Inhibition of 2-arachydonoylglycerol degradation attenuates orofacial neuropathic pain in trigeminal nerve-injured mice

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Abstract: Current therapeutics are not effective for orofacial neuropathic pain, and better options are needed. The present study used inferior orbital nerve (ION)-injured mice to investigate the effect of inhibiting monoacylglycerol lipase (MAGL), an enzyme that degrades the major endocannabinoid 2-arachydonoylglycerol (2-AG) in orofacial neuropathic pain. The head-withdrawal threshold to mechanical stimulation of the whisker pad was reduced on days 3, 5, and 7 after ION injury. Injection of JZL184, a selective inhibitor of MAGL, on day 7 after ION injury attenuated the reduction in head-withdrawal threshold at 2 h after administration. Moreover, the numbers of MAGL-immunoreactive neurons in the trigeminal subnucleus caudalis (Vc) and upper cervical spinal cord (C1-C2) were significantly greater in ION-injured mice than in sham-operated mice but were reduced after administration of JZL184. The increase in MAGL immunoreactivity suggests that increased 2-AG production is followed by rapid enzymatic degradation of 2-AG. JZL184 inhibited this degradation and thus increased 2-AG concentration in the brain, particularly in the Vc and C1-C2 regions, thus attenuating pain. Our findings suggest that inhibition of 2-AG degradation by MAGL inhibitors is a promising therapeutic option for treatment of orofacial neuropathic pain.

Keywords: orofacial neuropathic pain; 2-arachydonoylglycerol (2-AG); monoacylglycerol lipase (MAGL); JZL184.

Introduction

Neuropathic pain is a chronic condition that is difficult to treat and has debilitating effects on quality of life (1). It can result from nerve injury, inflammation, or diseases of the peripheral or central nervous system and is characterized by spontaneous pain (ongoing or paroxysmal) coincident with sensitization in the form of hyperalgesia or allodynia (2). Treatment of neuropathic pain is a major challenge, predominantly because of the heterogeneity of its origin.

Current pharmaceutical options have had limited success and can cause a variety of adverse effects (3,4). There is thus an urgent need for new therapeutic options that are more efficient for management of neuropathic pain. Natural and synthetic cannabinoids attenuated neuropathic pain in several experimental models (5-10). Agonists produce analgesic effects by acting directly on cannabinoid type 1 (CB1) and type 2 (CB2) receptors (7,11). The main disadvantage of using natural or synthetic cannabinoids for pain relief is their side effects,
which include sedation, dependency, cognitive impairment, and psychological problems (12,13). These side effects arise because of global activation of cannabinoid receptors, which are widely distributed throughout the brain and spinal cord.

Targeting of endocannabinoids (ECs) is a potential alternative approach for treating neuropathic pain and reduces the risk of side effects. ECs are the endogenous ligands for cannabinoid receptors, and the primary ECs in the human body are 2-arachydonoylglycerol (2-AG) and N-arachidonoyl ethanolamine (AEA). These molecules are synthesized and released on demand in response to physiological and pathological stimuli (14-16). ECs are released from postsynaptic neurons and act on presynaptic neurons to inhibit neurotransmitter release, a phenomenon known as retrograde suppression of synaptic transmission (7,15-16). Increased neuronal activity resulting from nerve injury or neuropathic pain can lead to increased synthesis of ECs (17,18). However, rapid cellular uptake and subsequent degradation of ECs by hydrolytic enzymes limits the level of analgesia induced by ECs (7,11,17,18).

Inhibiting degradation of ECs after endogenous release increases EC concentrations at body sites where ECs induce beneficial effects (17,18). Monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) are the main degradation enzymes for 2-AG and AEA, respectively (15,18). Multiple studies have investigated relief of neuropathic pain by using FAAH inhibitors to target endogenous AEA (19-23). However, far fewer studies have examined MAGL inhibitors in neuropathic pain models, owing to the later identification and development of these inhibitors (22,24,25). The effects of MAGL inhibitors were examined in a neuropathic pain model induced by spinal nerve injury (22,25). However, the effects of MAGL inhibitors on animal models of orofacial neuropathic pain have not been assessed. Injury to the trigeminal nerve in the orofacial area induced neuropathic pain in animal models (26-30). This type of nerve injury causes sensitization of the peripheral and central nervous systems (e.g., allodynia and hyperalgesia), which are typical characteristics of neuropathic pain (2,28,29). Furthermore, use of the synthetic cannabinoid WIN 55,212-2 was recently reported to attenuate allodynia and hyperalgesia caused by chronic constriction injury of the infraorbital nerve (ION) (31). The present study examined whether the MAGL inhibitor JZL184 relieved orofacial neuropathic pain associated with partial transection of the ION.

