Spinal Cord is the Primary Site of Action of the Cannabinoid CB$_2$ Receptor Agonist JWH133 that Suppresses Neuropathic Pain: Possible Involvement of Microglia

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Abstract: Neuropathic pain, a highly debilitating condition that commonly occurs after damage to the nervous system, is often resistant to commonly used analgesic agents such as non-steroidal anti-inflammatory drugs and even opioids. Several studies using rodent models reported that cannabinoid CB$_2$ receptor (CB$_2$R) agonists are effective for treating chronic pain. However, the analgesic mechanism of CB$_2$R agonists in neuropathic pain states is not fully understood. In this study, we investigated the role of CB$_2$Rs in the development and maintenance phases of neuropathic pain, and the mechanism of the CB$_2$R-mediated analgesic effect on neuropathic pain. In a rat model of neuropathic pain, systemic administration of JWH133, a CB$_2$R agonist, markedly improved tactile allodynia, and this effect was prevented by intrathecal pretreatment with AM630, a CB$_2$R antagonist. The antiallodynic effect of intrathecally administered JWH133 was inhibited by intrathecal pretreatment with pertussis toxin or forskolin. In the spinal cord, CB$_2$R expression was significantly increased on post-operative day 3, and persisted for 2 weeks. Furthermore, repeated intrathecal administration of JWH133 notably attenuated the development of tactile allodynia after peripheral nerve injury. In a culture of microglia activated by overexpressing interferon regulatory factor 8, a transcription factor crucial for neuropathic pain, JWH133 treatment suppressed the increased expression of interleukin-1β. Our findings suggest that activation of CB$_2$Rs upregulated in the spinal cord after nerve injury alleviates existing tactile allodynia through the G$_{i/o}$-adenylate cyclase signaling pathway and suppresses the development of allodynia. This process may reduce the inflammatory response of microglia. Therefore, spinal CB$_2$Rs may be a therapeutic target for the treatment of neuropathic pain.

Keywords: Allodynia, cannabinoid CB$_2$ receptor, microglia, neuropathic pain, spinal cord.

INTRODUCTION

Neuropathic pain is a highly debilitating chronic pain state that occurs after nerve damage associated with various diseases, such as cancer, diabetes mellitus, infection, autoimmune disease, and trauma. Tactile allodynia (pain hypersensitivity to normally innocuous stimuli) is one of the characteristic symptoms of neuropathic pain and is often refractory to currently available treatments such as non-steroidal anti-inflammatory drugs (NSAIDs), and even opioids. Therefore, new therapeutic agents with higher efficacy for the treatment of neuropathic pain are desirable.

Accumulating evidence from diverse animal models of neuropathic pain indicates that neuropathic pain is a reflection of the aberrant excitability of dorsal horn neurons evoked by peripheral sensory inputs [1, 2]. This hyperexcitability might result from multiple cellular and molecular alterations in the dorsal horn occurring after peripheral nerve injury (PNI). It has long been considered that there are damage-related changes in neurons, but recent studies provide compelling evidence indicating that spinal microglia, immune-like glial cells in the central nervous system (CNS), rapidly respond to PNI and become activated with changing morphology, increased numbers, and expression of a variety of genes [3, 4]. Activated spinal microglia secrete various biologically active signaling molecules, including proinflammatory cytokines [5], which induce hyperexcitability of dorsal horn neurons [4, 6]. Thus, it is important to control the activation of microglia for the treatment of neuropathic pain.

