CCL-1 in the spinal cord contributes to neuropathic pain induced by nerve injury

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Cytokines such as interleukins are known to be involved in the development of neuropathic pain through activation of neuroglia. However, the role of chemokine (C-C motif) ligand 1 (CCL-1), a well-characterized chemokine secreted by activated T cells, in the nociceptive transmission remains unclear. We found that CCL-1 was upregulated in the spinal dorsal horn after partial sciatic nerve ligation. Therefore, we examined actions of recombinant CCL-1 on behavioural pain score, synaptic transmission, glial cell function and cytokine production in the spinal dorsal horn. Here we show that CCL-1 is one of the key mediators involved in the development of neuropathic pain. Expression of CCL-1 mRNA was mainly detected in the ipsilateral dorsal root ganglion, and the expression of specific CCL-1 receptor CCR-8 was upregulated in the superficial dorsal horn. Increased expression of CCR-8 was observed not only in neurons but also in microglia and astrocytes in the ipsilateral side. Recombinant CCL-1 injected intrathecally (i.t.) to naive mice induced allodynia, which was prevented by the supplemental addition of N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801. Patch-clamp recordings from spinal cord slices revealed that application of CCL-1 transiently enhanced excitatory synaptic transmission in the substantia gelatinosa (lamina II). In the long term, i.t. injection of CCL-1 induced phosphorylation of NMDA receptor subunit, NR1 and NR2B, in the spinal cord. Injection of CCL-1 also upregulated mRNA level of glial cell markers and proinflammatory cytokines (IL-1ß, TNF-α and IL-6). The tactile allodynia induced by nerve ligation was attenuated by prophylactic and chronic administration of neutralizing antibody against CCL-1 and by knocking down of CCR-8. Our results indicate that CCL-1 is one of the key molecules in pathogenesis, and CCL-1/CCR-8 signaling system can be a potential target for drug development in the treatment for neuropathic pain.

Cell Death and Disease (2013) 4, e679; doi:10.1038/cddis.2013.198; published online 20 June 2013

Subject Category: Neuroscience

Neuropathic pain (also classified as chronic or malignant pain) is associated with nerve injury of multiple aetiology that includes acute trauma, diabetes, cancer, infection and autoimmune pathology.1 Pathogenesis of neuropathic pain reflects complex remodelling of the spinal cord, with primary role attributed to changes of synaptic transmission and activation of neuroglial cells, astrocytes and microglia.2–4 Glutamate, the major excitatory neurotransmitter in the brain and spinal cord, exerts its postsynaptic effects via a diverse set of ionotropic and metabotropic membrane receptors. The glutamate ionotropic N-methyl-D-aspartate receptors (NMDARs), specifically those localized in the dorsal horn of the spinal cord, are critically involved in nociceptive transmission and synaptic plasticity and have long been considered a target for the treatment of neuropathic pain.5,6 The native neuronal NMDAR is a tetramer that consists of two NR1 and two NR2 subunits.7 Phosphorylation of multiple sites in the cytoplasmic C terminus of the NR1 and NR2 subunits is known to modulate NMDAR activity and affect synaptic transmission.8–10

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Keywords: CCL-1; neuropathic pain; glutamate; spinal cord; NMDA receptor

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMV, avian myeloblastosis virus; ANOVA, analysis of variance; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CCL-1, chemokine (C-C motif) ligand 1; CCL-2, chemokine (C-C motif) ligand 2; CCL-21, chemokine (C-C motif) ligand 21; CCR-8, chemokine (C-C motif) receptor 8; CX3CL-1, chemokine (CX3C motif) ligand 1; DAPI, 4’, 6-diamidino-2-phenylindole, dihydrochloride; DMEM, Dulbecco’s modified eagle medium; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; GABA, D-amino butyric acid; GFAP, glial fibrillary acid protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Iba-1, ionized calcium-binding adapter molecule 1; IC 50, half maximal inhibitory concentration; IFN-γ, interferon γ; IL-17, interleukin 17; IL-1ß, interleukin 1ß; IL-6, interleukin-6; i.t., intrathecal injection; L5, lumbar 5; MAPK, mitogen activated protein kinase; MAP2, microtubule-associated protein 2; mEPSC, miniature excitatory postsynaptic current; mRNA, messenger ribonucleic acid; NeuN, neuron specific nuclear protein; NMDA, N-methyl-D-aspartate; NR1, NMDA receptor 1; NR2B, NMDA receptor 2B; PBS, phosphate buffered saline; pNR1, phosphorylated NR1; pNR2B, phosphorylated NR2B; PSNL, partial sciatic nerve ligation; PVD, polyvinylidene difluoride; RT-PCR, reverse transcription polymerase chain reaction; SE, standard error; Sg neuron, substantia gelatinosa neuron; siRNA, small interfering ribonucleic acid; sEPSC, spontaneous excitatory postsynaptic current; TCA-3, thymus-derived chemotactic agent 3; TNF-α, tumour necrosis factor-α; TTX, tetrodotoxin; VGAT, vesicular GABA transporter; VGLUT1, vesicular glutamate transport protein 1

