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# Activation of cannabinoid receptor 2 attenuates mechanical allodynia and neuroinflammatory responses in a chronic post-ischemic pain model of complex regional pain syndrome type I in rats

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# Abstract

Complex regional pain syndrome type 1 (CRPS-I) remains one of the most clinically challenging neuropathic pain syndromes and its mechanism has not been fully characterized. Cannabinoid receptor 2 (CB2) has emerged as a promising target for treating different neuropathic pain syndromes. In neuropathic pain models, activated microglia expressing CB2 receptors are seen in the spinal cord. Chemokine fractalkine receptor (CX3CR1) plays a substantial role in microglial activation and neuroinflammation. We hypothesized that a CB2 agonist could modulate neuroinflammation and neuropathic pain in an ischemia model of CRPS by regulating CB2 and CX3CR1 signaling. We used chronic post-ischemia pain (CPIP) as a model of CRPS-I. Rats in the CPIP group exhibited significant hyperemia and edema of the ischemic hindpaw and spontaneous pain behaviors (hindpaw shaking and lick-ing). Intraperitoneal administration of MDA7 (a selective CB2 agonist) attenuated mechanical allodynia induced by CPIP. MDA7 treatment was found to interfere with early events in the CRPS-I neuroinflammatory response by suppressing peripheral edema, spinal microglial activation and expression of CX3CR1 and CB2 receptors on the microglia in the spinal cord. MDA7 also mitigated the loss of intraepidermal nerve fibers induced by CPIP. Neuroprotective effects of MDA7 were blocked by a CB2 antagonist, AM630. Our findings suggest that MDA7, a novel CB2 agonist, may offer an innovative therapeutic approach for treating neuropathic symptoms and neuroinflammatory responses induced by CRPS-I in the setting of ischemia and reperfusion injury.

# Introduction

The management of complex regional pain syndrome (CRPS) is challenging and the outcomes are unsatisfactory because of lack of understanding of the underlying pathophysiological mechanism (s). CRPS is classified into type I (formerly called reflex sympathetic dystrophy) and type II (formerly termed causalgia) (Harden *et al.*, 2013). CRPS-I (de Mos *et al.*, 2007) usually occurs after fracture, soft tissue injury, or crush injury, without clinically verified nerve injury. The pathogenesis of CRPS-I is likely

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\*JX and YT contributed equally to this study. This work was conducted at Cleveland Clinic. multifactorial, including changes in both the peripheral and the central nervous system (CNS) (Bruehl, 2010; Barad *et al.*, 2014; Finch *et al.*, 2014; Tajerian *et al.*, 2014). The acute manifestations of CRPS-I are characterized by typical inflammatory signs such as increased skin temperature, edema, skin color changes, allodynia and hyperalgesia. This exaggerated inflammatory response plays an important role in the induction and development of CRPS-I (Bruehl, 2010).

In animal studies, peripheral inflammation/nerve injury induces microglial activation in the spinal cord (Svensson *et al.*, 2003; Tsuda *et al.*, 2003; Sun *et al.*, 2012). The activated microglia release proinflammatory mediators such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  which increase the excitability of nociceptive neurons in the spinal cord through the chemokine, cytokine and neuron-microglia

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interactions (Naguib *et al.*, 2012; Xu *et al.*, 2014). In nerve injury, the chemokine fractalkine is released from the primary neurons to act on its receptor CX3CR1, which is mainly expressed on microglia (Verge *et al.*, 2004; Lindia *et al.*, 2005). Fractalkine/CX3CR1 signaling appears to play a crucial role in mediating neuron-microglia interaction and microglial activation in the spinal cord during nociceptive transmission (Clark *et al.*, 2011; Mattison *et al.*, 2013). Expression of CX3CR1 in microglia is substantially upregulated following nerve injury in several neuropathic pain models (Lindia *et al.*, 2005; Zhuang *et al.*, 2007; Sun *et al.*, 2013), and impairment of spinal fractalkine/CX3CR1 signaling attenuates established neuropathic pain behaviors (Zhuang *et al.*, 2007; Staniland *et al.*, 2010). The role of fractalkine/CX3CR1 signaling and spinal microglial activation in the pathophysiology of CRPS-I remains unclear.

The endogenous cannabinoid system is a complex system consisting of two cannabinoid (CB) receptors (CB1 - expressed primarily in the brain and  $CB_2$  – expressed primarily in the peripheral immune system (Munro et al., 1993) and in the CNS (Van Sickle et al., 2005)), seven endogenous endocannabinoid ligands (arachidonic acid derivatives) including anandamide (N-arachidonoylethanolamine) and 2-arachidonoylglycerol (2-AG) (McPartland, 2004), and several proteins responsible for the regulation of endocannabinoid metabolic pathways, such as monoacylglycerol lipase and fatty acid amide hydrolase.(Ahn et al., 2008) CB1 and CB2 are seven transmembrane, G protein-coupled receptors, and they share 44% overall identity. Both receptors mediate inhibition of adenylyl cyclases and stimulation of mitogen-activated protein kinases (MAPK). The endocannabinoid 2-AG was found to act as a full agonist (whereas anandamide acts as a weak partial agonist) toward CB<sub>1</sub> and CB<sub>2</sub> receptors (Sugiura et al., 2000).

