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Involvement of cannabinoid type 1 receptor in fasting-induced analgesia

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Abstract

The endocannabinoid system (ECS) is known to modulate not only food intake but also pain, especially via the cannabinoid type 1 receptor (CB1R) expressed throughout the central nervous system and the peripheral tissues. Our previous study demonstrated that fasting produces an analgesic effect in adult male mice, which is reversed by intraperitoneal (i.p.) administration of CB1R antagonist (SR 141716). In the present study, we further examined the effect of CB1R expressed in the peripheral tissues. In the formalin-induced inflammatory pain model, i.p. administration of peripherally restricted CB1R antagonist (AM 6545) reversed fasting-induced analgesia. However, intraplantar administration of SR 141716 did not affect fasting-induced analgesia. Furthermore, mRNA expression of CB1R did not change in the formalin model by fasting in the dorsal root ganglia. The formalin-induced c-Fos expression at the spinal cord level was not affected by fasting, and *in vivo* recording from the superficial dorsal horn of the lumbar spinal cord revealed that fasting did not affect formalin-induced neural activity, which indicates minimal involvement of the spinal cord in fasting-induced analgesia. Finally, when we performed subdiaphragmatic vagotomy to block the hunger signal from the gastrointestinal (GI) system, AM 6545 did not affect fasting-induced analgesia, but SR 141716 still reversed fasting-induced analgesia. Taken together, our results suggest that both peripheral and central CB1Rs contribute to fasting-induced analgesic effects and the CB1Rs in the GI system which transmit fasting signals to the brain, rather than those in the peripheral sensory neurons, may contribute to fasting-induced analgesic effects.

Keywords

Fasting-induced analgesia, endocannabinoid system, cannabinoid receptor 1, vagotomy

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Introduction

Pain is a multidimensional experience, considered to have both sensory-discriminative and affective-motivational components.[1] Therefore, pain perception is affected by various psychological interventions.[2] Feeding is a complex behavior with psychological effects and is closely related to pain perception.[3–5] Our previous studies demonstrated that both food hedonics and hunger, innate factors for food-seeking, suppress pain signaling.[6,7]

The endocannabinoid system (ECS) has multiple functions in modulating both food intake and pain. Especially, the cannabinoid type 1 receptor (CB1R) expressed throughout the central nervous system and peripheral tissue regulates hedonic and homeostatic feeding.[8] The activation of CB1R is also well known to suppress pain signaling at supraspinal, spinal and peripheral levels.[9] Therefore, food intake and pain are likely to interact with each other via CB1R. In our

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previous study, the rats consistently displayed an increase in thermal withdrawal latency while drinking sucrose, which was reversed by intraperitoneal (i.p.) administration of CB1R antagonist (SR 141716).[7] We also demonstrated that 24 h fasting produces an analgesic effect in the formalin-induced acute inflammatory pain model, and SR 141716 (i.p.) inhibits the fasting-induced analgesia.[6]

Interestingly, recent works have reported that peripheral ECS is critical for food intake.[10] Acute food deprivation significantly increases anandamide (AEA, the endogenous ligand for CB1R) levels in the small intestine but not in the brain.[11] Furthermore, intracerebroventricular (i.c.v.) administration of SR 141716 did not affect food intake after fasting, whereas i.p. treatment of SR 141716 significantly decreased the amount of food intake in fasted rats.[11] Peripherally restricted CB1R antagonist (AM 6545, i.p.) also is well known to reduce food intake, body weight, and food-reinforced behavior.[12–14] Thus, CB1Rs expressed in peripheral organs might play an important role in transmitting feeding signals to the brain.

Besides, direct activation of CB1R in primary nociceptive sensory neurons is known to produce an analgesic effect in various animal pain models.[15–17] Peripheral administration of AEA attenuated thermal hyperalgesia in the carrageenan-induced inflammatory pain model, which was reversed by SR 141716.[15] Peripherally restricted inhibitor (URB937) of fatty acid amide hydrolase (FAAH), the enzyme for the degradation of AEA, reduced pain response in visceral, neuropathic, and inflammatory pain models.[16] In addition, sensory neuron-specific CB1 knockout mice showed increased basal pain sensitivity.[17] Accordingly, CB1Rs expressed in peripheral neurons could be targeted for endocannabinoid regulation of pain. However, little is known about how peripheral ECS contributes to fasting-induced analgesia. In this study, we thus sought to explore the involvement of peripheral CB1R in fasting-induced analgesia.

