analgesic effect of morphine in the normal state with that of morphine in neuropathic pain. Here, we demonstrated that the efficacy of systemically administered morphine against mechanical hyperalgesia after nerve injury was reduced compared with its acute antinociceptive effect. To our knowledge, this is the first study to reveal a relative lack of analgesia upon systemic administration of morphine in an animal model of neuropathic pain when compared with the normal state. In the current study, we also obtained other new findings. First, serotonergic descending inhibition of pain transmission was involved in morphine-induced analgesia under the normal state. However, the noradrenergic system was not activated by systemic morphine. Second, the character of serotonergic descending modulation changed from inhibitory to facilitatory after nerve injury through 5-HT3 receptors in the spinal cord.

The analgesic effects of systemically administered morphine have been shown to be mediated in part by descending bulbospinal projections that inhibit dorsal horn neuronal responses to noxious stimuli.[1,7–9] μ-Opioid receptors are densely expressed in the PAG and RVM.[32–34] The PAG projects to the RVM,[35] and morphine-induced PAG activation stimulates RVM output neurons synergistically with the direct effect of morphine on μ-opioid receptors in RVM neurons.[36–38] The PAG also projects to locus coeruleus,[39] and these supraspinal effects are then thought to project through descending serotonergic and noradrenergic neurons to the spinal cord.[40] In the current study, the 5-HT concentration in the lumbar spinal cord increased in normal and SNL rats after injection of intraperitoneal morphine when compared with saline treatment, which agrees with previous reports that also suggested that morphine induces spinal 5-HT increase.[7–9] Furthermore, in the current study, the systemic morphine-induced 5-HT increase in the spinal cord coincided with activation of serotonergic neurons in the RVM as determined by immunohistochemistry.

It is widely accepted that descending noradrenergic inhibition also plays a role in analgesia produced by opioids.[1,41,42] Systemic administration of opioids is believed to induce spinal noradrenaline release. However, only three studies have directly tested this hypothesis and the results are controversial. In a study in sheep, intravenous administration of morphine (1 mg/kg) increased noradrenaline levels in the cerebrospinal fluid and spinal dorsal horn.[43] However, in two recent studies in rats, intraperitoneal administration of morphine (10 mg/kg) reduced noradrenaline levels in the cerebrospinal fluid.[44,45] In the current study, the noradrenaline concentration in the lumbar spinal cord did not change, even though we previously used the same microdialysis technique to detect a three- to five-fold increase in noradrenaline levels after systemic administration of noradrenaline reuptake inhibitors, including antidepressants.[24,25] Therefore, our study suggests that spinal noradrenaline release actually plays a minimal role, if any, in systemic morphine-induced analgesia.

The RVM is recognized as a critical relay site for integrating descending modulatory inputs to the spinal cord.[3,4] Serotonergic cell bodies in the raphe magnus nucleus provide dense projections to the dorsal horn of the spinal cord, and this descending pathway has been shown to mediate the antinociceptive action of systemic morphine.[10,11,46] However, it has also been demonstrated that activation of supraspinal facilitatory pathways from the RVM maintains the abnormal, enhanced pain state associated with peripheral nerve injury.[12–15] Experimentally produced neuropathic pain can be reversed by lidocaine microinjection into the RVM, supporting the critical role of descending facilitation in chronic pain.[15,47] Although the function of serotonergic neurons in the RVM remains unclear, they may play both inhibitory and facilitatory roles in nociceptive transmission.[10,11] In SNL, Leong *et al.*[48] demonstrated that the number of RVM neurons, including serotonergic neurons, decreased, and the remaining serotonergic neurons mediated descending facilitation. Leong *et al.*[48] thus proposed that the loss of RVM neurons shifts the balance of descending control from pain inhibition to pain facilitation. In the current study, we also found that SNL significantly decreased the number of serotonergic neurons in the RVM. Therefore, degeneration of inhibitory serotonergic neurons in RVM may underlie the reduced efficacy of systemic morphine for neuropathic pain.

Chronic pain states are associated with enhanced descending facilitation of pain signaling, mediated in part through activation of excitatory spinal 5-HT3 receptors.[4,13,16] 5-HT3 receptors are expressed in the terminals of primary afferents[49] and also in the somadendritic regions and presynaptic terminals of γ-aminobutyric acid neurons in the spinal dorsal horn.[50] Stimulation of 5-HT3 receptors in the spinal cord can thus result in facilitation of pain transmission by increasing neurotransmitter release from the primary sensory afferents[51] or inhibition of pain transmission by increasing γ-aminobutyric acid release.[50,52] Animal studies have suggested that spinal 5-HT3 receptor blockade[4,13] attenuates nerve injury–induced hypersensitivity, and a clinical study demonstrated that ondansetron can attenuate chronic pain.[53] Paradoxically, 5-HT3 receptor agonists also reduce hypersensitivity in some animal pain models.[54,55] Thus, increased 5-HT levels in the spinal cord after systemic morphine administration likely result in activation of both inhibitory and facilitatory pathways through spinal 5-HT3 receptors, and the efficacy of morphine may therefore depend on the balance between these pathways.[56] Many studies have supported the dysfunction of γ-aminobutyric acidergic inhibition in the spinal cord after nerve injury, based on immunohistochemistry[57–60] or electrophysiological recordings.[58,59,61,62] We speculate that these functional changes in γ-aminobutyric acidergic interneurons in the spinal dorsal horn may underlie the switching of spinal 5-HT3 receptor activation from antinociceptive in

the normal state to prohyperalgesic in the setting of neuropathic pain.

The current ondansetron and 5,7-DHT experiments suggest that local activation of spinal 5-HT3 receptors by 5-HT released from spinally projecting RVM neurons upon systemic morphine administration facilitates analgesia in the normal state but blunts analgesia in neuropathic pain. Together with our observation of reduced numbers of putative opioid-activated descending inhibitory neurons in the RVM after SNL, these results support the idea that nerve injury leading to neuropathic pain shifts the balance of serotonergic nociceptive modulation toward descending facilitation of pain transmission and thus decreases the analgesic efficacy of morphine. Our results also suggest that this descending pain facilitation may at least in part be blocked by 5-HT3 antagonism, which is highly relevant because 5-HT3 antagonists, such as ondansetron, are already used clinically as antiemetic agents for patients treated with opioids. Therefore, 5-HT3 antagonists may be a promising tool to enhance the analgesic effects of morphine in neuropathic pain.

Acknowledgments

This work was supported by a grant (Grant-In-Aid for Scientific Research 23390373 to Dr. Obata and 24890036 to Dr. Kimura) from the Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan.

Competing Interests

The authors declare no competing interests.