Recently, cannabinoids have been shown to have potent analgesic effects in studies using several chronic pain models (including neuropathic pain) [7, 8]. For the treatment of chronic pain, cannabinoids have attracted much attention as potential new therapeutic alternatives to opioids. Cannabinoids activate their cognate receptors CB$_1$, CB$_2$, and GPR55 [9-11]. Among these cannabinoid receptors, the CB$_2$ receptor (CB$_2$R) may be a target for the development of novel analgesics because CB$_2$R agonists have been reported to exert their antinociceptive effects without causing neuropsychiatric side effects [8, 12, 13]. CB$_2$Rs are mostly expressed in peripheral tissues as well as immune cells, where they regulate cell activation and cytokine release [14-16]. In normal rats, systemic administration of AM1241, a CB$_2$R agonist, produces an antinociceptive effect, which can

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be prevented by intrapaw injection of naloxone [17]. These results suggest that CB₂R activation stimulates the release of β-endorphin from keratinocytes, which acts at local neuronal μ-opioid receptors to inhibit nociception. Under neuropathic pain conditions, intraplantar injection of the CB₂R agonist has no effect, but intrathecal administration produces an anti-allodynic effect [18]. Furthermore, CB₂Rs are expressed by activated microglia [19-21]. Thus, the mechanism of CB₂R-mediated pain inhibition might be different between normal and neuropathic conditions. However, it remains to be determined where the primary site of action of CB₂R agonists is when administered systemically to suppress neuropathic pain.

To address these issues, we investigated the antiallodynic effect of a CB₂R agonist administered systemically, the intracellular signaling pathway downstream of CB₂R, and the contribution of CB₂R to the development and maintenance of neuropathic pain. Furthermore, using cultured microglial cells activated by overexpressing interferon regulatory factor 8 (IRF8), a molecule previously identified as a transcription factor of the IRF family and crucial for microglial activation after PNI and neuropathic pain [22], we investigated the effect of a CB₂R agonist on IRF8-induced gene expression.

MATERIALS AND METHODOLOGY

Animals

Male Wistar rats (230–280 g) obtained from Japan SLC (Hamamatsu, Japan) were used. Rats were housed at a constant room temperature of 23 ± 1°C with a 12-h light-dark cycle (light on 8:00–20:00) and fed food and water *ad libitum*. All of the animals used in the present study were treated in accordance with the guidelines of Kyushu University.

Neuropathic and Inflammatory Pain Model

Preparation of both chronic pain models of rats was performed under isoflurane (2%) anesthesia. For the neuropathic pain model, a unilateral L5 spinal nerve of rats was tightly ligated with 5-0 silk suture and cut just distal to the ligature. For the inflammatory pain model, complete Freund's adjuvant (CFA; 0.1 mg/200 μL, suspended in an equal volume of phosphate-buffered saline (PBS); Sigma, St. Louis, MO, USA) was injected into the plantar surface of the left hindpaw of rats.

Behavioral Assays

To assess tactile allodynia, rats were placed individually in a wire mesh cage and habituated for 30 min to allow acclimatization to the new environment. Then, calibrated von Frey filaments (0.4–15.0 g; North Coast Medical, Morgan Hill, CA, USA) were applied to the plantar surface of the hindpaw from below the mesh floor. Paw withdrawal threshold (PWT) was determined using the up-down method [23].

Intrathecal Catheterization

Under isoflurane (2%) anesthesia, rats were implanted with a 32-gauge catheter (ReCathCo, Allison Park, PA,

USA) through the atlanto-occipital region and into the lumbar enlargement (close to the L4–5 segments) of the spinal cord [24]. After 3 days of recovery, the catheter placement was verified by the observation of transient hindpaw paralysis induced by intrathecal injection of lidocaine (2%, 7 μL). Animals that failed to show any paralysis were not used in experiments.

Drug Administration

To examine the acute effect, vehicle [PBS/1% dimethyl sulfoxide (DMSO)] or JWH133 (1 and 3 mg/kg or 0.1, 1, and 10 nmol in 10 μL PBS/1% DMSO; Tocris Bioscience, Bristol, UK) were administered intraperitoneally or intrathecally, through the implanted catheter, to nerve-injured rats 7 days after PNI. AM630 (20 nmol in 10 μL PBS/1% DMSO/1% Tween 80; Tocris Bioscience, Bristol, UK), pertussis toxin (PTx; 0.5 μg in 10 μL PBS; Sigma) and forskolin (10 μg in 10 μL PBS/1% DMSO; Sigma) were administered by intrathecal or intraplantar injection before JWH133 administration. The drug was administered just after pre-measurement of PWT. To examine the chronic effect, rats were administered vehicle or JWH133 intrathecally twice a day from day 0 (immediately after nerve injury) to 7 or from day 7 to 14. PWT was measured just before morning drug administration (at least 10 h after drug administration of the previous day).

