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Editorial

Opioid therapy for knee osteoarthritis and postoperative persistent pain after knee arthroplasty

Drugs and pain in revision total knee arthroplasty

Knee OA is a common musculoskeletal disorder in middle-aged and elderly adults. The number of patients with this disorder is increasing, as the population increases in age, especially in the US, Japan and Western Europe. In Japan, 60% of women >80 years of age have complained of osteoarthritic knee pain [1]. Knee OA, as well as other musculoskeletal disorders, are treated primarily with non-surgical methods such as rehabilitation and NSAIDs.

Recently, because of their potent analgesic effect as well as concerns about severe side effects due to NSAIDs, opioids are becoming increasingly prevalent in treating musculoskeletal disorders, including knee OA. In the USA, prescriptions for patients with chronic non-cancer pain doubled from 8% in 1980 to 16% in 2000 [2]. A large-scale retrospective study with 15 160 chronic pain patients treated consecutively with opioids showed that 2% of these patients were at risk of developing opioid dependence. Patients with psychiatric disorders had an especially high risk of developing opioid dependence (odds ratio 1.46) [3]. Previous research on opioid efficacy was <1–2 years in duration [4], and no study has investigated the long-term efficacy of opioids for chronic non-cancer pain.

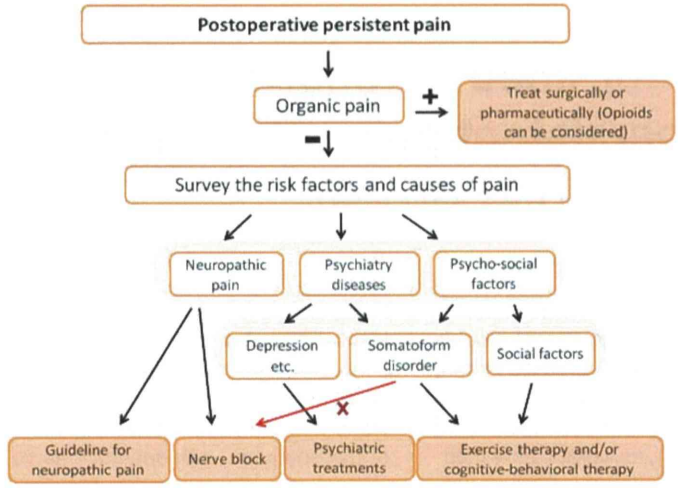
With the increase in the number of patients with knee OA worldwide, the number of total knee arthroplasty (TKA) cases has also increased. TKA is a useful surgical procedure for pain relief and improves the patient's ability to walk. Overall, the clinical outcomes of TKA are predominately positive; however, a patient may require revision surgery because of persistent postoperative pain, mechanical loosening or infection. Persistent postoperative pain can occur for a variety of reasons or may be idiopathic (i.e. pain of unknown origin). The number of patients who require revision arthroplasty is increasing and, unfortunately, revision arthroplasty does not produce promising results when compared with the primary surgery. Moderate to severe pain was reported by Singh and Lewallen [5] in 7.5% of patients 2 years after the initial knee arthroplasty, while 24% of patients reported pain after revision arthroplasty. In an article published in this issue [6], respondents to a questionnaire who underwent revision TKA reported NSAID usage of 13% at 2 years and 17% at 5 years after surgery, while 5% and 6% (at 2 and 5 years, respectively) required opioids to manage their pain post-surgery [6].

The risk associated with postoperative persistent pain includes pre-, peri- and postoperative factors. Preoperative factors include female gender, younger age, low income, low self-assessment of health, low educational background, preoperative pain and preoperative psychological factors (anxiety, catastrophe and depression). Perioperative factors include wound size, duration of the operation, intraoperative nerve injury and electrical scalpel use. Postoperative factors include severe perioperative pain, high doses of postoperative analgesia, surgery on a previously injured site and reoperation [7]. Because severe pain immediately after surgery is a risk factor for persistent postoperative pain, sufficient analgesics must be provided during and immediately after the surgery [8], but prolonged opioid administration should be avoided [4]. Patients with depression are more likely to develop postoperative persistent pain, and these patients require as much as 4.5 times more opioids than other patients, even 5 years after the revision knee arthroplasty [6]. Moreover, poor postoperative outcomes occur in patients who are treated with opioids prior to the primary surgery, with a significantly higher incidence of postoperative persistent idiopathic pain and a significantly higher rate of revision arthroplasty [9]. Postoperative pain can be alleviated by preoperative education, cognitive behavioural therapy for potential postoperative pain and adequately managing perioperative pain [10].

Fig. 1 indicates the therapeutic algorithm that was used to determine the postoperative persistent pain after knee arthroplasty. When mechanical loosening, infection in the joint or fractures around the joint are evident, surgical or pharmacological methods, including opioid treatment, should be employed. Neuropathic pain should be treated with NSAIDs and anticonvulsant drugs, such as pregabalin. A nerve block may also be used to control neuropathic pain. When the pain is idiopathic, however, other factors, including socio-psychological factors, should be examined and psychiatric approaches, such as antidepressant treatment as well as cognitive behavioural therapy, can be introduced. In actual clinical practice, however, few patients have pain due to only organic causes or completely lack these organic causes. Most patients have pain that is often believed to have both organic and idiopathic causes.

Today, opioids are prescribed without careful consideration when treating chronic non-cancer pain, including postoperative persistent pain after knee arthroplasty. Because the analgesic effects of opioids occur quickly and patients who believe opioids are the most potent analgesics may experience a strong placebo effect,

FIG. 1 Therapeutic algorithm for postoperative persistent pain



physicians find it easier to prescribe medication than to opt for non-pharmacological interventions, such as patient education, exercise and cognitive behavioural therapy. Patients with opioid dependence may think these drugs are effective because withdrawal symptoms appear as the drug effects wear off. At present, the long-term efficacy and adverse effects of opioid therapy for chronic non-cancer pain remain uncertain, therefore opioid administration without careful consideration is inappropriate, except when its potent analgesic effects are utilized for perioperative pain control.

Future studies should focus on whether opioid treatment for persistent postoperative pain actually improves the patient's quality of life. Furthermore, the consequences of knee arthroplasty in patients with depression should be investigated because depression is the most important risk factor for excessive opioid use [6]. These studies should determine whether treating depression prior to surgery improves the outcome of knee arthroplasty. While opioids are extremely effective as analgesics, caution should be exercised in their use to treat pain so that new problems do not develop.

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Short Communication

Interferon Regulatory Factor 8 Expressed in Microglia Contributes to Tactile Allodynia Induced by Repeated Cold Stress in Rodents

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Abstract. We investigated the role of interferon regulatory factor 8 (IRF8) in a model of chronic pain in which repeated cold stress (RCS) exposure produces tactile allodynia. RCS exposure produced a decrease in paw withdrawal threshold (PWT) to mechanical stimulation. Spinal microglia of RCS-exposed mice were morphologically activated. Expression of IRF8 was significantly increased in the spinal cord of RCS-exposed mice and was localized in microglia. IRF8-knockout mice failed to show the RCS-induced decrease in PWT. Thus, RCS exposure activates spinal microglia and upregulation of IRF8 in these cells is involved in the development of tactile allodynia after RCS exposure.

