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Involvement of central cannabinoid CB_2 receptor in reducing mechanical allodynia in a mouse model of neuropathic pain

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Abstract

We sought to examine the involvement of central cannabinoid CB_2 receptor activation in modulating mechanical allodynia in a mouse model of neuropathic pain. JWH133 was demonstrated to be a selective cannabinoid CB_2 receptor agonist in mice, reducing forskolin-stimulated cAMP production in CHO cells expressing mouse cannabinoid CB_2 and cannabinoid CB_1 receptors with EC_{50} values of 63 nM and 2500 nM, respectively. Intrathecal administration of JWH133 (50 and 100 nmol/mouse) significantly reversed partial sciatic nerve ligation-induced mechanical allodynia in mice at 0.5 h after administration. In contrast, systemic (intraperitoneal) or local (injected to the dorsal surface of the hindpaw) administration of JWH133 (100 nmol/mouse) was ineffective. Furthermore, the analgesic effects of intrathecal JWH133 (100 nmol/mouse) were absent in cannabinoid CB_2 receptor knockout mice. These results suggest that the activation of central, but not peripheral, cannabinoid CB_2 receptors play an important role in reducing mechanical allodynia in a mouse model of neuropathic pain.

Keywords: Cannabinoid CB_2 receptor; JWH133; Neuropathic pain; Allodynia; Central nervous system

1. Introduction

Two cannabinoid receptors, cannabinoid CB_1 and cannabinoid CB_2 receptors, have been cloned and characterized (for review, see Walker and Hohmann, 2005). Cannabinoid CB_1 receptors are abundant in the central nervous system (CNS) while cannabinoid CB_2 receptors are predominantly associated with the immune system (Herkenham et al., 1990, Galiegue et al., 1995). Cannabinoid CB_1 receptors are present at sites involved in pain processing, such as primary afferent fibers, spinal cord, thalamus, periaqueductal grey and rostral ventromedial medulla (Tsou et al., 1998). Activation of the cannabinoid CB_1 receptors induces antinociception (Scott et al., 2004, Yoon and Choi, 2003), however, it also produces many neurological effects that limit their therapeutic use (Scott et al., 2004, Sañudo-Peña et al., 2000).

Cannabinoid CB_2 receptor agonists have been also known to produce antinociception without overt behavioral effects in neuropathic, inflammatory, postoperative, and acute pain models (Malan et al., 2001, 2003, Quartilho et al., 2003 Sañudo-Peña et al., 2000, Valenzano et al., 2005). These studies assumed that the primary site of action is peripheral structures, mostly immune cells. However, the role of cannabinoid CB_2 receptor agonists in the CNS has not been well understood in connection to neuropathic pain. Increasing evidence suggests that cannabinoid CB_2 receptor activation in the CNS may also contribute to the analgesic effects of cannabinoids. The cannabinoid CB_2 receptor mRNA and protein are expressed in rat lumbar (L3–L4) spinal cord, though specific cell types were not determined (Beltramo et al., 2006, Walczak et al., 2005). Application of cannabinoid CB_2 receptor agonists inhibits capsaicin-mediated calcitonin gene-related peptide release in rat spinal cord slices (Beltramo et al., 2006) and KCl-stimulated increase in intracellular calcium in rat dorsal root ganglion neurons (Sagar et al., 2005). Recently, Romero-Sandoval and Eisenach (2007) reported that the intrathecally administered cannabinoid CB_2 receptor agonist, JWH015, reduced hypersensitivity in a rat postoperative pain model.

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JWH133 has been reported as being a cannabinoid CB_2 receptor-selective agonist with a K_i value against human cannabinoid CB_2 receptors of 3–20 nM (Huffman et al., 1999, Stern et al., 2006) and against whole brain cannabinoid CB_1 receptors of 670 nM (Huffman et al., 1999). Systemic administration of JWH-133 significantly reduced ipsilateral hind paw weight bearing deficit and paw volume in a rat carrageenan-induced inflammatory pain model (Elmes et al., 2005). These effects were abolished by the cannabinoid CB_2 receptor-selective antagonist, SR144528, suggesting that the effect of JWH133 was cannabinoid CB_2 receptor-mediated.

