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Research article

Attenuation of allodynia and microglial reactivity by inhibiting the degradation of 2-arachidonoylglycerol following injury to the trigeminal nerve in mice

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HIGHLIGHTS

- Microglia became reactive under neuropathic orofacial pain condition.
- An endocannabinoid degradation enzyme inhibitor, JZL184, effectively attenuated neuropathic pain.
- JZL184 attenuated microglial reactivity under neuropathic orofacial pain condition.

ABSTRACT

Endocannabinoids have an important role for the regulation of neuropathic pain. In our previous study, we observed that preventing the degradation of a endocannabinoid, 2-arachidonoylglycerol (2-AG), using an inhibitor of monoacylglycerol lipase (JZL184), attenuated neuropathic orofacial pain (NOP). The present study aimed to investigate mechanisms underlying JZL184-induced attenuation of NOP. We hypothesized that JZL184 may suppress microglial reactivity in the trigeminal spinal subnucleus caudalis (Vc) under NOP. The infraorbital nerve (ION) was hemisected to model NOP in mice, resulting in a significant reduction of mechanical head-withdrawal threshold (MHWT) on day 4 following the ION hemisection. Chronic systemic application of JZL184 at a concentration of 8 or 16 mg/kg/day for 4 days significantly attenuated the reduction of MHWT in mice exposed to NOP. Administering JZL184 at 4 mg/kg/day or its vehicle, however, did not attenuate the MHWT of mice with NOP. The reactivity of microglial cells in the Vc increased in mice with NOP compared to sham-operated controls. The application of JZL184 at 8 or 16 mg/kg/day for 4 days significantly reduced the increased microglial reactivity in the Vc. The changes of microglia under NOP were, by contrast, not reduced by application of the drug at 4 mg/kg/day or its vehicle. The results indicate that preventing 2-AG degradation may increase its accumulation in the Vc and normalize microglial reactivity under NOP, which may contribute to suppressing NOP.

1. Introduction

Reactive glial cells involve in central and peripheral sensitization [1, 2, 3, 4]. In particular, microglia involves in the initiation and maintenance of neuropathic pain [1, 2, 4, 5]. Several studies suggest that the endocannabinoid (eCB) system, an attractive target for managing microglial-mediated inflammation, may regulate many aspects of the central nervous system immune response [1, 2, 3, 4, 6].

Endocannabinoids, representing endogenous cannabinoid receptor (CBR) ligands, are considered a potential therapeutic target [7]. Previous studies reported an expression of CBR in the trigeminal ganglia and trigeminal spinal subnucleus caudalis (Vc) [8, 9]. The two eCBs that have been most studied are 2-arachidonoylglycerol (2-AG), and arachidonoylethanolamine (also named anandamide or AEA) [10]. Several preclinical studies reported that the eCB system affects the microglial reactivity [2, 4]. Reactive microglia can change their morphology from a

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ramified shape to an amoeboid shape, increase their proliferation, and modify their function [1, 2].

Studies over the past two decades have shown that the inflammation of central nervous system can be suppressed by inhibiting the degradation of eCB. A wealth of research has further demonstrated the potential of this approach for pain relief [4]. Accordingly, enhancing the action of eCB using endocannabinoid degradative enzyme inhibitors may be an attractive way for the neuropathic pain treatment [4]. The endocannabinoid 2-AG is degraded by an enzyme named as monoacylglycerol lipase (MAGL), while AEA is hydrolyzed by an enzyme named as fatty-acid amide hydrolase (FAAH) [11]. Preclinically, the MAGL inhibitor JZL184 is the most thoroughly characterized *in vivo*, notably in neuropathic pain. In our previous work, we showed that JZL184 successfully attenuates neuropathic pain in the orofacial area, following injury to the infraorbital nerve (ION) in mice. Microglia in the spinal cord were strongly reactive after peripheral nerve injury [13]. A previous study showed that the MAGL inhibitor UCM03025 reduces the number of Iba1-immunoreactive microglia among the cerebral cortex in a mouse model of multiple sclerosis [14]. Furthermore, JZL184 reduced number of Iba1-immunoreactive microglia among the hippocampus, as well as temporal and parietal cortices, in a model of Alzheimer's disease pathology [15]. In NOP studies, it has been reported that following trigeminal nerve injury, nociceptive neurons of the Vc are excessively activated, in tandem with microglial reactivity [2, 16, 17, 18]. Inhibition of microglial reactivity significantly reduces nocifensive behavior in NOP animal models, further supporting the concept that microglia are implicated in the mechanisms underlying NOP [19]. However, the contribution of the eCB system in modulating microglial function in NOP remains elusive. In the present study, we examined whether preventing the 2-AG degradation influences microglial reactivity in the Vc, as well as analgesia under NOP conditions.