Keywords: interferon regulatory factor-8, microglia, repeated cold stress

Microglia, the resident macrophages of the central nervous system (CNS), respond to a wide range of stimuli that may threaten CNS homeostasis. Peripheral nerve damage induces microglial activation in the spinal cord. This activation involves hypertrophy, proliferation, and altered gene expression (1 – 3). We have recently demonstrated that the transcription factor interferon regulatory factor 8 (IRF8) is expressed in microglia after peripheral nerve injury (4). Furthermore, we have shown that IRF8-knockout (IRF8-KO) mice are resistant to peripheral nerve injury–induced tactile allodynia (4), innocuous stimuli–mediated abnormal pain hypersensitivity. However, IRF8-KO mice show no major deficits in acute and inflammatory pain responses (4). Overall, these results indicate that microglial IRF8 plays a major role in the pathogenesis of neuropathic pain. However, the role of IRF8 in other animal models of chronic pain is unknown. In the present study, we used a model of chronic pain based on repeated cold stress (RCS) (5) [also called intermittent cold stress (ICS) (6)], which results in tactile allodynia. RCS (5) [and ICS (6)] has been previously used as an experimental animal model of fibromyalgia (5 – 7), which is highly debilitating chronic pain whose mechanisms are poorly understood.

Male C57BL/6 mice (Clea Japan, Tokyo), IRF8-KO mice (8) and their wild-type (IRF8-WT) littermates, and Sprague-Dawley rats (Japan SLC, Shizuoka) were used. Mice and rats were aged 9 – 12 and 8 weeks, respectively, and housed at 22°C ± 1°C, under a 12-h light-dark cycle, and fed food and water ad libitum before and after RCS. All experimental procedures were performed under the guidelines of Kyushu University and Nagoya University.

RCS exposure was performed as previously reported (5, 6). The temperature of the chamber was automatically controlled between 4°C and 22°C. After measuring the

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paw withdrawal threshold (PWT), animals were kept at 4°C from 16:30 (for mice) or 19:00 (for rats) on the first day to 10:00 and then exposed to 22°C (room temperature) followed by their respective cold temperatures at 30-min intervals from 10:00 to 16:30 (for mice) or 17:30 (for rats) (Fig. 1A). These procedures were repeated for 7 days (for mice) or 5 days (for rats). During the stress period, two mice were kept in each cage (12 × 15 × 10.5 cm) and two to three rats were housed per metal-mesh cage (22 × 43 × 19 cm), both receiving ad libitum access to food and water.

Mechanical sensitivity was assessed the day before and 1 – 14 days after RCS, as described in our previous study (4). Mice were individually placed in an opaque plastic cylinder (located on a wire mesh) for 1 h to allow for acclimatization to the new environment. Calibrated von Frey filaments (0.02 – 2.0 g; North Coast Medical, Gilroy, CA, USA) were then applied to the plantar surfaces of hindpaws of the mice. The 50% PWT was determined.

For immunohistochemistry, animals were deeply anesthetized after RCS exposure by pentobarbital and perfused transcardially with phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde/PBS. Lumbar spinal cord segments (mice: L3, 4, and 5; rats: L4, 5, and 6) were removed, postfixed in the same fixative, and placed in 30% sucrose solution for 24 h at 4°C. Transverse spinal cord sections (30 μm) were cut on a cryostat and incubated in blocking solution (3% normal goat or donkey serum), and then incubated for 48 h at 4°C in the primary antibodies: ionized calcium-binding adapter molecule-1 (Iba1; 1:5000; Wako, Osaka) and IRF8 (1:500; Santa Cruz Biotechnology, Dallas, TX, USA), followed by the secondary antibodies conjugated to Alexa Fluor™ 488 or 546 (1:1000; Life Technologies Japan, Tokyo) and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Three to five sections from the spinal cord segments of each mouse were randomly selected and analyzed using the LSM510 Imaging System (Carl Zeiss Japan, Tokyo).

For quantitative real-time PCR experiments, mice were deeply anesthetized with pentobarbital, perfused transcardially with PBS, and the L3, L4, and L5 dorsal spinal cords were removed immediately. The tissues were vertically separated by median, and hemisections of the dorsal spinal cord were subjected to total RNA extraction using Trisure (Bioline, London, UK) according to the protocol of the manufacturer and purified with an RNeasy mini plus kit (Qiagen, Valencia, CA, USA). The amount of total RNA was quantified by measuring OD260 using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For reverse transcription, 100 ng of total RNA was transferred to the reaction with Prime Script reverse transcriptase (Takara Bio, Otsu). Quantitative PCR was performed with Premix Ex Taq (Takara) using a 7500 real-time PCR system (Life Technologies Japan), and the data were analyzed using 7500 System SDS Software 1.3.1 (Life Technologies Japan). Expression levels were normalized to the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then results were presented relative to those of naïve mice. The sequences of TaqMan primer pairs and probe are described below. Mouse IRF8: 5′-GGATATGCCGCCTATGACACA-3′ (forward), 5′-CATCCGGCCCATACAACTTAG-3′ (reverse), 5′-FAM-CCATTCAGCTTTCTCCCAGATGGTCATC-TAMRA-3′ (probe) as well as the primers and probe for GAPDH, were obtained from Life Technologies Japan.

Statistical analyses (using Prism 4.03; GraphPad Software, San Diego, CA, USA) was performed using the Student's *t*-test or two-way ANOVA followed by the Bonferroni post hoc test. Significance was reached at values of $P < 0.05$, $P < 0.01$, or $P < 0.001$.

We first examined mechanical pain hypersensitivity following RCS by measuring PWT of mice before and after RCS. RCS produced a profound long-term decrease in PWT to mechanical stimulation (Fig. 1B), indicating the production of tactile allodynia following RCS. We determined whether spinal microglia were activated in RCS mice and found that immunoreactivity for Iba-1 was higher in the L4 dorsal horn of mice exposed to RCS for 7 days compared with controls (non-RCS mice) (Fig. 1C). This response was mainly observed in the medial part of the dorsal horn. Furthermore, dorsal horn microglia were hypertrophic and showed prominent immunoreactivity for Iba1 (Fig. 1C). These results suggest that RCS induces a switch in phenotype from the resting to the activated state in a population of microglia in the dorsal horn. In contrast, these changes were weak in the L3 and L5 segments (Fig. 1D), suggesting that microglial activation in the dorsal horn of RCS-exposed mice may occur in a segment-dependent manner. The spatial- and segment-specific activation of microglia in the dorsal horn of RCS-exposed mice was similarly observed in rats that were exposed to RCS (Fig. 1E), suggesting that RCS-induced microglial activation in the dorsal horn may not be a species-restricted phenomenon.