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Rats were deeply anesthetized intraperitoneally with pentobarbital (100 mg/kg), perfused transcardially with PBS, and the L5 segment of the spinal cord without L5 roots was removed immediately. The tissue was vertically separated at the median, and hemisections of the spinal cord were subjected to total RNA extraction using TRIsure (Bioline, Danwon-gu, South Korea) according to the manufacturer's protocol and purified with an RNeasy mini-plus kit (Qiagen, Valencia, CA, USA). Extraction of total RNA from a BV-2 mouse microglial-cell line was also performed using TRIsure. The amount of total RNA was measured using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE, USA). Complementary DNA was prepared *via* reverse transcription of total RNA using Prime Script reverse transcriptase (Takara, Kyoto, Japan). Quantitative PCR was performed with Premix Ex Taq (Takara) using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Expression levels were normalized to the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The TaqMan probe and the forward and reverse primers used in this study are shown in Table **1**. The primers and probe for GAPDH were obtained from Applied Biosystems.

Cell Culture

The BV-2 mouse microglial cell line was cultured as described previously [25]. Briefly, cells were cultured in Dulbecco's modified Eagle's medium with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. The lentiviral vector was prepared as described previously to overexpress the IRF8 gene [22]. In brief, full-length cDNAs for mouse IRF8 tagged with green fluorescent protein (IRF8-GFP) or GFP

Table 1. Sequences of forward primers, reverse primers, and TaqMan probes for quantitative real-time RT-PCR.

		Primer	Probe
rCB2R	Forward	5′-CATGCTGTCTCTGGCAGATTCA-3′	5′-FAM-CCAACAGACAAGACGTCCGTCCCC-TAMRA-3′
	Reverse	5′-ATTAGGGAGGCTGATTGGTCTTCT-3′	
mCB2R	Forward	5′-GGATGCCGGGAGACAGAA-3′	5′-FAM-CCAACGGCTCCAACGGTGGCTT-TAMRA-3′
	Reverse	5′-TGCTCAGGATCATGTACTCCTTCA-3′	
mIRF8	Forward	5′-GGATATGCCGCCTATGACACA-3′	5′-FAM-CCATTCAGCTTTCTCCCAGATGGTCATC-TAMRA-3′
	Reverse	5′-CATCCGGCCCATACAACTTAG-3′	
mP2X4R	Forward	5′-ACAACGTGTCTCCTGGCTACAAT-3′	5′-FAM-CAATGAGCAACGCACACTCACCAAGG-TAMRA-3′
	Reverse	5′-GTCAAACTTGCCAGCCTTTCC-3′	
mP2Y12R	Forward	5′-TGAAGACCACCAGGCCATTT-3′	5′-FAM-AAACGTCCAGCCCCAGCAATCTCTTG-TAMRA-3′
	Reverse	5′-AGGCCCAGATGACAACAGAAA-3′	
mTNF-α	Forward	5′-GTTCTCTTCAAGGGACAAGGCTG-3′	5′-FAM-TACGTGCTCCTCACCCACACCGTCA-TAMRA-3′
	Reverse	5′-TCCTGGTATGAGATAGCAAATCGG-3′	
mIL-1β	Forward	5′-GAAAGACGGCACACCCACC-3′	5′-FAM-TGCAGCTGGAGAGTGTGGATCCCAA-TAMRA-3′
	Reverse	5′-AGACAAACCGCTTTTCCATCTTC-3′	
mIL-6	Forward	5′-GGGACTGATGCTGGTGACAA-3′	5′-FAM-TCACAGAGGATACCACTCCCAACAGACCTG-TAMRA-3′
	Reverse	5′-TGCCATTGCACAACTCTTTTCT-3′	

r, rat; m, mouse; CB2R, cannabinoid receptor 2; IRF, interferon regulatory factor; P2X4R, P2X purinoceptor 4; P2Y12R, P2Y12 receptor; TNF, tumor necrosis factor; IL, interleukin.

