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

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ABSTRACT

Although there is growing evidence showing that the involvement of chemokines in the pathogenesis of neuropathic pain is associated with neuroinflammation, the details are unclear. We investigated the C-X-C chemokine ligand type 2 [macrophage inflammatory protein 2 (MIP-2)/C-X-C chemokine receptor type 2 (CXCR2)] axis and epigenetic regulation of these molecules in neuropathic pain after peripheral nerve injury. Expression of MIP-2 and CXCR2 was increased in the injured peripheral nerve elicits chronic neuroinflammation and presents as severe chronic neuropathic pain (Marchand et al., 2005). The typical symptoms of neuropathic pain include tactile allodynia (a burning pain caused by noxious stimuli), hyperalgesia, and spontaneous pain (Baron, 2006). Because it is difficult to mitigate these symptoms by using standard analgesics (Finnterup et al., 2010), an effective remedy needs to be established. Accumulating evidence indicates that diverse types of immune cells, such as neutrophils and macrophages, infiltrate into the damaged nervous system, and several inflammatory molecules originating from these cells cause the chronic neuroinflammation, leading to neuropathic pain. Neutrophil depletion by intraperitoneal injection of Ly6G antibody attenuated PSL-induced neuropathic pain. Both anti-MIP-2 and SB225002 suppressed up-regulation of inflammatory cytokines and chemokines in the injured SCN. In addition, acetylation of histone H3 [lysine (Lys9)-acetylated histone H3 (AcK9-H3)] on the promoter region of MIP-2 and CXCR2 was increased in the injured SCN after PSL. Expression of AcK9-H3 was observed in the nuclei of neutrophils and macrophages surrounding the epineurium. Administration of the histone acetyltransferase inhibitor anacardic acid suppressed the up-regulation of MIP-2 and CXCR2 in the SCN after PSL and resulted in the prevention of PSL-induced neuropathic pain. Taken together, these results show that augmentation of the MIP-2/CXCR2 axis by hyperacetylation of histone H3 on the promoter region of MIP-2 and CXCR2 located in the injured peripheral nerve elicits chronic neuroinflammation through neutrophil accumulation, leading to neuropathic pain.

Introduction

Neuropathic pain occurs because of damage and/or inflammation in the nervous system and presents as severe chronic pain (Marchand et al., 2005). The typical symptoms of neuropathic pain include tactile allodynia (a burning pain caused by noxious stimuli), hyperalgesia, and spontaneous pain (Baron, 2006). Because it is difficult to mitigate these symptoms by using standard analgesics (Finnterup et al., 2010), an effective remedy needs to be established. Accumulating evidence indicates that diverse types of immune cells, such as neutrophils and macrophages, infiltrate into the damaged nervous system, and several inflammatory molecules originating from these cells cause the chronic neuroinflammation, leading to neuropathic pain (Scholz and Woolf, 2007). Cyto-
kines, including interleukin (IL)-1β and tumor necrosis factor (TNF) α, are major inflammatory molecules that have been well investigated as key mediators of neuropathic pain (Moalem and Tracey, 2006).

Chemokines are primary regulators of the inflammatory response, and their receptors are widely expressed in most tissues (Ransohoff, 2009). Several lines of evidence demonstrate that the enhancement of inflammatory chemokine signals aggravates inflammatory diseases (Charo and Ransohoff, 2006). There is recent evidence indicating that chemokine systems modulate acute and chronic pain. Indeed, up-regulation of the CC-chemokine ligand 2 and CC-chemokine ligand 3/macrophage inflammatory protein (MIP)-1α after nerve injury contributes to the development of neuropathic pain via peripheral and central mechanisms (White et al., 2007; Ren and Dubner, 2010). Nevertheless, the comprehensive roles of chemokines underlying the pathogenesis of neuropathic pain are poorly understood.

Prolonged gene expression in human disease has been associated with epigenetic alterations that affect chromatin dynamics (Egger et al., 2004). It is noteworthy that histone modifications, such as acetylation, methylation, and phosphorylation can affect transcriptionally active euchromatin and silencing heterochromatin (Khorasanizadeh, 2004). Among the core histones, modifications of histone H3 and H4 have been well investigated. Euchromatin is characterized by hyperacetylation of lysine residues, such as Lys9, Lys14, and Lys23 of histone H3 (Kondo et al., 2004; MacDonald and Howe, 2009). Lys9-H3 is also modified by methylation, which results in the conversion to heterochromatin, and the modification of Lys9-H3 has an important role in gene expression. Because cytokine up-regulation is also affected by the degree of histone acetylation (Schmeck et al., 2008), involvement of histone modification was demonstrated in various cytokine-related diseases (Portela and Esteller, 2010). However, the relationship between epigenetic alterations and the pathogenesis of neuropathic pain have not been conducted.

