presumably secreted

### Introduction

Neuropathic pain, which is characterized by spontaneous burning pain, hyperalgesia (exaggerated pain in response to painful stimuli) and allodynia (pain caused by normally innocuous stimuli), is the most difficult pain to manage in the pain clinic. To date, although several animal models of chronic pain have been created to investigate the mechanisms that underlie the development of neuropathic pain (Tsuda et al., 2003; Narita et al., 2006; Kawasaki et al., 2008), those that underlie the maintenance of neuropathic pain accompanied by lasting spinal plasticity and neuronal adaptive responses are not fully understood. An important step towards unravelling the complex machinery of neuropathic pain is a multiplex analysis for gene expression profiling along with studies on epigenetic modifications, which exert lasting control over gene expression without altering the genetic code. DNA microarray and chromatin immunoprecipitation (ChIP) studies are likely to be useful tools for this purpose (Costigan et al., 2002; Wang et al., 2002; Coyle, 2007, Persson et al., 2009). However, to date, few, if any, studies have used this approach in animal models with persistent neuropathic pain.

Epigenetic modulation is mainly controlled by histone-tail modification. Modification of the histone-tail alters the compact chromatin structure and changes the degree to which regulatory proteins can access DNA. There is increasing evidence that regulation of the chromatin structure through histone modifications might mediate long-lasting behavioural changes in the context of learning and memory. This idea is fascinating because similar mechanisms are used to trigger and store long-lasting memories at the cellular level in, for example, spinal plasticity and neuronal adaptive responses under neuropathic pain.

In the present study, we found for the first time that a long-lasting increase in the expression of monocyte chemotactic protein 3 (MCP-3, known as CCL7) along with interleukin 6 (IL6) signalling-dependent histone modification at the MCP-3 promoter after partial sciatic nerve ligation promotes pain sensation that is associated with enhanced interaction between astrocytes and microglia in the spinal cord. This phenomenon may, at least in part, explain the complex machinery of neuropathic pain.

### Materials and methods

#### Mouse model of neuropathic pain

In the present study, we used male ICR mice (Tokyo Laboratory Animals Science Co Ltd), male IL6 knockout mice (Jackson Laboratory), male C-C chemokine receptor type 2 (CCR2) knockout mice (Jackson Laboratory), their wild-type C57BL/6J mice (Jackson Laboratory), and male protein kinase C-γ (PKC-γ) knockout mice (Jackson Laboratory) and their wild-type mice (Jackson Laboratory). PKC-γ knockout mice were maintained on a mixed genetic background of C57BL/6J and 129Sv. We produced a partial sciatic nerve ligation model as described previously (Malmberg and Basbaum, 1998). To assess the sensitivity to thermal stimulation, each of the hind paws of mice was tested individually using a thermal stimulus apparatus (IITC Inc/Life Science Instruments). To quantify the sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using von Frey filaments (North Coast Medical Inc).

#### DNA microarray

Total RNA of the spinal cord was prepared using an RNeasy® Lipid Tissue Mini Kit (Qiagen). For microarray-based expression analysis, synthesized complementary RNA labelled with cyanine 3 was hybridized to G4122F whole-mouse genome microarrays (Agilent Technologies).

#### Western blotting

The lumbar spinal cord was dissected, and proteins (cytosol fraction) were prepared as described previously (Narita et al., 2001a, b). For immunoblot detection, nitrocellulose membrane was incubated with the following primary antibodies: anti-signal transducer and activator of transcription 3 (STAT3) (Cell Signaling Technology), anti-phosphorylated STAT3 (p-STAT3) (Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon) overnight at 4°C, and then incubated to secondary antibody and detected by enhanced chemiluminescence and visualized by exposure to Amersham Hyperfilm.
Semi-quantitative analysis by reverse transcription-polymerase chain reaction and quantitative analysis by real-time reverse transcription-polymerase chain reaction

Total RNA in the lumbar spinal cord of mice was extracted using SV Total RNA Isolation System (Promega) following the manufacturer’s instruction. Purified 1 μg of RNA was used to prepare first-strand complementary DNA as described previously (Narita et al., 2001b). Each gene was amplified in PCR solution with synthesized primers as described in the online Supplementary material.

Chromatin immunoprecipitation

A chromatin immunoprecipitation assay was performed as described previously (Tsankova et al., 2004; Takeshima et al., 2009) with minor modifications. Soluble chromatin extracted from the mouse spinal cord was incubated with specific antibodies against acetylated histone H3 (H3Ac) (Millipore), histone 3 trimethylated at Lys4 (H3K4me3) (Wako Pure Chemicals), at Lys9 (H3K9me3) (Millipore) and at Lys27 (H3K27me3) (Millipore). The immunocomplex was collected by Dynabeads® Protein A (Invitrogen Dynal), and DNA was recovered by isopropanol precipitation. Quantitative PCR was performed as described previously (Nakajima et al., 2004).

Immunohistochemistry

Mice were deeply anesthetized with 3% isoflurane and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde. Lumbar spinal cord sections (10 μm) were incubated in blocking solution for 1 h at room temperature, and then incubated for 48 h at 4°C with primary antibodies; antibodies: anti-MCP-3 (R&D), anti-NeuN (Millipore), anti-glial fibrillary acidic protein (GFAP) (Nichirei), anti-Iba-1 (Wako Pure Chemicals). The antibody was then rinsed and incubated with an appropriate secondary antibody for 2 h at room temperature. Fluorescence of immunolabelling was detected using a light microscope and digitized images.