We sought to examine the involvement of central cannabinoid CB_2 receptor activation in modulating mechanical allodynia in a mouse model of neuropathic pain induced by partial sciatic nerve ligation using JWH133. We first evaluated the *in vitro* agonist activity and selectivity of JWH133 for mouse cannabinoid CB_2 and cannabinoid CB_1 receptors. We next examined and compared the effects of JWH133 following intrathecal (i.t.), systemic (intraperitoneal; i.p.) and local (injected to the dorsal surface of the hindpaw; i.pw.) administration on partial sciatic nerve ligation-induced mechanical allodynia to determine a target site of action of cannabinoid CB_2 receptor agonist. Finally, we used cannabinoid CB_2 receptor knockout (KO) mice to confirm that the effect of JWH133 was mediated by the cannabinoid CB_2 receptor.

2. Materials and methods

2.1. Reagents

Selective cannabinoid CB_2 ligands JWH133 (3-[1.1-Diethylbutyl]-1deoxy-Δ8-tetrahydrocannabinol) and AM1241 ((R,S)-3-(2-Iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole) were purchased from Sigma (St. Louis, Mo., USA) and synthesized by Pfizer Global Research and Development Nagoya Laboratories, respectively. Non-selective cannabinoid agonists WIN55212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) and CP55940 ((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol) were synthesized by Pfizer Global Research and Development Nagoya Laboratories. The test compounds were dissolved in 100% dimethylsulfoxide (DMSO), and were prepared as DMSO solutions at a final concentration of 10 mM for the *in vitro* experiments and either 10 mM or 20 mM for the *in vivo* experiments. For i.t. administration, the drugs were injected into the subarachnoid space through the intervertebral foramen between L5 and L6 according to the method described by Hylden and Wilcox (1980). For i.pw. administration, the drugs were injected into the dorsal surface of the right hindpaw using a 29-gauge needle. For systemic administration, the JWH133 was injected intraperitoneally in mice. The injection volume was 5 μl for i.t. and i.pw., and 10 μl for i.p.

2.2. Construction of expression vectors

Total RNA was isolated from mouse brain using RNeasy® Mini kit (QIAGEN, Valencia, CA). The poly(A)$^+$ RNA was extracted with oligo(dt)$_{12-18}$ primer (Invitrogen, Carlsbad, CA) and reverse transcribed by SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA). Mouse cannabinoid CB_1 cDNA was generated by amplifying mouse brain cDNA using the following designed primers: 5'-ACCATGAAGTCGATCTTAGACGGC-3' (sense primer) 5'-TCACAGAGCCTCGGCAGA-3' (antisense primer) with the PCR conditions: 96 °C for 5 min, followed by 30 cycles of denaturation (96 °C for 30 s), annealing (60 °C for 30 s), and elongation (72 °C for 1 min) using Taq DNA polymerase. Mouse cannabinoid CB_2 cDNA was generated by amplifying mouse brain cDNA using the following designed primers: 5'-GCCACCATGGAGGGATGCCGGGAGAC-3' (sense primer) and 5'-TCCAGCCAACCAGCATTCC-3' (antisense primer) with the PCR conditions: 94 °C for 2 min, followed by 33 cycles of denaturation (94 °C for 15 s), annealing (53 °C for 30 s), and elongation (68 °C for 2 min). PCR products were ligated into the pcDNA2.1-TOPO vector using TOPO™ TA cloning kit (Invitrogen, Carlsbad, CA). Nucleotide sequences were verified by TOYOBO Gene Analysis. The cDNAs for mouse cannabinoid CB_1 and cannabinoid CB_2 were then ligated into the pcDNA3.1(+) vector.

2.3. Cell culture and transfection

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. mCB_1/pcDNA3.1(+) and m CB_2/pcDNA3.1(+) were initially transfected into CHO cells using FuGENE6 (Roche). Stably transfected cells were established by selection of transfected cells with Geneticin. Approximately 100 clones were isolated and expanded. Ten robust clones were subjected to the cAMP assay to see if they responded to WIN55212-2 and CP55940 that inhibit cAMP production by forskolin stimulation. Transfected cell lines were maintained in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, 20 mM HEPES, 500 μg/ml geneticin, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C.