CB2 receptors play an important role in modulating central immune response in neuropathic pain syndromes (Naguib *et al.*, 2012). Peripheral inflammation or nerve injury induces a significant increase in CB2 receptor expression on the activated microglia in the spinal cord (Wotherspoon *et al.*, 2005; Cheng & Hitchcock, 2007) and CB2 agonists suppress mechanical allodynia by inhibiting microglial activation (Racz *et al.*, 2008; Romero-Sandoval *et al.*, 2008; Toth *et al.*, 2010; Naguib *et al.*, 2012). Our previous studies have demonstrated that the selective CB2 agonist, MDA7, effectively alleviates mechanical allodynia induced by nerve injury or chemotherapy in animals (Naguib *et al.*, 2008, 2012).

The role of CB1 agonists in treating different neuropathic pain syndrome has been debated (Naguib & Foss, 2015) and the evidence provided by animal studies are not conclusive (Scott *et al.*, 2004; Agarwal *et al.*, 2007; Khasabova *et al.*, 2012). Synthetic  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) analogs (e.g. Marinol<sup>®</sup>, Cesamet<sup>®</sup>) and medicinal cannabis preparations containing both  $\Delta^9$ -THC and cannabidiol (e.g. Sativex<sup>®</sup>, Cannador<sup>®</sup>) have been tried clinically in patients with neuropathic pain. However, these drugs in all trials failed to show efficacy compared with placebo, and their use resulted in a high incidence of psychotropic adverse effects (Attal *et al.*, 2004; Selvarajah *et al.*, 2010). In contrast, CB<sub>2</sub> agonists are neuroprotective and are emerging as treatments for neuropathic pain. Unlike CB1 agonists, CB2 agonists lack psychoactive side effects (Beltramo *et al.*, 2006; Yiangou *et al.*, 2006; Naguib *et al.*, 2008; Xu *et al.*, 2010).

The chronic post-ischemia pain (CPIP) animal model produces an inflammatory response and pain syndrome that resembles the CRPS-I in humans (Coderre *et al.*, 2004). In this study, we hypothesized that early activation of CB2 by selective agonist can blunt neuroinflammatory reaction and mechanical allodynia mediated by CPIP.

# Materials and methods

# Drugs

MDA7 ( $hCB_1$  Ki value > 10 1000 nm;  $hCB_2$  Ki value = 422 nm;  $rCB_1$  Ki value = 2565 nm;  $rCB_2$  Ki value = 238 nm;  $EC_{50}$  at  $hCB_1$  = not active,  $EC_{50}$  at  $hCB_2$  = 128 nm;  $EC_{50}$  at  $rCB_1$  = not active;  $EC_{50}$  at  $hCB_2$  = 67 nm) was synthesized as previously described (Naguib *et al.*, 2008; Diaz *et al.*, 2009) and was administered in a vehicle consisting of 25% *N*-methylpyrrolidone (International Specialty Products, Wayne, NJ, USA), 25% propylene glycol (The Dow Chemical Company, Midland, MI, USA), 10% Cremophor, EL (BASF, Florham Park, NJ, USA) and purified water (Baxter, Deerfield, IL, USA). The vehicle alone has no effect on pain threshold and spinal microglial activation (Naguib *et al.*, 2008, 2012). AM630 (a selective CB2 antagonist) was purchased from Tocris Bioscience (Bristol, UK) and was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma) (Final concentration > 95%).

# Animals

Adult male Sprague–Dawley rats weighing 300–320 g (9 to 10week old; Charles River Laboratory) were used in experimental procedures that were approved by the IACUC at the Cleveland Clinic Lerner College of Medicine. The animals were housed in a temperature-controlled room  $(23 \pm 1 \text{ °C})$  on an automatic 12-h light/dark cycle (06:00–18:00 h) and were fed and watered *ad libitum*.

#### Animal model

We used the CPIP as an animal model of CRPS-I (Coderre *et al.*, 2004). Rats were anesthetized with intraperitoneal (IP) administration of 60 mg/kg sodium pentobarbital. Additional doses of 20 mg/kg sodium pentobarbital were administered IP every 60 min during the 3 h ischemic period, depending on the response to anesthesia in each animal. A Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA, USA) with 7/32 inch internal diameter was placed just proximal to the ankle joint of the rat's right hind limb by sliding the O-ring off a 3-mL syringe (that was cut in half), after the hind paw was inserted into the syringe barrel as far as possible (Coderre *et al.*, 2004).The O-ring was then left on the limb for 3 h and cut before the emergence from anesthesia to allow reperfusion.