We have previously shown that the transcription factor IRF8 plays a crucial role in microglial activation and pain hypersensitivity (4). Therefore, in the present study, we investigated the involvement of IRF8 in RCS-induced tactile allodynia. RT-PCR revealed that IRF8 mRNA was significantly increased in the dorsal spinal cord immediately 7 days after RCS exposure ($P < 0.001$), but not in the L3 and L5 dorsal horn (Fig. 2A). Immuno-

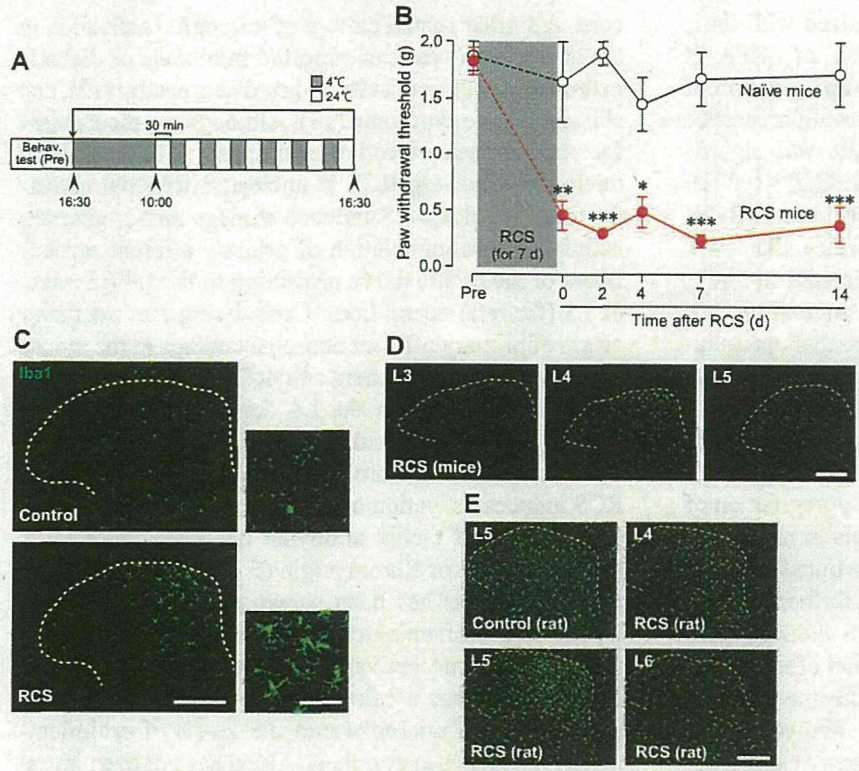

Fig. 1. Morphological changes of microglia in the dorsal horn after repeated cold stress (RCS) exposure. A) Schematic of RCS exposure in mice. B) The withdrawal threshold of mechanical stimulation to the hindpaw was examined in mice with and without RCS. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. naïve control group. n = 4. C) Immunofluorescence of Iba1 (a microglia marker) in L4 dorsal horn of mice with or without RCS exposure (scale bar = 200 μm). Insets: high-magnification images of Iba1 immunostaining in the dorsal horn (scale bar = 40 μm). D, E) Segmental pattern of microglial activation in the dorsal horn after RCS exposure in mice (D) and rats (E). Immunofluorescence of Iba1 in the lumbar segments (mice: L3, 4, and 5; rats: L4, 5, and 6) of the dorsal horn after RCS exposure [scale bar = 200 μm (D, E)].

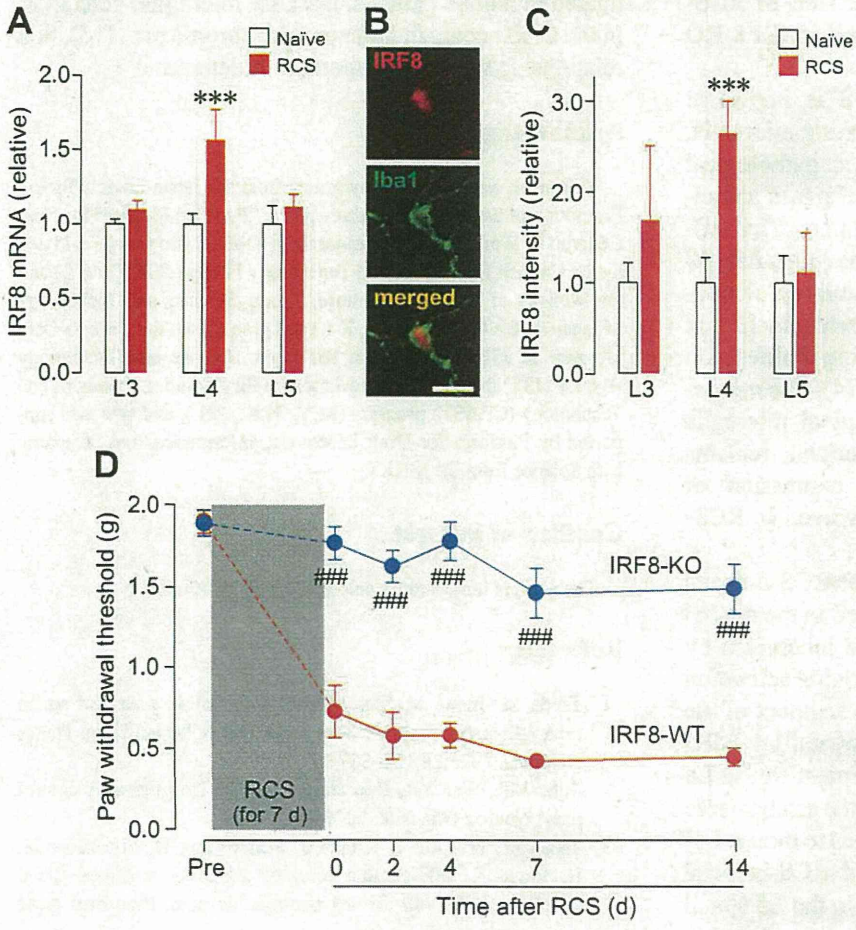

Fig. 2. IRF8 upregulated in spinal microglia after RCS exposure contributes to RCS-induced tactile allodynia in mice. A) Real-time polymerase chain reaction analysis of IRF8 mRNA in the spinal dorsal horn of non-RCS (Naïve)- and RCS-exposed mice. Values represent the relative ratio of IRF8 mRNA (normalized to GAPDH mRNA) to naïve mice (n = 4) ($***P < 0.001$ vs. naïve mice). B) Double immunolabeling of IRF8 with Iba1 in the spinal dorsal horn (scale bar = 25 μm). C) Intensity of IRF8 immunofluorescence per microglia (normalized to naïve mice) (n = 4) ($***P < 0.001$ vs. naïve mice). D) Paw withdrawal threshold of wild-type littermates (IRF8-WT) and IRF8-KO mice before (Pre) and after RCS exposure (n = 8 – 10) ($^{###}P < 0.001$ vs. the value of each time point of IRF8-WT mice).

fluorescence revealed that IRF8 co-localized with Iba1, suggesting that the nuclear localization of IRF8 in microglia (Fig. 2B). Consistent with the upregulation of IRF8 mRNA expression (Fig. 2A), immunofluorescence intensity of IRF8 in individual microglia was significantly enhanced after RCS exposure (Fig. 2C, $P < 0.001$). We then investigated whether IRF8 contributed to RCS-induced tactile allodynia using IRF8-KO mice. IRF8-WT mice exhibited a profound long-term decrease in PWT, which was not observed in IRF8-KO mice (Fig. 2D, $P < 0.001$). These behavioral data indicate that upregulation of IRF8 in the spinal cord is required for the development of tactile allodynia after RCS exposure.

In the present study, activation of dorsal horn microglia occurred in mice and rats exposed to RCS. In mice, the activation was shown by the upregulation of IRF8 as well as morphological alterations in microglia, both of which have been implicated in the transformation of microglia to the reactive state. We further showed that the loss of IRF8 suppressed RCS-induced pain hypersensitivity. Therefore, the upregulation of microglial IRF8 may play an important role in RCS-induced pain hypersensitivity in mice. However, the involvement of other cell types (e.g., DRG macrophages) expressing IRF8 cannot be excluded in the suppression of RCS-induced tactile allodynia that we observed in IRF8-KO mice.