2. Materials and methods

2.1. Animals

Approval of animal care and experimental procedures obtained from the Matsumoto Dental University Intramural Animal Care and Veterinary Science Committee. All animals treated humanely according to the guidelines developed by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research, ARRIVE (Animal Research: Reporting of In Vivo Experiments) and the International Association for the Study of Pain [20].

2.2. Treatment

We made great efforts to reduce the number of animals used, together with animal distress and suffering. Mice (male C57Bl/6J; SLC Inc., Japan) were used for all experiments. Mice were 8–9 weeks old (20–26 g) at the beginning of the experiments. Animals were kept in animal center of the Matsumoto Dental University under conditions with a 22 ± 2 °C temperature, $40 \pm 5\%$ relative humidity, and photoperiod of 12-hour day and 12-hour dark. Food and water were provided *ad libitum*.

2.3. Experimental neuropathic pain model and study protocol

The model of neuropathic pain involved a surgical procedure described previously [21, 22]. In brief, after proper anesthesia (pentobarbital sodium; 50 mg/kg, i.p.), a small incision made intraorally along the bucco-gingival margin (proximal to the first molar) to expose the ION. The nerve was then hemisected. For sham control model the bucco-gingival margin was scratched but the nerve was not exposed.

2.4. Assessment of nocifensive behavior

Von Frey filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.40 and 2.0 g) (Touch Test Sensory Evaluator, North Coast Medical Inc., CA, USA) were used to measure mechanical head-withdrawal threshold (MHWT).

The MHWT assessment method was adapted from a published study [25]. The mice received training for 5–7 days before the assessment of MHWT. The investigator held a mouse in a way that allowed free movement of the animal head. Mice were trained to remain quiet in the investigator's hand by the training. The measurement of MHWT was conducted by two investigators. Each mouse was held by one investigator's hand, while the other investigator applied the filament onto the center of the whisker pad among a particular region, with each hair positioned perpendicularly (i.e., same side to the sham operation/ION hemisection). An up-down method was used to apply the von Frey filaments [23]. If a positive response to a stimulus was observed, the next weaker filament in the series was applied. If there was no positive response, the next stronger filament in the series was instead applied. The experiment followed this protocol until a positive response to a von Frey stimulus was detected in each mouse. For each mouse, the MHWT was measured twice: before performing the surgery and on day 4 following the sham operation/nerve hemisection. On day 4, the MHWT was measured 2 h following injection of the JZL184 drug or vehicle [24], to investigate outcomes of the treatment on behavior.

2.5. Drug treatment

The drug JZL184 [4-nitrophenyl-4-(dibenzo[d] [1, 3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate] (Cayman Chemical, MI, USA) prepared by dissolving it in a combination of saline and ethanol-dimethylsulfoxide-Cremophor (Calbiochem, Darmstadt, Germany) (17:1:1:1) [27], which also served as a vehicle control. Intraperitoneal (i.p.) injection of the drug administered at a concentration of 4, 8 or 16 mg/kg of body weight, 3 h after ION hemisection or sham operation [25], then once daily until experimental day 4. The concentration range was selected based on previous studies [12, 26].