The animals were randomly assigned into four groups. The sham group received anesthesia and a cut O-ring was placed loosely around the ankle for 3 h. This cut O-ring did not occlude blood flow to the hind paw. The CPIP group received anesthesia and ischemia produced by a tight-fitting O-ring for 3 h. The O-rings were then cut to relieve the ischemia and allow for reperfusion of the hind paw at the conclusion of anesthesia. The CPIP + MDA7 group received anesthesia and IP injection of 15 mg/kg MDA7 (the injection volume was 1 mL/kg) 30 min prior to ischemia followed by daily administration of 15 mg/kg MDA7 IP for 14 days (post-ischemia). The CPIP + MDA7 + AM630 group received anesthesia and IP injection of 5 mg/kg MDA7 administration. The daily injection of AM630 and MDA7 was continued for 14 days. All injections were administered in the afternoon by a researcher who was not involved in the behavioral testing.

# Assessment of local inflammation and spontaneous pain after ischemia and reperfusion

Local inflammation was assessed by observing color discoloration of the hind limb (cyanosis during ischemia and hyperemia after reperfusion). Hind paw edema was assessed by quantifying mid-paw thickness and circumference in animals (n = 5 for CPIP group; n = 6 for sham, CPIP + MDA7, and CPIP + MDA7 + AM630 groups) before, 15 min, 24 h, 48 h and 72 h after reperfusion. Local inflammation was assessed in a separate experimental group in order to minimize the stress which could affect the behavioral testing. Mid-paw thickness was measured with an UltraTech digital caliper ( $\pm 0.01$  mm, General Tools and Instruments, Secaucus, NJ) as described previously (Chatterjea *et al.*, 2012; Hussein *et al.*, 2012). The mid-paw circumference was measured by wrapping a 4-0 surgical suture around the mid-paw. The suture was then cut and its length, representing the circumference, was measured with the UltraTech digital caliper.

Spontaneous pain after ischemia was assessed by quantifying the accumulated licking time on the ischemic side paw. Sham (n = 6), CPIP (n = 6), MDA7 + CPIP (n = 5) and AM630 + MDA7 + CPIP (n = 6) animals were individually housed and video recorded for 1 h before, 1, 2, 3 and 24 h after ischemia. The videos were then reviewed to retrieve the accumulated licking time for each animal.

# Assessment of mechanical allodynia of hind paws

Mechanical allodynia was assessed in different groups of animals (n = 18 in each group) before conducting the experiment (baseline) and then daily for 14 days post ischemia, as described before (Naguib et al., 2012). All behavioral tests were conducted in the morning by an investigator who was unaware of the treatment regimen. Rats were placed in a compartment with a wire mesh bottom and allowed to acclimate for 30 min before testing. Sensory thresholds for the development of allodynia to mechanical stimuli were assessed. As previously described (Chaplan et al., 1994), mechanical sensitivity was assessed by using a series of Von Frey filaments with logarithmic incremental stiffness (Stoelting Co., Wood Dale, IL, USA). Filaments were applied to the plantar surface of the hind paws for about 6 s in an ascending or descending order after a negative or positive withdrawal response, respectively. Six consecutive responses after the first change in response were used to calculate the paw withdrawal threshold (in grams). If the response thresholds occurred outside the range of detection, the paw withdrawal threshold was assigned at 15.00 g for continuous negative responses and at 0.25 g for continuous positive responses. 50% probability paw withdrawal thresholds were calculated with the up-down method (Crocker & Russell, 1984).

# The immunofluorescence analysis of microglial activation, CB2 and CX3CR1 receptor expression, and intraepidermal nerve fibers

Rats (n = 7 in each group) from aforementioned groups were killed at day 7, which represents the peak of allodynic behaviors. Animals were deeply anesthetized with 60 mg/kg sodium pentobarbital i.p. and perfused transcardially with 200 mL heparinized normal saline followed by 200 mL of 4% formaldehyde solubilized in 0.1 M phosphate-buffered saline (PBS). The lumbar spinal cord was then removed by cutting open the canalis vertebralis from the cauda; postfixed in 4% formaldehyde for 3 h and then cryopreserved in 30% sucrose in PBS for 48–72 h at 4 °C. The plantar skin of the ipsilateral hind paw was also excised and postfixed overnight and cryopreserved for 24 h in 30% sucrose in PBS at 4 °C(Liu *et al.*, 2010). The lumbar spinal cord tissues and the paw skin were then cut to 25 and 16 µm in thickness, respectively, and collected free floating in 0.01 M PBS. Tissue sections