Experimental procedures

Animals

Male C57BL/6 mice weighing 18–25 g were used for the experiments and purchased from DooYeol Biotech (Korea). The mice were housed 3–5 per cage at a temperature-controlled room ($23 \pm 1\,^{\circ}C$, 12 h/12 h light/dark cycle with lights on at 08:00) and maintained with standard lab chow (pellet diet) and water ad *libitum* except when food was removed for deprivation experiments. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University and were consistent with the International Association for the Study of Pain (IASP) guidelines.

Formalin-induced pain model

All procedures were prepared as previously described.[18] 20 µl of 1% formalin (formaldehyde solution, 36∼38%, Junsei) was intraplantarly (i.pl) injected into the right hind paw with a 0.3 ml insulin syringe. Following formalin injection, the animals were immediately placed in a test chamber and recorded using a video camera for 40 minutes. The time mice spent licking was measured during each 5 minutes by an observer who was blinded to the treatment. Formalin-induced pain behaviors during 0–10 minutes after formalin injection represented the first phase and during 10–40 minutes after formalin injection represented the second phase.

Administration of drugs

Both AM 6545 (peripherally restricted CB1R antagonist, Tocris) and SR 141716 (CB1R antagonist, Tocris) were diluted in 0.9% saline with 10% DMSO and 1% tween 80 and then sonicated. Either AM 6545 or SR 141716 was injected at a dose of 10 mg/kg (i.p.) in a volume of 10 ml/kg body weight 30 min before formalin injection. To evaluate the peripheral effects of SR 141716, formalin test was performed after i.pl. injection of SR 141716 (10 µg per mouse), which was diluted in 1% formalin (20 µl/0.9% saline).

Quantitative reverse transcription PCR

Mice were sacrificed 1 h after formalin injection. Quantitative reverse transcription PCR was performed as previously described.[19] The primer pairs for activating transcription factor 3 (ATF3) was CCAGGTCTCTG CCTCAGAAG, CCGATGGCAGAGGTGTTTAT. The primer pairs for c-Fos was GGTGAAGACCGTGTCA GGAG, CCTTCGGATTCTCCGTTTCTCT. The primer pairs for cnr1 was ACGGTGTTTGCCTTCTGTAGT, CTGTGTTATTGGCGTGCTTGT. The primer pairs for the synthesizing enzyme N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) was GACGCTGATGG TGGA AATGGA, AGGTGGTCGTAGTGGTTGTGA. The primer pairs for GAPDH was ATGGTGAAGGTC GGTGTGAAC, CATGGTGGTGAAGACACCAGT AG. All experimental groups relative to the naïve control group were calculated by the $\Delta\Delta$CT method with GAPDH as the reference gene.

c-Fos immunohistochemistry

All procedures were prepared as previously described.[18] Animals were sacrificed 2 h after formalin injection. Sections were pre-blocked with 5% normal goat serum

(NGS). Sections were incubated in 1st antibody (PC38, Calbiochem, USA; 1:1000) for 48 h at 4 °C, and incubated in biotinylated goat anti-rabbit (BA1000, Vector laboratories, USA; 1:400) for 2 h at RT. Sections were processed with ABC kit (PK-6100, Vectastain ABC kit, Vector Laboratories, USA), visualized with DAB kit (DAB substrate kit for peroxidase, Vector laboratories, USA). After dehydration steps, all sections were mounted on slide grass with hardening mounting medium (Sigma, Germany) and examined under the bright-field microscope (DM5000B, Leica, Germany)

Cell counting and image analysis

For the quantification of c-Fos expression, we confirmed that most c-Fos expression induced by injection of 1% formalin (i.pl.) localized in Lumbar (L) 4–5 segment. 4–6 sections with the highest expression of c-Fos per animal were chosen, and lamina I-VI and lamina I-II (superficial dorsal horn) of L 4–5 segments were selected for analysis. The number of c-Fos positive neurons was counted blindly, and the mean value was used as representative counts. Using image J, the image of selected sections was converted to a greyscale, background subtracted, enhanced and sharpened. The intensity threshold was adjusted and then analyzed using the "analysis particles" function.