alone were cloned into the lentiviral CS2-EF-MCS vector (RIKEN, Saitama, Japan). Each vector with pCAG-HIVgp (packaging plasmid; RIKEN) and pCMV-VSV-G-RSV-Rev (RIKEN) was co-transfected into HEK293T cells. After mixing with polyethylene glycol, viral particles and polybrene were added onto BV-2 microglial cells plated on 24-well plates. After a 12-h treatment with the lentivirus, culture medium was changed to new medium, and cells were cultured further for 60 h. For the gene expression experiments, the transduced cells were subjected to total RNA extraction as described above.

cAMP Assay

BV-2 cells were seeded in 24-well plates. After treatment with reagents, the intracellular concentration of cAMP was estimated using an Amersham cAMP Biotrak Enzymeimmunoassay system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol.

Statistical Analysis

All data were presented as the mean ± SEM. The statistical significance of differences between the values was evaluated by the Student's *t*-test, one-way analysis of variance (ANOVA) with the Dunnett's *post hoc* test, or two-way ANOVA with the Bonferroni's *post hoc* test. Differences were considered significant when the p-value was <0.05.

RESULTS

Primary Site of Action of JWH133 on Tactile Allodynia Induced by PNI

Consistent with our previous studies [24], the PWT of the ipsilateral hindpaw to PNI in rats markedly decreased. The peak of threshold decrease was observed on days 7 or 14 after PNI (data not shown). On day 7, we intraperitoneally administered JWH133 (3 mg/kg), a selective CB_2R agonist, and found that JWH133 remarkably alleviated tactile allodynia (Fig. **1A**). The effect of JWH133 occurred in a dose-dependent manner (Fig. **1B**). To determine the site of action of the JWH133-induced antiallodynic effect, we intrathecally administered the selective CB_2R antagonist AM630 (20 nmol) prior to intraperitoneal (i.p.) administration of JWH133. AM630 completely prevented the antiallodynic effect of JWH133 (Fig. **1C**). In contrast, the JWH133 (i.p.)-induced antiallodynic effect was not affected by intraplantar pretreatment with AM630 (Fig. **1C**). Furthermore, intrathecal administration of JWH133 produced an antiallodynic effect in a dose-dependent manner (Fig. **1D**, **E**), and the effect was completely abolished by intrathecal pretreatment with AM630 (Fig. **1D**). These results indicated that CB_2Rs in the spinal cord are the primary site of action for the suppressive effect of JWH133 administered systemically for treatment of PNI-induced tactile allodynia.

Involvement of the $G_{i/o}$ Signaling Pathway in Suppressing Tactile Allodynia *via* CB_2Rs

We investigated the intracellular signaling pathway involved in CB_2R-mediated mitigation of tactile allodynia. Because CB_2Rs are coupled with $G_{i/o}$-proteins, we examined the effect of the $G_{i/o}$-protein inhibitor PTx. Rats subjected to PNI were injected intrathecally with PTx (0.5 µg) and JWH133 (10 nmol). As shown in Fig. (**2A**), the ability of JWH133 to improve tactile allodynia was almost completely inhibited by PTx (Fig. **2A**). There was no change in PWT by

intrathecal administration of PTx alone (data not shown). Next, we tested the effect of forskolin, an activator of adenylate cyclase (AC), a downstream enzyme of CB_2R-$G_{i/o}$ signaling, because JWH133 suppresses the activation of AC *via* CB_2R-$G_{i/o}$ signaling. By administering forskolin (10 µg) intrathecally with JWH133 to PNI rats, the antiallodynic action of JWH133 was significantly inhibited (Fig. **2B**). These results suggest that JWH133 exerts an allodynia-mitigating effect through the CB_2R-$G_{i/o}$-AC pathway.