were washed with 0.1% Triton-X 100 in PBS and blocked with donkey serum followed by washing with PBS. A mouse monoclonal anti-CD11b antibody (marker for microglial activation, ab8879, 1:100; Abcam, Cambridge, MA, USA) was co-incubated with a rabbit monoclonal anti-CB2 primary antibody (sc-25494, 1: 200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or a rabbit polyclonal anti-CX3CR1 primary antibody(TP502, 1:500; Torrey Pines Biolabs, Secaucus, NJ, USA) in the spinal cord sections. A rabbit polyclonal anti-protein gene product 9.5 primary antibody (RB-9202, 1:200; ThermoScientific, Rockford, IL, USA) was used in the paw sections. Primary antibodies were incubated for 2 h at room temperature and then overnight at 4 °C. Sections were then washed with PBS and incubated with fluorescein isothiocyanate (FITC, donkey anti-mouse, 715-096-151, 1:200) or Cy3 (goat anti-rabbit, 111-165-144, 1:200) conjugated secondary antibodies (Jackson Immuno Laboratories, West Grove, PA, USA) for 2 h at room temperature. Sections were then rinsed with wash buffer and mounted with Slow Fade antifade reagents (Invitrogen, Carlsbad, CA, USA). Omission of primary or secondary antibodies resulted in no immunostaining. Five optical sections from each rat were randomly selected and analyzed using a Leica SP5 confocal microscope by an investigator who was unaware of the origin of tissue being examined. The staining intensities were examined in a standardized area of laminae I-II in the spinal cord with four sections examined per animal in each group. The exposure and capture conditions for each channel were adjusted to ensure that images were captured within the dynamic range for all samples. Images were quantified using Image Pro Plus (Rockville, MD, USA). Negative control sections processed with the secondary antibody alone were used to account for the autofluorescence from the spinal cord itself and non-specific fluorescence from secondary antibody. Fluorescence image stacks were quantified for fluorescence intensity after the background fluorescence was subtracted.

Intraepidermal nerve fiber density was assessed as described before (Liu *et al.*, 2010). Five plantar skin sections per animal were chosen randomly to quantify the intraepidermal nerve fiber density using a confocal microscope. All nerve fibers crossing into the epidermis were counted and fibers that branched within the epidermis were counted as one. The number of intraepidermal nerve fibers per viewing field was counted.

#### Statistical analysis

Data were analyzed with one- or two-way analysis of variation (ANOVA) followed by *post hoc* Bonferroni test. All statistical analyses were performed with BMDP statistical software (Statistical Solutions, Saugus, MA, USA), GRAPHPAD PRISM or GRAPHPAD INSTAT software (GraphPad, Prism, Inc., La Jolla, CA). All data are expressed as mean  $\pm$  SEM. For all tests, a two-tailed P < 0.05 was considered statistically significant.

#### Results

# MDA7 alleviates the inflammatory manifestations and spontaneous pain behaviors in CPIP rats

We first assessed the acute inflammatory changes induced by ischemia and reperfusion. Compared to the contralateral hind paw, the ipsilateral hind paw with an O-ring tourniquet showed clear evidence of ischemia (cold and cyanosis) shortly after the initiation of the ischemia (Fig. 1a). Within 10 min after reperfusion, the



FIG. 1. MDA7 reduces acute inflammation and spontaneous pain in the CPIP rat model of CRPS-I. CPIP was induced using an ischemia-reperfusion injury of the rodent hind paw. (a) A tight-fitting O-ring was placed on the right hindlimb of an anesthetized rat just proximal to the ankle joint for 3 h, and was removed prior to termination of the anesthesia to allow reperfusion. Sham rats were anesthetized but a cut O-ring was placed on the right hindlimb (no ischemia was induced). (b) CPIP rats but not pretreated with MDA7 (c) exhibited hyperemia and edema of the ischemic hind paw after reperfusion. (d–g) Mid-paw thickness and circumference were measured before and after hindpaw ischemia in sham (n = 6), CPIP (n = 5), CPIP + MDA7 (n = 6) and CPIP + MDA7 + AM630 (n = 6) rats. (d) Two-way ANOVA, effect of group ( $F_{3,19} = 0.05$ , P = 0.98), effect of time (P = 0.25). (e) Two-way ANOVA, effect of group ( $F_{3,19} = 26.5$ , P < 0.0001), effect of time (P < 0.0001). (f) Two-way ANOVA, effect of group ( $F_{3,19} = 2.2.8$ , P < 0.0001), effect of time (P < 0.0001). (g) Two-way ANOVA, effect of group ( $F_{3,19} = 2.5.9.4$  (a) CPIP (n = 6), CPIP + MDA7 (n = 6) and CPIP + n = 0.25. (e) Two-way ANOVA, (fiect of group ( $F_{3,19} = 1.22$ , P = 0.32), effect of time (P = 0.37).AM630 is a selective CB2 antagonist and was administrated 15 min before MDA7 injection. Baseline values were measured immediately before tourniquet application. \*\*\*P < 0.01, two-way ANOVA, (h) Spontaneous pain behavior was assessed by calculating the cumulative licking time (sec/h) of the right hind paw before and after ischemia from video recordings. Sham (n = 6), CPIP (n = 6), CPIP + MDA7 (n = 6) and CPIP + MDA7 + AM630 (n = 6) rats. Two-way ANOVA, effect of group ( $F_{3,19} = 22.8$ , P = 0.02), effect of