In vivo electrophysiological recording

The methods for making in vivo preparation were similar to those in our previous studies.[20–22] Briefly, mice were anesthetized with urethane (1.2–1.5 g/kg, i.p.) and placed on a warm (~40 °C) plate. A thoracolumbar laminectomy at T13-L2 levels was performed to expose the dorsal surface of the lumbar enlargement of the spinal cord at L3-L5 levels. The mouse was then placed in a stereotaxic apparatus (ST-7M-HT, Narishige, Tokyo, Japan). The dura matter was removed, and the pia-arachnoid membrane was cut for making a small window to insert a tungsten electrode with an impedance of 10 MΩ (FHC, Bowdoin, ME, USA) using a micromanipulator (MHW-4–1, Narishige) at a fixed angle. The electrode was placed into the spinal dorsal horn, and multiunit neuronal firings were amplified with a differential extracellular amplifier (EX1, Dagan, Minneapolis, MN, USA). The signal was bandpass-filtered at 300–3 kHz and sampled at 25 kHz. Recorded signals were spike-sorted with a software (Spike2 ver. 6, Cambridge Electronic Design, Cambridge, UK) as previously reported.[23,24] We detected unit firings if they had basically biphasic shapes, and the amplitude was three times higher than SD of the baseline. If the amplitude and shape of firings were almost the same (see *inset* in Figure 3(Cb)), we used the firings as a single unit. Formalin (20 µl, 1%, i.pl) was injected into the paw ipsilateral to the recording site. We continuously recorded more than 40 min after the formalin injection.

Bilateral subdiaphragmatic vagotomy

All procedures were prepared as previously described.[25,26] Mice were anesthetized by pentobarbital (i.p., 50 mg/kg) and placed in dorsal recumbency. A 1 cm skin incision was made immediately caudal to the xiphoid process. The liver was retracted with a saline dampened cotton swab, and gentle traction was applied to the esophagus by lifting the stomach out of the peritoneal cavity. Esophagogastric junction was visualized with the aid of a surgical microscope, and a vagal segment was isolated then excised bilaterally. The incision in the abdominal wall and skin were closed separately. In sham-operated mice, the vagus was exposed but not excised. A formalin test was performed at least 1 week after vagotomy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, USA). A comparison between the two groups was made using the unpaired Student's t-test. For multiple comparisons, data were analyzed using the one-way ANOVA or two-way ANOVA followed by the Tukey test. Detailed statistics for each experiment were shown in the figure legend. Data are presented as mean ± SEM. Differences with $p < 0.05$ were considered significant.

Results

The peripheral CB1R contributes to fasting-induced analgesia

In a previous study, we showed that 24 h acute fasting suppressed formalin-induced spontaneous pain only in the second phase, which was reversed by systemic administration of SR 141716 (i.p., 10 mg/kg), a CB1R antagonist.[6] To determine the specific role of peripheral CB1R in this study, we used AM 6545 (i.p., 10 mg/kg), which shows markedly reduced brain penetration whereas SR 141716 (i.p., 10 mg/kg) are detected high concentration in the brain at 1 h after treatment of AM 6545 and SR 141716.[12] Both AM 6545 (i.p., 10 mg/kg) and SR 141716 (i.p., 10 mg/kg) were treated 30 minutes before formalin injection (Figure 1(A)), and we compared the effect of AM 6545 and SR 141716 (Figure 1(B)). As compared with the vehicle-treated group, both AM 6545 and SR 141716 significantly reversed fasting-induced analgesia (Figure 1(B) and (C)). These results suggest that the peripheral CB1Rs contribute to fasting-induced analgesia.