Received 11.1.13; revised 03.4.13; accepted 24.4.13; Edited by D Bano
There are many reports that nerve injury triggers reactive changes in peripheral immune system and in neuroglial cells in both peripheral and central nervous systems. In the periphery, activation of Schwann cells and resident macrophages recruit hematogenous immune cells, which subsequently invade the injured nerves. In the spinal dorsal horn receiving the injured sensory afferents, activation of microglia is commonly associated with early establishment stages of neuropathic pain. Cytokines such as interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and IL-6, which originate from the peripheral injured area or from activated neuroglial cells in the spinal cord, are known to alter synaptic transmission in the dorsal horn.

Chemokines and their receptors are widely expressed in immune cells and in the nervous system. Recent studies have shown that chemokines C-C motif ligand 2 (CCL-2) and CX3CL-1 induce tactile allodynia (when non-painful innocuous stimuli become painful) through activation of spinal cord microglia. These reactive changes mediated through cytokines and chemokines develop in parallel with tactile allodynia, the latter being the main symptom of neuropathic pain.

CCL-1 (also known as thymus-derived chemotactic agent 3) is one of the well-characterized chemokines and is classified into the same CC chemokine group as CCL-2. CCL-1 is secreted by activated T cell, mast cells and endothelial cells and has important role as the chemoattractant for neutrophils and monocytes. Although effects of CCL-1 on the immune cells are well characterized, the roles of CCL-1 in the central nervous system, and especially in the development of neuropathic pain, remain unclear. In this report, we found that CCL-1 is involved in the development of tactile allodynia following peripheral nerve injury through enhancement of spinal nociceptive transmission and activation of glial cells with subsequent release of cytokines.

Results

Expression of CCL-1 in the spinal cord in neuropathic mice. We first examined the development of allodynia in a partial sciatic nerve ligation (PSNL) model. Mice showed a significant increase in withdrawal response to mechanical stimulus in the ipsilateral hind-paw (tactile allodynia) at 7 days after PSNL. The pain score in the ipsilateral hind-paw was 7.5 ± 0.8 (n = 8), while contralateral hind-paw was 1.9 ± 0.6 (n = 8) (Figure 1a). In parallel, the protein level of CCL-1 significantly increased 7 days after PSNL in the ipsilateral spinal cord (relative protein level, 0.14 ± 0.01; n = 4) compared with the contralateral spinal cord (relative protein level, 0.10 ± 0.01; n = 4) (Figure 1b). On the other hand, levels of other cytokines, TNF-α, interferon-γ and IL-1β...
were not significantly changed 7 days after nerve ligation (Figure 1b).

Time course of CCL-1 mRNA expression in the spinal cord and dorsal root ganglion (DRG) after nerve ligation.
First, we compared CCL-1 mRNA between the spinal cord and DRG before nerve ligation. CCL-1 mRNA was expressed in DRG ($12428 \pm 3606.7\%$; $n = 4$) more than the spinal cord (Figure 1c). Next, we examined the expression of CCL-1 mRNA in a PSNL model. CCL-1 mRNA level significantly increased at 3 h, 1 and 3 days ($194.4 \pm 19.9\%$, $147.3 \pm 17.3\%$, $142.0 \pm 2.0\%$, respectively; $n = 4$) after nerve ligation in the DRG (Figure 1d). In the spinal cord, CCL-1 mRNA level significantly increased at 3 h after nerve ligation ($361.1 \pm 74.5\%$; $n = 6$; Figure 1e).