FIG. 2. MDA7 alleviates CPIP-induced mechanical allodynia. Mechanical allodynia of the ipsilateral (a) and contralateral (b) hindpaw was assessed using the Von Frey method. We started with 15 animals per group, seven of which were killed for immunofluorescence studies at day 7 and the remaining eight animals continued with behavioral testing until day 14. Groups of rats were treated with IP MDA7 (15 mg/kg, IP) 30 min prior to and daily after CPIP induction or IP AM630 (5 mg/kg, a CB2 antagonist) 15 min prior to MDA7 administration. Compared with sham group, mechanical allodynia in the ipsilateral paw developed at 48 h after reperfusion, peaked at 7 day, and persisted for 2 weeks after reperfusion (*n* = 18 rats per group, two-way ANOVA, effect of group ( $F_{3,68} = 30.1$ , P < 0.0001), effect of time ( $F_{3,11} = 29.5$ , P < 0.0001). The anti-allodynic effect of MDA7 was blocked by AM630. A similar pattern was noted in the contralateral paw (*n* = 18 rats per group, two-way ANOVA, effect of group ( $F_{3,68} = 17.6$ , P < 0.0001), effect of time ( $F_{3,11} = 28.1$ , P < 0.0001). Data are shown as mean  $\pm$  SEM. \*P < 0.05 and \*\*P < 0.01, CPIP + MDA7 and sham groups vs. CPIP and CPIP + MDA7 + AM630 groups. CB, cannabinoid; CPIP, chronic post-ischemia pain; IP, intraperitoneal.



FIG. 3. MDA7 reduces expression of CX3CR1 receptor and microglial activation induced by CPIP in ipsilateral spinal cord dorsal horn lamina I to III (area in exemplary low power spinal cord image shown on the top right). CD11 and CX3CR1 immunofluorescence intensities were significantly increased at 7 day after reperfusion compared with sham group. Pretreatment with MDA7 significantly inhibited the increased immunofluorescence intensities of CD11 (n = 4 sections per animal from five animals in each group were randomly chosen, one-way ANOVA,  $F_{3,16} = 12.2$ , P = 0.0002) and CX3CR1 (n = 4 sections per animal from five animals in each group were randomly chosen, one-way ANOVA,  $F_{3,16} = 5.0$ , P = 0.01) induced by CPIP, and the effect of MDA7 was reversed by pre-administration of by the CB2 antagonist, AM630. Data are shown as mean  $\pm$  SEM; scale bar = 50 µm. \*\*P < 0.01 and \*P < 0.05 – sham and CPIP + MDA7 vs. CPIP and CPIP + MDA7 + AM630 groups. CB, cannabinoid.

ipsilateral hind limb became hyperemic and edematous (Fig. 1b). After tourniquet removal, mid-paw circumference (Fig. 1d and e) and thickness (Fig. 1f and g) were significantly increased on the ipsilateral side, but not on the contralateral side. Compared with the baseline, mid-paw circumference and thickness in CPIP and CPIP + MDA7 groups were significantly increased at 15 min, 24 h

and 48 h after tourniquet removal and returned to baseline level at 72 h after reperfusion. Compared with CPIP group, pre-administration of MDA7 significantly attenuated the increase in mid-paw thickness and circumference at 15 min after reperfusion (Fig. 1 c, d and f). There is no change noted in the ipsilateral and contralateral hind paws in sham group. After ischemia, CPIP + MDA7 animals



FIG. 4. MDA 7 inhibits CPIP-mediated CB2 receptor upregulation in the ipsilateral dorsal hom lamina I to III. Immunointensity of CB2 receptors located on the activated microglial cells (arrows and high magnification inserts) was significantly increased after CPIP (n = 4 sections per animal from five animals in each group were randomly chosen, one-way ANOVA,  $F_{3,16} = 9.3$ , P = 0.0009). Pretreatment with MDA7 significantly inhibited the increased immunofluorescence intensities of CD11 (n = 4 sections per animal from five animals in each group were randomly chosen, one-way ANOVA,  $F_{3,16} = 9.3$ , P = 0.0009). Pretreatment with MDA7 significantly inhibited the increased immunofluorescence intensities of CD11 (n = 4 sections per animal from five animals in each group were randomly chosen, one-way ANOVA,  $F_{3,16} = 27.9$ , P < 0.0001). The upregulation of GB2 receptors was blocked by pre-administration of MDA7. No significant difference of the staining intensity of CB2 receptors was detected between CPIP + MDA7 Group and sham group. The effects of MDA7 were reversed by the CB2 antagonist, AM630. Data are shown as mean  $\pm$  SEM, scale bar = 50 µm. \*\*P < 0.01 and \*P < 0.05 – sham and CPIP + MDA7 vs. CPIP and CPIP + MDA7 + AM630 groups. CB, cannabinoid; CPIP, chronic post-ischemia pain.

expressed significantly less spontaneous pain behaviors (accumulative time of hind paw licking) than CPIP alone animals during the first hour after reperfusion (Fig. 1h). These data suggest an important role of MDA 7 in reducing acute inflammation and spontaneous pain mediated by ischemia and reperfusion.