Thermal hyperalgesia and tactile allodynia

To quantify the sensitivity to thermal or tactile stimulation after intrathecal treatment with MCP-3 and anti-MCP-3 antibody, paw withdrawal was measured using a thermal stimulus apparatus or von Frey filaments, respectively.

Functional imaging

A continuous functional MRI scanning protocol was used to study changes in brain signal intensity using T2-weighted blood oxygenation level-dependent contrast. Data analysis was carried out using FEAT (http://www.fmrib.ox.ac.uk) software packages.

All methods are described in detail in the Supplementary material.

Results

Expression of the MCP-3 gene is dramatically increased after partial sciatic nerve ligation

To determine the key modulator of pain-processing, we performed gene expression profiling in the ipsilateral side of the spinal cord of nerve-ligated mice at 7 days after surgery using whole-genome oligonucleotide microarrays. To improve the accuracy of microarray data analysis, we prepared RNA from three independent groups consisting of three mice each as three replicates. We then extracted genes that were commonly changed in all three replicates by >2.0-fold relative to the control sample (Fig. 1A and B). Among the 33 541 genes that could be detected in both sham-operated and nerve-ligated mice, the level of MCP-3 (a Cys–Cys chemokine) gene expression in the spinal cord was most dramatically increased at 7 days after partial sciatic nerve ligation (n = 3; Fig. 1A and B). On the other hand, partial sciatic nerve ligation caused persistent neuropathic pain that lasted for 4 weeks in mice (n = 8; Fig. 2A). Consistent with the results of the DNA microarray analysis, the present reverse transcription–PCR assay demonstrated that the expression of MCP-3 messenger RNA on the ipsilateral side of the spinal cord obtained from nerve-ligated mice was significantly increased at 1 day to 4 weeks after partial sciatic nerve ligation (n = 4, 1 day: P < 0.05; 3 days: P < 0.01; 5–28 days: P < 0.001 versus sham-operated mice; Fig. 2B). The messenger RNA levels of another Cys–Cys subfamily of chemokines (MCP-1 and RANTES) were also significantly, albeit slightly, increased in the spinal cord of nerve-ligated mice (n = 4, MCP-1 5 and 7 days: P < 0.05; RANTES 5 days: P < 0.05, 7 days: P < 0.01 versus sham-operated mice; Fig. 2C and D). Furthermore, this enhanced expression of MCP-1 or RANTES resolved within 2 weeks after nerve injury.

A recent bioinformatics analysis of the MCP-3 gene predicted putative binding sites for transcriptional factors, such as STAT3, NFIL6 and Ets-family transcription factor PU.1, within the MCP-3 promoter region (Murakami et al., 1997; Aung et al., 2006; Rafei et al., 2008; Sobota et al., 2008). In the present study, the level of p-STAT3, but not that of STAT3, was significantly increased on the ipsilateral side of the lumbar spinal cord obtained from nerve-ligated mice at 1–5 days after partial sciatic nerve ligation (n = 4, 1 and 3 days: P < 0.001; 5 days: P < 0.01 versus sham-operated mice; Fig. 2E). In addition, the expression level of PU.1, but not NFIL6, messenger RNA was significantly increased in the ipsilateral side of the lumbar spinal cord at 3–5 days after partial sciatic nerve ligation (n = 4, 3 days: P < 0.01; 5 days: P < 0.05 versus sham-operated mice; Fig. 2F).

Involvement of interleukin 6 signalling in the increased expression of MCP-3 messenger RNA in the spinal cord of nerve-ligated mice