Time course of CCR-8 protein expression in the spinal cord. CCR-8 is a specific receptor for CCL-1. Nerve ligation increased protein levels of CCR-8 in the ipsilateral dorsal spinal cord 6 h after surgery (Figures 2a and c). The relative protein level of CCR-8 compared with $\beta$-actin also showed significant increase at 6 h after ligation (relative protein level, $0.9 \pm 0.1$; $n = 9$; Figure 2b). We performed co-staining of CCR-8 with neuronal or glial markers 6 h after nerve ligation in the spinal cord. Increased expression of CCR-8 was observed not only in neurons but also in microglia and astrocytes in the ipsilateral side (Figure 3). In addition, we showed that CCR-8 was expressed not only on glutamatergic neurons but also on GABAergic neuronal fibers in primary cultured neurons (Supplementary Figure S1).

Injection of a neutralizing antibody against CCL-1 inhibited nerve ligation-induced allodynia. To examine the prophylactic effect of blocking CCL-1 against tactile allodynia, a neutralizing antibody against CCL-1 (anti-CCL-1, 2.5, 25 and 50 ng) was injected i.t. 10 min before nerve ligation and for 6 consecutive days after nerve ligation. The injection of 50 ng anti-CCL-1 antibody significantly reduced tactile allodynia in 3, 5 and 7 days after nerve ligation (pain score at each time point, $5.2 \pm 0.8$, $6.0 \pm 0.8$ and $6.0 \pm 0.9$; $n = 15$, respectively; Figure 4a). The inhibition by prophylactic administration of anti-CCL-1 antibody was concentration-dependent (Figure 4b). In order to test the effects of post-chronic administration, daily injection of anti-CCL-1 antibody was started 3 days after nerve ligation and continued for 7 days. Unlike prophylactic administration, the tactile allodynia was not blocked once it was induced by nerve ligation (Figure 4c).

Injection of CCL-1 induced allodynia, which was attenuated by NMDAR inhibitor. We next examined the

![Figure 2](image-url)

**Figure 2** Expression of CCR-8 in the spinal cord following nerve ligation. (a and b) Protein levels of CCR-8, a selective receptor for CCL-1, in the spinal cord at different time points after nerve ligation were evaluated by western blotting. C, contralateral; I, ipsilateral. Data represent mean ± S.E. (b, $n = 9$). *$P < 0.05$: versus ipsilateral before ligation, **$P < 0.01$: versus contralateral (two-way analysis of variance followed by Tukey–Kramer tests). (c) Immunostaining for CCR-8 in contralateral (Contra-) and ipsilateral (Ipsi-) spinal cord before and 6 h after nerve ligation. Bars = 200 μm (upper panels), 100 μm (lower panels).
**Figure 3**  
CCR-8 expression in neurons, microglia and astrocytes after nerve ligation.  

(a) Immunostaining for NeuN (red) and CCR-8 (green) 6 h after nerve ligation in the spinal cord. Bars = 10 μm.  

(b) Immunostaining for Iba1 (red) and CCR-8 (green) 6 h after nerve ligation in the spinal cord. Bars = 10 μm.  

(c) Immunostaining for GFAP (red) and CCR-8 (green) 6 h after nerve ligation in the spinal cord. Bars = 10 μm.
effect of an intrathecal (i.t.) injection of CCL-1 on nociceptive behaviour. The effect of CCL-1 was dose- and time-dependent; 25 ng CCL-1 significantly increased the pain score after 10, 20, 30, 40 and 80 min (pain score at each time point, 8.6 ± 1.2, 11.3 ± 1, 11.4 ± 1.7, 11.6 ± 0.9, 9.2 ± 1.4 and 7.4 ± 0.9, respectively; n = 8; Figure 5a). The pain score 20 min after i.t. injection of different concentration of CCL-1 is presented in Figure 5b. NMDARs are critical in pathogenesis of allodynia. In our model, the allodynia induced by i.t. injection of CCL-1 (25 ng) was dose-dependently blocked by co-injection of MK-801, a non-competitive use-dependent NMDAR antagonist, at concentration of 125, 250 and 500 pM (Figure 5c). MK-801 showed dose-dependent inhibition of allodynia induced by CCL-1 at 10, 20, 30, 40 and 50 min after injection (pain score at each time point, 3.0 ± 0.7, 4.6 ± 0.7, 6.4 ± 1.0, 4.3 ± 0.8, 4.0 ± 0.7, respectively; n = 8; Figure 5c). The IC_{50} for MK-801 effects was 354 pM (n = 8) when determined in 20 min after i.t. injection of CCL-1 (Figure 5d), suggesting the role of NMDA-mediated transmission in allodynia.