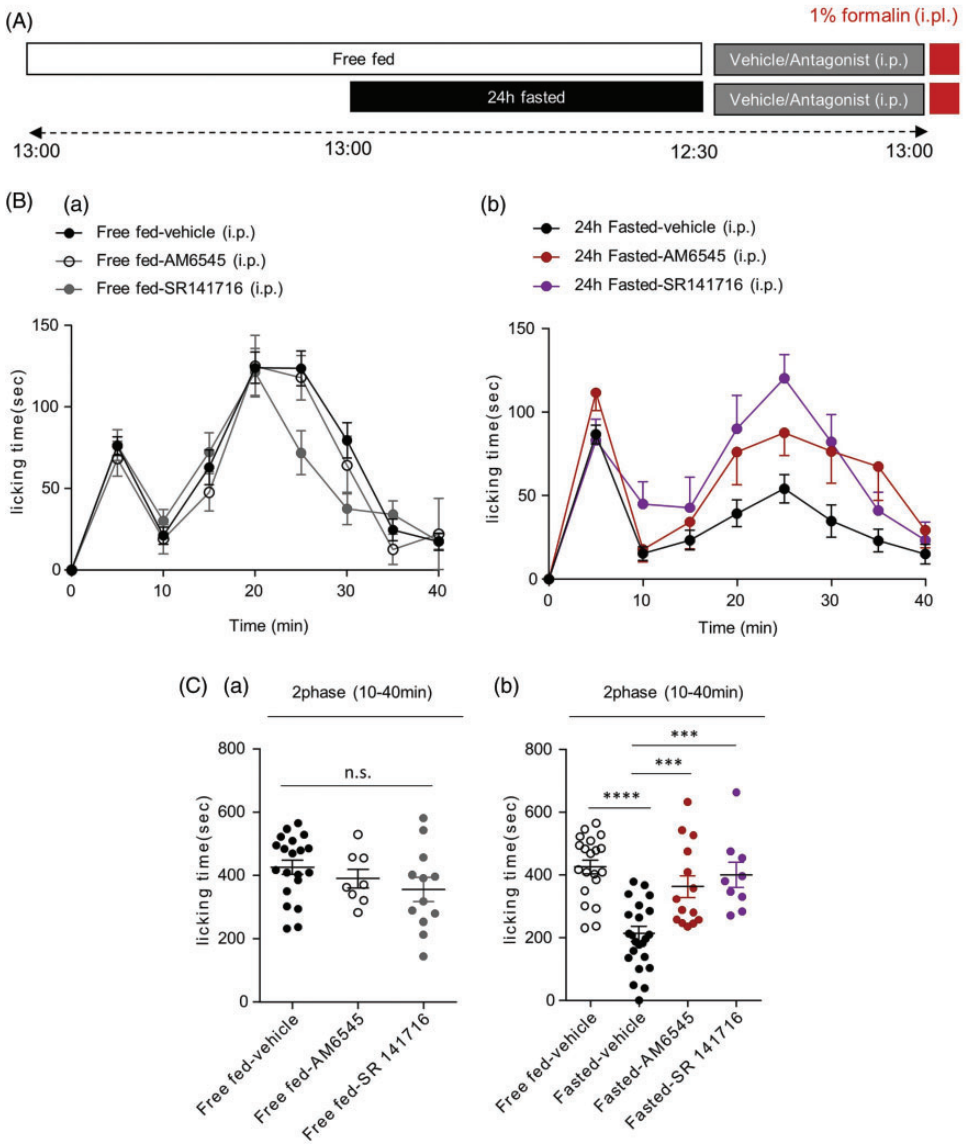

Figure 1. The effect of peripherally restricted CB1R antagonist on fasting-induced analgesia in formalin-induced acute inflammatory pain model. (A) Experimental design and schedule for formalin test. (B) Time course of spontaneous pain behavior following intraplantar (i.pl) injection of formalin in free-fed and 24 h fasted mice who received either AM 6545 or SR 141716, respectively. We adopted the result of SR 141716 from our recent publication ("The analgesic effect of refeeding on acute and chronic inflammatory pain" by Jeong-Yun Lee and Grace J. Lee et al. is licensed under CC BY 4.0). (C) Formalin-induced pain behavior was divided into two phases and analyzed. As compared with free fed-vehicle groups, formalin-induced pain behavior decreased in the 24 h fasted-vehicle group only at 2 phase. As compared with 24 h fasted-vehicle group, AM 6545 and SR 141716 reversed fasting-induced analgesia. ***$p<0.001$, ****$p<0.0001$ ((C) one-way ANOVA followed by Tukey test).

The CB1R expressed in DRG is unlikely to be involved in fasting-induced analgesia

To identify the role of peripheral CB1R in dorsal root ganglion (DRG), we administrated CB1R antagonist into the hind paw. In the previous study, i.pl injection of SR 141716 has been shown to significantly inhibit the analgesic effect of AEA, even at a dose of 100 ng.[15] However, although we co-administrated much higher concentration of SR 141716 (i.pl., 10 μg) with 1% formalin, SR 141716 did not block the analgesic effect of fasting (Figure 2(A)). Furthermore, in the formalin model, there was no difference in mRNA expression of CB1R (encoded by the cnr1 gene) in DRG between the free-fed group and 24 h fasted group (Figure 2(Ba)). Even after 24 h fasting, the formalin-induced ATF3 expression was not different between the two groups (Figure 2(Bb)).