In the present study the roles of the CXC-chemokine CXCL2 (MIP-2; a murine functional homolog of IL-8, which is a mammalian neutrophil migration factor) (Sonoda et al., 1998) and its receptor, CXCR2, were investigated in nerve injury-induced neuropathic pain. Moreover, the epigenetic regulation of chemokines was also examined for the targets of neuropathic pain.

Materials and Methods

Animals and Surgery. All experimental procedures used in this study were approved by the Animal Research Committee of Wakayama Medical University and complied with the Ethical Guidelines of the International Association for the Study of Pain. Male ICR mice (20–22 g) were obtained from SLC (Osaka, Japan). They were housed in plastic cages in a temperature-controlled (23–24°C, 60–70% relative humidity) vivarium with a 12-h dark/light cycle and fed water and food ad libitum. To induce neuropathic pain, mice were subjected to partial sciatic nerve ligation (PSL) using a method described previously (Seltzer et al., 1990). Under sodium pentobarbital (72 mg/kg i.p.) anesthesia, the agents (10 μl) were injected without a skin incision into the region surrounding the SCN, using a microsyringe fitted with a 30-gauge needle. Mice were kept for at least 3 h, and behavioral tests were performed after anesthetic effects completely disappeared. For the depletion of circulating neutrophils, as shown in previous reports (Stirling et al., 2009), functional grade antibody for Ly6G (eBioscience, San Diego, CA) and control rat IgG2b (eBioscience) were dissolved in sterile PBS and administered at 4 mg/kg i.p. to naive mice 1 day before surgery.

Quantitative and Semiquantitative RT-PCR. Mice were euthanized by rapid decapitation, and a 10-mm length of SCN was collected. TRIzol reagent (Invitrogen, Carlsbad, CA) was used for the isolation of total RNA from the SCN, and then 1 μg of total RNA was converted to cDNA by reverse transcription, using PrimeScript (Takara Bio, Otsu, Japan) and random primers (Invitrogen). The cDNA was used for the template for quantitative real-time PCR with qPCR Mastermix Plus for SYBR Green I (Eurogentec, Seraing, Belgium) containing deoxyoxynucleoside-5’-triphosphates, DNA polymerase, using iCycler and iQ Real-Time PCR systems (Bio-Rad, Tokyo, Japan). PCR was performed under the following conditions: 10 min at 95°C, followed by 50 cycles of two steps, 15 s at 95°C and 1 min at 60°C. The fluorescent intensity of the intercalated SYBR Green I was analyzed and normalized by GAPDH. For the semiquantitative PCR, GoTaq DNA polymerase (Promega, Madison, WI) and a mixture of deoxyoxynucleoside-5’-triphosphates (Promega) were used. The PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The fluorescent intensities of the PCR products were analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD) and normalized by GAPDH. Data are presented as the expression ratio to GAPDH. All primers used in both analyses were purchased from Operon Biotechnology (Tokyo, Japan), and these sequences are shown in Table 1.

Immunohistochemistry. Mice were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially with ice-cold PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The collected SCN was postfixed and dehydrated in 25% sucrose at 4°C overnight. Tissue was frozen in a freezing compound (SAKURA, Tokyo, Japan) and cut longitudinally at 10 μm by using a cryostat, and the sections were mounted on silane-coated glass slides. The sections were washed with PBS containing 0.1% Triton X-100 and incubated with blocking buffer (4% BSA, 0.1% Triton X-100 in PBS) for 2 h at room temperature. The sections were incubated in specific primary antibodies against MIP-2 (20 μg/ml; R&D Systems), CXCR2 (1:100, Abcam Inc., Cambridge, MA), F4/80 (macrophage marker, 1:200; Cedarlane, Ontario, Canada), Iba-1 (macrophage marker, 1:200; Wako Pure Chemicals, Osaka, Japan), Ly-6G (neutrophil marker, 1:100; eBioscience), and lysine (Ly6S)-acylated histone H3 (AcK9-H3; 1:600; Abcam Inc.) at 4°C overnight. Antibodies were diluted in reaction buffer (1% BSA and 0.025% Triton X-100 in PBS). The sections were washed with PBS containing 0.1% Triton X-100 and incubated in secondary antibodies conjugated with fluorescent markers (Alexa Fluor-488 or Alexa Fluor-594, 1:200; Invitrogen) for 2 h at room temperature, followed by nuclear staining using Hoechst33342 solution (1:1000; Invitrogen). A cover slip with Perma Fluor (Thermo Fisher Scientific, Waltham, MA) was placed over the sections, and immunoreactivity was detected by using a confocal laser scanning microscope. Micro-
50 mM sodium cacodylate, pH 7.4). The excised SCN was fixed in Glutaraldehyde 2% and osmium tetroxide 1%. After dehydration in ethanol, the SCN was embedded in Quetol 812 resin (Nissin EM, Tokyo, Japan). The SCN was cut transversely at 90 nm thickness by using an ultramicrotome (Reichert, Depew, NY), and sections were mounted on a polycarbonate grid. The sections were stained with uranyl acetate and lead citrate and examined with a JEM-1400 electron microscope (JEOL, Tokyo, Japan) at a voltage of 60 kV.