Our findings also suggest that IRF8 is important for relaying signals responsible for activating microglia, a process that may contribute to subsequent pathological states. IRF8 activates the transcription of genes associated with the reactive state of microglia [e.g., *Tlr2* (encoding Toll-like receptor 2)], chemotaxis [e.g., *P2ry12* (encoding P2Y12 receptor)], and inflammatory components [e.g., *Il1b* and *Ctss* (encoding interleukin-1β and cathepsin S, respectively)] without affecting proliferation of microglia after peripheral nerve injury (4). The mechanism by which upregulated IRF8 in spinal microglia contributes to RCS-induced tactile allodynia remains unknown. However, IRF8-dependent expression of genes in activated microglia may be involved in RCS-induced tactile allodynia.

Interestingly, our findings showed that RCS-induced microglial activation was mainly observed in the medial side of the dorsal horn, which is an area innervated by myelinated fibers (9). Furthermore, microglial activation in mice was more pronounced in the L4 segment of the dorsal horn than in the L3 or L5 segments. L4 DRG neurons, whose central fibers mainly project to the L4 dorsal horn, predominantly contribute to the sciatic nerve (10). In rats, L5 DRG neurons (corresponds to mouse L4) contribute to the sciatic nerve (10), and RCS-induced microglial activation in rats was intense in the L5 spinal cord. A similar spatial pattern of microglial activation in the dorsal horn has been reported in models of diabetic neuropathy (11), paclitaxel-induced neuropathy (12), and chronic fatigue syndrome (13). Although the mechanism for the segment-selective activation of dorsal horn microglia following RCS is unclear, microglial activation may involve RCS-induced damage and/or aberrant activity of a subpopulation of primary afferent sensory fibers of the sciatic nerve projecting to the L4 (in mice) or L5 (for rats) dorsal horn. Considering that the major origin of primary afferent neuronal endings in the mouse hindpaw is the L4 segment of DRG neurons, microglial activation observed in the L4 dorsal spinal cord may play a role in RCS-induced tactile allodynia. Future studies should therefore focus on understanding how RCS induces activation of dorsal horn microglia.

RCS-induced tactile allodynia has been reported as a rodent model of fibromyalgia (5 – 7). Blood of fibromyalgia patients has been shown to contain increased levels of proinflammatory cytokines (14). Our study may suggest that activated microglia are involved in fibromyalgia, but a relationship between the reactive states of spinal microglia and the levels of proinflammatory cytokines in peripheral blood has not been investigated in human patients. Because microglial activation in the CNS occurs in humans with chronic pain (15), this relationship would be important to determine.

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Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Involvement of the chemokine CCL3 and the purinoceptor P2X7 in the spinal cord in paclitaxel-induced mechanical allodynia

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Abstract

Background: Paclitaxel is an effective chemotherapeutic agent widely used for the treatment of solid tumors. The major dose-limiting toxicity of paclitaxel is peripheral neuropathy. The mechanisms underlying the development and maintenance of paclitaxel-induced peripheral neuropathy are still unclear, and there are no currently established effective treatments. Accumulating evidence in models of neuropathic pain in which peripheral nerves are lesioned has implicated spinal microglia and chemokines in pain hypersensitivity, but little is know about their roles in chemotherapy-induced peripheral neuropathy. In the present study, we investigated the role of CC-chemokine ligand 3 (CCL3) in the spinal cord in the development and maintenance of mechanical allodynia using a rat model of paclitaxel-induced neuropathy.

Findings: Repeated intravenous administration of paclitaxel induced a marked decrease in paw withdrawal threshold in response to mechanical stimulation (mechanical allodynia). In these rats, the number of microglia in the spinal dorsal horn (SDH) was significantly increased. Paclitaxel-treated rats showed a significant increase in the expression of mRNAs for CCL3 and its receptor CCR5 in the SDH. Intrathecal administration of a CCL3-neutralizing antibody not only attenuated the development of paclitaxel-induced mechanical allodynia but also reversed its maintenance. Paclitaxel also upregulated expression of purinoceptor P2X7 receptors (P2X7Rs), which have been implicated in the release of CCL3 from microglia, in the SDH. The selective P2X7R antagonist A438079 had preventive and reversal effects on paclitaxel-induced allodynia.

Conclusions: Our findings suggest a contribution of CCL3 and P2X7Rs in the SDH to paclitaxel-induced allodynia and may provide new therapeutic targets for paclitaxel-induced painful neuropathy.

Keywords: Paclitaxel, Microglia, Chemokines, Spinal cord, Allodynia, Rats

Findings

Introduction

Paclitaxel is an effective chemotherapeutic agent widely used for the treatment of breast, ovarian, and non-small-cell lung cancer [1]. Paclitaxel often induces peripheral neuropathy, which is characterized by a sensory abnormality of extremities usually occurring in a stocking-and-glove distribution in addition to motor dysfunction in patients [2]. Peripheral neuropathy is the major dose-limiting toxicity of paclitaxel and may persist for months to years [3], having a long-term negative impact on patients' quality of life. However, the mechanisms underlying paclitaxel-induced peripheral neuropathy remain unknown, and there are no established treatments.

A growing body of evidence indicates that activated microglia in the spinal dorsal horn (SDH) play important roles in pathological chronic pain in different animal models [4-6]. Activated microglia produce many types of inflammatory mediators, such as proinflammatory cytokines and chemokines, which contribute to the initiation and maintenance of pain hypersensitivity [6-8]. It was recently reported that paclitaxel treatment induces activation of microglia in the SDH in rats and mice [9] and that intrathecal administration of minocycline, a reagent that can inhibit microglial activation attenuates paclitaxel-induced pain hypersensitivity [10]. We have previously

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shown that microglia produce and release CC-chemokine ligand 3 (CCL3; also known as MIP-1α) [11] and that in a model of spinal nerve injury, spinal CCL3 expression is upregulated in microglia and contributes to mechanical allodynia [12]. Furthermore, intrathecal administration of CCL3 to naïve animals produces mechanical allodynia [12,13]. CCL3 is known to activate its cognate receptors CCR5 and CCR1 [14], and a pharmacological blockade of spinal CCL3 signaling by intrathecal administration of CCR5 antagonists reduces pain hypersensitivity after traumatic nerve injury [12,15]. These results suggest that spinal CCL3 plays an important role in traumatic nerve injury-induced allodynia. However, there are no reports demonstrating whether CCL3 in the spinal cord contributes to chemothe rapy-induced neuropathic pain. Thus, the aim of the present study was to investigate the role of spinal CCL3 in mechanical allodynia using a rat model of paclitaxel-induced neuropathy.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use committee review panels at Kyushu University.

Male Sprague–Dawley rats (8–11 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The rats were housed at a temperature of 22 ± 1°C with a 12-hour light–dark cycle and had *ad libitum* access to food and water.

Paclitaxel (LKT Laboratories, St. Paul, USA) was dissolved in a 1:1 mixture of ethanol and Cremophor EL (Sigma-Aldrich, St. Louis, USA) to make a stock solution of 12 mg/mL. Prior to administration, the paclitaxel solution was further diluted with sterile saline (1:3). Under isoflurane (2%) anesthesia, rats were administered the solution via the tail vain on days 0 and 3 after paw withdrawal threshold was measured. We used a previously characterized model of paclitaxel-induced peripheral neuropathy produced by repeated infusions of paclitaxel at a cumulative dose of 36 mg/kg (2 × 18 mg/kg, 3 days apart) [9]. Control rats received equivalent volumes of the Cremophor/ethanol vehicle.