Materials and Methods
The protocol of this study was approved by the Intramural Animal Care and Veterinary Science Committee of Matsumoto Dental University (No. 277; 2015). All experiments were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 8023) (32), as revised in 1996, and the International Association for the Study of Pain in Conscious Animals. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Animals
All experiments involved male C57Bl/6J mice purchased from SLC Inc., Japan. At the beginning of the experiments, all animals were 8 to 9 weeks of age and weighed 20 to 26 g. The mice were housed under standard conditions at a room temperature of 22 ± 2°C, a relative humidity of 40 ± 5%, and a photoperiod of 12L:12D. Food and water were provided ad libitum.

Preparation of the animal models
Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the ION was exposed with a small intraoral incision along the gingivobuccal margin proximal to the first molar. This method was adapted from a previously described model of neuropathic pain (33,34). Once visible, the nerve was freed from connective tissue and hemisected. This intraoral approach left intact the hair and skin on the snout and the vibrissae of the animal but partially transected the ION.

For comparison, a sham model was produced by scratching the gingivobuccal margin proximal to the first molar under pentobarbital anesthesia (50 mg/kg i.p.), without exposing the ION.

Behavioral assessment
The head-withdrawal threshold (HWT) to mechanical stimulation was assessed in the whisker pad area (Fig. 1A) by using a series of von Frey filaments (0.008 g, 0.02 g, 0.04 g, 0.07 g, 0.16 g, 0.4 g, 0.6 g, 1.0 g, 1.4 g, and 2.0 g) (Touch Test Sensory Evaluator; North Coast Medical Inc., Gilroy, CA, USA). The method for detecting HWT was adopted from a previous study (35). Mice were trained for 5 to 7 days before determining HWT. During training, each mouse was held by the experimenter in such a manner that the animal could move its head freely in the experimenter’s hand. After 5 to 7 days of training, the mice remained calm in the experimenter’s hand with its head exposed. HWT could then be assessed by two experimenters: one held each mouse in his/her
hand with its head exposed while the other experimenter applied the von Frey filament to the center of the whisker pad in a region ipsilateral to the ION hemisection/sham operation. von Frey filaments were applied by using an up-down method, as described previously (35). Whenever a positive response to a von Frey stimulus occurred, the next-weakest filament was applied. If no positive response was observed, the next-strongest filament was applied. Experiments proceeded in this manner until a positive response to mechanical stimulation was achieved. The HWT was measured in each mouse before the surgical procedure was carried out and then on days 3, 5, and 7 after the ION hemisection/sham operation. On day 7 postoperatively, the threshold was measured before and 2 h after the intraperitoneal injection of JZL184 or vehicle solution (36), to investigate the effects of the drugs on behavioral changes.

**Drugs and chemicals**

JZL184 [4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl)(hydroxy)methyl)piperidine-1-carboxylate] (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in a mixture of ethanol, cremophor (Calbiochem, Darmstadt, Germany), and saline (ethanol:cremophor:saline, 1:1:18) (37). The 1:1:18 mixture was used as the vehicle solution. JZL184 was administered intraperitoneally (i.p.) at a dose of 1, 4, and 16 mg/kg of body weight on day 7 after ION hemisection. This dose range was selected because it was used in earlier studies (24,36).

**Immunohistochemistry**

Fluorescent immunohistochemistry was used to investigate relative MAGL immunoreactivity in tissues from the trigeminal spinal subnucleus caudalis (Vc) and upper cervical spinal cord (C1-C2) in the treatment groups. Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 2 h after administration of JZL184 or vehicle solution. Whole brains were rapidly dissected, postfixed at 4°C overnight in the same fixative solution, and then transferred into 30% sucrose in 0.1 M phosphate buffer saline (pH 7.2) at 4°C for 24 h for cryoprotection. Tissues from the brainstem were then systematically cut with a microtome into a series of 20-μm thin sections. Every fifth section was mounted on amino silane-coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan) and then dried for 12 h at room temperature. After washing three times, sections were blocked with 5% normal goat serum in Triton X (0.3%) in 0.01 M PBS for 1 h, after which sections were incubated with primary antibody (rabbit anti-MAGL, 1:100; Abcam, Cambridge, UK) for 24 h. Sections were washed and then incubated with secondary antibody (Goat Anti-Rabbit Alexa Fluor 594, 1:200; Molecular Probes, Eugene, OR, USA) for 1.5 h. Sections were then washed three times and cover-slipped with ProLong Gold Antifade Reagent (Life Technologies, Carlsbad, CA, USA). Finally, stained sections were viewed and imaged with a camera attached to a BZ-X700 fluorescence microscope (Keyence Corp., Osaka, Japan). To investigate relative MAGL immunoreactivity, we specifically analyzed an area measuring 720 × 540 μm within each thin section of Vc or C1-C2 tissue. In each mouse, three sections (one with the largest number of labeled cells and the following two serial sections) were selected for quantitation with ImageJ software (NIH Image, Bethesda, MD, USA). An immunofluorescent intensity was defined as positive when it was greater than twice the background intensity. No specific immunoreactivity was observed in sections when the primary antibody was omitted from the immunohistochemistry procedure.
Statistical analysis
One-way analysis of variance (ANOVA) followed by the Dunnett test was used to compare postoperative and preoperative behavioral data. The Student t-test was used to compare pre-injection and post-injection behavioral data obtained on day 7 postoperatively. One-way ANOVA followed by the Tukey test was used to compare behavioral data between the sham and other groups on day 7 postoperatively. Statistical analysis of the number of MAGL-immunoreactive neurons was done with one-way ANOVA followed by the Tukey test. A P value of <0.05 was considered to indicate statistical significance.