2.4. cAMP assay

CHO cells expressing cannabinoid receptors were harvested and plated at a density of 5×10^3 cells per well in a 96-well culture plate. After an 18-h incubation in 5% CO_2 at 37 °C, cells were washed with phosphate-buffered saline (−) and incubated in 5% CO_2 at 37 °C for 15 min in Ham's F-12 medium containing 20 mM HEPES, 1 mM 3-isobutyl-1-methylxanthine and 1 mg/ml bovine serum albumin (BSA) in the presence or absence of test compounds. Cells were then incubated in 5% CO_2 at 37 °C for 15 min with 1 μM and 10 μM forskolin for mouse cannabinoid CB_1 and cannabinoid CB_2 receptors, respectively. After the forskolin incubation, 1% Triton X-100 was added into each well to stop the reaction. The wells were agitated for 60 min at room temperature. cAMP concentration in the mixture of each well was measured by the HTRF-cAMP dynamic kit (CisBio International, Bedford, MA).

2.5. Animals

Male ddY mice (14–16 g) were obtained from Japan SLC Inc. (Hamamatsu, Japan). Female cannabinoid CB_2 receptor KO mice (18–24 g) in a C57BL/6 background and their age-matched wild-type littermates were produced, and breeding and genotyping were performed as described by Buckley et al. (2000). The animals were acclimatized to the laboratory conditions during experiments. The experiments were carried out according to a protocol approved by the animal ethics committee at the Nagoya Laboratories of Pfizer Global Research and Development.

2.6. Partial sciatic nerve ligation

The mice were anesthetized with isoflurane in oxygen during surgery. Right sciatic nerve was exposed and ligated with 9–0 silk suture around approximately one-third to one-half of the nerve diameter under light microscope as described previously (Malmberg and Basbaum, 1998). In sham-operated mice, the nerve was exposed without ligation.

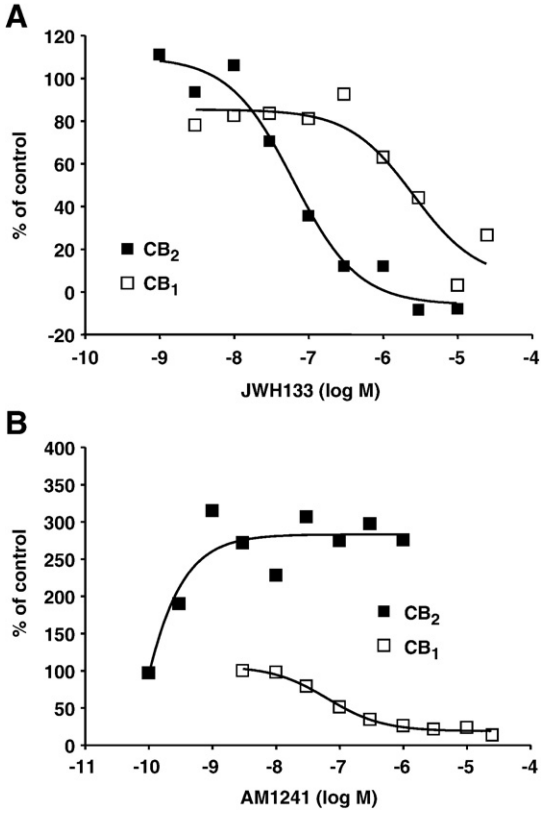

Fig. 1. The ability of JWH133 (A) and AM1241 (B) to activate mouse cannabinoid CB_2 and cannabinoid CB_1 receptors in a functional cAMP assay using CHO cells expressing recombinant mouse cannabinoid CB_2 and cannabinoid CB_1 receptors. The assay was performed in duplicate, and the data are expressed as the mean values. The forskolin-stimulated cAMP production (pmol/ml) in the absence and presence of Win55212-2 was 40.4 and 25.0 in mouse cannabinoid CB_2 receptors; 34.5 and 17.8 in mouse cannabinoid CB_1 receptors, respectively ($n=2$).