NMDAR phosphorylation after injection of CCL-1 in the spinal cord. We examined whether CCL-1 induced phosphorylation of NR1 and NR2B subunits of NMDAR in the spinal cord using western blotting (Figures 6a and b). CCL-1 affected phosphorylation levels of NR1 subunit at Ser896 (p-NR1) in the spinal cord at 3 h, 1 and 3 days after injection of CCL-1 (25 ng, i.t.) (relative protein levels, 2.7 ± 0.75, 4.5 ± 1.16, 3.5 ± 0.88, respectively; n = 3; Figure 6c). After immunoblotting, the membrane was stripped and reprobed with anti-NR1 antibody (Figure 6a). However, CCL-1 had no effect on the increase of NR1 protein level (Figure 6d). The protein level of p-NR1/NR1 was increased at 1, 3 h, 1 and 3 days after injection of CCL-1 (25 ng, i.t.) (relative protein levels, 5.5 ± 0.69, 5.5 ± 1.32, 9.8 ± 1.84, 7.3 ± 1.46, respectively; n = 3; Figure 6e). CCL-1 induced phosphorylation of
NR2B subunit at Tyr1472 (p-NR2B) in the spinal cord at 1 and 3 h after injection of CCL-1 (25 ng, i.t.) (relative protein levels, 2.4 ± 0.24, 4.7 ± 0.18, respectively; n = 3; Figure 6f). After immunoblotting, the membrane was stripped and reprobed with anti-NR2B antibody (Figure 6b). CCL-1 increased level of NR2B protein at 1, 3 and 7 days after injection of CCL-1 (25 ng, i.t.; relative protein levels, 2.9 ± 0.33, 2.9 ± 0.35, 3.2 ± 0.65, respectively; n = 3; Figure 6g). The protein level of p-NR2B/NR2B was increased at 1 and 3 h after injection of CCL-1 (25 ng, i.t.; relative protein levels, 0.90 ± 0.34, 0.97 ± 0.34, respectively; n = 3; Figure 6h).