2.6. Fluorescent immunohistochemical staining of microglia

The mice were sacrificed on day 4, 2 h following the application of JZL184 or vehicle. The animals with deep anesthesia were perfused intracardially with saline, and then, with paraformaldehyde (4%) dissolved in phosphate buffer (0.1 M). Whole brains removed and placed in the same fixative (4 °C; overnight). Then, the whole brains immersed into sucrose (30% dissolved in phosphate-buffered saline (PBS; 0.1 M) until sinking. The brainstem was then cut into series of 20-μm sections with a freezing microtome, mounting every fifth section onto glass slides (Aminosilane-coated; Matsunami Glass Ind., Ltd., Osaka, Japan).

Immunohistochemistry conducted on tissues containing the Vc to investigate microglial immunoreactivity against Iba1 in each treatment group. The sections were washed and were blocked for 1 h with normal goat serum (5%) diluted in PBS (0.01 M) with Triton X (0.3%). After that, the sections were incubated (24 h) with a primary antibody (rabbit anti-Iba1; 1:500; Wako Pure Chemical Corporation, Osaka, Japan). The sections were washed and incubated (1.5 h) with goat anti-rabbit secondary antibody (Alexa fluor 594; 1:1000; Molecular Probes, USA). After washing, sections were coverslipped with antifade reagent (ProLong Gold; Life Technologies, USA). Images of slides were captured using a BZ-X700 fluorescent microscope (Keyence Corp., Japan) with an attached camera. Three sections from a mouse containing the Vc (the section containing the highest number of microglial cells and the immediate two serial sections) were used to quantify the microglial immunoreactivity against Iba1. In each section, a 720×540 μm area was analyzed using a software (ImageJ; NIH Image, USA). Microglial immunofluorescent intensity at least two times above background was considered positive. We did not observe positive immunoreactivity for microglia within tissue sections when the sections were incubated without the primary antibody.

2.7. Statistical analysis

Comparison of post-operation behavioral data on day 4 with pre-operation behavioral data was conducted using a *t*-test. An ANOVA

(one-way) with a Tukey's post-hoc test was conducted to compare of behavioral data between sham and other groups on postoperative day 4, following application of JZL184 or vehicle. Comparison of the number of Iba1-immunoreactive cells among the groups was conducted with an ANOVA (one-way) and a Tukey's post-hoc test. The threshold for statistical significance was set to $p < 0.05$.

3. Results

3.1. Treatment with JZL184 attenuates the reduction of MHWT in mice upon ION hemisection

The C57Bl/6J mice were subjected to ION hemisection or sham operation, then treated with various concentrations of JZL184 or vehicle. The MHWT was measured before ION hemisection or sham operation (day 0), then 2 h following i.p. application of JZL184 or vehicle on day 4 post-surgery. The drug JZL184 was administered at 4, 8 or 16 mg/kg of body weight/day for 4 days (n = 5 mice per treatment group). The MHWT was above 1 g before ION hemisection. It decreased significantly on day 4 post-operation ($p < 0.05$, t-test), suggesting the development of NOP (Figure 1A). No such decrease of MHWT was observed in sham mice ($p > 0.05$). Four days following ION hemisection, daily treatment with JZL184 at 8 or 16 mg/kg resulted in a significant attenuation of the MHWT reduction ($p < 0.05$, ANOVA (one-way) with Tukey's post-hoc test) compared to the sham group. In hemisected mice, injection of

JZL184 at 4 mg/kg did not induce a significant change of the MHWT compared to vehicle-treated mice. Injection of the vehicle also failed to attenuate the reduction of MHWT in mice receiving ION hemisection operation. Similarly, i.p. injection of vehicle in sham mice did not modify the MHWT. Finally, no significant difference observed ($p > 0.05$) for the MHWT of mice with sham operation followed by 4 days of daily vehicle injection compared to mice with hemisection followed by 4 days of daily JZL184 injection at 8 or 16 mg/kg (Figure 1A).