Results
Nocifensive behavior
HWT was measured before ION hemisection or sham operation and on days 3, 5, and 7 postoperatively. On day 7 postoperatively, we injected JZL184 or vehicle solution, and HWT was measured before and 2 h after the injection. JZL184 was administered at a dose of 1, 4, and 16 mg/kg of body weight (n = 5 for each dose group).

Fig. 2 (A) Immunohistochemical photomicrograph showing MAGL-IR neurons (arrowheads) in the Vc of mice, and (B) the numbers of MAGL-IR neurons in the Vc of mice. Data indicate means ± standard error of the mean (SEM).

Fig. 3 (A) Immunohistochemical photomicrograph showing MAGL-IR neurons (arrowheads) in the C1-C2 of mice, and (B) the numbers of MAGL-IR neurons in the C1-C2 of mice. Data indicate means ± standard error of the mean (SEM).
MAGL immunoreactivity

Fluorescent immunohistochemistry for MAGL in Vc and C1-C2 was used to investigate MAGL activity in the brainstem terminals of trigeminal afferent nerves. In sham and ION mice, MAGL immunohistochemical analysis was performed 2 h after vehicle or JZL184 (16 mg/kg, i.p.) administration. The numbers of MAGL-IR cells in the Vc and C1-C2 were significantly greater ($P < 0.05$) in neuropathic mice than in those receiving the sham operation (Figs. 2, 3). Two hours after injection of JZL184 16 mg/kg, the numbers of immunoreactive cells in the Vc and C1-C2 had significantly decreased ($P < 0.05$), as compared with neuropathic mice (Figs. 2, 3).

Discussion

The present results show that orofacial neuropathic pain was successfully attenuated in ION-injured mice by inhibiting the enzyme responsible for degrading 2-AG, a dominant form of endocannabinoid. In addition, the numbers of MAGL-IR cells in the Vc and C1-C2 were significantly higher after ION injury and significantly lower 2 h after JZL184 administration.

Orofacial neuropathic pain is a chronic condition of the head, neck, face, and oral and perioral regions. Multiple studies reported that injury to the peripheral nerves induces chronic pain in extensive areas of the body innervated by both injured and uninjured nerve fibers (2,37). Injury to peripheral orofacial nerves may occur during tooth extraction, root canal treatment, and dental implant surgery (37). However, the mechanisms underlying neuropathic pain are not well understood (1,2). Moreover, present treatments are associated with a number of adverse effects and are not effective; therefore, effective therapeutic options are needed (3).

In the present study, administration of the MAGL inhibitor JZL184 attenuated neuropathic pain behavior in mice. MAGL is the predominant serine hydrolase responsible enzyme for degrading 2-AG (38), which was reported to be the most abundant endocannabinoid in the central nervous system (39). This endocannabinoid is thus a promising treatment option for neuropathic pain and other pain-related disorders. MAGL inhibitors block degradation of 2-AG, thereby increasing its concentration and consequent activity on cannabinoid receptors. Initial MAGL inhibitors, such as methyl arachidonoyl fluorophosphonate and N-arachidonoyl maleimide, were not potent and were poorly selective. In addition, it was difficult to determine whether the observed pharmacological effects of these inhibitors were due to MAGL inhibition or inhibition of another serine hydrolase (40,41). However, JZL184 is more selective for MAGL than for FAAH (36). Earlier studies reported that 2-AG level in the mouse brain was increased by 8-fold, without a change in anandamide level, after acute (single) administration of JZL184 (36), and that MAGL inhibition by JZL184 was rapid and persistent. Maximal inhibition was achieved within 0.5 h of acute administration of JZL184 at a dose of 16 mg/kg and persisted approximately 24 h (36).