Effects of CCL-1 injection on glial cells and cytokines in the spinal cord. Activation of glial cells contributes to the development and maintenance of tactile allodynia.11,13 We examined microglia and astrocytes in the spinal cord after injection of CCL-1 (25 ng, i.t.). Iba1 mRNA levels significantly increased in the spinal cord at 1, 3 h and 1 day after injection of CCL-1 (25 ng, i.t.; 179.8 ± 12.0, 168.4 ± 20.0, 149.5 ± 20.0%, respectively; n = 4; Figure 7a). And we showed that CCL-1 (25 ng, i.t.) induced increase and morphological change of Iba1-positive microglia 1 day after injection (Supplementary Figure S3). CD11b mRNA levels also significantly increased in the spinal cord at 1 day after injection of CCL-1 (25 ng, i.t.; 249.5 ± 51.2%; n = 4; Figure 7b). It was reported that P2X4R on microglia are important for the development of neuropathic pain.2,3 We examined the expression of P2X4R mRNA on microglia after injection of CCL-1 (25 ng, i.t.). However, P2X4R mRNA had not changed after injection of CCL-1 (Figure 7c). Glial fibrillary acid protein...
(GFAP) mRNA level significantly increased in the spinal cord at 1, 3 h and 1 day after injection of CCL-1 (25 ng, i.t.; 199.1 ± 19.0, 163.5 ± 16.6, 169.7 ± 20.8%, respectively; n = 4; Figure 7d). In addition, we observed that CCL-1 (25 ng, i.t.) induced morphological change and the number of GFAP-positive astrocytes 1 day after injection (Supplementary Figure S3). Furthermore, we examined activation of microglia and astrocytes after injection of CCL-1 (25 ng, i.t.) using phosphor-p38 MAPK antibody (p-p38). CCL-1 induced phosphorylation of p38 MAPK on microglia and astrocytes (Supplementary Figure S2). Cytokines and growth factors are important mediators of neuronal–glial interactions.12,23 We examined cytokine (IL-1β, TNF-α, IL-6) and brain-derived neurotrophic factor (BDNF) in the spinal cord after injection of CCL-1 (25 ng, i.t.). Though protein levels of IL-1β, TNF-α and IL-6 were not changed after 7 days of nerve ligation (Figure 1b), IL-1β mRNA significantly increased in the spinal cord at 1 and 3 h after injection of CCL-1 (25 ng, i.t.; 688.0 ± 150.8, 609.2 ± 148.1%, respectively; n = 4; Figure 7e), as well as TNF-α mRNA at 30 min, 1 and 3 h (25 ng, i.t.; 368.1 ± 150.1, 485.8 ± 94.4, 408.1 ± 97.4%, respectively; n = 4; Figure 7f), and IL-6 mRNA at 1 and 3 h (25 ng, i.t.; 248.7 ± 40.8, 318.0 ± 68.8%, respectively; n = 4; Figure 7g) after injection of CCL-1. On the other hand, BDNF mRNA did not change after injection of CCL-1 (Figure 7h).
Effects of i.t. CCR-8 siRNA treatment on allodynia. We next examined the effects of CCR-8 downregulation on tactile allodynia. We made CCR-8 knockdown model using transfection of siRNA against CCR-8. We confirmed that protein level of CCR-8 was reduced in the spinal cord 5 days after injection of CCR-8 siRNA (Figure 8a). The CCR-8 knockdown mice were subjected to PSNL 5 days after injection of CCR-8 siRNA. We examined the effect of CCR-8 knockdown on allodynia and coordinated motion using von Frey filament test and rota-rod test. Downregulation of CCR-8 reduced PSNL-induced tactile allodynia for 2 weeks (n = 5; Figure 8c). By contrast, downregulation of CCR-8 had no effect on coordinated motion (n = 5; Figure 8d).

Discussion
The present study, for the first time, demonstrates that expression of CCL-1 in the spinal cord and DRG at the mRNA and protein levels is increased after nerve ligation, and the nerve ligation-induced tactile allodynia is attenuated by treatment with neutralizing antibody against CCL-1 and in CCR-8 knockdown mice. We further show that CCL-1 has an excitatory action on the glutamatergic synaptic transmission and phosphorylation of NMDAR subunits, NR1 and NR2B, in the spinal dorsal horn and increase expression of glial cells activation markers (Iba1, CD11b and GFAP) and cytokines (IL-1β, TNF-α and IL-6). Our results therefore indicate that CCL-1 may be one of the key mediators of chronic tactile pain.
allodynia acting through an acute modulation of nociceptive synaptic transmission, phosphorylation of NR1 and NR2B, glial activation and increase of cytokines in the spinal cord. In this study, we showed that CCL-1-specific receptor, CCR-8, was expressed in not only neurons but also glial cells 6 h after nerve ligation in the ipsilateral side (see Figure 3). We suggested that increase of total CCR-8 protein after nerve ligation in the spinal cord by increase of CCR-8 expression on glial cells. CCL-1 mRNA showed only a transient increase in the spinal cord (see Figure 1e) and at 3 h, 1 and 3 days after nerve ligation in DRG (see Figure 1d). This indicates that CCL-1 may be mainly produced in DRG after nerve ligation. In the case of CCL-21 and CCL-2, they were produced in DRG after nerve ligation and were transported to neuronal terminals in the dorsal horn.24,25 CCL-2 was released from neuronal synaptic vesicles in the spinal cord.26 Similarly, we assume that CCL-1, which is produced in DRG neurons after nerve ligation, is subsequently transported to the spinal cord and then released. CCL-1 mRNA in the spinal cord at 3 h after nerve ligation was also produced in glial cells albeit only transiently; this however, requires further investigation.