For immunohistochemical experiments, rats were deeply anesthetized by pentobarbital and perfused transcardially with phosphate-buffered saline (PBS, composition in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.5, NaH_2PO_4 8.1; pH 7.4) followed by ice-cold 4% paraformaldehyde/PBS. The L5 segment of the lumbar spinal cord was removed, postfixed in the same fixative, and placed in 30% sucrose solution for 24 hr at 4°C. Transverse L5 spinal cord sections (30 μm) were cut on a Leica CM 1850 cryostat (Leica Biosystems, Wetzlar, Germany) and incubated for 2 hr at room temperature in a blocking solution (3% normal goat serum), and then incubated for 48 hr at 4°C in the primary antibody for ionized calcium-binding adapter molecule 1 (Iba1, 1:2000, Wako, Osaka, Japan), a marker of microglia. Spinal sections were incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:1000, Life Technologies Japan, Tokyo, Japan) and mounted in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, USA). Two to three sections from the L5 spinal cord segments of each rat were randomly selected and analyzed using an LSM510 Imaging System (Carl Zeiss Japan, Tokyo, Japan). The numbers of $Iba1^+$ cells in the SDH (lamina I – IV) were counted.

For quantitative real-time PCR, rats were deeply anesthetized with pentobarbital, perfused transcardially with PBS, and the L5 spinal cord was removed immediately. The tissues were separated into ventral and dorsal horn. The sample was homogenized with TRIsure (Bioline, London, UK) and RNA was purified using an RNeasy mini plus kit (Qiagen, Valencia, USA). The amount of RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). RNA was transcribed using PrimeScript Reverse Transriptase (Takara Bio, Otsu, Japan). Quantitative PCR was performed using Premix Ex *Taq* (Takara) together with a 7500 real-time PCR system (Life Technologies Japan, Tokyo, Japan), and the data were analyzed using 7500 System SDS Software 1.3.1 (Life Technologies Japan, Tokyo, Japan). Expression levels of genes of interest were normalized to the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were expressed as fold change over control rats. The sequences of TaqMan primer pairs and probes are described below: rat Iba1, 5′-GATTTGCAGGGAGGAAAAGCT-3′ (forward), 5′-AACCCCAAGTTTCTCCAGCAT-3′ (reverse), 5′-CAGGAAGAGAGGTTGGATGGGATCAA-3′ (Taqman probe); rat CCL3, 5′-CCACTGCCCTTGCTGTTCTT-3′ (forward), 5′-GCAAAGGCTGCTGGTTTCAA-3′ (reverse), 5′-CGCCATATGGAGCTGACACCCCG-3′ (Taqman probe); rat CCR1, 5′-CTAAGATGGCTAGGGCCCAAATA-3′ (forward), 5′-TCCCTGAGGGCCCGAACTGTCA-3′ (reverse), 5′-CCTGGGCTTATACAGTGAGATCTTC-3′ (Taqman probe); rat CCR5, 5′-GACCGGGTATA GACTGAGCTTACAC-3′ (forward), 5′-ACTCTTGG GATGACACACTGCTGCCTC-3′ (reverse), 5′-CAGG CAATGCAGGTGACAGA-3′ (Taqman probe); and rat purinoceptor P2XR7, 5′-CATGGAAAAGCGGACAT TGA-3′ (forward), 5′-CCAGTGCCAAAAACCAGGAT-3′ (reverse), 5′-AAAGCCTTCGGCGTGCGTTTTGA-3′ (Taqman probe).

Mechanical allodynia was assessed using von Frey filaments (North Coast Medical, Gilroy, USA). Rats were placed in an aluminum cage with a wire mesh grid floor in a quiet room, 30 min before the start of testing. The von Frey filament (1.0–15.0 g) was inserted through the mesh floor bottom and was applied to the middle of the plantar surface of the hindpaw. The 50% paw withdrawal

threshold (PWT) was determined using the up-down method [16].

For intrathecal administration, under isoflurane (2%) anesthesia, rats were implanted with a 32-gauge intrathecal catheter (ReCathCo, Allison Park, USA) through the atlanto-occipital region into the lumbar enlargement of the spinal cord. Seven days after implantation, the catheter placement was verified by the observation of transient hindpaw paralysis induced by intrathecal injection of lidocaine (2%, 5 μL). Animals that failed to display paralysis following lidocaine administration were not included in the experiments. To test for possible effects on the development of paclitaxel-induced mechanical allodynia, rats were injected with a CCL3-neutralizing antibody (4 ng/10 μL; R&D Systems, Minneapolis, UAS) (or control IgG2A) and the selective P2X7R antagonist A438079 (1 μg/10 μL, Tocris Bioscience, Bristol, UK) (or PBS) once a day for 7 days starting from 1 day before the first injection of paclitaxel. Intrathecal administration of these drugs was done soon after the completion of behavioral measurement (10:00 ~ 12:00). For experiments testing their effects on maintenance of paclitaxel-induced mechanical allodynia, these agents were administered intrathecally to paclitaxel-treated rats, once, on day 7.

Data are expressed as the means ± SEM. Statistical analyses of the results were conducted with the Student's t test, one-way ANOVA with post hoc Dunnett's multiple comparisons, and two-way ANOVA with Bonferroni's post hoc analysis. The threshold for statistical significance was set at a P value < 0.05.

Results

First, to confirm mechanical pain hypersensitivity in rats that had been administered paclitaxel via the dosing regimen used in this study, we measured PWT in response to mechanical stimulation of paclitaxel-treated rats. As shown in Figure 1A, paclitaxel produced a profound, long-term decrease in PWT. We next immunohistochemically examined activation of microglia in the SDH using an antibody against Iba1, a marker of microglia, in paclitaxel-treated rats. In the L5 SDH of paclitaxel-treated rats, Iba1 immunofluorescence was clearly increased (Figure 1B). At high magnification, Iba1-positive microglia showed an activated morphology such as thickened cell bodies and retracted processes (Figure 1B insets). The number of Iba1-positive microglia in the L5 SDH was significantly increased on days 7 and 14 after the first injection of paclitaxel (Figure 1C). In addition, we also observed a significant increase in the level of mRNA for Iba1 in the SDH (data not shown). These results together indicate that microglia become activated in the SDH of paclitaxel-treated rats.

To examine changes in expression of CCL3 in the SDH, we performed real-time RT-PCR analysis. Seven days after the first paclitaxel injection, the expression of CCL3 mRNA was markedly increased in the SDH (Figure 2A). The increase in CCL3 mRNA in the SDH was detected as early as day 3 (but was not statistically significant at this time), and the highest level was observed on day 7. The upregulation of CCL3 was still evident, to a slightly lesser extent, on day 14 (Figure 2A). The time course of CCL3 upregulation paralleled that of the number of microglia in the SDH (Figure 1C). We also examined the expression of CCR5 and CCR1 (receptors for CCL3 [14]) mRNAs in the SDH. CCR5 expression was significantly increased in the SDH on days 7 and 14 after the first paclitaxel administration (Figure 2B). By contrast, the CCR1 mRNA expression level was unchanged at all time points tested (Figure 2C).