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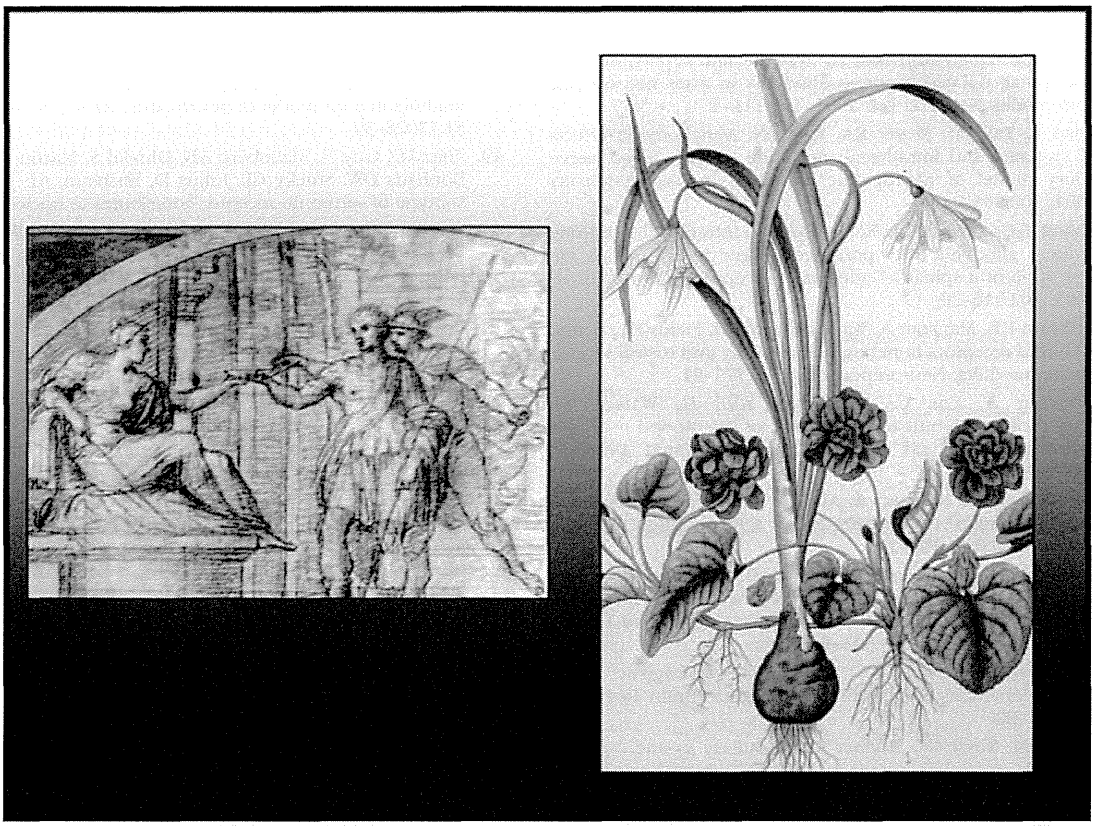

Above the double-flowering sweet violets (ca.1870, *right*), W. H. Prestele depicted Snowdrops from the genus *Galanthus* of winter/spring blooming plants with dark roots and down-facing white flowers. Snowdrops were linked (1722) by British poet Thomas Tickell to the herb Moly that wing-helmeted Hermes (*left*, A. Carracci's ca.1590 *Mercury Protecting Ulysses from the Charms of Circe*) gave Odysseus (Ulysses) to counteract the potion of the witch-goddess Circe. Plaitakis and Duvoisin (1983) have suggested that the Snowdrop's anticholinesterase inhibitor, galant(h)amine, could be apotropaic (warding off evil) against the tropane alkaloidal effects of Circe's anticholinergic potion. (Copyright © the American Society of Anesthesiologists, Inc.)

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Section Editor: **Jianren Mao**

The Antihyperalgesic Effects of Intrathecal Bupropion, a Dopamine and Noradrenaline Reuptake Inhibitor, in a Rat Model of Neuropathic Pain

Hajime Hoshino, MD, Hideaki Obata, MD, PhD, Kunie Nakajima, MD, PhD, Rie Mieda, MD, and Shigeru Saito, MD, PhD

BACKGROUND: Antidepressants are often used for the treatment of neuropathic pain, and their analgesic effects rely on increased noradrenaline and serotonin levels in the spinal cord. Clinical studies have also shown that bupropion, a dopamine and noradrenaline reuptake inhibitor, has strong efficacy in neuropathic pain; however, the role of spinal cord dopamine in neuropathic pain is unknown. We hypothesized that bupropion inhibits neuropathic pain by increasing noradrenaline and dopamine in the spinal cord. In the present study, we determined the efficacy and underlying mechanisms of intrathecal administration of bupropion in a rat model of neuropathic pain.
METHODS: Male Sprague-Dawley rats were anesthetized, and right L5 spinal nerve ligation (SNL) was performed to produce mechanical hyperalgesia of the hindpaw. Withdrawal threshold to a paw pressure test was measured before and after intrathecal administration of bupropion, without or with intrathecal antagonists for α_2-adrenoceptors and dopamine D_2 receptors. In vivo microdialysis was performed in the dorsal horn of the lumbar spinal cord to measure noradrenaline and dopamine concentrations after intrathecal injection of bupropion. We also measured the noradrenaline and dopamine contents in the ipsilateral dorsal lumbar spinal cord in normal rats and in rats 2, 3, and 4 weeks after SNL.
RESULTS: Intrathecal injection of bupropion produced a dose-dependent antihyperalgesic effect (3, 10, 30, and 100 μg, $P < 0.001$). The effect (30 μg) was dose-dependently reversed by intrathecal pretreatment (15 minutes before bupropion injection) with the α_2-adrenoceptor antagonist idazoxan (3, 10, and 30 μg, $P < 0.001$) and D_2 receptor antagonist sulpiride (3, 10, and 30 μg, $P < 0.001$). Microdialysis revealed that noradrenaline and dopamine concentrations in the spinal dorsal horn were increased after intrathecal injection of bupropion (30 μg, $P < 0.001$ and $P = 0.001$, respectively). Furthermore, the noradrenaline and dopamine contents in the spinal dorsal horn were increased 2 weeks after SNL ($P < 0.001$ and $P = 0.044$, respectively) and then decreased gradually.
CONCLUSIONS: These findings suggest that plasticity of descending inhibitory pathways such as the noradrenaline and dopamine systems contributes to the maintenance of neuropathic pain and that spinal cord noradrenaline and dopamine both play an inhibitory role in neuropathic pain.
(Anesth Analg 2015;120:460–6)

Peripheral nerve injury leads to neuropathic pain, which is associated with various changes in sensory processing from the primary afferent neurons to the spinal cord and on to supraspinal and cortical regions. Brainstem-spinal descending inhibitory pathways, including noradrenergic neurons, suppress nociceptive signals from primary afferent neurons to the spinal dorsal horn.[1] Antidepressants such as tricyclic antidepressants and serotonin-noradrenaline reuptake inhibitors are thus recommended as first-line drugs for the treatment of neuropathic pain,[2] as a recent study showed that increased noradrenaline levels in the spinal cord underlie the therapeutic effect of antidepressants in neuropathic pain.[3]

Dopamine also plays a crucial role in nociceptive transmission in the central nervous system.[4] Animal studies have shown that in the brain the dopaminergic system is involved in pain modulation[5,6] and that dopamine receptor agonists predominantly suppress pain-related responses via dopamine D_2 receptors.[7,8] Furthermore, focal electrical stimulation of the A11 area in the brain suppresses the nociceptive responses of spinal dorsal horn neurons.[9] These findings suggest that activation of descending dopaminergic pathways and subsequent release of dopamine in the spinal cord play an important role in the control of nociceptive transmission. However, the role of spinal cord dopamine in neuropathic pain is not clear.

Bupropion is a noradrenaline and dopamine reuptake inhibitor that has been reported to show strong efficacy against neuropathic pain.[10,11] We hypothesized that bupropion suppresses neuropathic pain by increasing noradrenaline and dopamine levels in the spinal cord. To test this

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Accepted for publication September 26, 2014.

Funding: This work was supported by Grants-in-Aid for Scientific Research (grant number 20591823 to HO, 42791578 to RM, and 24791581 to KN) from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (Tokyo, Japan).

The authors declare no conflicts of interest.

Reprints will not be available from the authors.

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DOI: 10.1213/ANE.0000000000000540

hypothesis, we examined the efficacy and mechanisms of the antihyperalgesic effects of intrathecally administered bupropion using a rat model of neuropathic pain produced by spinal nerve ligation (SNL). Because previous studies have suggested that the descending noradrenergic system shows plastic changes after nerve injury,[3,12,13] we also examined the change of noradrenaline and dopamine levels in the lumbar spinal cord over time after SNL.