**Chromatin Immunoprecipitation Assay.** ChiP assay was performed as described previously, with several modifications (Kondo et al., 2004; Maeda et al., 2009). The SCN was freshly isolated from two euthanized mice, and tissues were combined and analyzed as one sample. The SCNs were cross-linked in PBS containing 1% formaldehyde for 10 min at room temperature. To quench the reaction, glycine was added to adjust the final concentration to 400 mM and incubated for 10 min. The SCN was washed with PBS, resuspended in SDS lysis buffer (50 mM Tris, 10 mM EDTA, and 1% SDS, pH 8.0), and homogenized. After the addition of ChIP dilution buffer (50 mM Tris, 167 mM NaCl, 1% Triton X-100, and 0.1% deoxycholic acid, pH 8.0) with protease inhibitor cocktails, SCNs were sonicated on ice. Lysates were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was collected, and 1/10 of the supernatant was separated as an input sample. For immunoprecipitation (IP), a 30-μl aliquot of protein-G agarose beads (Calbiochem) was washed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 0.1% deoxycholic acid, pH 8.0) and incubated with 1 μg of antibody for lysine (Lys9)-acetylated histone H3 (Abcam Inc.) or normal H3 (Abcam Inc.) in RIPA buffer containing 1% BSA, for at least 4 h at 4°C. Chromatin samples diluted in RIPA buffer were diluted 10-fold before immunoprecipitation. DNA was precipitated with ethanol, washed with 70% ethanol, and resuspended in 0.2× Tris-EDTA buffer. A ChiP assay was performed as described previously, with several modifications (Kondo et al., 2004; Maeda et al., 2009). The genomic region and the histone modification were identified by ChIP-seq analysis.

**Behavioral Test.** PSL-induced tactile allodynia and thermal hyperalgesia were assessed by the von Frey test and the Hargreaves test (Hargreaves et al., 1988), mice were placed on a 5-mm wire-mesh grid floor and covered with an opaque cup. Mice were allowed to adapt for 2 to 3 h before the test. The von Frey filaments (Neuroscience, Tokyo, Japan) were inserted through the mesh floor bottom and applied to the middle of the plantar surface of the hind paw with a weight of 0.07 or 0.16 g. Withdrawal responses were measured 10 times for each hind paw. Tactile allodynia was considered the number of withdrawal responses to stimulation. In the Hargreaves test (Hargreaves et al., 1988), mice were placed on top of a glass sheet and covered with a clear cage. Mice were allowed to adapt for 2 to 3 h before the test. The radiant heat source (IITC 390 Plantar Test Analgesia Meter (Neuroscience), was positioned under the glass sheet and applied to the plantar surface of the hind paw. Withdrawal latencies were measured three times for each hind paw, and thermal hyperalgesia was considered the mean latency of three stimulations. A cutoff latency of 15 s was set to avoid tissue damage.

**Transmission Electron Microscopy.** Deeply anesthetized mice were perfused transcardially with ice-cold saline, followed by 50% Karnovsky solution (5% glutaraldehyde, 4% paraformaldehyde, and 50 mM sodium cacodylate, pH 7.4). The excised SCN was fixed in Karnovsky solution for 2 h and rinsed with 0.1 M sodium cacodylate at 4°C overnight. The SCN was postfixed in 2% osmium tetroxide for 2 h at 4°C and dehydrated through graded (50, 70, 80, 90, 95, and 100%) ethanol. After the replacement of ethanol for propylene oxide, SCN was embedded in Quetol 812 resin (Nissin EM, Tokyo, Japan). The SCN was cut transversely at 90 nm thickness by using an ultramicrotome (Reichert, Depew, NY), and sections were mounted on a copper grid. The sections were stained with uranyl acetate and lead citrate and examined with a JEM-1400 electron microscope (JEOL, Tokyo, Japan) at a voltage of 60 kV.