# MDA7 mitigates the mechanical allodynia in CPIP rats

We then investigated whether CPIP contributes to the neuropathic behavior and whether MDA7 can modulate the neuropathic behaviors by measuring the mechanical withdrawal thresholds. There was no significant change in mechanical withdrawal thresholds in ipsilateral or contralateral hind paws in the sham group during the 14-day study period (Fig. 2a and b). Compared with the sham group and the baseline value, the mechanical withdrawal threshold in the ipsilateral hind paws in the CPIP group decreased significantly at 48 h after reperfusion, indicating development of the mechanical allodynia. This decrease in the mechanical threshold persisted for 2 weeks after reperfusion (Fig. 2a). Administration of MDA7 attenuated the mechanical allodynia induced by CPIP. The effects of MDA7 were reversed by pre-administration of a selective CB2 receptor antagonist, AM630 (Fig. 2). In accordance with the data reported by Coderre et al. (2004), a less pronounced mechanical allodynia was noted in the contralateral side (Fig. 2b) on day 5 and lasted for 2 weeks. These data indicated that pre-administration of MDA7 could alleviate CPIP-mediated mechanical allodynia through the CB2 receptor.

# MDA7 attenuates activation of spinal microglia as well as upregulation of CB2 and CX3CR1 receptors in CPIP rats

To determine the role of CX3CR1 and CB2 signaling and spinal microglial activation in CPIP-mediated mechanical allodynia, we analyzed the expression of CD11b (a marker of microglial activation), CX3CR1 and CB2 receptors in the spinal cord dorsal horn. On the seventh day after ischemia, the immunoreactivity of spinal CD11b, CX3CR1 and CB2 receptors in CPIP group were significantly increased compared with sham animals (Figs 3 and 4). Treatment with MDA7 alleviated the microglial activation and upregulation of CX3CR1 and CB2 receptor caused by CPIP (Figs 3 and 4). The decreased immunoreactivity of CD11b, CX3CR1 and CB2 receptors were partially reversed by prior administration of the selective CB2 receptor antagonist, AM630 (Figs 3 and 4).

# MDA7 reduces the loss of intraepidermal nerve fiber induced by CPIP

We then asked whether CPIP induces intraepidermal nerve fiber loss and whether the nerve loss can be restored by MDA7. In the sham group, the PGP9.5 (a pan-neuronal marker)-labeled nerve fibers originate from cutaneous layer and extend into the intraepidermis as long nerve fibers. CPIP caused a significant decrease in the number of the intraepidermal nerve fibers (Fig. 5). Treatment with MDA7 prevented the reduction in the intraepidermal nerve fibers induced by ischemia and the protective effect of MDA7 was attenuated by pre-administration of AM630 (Fig. 5).

# Discussion

Using the post-ischemia pain (CPIP) animal model for CRPS-I (Coderre *et al.*, 2004), we demonstrated that a selective CB2 receptor agonist, MDA7, alleviated ischemia-mediated peripheral edema and mechanical allodynia, inhibited CX3CR1 upregulation and microglial activation in the spinal dorsal horn, and preserved hind paw intraepidermal nerve fibers after ischemia and reperfusion injury.

Rats exposed to the prolonged ischemia (3 h) demonstrated hind paw hyperemia and edema upon releasing of the O-ring tourniquet during reperfusion (Fig. 1). Peripheral edema and mechanical allodynia were ameliorated by administration of the CB2 agonist, MDA7. As MDA7 was administered intraperitoneally prior to tourniquet application, this might represent a potential systemic effect of MDA7. CB2 receptors appear to modulate macrophages (Chiurchiu *et al.*, 2014) and other inflammatory mediators involved in skin wound healing (Zheng *et al.*, 2012), and it is not unexpected to see that MDA7 plays a potential therapeutic role in inflammation. In fact, activation of the CB<sub>2</sub> receptor has been reported to reduce post-traumatic inflammation in mice (Amenta *et al.*, 2014). Rats that underwent CPIP also developed acute spontaneous pain behaviors

# Intraepidermal Innervation Sham Sham Ischemia Ischemia + MDA7 Ischemia + MDA7 + AM630 The number of intraepidermal Ischemia 10 nerve fibers Ischemia + MDA7 2-0 Ischemia + AM630 + MDA7