METHODS

Surgical Preparation

The experiments were approved by the Animal Care and Use Committee of the Gunma University Graduate School of Medicine. Adult male Sprague-Dawley rats (250 g) were used (99 rats). The animals were housed on soft bedding in a temperature-controlled environment under a 12-hour light-dark cycle with free access to food and water. The animals were allowed to habituate to the housing facilities before surgery or behavioral testing.

SNL was performed as previously described.[14] In brief, animals were anesthetized with isoflurane (2%) in oxygen, and the right L5 spinal nerve was tightly ligated with 5-0 silk and cut just distal to the ligature. The wound was then closed. Ten days after SNL surgery, an intrathecal catheter was inserted for drug administration. A sterilized 32-gauge polyethylene catheter (RecathCo, Allison Park, PA) connected to 8.5 cm of Tygon external tubing (Saint-Gobain Performance Plastics, Akron, OH) was inserted through the cisterna magna while the rat was under isoflurane anesthesia, as previously described.[15] The catheter was passed caudally 7.5 to 8.0 cm from the cisterna magna to the lumbar enlargement. The animals were allowed to recover for 7 days before drug testing.

Behavioral Testing

The withdrawal threshold to pressure applied to the hindpaw, expressed in grams, was measured using an analgesimeter (Ugo Basile, Comerio, Italy), as previously described.[16] In brief, the analgesimeter device is used to apply increasing pressure to the hindpaw. When the animal withdraws its paw, the pressure is immediately released, and the withdrawal threshold is output in grams. A cutoff of 250 g was used to avoid tissue injury. Animal training with the analgesimeter was performed 3 times to familiarize the animals to the test before drug treatment.

Drug Testing and Their Administration

The first series of experiments was performed to examine the time course and dose response of the antihyperalgesic effects of intrathecally administered bupropion (0, 3, 10, 30, and 100 μg). The withdrawal thresholds were determined before (before SNL surgery), at 0 (before drug injection), 15, 30, and 60 minutes after injection, and then at 60-minute intervals until 480 minutes after injection. The second series of experiments was performed to determine the effects of intrathecal pretreatment with the α_2-adrenoceptor antagonist idazoxan or the dopamine D_2 receptor antagonist sulpiride. Each antagonist (0, 3, 10, and 30 μg) was administered intrathecally 15 minutes before bupropion (30 μg) injection. Adverse behavioral effects, such as sedation or agitation, were carefully observed and graded as 0 (normal), 1 (moderate), or 2 (severe). Motor function was assessed in terms of the placing reflex and righting reflex with scores of 0 (normal), 1 (impaired), or 2 (absent). To evaluate the placing reflex, the hind limbs of the rat were held and the dorsal surface of the hindpaws was brought into contact with the edge of a table. The experimenter recorded whether the hindpaws were placed on the surface reflexively. To evaluate the righting reflex, the rat was placed on its back on a flat surface and the experimenter noted whether it immediately assumed the normal upright position. Drug tests were performed 17 to 22 days after SNL surgery. To reduce the number of animals, we used the rats 2 times at 2- to 3-day intervals. The experimenter performing the behavioral test was blinded to the drug treatment and dose.

For intrathecal injection, bupropion and idazoxan were dissolved in saline, and sulpiride was dissolved in a mixture of 67% dimethylsulfoxide and 33% saline. The drugs were injected intrathecally in a volume of 5 μL, followed by a 15-μL injection of saline to flush the catheter. Bupropion and idazoxan were purchased from Sigma (St. Louis, MO), and sulpiride was purchased from Tocris (Ellisville, MO).

Microdialysis

Microdialysis was performed as previously described.[17] Anesthesia was induced with 3% isoflurane and maintained with 1.5% isoflurane in 100% oxygen through a nose cone. The left femoral vein was cannulated for fluid infusion. The rectal temperature was maintained at 37°C to 38°C with a heating pad placed beneath the animal. The L3-6 level of the right spinal cord was exposed by thoracolumbar laminectomy, and then the rat was placed in a stereotaxic apparatus. After the dura was punctured with a 30-gauge needle, the microdialysis probe (outer diameter = 0.22 mm, inner diameter = 0.20 mm, length = 1 mm; A-I-8-01; Eicom Co., Kyoto, Japan) was inserted from just lateral to the dorsal root and advanced at an angle of 15° to 30° to a depth of 1 mm using a micromanipulator (model WR-88; Narishige, Tokyo, Japan). The microdialysis probe was perfused with Ringer's solution (147 mmol/L NaCl, 4 mmol/L KCl, and 2.3 mmol/L $CaCl_2$) at a constant flow rate (1 μL/min) using a microsyringe pump (ESP-64; Eicom Co.). After 120 minutes of constant perfusion, 2 consecutive samples were collected to determine basal noradrenaline and dopamine concentrations in the dialysate (baseline). The effective dose of bupropion (30 μg) or saline (5 μL) was administered through the intrathecal catheter, and 15-minute perfusate fractions were collected into an auto injector (EAS-20; Eicom Co.). The noradrenaline and dopamine concentrations in the perfusate were analyzed using high-performance liquid chromatography with electrochemical detection using an HTEC-500 analyzing system (Eicom Co.). The chromatographic conditions were as follows: The mobile phase comprised 0.1 mol/L ammonium acetate buffer (pH 6.0), 0.05 mol/L sodium sulfonate in methanol (7:3 vol/vol), and 50 mg/L Na_2-EDTA, and the column was an EICOMPAC CAX (2.0 mm × 200 mm; Eicom Co.). The working electrode was glassy carbon (WE-3G; Eicom Co.) with a flow rate of 0.25 mL/min. The detector voltage was set to 0.45 V. The detector temperature was set to 35.0°C. The retention time for noradrenaline and dopamine was 5.4 minutes and 7.1 minutes, respectively.

Noradrenaline and Dopamine Contents in the Spinal Cord

We also measured the noradrenaline and dopamine contents in the spinal dorsal horn in normal and SNL rats at 2, 3, and 4 weeks after SNL surgery. To isolate the dorsal horn of the spinal cord, the portion corresponding to segments L4-6 was divided into 4 constituent quadrants: dorsal right, dorsal left, ventral right, and ventral left. The dorsal right (ligation side) portion of the spinal cord was weighed and homogenized in 500 μL of 0.2 mol/L perchloric acid containing 0.1 mmol/L Na_2-EDTA and isoproterenol (0.02 mg/mL) as an internal standard, and centrifuged at 20,000*g* at 0°C for 15 minutes. The supernatants were adjusted to pH 3.0 by adding 1 mol/L sodium acetate and then filtered through a centrifugal filter with a pore size of 0.45 μm (Millipore, Bedford, MA). Samples (10 μL) were injected into an HTEC-500 analyzing system (Eicom Co.), and the concentrations of noradrenaline and dopamine were analyzed using high-performance liquid chromatography with electrochemical detection. The chromatographic conditions were as follows: The mobile phase comprised 0.1 mol/L phosphate buffer (pH 6.0) containing 5 mg/L Na_2-EDTA, 190 mg/L sodium 1-octanesulfate acid, and 17% methanol, and the column was an EICOMPAK SC-5ODS (3.0 × 150 mm; Eicom Co.). The working electrode was glassy carbon (WE-3G; Eicom Co.), with a flow rate of 0.5 mL/min. The detector voltage was set at 0.75 V. The detector temperature was set at 35.0°C. The retention time for noradrenaline and dopamine was 4.43 minutes and 9.56 minutes, respectively.