To determine the role of spinal CCL3 in paclitaxel-induced mechanical allodynia, we tested the effect of a CCL3-neutralizing antibody on the PWT in paclitaxel-treated rats. Repeated intrathecal injection of an anti-CCL3 antibody [once a day from day 0 (before the first paclitaxel injection) until day 6] prevented the decrease in PWT in paclitaxel-treated rats (Figure 3A). We also tested the effect of a single intrathecal injection of the CCL3 antibody on day 7 after the first paclitaxel injection, a time corresponding to the highest expression level of CCL3. Intrathecal CCL3 antibody injection significantly increased the PWT in paclitaxel-treated rats (Figure 3B). These results indicate that spinal CCL3 contributes to the development and maintenance of paclitaxel-induced mechanical allodynia.

We have previously demonstrated that activating purinergic P2X7Rs in cultured microglia causes release of CCL3. Thus, we further investigated the involvement of spinal P2X7Rs. We examined expression of P2X7Rs in the SDH and found that paclitaxel treatment significantly increased expression of P2X7R mRNA in the SDH on day 7. To determine the role of spinal P2X7Rs in the development of mechanical allodynia, we repeatedly administered A438079, a selective antagonist for P2X7Rs [17,18], into the intrathecal space. As shown in Figure 4B, repeated intrathecal administration of A438079 prevented the decrease in the PWT in paclitaxel-treated rats (Figure 4B). We also found that a single intrathecal administration of A438079 to paclitaxel-treated rats on day 7 reversed mechanical allodynia (Figure 4C).

Discussion

In the present study, we demonstrated for the first time that the chemokine CCL3 in the spinal cord is involved in the mechanical allodynia caused by paclitaxel treatment in rats. Our behavioral data showing both the preventive and reversal effects of chronic and single intrathecal administration, respectively, of a CCL3-neutralizing antibody on the paclitaxel-induced allodynia suggest that CCL3 plays important roles not only in the development of paclitaxel-induced allodynia, but also in its maintenance. Although

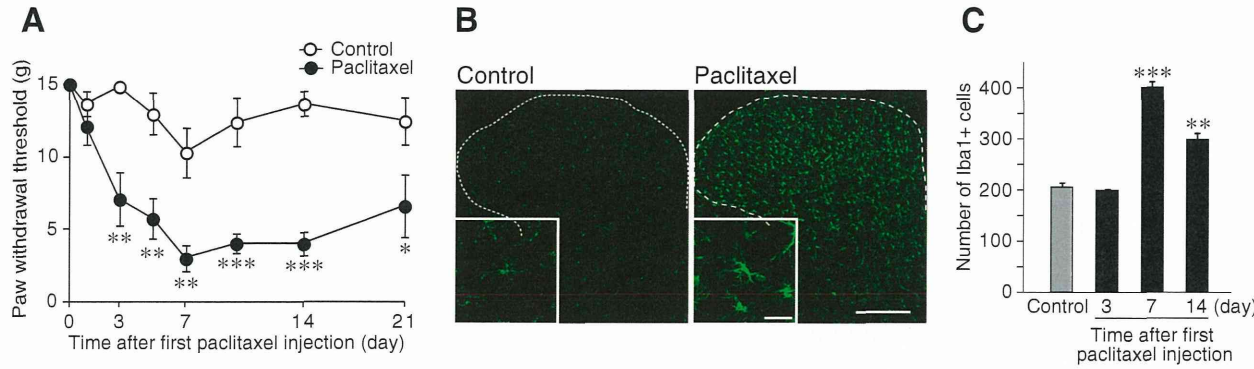

Figure 1 Administration of paclitaxel causes activation of microglia in the SDH. (A) Threshold for withdrawal in response to mechanical stimulation to the hindpaw before and after injection of vehicle or paclitaxel (**$P < 0.01$, *** $P < 0.001$ vs. vehicle group, $n = 6$). **(B)** Immunofluorescence for Iba1, a marker of microglia, in the SDH of vehicle (control)- or paclitaxel-treated rats (Scale bar, 200 μm). Insets: high magnification images (Scale bar, 20 μm). **(C)** The number of microglia labeled with Iba1 per 6×10^5 μm in the SDH of paclitaxel-treated rats (n = 3, **$P < 0.01$, ***$P < 0.001$, vs. naïve control rats). Values are means ± s.e.m.

the type of cells responsible for upregulating CCL3 expression in the SDH of paclitaxel-treated rats remains to be determined, the temporal correlation between the upregulation of CCL3 expression and the increase in the number of microglial cells in the SDH leads us to hypothesize that the CCL3 may be derived from activated microglia. Indeed, the morphological hypertrophy of microglia and the increase in the number of microglia, both of which are major immunohistochemical features of microglial activation, were evident in the SDH of paclitaxel-treated rats. Furthermore, our previous study revealed production and release of CCL3 from cultured microglia [11]. In a recent study using rat brain slices *in vitro*, neuronal injury was shown to induce the microglial production of CCL3 [19]. We have also recently demonstrated upregulation of CCL3 expression in spinal microglia after traumatic nerve injury in rats [12], which strongly supports our hypothesis. Moreover, P2X7Rs have been implicated in CCL3 release from microglia [11], and our study showed that pharmacological blockade of spinal P2X7Rs suppressed paclitaxel-induced mechanical allodynia, the effect of which was similar to that of the CCL3-neutralizing antibody. P2X7R expression has been reported to be upregulated predominantly in microglia in the SDH after traumatic nerve injury [20,21]. Thus, microglial cells could be a candidate for the source of CCL3 in the SDH of paclitaxel-treated rats. However, we can not exclude a possible involvemen t of other cell types expressing CCL3 in the SDH. Indeed, in addition to microglia, cultured astrocytes also express CCL3 [22]. Consistent with previous studies, we also found that paclitaxel treatment changed the morphology and expression of GFAP in the SDH (data not shown). Recent studies have implicated spinal astrocytes in chemotherapy-induced mechanical hypersensitivity [23,24].

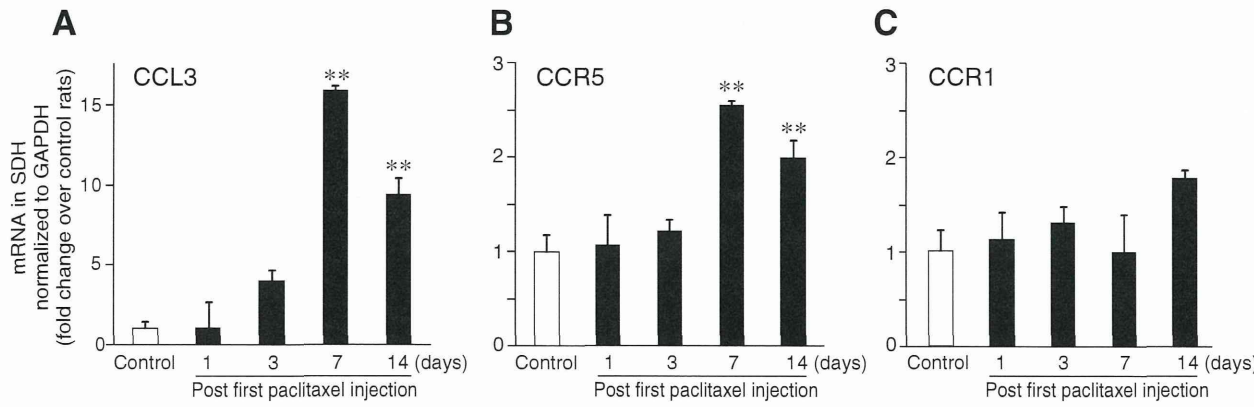

Figure 2 Upregulation of CCL3 and CCR5 mRNAs in the SDH following administration of paclitaxel. Real-time RT-PCR analyses of mRNA levels for CCL3 **(A)**, and the chemokine receptors CCR1 **(B)** and CCR5 **(C)** in the SDH of paclitaxel- and vehicle-treated rats at each time point after the first injection of paclitaxel (n = 4–6, **$P < 0.001$ vs. control). Values represent the relative ratio of each mRNA (normalized to the level of GAPDH mRNA) in paclitaxel-treated rats to that in naïve control rats (fold change over control rats). Values are means ± s.e.m.