We found that the messenger RNA level of IL6 on the ipsilateral side of the mouse dorsal root ganglia was particularly increased at
Figure 1 Changes in gene expression in the spinal cord of nerve-ligated mice. (A) Gene expression analysis using whole-genome oligonucleotide microarrays. Samples were prepared 7 days after partial sciatic nerve ligation or sham operation. Each column shows the relative expression corresponding to a separate sample (n = 3 for ligation and n = 3 for sham control). The level of expression is represented on a logarithmic scale; red corresponds to high expression and dark green to low expression (see colour scale in the bottom-left corner). (B) Selected upregulated genes (>2.0-fold increase relative to the control) ranked in order of the magnitude of increase.
Figure 2  Time-course changes in the pain threshold and the expression of messenger RNA of Cys–Cys chemokine and its related transcriptional factors on the ipsilateral side of the lumbar spinal cord after nerve ligation. (A) Changes in the latency of paw withdrawal in response to a thermal (upper) or tactile stimulus (lower) in mice after partial sciatic nerve ligation. Each point indicates the mean ± standard error of the mean (SEM) of eight mice. **P < 0.01, ***P < 0.001 versus sham-operated mice. (B–D) Changes in the expression of Cys–Cys chemokine messenger RNA on the ipsilateral side of the lumbar spinal cord after nerve ligation. Each point represents the mean ± SEM of four samples. *P < 0.05, **P < 0.01, ***P < 0.001 versus sham-operated mice. (E) Changes in the levels of STAT3 and p-STAT3 in the mouse spinal cord after nerve ligation. Each point represents the mean ± SEM of four samples. **P < 0.01, ***P < 0.001 versus sham-operated mice. (F) Changes in the expression of PU.1 and NFIL6 messenger RNA in the mouse spinal cord after nerve ligation. Each point represents the mean ± SEM of four samples. *P < 0.05, **P < 0.01 versus sham-operated mice.
1–7 days after partial sciatic nerve ligation, whereas we did not detect IL6 messenger RNA expression on the ipsilateral side of the mouse spinal cord under the same conditions (n = 4, P < 0.001 versus sham-operated mice; Supplementary Fig. 1). It has been widely accepted that the binding of IL6 to gp130 induces the activation of janus kinase 1 in the cell membrane, which subsequently phosphorylates STAT3 at Tyr705 in the cytoplasm (Wegenka et al., 1994; Taga and Kishimoto, 1997). In addition, IL6 stimulation activates the Ets-family transcription factor PU.1, which regulates macrophage-specific gene expression (Panopoulos et al., 2002). Thus, we hypothesized that the increased expression of MCP-3 messenger RNA in the spinal cord of mice with nerve injury may be accompanied by increased IL6 in the dorsal root ganglia and/or spinal cord of mice in response to a noxious stimulus. In IL6 knockout mice, the increased levels of MCP-3, MCP-1 and RANTES messenger RNAs in the spinal cord after partial sciatic nerve ligation were significantly decreased at 7 days after ligation, as detected by reverse transcription–PCR assay (n = 3, MCP-3: P < 0.05; MCP-1: P < 0.001; RANTES: P < 0.01 versus nerve-ligated wild-type mice; Fig. 3A). An additional experiment by real-time PCR assay showed that the increase in the expression of MCP-3 observed in the spinal cord of wild-type (C57BL/6) mice after partial sciatic nerve ligation was dramatically greater than that in IL6 knockout mice with partial sciatic nerve ligation (n = 3, P < 0.05 versus nerve-ligated wild-type mice; Fig. 3B). Furthermore, a single intrathecal injection of recombinant IL6 (1 pmol/mouse) significantly increased MCP-3 messenger RNA in the spinal cord of normal mice at 3 days after injection (n = 4, P < 0.001 versus PBS-treated mice; Supplementary Fig. 2A). To gain further insight into these phenomena, we next investigated whether the IL6-induced transcription of MCP-3 in the spinal cord of nerve-ligated mice could be associated with the activation of its
Long-lasting interleukin 6-dependent histone modifications at the MCP-3 promoter after partial sciatic nerve ligation

In the present study, we performed chromatin immunoprecipitation assays with antibodies against AcH3, H3K4me3, H3K9me3 and H3K27me3 and quantified the amount of DNA associated with the modified histones using real-time PCR. As shown in Fig. 4A, the levels of histone acetylation and trimethylation at the promoter region of the MCP-3 or RANTES gene were similar in the spinal cords of sham-operated and nerve-ligated mice at 7 days after surgery. Likewise, there were no differences in the levels of H3 acetylation or K4 and K9 trimethylation at the promoters of the MCP-3 gene between sham-operated and nerve-ligated mice (Fig. 4A). The key finding of the present study was that partial sciatic nerve ligation significantly decreased H3K27me3 at the MCP-3 promoter in the spinal cord of mice with partial sciatic nerve ligation compared with that in sham-operated mice (n = 3, P < 0.05 versus sham-operated mice; Fig. 4A). Furthermore, these decreases in H3K27me3 at the MCP-3 promoter in the spinal cord of mice with partial sciatic nerve ligation lasted for 4 weeks after nerve ligation (n = 3, 1 week: P < 0.01; 2 weeks: P < 0.05; 4 weeks: P < 0.01 versus sham-operated mice; Fig. 4B). In addition, the epigenetic modification at the MCP-3 promoter observed in the spinal cord of wild-type mice was not seen in IL6 knockout mice (n = 3: Fig. 4C). In agreement with these data, a single intrathecal injection of recombinant proteins of IL6 caused a significant decrease in the level of K27 trimethylation at the MCP-3 promoter in the spinal cord of mice at 3 days after injection (n = 3, P < 0.05 versus PBS-treated mice; Fig. 4D).

Cellular distribution of MCP-3 in the spinal cord of mice with partial sciatic nerve ligation