**TABLE 1**

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**ChiP assay**

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**Fig. 1.** Neutrophil accumulation in the injured sciatic nerve. A, accumulation of Ly6G+ neutrophils in the SCN was visualized by immunohistochemistry. Representative micrographs of longitudinal SCN after sham operation or PSL are shown. Scale bars, 20 μm. B, quantitative analysis of neutrophils was calculated in a square area of 4 × 104 μm². Each column shows the mean number of neutrophils. Data are presented as the mean ± S.E.M. (n = 4–5). **+, P < 0.001 versus sham. C, association between Ly6G+ neutrophils and Iba-1+ macrophages in the SCN at 12 h after PSL was evaluated by double-immunostaining. Green, Ly6G; red, Iba-1. Scale bar, 20 μm.
added in protein-G agarose beads combined with antibody and incubated at 4°C overnight. After gentle centrifugation at 1000 rpm for 1 min, the beads were washed with RIPA buffer and TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). To elute the chromatin complex and reverse cross-link, beads were resuspended in elution buffer (10 mM Tris, 300 mM NaCl, 5 mM EDTA, and 0.5% SDS, pH 8.0) and incubated for 6 h at 65°C followed by incubation with 0.05% proteinase K for 1 h at 55°C. Input chromatin samples were also processed in the same way. The DNA was purified with conventional phenol-chloroform extraction and isopropyl-alcohol precipitation. Purified DNA was dissolved in Tris/EDTA buffer and used as a template for quantitative or semiquantitative PCR analysis with a particular transcription factor binding site on promoter regions for MIP-2 and CXCR2 gene-specific primers. After electrophoresis on agarose gel, the intensities of the PCR products were analyzed by using ImageJ software. Data are presented as the expression ratio to input control. All primers were purchased from Operon Biotechnology, and the sequences are shown in Table 1.

Statistical Analysis. Data are presented as the mean ± S.E.M. Statistical analysis was performed by using a two-way analysis of variance followed by Bonferroni multiple comparisons test, one-way analysis of variance followed by Tukey multiple comparisons test, or Student’s t test. Significance was established at P < 0.05.

Results

Up-Regulation of MIP-2 and CXCR2 in Infiltrating Neutrophils and Macrophages. To profile the accumulation of neutrophils after PSL, these cells were visualized by antibody for Ly6G, a neutrophil marker. Ly6G-positive (Ly6G⁺) neutrophils were prominently increased in the injured SCN at 12 h to 3 days after PSL, whereas no Ly6G⁺ neutrophils were observed in the sham-operated SCN (Fig. 1A). Quantitative analysis revealed that the number of Ly6G⁺ neutrophils in an area of 4 × 10⁴ μm² in size in the PSL-operated SCN was significantly greater than that in the sham-operated SCN (Fig. 1B). Quantitative RT-PCR revealed that mRNA expression of myeloperoxidase (MPO), a neutrophil-specific molecule, in the SCN was up-regulated at 12 h and 1 day after PSL (Supplemental Fig. 1). Iba-1-positive (Iba-1⁺) macrophages were observed around the accumulated Ly6G⁺ neutrophils at 12 h after PSL (Fig. 1C). The mRNA expression of MIP-2 in the injured SCN was up-regulated at 6 h to 1 day after PSL (Fig. 2A). The mRNA expression of MIP-2 and CXCR2 gene-specific primers. After electrophoresis on agarose gel, the intensities of the PCR products were analyzed by using ImageJ software. Data are presented as the expression ratio to input control. All primers were purchased from Operon Biotechnology, and the sequences are shown in Table 1.

Fig. 2. Up-regulation of MIP-2 and CXCR2 in accumulating neutrophils and macrophages in the injured SCN. A and D, time course of MIP-2 (A) and CXCR2 (D) up-regulation in the SCN after PSL was evaluated by real-time RT-PCR. Each column shows the mean ratio of MIP-2/GAPDH (A) or CXCR2/ GAPDH (D). Data are presented as the mean ± S.E.M. (n = 5–6), **, P < 0.01; ***, P < 0.001 versus Pre (naive). B and E, expression of MIP-2 (B) and CXCR2 (E) in the SCN was visualized by immunohistochemistry. Representative micrographs of longitudinal SCN at 12 h after sham or PSL are shown. Scale bars, 20 μm. C and F, localization of MIP-2 (C) and CXCR2 (F) in F4/80⁺ macrophages or Ly6G⁺ neutrophils in the SCN at 12 h after PSL were evaluated by double-immunostaining. Green, MIP-2 or CXCR2; red, F4/80 or Ly6G. Scale bars, 10 μm.
ined. Long-lasting tactile allodynia and thermal hyperalgesia estimated by von Frey and Hargreaves tests, respectively, were elicited in the PSL-operated (ipsilateral) side, but not the untreated (contralateral) side. No pain behavior was observed after the sham operation (Fig. 3, A–D). PSL-induced tactile allodynia and thermal hyperalgesia were significantly prevented by the perineural administration of anti-MIP-2 (1 ng; every 3 days, 0–6 days). Normal IgG given as a control had no effect on the PSL-induced neuropathic pain (Fig. 3, A and B). Preventive effects of anti-MIP-2 were observed in a dose-dependent manner in both tests (Supplemental Fig. 2, A and B). Next, we evaluated the effects of SB225002, an antagonist for CXCR2 (Bento et al., 2008), on PSL-induced neuropathic pain. PSL-induced tactile allodynia and thermal hyperalgesia in the ipsilateral side were significantly prevented by the perineural administration of SB225002 (1 μg; every day, 0–6 days). SB225002 administration had no effect on the contralateral side (Fig. 3, E and F). Preventive effects of SB225002 were dose-dependent in both tests (Supplemental Fig. 2, C and D). To examine whether exogenous MIP-2 elicits neuropathic pain-like behavior, rMIP-2 was perineurally administered in naive mice. A single injection of rMIP-2 (1 μg; 0 day) elicited tactile allodynia- and thermal hyperalgesia-like prolonged pain behaviors in the ipsilateral side at 4 to 7 days after administration (Fig. 3, G and H).