2.7. Measurement of mechanical allodynia

The effect of the drugs on mechanical allodynia was measured two weeks after the surgery. Mechanical allodynia was detected as described previously (Tal and Bennett, 1994) with minor modifications. Briefly, the animals were habituated to mesh bottom cages prior to the experiment. Mechanical allodynia was evaluated by the application of von Frey hairs (VFHs) (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0 and 1.4 g, Semmes–Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA) in ascending order of force to the plantar surface of the hind paw. Each VFH was applied until a withdrawal response was observed with ten times at maximum at intervals of 1–4 s. Once the withdrawal response was observed, the paw was re-tested with the same VFH to confirm the response. The lowest amount of force required to elicit a response was recorded as the paw withdrawal threshold (PWT, g). Mechanical allodynia was defined when animals responded to 0.04 g VFH or below, which was innocuous to normal or sham-operated mice. All the VFH measurements were performed in a blinded manner.

2.8. Analysis of data

The data relating to mechanical allodynia were expressed as the median force (g) with vertical bars in the 1st and 3rd quartiles and were subjected to a Kruskal–Wallis test followed by an individual Dunn's test (or just an individual Mann–Whitney *U*-test where there were only two groups). p values less than 0.05 were regarded as significant. All calculations were performed by the exact test using SigmaPlot ver. 3.1 (San Jose, CA, USA).

3. Results

3.1. cAMP assay

The ability of JWH133 to activate mouse cannabinoid CB_2 and cannabinoid CB_1 receptors was assessed in a functional cAMP assay using CHO cells expressing recombinant mouse cannabinoid receptors. In mouse cannabinoid CB_2 receptors, JWH133 showed a concentration-dependent inhibition of forskolin-induced cAMP accumulation with an EC_{50} value of 63 nM and a maximum inhibition of 106% (Fig. 1A). In mouse cannabinoid CB_1 receptors, JWH133 demonstrated a weak potency with an EC_{50} value of 2500 nM and a maximum inhibition of 94% (Fig. 1A). JWH133 exhibited a 40-fold selectivity for mouse cannabinoid CB_2 receptors over mouse cannabinoid CB_1 receptors.

In contrast to JWH133, AM1241 behaved as an inverse agonist in mouse cannabinoid CB_2 receptors, increasing forskolin-induced cAMP levels by 76% (Fig. 1B), whereas it showed agonist activity on mouse cannabinoid CB_1 receptors with an EC_{50} value of 64 nM and a maximum inhibition of 81% (Fig. 1B).

Both reference compounds, WIN55212-2 and CP-55940, exhibited agonist activities on cannabinoid CB_2 and cannabinoid CB_1 receptors with EC_{50} values of 0.7 nM and 0.2 nM for cannabinoid CB_2 receptors, and 7.3 nM and 0.51 nM for cannabinoid CB_1 receptors, respectively.