To clarify the cellular distribution of MCP-3 in the spinal cord of nerve-ligated mice, the L4 lumbar spinal cord of mice was immunostained with anti-MCP-3 antibody and double-stained for neuronal and glial markers. MCP-3 immunoreactivities were weakly detected in either the ipsilateral or contralateral side of the L4 lumbar spinal dorsal horn of sham-operated mice at 7 days after sham operation (Fig. 5A). Consistent with the results of DNA microarray and PCR analysis, partial sciatic ligation caused a visually marked increase in MCP-3 immunoreactivity in the ipsilateral side, compared with the contralateral side, of the dorsal horn of the spinal cord obtained from nerve-ligated mice at 7 days after nerve ligation (Fig. 5Bi). These increased MCP-3 immunoreactivities in the ipsilateral side of the dorsal horn of the spinal cord obtained from nerve-ligated mice lasted for up to 2 weeks after nerve injury (Fig. 5Bii). Under these conditions, MCP-3 immunoreactivity was not co-localized with neuronal nuclei (NeuN; Fig. 5C), anti-metabotropic glutamate subtype 5 receptor (mGluR5) (Supplementary Fig. 3A), which is located mostly in neurons of laminae I and II on the dorsal horn of the spinal cord, PKC-γ (Supplementary Fig. 3B), which is densely found in neurons of lamina II, or microtubule-associated protein 2, which is widely distributed in neural dendrites and axon terminals (Supplementary Fig. 3C). In contrast, increased MCP-3 immunoreactivity in the dorsal horn of the mouse spinal cord at 7 days after partial sciatic nerve ligation was highly co-localized with the astrocyte marker GFAP (Fig. 5D), but not with the microglial marker, Iba-1 (Fig. 5E). Although partial sciatic nerve ligation induced a marked activation of spinal astrocytes as indicated by a robust increase in GFAP immunoreactivity on the ipsilateral side of the dorsal horn of the spinal cord obtained from nerve-ligated wild-type mice (Fig. 5Fii), this increase in GFAP immunoreactivity observed in nerve-ligated wild-type mice was abolished in the same area in IL6 knockout mice (Fig. 5Gii), indicating that inducible MCP-3 protein after nerve injury is found mainly in IL6-sensitive activated astrocytes. On the other hand, Iba-1 immunoreactive microglia in the spinal cord was significantly increased by a single intrathecal injection of IL6 (n = 3, P < 0.001 versus PBS-treated mice; Supplementary Fig. 4A). Furthermore, Iba-1 immunoreactivity was not increased in the spinal cord after nerve injury in IL6 knockout mice (Supplementary Fig. 4Eii).

Involved of MCP-3/CCR2 signalling in the activation of spinal microglia or spinal neurons under the neuropathic pain

Either partial sciatic nerve ligation or intrathecal injection of IL6 significantly increased the expression of CCR2 messenger RNA in the dorsal horn of the spinal cord (n = 4, ligation: P < 0.001 versus sham-operated mice, IL6 injection: P < 0.001 versus PBS-treated mice; Supplementary Figs. 5A and B). Furthermore, the increase in Iba-1 immunoreactivity in the dorsal horn of the spinal cord after partial sciatic nerve ligation was almost abolished in CCR2 knockout mice (Fig. 6Cii and Dii), whereas partial sciatic nerve ligation caused the increase in GFAP-positive astrocytes in the same region of both CCR2 knockout and wild-type mice (Fig. 6Aii and Bii). We further found that a single intrathecal injection of MCP-3 at 1 pmol caused a marked increase in Iba-1 levels in the spinal cord at both 3 h and 1 day after the injection (n = 3, 3h: P < 0.001; 24h: P < 0.05 versus saline-treated mice; Supplementary Figs. 6Bii and Cii). Likewise, the increased expression of Iba-1 in the ipsilateral side of the spinal cord obtained from nerve-ligated mice was markedly suppressed by the
Figure 4 IL6-dependent long-lasting histone modification at MCP-3 promoter in the lumbar spinal cord after nerve ligation. (A) Quantitative chromatin immunoprecipitation (qChIP) analysis of acetylated histone 3 (AcH3) and histone 3 trimethylated at lysine 4 (H3K4me3), lysine 9 (H3K9me3) and lysine 27 (H3K27me3) at MCP-3, MCP-1 and RANTES loci in the lumbar spinal cord of nerve-ligated ICR mice at 7 days after nerve ligation. (B) Time-course changes in AcH3, H3K4me3, H3K9me3 and H3K27me3 at the MCP-3 promoter in the lumbar spinal cord of nerve-ligated ICR mice. (C and D) Quantitative chromatin immunoprecipitation (qChIP) analysis of AcH3, H3K4me3, H3K9me3 and H3K27me3 at the MCP-3 promoter in the lumbar spinal cord of wild-type/IL6 knockout (KO) mice at 7 days after nerve ligation (C) and IL6-treated mice at 3 days after intrathecal injection (D). (A–D) Each column represents the mean ± SEM of three samples. *P < 0.05, **P < 0.01 versus sham or PBS groups.
Immunofluorescent staining for MCP-3 in the dorsal horn of the L4 lumbar spinal cord in mice with nerve ligation. (A and B) MCP-3 immunoreactivity was increased in the ipsilateral side of the dorsal horn of the L4 spinal cord at 1 to 2 weeks after nerve injury. (C–E) Cellular distribution of MCP-3 in the L4 lumbar spinal dorsal horn of nerve-ligated mice. Coronal sections of the L4 lumbar spinal dorsal horn were double-immunostained for MCP-3 and NeuN (C), GFAP (D) or Iba-1 (E). MCP-3 immunoreactivity (red) did not appear to be co-localized with NeuN- (C, green) or Iba-1 immunoreactivity (E, green) in the spinal dorsal horn of nerve-ligated mice at 7 days after surgery. MCP-3 immunoreactivity (red) was highly co-localized with GFAP immunoreactivity (D, green) in the same region of the mouse spinal cord at 7 days after nerve ligation. (F and G) Increased GFAP immunoreactivity in the spinal cord of nerve-ligated wild-type (WT) mice (Fii) was abolished in the same area in IL6 knockout (KO) mice at 7 days after surgery (Gii). Scale bars = 50 μm.
repeated intrathecal injection of a function-blocking antibody of MCP-3 at 6 h before and once a day from Day 1 to Day 6 after surgery \((n = 6, P < 0.05\) versus ipsilateral side of nerve-ligated mice after IgG treatment; Supplementary Fig. 7). To ascertain the effect of treatment with MCP-3 on purified mouse spinal microglia, we conducted immunohistochemical staining with a specific antibody for Iba-1 using primary spinal microglia cultures. As shown in Supplementary Fig. 8, in vitro treatment with MCP-3 (100 ng/ml) for 1 day caused a robust microglia activation, as detected by a hypertrophy and an increase in the level of Iba-1 immunoreactivity compared with that in normal medium-treated cells. This activation of purified spinal microglia after MCP-3 treatment was attenuated by co-treatment with a selective CCR2 antagonist RS102895 (10 μM). On the other hand, it has been reported that the spinal application of MCP-1, which is also endogenous CCR2-ligand, produced the activation of ERK in dorsal horn neurons via CCR2, which is closely related to the induction of central sensitization (Gao et al., 2009). In agreement with this report, we demonstrated that intrathecal injection of MCP-3 increased the level of p-ERK in the dorsal horn of the mouse spinal cord at both 3 h and 1 day after the injection \((n = 3, P < 0.05\) versus saline-treated mice; Supplementary Fig. 6Bii and Ci).