Statistical Analysis

We selected a minimal sample size of 6 based on our previous study.[18] The data are presented as the mean ± SEM. The effects of the drug treatments on withdrawal thresholds in the behavioral studies and on spinal cord noradrenaline and dopamine levels in the microdialysis studies were analyzed using 2-way repeated-measures analyses of variance (ANOVA), followed by the Student *t* test with Bonferroni correction for dose-response analysis. Independent analyses were performed for each time point by the Student *t* test and report *P* values that are Bonferroni corrected; differences between time points were not compared statistically. The change in the noradrenaline and dopamine contents in the spinal cord over time after SNL, with the level in normal (control) rats included as a representative baseline, was evaluated using a 1-way ANOVA, followed by the Student *t* test and report *P* values that are Dunnett corrected because the focus of the analysis was the change in the injured animals at 2, 3, and 4 weeks after SNL surgery. Before ANOVA, the data were first assessed for normality (Shapiro-Wilk test) and equal variance (*F* test). Because some data from the behavioral studies did not pass these tests, a conservative approach was chosen for the behavioral studies; $P < 0.01$ was defined as statistically significant. The residuals of each of the other 4 ANOVA models followed normal distributions (all 4 $P > 0.251$) and maintained equality of variance (all 4 $P > 0.284$); therefore, $P < 0.05$ was defined as statistically significant. The statistical analysis was conducted using SigmaPlot 12 (Systat Software Inc., San Jose, CA).

RESULTS

Antihyperalgesic Effects of Bupropion

Intrathecal injection of bupropion (3, 10, 30, and 100 μg) produced dose-dependent antihyperalgesic effects ($F_{4,275}$ = 36.77, $P < 0.001$ by 2-way repeated-measures ANOVA followed by the Student *t* test with Bonferroni correction); the effect was observed at 15 minutes and continued to 480 minutes after injection of the 100-μg dose, which was the end of the measurement period in the experiment (Bonferroni-adjusted $P = 0.007$ and 0.001, respectively; Fig. 1). No adverse behavioral effects, such as sedation or agitation, were observed, and the placing reflex and righting reflex were normal (the score was 0 in all rats). Intrathecal pretreatment with idazoxan, an α_2-adrenoceptor antagonist (3, 10, and 30 μg), dose-dependently reversed the antihyperalgesic effect of bupropion (30 μg) ($F_{5,240}$ = 28.82, $P < 0.001$ by 2-way repeated-measures ANOVA followed by the Student *t* test with Bonferroni correction). The maximal dose of idazoxan by itself (30 μg) did not alter withdrawal thresholds compared with the saline group (Fig. 2A). Intrathecal pretreatment with sulpiride, a dopamine D_2 receptor antagonist (3, 10, and 30 μg), dose-dependently reversed the antihyperalgesic effect of bupropion (30 μg) ($F_{5,240}$ = 17.01, $P < 0.001$ by 2-way repeated-measures ANOVA followed by the Student *t* test with Bonferroni correction). The maximal dose of bupropion itself (30 μg) did not alter withdrawal thresholds compared with the saline group (Fig. 2B).

Increased Noradrenaline and Dopamine Levels in the Spinal Cord After Injection of Bupropion Revealed by Microdialysis

Figure 3 shows the time course of the change of the noradrenaline and dopamine concentrations in the dorsal horn of the spinal cord in SNL rats after bupropion injection. The baseline noradrenaline and dopamine concentrations before bupropion injection were 2.48 ± 0.38 pg/15 μL and 0.89 ± 0.19 pg/15 μL, respectively ($n = 6$). After intrathecal

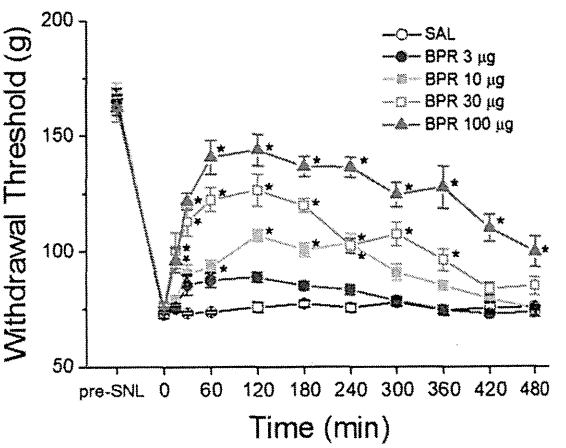

Figure 1. Time course of the antihyperalgesic effect of intrathecally injected bupropion (BPR) in rats with spinal nerve ligation. Intrathecally injected bupropion (3–100 μg) produced a dose-dependent antihyperalgesic effect ($P < 0.001$ by 2-way repeated-measures analysis of variance). All values represent the mean ± SEM for 6 rats. *$P < 0.01$ compared with the saline (SAL)-treated group by the Student *t* test with Bonferroni correction at each time point. SNL = spinal nerve ligation.

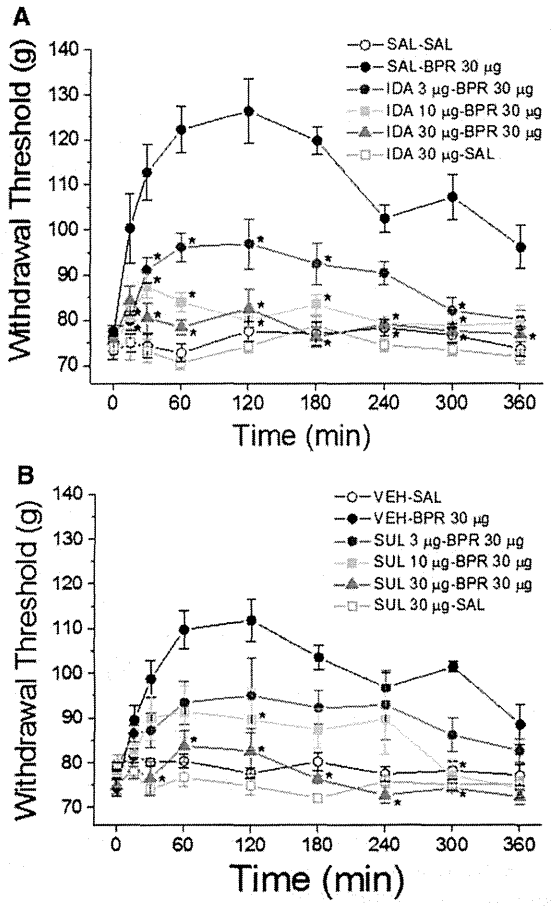

Figure 2. Intrathecal pretreatment with an α_2-adrenoceptor antagonist and a D_2 receptor antagonist dose-dependently reversed the antihyperalgesic effect of 30 μg of bupropion (BPR, $P < 0.001$, respectively, by 2-way repeated-measures analysis of variance). A, After the baseline threshold was determined, rats were intrathecally administered saline (SAL) or idazoxan (IDA, 3–30 μg), an α_2-adrenoceptor antagonist, followed by bupropion injection 15 minutes later. B, After the baseline threshold was determined, rats were intrathecally administered vehicle (VEH) or sulpiride (SUL, 3–30 μg), a D_2 receptor antagonist, followed by bupropion injection 15 minutes later. All values represent the mean ± SEM for 6 rats. *$P < 0.01$ compared with the control (SAL-BPR 30 μg or VHE-BPR 30 μg) group by the Student *t* test with Bonferroni correction at each time point.

injection of bupropion (30 μg), the concentrations of both noradrenaline and dopamine were increased ($F_{1,60}$ = 37.14, $P < 0.001$ and $F_{1,60}$ = 19.55, $P = 0.001$, respectively, by 2-way repeated-measures ANOVA). The noradrenaline concentration increased within 15 minutes (Bonferroni-adjusted $P = 0.013$), and the increase continued for >90 minutes (Bonferroni-adjusted $P < 0.001$) compared with the saline-treated group. The concentration of dopamine also increased within 15 minutes (Bonferroni-adjusted P = 0.023), and the increase was maintained at 45 minutes after injection (Bonferroni-adjusted $P < 0.001$).