FIG. 5. MDA7 reduces intraepidermal nerve fibers loss induced by ischemia and reperfusion injury. Immunofluorescence staining intensity of intraepidermal nerve fibers in the ipsilateral paw indicated that CPIP caused significant injuries to the intraepidermal nerve ending, with reduced nerve ending numbers and damaged nerve structures. Shown here is the number of the nerve fibers per section per representative animal. There was a greater decrease in the number of intraepidermal nerve fibers in CPIP group compared with those in the sham group (n = 4 sections per animal from five animals in each group were randomly chosen, one-way ANOVA,  $F_{3,16} = 27.9$ , P < 0.0001). Pretreatment with MDA7 preserved the intraepidermal nerve fiber architecture both in nerve ending numbers and structures compared with CPIP group. However, this neuroprotective effect of MDA7 was blocked by pre-administration of a CB2 antagonist, AM630. Data are shown as mean  $\pm$  SEM, scale bar = 50  $\mu$ m. \*\*P < 0.01 – sham and CPIP + MDA7 vs. CPIP and CPIP + MDA7 + AM630 groups. CB, cannabinoid; CPIP, chronic postischemia pain.

(hind paw licking and biting) (Fig. 1h). It is interesting that although spontaneous pain is common in CRPS patient, there was no significant difference in the duration of hind paw licking 24 h after the reperfusion in majority of the animals, indicating that the spontaneous pain likely resolved after 24 h, consistent with the previous report (Coderre et al., 2004). Mechanical allodynia was observed through the 14-day experimental period (Fig. 2). The CB2 selective agonist, MDA7, alleviated the mechanical allodynia and reduced expression of CB2 and CX3CR1 induced by CPIP (Figs 2 and 4) and the protective effect of MDA7 was partially abolished by pre-administration of a CB2 antagonist, AM630 (Figs 2 and 4). This study demonstrates for the first time that modulation of CB2 activity plays an important role in the pathogenesis of CRPS-I. These findings confirmed our hypothesis that the CB<sub>2</sub> receptor functions in the immunomodulatory negative-feedback loop and that CB2 receptor activation can blunt neuroinflammatory responses and neuropathic pain in this CPIP model of CRPS-I.CB2 receptor expression and function in the spinal cord tissue have been studied in neuropathic pain. The use of a CB2 receptor antibody in detecting the protein expression has been an issue due to lack of specificity of the antibody (Atwood & Mackie, 2010; Marchalant et al., 2014). We compared CB2 receptor antibodies from several resources and chose the current one to be used in the immunofluorescence staining. The staining quality was affected by the limited specificity of the available CB2 receptor antibody but we were able to make comparison among groups.

Activation of spinal cord microglial cells appears to be the upstream common process leading to neuropathic pain from different etiologies (Scholz & Woolf, 2007; Milligan & Watkins, 2009; Naguib *et al.*, 2012). Studies have shown that activated microglial cells synthesize the most abundant endocannabinoid, 2-AG, which activates CB2 receptors (Carrier *et al.*, 2004; Witting *et al.*, 2004). CB<sub>2</sub> receptors appear to function in a negative-feedback loop and that early MDA7 administration can blunt the neuroinflammatory response and can decrease cytokine release from microglial cells (Merighi *et al.*, 2012; Naguib *et al.*, 2012). In the paclitaxel-induced peripheral neuropathy model, gene expression profiling and pathway analysis showed that MDA7 down-regulates inflammatory pathways following microglial activation, including fractalkine/CX3CR1 signaling pathway (Xu *et al.*, 2014).

Fractalkine/CX3CR1 signaling is crucial in mediating neuronmicroglia interaction and microglial activation in the spinal cord dorsal horn during nociceptive transmission (Milligan et al., 2008; Staniland et al., 2010; Yang et al., 2012; Sun et al., 2013; Clark & Malcangio, 2014). Fractalkine is mainly expressed by neurons while CX3CR1, the only receptor for fractalkine, is mostly expressed by microglia in the spinal cord dorsal horn (Lindia et al., 2005; Yang et al., 2012). Expression of CX3CR1 on microglia is extensively upregulated following nerve injury in several neuropathic models (Lindia et al., 2005; Zhuang et al., 2007; Hu et al., 2012; Yang et al., 2012) and impairment of spinal fractalkine/CX3CR1 signaling attenuates neuropathic pain behaviors (Zhuang et al., 2007; Milligan et al., 2008; Staniland et al., 2010; Clark & Malcangio, 2014; Old et al., 2014). CX3CR1 mRNA expression is increased in rat spinal cord dorsal horn laminae I-III after chronic constriction injury and sciatic inflammatory neuropathy, which correlates well with clustering of OX-42-positive cells (activated microglia) (Verge et al., 2004). Intrathecal (Milligan et al., 2005), but not intra-neural (Holmes et al., 2008) administration of fractalkine can induce hyperalgesia and mechanical allodynia. On the other hand, intrathecal administration of CX3CR1 neutralizing antibody can inhibit or reverse inflammatory and neuropathic pain (Milligan *et al.*, 2005; Zhuang *et al.*, 2007). Mechanical allodynia and thermal hyperalgesia are less severe in CX3CR1 knock-out (KO) than in wild-type (WT) mice when induced by partial sciatic nerve ligation (Staniland *et al.*, 2010). Spinal Iba1 (a microglial marker) expression and phosphorylation of p38 MAPK increase after nerve injury in WT but not CX3CR1 KO mice (Staniland *et al.*, 2010). Consistent with this data, this study showed that CX3CR1 expression in rat spinal cord is increased after CPIP and this upregulation is associated with microglial activation (Fig. 3). Importantly, we also noted that these effects are attenuated by pre-administration of MDA7.