We next investigated whether the increased expression of MCP-3 mRNA in the spinal cord could be initiated after the activation of spinal microglia or spinal neurons after partial sciatic nerve ligation. After confirming that CCR2 and PKC-γ messenger RNAs were not expressed in CCR2 and PKC-γ knockout mice, respectively (Supplementary Fig. 9A), we found that deletion of the CCR2 or PKC-γ gene did not affect the acceleration of MCP-3 expression in the spinal cord after nerve injury \((n = 4; \) Supplementary Fig. 9B).

**Direct pain signal induced by spinal MCP-3**

The next study was undertaken to investigate whether the long-lasting, IL6-dependent enhanced MCP-3 production in spinal astrocytes could directly contribute to the development of neuropathic pain induced by partial sciatic nerve ligation. A single intrathecal injection of MCP-3 at 1 pmol caused a painful state in normal mice at 3 h to 2 days after the injection \((n = 6–10; \) Fig. 7A and Supplementary Fig. 6A). Repeated intrathecal treatment with RS102895 (100 ng/mouse) significantly suppressed the painful state as detected by decreased thermal and tactile thresholds after a single intrathecal injection of MCP-3 \((n = 6, \) ANOVA, thermal threshold: \(P < 0.01\), tactile threshold: \(P < 0.05\) versus saline–MCP-3-treated mice; Fig. 7B). Behaviours associated with a neuropathic pain-like state were significantly suppressed by repeated intrathecal injection of MCP-3 antibody twice a day from Day 1 to Day 7 after nerve injury \((n = 6, \) ANOVA, tactile threshold: \(P < 0.05\) versus IgG-MCP-3-treated mice; Fig. 7C). In addition, repeated intrathecal treatment with the MCP-3 antibody twice a day from Day 7 to Day 15 after nerve injury also significantly reversed the decreased thermal thresholds on the ipsilateral side in nerve-ligated mice \((n = 6, \) ANOVA, \(P < 0.01\) versus ligation–IgG mice; Supplementary Fig. 10). As shown in Supplementary Fig. 11, we further demonstrated that the neuropathic pain induced by partial sciatic nerve ligation in wild-type mice was significantly suppressed in CCR2 knockout mice after nerve injury \((n = 5, \) ANOVA, thermal threshold: \(P < 0.001\), tactile threshold: \(P < 0.001\) versus nerve-ligated wild-type mice).

Furthermore, the decreased thermal and tactile thresholds on the ipsilateral side in wild-type mice with partial sciatic nerve ligation were significantly suppressed in IL6 knockout mice \((n = 7–
Figure 7  Involvement of MCP-3 signalling pathways in the spinal cord in the neuropathic pain-like state induced by partial sciatic nerve ligation in mice. (A) Changes in the latency of paw withdrawal in response to a thermal (left) or tactile stimulus (right) in mice with a single intrathecal injection of MCP-3. Each point indicates the mean ± SEM of 6–8 mice. ***P < 0.001 versus PBS group. (B and C) Effect of repeated intrathecal (i.t.) pre-treatment with a selective CCR2 antagonist, RS102895 (B) or a function-blocking antibody of MCP-3 (C) on the development of thermal hyperalgesia (left) and tactile allodynia (right) after intrathecal injection of MCP-3 (B) or partial sciatic nerve ligation (C) in mice. (B) RS102895 was injected intrathecal 1 h before and once a day for three consecutive days after intrathecal injection
Hind paw caused a significant increase in signal activity in these regions of mice (all n = 4, the somatosensory cortex: P < 0.01, cingulate cortex: P < 0.05, medial thalamic nuclei: P < 0.01, lateral thalamic nuclei: P < 0.05 versus PBS-treated mice; Fig. 7H–K).