3.2. JZL184 attenuates microglial reactivity in mice with ION hemisection

We next investigated the reactivity of microglia in the trigeminal afferent nerve's terminals located in the brainstem. We performed fluorescence immunohistochemistry against the microglial marker Iba1 in tissue containing the Vc region. In the sham mice, immunohistochemical study was performed 2 h after the injection of vehicle on day 4. In the hemisected mice, immunohistochemical study was performed 2 h after the injection of vehicle or JZL184 at 4, 8 or 16 mg/kg on day 4. The number of immunoreactive cells for Iba1 was significantly increased ($p < 0.05$) in the Vc of hemisected mice compared to sham-operated mice (Figure 1B and C). Hemisected mice given 4 daily injections of JZL184 at 8 or 16 mg/kg also displayed significantly fewer ($p < 0.05$) Iba1-immunoreactive cells compared to hemisected mice receiving vehicle (Figure 1B and C). This reduction was concentration dependent.
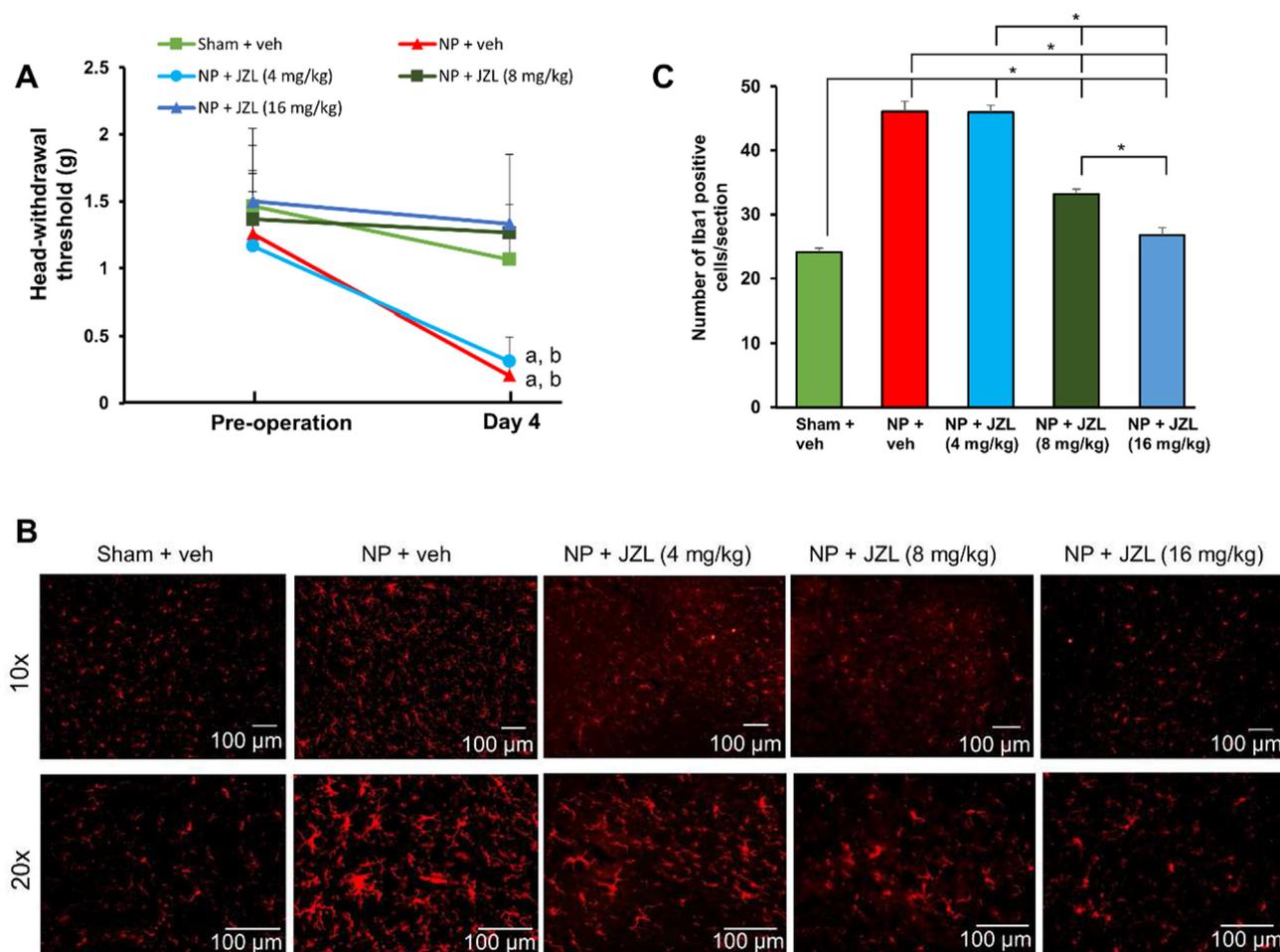
Figure 1. Attenuation of neuropathic pain and microglial density upon application of JZL184. (A) Mechanical head-withdrawal threshold (MHWT) in different groups of mice. The neuropathic (NP) mice receiving ION hemisection or sham mice were injected with vehicle (veh) or JZL184 (JZL) for 4 days. a: Significant difference ($p < 0.05$) when postoperative day 4 data were compared with pre-operation data; b: Significant difference ($p < 0.05$) when data between sham and other groups compared on postoperative day 4. (B) Iba1 reactive microglia in the Vc area of different groups of mice. (C) The number of Iba1-immunoreactive microglia in the Vc area of different groups of mice. Data are presented as mean ± standard error of the mean (SEM). *$p < 0.05$. NP: neuropathic pain.