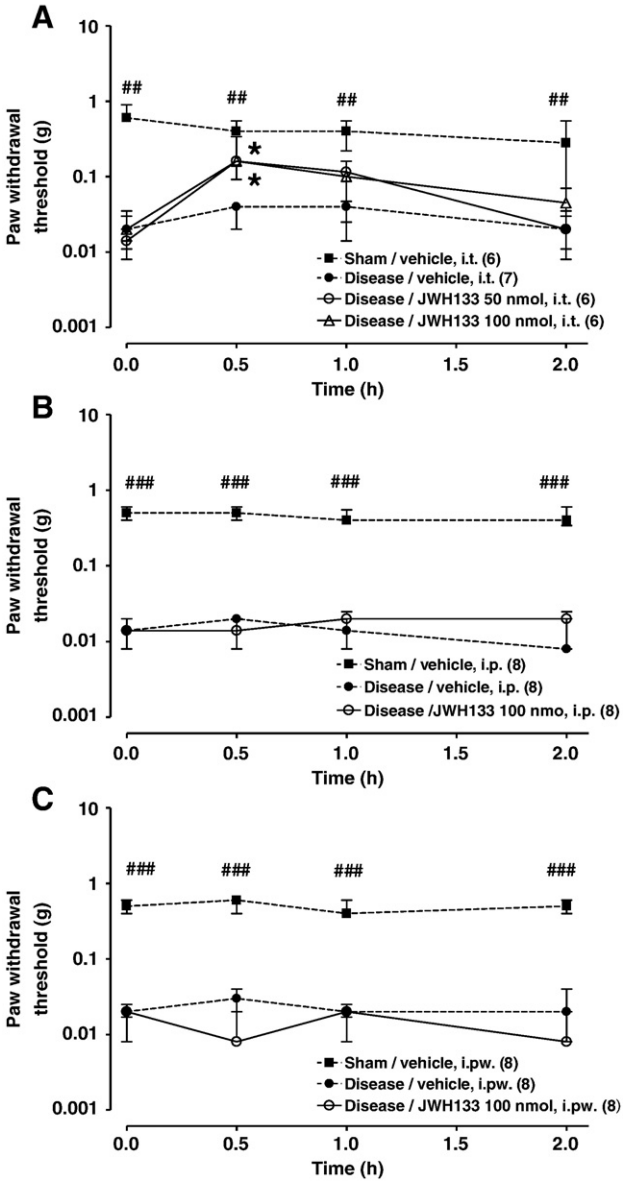

Fig. 2. Intrathecal (i.t.; A), but not systemic (i.p.; B) and local (i.pw.; C) administration of JWH133 reduced partial sciatic nerve ligation-induced mechanical allodynia in mice (disease). The paw withdrawal threshold to von Frey hairs was determined in sham and disease (partial sciatic nerve ligation) mice before and 0.5, 1, 2 h after administration. (A) For i.t. administration, drugs were injected into the subarachnoid space through the intervertebral foramen between L5 and L6. (B) For systemic administration, drugs were injected intraperitoneally (i.p.). (C) For local administration, drugs were injected into the dorsal surface of the left hind paw (i.pw.). Data are expressed as the median force (g) required to induce paw withdrawal in 6–8 animals per group (parenthesis indicate the number of animals used), and vertical bars represent 1st and 3rd quartiles. ## $p<0.01$, ### $p<0.001$ (Mann–Whitney *U*-test) compared with disease/vehicle group. *$p<0.05$ (Kruskal–Wallis test followed by Dunn's test) compared with disease/vehicle group.

3.2. Anti-allodynic effect of JWH133 in a mouse model of neuropathic pain

A total of 70 mice underwent partial sciatic nerve ligation. Two weeks after the surgery, approximately 60 mice showed mechanical allodynia (100% response to 0.04 g or lower VFH) and these mice were divided into subgroups for drug evaluation. Each subgroup consisted of 6–8 mice. The median PWT of each subgroup was 0.02–0.04 g in partial sciatic nerve ligation mice in contrast to 0.4–1.0 in sham-operated mice.

I.t. administration of JWH133 (50 and 100 nmol/mouse) significantly reversed mechanical allodynia compared with the vehicle group at 0.5 h after administration (Fig. 2A), whereas JWH133 at 1 and 10 nmol/mouse did not show significant effects (data not shown). In contrast, systemic (i.p.) or local (i.pw.) administration of JWH133 (100 nmol/mouse) was ineffective in the partial sciatic nerve ligation mice (Fig. 2B and C). Vehicle (100% DMSO) injections with all routes did not affect pain thresholds and behaviors, and no behavioral abnormalities such as motor dysfunction were observed for all routes and doses during the 2-h observation period.

3.3. Effect of JWH133 in cannabinoid CB_2 receptor KO mice

Two weeks after the surgery, both wild-type and CB_2 KO mice showed mechanical allodynia and there were significant reductions in PWTs compared to pre-surgery in each group. In our previous experiments, no significant differences in PWTs between sham and normal mice in both wild-type and cannabinoid CB_2 receptor KO mice were observed (data not shown), therefore, development of mechanical allodynia was confirmed and compared to the pre-surgery PWTs. I.t. administration of JWH133 (100 nmol/mouse) significantly reversed mechanical allodynia in wild-type mice at 0.5 h and 1 h after administration. In contrast, the same dose of JWH133 did not show any significant anti-allodynic effect in cannabinoid CB_2 receptor KO mice (Fig. 3). No behavioral abnormalities such as motor dysfunction were observed during the 2-h observation period.