**Discussion**

It has been considered that long-lasting neuropathic pain may result from hypersensitivity because of the alteration of primary afferent neurons and/or spinal dorsal horn neurons, followed by the facilitation of ascending pain transmission to several brain regions after nerve injury (Ji and Woolf, 2001). This spinal plasticity and neuronal adaptive responses after nerve injury have been believed to require diverse alterations in gene expression. The novel essence of the present study was the identification of long-lasting DNA modifications after nerve injury. Among 33,541 genes for mice, the expression of MCP-3 was the most dynamically changed after nerve ligation. Indeed, it was increased by a factor of almost four to five times more than that of other molecules, such as MCP-1, which has been an important subject in the pathogenesis of neuropathic pain (Gao et al., 2009; Gao and Ji, 2010; Van Steenwinckel et al., 2011). Furthermore, the present findings clearly indicate that the expression of MCP-3 is dramatically increased in the ipsilateral side of the spinal cord obtained from nerve-ligated mice. The critical point of the present study was the ‘duration of action’ of the increased MCP-3. We demonstrated here that MCP-3 gene transcription was dramatically accelerated for 4 weeks after nerve injury, whereas enhanced MCP-1 expression resolved within 2 weeks after partial sciatic nerve ligation. Consistent with reverse transcription–PCR assay showing the long-lasting upregulation of MCP-3 messenger RNA after partial sciatic nerve ligation, present immunohistochemical analysis revealed that MCP-3 immunoreactivity was dramatically increased in the ipsilateral side of the dorsal horn of the mouse spinal cord at 1–2 weeks after partial sciatic nerve ligation. To our knowledge, the present data are the first to indicate that partial ligation of the sciatic nerve caused a marked and long-lasting increase in the expression of MCP-3 in the ipsilateral side of the spinal cord of mice.

The ciliary neurotrophic factor family of cytokines consists of IL6, IL11, ciliary neurotrophic factor, leukaemia inhibitory factor, oncostatin M (Sims et al., 1988), cardiotrophin 1 and...
cardiotrophin-like cytokine. Among this family of cytokines, it has been demonstrated that IL6 is dramatically upregulated after peripheral nerve injury at the site of injury, in both the dorsal root ganglia and the spinal cord (DeLeo et al., 1996; Flatters et al., 2003). Furthermore, IL6 has been shown to be a potent inducer of the Cys–Cys subfamily of chemokines in peripheral tissues (Sobota et al., 2008). In the present study, we demonstrated that the level of IL6 messenger RNA on the ipsilateral side of the mouse dorsal root ganglia was particularly increased at 1 and 7 days after partial sciatic nerve ligation, whereas we did not clearly detect IL6 messenger RNA expression on the ipsilateral side of the mouse spinal cord under the same conditions. Furthermore, the robust increase in MCP-3 messenger RNA expression observed in the spinal cord of nerve-ligated wild-type mice was significantly reduced in the spinal cord of IL6 knockout mice at 7 days after partial sciatic nerve ligation. These findings indicate that increased IL6 is critical for increased MCP-3 expression in the spinal cord after nerve injury.

Several studies have recently demonstrated that glial cells in the spinal cord are key players in the induction and maintenance of pathological pain (Watkins et al., 2001; McMahon et al., 2005; Narita et al., 2006). After activation, microglia and astrocytes are endowed with the ability to secrete soluble mediators (chemokines, cytokines and neurotrophins), which may help to regulate the interaction between the nervous and immune systems. In the present study, the increased MCP-3 immunoreactivity in the ipsilateral side of the dorsal horn of the mouse spinal cord was highly co-localized with the astrocyte marker GFAP, but not with the microglial marker iba-1 or neuronal markers. In addition, the robust increase in GFAP immunoreactivity observed in the ipsilateral side of the dorsal horn of the spinal cord obtained from nerve-ligated wild-type mice was abolished in IL6 knockout mice. These findings suggest that IL6 signalling within the spinal cord may play a critical role in the acceleration of MCP-3 production through the activation of astrocytes on the ipsilateral side of the spinal cord after partial sciatic nerve injury.

A growing body of evidence has suggested that IL6 downstream transcriptional factors, such as STAT3, NFIL6 and PU.1, are critical regulators of the expression of numerous genes involved in innate immunity, including IL1β, toll-like receptor 4 and CD14 (Berczak et al., 2007; Rafei et al., 2008; Tissieres et al., 2009). Interestingly, a recent bioinformatics analysis of the MCP-3 gene predicted putative binding sites for these transcriptional factors within the MCP-3 promoter region (Murakami et al., 1997; Aung et al., 2006; Rafei et al., 2008; Sobota et al., 2008). In the present study, we demonstrated that the level of p-STAT3 and the expression level of PU.1 were significantly increased on the ipsilateral side of the lumbar spinal cord obtained from nerve-ligated mice at 1–3 days and 3–5 days after surgery, respectively. Consistent with these findings, the levels of p-STAT3 and PU.1 messenger RNA were significantly increased in the dorsal horn of the spinal cord after a single intrathecal injection of IL6. These findings, together with the proximal locations of the STAT3 and PU.1 binding elements in the MCP-3 promoter, allow us to conclude that these transcription factors are likely candidates for the regulation of MCP-3 expression in the mouse spinal cord under neuropathic pain.