**Contribution of Infiltrating Neutrophils to PSL-Induced Neuropathic Pain.** Expression of CXCR2 was found in neutrophils, which was in agreement with a previous report suggesting that MIP-2 plays a key role in neutrophil migration (Gouwy et al., 2005). Therefore, we investigated the effect of anti-MIP-2 on neutrophil infiltration in the injured SCN after PSL. Immunohistochemistry revealed that the infiltration of Ly6G+ neutrophils on day 1 after PSL was reduced by perineural administration of anti-MIP-2 (1 ng; 0 day), which was confirmed by quantitative analysis in an area of 4 × 10^4 μm^2 in size in the SCN (Fig. 4A). Transmission electron microscopy revealed intact myelin and nerve fibers in the SCN after the sham operation and no neutrophil infiltration. Infiltrating neutrophils and morphological alterations of myelin were observed in the injured SCN on day 1 after PSL, which were suppressed by the perineural administration of anti-MIP-2 (Fig. 4B). Moreover, neutrophil accumulation surrounding the epineurium was observed, but only a few neutrophils and macrophages were observed after the sham operation. In contrast, many accumulating neutrophils and macrophages were observed on day 1 after PSL. The number of accumulated neutrophils surrounding the epineurium was also reduced by the anti-MIP-2 antibody treatment (Fig. 4C). To assess the contribution of neutrophils on PSL-induced neuropathic pain, circulating neutrophils were depleted by intraperitoneal injection of antibody for Ly6G (4 mg/kg), as mentioned in a previous report (Daley et al., 2008). Rat IgG2b (4 mg/kg) was injected as a control. Accumulation of Ly6G+ neutrophils in the injured SCN on day 1 after PSL was markedly reduced by Ly6G antibody administration (Fig. 4D). PSL-induced tactile allodynia and thermal hyperalgesia in the ipsilateral side were significantly attenuated.
by the Ly6G antibody. Neutrophil depletion had no effect on pain behavior in the contralateral side (Fig. 4, E and F).

Suppression of Neuroinflammation after PSL Caused by Inhibition of the MIP-2/CXCR2 Axis. The involvement of MIP-2/CXCR2 axis was tested on PSL-induced neuroinflammation, which is observed by the up-regulation of inflammatory molecules. RT-PCR revealed that PSL-induced up-regulation of IL-1β, TNFα, MIP-1α, and MIP-1β were significantly suppressed by perineural administration of anti-MIP-2 (1 ng; every 3 days, 0–6 days) in the injured SCN, 7 days after PSL. These molecules were hardly expressed in the SCN after the sham operation (Fig. 5, A–D). PSL-induced up-regulation of all of these molecules in the injured SCN at 7 days was also suppressed by perineural administration of SB225002 (1 µg; every day, 0–6 days) (Fig. 5, E–H). Moreover, mRNA for F4/80 and CD3γ, which is a T-lymphocyte-specific molecule, was increased in the injured SCN after PSL, indicating the accumulation of macrophages and T-lymphocytes. Immunohistochemistry confirmed the accumulation of F4/80+ macrophages, and CD3+ T-lymphocytes on day 7 after PSL (Supplemental Fig. 3). Increased expression of these molecules was suppressed by SB225002. The expression of these molecules was lower in the contralateral side (Fig. 5, I and J).