Noradrenaline and Dopamine Contents in the Spinal Cord of Normal and SNL Rats

The noradrenaline and dopamine contents in homogenized tissue from the ipsilateral dorsal spinal cord of normal rats (control) and SNL rats were also determined (Fig. 4). The

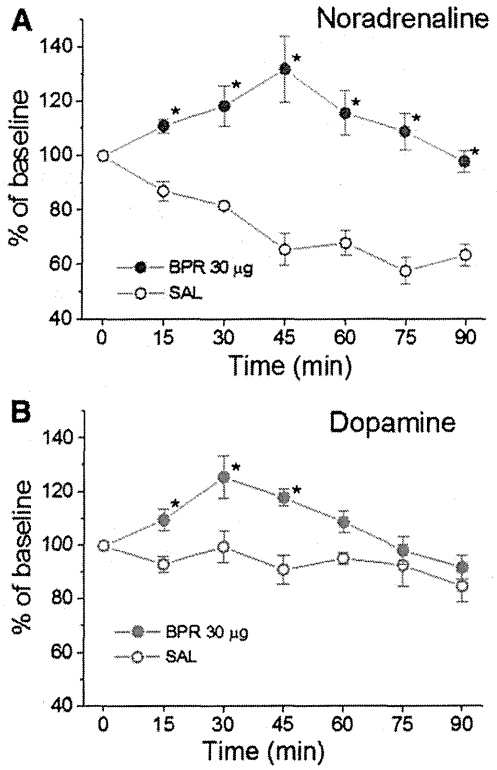

Figure 3. Microdialysis in the dorsal horn of the lumbar spinal cord to determine noradrenaline (A) and dopamine (B) levels after bupropion injection. Rats with spinal nerve ligation were intrathecally administered saline (SAL) or 30 μg of bupropion (BPR). Levels of both noradrenaline and dopamine were increased after bupropion injection ($P < 0.001$ and $P = 0.001$, respectively, by 2-way repeated-measures analysis of variance). Data are presented over time as a percentage change relative to the baseline. All values represent the mean ± SEM for 6 rats. *$P < 0.05$ compared with the control (SAL) group by the Student *t* test with Bonferroni correction at each time point.

noradrenaline concentration in SNL rats 2 weeks after nerve ligation was higher (1617.5 ± 104.0 pg/g, $n = 6$) than that in normal rats (1196.3 ± 117.6 pg/g, $n = 6$, $F_{3,20} = 64.55$, $P < 0.001$ by 1-way ANOVA, Dunnett-adjusted $P < 0.001$). However, the noradrenaline concentration in SNL rats 3 weeks (925.6 ± 49.9 pg/g) and 4 weeks (1037.5 ± 100.3 pg/g, $n = 6$) after ligation was decreased compared with that in normal rats (Dunnett-adjusted $P < 0.001$ and $P = 0.023$, respectively).

At 2 weeks after SNL, an increase in the dopamine concentration in the ipsilateral dorsal spinal cord (220.7± 33.8 pg/g, $n = 6$) was observed relative to normal rats (142.9 ± 23.9 pg/g, $n = 6$, $F_{3,20} = 3.593$, $P = 0.032$ by 1-way ANOVA, Dunnett-adjusted $P = 0.044$), and the dopamine concentration subsequently returned to a level similar to that in normal rats.

DISCUSSION

Bupropion is a dopamine-noradrenaline reuptake inhibitor whose acute administration decreases the reuptake of dopamine and noradrenaline into synaptosomes,[19] reduces the firing rate of central noradrenaline- and dopamine-containing neurons,[20] and increases extracellular striatal dopamine levels.[21] We hypothesized that intrathecal administration of bupropion would suppress neuropathic pain symptoms

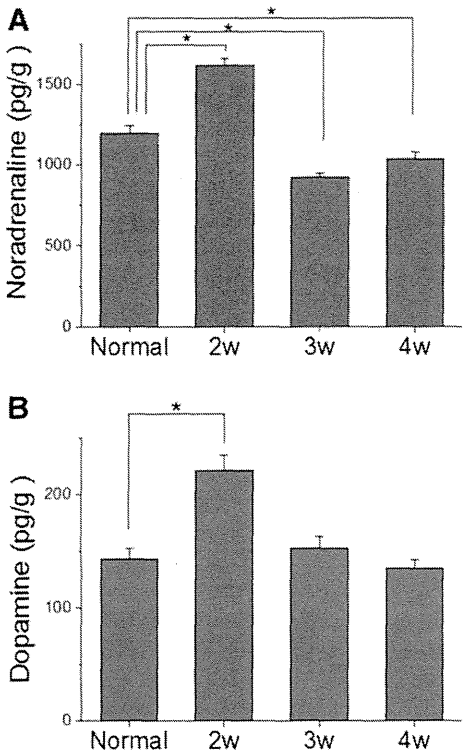

Figure 4. The noradrenaline (A) and dopamine (B) content in the ipsilateral dorsal half of the lumbar spinal cord was measured in normal rats (control) and rats with spinal nerve ligation (2, 3, and 4 weeks after ligation). All values represent the mean ± SEM for 6 rats. *$P < 0.05$ compared with normal rats by the Student t test with the Dunnett correction after 1-way analysis of variance.

through increased noradrenaline and dopamine levels in the spinal cord. We found that intrathecal administration of bupropion indeed produced dose-dependent antihyperalgesia through increased noradrenaline and dopamine levels in the spinal cord, with the effects mediated by spinal α_2-adrenoceptors and dopamine D_2 receptors. Furthermore, the antihyperalgesic effect of the maximal dose of bupropion (100 μg) continued for >8 hours without any adverse effects.

We recently demonstrated that increased noradrenaline levels in the spinal cord play a critical role in the inhibitory effect of antidepressants on neuropathic pain symptoms.[3] Previous studies demonstrated increased potency and efficacy of intrathecal injection of α_2-adrenoceptor agonists such as dexmedetomidine and clonidine, which mimic the effects of spinally released noradrenaline, in neuropathic pain states.[18,22] These pharmacologic effects in neuropathic pain may be associated with spinal cord plasticity because animal models of neuropathic pain have shown increased expression of inhibitory α_2-adrenoceptors in C-fibers,[23] increased G protein coupling of spinal α_2-adrenoceptors,[24] and increased α_2-adrenoceptor–mediated activation of inhibitory cholinergic interneurons.[18,25] Consistent with these reports, intrathecal injection of bupropion in the present study attenuated SNL-induced hyperalgesia, and the effect was dose-dependently reversed by idazoxan, an α_2-adrenoceptor antagonist. In the microdialysis studies, noradrenaline levels were increased in the dorsal horn of the spinal cord after intrathecal bupropion injection. These results indicate that the antihyperalgesic effect of bupropion depends on increased noradrenaline levels in the spinal cord.