As described earlier in the text, as activated STAT3 and PU.1 can act as transcriptional factors to promote MCP-3 gene expression, one wonders why there is a discrepancy between the long-lasting enhancement of MCP-3 gene expression and the short duration of the increase in these transcriptional factors. A growing body of evidence suggests that the long-lasting alteration of the transcription of some genes is accompanied by epigenetic modification at the promoter. Such epigenetic modulation is mainly controlled by histone-tail modification. Modification of the histone-tail alters the compact chromatin structure and changes the ability of regulatory proteins to access DNA. The acetylation of H3 and H4 at gene promoters reduces the electrostatic interaction between histone proteins and DNA, which is thought to relax the chromatin structure and make DNA more accessible to transcriptional regulators (Francis et al., 2004; Ringrose et al., 2004; Kouzarides, 2007; Li et al., 2007). Histone methylation is particularly complex and can exist in mono-, di- or trimethylated states, which enables each state to recruit unique co-regulators and exert distinct effects on transcriptional activity (Francis et al., 2004; Ringrose et al., 2004; Kouzarides, 2007; Li et al., 2007). Histone methylation is also unique because each lysine residue has distinct, and often opposite, effects on transcription. For example, H3K4me3 is highly associated with gene activation, whereas H3K9me3 or H3K27me3 is usually associated with gene repression (Francis et al., 2004; Ringrose et al., 2004; Kouzarides, 2007; Li et al., 2007). In the present study, we clearly found that partial sciatic nerve ligation caused a robust and persistent decrease in IL6-dependent H3K27 trimethylation at the MCP-3 promoter in the mouse spinal cord. This phenomenon would help to loosen the structural interactions between DNA and histones and enhance access to IL6 downstream targets, STAT3 and PU.1, for the MCP-3 promoter, leading to a long-lasting increase in MCP-3 expression. Thus, our results provide new insight into the molecular mechanisms at the DNA level that are related to spinal plasticity under neuropathic pain.

Several lines of evidence suggest that CCR2, which has been classified as a receptor for monocyte chemotactic proteins, including MCP-3, is not normally expressed under healthy conditions but is dramatically upregulated in both spinal cord neurons and spinal microglia after peripheral nerve injury (Zhang et al., 2007). Consistent with these reports, we found that the expression of CCR2 messenger RNA was significantly increased in the dorsal horn of the spinal cord after either partial sciatic nerve ligation or the spinal injection of IL6.

Of particular interest is a direct correlation between MCP-3/CCR2 signalling-dependent spinal microglia activation and the pathogenesis of neuropathic pain induced by partial sciatic nerve ligation. In the present study, we found that the spinal application of exogenous MCP-3 causes a painful state and marked increase in iba-1 expression in the spinal cord of normal mice at 3 h and 1 day after the injection. Likewise, in vitro treatment with MCP-3 caused a robust activation of primary spinal microglia cultures, as detected by a hypertrophy and an increase in the level of iba-1 immunoreactivity, via CCR2 expressed mainly in spinal microglia. The increased iba-1 expression in the ipsilateral side of the spinal cord obtained from nerve-ligated mice was significantly suppressed by the repeated intrathecal injection of a function-
blocking antibody of MCP-3. We also found that the robust increase in Iba-1 immunoreactivity observed in the ipsilateral side of the dorsal horn of the spinal cord obtained from nerve-ligated wild-type mice was abolished in CCR2 knockout mice, whereas deletion of the CCR2 gene did not affect the increase in GFAP-positive astrocytes under the neuropathic pain. More importantly, the decreased thermal and tactile thresholds on the ipsilateral side in wild-type mice after partial sciatic nerve ligation were significantly attenuated in CCR2 knockout mice. Our present findings are supported by previous reports by Abbadie et al. (2003) and Zhang et al. (2007). In the report by Zhang et al. (2007), the activation of resident microglia on the ipsilateral side of the dorsal horn of the spinal cord obtained from wild-type mice, which lasted for up to 30 days after partial sciatic nerve ligation, was almost completely abolished in CCR2 knockout mice. They also clearly showed that CCR2 is associated with the infiltration of circulating macrophage into the spinal cord parenchyma of mice and differentiation of migrated cells into the activated microglia. These findings provide critical evidences that MCP-3/CCR2 signalling-dependent reciprocal communication between astrocytes and microglia within the spinal cord may be a key aspect of the pathogenesis of the neuropathic pain induced by partial sciatic nerve ligation.

Gao et al. (2009) have demonstrated that the spinal application of MCP-1 produces the activation of ERK in dorsal horn neurons via CCR2, which is closely related to the induction of central sensitization. In agreement with their report, we found that intrathecal injection of exogenous MCP-3 increased the level of p-ERK in the dorsal horn of the mouse spinal cord at 3 h and 1 day after injection. Although further investigation is still needed, these results support the idea that inducible MCP-3 in spinal astrocytes after nerve injury may act on CCR2 receptors expressed in spinal cord neurons, as well as spinal microglia, to enhance neuronal sensitivity.

On the other hand, it has been reported that mice lacking PKC-γ displayed normal responses to acute pain stimuli, but they almost completely failed to develop a neuropathic pain after partial sciatic nerve section (Malmberg et al., 1997). In this report, they have also shown the restricted distribution of PKC-γ in dorsal horn neurons. Furthermore, we previously demonstrated that the ipsilateral hyperalgesia induced by sciatic nerve ligation was significantly attenuated in PKC-γ knockout mice (Ohsawa et al., 2001). In the present study, we found no change in the increased levels of MCP-3 messenger RNA in PKC-γ knockout mice with partial sciatic nerve ligation. Furthermore, there were no differences in levels of MCP-3 messenger RNA between nerve-ligated wild-type and CCR2 knockout mice at 7 days after nerve injury. These results indicate that the increased expression of MCP-3 messenger RNA in spinal astrocytes can be observed before the activation of spinal microglia and spinal neurons in the dorsal horn of the spinal cord after sciatic nerve injury.