Hyperacetylation of Histone H3 in the Promoter Region of MIP-2 and CXCR2. Normally, gene transcription is enhanced by the acetylation of histones in the promoter region, because transcription factors easily come into contact with DNA in the euchromatin form, a loose state of the chromatin complex (Khorasanizadeh, 2004). Then we determined the involvement of histone H3 acetylation in the PSL-induced up-regulation of MIP-2 and CXCR2 in the injured SCN. Promoter regions of both MIP-2 and CXCR2 were immunoprecipitated by antibody for AcK9-H3, which was increased at 12 h to 1 day after PSL, showing that histone H3 acetylation was increased by PSL. There was no change in AcK9-H3 after the sham operation (Fig. 6, A and B). These
results were confirmed by use of primers for other sequences of MIP-2 and CXCR2 promoter regions in the ChIP assay (Supplemental Fig. 4). The results of qPCR were similar to that of semi-qPCR (Supplemental Fig. 5). Immunostaining for AcK9-H3 was markedly increased in the surrounding epineurium at 12 h after PSL compared with the sham operation control, and staining was localized in the nuclei of F4/80$^{+}$ macrophages and Ly6G$^{+}$ neutrophils (Fig. 6, C and D). By comparison, DNA immunoprecipitated by antibody for normal histone H3 did not change, indicating that the expression level of histone H3 was constant after PSL. To clarify whether total histone expression level is altered by PSL, mRNA expression of core histone molecules (H2, H3, and H4) and linker histone H1 in the SCN were evaluated. Expression levels of histone H3, H4, H2a, and H1 1 day after PSL were similar to those after the sham operation (Fig. 7).

**Involvement of Histone H3 Acetylation in MIP-2/CXCR2 Axis-Mediated Neuropathic Pain.** Histone acetylation is regulated by HAT and histone deacetylase. Thus, we examined the effects of the HAT inhibitor ACA (Sung et al., 2008) on MIP-2/CXCR2 axis-mediated neuropathic pain after PSL. ACA (30–100 μmol/kg) was administered intraperitoneally 30 min before PSL. Up-regulation of MIP-2 and CXCR2 was suppressed by ACA in a dose-dependent manner in the SCN on day 1 after PSL (Fig. 8, A and B). Moreover, MPO expression was also suppressed by ACA, indicating that ACA prevented neutrophil accumulation in the injured SCN (Fig. 8C). The preventive effects of ACA on the hyperacetylation of histone H3 in the promoter regions of MIP-2 and CXCR2 was examined on day 1 after PSL by using the ChIP assay. Promoter DNA of both MIP-2 and CXCR2 was immunoprecipitated by anti-AcK9-H3 in the SCN after ACA treatment and PSL. ACA had no effect on the amount of DNA immunoprecipitated by antihistone H3 antibody (Fig. 8, D and E). Furthermore, PSL-induced tactile allodynia and thermal hyperalgesia on day 7 after PSL were significantly prevented by ACA, whereas ACA by itself had no effect on behaviors after the sham operation (Fig. 8, F and G).

**Discussion**

In the present study, we show that activation of MIP-2/CXCR2 axis in the injured SCN elicits neuropathic pain through neutrophil accumulation, which is associated with neuroinflammation. Because the expression of MIP-2 was rapidly and transiently up-regulated in macrophages and neutrophils,
MIP-2 may act in the early phase of PSL-induced neuropathic pain. Previous reports showed that resident macrophages and Schwann cells were activated in the injured peripheral nerves, and they release several inflammatory mediators (Mueller et al., 2001; Thacker et al., 2007), which initiate neuroinflammation. Then, circulating immune cells such as macrophages, neutrophils, and lymphocytes are recruited by chemokines derived from activated macrophages and Schwann cells (Gouwy et al., 2005; Scholz and Woolf, 2007; Soehnlein et al., 2009). Despite several lines of evidence showing the involvement of macrophages, the detailed roles of neutrophils in neuropathic pain were unknown. In this study, neutrophils were found to accumulate after PSL with macrophages, as an early event preceding various other pathological features in the injured SCN. On the other hand, MIP-2 is a principal chemotactic factor for neutrophils (Sonoda et al., 1998). Therefore, we hypothesized that up-regulated MIP-2 elicited neuropathic pain through the accumulation of neutrophils. Indeed, perineural administration of anti-MIP-2 prevented PSL-induced tactile allodynia and thermal hyperalgesia, which was accompanied by the suppression of neutrophil accumulation. Histological analysis revealed that the neutrophils accumulated in the surrounding epineurium and penetrated into the SCN. Moreover, infiltrating neutrophils were localized near denatured myelin, which is in agreement with evidence indicating the relationship between neutrophils and myelin morphology (Morin et al., 2007). These events were suppressed by anti-MIP-2. Neutrophil depletion by Ly6G antibody attenuated PSL-induced neuropathic pain. Because the effect of Ly6G antibody treatment lasts for several days, neutrophil functions might be blocked in the early phase after nerve injury. These results suggest that MIP-2 and the accumulated neutrophils in the injured SCN strongly contribute to the development of neuropathic pain. This hypothesis is supported by results showing that perineural administration of rMIP-2 caused neuropathic pain behaviors. Although it is reported that sensory neurons have some chemokine receptors, such as C-C chemokine receptor type 1 and CXCR4, there is no report showing the expression of CXCR2 in the sensory neurons. Thus, delayed effects of rMIP-2 might be based on the migration of neutrophils and a part of macrophages.