Mechanical- and acetone-induced cold allodynia from chronic constriction injury of the sciatic nerve was attenuated within 2 h by JZL184 administration in neuropathic mice (42). JZL184 had analgesic effects in a variety of pain assays, including the acetic acid writhing test of visceral pain (36), formalin-induced pain (43), carrageenan-induced pain (44), cisplatin-induced pain (24) and capsaicin-induced thermal hyperalgesia (45). However, no previous study investigated the relative effects of JZL184 on orofacial neuropathic pain. In the present study, we observed that enhanced nocifensive behavior associated with trigeminal nerve injury was attenuated after acute administration of JZL184.

As compared with sham mice, MAGL immunoreactivity was upregulated in the Vc and C1-C2 of neuropathic mice. The terminals of primary afferent neurons receiving sensory inputs from the whisker pad are predominantly localized to the Vc and C1-C2 areas (2,26,38,46,47). Additionally, injury to the trigeminal nerve increases activity in VC and C1-C2 neurons (38,48,49). Because endocannabinoids are synthesized in response to both physiological and pathological stimuli, increased neuronal activity in response to nerve injury may therefore increase endocannabinoid levels in VC and C1-C2 neurons. In a spinal nerve injury model, concentrations of major endocannabinoids (2-AG and AEA) were increased in the dorsal root ganglia and spinal cord (50-53) after sciatic nerve and spinal nerve injury. We did not directly measure 2-AG levels in the Vc and C1-C2; however, the observed upregulation of MAGL immunoreactivity may be indirectly indicated the elevation of 2-AG levels in these areas. The elevated levels of 2-AG may then have been rapidly degraded by increased levels of MAGL. Previous studies found that endocannabinoids had a relatively short effect, largely because of efficient metabolic action by degrading enzymes (15,18,36). Because of the rapid degradation of 2-AG by MAGL, it had no apparent analgesic effect in neuropathic mice. Moreover, when the action of MAGL is inhibited by JZL184 administration, 2-AG concentration might persistently increase in the brain, including the Vc and C1-C2, thus attenuating neuropathic pain. Consistent with our findings, a previous study found that
upregulation of MAGL immunoreactivity in the spinal cord dorsal horn after chronic constriction injury of the sciatic nerve was subsequently downregulated by intrathecal application of a cannabinoid receptor agonist (54).

The side-effect profile of targeting ECs for pain control may be better than that for synthesized cannabinoids (CB1 and CB2 ligands). Although we did not evaluate potential side effects in this study, several previous studies reported that, unlike direct cannabinoid agonists, ECs degrading enzyme inhibitors produced antinociception without inducing cannabimimetic side effects like hypomotility, hypothermia, catalepsy, and hyperphagia (25,55-57). Previous studies reported no or fewer signs of physical dependency in patients treated with these inhibitors (58,59). Recently, MAGL inhibition with JZL184 showed antinociceptive and anti-inflammatory effects but did not result in functional tolerance or cannabinoid dependency (60). Inhibition of degradation may enhance ECs specifically at sites where they are produced on demand, which results in more-localized activation of cannabinoid receptors as compared with globally acting exogenous cannabinoid receptor agonists. Selectively increasing endocannabinoid tone in neural circuits of nociception can attenuate pain, with no or few adverse effects (61). Similarly, enhancement of endocannabinoid tone in localized areas of the orofacial pain pathway (as observed in our study) may produce analgesia without major side effects. However, this hypothesis requires evaluation in future studies.

Although the mechanisms responsible for biosynthesis and degradation of ECs are not fully understood, accumulating evidence (7,14-16) suggests that 2-AG is produced and released from postsynaptic neurons in an activity-dependent manner (often referred to as “on demand”) and diffuses locally toward the presynaptic terminal to act on cannabinoid receptors. Inactivation of 2-AG results from rapid uptake into neurons (by putative endocannabinoid transporters), followed by intracellular degradation by degrading enzymes (mainly MAGL) (62,63). Although our study design did not allow us to determine whether the MAGL-IR neurons observed in VC and C1-C2 areas were presynaptic or postsynaptic, previous electron microscopic studies reported MAGL expression on presynaptic neurons (64,65). MAGL was also found to be expressed in microglia and astrocytes and may be involved in inactivation of released ECs (66,67).

In conclusion, this is the first systematic study of the effect of MAGL inhibitors on neuropathic pain in ION-injured mice. The findings suggest that inhibition of the degradation of the major endocannabinoid 2-AG is a promising therapeutic option for orofacial neuropathic pain.

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Conflict of interest

The authors have no conflict of interest to declare.

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