Dopamine plays an important role in nociceptive transmission, and several reports have described direct analgesic actions of dopamine in regions of the brain, such as the striatum,[7,26] the basal ganglia,[27] and the nucleus accumbens.[8,28] Dopamine also plays a critical role in nociceptive transmission in the spinal cord through descending inputs from the brain, as a previous study reported that no dopaminergic cell bodies are present in the spinal cord.[29] Electrophysiologic studies have shown that focal electrical stimulation of the A11 area of the brain suppresses the nociceptive responses of neurons in the dorsal horn of the spinal cord.[9] Furthermore, an in vivo patch clamp analysis revealed that dopamine suppressed the synaptic response to noxious stimuli in substantia gelatinosa neurons in the spinal cord.[30] Behavioral studies have also demonstrated that intrathecal administration of a dopamine agonist has thermal antinociceptive effects.[31,32]

Previous studies have shown that dopamine D_2 receptors in the brain contribute to dopamine-induced analgesia for pathologic pain such as inflammatory pain[7,8,33] and neuropathic pain.[26] D_2 receptors are also involved in dopaminergic suppression of nociceptive transmission in the spinal dorsal horn.[30] Therefore, we speculate that D_2 receptors in the spinal dorsal horn are involved in the attenuation of not only acute nociception but also pathologic pain. In the present study, bupropion-induced antihyperalgesia was dose-dependently reversed by sulpiride, a D_2 receptor antagonist. Furthermore, in the microdialysis studies, dopamine levels were increased in the dorsal horn of the spinal cord after bupropion injection. These results indicate that increased dopamine levels and subsequent activation of D_2 receptors in the spinal cord strongly contribute to the antihyperalgesic effect of bupropion.

The change over time of the noradrenaline and dopamine contents in the homogenized tissue from the ipsilateral dorsal lumbar spinal cord after SNL was intriguing. A previous study demonstrated plastic changes in descending noradrenergic neurons after nerve injury,[12] where the density of the descending noradrenergic fibers and noradrenaline content in the ipsilateral lumbar spinal cord were increased 10 days after SNL in rats. In contrast, Hughes et al.[13] reported a loss of noradrenergic fibers in the ipsilateral lumbar spinal cord 19 to 21 days after tibial nerve transection in rats. Although the animal models were different, these results suggest that the tone of the descending noradrenergic system dynamically changes over time after nerve injury. Consistent with these findings, the noradrenaline content in the spinal cord in the present study was increased 2 weeks after SNL, followed by a subsequent decrease to preinjury levels at 3 to 4 weeks. The dopamine content in the ipsilateral lumbar spinal cord was also increased 2 weeks after SNL but then returned to a level similar to that in normal rats. No previous study has examined the plasticity of the descending dopaminergic system after injury; however, our data indicate that the changes in the descending noradrenaline and dopamine systems over time after nerve injury are similar. Several clinically approved treatments for neuropathic pain, including gabapentin[34] and antidepressants,[3] modulate or mimic

the activation of descending noradrenergic pathways to produce analgesia and may overcome or compensate for decreased function of descending inhibitory pathways. We performed behavioral experiments at approximately 3 weeks after SNL surgery. Although we did not compare the efficacy of bupropion across various time points after nerve injury, the plasticity of descending inhibitory systems over time may contribute to the efficacy of antidepressants for neuropathic pain.

Antidepressants, particularly tricyclic antidepressants and serotonin-noradrenaline reuptake inhibitors, are widely used for the management of neuropathic pain,[2] and it is well known that their analgesic effects are mediated by recruitment of descending inhibitory pathways, such as noradrenergic and serotonergic systems.[35] Compared with the large amount of literature on the noradrenergic and serotonergic descending inhibitory systems, however, little information is available regarding the analgesic effects of dopamine. It has been reported that mechanical allodynia is attenuated by systemic administration of bupropion in animal models of neuropathic pain.[36,37] In a small trial of 41 human patients with neuropathic pain of different etiologies, bupropion showed strong efficacy for pain reduction,[10] and the number needed to treat was calculated as 1.6.[11]

Taken together with these previous studies, the present findings provide strong evidence of the therapeutic effect of bupropion, a noradrenaline and dopamine reuptake inhibitor, against neuropathic pain symptoms. Bupropion may thus be useful for the treatment of neuropathic pain through a spinal mechanism. ■

DISCLOSURES

Name: Hajime Hoshino, MD.
Contribution: This author helped conduct the study, collect data, analyze the data, and prepare the manuscript.
Attestation: Hajime Hoshino approved the final manuscript and reviewed the original study data and data analysis. This author attests to the integrity of the original data and the analysis.
Name: Hideaki Obata, MD, PhD.
Contribution: This author helped design and conduct the study, analyze the data, and prepare the manuscript.
Attestation: Hideaki Obata approved the final manuscript and reviewed the original study data and data analysis. This author attests to the integrity of the original data and the analysis.
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Attestation: Kunie Nakajima approved the final manuscript and attests to the integrity of the original data and the analysis.
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Contribution: This author helped to analyze the data and prepare the manuscript.
Attestation: Rie Mieda approved the final manuscript and attests to the integrity of the original data and the analysis.
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Contribution: This author helped to design the study, analyze the data, and prepare the manuscript.
Attestation: Shigeru Saito approved the final manuscript, attests to the integrity of the original data and the analysis, and is the archival author.
This manuscript was handled by: Jianren Mao, MD, PhD.

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World Journal of Neuroscience, 2015, 5, 1-6
Published Online February 2015 in SciRes. http://www.scirp.org/journal/wjns
http://dx.doi.org/10.4236/wjns.2015.51001

Scientific Research Publishing

The Endocannabinoid, 2-Arachidonoyl Glycerol, Induces Growth Cone Collapse and Neurite Retraction in Growing Peripheral Sensory Neurons

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Received 19 November 2014; revised 10 December 2014; accepted 17 December 2014

Academic Editor: Anamitra Ghosh, Van Andel Research Institute, USA

Abstract

Cannabis has a detrimental impact on the developing nervous system. Therefore, regular consumption of cannabis by pregnant and lactating woman poses a potential risk to neuronal growth in fetuses and infants. Indeed, endogenous cannabis-like molecules called endocannabinoids regulate many physiological processes, including neurogenesis, axon guidance, and synaptic plasticity through CB1 receptors. To investigate the physiological role of CB1 receptors on peripheral sensory nerve growth, the endocannabinoid 2-arachidonoyl glycerol was added to cultured chick dorsal root ganglion neurons. This compound inhibited neurite elongation and induced growth cone collapse in a dose- and time-dependent manner. These data suggest that caution should be exercised regarding maternal cannabis use during pregnancy. Because ectopic sprouting and abnormal neuronal network connections are considered to be a cause of neuropathic pain, our current data imply an additional role of endocannabinoids as inhibitors of the formation of pain-maintenance networks.

Keywords

Endocannabinoid, CB1 Receptors, Dorsal Root Ganglion Neurons, Growth Cone

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How to cite this paper: Aso, C., Takazawa, T., Horiuchi, T. and Saito, S. (2015) The Endocannabinoid, 2-Arachidonoyl Glycerol, Induces Growth Cone Collapse and Neurite Retraction in Growing Peripheral Sensory Neurons. *World Journal of Neuroscience*, **5**, 1-6. http://dx.doi.org/10.4236/wjns.2015.51001

1. Introduction

Cannabis is considered a relatively harmless recreational agent despite gathering evidence of its detrimental impact on both the adult and the developing central nervous system (CNS) [1]. This attitude may simply reflect people's recognition that the risk of cannabis is relatively low, particularly when compared to that of tobacco and alcohol [2].

Cannabis has a small molecular size and is generally lipophilic, enabling the compound to readily cross the blood-brain or other cellular (for example, placental) barriers. Thus, regular cannabis use during pregnancy may result in relatively high concentrations of active cannabinoids in the developing fetus [3]. Moreover, cannabis and its metabolites readily pass into breast milk. When cannabis is regularly consumed by breast-feeding mothers, human milk concentrations of delta-9-tetrahydrocannabinol (THC), the main component of cannabis with psychoactive properties, may be up to 8-fold higher than simultaneously measured maternal plasma concentrations [4]. The effects of cannabis on the developing fetus and infant remain uncertain, although some evidence suggests that perinatal cannabis exposure can negatively affect fetal growth [5].