However, there was no reduction of Iba1-immunoreactive cells in the Vc of hemisected mice administered JZL184 at 4 mg/kg.

4. Discussion

In response to injury to the nerve, immune cells including glial cells become reactive and play a role in initiating pain [2, 27]. There is general agreement that glial cells of the spinal dorsal horn can control pain in their reactive state by producing various pain mediators [2, 13]. Microglial reactivity may be involved in the neuropathic pain through the release of several pro-inflammatory mediators known to produce neuronal sensitization, leading to the initiation of neuropathic pain (Figure 2). In the current study, we found that ION hemisection increases microglial density on day 4 in the Vc, a region playing a pivotal role in analgesia and contributing to pain hypersensitivity. This finding is consistent with a previous study showing the presence of reactive microglia, evidenced by an increased expression of Iba1 observed after L5 nerve transection [28]. An accumulating number of studies suggest the existence of a crosstalk between endocannabinoids and glial cell signaling in the nervous system [29, 30, 31]. Upon injury endocannabinoids increased. And this upregulation may have protective roles by reducing mediators for inflammation, microglial reactivity, and promoting brain homeostasis [31]. 2-AG acts as a full agonist of both CBR1 and CBR2 receptors, and is mainly hydrolyzed by MAGL [11, 32, 33] that accounts for 85% of 2-AG hydrolytic activity in brain tissues [34]. Inactivation of MAGL by systemic application of JZL184 results in an eight-fold elevation of 2-AG brain levels without altering AEA levels [35]. After treatment with JZL184, nociceptive behavior attenuated in various animal models of pain, involving formalin, mechanical or cold allodynia [24, 36, 37]. Our previous study in the mouse model of ION hemisection demonstrated a pain attenuating effect of JZL184 [12]. Acute application of JZL184 at a concentration of 4 or 16 mg/kg of body weight showed significant pain suppressive effect, when measured 2 h after the application. In the present study, chronic daily application of JZL184 at a concentration of 4 mg/kg body weight did not induce significant pain suppressive effects. This apparent discrepancy possibly due to the fact that the mice used in the current study might have developed a habituation to the drug due to its chronic application, leading to a lack effectiveness at low concentration. Previously, we also found increased numbers of MAGL-positive cells in the Vc of ION-hemisected mice [12]. This finding may be related to enhanced 2-AG levels, because these lipids are produced when needed without any substantial storage [38]. Although the beneficial outcome of blocking the degradation of 2-AG on pain modulation has been clearly demonstrated [39], the microglial mechanisms underlying its analgesic action remains unclear. In an Alzheimer's animal model, the inactivation of MAGL using JZL184 decreased reactivity of microglial in the hippocampus [15]. It is also well-documented that microglia express CBRs [40, 41, 42]. In line with these observations, we found here that administering JZL184 for 4 days to mice with ION hemisection resulted in a normalized microglial density among the Vc, in a concentration-dependent manner, which was accompanied by peripheral antinociception (Figure 2).