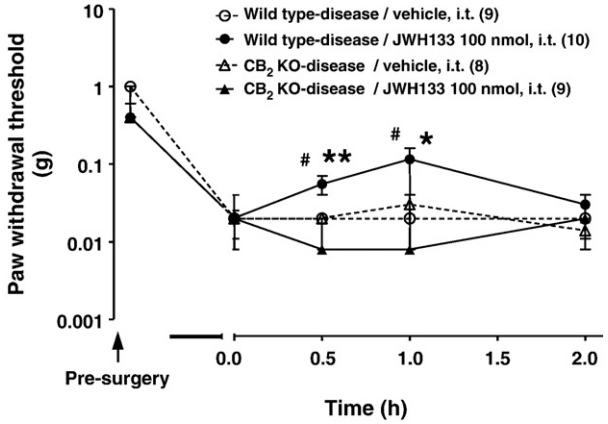

Fig. 3. The efficacy of i.t. administration of JWH133 was abolished in cannabinoid CB_2 receptor knockout (KO) mice. The paw withdrawal threshold to von Frey hairs was determined pre-surgery, and before and 0.5, 1, 2 h after drug administration. Data are expressed as the median force (g) required to induce paw withdrawal in 8–10 animals per group (parenthesis indicate the number of animal used), and vertical bars represent 1st and 3rd quartiles. # $p<0.05$ (Mann–Whitney *U*-test) compared with each disease/vehicle group. *$p<0.05$, **$p<0.01$ (Mann–Whitney *U*-test) compared with CB_2 KO-disease/vehicle group.

4. Discussion

Cannabinoid CB_2 receptor agonists reduce hypersensitivity in neuropathic pain models (for review, see Whiteside et al., 2007) such as spinal nerve ligation and partial sciatic nerve ligation models. In this report we used a mouse model of neuropathic pain induced by partial sciatic nerve ligation. The reasons why we used this model and detected the mechanical allodynia instead of mechanical hyperalgesia were as follows: (1) preliminary experiments conducted in our laboratory demonstrated that pregabalin, the gold standard for the treatment of neuropathic pain, significantly suppressed mechanical allodynia in partial sciatic nerve ligation mice, (2) we could perform this study with consistent experimental conditions in terms of species (mouse) between the *in vitro* and *in vivo* assays, (3) we were able to utilize cannabinoid CB_2 receptor KO mice to confirm that JWH133 acts on cannabinoid CB_2 receptors *in vivo*. We typically used male ddY mice for drug evaluation as this strain shows passive movement, which helps in detecting pain behavior. On the other hand, male C57BL mice (the genetic background of the cannabinoid CB_2 knockout mouse) are more fidgety than the ddY strain. Although it was already observed in our laboratory that male C57BL mice can be used for the evaluation of paw withdrawal thresholds in this model, we chose to use female C57BL mice, which are expected to be more passive than males. In addition, we observed no difference in the withdrawal thresholds between males and females compared to our previous data (data not shown). Thus, gender difference did not seem to affect the PWTs in this experimental model.

AM1241 has been widely used as a cannabinoid CB_2 receptor-selective agonist *in vitro* and *in vivo* (Hohmann et al., 2004, Ibrahim et al., 2003, Malan et al., 2001, Nackley et al., 2003, 2004, Quartilho et al., 2003). However, Yao et al. (2006) reported that AM1241 is a protean agonist at the cannabinoid CB_2 receptor, which means AM1241 could exhibit distinct functional properties depending on the assay conditions employed. In fact, a recent report showed that AM1241 behaved as an inverse agonist in mouse cannabinoid CB_2 receptors (Bingham et al., 2007), and our result was consistent with this report. These *in vitro* data can complicate interpretations with regard to *in vivo* efficacies with this compound.

JWH133 has also been reported to be a selective cannabinoid CB_2 receptor agonist (Huffman et al., 1999, Stern et al., 2006). In the first set of experiments, we evaluated JWH133 *in vitro* using mouse cannabinoid CB_2 and cannabinoid CB_1 receptors, and confirmed that JWH133 is a selective cannabinoid CB_2 receptor agonist in our assay conditions.