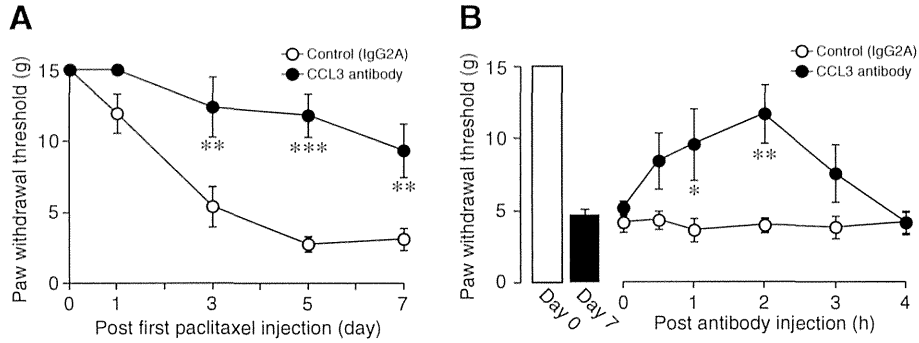

Figure 3 A CCL3-neutralizing antibody suppresses the development and maintenance of paclitaxel-induced mechanical allodynia. Intrathecal administration of a CCL3-neutralizing antibody (4 ng/10 μL) or control IgG2A was done once a day for 7 days (from a day before the first paclitaxel injection until day 6) **(A)** or once on day 7 after the first paclitaxel injection **(B)** (n = 6, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vs. control group). Values are means ± s.e.m.

The present study showed that a single intrathecal administration of either a CCL3-neutralizing antibody or the selective P2X7R antagonist reversed the established mechanical allodynia in paclitaxel-treated rats. Considering that microglia release CCL3 in response to P2X7R activation [11], the most parsimonious hypothesis is that ongoing signaling via CCL3 (presumably released from P2X7R-stimulating microglia) is crucial for maintaining paclitaxel-induced pain hypersensitivity. Although the detailed mechanisms by which CCL3 mediates paclitaxel-induced mechanical allodynia are not clear, spinal CCL3 might affect spinal pain processing. The ability of spinal CCL3 to produce mechanical allodynia has been demonstrated by our and other studies showing that intrathecal administration of CCL3 to naïve animals produces pain hypersensitivity in response to mechanical stimulation [12,25]. The expression of CCR5 in the SDH was markedly upregulated, and the time course of its upregulation matched that of CCL3 expression in the SDH. CCR5 was implicated in pain hypersensitivity in a model of neuropathic pain caused by traumatic nerve injury [12,15]. CCR5 expression has been reported to be localized to activated microgila in the spinal cord in response to nerve injury [12,26]. It is thus speculated that spinal CCL3 released from microglia in response to activation of P2X7Rs by extracellular ATP (which is presumably released from neighboring neurons [27] or glial cells [28]) further activates microglia via CCR5 in an autocrine manner, which may in turn lead to an alteration of dorsal horn pain processing. On the other hand, there was a lack of changes in CCR1 expression in the SDH of pactitaxel-treated rats, although a previous study has reported the upregulation of CCR1 in the spinal cord after traumatic nerve injury [13]. Considering recent findings showing the failure of a selective CCR1 antagonist to

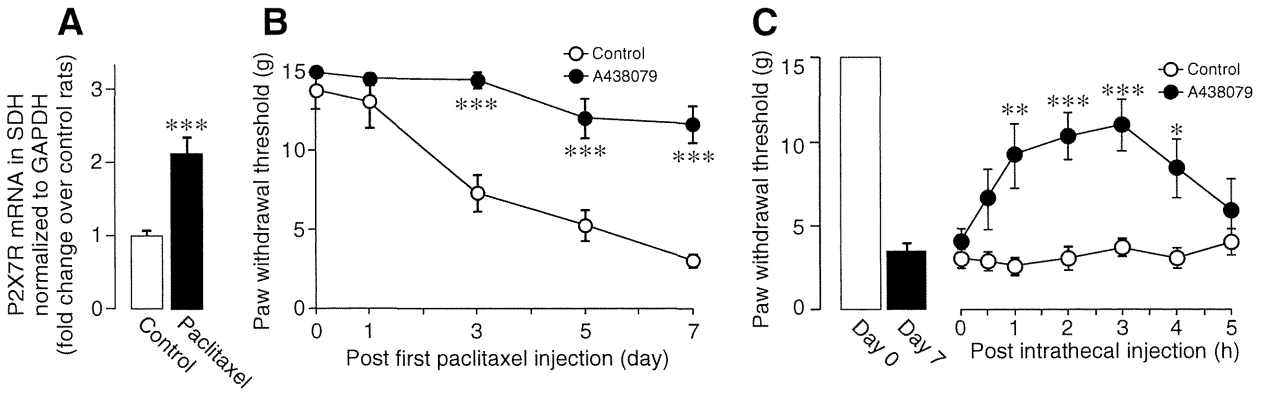

Figure 4 P2X7Rs are upregulated by paclitaxel treatment and contribute to mechanical allodynia. (A) Real-time RT-PCR analysis of P2X7R mRNA in the SDH of paclitaxel- and vehicle-treated rats 7 days after the first injection of paclitaxel (n = 6, ***$P < 0.001$). Values represent the relative ratio of P2X7R mRNA (normalized to the level of GAPDH mRNA) in paclitaxel-treated rats to that in vehicle control rats (fold change over control rats). **(B)** A438079 (a selective P2X7R antagonist; 1 μmol/10 μL) was intrathecally administered once a day for 7 days (from a day before the first paclitaxel injection to day 7) (n = 6, ***$P < 0.001$, vs. vehicle-treated control group). **(C)** A438079 (1000 nmol/10 μL) was intrathecally administered once on day 7 (n = 6, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle-treated control group). Values are means ± s.e.m.

suppress paclitaxel-evoked cold hypersensitivity [29], spinal CCL3-CCR5 signaling may play an important role in paclitaxel-induced neuropathic pain. However, how CCL3-stimulated microglia affect pain processing in the dorsal horn remains to be determined.