significantly increased in the L5 segment of the ipsilateral spinal cord of PNI rats compared with that in the spinal cord of naïve rats and the contralateral spinal cord after PNI (days 3, 7, and 14; Fig. **3A**). In contrast, there was no change in the mRNA expression of CB_2R in the spinal cord (L5) of rats administered CFA into the hindpaw, a model of chronic inflammatory pain (Fig. **3B**). These results suggest that spinal CB_2R expression is upregulated by PNI but not by peripheral tissue inflammation.
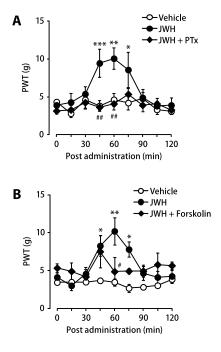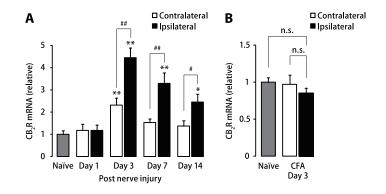

Fig. (1). Stimulation of spinal CB_2Rs alleviates tactile allodynia caused by PNI. Drug administration and measurements of the PWT of hindpaws were performed at day 7 after PNI. (**A**) Rats were subjected to intraperitoneal administration of vehicle or JWH133 (3 mg/kg), a selective CB_2R agonist (n=3–9; *p<0.05, ***p<0.001 *vs.* vehicle). (**C**) Intrathecal or intraplantar administration of 20 nmol AM630 in conjunction with intraperitoneal administration of 3 mg/kg of JWH133 (n=3–4; **p<0.01, ***p<0.001 *vs.* JWH). (**D**) Vehicle or JWH133 (10 nmol) was administered intrathecally and AM630 (20 nmol), a selective CB_2R antagonist, was administered intrathecally 15 min before JWH133 administration (n=5–11; *p<0.05, ***p<0.001 *vs.* vehicle; ###p<0.001 *vs.* JWH). (**B, E**) PWT was estimated 105 min after intraperitoneal administration (**B**; n=3–9) or 60 min after intrathecal administration (**E**; n=5–11) of JWH133 (***p <0.001 *vs.* vehicle).

Fig. (2). $G_{i/o}$ signaling is involved in the improvement of tactile allodynia by spinal CB_2R activation. Measurements of the PWT of the hindpaw were performed at day 7 after PNI. (**A**) Pertussis toxin (0.5 µg), a $G_{i/o}$ inhibitor, was injected intrathecally 1 day after PNI, and JWH133 (10 nmol) was administered intrathecally 7 days after PNI (n=3–4; *p<0.05, **p<0.01, ***p<0.001 *vs.* vehicle; ##p<0.01 *vs.* JWH133). (**B**) Forskolin (10 µg), an activator of adenylate cyclase, was administered intrathecally 15 min before JWH133 (10 nmol) (n=3–5; *p<0.05, **p<0.01 *vs.* vehicle; #p<0.05, ##p<0.01 *vs.* JWH133).

Changes in CB_2R Expression in the Spinal Cord after PNI

Our findings imply a contribution of spinal CB_2Rs in the analgesic effect. The expression of CB_2R mRNA was

Effect of Chronic Administration of JWH133 During the Early and Late Phase in the Development and Maintenance of Allodynia

To further test the antiallodynic effect of the CB_2R agonist in the development and maintenance of neuropathic pain, we intrathecally administered JWH133 (10 nmol) twice a day (10:00 and 20:00) during the development phase of neuropathic pain (from day 0 to 7) and PWT was measured on days 0, 1, 3, and 7 post-PNI before intrathecal injection of JWH133. Compared with the vehicle-treated group, PWT was significantly attenuated in the JWH133-treated group after PNI (Fig. **4A**). In contrast, PWT in the contralateral side was not affected by JWH133. To examine the effect on the maintenance phase, JWH133 (10 nmol) was administered intrathecally twice a day from day 7 to 14 post-PNI. JWH133-treated rats also showed a tendency for increased PWT although this was not statistically significant (Fig. **4B**).