CB1 receptors (CB1Rs) are widely distributed within the brain including cerebral cortex, hippocampus, basal ganglia, and amygdala, as major targets of exogenous cannabinoids such as THC. Moreover, the most abundant endocannabinoid (endogenous cannabinoid: eCB) in the CNS, 2-arachidonoyl glycerol (2-AG), also triggers a broad range of signaling events by acting on CB1Rs. The eCB system regulates many physiological processes including neurogenesis, axon guidance, and synaptic plasticity [6]-[8]. In addition to the CNS, CB1Rs are also expressed in the peripheral nervous system [9] [10]. However, few studies have demonstrated a physiological role for CB1Rs that are expressed in peripheral neurons [11]. Therefore, whether the activation of CB1Rs in developing peripheral sensory neurons affects neuronal growth and function should be determined.

Here we study dorsal root ganglion (DRG) neurons, which are peripheral sensory neurons. The afferents of DRG neurons relay sensory information that originates from the skin to the brain. Therefore, they play a critical role in nociception including inflammatory and neuropathic pain [12]. Cultured DRG neurons isolated from chick embryos can be utilized to evaluate the effects of drugs on neuronal growth during development [13]. We previously show that several local anesthetics have direct neurotoxic effects on cultured DRG neurons [14] [15]. The purpose of the present study is to elucidate the effects of cannabinoids on peripheral sensory nerve growth and regeneration by examining neurite extension and growth cone collapse in particular. For this purpose, cultured DRG neurons are exposed to 2-AG.

2. Materials and Methods

This study was approval by the Institutional Animal Care Committee. Chick neural tissues were isolated from day 7 embryos. To prepare peripheral neurons, DRGs were dissected from lumbar paravertebral sites. After removing the original neurites, the explants were plated on laminin-coated coverslips and cultured in F-12 medium supplemented according to Bottenstein's method [13], containing 100 μg/ml bovine pituitary extract, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml mouse 7S nerve growth factor. Cultures were maintained at 37˚C in 5% carbon dioxide. 2-arachidonoyl glycerol (2-AG) was purchased from Sigma Co. Ltd. (10 mM stock in ethanol).

After culturing for 20 hr, DRGs were exposed to the agents. The neurite length and percent collapse were determined after exposure for 2, 6, or 24 hr. 2-AG was prepared in pre-warmed fresh culture medium and was gently added to the culture medium. The volume of the added solution was 1/100 of the total volume of the culture medium. Cell viability was determined by exposing the cells to vehicle solution for identical durations.

The tissues were fixed with 4% paraformaldehyde in PBS (pH 7.4) containing 10% sucrose as described previously [14] [15] and viewed with a 40× phase objective using a phase-contrast microscope (Axiovert; Zeiss, Germany). Growth cones at the periphery of explants were scored for collapse if they were not in contact or close proximity to other growth cones or neurites. Fifty growth cones were viewed and scored per coverslip. Growth cones without filopodia and lamellipodia were counted as collapsed [14] [15]. The counting was performed by a trained examiner who was blinded to the experimental protocol.

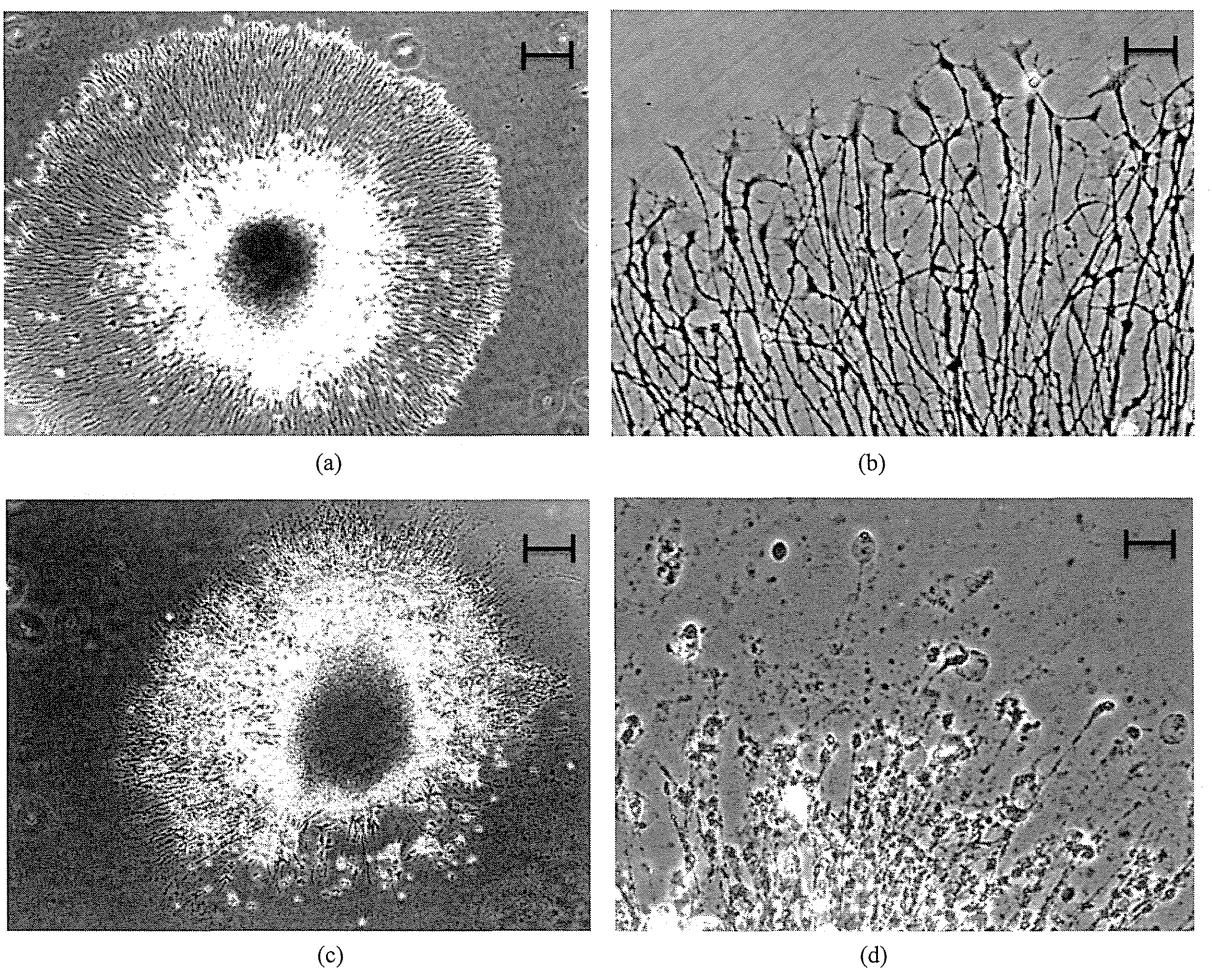
The data are expressed as the mean ± SD of six independent measurements. Each data point for the neurite length and growth cone collapse assays was statistically analyzed with two-way analysis of variance with Bonferroni's method using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). P values less than 0.05 were considered significant.

3. Results

Significant neurite elongation of cultured DRG neurons was observed over time under control condition (*i.e.* without 2-AG). However, the endogenous CB1R agonist 2-AG induced significant inhibition of neurite elongation with growth cone collapse as shown in Figure 1. These effects were dose- and time-dependent (two-way ANOVA, Figure 2). 2-AG (10 μM) blocked axonal elongation at 6 and 24 hr after exposure ($P < 0.0001$, Figure 2(a)). The total neurite length 24 hr after exposure of 2-AG (10 μM) was even shorter than that before exposure suggesting severe neurotoxicity. 2-AG (10 μM) significantly increased the rate of growth cone collapse 2, 6, and 24 hr after exposure ($P < 0.0001$, Figure 2(b)). Even low concentrations of 2-AG (1 and 0.1 μM) increased the rate of collapse in a dose-dependent manner (Figure 2(b)). The effects of 2-AG on neurite length and rate of collapse 24 hr after exposure are summarized in Figure 3(a) and Figure 3(b), respectively.