Immune cells and glial cells interact with neurons to alter pain sensitivity and to mediate the transition from acute to chronic pain.11,14,18 It is generally believed that microglia are involved in the development and astrocytes in the maintenance of neuropathic pain.27–29 Cytokines are important messengers for the communication between neurons and glia.11,13,30 Injection of CCL-1 into the spinal cord increased the mRNA level of microglial (CD11b or Iba1) and astroglial (GFAP) markers, as well as that of cytokines (IL-1β, TNF-α, and IL-6) (see Figure 7). We also observed double staining of GFAP or CD11b with p-P38 after i.t. injection of CCL-1, suggesting that microglia and astrocytes were in reactive states (see Supplementary Figure S2). Activation of microglia in the spinal dorsal horn following peripheral nerve injury is well characterized.2,31 The mechanisms of nerve injury-induced microglial activation are complex and involve several signaling molecules.32 Purinergic signaling mediated through P2X4, P2X7 and P2Y12 receptors seems to contribute to the initial activation of microglia following acute nerve injury.2,3,16,31,33,34 We, however, failed to identify changes in P2X4 receptor mRNA in the spinal cord after injection of CCL-1. This suggests that CCL-1-mediated activation of microglia does not involve the upregulation of P2X4 receptors but engages distinct signaling cascade(s). Several reports have previously demonstrated that cytokines and chemokines activate microglia and astrocytes, inducing secretion of proinflammatory factors (e.g., IL-1β, TNF-α, BDNF, IL-6, IL-17, CCL-2).2,12,27 Several proinflammatory cytokines and chemokines have been implicated in altered nociceptive processing.12 TNF-α enhanced synaptic efficacy by increasing surface expression of AMPA receptors.35 IL-1β modulates sensory neuron transmission via increased release of the nociceptive neuropeptides substance P and calcitonin gene-related peptide.36–38 IL-1β and TNF-α induced IL-6 expression in cultured neurons and astrocytes.42 According to these reports, it might be reasonable that a peak expression of IL-6 in the spinal cord occurred later compared with that of TNF-α and IL-1β after injection of CCL-1 (see Figure 7g). These results indicate that CCL-1 is involved in the development of allodynia through glial activation and expression of cytokines. However, other signaling molecules can be also involved in the pathogenesis of neuropathic pain. Effects of CCL-1 on glial function need to be investigated in more detail in the future.

We showed that allodynia induced by injection of CCL-1 was blocked by co-injection of NMDAR antagonist.
We assumed that CCL-1 enhanced glutamate release from presynaptic terminal or affected postsynaptic NMDARs. We confirmed that CCL-1 affected both, increasing glutamate release transiently and inducing phosphorylation of NR1 and NR2B gradually. As for the presynaptic effect, we showed that CCL-1 increased glutamate release only in some but not all neurons (see Figure 5e). Similarly, double immunostaining with anti-CCR-8 and anti-NeuN showed that CCR-8 expression was only in sub-population of neurons (see Figure 3), the reason of which requires further investigation. As for the postsynaptic effect, it is already reported that activation of NMDAR is important for initiating long-lasting changes in synapses and has been implicated in persistent pain by strengthening glutamatergic sensory transmission. Though we did not directly check whether postsynaptic NMDA currents were enhanced by CCL-1 or not, we observed that both phosphorylation of NMDAR and increase in the number of NMDARs take place in response to CCL-1. Phosphorylation of NMDAR is known to modulate NMDAR activity and facilitate transmission of nociceptive inputs in inflammatory and neuropathic pain models. Partial ligation of sciatic nerve significantly increased the phosphorylated proportion of NR1 and NR2B subunits in the dorsal horn. The phosphorylation of NR1 subunit is related to enhancement of synaptic currents were enhanced by CCL-1 or not, we observed that phosphorylation of NMDAR subunits, NR1 and NR2B. As for the presynaptic effect, we showed that CCL-1 is particularly important for pain perception, we speculate that phosphorylation of NR2B would be more important than phosphorylation of NR1 together with upregulation of NR2B. As for the phosphorylation of NR1, IL-1β was reported to induce phosphorylation of NR1. As IL-1β mRNA was increased in the spinal cord after injection of CCL-1 at 1 and 3 h (Figure 7e), we speculate that NR1 would be phosphorylated not only by CCL-1 but also by IL-1β.

In conclusion, our study provides evidence that CCL-1 is produced mainly in DRG after nerve ligation, supposed to be transported to the spinal cord, where it increases glutamate release together with local upregulated CCL-1, and then activates glial cells and cytokine release. CCL-1 also induced phosphorylation of NMDAR subunits, NR1 and NR2B. Treatment with neutralizing antibody against CCL-1 and CCR-8 siRNA blocked nerve ligation-induced tactile allodynia. Therefore CCL-1 is one of the key molecules in pathogenesis of allodynia, and CCL-1/CCR-8 signaling system can be a potential target for drug development in the treatment for neuropathic pain.