Because CXCR2 is considered the dominant MIP-2 receptor (Cacalano et al., 1994), regulation of CXCR2 expression may be important for MIP-2 functions. CXCR2 expression was also up-regulated after PSL and localized in accumulating macrophages.

**Fig. 6.** Epigenetic augmentation of histone H3 acetylation in the promoter region of MIP-2 and CXCR2. A and B, time course of histone H3 acetylation in the promoter region of MIP-2 (A) and CXCR2 (B) in the SCN after PSL was evaluated by ChIP assay using ChIP primer. A representative image is shown; each column shows the mean ratio of AcK9-H3 IP/input or normal H3 IP/input. Data are presented as the mean ± S.E.M. (n = 4), ***, P < 0.001 versus Pre (naive).** C, expression of AcK9-H3 in the SCN was visualized by immunohistochemistry. Representative micrographs of longitudinal SCN at 12 h after sham operation or PSL are shown. Scale bars, 20 μm. D, localization of AcK9-H3 in the nuclei of F4/80− macrophages and Ly6G+ neutrophils in the SCN at 12 h after PSL were evaluated by double-immunostaining. Green, AcK9-H3; red, F4/80 or Ly6G; blue, Hoechst33342. Scale bars, 10 μm.

**Fig. 7.** No change of total histone expression in the SCN. Expression of histone mRNA in the SCN 1 day after PSL was evaluated by real-time RT-PCR. Each column shows the mean ratio of histone H1/GAPDH (A), H2a/GAPDH (B), H3/GAPDH (C), and H4/GAPDH (D). Data are presented as the mean ± S.E.M. (n = 5–6).
phages and neutrophils. According to our results, perineural administration of the CXCR2 antagonist SB225002 significantly prevented PSL-induced neuropathic pain. Because the protein expression occurred after the up-regulation of mRNA, inhibitory agents were administered until day 6 after PSL to significantly prevent PSL-induced neuropathic pain. Because macrophages also express CXCR2, its activation was suppressed by inhibition of the MIP-2/CXCR2 axis. According to the close relationship among immune cells, it is hypothesized that the MIP-2/CXCR2 axis may recruit these immune cells, which are responsible for neuropathic pain (Moalem and Tracey, 2006).

Although the upstream regulators of MIP-2 and CXCR2 are not well known, we showed that epigenetic regulation of histone H3 acetylation in the promoter regions was underlying the up-regulation of MIP-2 and CXCR2, leading to neuropathic pain. Recently, histone modifications, including acetylation, have been associated with the pathogenesis of several diseases based on the activation of immune cells (Uchida et al., 2010). Epigenetic mechanisms controlling pain are poorly understood, but evidence is emerging (Doehring et al., 2011). For instance, contributors to neuropathic pain, such as sensory fiber dysfunction and opioid resistance, are known to be modified by histone acetylation-dependent up-regulation of a silencing factor (Uchida et al., 2010). Epigenetic modification of glutamate receptors can aid in the treatment of inflammatory pain (Chiechio et al., 2009). In addition, general up-regulation of inflammatory mediators in immune cells is associated with histone acetylation (Schmeck et al., 2008). These lines of evidence suggest that uncultivated epigenetic mechanisms may be used as novel therapeutic strategies for neuropathic pain.

The time course of histone H3 acetylation in the promoter regions of MIP-2 and CXCR2 was similar to that of mRNA expression of these molecules in the SCN after PSL. Moreover, immunohistochemical analysis revealed that AcK9-H3 expression was increased surrounding the epineurium of the
SCN after PSL, and it was observed mainly in the nuclei of macrophages and neutrophils, which are sources of MIP-2 and CXCR2. These results strongly indicate that the expression of MIP-2 and CXCR2 were regulated by histone H3 acetylation. The total amount of histone expression was constant, as shown by RT-PCR and ChIP assays. The degree of histone acetylation is controlled by the enzymes HAT and histone deacetylase (Khorasanizadeh, 2004; MacDonald and Howe, 2009). These enzymes are directly associated with the pathogenesis of several diseases, such as cancer, Parkinson’s disease, and amyotrophic lateral sclerosis (Egger et al., 2004; Portela and Esteller, 2010). To assess whether hyperacetylation of histone H3 after PSL is regulated by HAT and involved in neuropathic pain, we examined the effect of ACA, an inhibitor of HAT, on PSL-induced neuropathic pain-related events (Sung et al., 2008). Hyperacetylation of histone H3 in the promoter region of both MIP-2 and CXCR2 after PSL was suppressed by ACA. PSL-induced up-regulation of MIP-2 and CXCR2 in the injured SCN was attenuated by ACA. Moreover, up-regulation of MPO was also suppressed by ACA. Finally, PSL-induced neuropathic pain was also prevented by ACA. These results together suggest that the MIP-2/CXCR2 axis, which is positively regulated by histone H3 acetylation, recruits neutrophils to the injured SCN and elicits neuropathic pain. As a result, various events after PSL were suppressed by ACA, indicating that HAT may be activated by PSL and may play a crucial role in neuropathic pain. Although inflammatory molecules are responsible for the histone modification enzymes (Aung et al., 2006), the detailed mechanisms of HAT activation in the injured SCN are unknown. Thus, it is important to clarify the mechanisms underlying neuroinflammation, including the interactions among neurons, glial cells, and immune cells in the injured SCN.