We further investigated whether the increased MCP-3 expression along with epigenetic modification at the MCP-3 promoter in response to IL6 signalling within the spinal cord could directly contribute to the development of neuropathic pain induced by nerve ligation. A single intrathecal injection of MCP-3 caused a painful state in normal mice. In addition, repeated intrathecal treatment with the specific antibody of MCP-3 from Day 1 to Day 7 after surgery or the selective CCR2 antagonist RS102895 1 h before and once a day for three consecutive days after intrathecal injection of MCP-3 significantly attenuated the neuropathic pain-like behaviours induced by nerve injury or MCP-3 injection, respectively. Interestingly, repeated intrathecal treatment with the MCP-3 antibody from Day 7 to Day 15 after surgery was still effective for reversing the neuropathic pain-like behaviours induced by nerve injury, indicating the possibility that blocking MCP-3/CCR2 signalling within the spinal cord may be an effective approach to relieve neuropathic pain. We also found that repeated intrathecal pretreatment with gp130/Fc suppressed the neuropathic pain-like state induced by nerve injury or a single intrathecal injection of IL6 in normal mice. Gp130/Fc is a bivalent homodimer that contains the extracellular ligand-binding domain of a given gp130, followed by the hinge and FCy region of human IgG1 (Sims et al., 1988; Lewis et al., 1991; Kumar et al., 1995). This receptor chimera protein acts at IL6 receptor bodies to sequester endogenous IL6 and is a highly potent and specific inhibitor of endogenous IL6. Taken together, these findings provide, for the first time, critical evidence that, in response to IL6, MCP-3 modulating astrocyte-to-microglia signalling within the spinal cord may contribute to the induction of central sensitization related to the pathogenesis of a neuropathic pain-like state induced by nerve injury in mice.

A better understanding of the direct linkage between activated glia-dependent spinal plasticity and the acceleration of brain activation related to pain recognition may pave the way for the development of new therapies for neuropathies. Another key finding of the present study was that we were able to assess whether the spinal application of exogenous MCP-3 could facilitate neuronal activity in several brain regions, which is associated with the pain network. Functional MRI can be used to investigate the spatial and temporal aspects of brain activation. Functional MRI has been used to objectivley evaluate pain perception in the CNS in healthy humans and in those with various kinds of pain (Honore et al., 2000; Zhang et al., 2004). Noxious heat stimulation in humans or repetitive heat stimulation through peltier elements in animals activates several brain regions; the so-called pain matrix (Tracey et al., 2000; Wise et al., 2004). It has recently been demonstrated that neuroimaging in humans and animals can be used to detect changes in regional activity initiated by the administration of drugs that induce/modulate pain, such as morphine, ketamine, formalin and capsaicin (Leslie and James, 2000; Malisz and Docherty, 2001; Honey et al., 2008; Shih et al., 2008). Furthermore, these studies have introduced the new concept of pharmacological functional MRI for this technique, which promises to become an important new tool for researchers who are interested in mapping and understanding the mechanism of pain. We previously demonstrated using a mouse pharmacological functional MRI assay that neuropathic pain-like transmission evoked by the spinal activation of protein kinase C caused a significant increase in the activity of several brain regions (Niikura et al., 2008). In the present study, we demonstrated that the spinal application of exogenous MCP-3, as well as partial sciatic nerve ligation, followed by the application of heat stimuli to the mouse hind paw caused a significant increase in blood oxygenation...
level-dependent signal intensity in the somatosensory cortex, cingulate cortex and medial and lateral thalamic nuclei of mice. As cortical areas are activated by receiving noxious information from the spinothalamic tract, neuroimaging studies may be able to reveal their activities by demonstrating brain circuitry (Talbot et al., 1991; Borsook et al., 2007). The cingulate cortex area is an affective-motivational component of pain and mainly receives information from the medial system of the spinothalamic tract (Melzack, 1999; Rorden and Karnath, 2004). On the other hand, the somatosensory cortex area is a sensory-discriminative component of pain and mainly receives information from the lateral system of the spinothalamic tract. The medial and lateral thalamic nuclei are also categorized as centres for pain perception, which relay sensory information to those cortical areas. Collectively, activation of the MCP-3/CCR2 signalling network in the spinal cord after nerve injury may cause spinal plasticity, which leads to the sensitization of ascending pain transmission to several brain regions.

In conclusion, we demonstrated that, in response to IL6 signalling within the spinal cord after dorsal root ganglia activation, a dramatic and long-lasting increase in MCP-3 gene expression occurred mostly in astrocytes on the ipsilateral side of the mouse spinal cord after partial sciatic nerve ligation. This long-lasting enhancement of MCP-3 gene transcription was because of a decrease in H3K27 trimethylation at the MCP-3 promoter. This phenomenon could activate spinal microglia that expresses CCR2, which may in turn promote the spinal sensitization associated with the facilitation of ascending pain transmission. Although further molecular experiments are needed, we propose here that the re-setting of enhanced spinal MCP-3 signalling accompanied by epigenetic repressive modification after nerve injury may be a novel target for therapeutic intervention in neuropathic pain states.

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Supplementary material

Supplementary material is available at Brain online.

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