4. Discussion

In the present study, we showed that 2-AG induced growth cone collapse and inhibited axonal elongation in cultured peripheral sensory neurons. These actions were dose dependent and seemed to be pharmacological. However, we cannot completely exclude the possibility that the effect of 2-AG was not through CB1Rs, because we did not test whether the action of 2-AG was reversed by a CB1R antagonist such as AM251. Whether CB1R antagonists alone have any effect on neuronal growth of developing DRG neurons is also unclear. Testing with CB1R antagonists with neither independent neurotoxic nor neuro-pharmaceutical actions may be useful for



(a) (b)

(c) (d)

Figure 1. Typical neurite retraction and growth cone collapse induced by 2-AG in cultured DRG neurons. (a) (b) The neuron before the application of 2-AG (preexposure); (c) (d) The neuron after exposure to 10 μM 2-AG for 24 hr. Scale bars in (a) and (c) = 10 μm; bars in (b) and (d) = 60 μm.

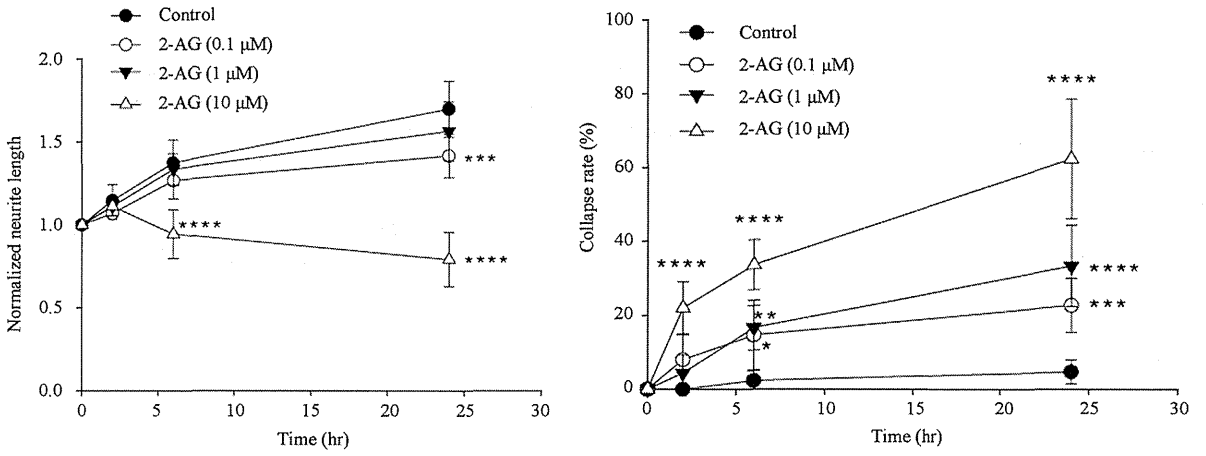


Figure 2. The effect of 2-AG on neurite length (a) and collapse rate of cultured DRG neurons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, two-way ANOVA with a post-hoc Bonferroni test.

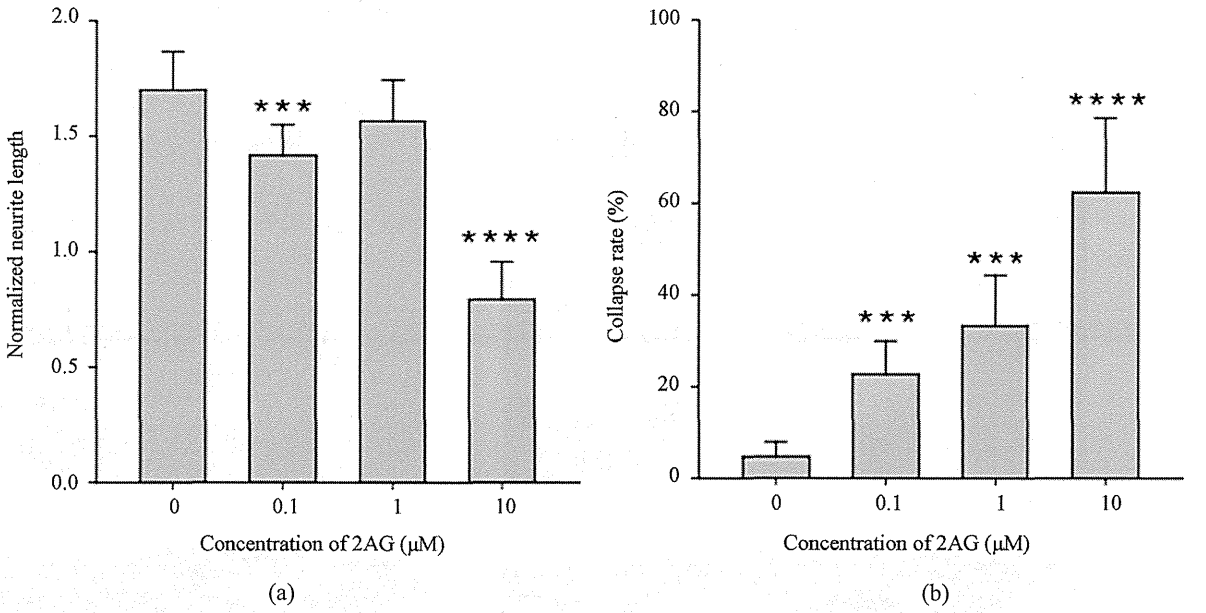


Figure 3. The effect of exposure to 2-AG for 24 hr on neurite length (a) and the rate of growth cone collapse (b) of cultured DRG neurons. $^{***}P < 0.001$, $^{****}P < 0.0001$, two-way ANOVA with post-hoc Bonferroni test.

elucidating the pharmacological action of cannabinoids on developing neurons. Further studies are needed to clarify the mechanisms underlying the inhibitory effect of 2-AG on DRG neurons.

We used chick explant cultures for analysis of the pharmacological actions of 2-AG because growth cones in chick explant cultures have wide fan-like shapes that are suitable for morphological analysis. The growth cone collapse assay is often used for biological analysis of endogenous factors and externally applied substances, both in developmental and regeneration studies. We previously reported that this system is applicable for pharmacological and toxicological studies of neurotropic factors, lipid mediators, and analgesic drugs [14]-[17]. However, the action of eCB may be species specific. Studies with human and other mammalian nervous tissues will be necessary for further analysis of the therapeutic use of cannabinoids.

CB1Rs are targets of exogenous cannabinoids such as cannabis. The current study suggested that activation of CB1Rs during development led to serious negative effects on neuronal growth in the peripheral nervous system. Therefore, high cannabis consumption by pregnant and lactating women may result in sensory system dysfunction in fetuses and infants, although the critical period(s) and doses of cannabis consumption in such situations

are unclear.

The expression of CB1Rs, as well as endocannabinoid levels in the spinal cord, increased in a rat model of neuropathic pain [18] [19]. The authors speculated that eCBs function as endogenous analgesics in neuropathic pain. Clinical studies showed that cannabinoids are applicable in both acute and chronic pain patients as analgesics [20]-[22]. Because ectopic sprouting and abnormal neuronal network connections are considered a cause of neuropathic pain, the actions of eCBs on neurite extension may be crucial for inhibiting painful conditions.

5. Conclusion

Although the precise physiological mechanism is still unknown, our current data imply an additional action of eCBs in inhibiting formation of pain-maintenance networks. Further studies should be performed to clarify the underlying signaling cascades and morphological regulatory roles of eCBs in pain.

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