In conclusion, expression of MIP-2 and CXCR2 was up-regulated by epigenetic histone H3 acetylation in recruited macrophages and neutrophils localized in the injured peripheral nerves. Augmentation of the MIP-2/CXCR2 axis recruited neutrophils into the injured nerves and elicited neuroinflammation. Initially, various inflammatory molecules are responsible for the histone modification enzymes (Aung et al., 2006), the detailed mechanisms of HAT activation in the injured SCN are unknown. Thus, it is important to clarify the mechanisms underlying neuroinflammation, including the interactions among neurons, glial cells, and immune cells in the injured SCN. We propose that novel critical molecules involving the MIP-2/CXCR2 antagonist SB225002 ameliorate acute inflammatory colitis in mice. J Leukoc Biol 83:84–82.


tion of the inhibitory subunit of nuclear factor-κB kinase, leading to potentiation of apoptosis. Blood 111:4880–4891.


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Supplementary figure 1

Norikazu Kiguchi, Yuka Kobayashi, Takehiko Maeda, Yohji Fukazawa, Kazuo Tohya, Michio Kimura, Shiroh Kishioka

Epigenetic augmentation of the MIP-2/CXCR2 axis through histone H3 acetylation in injured peripheral nerves elicits neuropathic pain

*J. Pharmacol. Exp. Ther.*

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**Fig. S1. Upregulation of myeloperoxidase in the injured SCN.**

Time course of myeloperoxidase (MPO) expression in the SCN after PSL was evaluated by real-time RT-PCR. Each column shows the mean ratio of MPO/GAPDH. Data are presented as the mean ± SEM. n = 5-6. **P<0.01 vs. Pre (Naïve).**
Supplementary figure 2

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**Fig. S2. Prevention of neuropathic pain by MIP-2/CXCR2 axis inhibitory agents.**

Dose-dependent effects of MIP-2/CXCR2 axis-inhibitory agents on tactile allodynia and thermal hyperalgesia were evaluated on day 7 after PSL by the von Frey test (A, C) and the Hargreaves test (B, D), respectively. (A, B) The anti-MIP-2 (0.3-3 ng) of Veh was perineurally-administered three times, just after PSL, 3 and 6 days after PSL. (C, D) SB (0.3-3 μg) or Veh were perineurally-administered seven times, just after PSL, 1 to 6 days after PSL. (A-D) Data are presented as the mean ± SEM. n=4-11. ***P<0.001 vs. Veh/contra. ###P<0.001, ##P<0.01, #P<0.05 vs. Veh/ipsi.
Supplementary figure 3

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**Fig. S3. Accumulation of immune cells in the injured SCN.**

Accumulation of F4/80⁺, Iba-1⁺ macrophages and CD3⁺ lymphocytes in the injured SCN was evaluated by immunostaining. Representative micrographs of longitudinal SCN on day 7 after PSL are shown. Green; F4/80 or CD3, red; Iba-1. Scale bars = 10 μm.
Supplementary figure 4

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Fig. S4. Hyperacetylation of histone H3 in the promoter region of MIP-2 and CXCR2.
(A, B) Time course of histone H3 acetylation in the promoter region of MIP-2 and CXCR2 in the SCN after PSL was evaluated by ChIP assay using ChIP primer (2). A representative image is shown; each column shows the mean ratio of lysine (K9)-acetylated H3 (AcK9-H3) IP/input or normal H3 IP/input. Data are presented as the mean ± SEM. n = 4. ***P<0.001 vs. Pre (Naive).
Supplementary figure 5

Norikazu Kiguchi, Yuka Kobayashi, Takehiko Maeda, Yohji Fukazawa, Kazuo Tohya, Michio Kimura, Shiroh Kishioka

Epigenetic augmentation of the MIP-2/CXCR2 axis through histone H3 acetylation in injured peripheral nerves elicits neuropathic pain

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**Fig. S5. Hyperacetylation of histone H3 in the promoter region of CXCR2.**

Time course of histone H3 acetylation in the promoter region of CXCR2 in the SCN after PSL was evaluated by ChIP assay (quantitative PCR using ChIP primer (1)). Each column shows the mean ratio of lysine (K9)-acetylated H3 (AcK9-H3) IP/input. Data are presented as the mean ± SEM. n = 4. *P<0.001 vs. Pre (Naïve).