Fig. (3). Expression of spinal CB$_2$R increases specifically with neuropathic pain. CB$_2$ mRNA in the L5 spinal cord from naïve rats and on day 1, 3, 7, and 14 from PNI-treated neuropathic rats (**A**; n=5–6) or day 3 from CFA-injected inflammatory rats (**B**; n=3) were measured by quantitative real-time RT-PCR (*p<0.05, **p<0.01 *vs.* naïve; [#]p<0.05, [##]p<0.01 *vs.* contralateral).
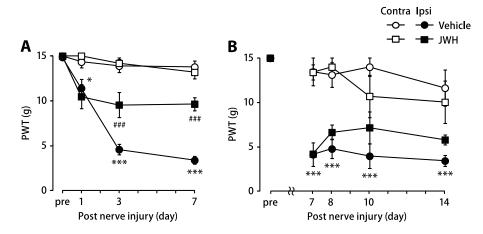

Fig. (4). Development of neuropathic pain after PNI is suppressed by chronic activation of spinal CB$_2$Rs. JWH133 (10 nmol) was administered intrathecally twice a day from day 0 to 7 (**A**; n=5–7) or from day 7 to 14 (**B**; n=3) after nerve injury. The PWT of the intact (contralateral) and injured sides (ipsilateral) were determined just before the first administration of each day (*p<0.05, ***p<0.001 *vs.* vehicle-contra; [###]p<0.001 *vs.* vehicle-ipsi).

Thus, the chronic administration of JWH133 produced a suppressive effect on the development of PNI-induced tactile allodynia and, to a lesser extent, on its maintenance.
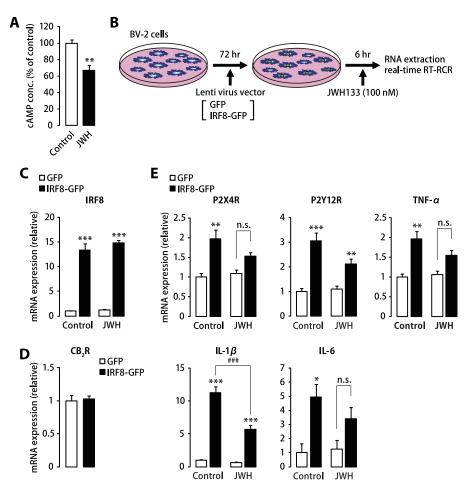
Effect of JWH133 on Gene Expression Induced by IRF8 in Cultured Microglial Cells

Because CB$_2$R in the spinal cord is expressed in activated microglia after PNI [21, 26, 27], we investigated the function of CB$_2$Rs on microglial activation *in vitro* using BV-2 cells, a well-known murine microglial cell line [28]. First, we performed a cAMP assay to confirm the expression of functional CB$_2$Rs in BV-2 cells and found that JWH133 (100 nM) significantly decreased cAMP concentrations (Fig. **5A**). We previously showed that the transcription factor IRF8 critically contributed to neuropathic pain through the expression of microglial genes encoding purinergic receptors and proinflammatory cytokines [22]. We thus examined whether JWH133 affected gene expression in microglia activated by overexpressing IRF8. We transduced BV-2 cells with a lentiviral vector encoding IRF8-GFP or a control vector encoding GFP alone, and examined the levels of gene transcripts in microglia by RT-PCR (Fig. **5B**). Compared with the control group (GFP), a marked increase of IRF8 expression was confirmed in the IRF8-GFP group (Fig. **5C**).

IRF8 expression was not changed by treatment with JWH133. The levels of CB$_2$Rs were indistinguishable between GFP-control and IRF8-GFP-transduced BV-2 cells (Fig. **5D**). Consistent with our previous data, expression of mRNA encoding the purinergic receptors P2X4R and P2Y12R, and the proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 increased (Fig. **5E**). The IRF8-induced upregulation of IL-1β expression was significantly decreased following treatment with JWH133 (Fig. **5E**). Furthermore, the upregulation of P2X4R, TNF-α, and IL-6 mRNA tended to be reduced by JWH133 (Fig. **5C**).