Materials and Methods

Animals. Male ddY mice (20–30 g, Kyudo Co. Ltd., Tosu, Saga, Japan) were used. Mice were housed at 23 ± 2 °C with 12 h light: 12 h dark cycle (light on from 0700 to 1900 hours) and were given free access to commercial food and tap water. Experimental procedures were based on the Guidelines of the Committee for Animal Care and Use of Kyushu University, Fukuoka University and National Institute for Physiological Sciences.

Drugs. Recombinant mouse CCL-1 and neutralizing antibody CCL-1 were purchased from R&D Systems (Minneapolis, MN, USA). MK801 was purchased from Sigma Aldrich (St. Louis, MO, USA).

PSNL. The mice were anesthetized with a intraperitoneal (i.p.) injection of pentobarbital (65 mg/kg). A PSNL was produced by tying a tight ligature with a No. 8 nylon thread around approximately 1/3–1/2 the diameter of the sciatic nerve located on the left side (ipsilateral side) based on the Seltzer method. On the right side (contralateral side), the nerve was exposed without ligation.

Behavioural analysis. Behavioural analysis was performed to assess the development of tactile allodynia in mice. Tactile allodynia was measured using a von Frey filament (North Coast Medical, Gilroy, CA, USA). Mice were placed in glass cages with a wire mesh bottom and allowed to habituate for at least 1 h. A von Frey filament with bending forces of 0.16 g (innocuous stimulation) was pressed perpendicularly against the plantar skin of a hind-paw. The paw withdrawal responses to the mechanical stimulus were evaluated by scoring as follows: 0, no response; 1, lifting the hind-paw within 3 s; 2, lifting the hind-paw over 3 s; and 3, immediate flinching or licking of the hind-paw. The stimulation of the same intensity was applied seven times to each hind-paw at several seconds interval and the total served as the pain-related score (score) in the SNL model. The behavioural data were analyzed using Origin (Microcal software Inc., Sunnyvale, MA, USA) to determine the IC50 values.

We performed a rota-rod (KN-75 Rota-rod, Natsume, Japan) test to measure motor balance and coordination. Mice were pre-trained on rota-rod apparatus for 3 days and then tested on the accelerating rod in which the speed of the spindle was 10 r.p.m. Latency until fall was automatically recorded. To eliminate stress and fatigue, mice were given a maximum cutoff latency of 120 s.

Intrathecal injection. Intrathecal (i.t.) injection was performed using a 25-μl Hamilton syringe with a 28-gauge needle (Muranaka Medical Instruments co. LTD., Osaka, Japan), as described previously. The needle was inserted into the intervertebral space of a conscious mouse between the lumbar 5 (L5) and 6 (L6) regions of the spinal cord. A reflexive flick of the tail was considered to be an indicator of the accuracy of each injection. A volume of 5 μl was used for the i.t. injections.

Immunohistochemical analyses. Mice were anesthetized by pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS: 80 mM Na2HPO4, 20 mM KH2PO4, 150 mM NaCl, pH 7.4). The L4–L6 segment of the lumbar spinal cord was removed, postfixed in the same fixative and placed in 20% sucrose solution for 24 h at 4 °C. Transverse L4–L6 spinal cord section (20 μm) were sliced by a HM 550 cryostat (Micro-edge Instruments Co., Tokyo, Japan) and incubated for 1 h at room temperature in 5% donkey serum (Jackson Immuno Research, West Grove, PA, USA). Then, the sections were incubated with the following: NeuN (1:200, Millipore, Billerica, MA, USA), Iba1 (ionized calcium-binding adapter molecule-1, 1:2000, Wako, Osaka, Japan), CD11b (1:500, Serotec, Oxford, UK), GFAP (1:800, Millipore) or p-p38 (phospho-p38 MAPK, 1:100, Cell Signaling Technology, Danvers, MA, USA) for 24 h at 4 °C. The sections were incubated for 4 h at room temperature in the secondary antibody (IgG-conjugated Alexa Fluor 488 or 594, 1:1000, Molecular Probes, Eugene, OR, USA). Sections were mounted on coverslips with pentafluor aqueous mounting medium (Thermo Scientific, Yokohama, Japan). The sections were analyzed using a fluorescence microscope (BX2900, Keyence, Japan) and a confocal laser scanning microscope (LSM510META, Carl Zeiss, Heidelberg, Germany). In confocal imaging, we used a series of Z-stack images (images taken at multiple focal points of an objective lens through the z-axis) for 3D analysis of each slice.