Systemic, or local (peripheral) administration of cannabinoid CB_2 receptor agonists has been known to produce antinociception without overt behavioral effects in several pain models (Malan et al., 2001, 2003, Quartilho et al., 2003, Sañudo-Peña et al., 2000, Valenzano et al., 2005). Although these studies assumed a primary mechanism of action on peripheral structures, mostly immune cells, CNS actions of cannabinoid CB_2 receptor agonists cannot be ruled out. Recently, Romero-Sandoval and Eisenach (2007) reported that i.t.-administered cannabinoid CB_2 receptor agonist JWH015 reduced hypersensitivity in a rat postoperative pain model. However, the effects of i.t. administration of cannabinoid CB_2 receptor agonists in neuropathic pain models have not been studied yet. Therefore, in the second set of experiments, we performed a comparative study of intrathecal, systemic and local administration of JWH133 to determine a target site of action of cannabinoid CB_2 receptor agonists in a mouse partial sciatic nerve ligation model. JWH133 exhibited an anti-allodynic effect following i.t., but not systemic (i.p.) or local (i.pw.) administration. Our results from the local administration of JWH133 are contrary to those of Elmes et al. (2004). They showed that intraplantar injection of JWH133 inhibited mechanically evoked responses of wide dynamic range in dorsal horn neurons in a rat spinal nerve ligation and carrageenan-induced inflammation models. This discrepancy between Elmes et al. and our results in terms of the effect of intraplanter injection of JWH133 could be attributed to methodological differences, such as the nerve lesion models used, e.g., pro-inflammatory immune cell responses might not contribute to hypersensitivity in our neuropathic pain model, and the sensitivity of endpoint measurement, e.g., behavioral assessment by VFH application versus electrophysiological recording of the spinal wide dynamic range neurons. Taken together, these data suggest that central cannabinoid CB_2 receptor activation mediates analgesic effects in a neuropathic pain model.

In the third set of experiments, JWH133 was evaluated in cannabinoid CB_2 receptor KO mice by i.t. administration. There was a possibility that JWH133 acted on target molecules other than cannabinoid CB_2 receptors (such as cannabinoid CB_1 receptors) which might also be involved in pain modulation. Although selective cannabinoid CB_2 receptor antagonists such as SR144528 are useful tools, using cannabinoid CB_2 receptor KO mice can give us direct evidence of cannabinoid CB_2 receptor involvement *in vivo*. The data from the present study show that the effect of JWH133 was completely abolished in cannabinoid CB_2 receptor KO mice, indicating the important role of central cannabinoid CB_2 receptors in reducing mechanical allodynia in a mouse model of neuropathic pain.

As described earlier, central cannabinoid CB_2 receptors may play an important role in pain responses. It has been demonstrated that the expression of cannabinoid CB_2 receptors increased in the dorsal and ventral horn of the spinal cord in response to nerve damage (Zhang et al., 2003), possibly on microglia. There was a possibility that JWH133 acted on efferent neurons in ventral horn, which might cause motor dysfunction. However, no significant changes in locomotor behavior were observed during our experiments. Although we did not actually detect the expression of the cannabinoid CB_2 receptors in the spinal cord, the lack of significant changes in locomotor behavior during the experiments suggests little involvement of cannabinoid CB_2 receptors in the ventral horn in our behavioral assays.

It is not known how cannabinoid CB_2 receptor agonists inhibit pain at the spinal and/or supraspinal level. Mechanistically it is now known that blockade of either microglial function or the activity of glia-derived substances, such as cytokines and excitatory amino acids, results in the prevention and reversal of a diverse range of pain signals (for review, see Wieseler-Frank et al., 2004). These results imply that cannabinoid CB_2 receptor agonist-

mediated inhibition of activated microglia located within the spinal cord could reverse neuropathic pain. Furthermore, the spinal action of cannabinoid CB_2 receptor agonists is also supported by the findings of Sagar et al. (2005) and Beltramo et al. (2006). Taken together, these mechanism(s) may contribute to the cannabinoid CB_2 receptor-mediated anti-allodynic effect in partial sciatic nerve ligation mice. Our present data suggest the existence and the role of central cannabinoid CB_2 receptors in the neuropathic pain status.

In conclusion, we have provided evidence based on results from local and systemic administration of cannabinoid CB_2 agonists and from cannabinoid CB_2 KO mice that central cannabinoid CB_2 receptors are required for the anti-allodynic effects in partial sciatic nerve ligation-induced neuropathic pain in mice.

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