DISCUSSION

In the present study, we investigated the molecular and pharmacological action by which the CB$_2$R agonist JWH133 suppressed neuropathic pain. Behavioral data showed that the reversal effect of JWH133 administered systemically on PNI-induced allodynia was blocked by intrathecal pretreatment with AM630, a CB$_2$R-selective antagonist. This indicated that spinal CB$_2$Rs are the primary site of action for CB$_2$R agonist treatment of neuropathic pain. Indeed, activating CB$_2$Rs directly by intrathecal administration of JWH133 produced a profound antiallodynic effect,

Fig. (5). The CB_2R signal regulates the expression of pain-related molecules and suppresses inflammatory responses. (**A**) The concentration of cAMP was estimated 30 min after treatment with JWH133 (100 nM) on BV-2 cells (n=4; **p<0.01 *vs.* control). (**B**) Scheme of gene transfer experiments. Activated microglial cell cultures were prepared by overexpression of GFP-labeled IRF8 (IRF8-GFP) using lentiviral vectors. Changes in the expression of pain-related molecules were analyzed after JWH133 treatment. (**C-D**) After gene transduction with lentiviral vectors, the expression changes of IRF8 (**C**) and CB2R (**D**) mRNA were confirmed by real-time RT-PCR (n=4; ***p<0.001 *vs.* GFP). E. The expression changes of P2X4R, P2Y12R, TNF-α, IL-1β, IL-6, and IL-10 mRNA were analyzed by real-time RT-PCR after JWH133 (100 nM, 6h) treatment (n=4; ***p<0.001 *vs.* GFP; ###p<0.001 *vs.* control).

consistent with previous reports [18, 26]. A previous study in normal rats reported that activating CB_2Rs in keratinocytes induced the release of β-endorphin and that an antinociceptive effect on noxious heat stimuli by the CB_2R agonist AM1241 administered intraperitoneally was prevented by naloxone treatment in the hindpaw [17]. However, our study showed that intraplantar pretreatment with AM630 had no effect on the antiallodynic action of JWH133 given systemically in neuropathic rats. This suggested a minor role of CB_2Rs in the skin in suppressing allodynia by JWH133 during neuropathic pain states. Interestingly, the level of CB_2R expression in the spinal cord was very low under normal conditions [21], but it was upregulated after PNI, and was observed for at least 2 weeks after PNI. The change in CB_2R expression in the spinal cord appears to parallel the behavioral effect of JWH133 in neuropathic rats because its effect on PWT was observed in the ipsilateral, but not the contralateral side. Furthermore, consistent with previous studies [21, 29], the expression of CB_2R mRNA in the spinal cord was not increased following peripheral tissue inflammation caused by intraplantar injection of CFA. When CB_2R agonists are

administered intrathecally, they do not show profound antinociceptive effects in CFA-induced inflammatory pain [29]. Thus, it is assumed that upregulation of CB_2R expression may be involved in producing an efficient cannabinoid action during neuropathic pain states and strongly supports our view that following PNI, the primary site of action of CB2R agonist is the spinal cord where they modulate agonist pain.

In addition to the acute reversal effect, we demonstrated that repeated intrathecal administration of JWH133 prevented the development of tactile allodynia after PNI. It seems likely that this preventive effect is not simply due to the residual acute antiallodynic effect of JWH133 because each behavioral measurement was performed when the effect of JWH133 on PWT was no longer observed (Fig. **1D**). However, when JWH133 was administered intrathecally from day 7 post-PNI, the CB_2R agonist produced a slight increase in PWT. The reduction in JWH133 efficacy may be associated with the upregulation of CB_2R expression during the maintenance phase. Together, our results suggest that CB_2R agonists induce a potent reversal effect on established

tactile allodynia and prevent the development of tactile allodynia after PNI.