In summary, the present study revealed CCL3 as an important player in the development and maintenance of mechanical allodynia following paclitaxel treatment. Therefore, our findings not only provide evidence for a new mechanism underlying the pathogenesis of chemotherapy-induced peripheral sensory neuropathy, but also suggest a novel therapeutic approach to neuropathic pain.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RO designed, performed the experiments, analyzed the data, and wrote the manuscript; KN and TI designed and performed the experiments; HS-T analyzed the data; MT supervised the overall project, and wrote the manuscript. KI conceived the study, supervised the overall project, and wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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キーワード解説

神経障害性疼痛

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1. 神経障害性疼痛とは

痛みは，急性疼痛と慢性疼痛に大別される．前者は，身体に傷や何らかの障害が起きたことを生体に認識させる重要な警告信号である．後者は，炎症性疼痛と神経障害性疼痛に分類される．炎症性疼痛は，皮膚などの末梢組織の炎症に起因する疼痛で，非ステロイド性抗炎症薬やオピオイドなどで治療できる．また，炎症性疼痛は炎症患部への物理的な接触を避る行動につながり，結果的に治癒を促すという側面もある．しかし，神経障害性疼痛の場合，そのような生体警告系としての役割がなく，慢性化する堪え難い痛みから患者のQOLは極度に低下してしまう．

国際疼痛学会IASPでは，2011年に神経障害性疼痛を「体性感覚神経系の損傷や疾患によって引き起こされる痛み（Pain caused by a lesion or disease of the somatosensory nervous system）」と定義している．臨床的には，末期がん，糖尿病，帯状疱疹後，脊髄損傷，手術の後遺症，HIV感染，多発性硬化症など多くの疾患で発症することが知られている．また，がんの化学療法薬であるパクリタキセルやオキサリプラチンなどによっても発症する．神経障害性疼痛の主な症状としては，持続的な自発痛，痛覚過敏（侵害刺激による痛みが正常時より強くなる状態），そしてアロディニア（異痛症）（通常では痛みを引き起こさないような非侵害刺激で誘発される痛み）などが挙げられる．

我が国における神経障害性疼痛の薬物治療は，2011年に日本ペインクリニック学会から提示された「神経障害性疼痛薬物療法ガイドライン」に記載されているアルゴリズムが推奨されている．第一選択薬としては，三環系抗うつ薬であるノルトリプチリン，アミトリプチリン，イミプラミン，カルシウムチャネル$\alpha 2\delta$リガンドであるプレガバリンやガバペンチンがある．また，帯状疱疹後神経痛にはノイロトロピン（ワクシニアウイルス接種家兎炎症皮膚抽出液含有製剤）が，有痛性糖尿病性ニューロパチーにはデュロキセチン（抗うつ薬），メキシレチン（抗不整脈薬），エパルレスタット（アルドース還元酵素阻害薬）が，上記薬物に加え，第一選択薬として考慮と記されている．ただ，三叉神経痛だけは特殊な薬物療法が必要とされ，抗てんかん薬のカルバマゼピンが推奨されている．モルヒネやフェンタニルなどのオピオイドは第三選択として推奨されている．しかし，オピオイドの使用には，他の薬物治療の可能性や乱用・依存リスクの少ない患者の選択など，慎重な配慮が必要とされ，2012年に日本ペインクリニック学会から「非がん性慢性［疼］痛に対するオピオイド鎮痛薬処方ガイドライン」が提示されている．

2. 神経障害性疼痛のメカニズム（基礎研究から）

上述ガイドラインに沿った薬物治療が行われているが，その有効性や副作用の観点からまだまだ十分とは言えないのが現状である．そのため，神経障害性疼痛の発症維持における新しいメカニズムを解明し，それをターゲットにした有効な治療薬を創製することが極めて重要である．

病態メカニズムの解明や治療薬開発には，病態モデル動物が必要であるが，神経障害性疼痛モデルとしては，末梢神経である坐骨神経や脊髄神経を結紮あるいは切断するモデルが汎用されている(1,2)．また，より臨床に近いモデルとして，帯状疱疹痛モデル，がん性疼痛モデル，術後痛モデル，糖尿病モデル，脊髄損傷モデルなども用いられている．

それらの動物モデルを用いて数多くの研究が精力的に行われており，多くの重要な成果が出ている．そのメカニズムとして，痛覚伝達系のニューロン機能の過活動や脊髄後角でのニューロン回路の変調などが明らかとなっている(3)．さらに，ニューロンだけでなく，脳や脊髄に数多く存在するグリア細胞も極めて重要な役割を担っていることが明らかになり，神経障害性疼

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痛の新しいメカニズムとして注目されている．中枢神経系におけるグリア細胞は，アストロサイト，オリゴデンドロサイト，そしてミクログリアの3種類に大別される(4)．神経障害性疼痛モデルの脊髄においては，特にミクログリアとアストロサイトの著明な形態学的変化が認められる．そこで，以下ではミクログリアの役割について概説する．

ミクログリアは，胎生期の卵黄嚢で発生する原始マクロファージを起源とし，それが脳や脊髄に浸潤して成熟を遂げた細胞と考えられている(5)．成熟動物での正常脳や脊髄のミクログリアは，分岐の多い突起を長く伸ばした，いわゆるラミファイド（ramified）型で存在している．上述した末梢神経を損傷させた神経障害性疼痛動物モデルの脊髄において，ミクログリアは活性化型の形態学的特徴である細胞体の肥大化を示すとともに細胞分裂を起こして細胞数を2〜3倍に増加させる(6)．さらに，ミクログリアは細胞膜受容体などの機能分子を発現し，細胞外からのシグナル分子に応答し，炎症性サイトカインなどの液性因子を産生放出する．神経障害性疼痛におけるミクログリアの役割は，神経損傷後の脊髄でミクログリア特異的に発現する様々な分子の研究によって徐々に明らかになってきている．

脊髄ミクログリアの重要性を明らかにする突破口となった分子は，細胞外ATPで活性化する非選択的陽イオンチャネルの一つであるP2X4受容体（P2X4R）である．神経障害性疼痛動物モデルの脊髄後角では，P2X4Rがミクログリアで特異的に高発現し，その受容体を遮断すること，あるいは遺伝子を欠損させることで，アロディニアが著明に抑制された(7)．ミクログリアのP2X4R刺激により，脳由来神経栄養因子BDNFが産生放出され，BDNFは脊髄後角痛覚ニューロンのTrkBを介して，このニューロンを抑制するGABAの作用を興奮性へと変化させ，結果として異常興奮を起こす(8)．この結果は，ミクログリアのP2X4Rの活性化に端を発するグリア－ニューロン連関の形成が，ニューロンの過剰興奮，さらには神経障害性疼痛の原因であることを示唆している．

活性化したミクログリアではさまざまな遺伝子の発現が変化するが，最近我々は，その遺伝子発現に重要な転写因子としてinterferon regulatory factor 8（IRF8）を特定した(9)．IRF8は，神経障害性疼痛に関与するミクログリア遺伝子（TLR2，P2Y12R，CX3CR1，IL-1β，カテプシンS，P2X4R）の発現を制御しており，IRF8欠損マウスは，神経損傷後のアロディニアも抑制されていた．以上の結果から，IRF8が，ミクログリアを活性化状態へと誘導し，神経障害性疼痛の発症と維持に重要な役割を担っていることが示唆される．

3. おわりに

2003年以降(7)，ミクログリア分子の発現や機能を抑制することで神経障害性疼痛を緩解するという，一貫したエビデンスが基礎研究レベルで数多く蓄積しており，神経障害性疼痛の発症維持の新しいメカニズムとして脚光を浴びている(10, 11)．そのメカニズムについては，神経損傷後に活性化するグリア，それを起点として新たに形成されるニューロンとの病的な連関が原因と考えられている(11)．しかし，活性化グリア細胞が作り上げるニューロンの機能異常については不明な点が多く残されているのも事実である．したがって，グリア細胞の役割を今後詳細に研究し，その全容を明らかにすることで，それらを標的にした薬剤が，将来的に有効な治療薬となることを期待したい．また，ニューロンやアストロサイトに関する神経障害性疼痛に関する重要な成果は，誌面の制約上，割愛したがそれらについては他の総説等(12, 13）を参照していただきたい．

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