Activation of CBR2 on immune cells by 2-AG may synergize with activation of CBR1 on peripheral nociceptors at various sites of pain pathway [43]. Although we could not clarify how the 2-AG-induced activation of cannabinoid receptors (either CBR1 or CBR2) inhibited the ION hemisection-induced changes in microglial density in the current study, previous findings have demonstrated that an increased expression of CBR2 after nerve injury is related to microglial reactivity [44]. Indeed, CBR2 is upregulated in the dorsal horn after peripheral nerve injury, when microglia play a key role in the development of pain [40]. Additionally, CBR2 agonists produce analgesic effects, normalize microglial states in neuropathic pain models, and reduce microglial release of inflammatory cytokines [45]. Treatment with a CBR2 agonist in a mouse model of spinal cord injury decreased mechanical allodynia and thermal hyperalgesia, as well as reduced microglial hypertrophy in the ipsilateral dorsal horn [44].
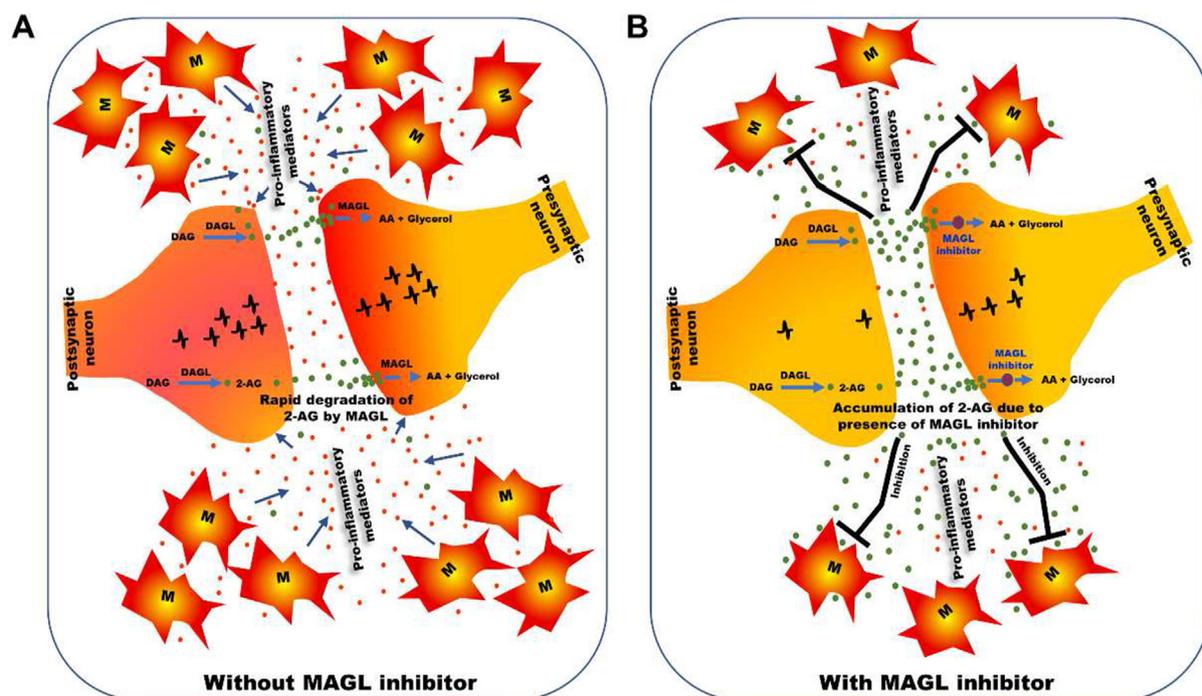
Figure 2. Schematic illustration of a possible mechanism of attenuation of microglial reactivity and neuropathic pain by inhibiting the degradation of 2-AG following the trigeminal nerve injury. (A) In the Vc, microglial reactivity increases under neuropathic orofacial pain. Reactive microglia release pro-inflammatory mediators causing neuronal sensitization. Under this condition, 2-AG production may be increased by postsynaptic neurons which rapidly degraded by MAGL. (B) Preventing the degradation of 2-AG by a MAGL inhibitor (JZL184) may increase its localized accumulation which reduces microglial reactivity and release of pro-inflammatory mediators thereby attenuates neuronal sensitization and neuropathic pain. 2-AG: 2-arachydonoylglycerol; AA: arachidonic acid; DAG: diacylglycerol; DAGL: diacylglycerol lipase; MAGL: monoacylglycerol lipase; M: microglia.