The ability to suppress PNI-induced allodynia by stimulating CB_2Rs suggests that CB_2Rs upregulated in the spinal cord may function as endogenous suppressors of neuropathic pain. However, intrathecal administration of CB_2R antagonists did not exacerbate PNI-induced allodynia. It was also shown that inhibitors of fatty acid amide hydrolase and monoacylglycerol lipase enzymes, involved in the metabolism of endocannabinoids, attenuate neuropathic pain [30, 31]. Therefore, the levels of endocannabinoids that bind CB_2Rs may not be sufficient to suppress PNI-induced neuropathic pain even though CB_2R expression is upregulated. Thus, enhancing CB_2R activity in the spinal cord by administering exogenous CB_2R agonists might be an effective way to relieve neuropathic pain. As pharmacological stimulation of CB_2Rs in the spinal cord did not affect the mechanical threshold in the contralateral side, a predicted therapeutic benefit of spinal CB_2R activation is that normal pain sensitivity might be unaffected.

Several lines of evidence indicate that glial cells are the predominant cell type expressing CB_2Rs in the spinal cord [21, 26, 27]. The time course of increased CB_2R expression is similar to that of microglial activation, and the upregulation of CB_2Rs also occurs in other diseases associated with microglial activation [32, 33]. It was reported that microglial activation in the spinal cord after PNI is suppressed by administration of CB_2R agonists and in mice overexpressing CB_2Rs [26, 34]. Furthermore, CB_2R agonists decrease the level of the proinflammatory cytokine IL-1β in the spinal cord [27]. These findings suggest that glial cells, especially microglia, are the target cells involved in suppressing PNI-induced tactile allodynia by CB_2R agonists. However, the underlying mechanisms remain to be fully determined. We recently demonstrated that the transcription factor IRF8 is a critical regulator for the reactive state of microglia after PNI and for neuropathic pain. In the present study, we demonstrated that JWH133 reduced the expression of IL-1β and also showed a tendency to decrease P2X4R, a purinergic receptor, and other proinflammatory cytokines (TNF-α and IL-6). P2X4R is a member of the ionotropic purinergic receptor [35] and is expressed in activated microglia after PNI [24] and other neurodegenerative diseases [36]. Importantly, P2X4Rs are essential for the pathogenesis of neuropathic pain [6, 24, 37]. Furthermore, IL-1β, IL-6, and TNF-α can enhance neuronal excitability in the spinal cord and contribute to neuropathic pain [1, 4, 38]. Although the present study did not examine the expression of these molecules at the protein level, it is speculated that activating spinal CB_2Rs reduces the inflammatory responses derived from reactive microglia following PNI, which may be involved in suppressing the development of neuropathic pain. However, it seems unlikely that the acute reversal effect of JWH133 on established tactile allodynia is similarly explained by its effect on IRF8-induced gene expression because the antiallodynic effect of intrathecal administration of JWH133 was already observed 30 min after the injection. As the reversal of PNI-induced allodynia was cancelled by either the $G_{i/o}$ inhibitor PTx or AC activator forskolin, it implies the involvement of the CB_2R-$G_{i/o}$-AC signaling pathway. As there is increasing evidence that certain types of cannabinoid

ligands also influence activity of T-type calcium channels at primary afferent sensory neurons *via* both CB_2R-dependent and independent manners [39-41], it is possible that these effects may be involved in the reversal of PNI-induced allodynia by JWH133. This possibility should be investigated in future studies.

CONCLUSION

We demonstrated that CB_2R in the spinal cord is upregulated after PNI and is the primary site of action for CB_2R agonists administered systemically to improve established neuropathic pain through the $G_{i/o}$-AC signaling pathway. Activating spinal CB_2Rs also suppressed the development of neuropathic pain. Furthermore, treatment with the CB_2R agonist reduced the inflammatory response of microglia activated by overexpressing IRF8. Thus, spinal CB_2R may be a therapeutic target to suppress the development and maintenance of neuropathic pain.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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PATIENT'S CONSENT

Declared none.

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