Besides fractalkine/CX3CR1 signaling, other mechanisms may be modulated by MDA7 in the CPIP model. The transcription factor nuclear factor kappa B (NFkB) was elevated in the CPIP (de Mos et al., 2009) and paclitaxel neuropathic pain models (Xu et al., 2014). Proteomic analysis indicated that cerebral proteins related to reactive oxygen species (ROS), cell signaling, synaptic plasticity and cell proliferation may also be involved in the pathogenesis of CRPS using the CPIP model (Perez et al., 2003; Coderre et al., 2004; Nahm et al., 2014; Schiller et al., 2015). Endothelin (ET)-1 level in plasma and spinal cord increased after CPIP (Kim et al., 2015). Blocking the transient receptor potential ankyrin 1 (TRPA1) reduced mechanical and cold allodynia after CPIP (Klafke et al., 2016). In addition, clinical CRPS is more predominant in female than male patients (de Mos et al., 2007). Animal studies showed that female rats displayed lower nociceptive thresholds but no differences in ongoing or spontaneous pain in the tibial fracture CRPS model (Tajerian et al., 2015). The mechanism underlying the role of gender in CRPS remains unclear. Further studies may help to elucidate that whether CB2 agonists could modulate these signals in CPIP.

We investigated the spinal microglial activation on day 7 after CPIP because it seems that the decrease in threshold stabilized by day 7 through day 14. Others have reported early microglial activation in the spinal cord dorsal horn in neuropathic and inflammatory pain models (Cao & Zhang, 2008; Gwak *et al.*, 2012). It is possible that spinal microglia activation developed at the early stage after CPIP and persisted due to the prolonged ischemic insult and the neuroinflammatory cascade initiated after reperfusion.

Loss of intraepidermal nerve fibers (IENFs) has been reported to play a critical role in the development of various neuropathic pain syndromes including chemotherapy-induced peripheral neuropathy (Boyette-Davis et al., 2011), diabetic and non-diabetic neuropathy (Pittenger et al., 2004), autoimmune diseases-associated neuropathy (Goransson et al., 2006), HIV-associated sensory neuropathy (Polydefkis et al., 2002) and ischemic pain (Grone et al., 2014). Immunohistochemical analysis of both upper and lower extremity skin biopsies from amputated CRPS patients revealed reduction of epidermal innervation supplied by c fibers and Aδ fibers as compared to control skin (Albrecht et al., 2006). We noted that the number of intraepidermal nerve fibers from the ipsilateral plantar skin remarkably decreased after CPIP. Pre-administration of MDA7 preserved the intraepidermal nerve fibers. The mechanisms by which MDA7 preserves nerve fibers need to be examined, but could be attributed to the anti-inflammatory systemic effects of MDA7. There are several limitations to this study. We only observed the plantar intraepidermal nerve fibers. There are other peripheral agents such as immune cells, cytokine and chemokines which may play an important role in the inflammation after ischemia. Immune cells and chemokines other than CX3CL1 may be also involved in the neuroinflammation at the spinal cord. Further studies are needed to explore their role in CPIP.

In summary, the CPIP rat model mimics the clinical picture of CRPS-I in humans. CPIP causes activation of spinal microglia with increased expression of CB2 and CX3CR1 receptors. CPIP also results in loss of plantar intraepidermal nerve fibers. Pre-administration of the selective CB2 agonist MDA7 alleviates CPIP-mediated mechanical allodynia by inhibiting microglial activation via suppression of CX3CR1 signaling. MDA7 also mitigated the loss of intraepidermal nerve fibers. This study is a novel step toward understanding the molecular mechanisms underlying CRPS using the ischemia and reperfusion animal model. Our findings suggest that selective CB2 agonist may offer an innovative therapeutic approach for attenuating neuropathic symptoms and neuroinflammatory responses induced by CRPS-I in the setting of ischemia and reperfusion injury.

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#### Abbreviations

2-AG, 2-arachidonoylglycerol; ANOVA, analysis of variation; CB2, cannabinoid type 2 receptor; CNS, central nervous system; CPIP, chronic post-ischemia pain; CRPS, complex regional pain syndrome; DMSO, dimethyl sulfoxide; ET, endothelin; IENFs, intraepidermal nerve fibers; IP, intraperitoneal; KO, knock-out; MAPK, mitogen-activated protein kinases; NF $\kappa$ B, nuclear factor kappa B; PBS, phosphate buffered saline; WT, wild-type;  $\Delta^9$ -THC,  $\Delta^9$ -tetrahydrocannabinol.

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