Cytokine array. Mouse cytokine array kit was purchased from R & D Systems and was assessed as described previously. The L4–L6 segment of the lumbar spinal cord was isolated and homogenized in the lysis buffer (10 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, pH 7.4) containing protease inhibitor cocktail (Sigma) and centrifuged at 15,000 r.p.m. for 20 min at 4 °C. Each
reaction was performed according to the protocol of the manufacturer using 20 μg of protein collected from the sample. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C. The protein sample was incubated with a blot array precoated with 40 cytokine/chemokine antibodies overnight at 4°C.

**Transfection of CCR-8 siRNA by electroporation.** SiRNA against CCR-8 were obtained from Bionac Corporation (Kurume, Japan) (GenBank accession number: NM_007720, mCCR8 sense 5'-CCAGAACACAGGAGGACAU-3', anti-sense 5'-AUGUGUCCUCAGUUCUGUGC-3'), mCCR8 sense 5'-GGACAGACUGUGAGGUGA-3', anti-sense 5'-UCCACUCAAGAGCGUCGUC-3', and mCCR8 sense 5'-GAGAGAGAUUGAAAGCA-3', anti-sense 5'-UGUUCUUCACACACUCUC-3'). Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). SiRNA (0.2 μg/μl, 10 μl) was injected i.t. using a 28-gauge needle (Murakana Medical Instruments Co., LTD.). Scrambled siRNA (5'-UACUAUUGACAGAAGCAAG-3', anti-sense 5'-CUUUUGGUGUGAUA-3', Bonac Corporation) was used as a negative control. A volume of 5 μl was used for the i.t. injections at each points. Electric pulses (pulsing pulse: 75 V, 5 ms of length with 50-ms interval, transfer pulse: 20 V, 50 ms of length with 50-ms interval) were delivered using NEPA21 (Nepa Gene, Ichikawa, Japan). Knock-down effects were evaluated by western blotting.

**Preparation of spinal cord acute slices.** The mice were anesthetized with urethane (1.2–1.5 g/kg, i.p.), and a thoracolumbar laminectomy was performed. The lumbarosacral segments of the spinal cord (L1–S1) were placed in an ice-cold Krebs solution equilibrated with 95% O2 and 5% CO2 in a 10% CO2 incubator for 5–7 days with neurobasal medium (GIBCO, Grand Island, NY, USA) containing 2% B27 supplement (GIBCO) and 0.5 mM L-glutamine (GIBCO).

**Immunocytochemical analysis.** Primary cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton-X100 in PBS for 5 min, followed by treating with blocking solution (Block Ace; Dainippon Pharmaceutical, Osaka, Japan) for 30 min at room temperature. Cells were incubated with primary antibodies: CCR-8 (1: 200, Epitomics), MAP2 (1: 1000, Sigma, St. Louis, MO, USA), VGLUT1 (1: 1000, Synaptic Systems, Göttingen, Germany) or VGAT (1: 300, Synaptic Systems) overnight at 4°C. The cells were washed in PBS and then incubated for 3 h at room temperature with secondary antibodies (goat-conjugated Alexa Fluor 488 or 568 or 633, 1: 1000, Molecular Probes). The cells were treated with 4% paraformaldehyde for 10 min and then washed with PBS. Slides were cover-slipped with permaltraeous aqueous mounting medium. The slides were confirmed by the use of a confocal laser scanning microscope (LSM510META, Carl Zeiss).

**Statistical analysis.** All data are presented as mean ± S.E.M. The statistical analysis of the results were evaluated by using two-tailed Student’s paired or unpaired t-test, one-way ANOVA followed by Student–Newman–Keuls test, one-way ANOVA followed by Bonferroni’s test or two-way ANOVA followed by Tukey–Kramer test. P < 0.05 was considered statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.
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