Cannabinoid receptors are expressed by many types of cells, including microglia, astrocytes, and neurons in the rodent spinal cord [44]. Accumulating data from models of neuropathic pain also indicate that microglia become reactive before astrocytes and may contribute for the initial pain [45]. Reactive microglia, which release inflammatory molecules, could underlie the subsequent transformation of astrocytes that leads to a consolidation of the neuropathic pain state [46]. Although additional studies are required, the eCB system has an ample potential as a therapeutic target for the treatment of neuropathic pain syndromes.

A limitation of this study pertains to its focus on the contribution of Vc microglia only, as a mechanism underlying the NOP attenuation resulting from the inhibition of 2-AG degradation with the MAGL inhibitor JZL184. Other mechanisms could participate in the attenuation of NOP. For example, accumulated 2-AG in the Vc area may directly reduce neuronal excitability in neuropathic pain. Furthermore, 2-AG may act on neuronal CBR1 to reduce excitatory neurotransmitter release from the neurons and thereby attenuate the pain under neuropathic pain condition [4]. However, in the current research, we did not investigate the types of receptors involved in the mechanism of JZL184-induced attenuation of NOP. Additionally, 2-AG may act on microglial CBR. Previous studies have reported that CBR2 is predominantly expressed by microglia [41, 42]. This receptor may also inhibit astrocytic activity in the Vc [47]. Another limitation of this study is that we did not quantify 2-AG from the Vc area after administering the different concentrations of JZL184. However, in our previous study with the same animal model of NOP [12], we investigated MAGL immunoreactivity in the Vc regions to understand the 2-AG levels indirectly. In that study, MAGL-immunoreactivity increased in the Vc regions under the NOP indirectly indicated an increase of 2-AG production [12], because, the increased 2-AG might rapidly degrade by increased levels of MAGL. In this context, it is well-recognized that the actions of endocannabinoids are relatively short due to their quick metabolic degradation. Additionally, in that study, acute administration of a MAGL inhibitor (JZL184) decreased the MAGL-immunoreactivity in the Vc which indirectly suggested a reduction of degradation of 2-AG, thereby increased the accumulation of 2-AG [12]. Future research is warranted to further explore from various perspectives the mechanisms underlying the attenuation of NOP by endocannabinoids.

In summary, in this study, we showed that inhibiting the degradation of 2-AG with the MAGL inhibitor JZL184 suppressed ION hemisection-induced microglial reactivity in the Vc, which may contribute to alleviating NOP (Figure 2). Our current findings provide a insight into the mechanisms, by which eCB-targeting drugs could be useful for the treatment of NOP.

Declarations

Author contribution statement

Rantaro Kamimura, Mohammad Zakir Hossain, Junichi Kitagawa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kojiro Takahashi, Isao Saito: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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