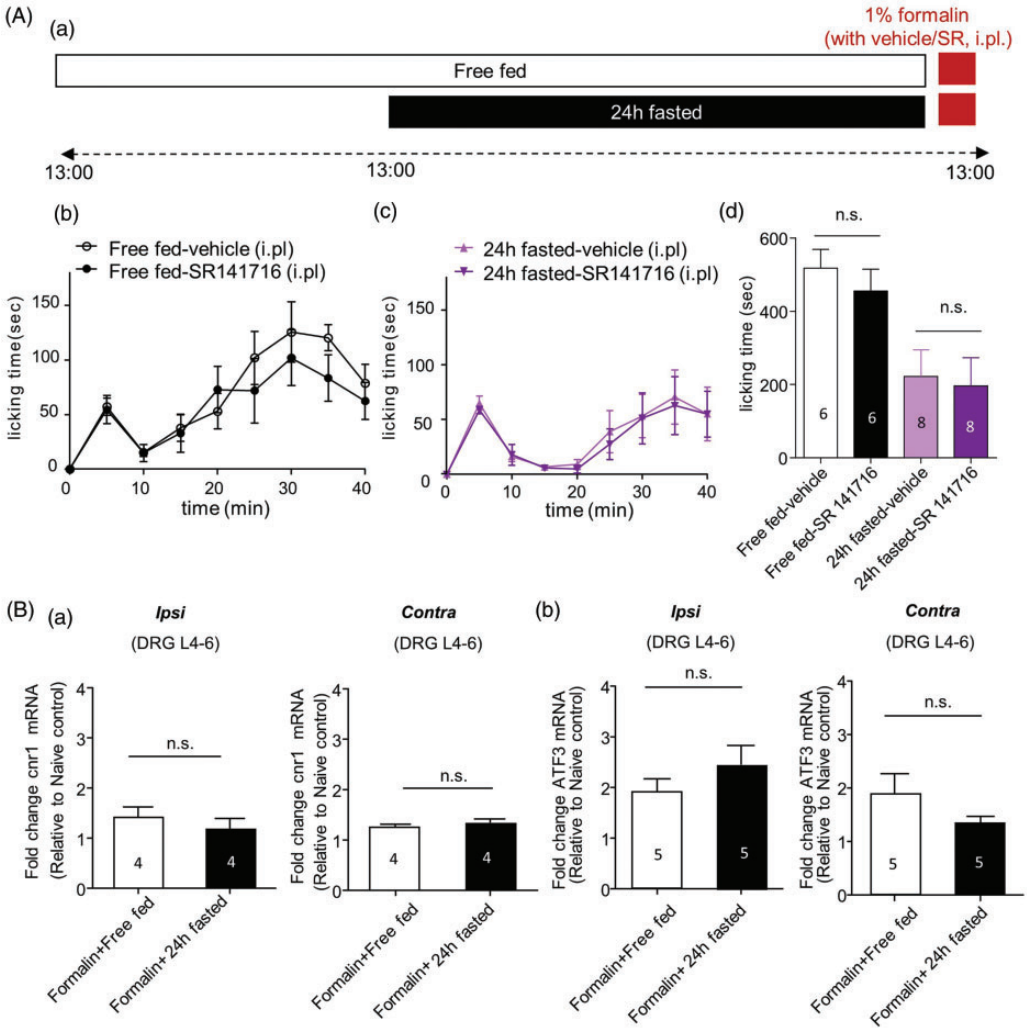

Figure 2. The effect of fasting on nociceptive signaling in the peripheral sensory neurons in formalin-induced acute inflammatory pain model. (A) The effect of intraplantar (i.pl) injection of CB1R antagonist on fasting-induced analgesia. Experimental design and schedule for formalin test (a). Time course of spontaneous pain behavior following injection of formalin (b, c). Formalin-induced pain behavior was divided into two phases, and the second phase was analyzed (d). SR 141716 (i.pl., 10 μg) did not affect the analgesic effect of fasting in the formalin-induced acute inflammatory pain model. (B) The mRNA expression of CB1R (cnr1) (a) and activating transcription factor 3 (ATF3) (b) in DRG. 24 h fasting did not affect the mRNA expression of cnr1 and ATF3 in formalin-induced acute inflammatory pain model ((A), (B) unpaired t-test).

Next, we examined whether fasting suppresses formalin-induced pain signals at the spinal cord level. The protein expression of formalin-induced c-Fos, an important marker of nociceptive neuronal activation,[27] was not different between the free-fed group and 24 h fasted group (Figure 3(A)). The mRNA expression of c-Fos also did not differ between the two groups (Figure 3(B)). Furthermore, the mRNA expression of cnr1 and NAPE-PLD (the major enzyme responsible for AEA) at the lumbar spinal cord remained unchanged after 24 h fasting in the formalin-induced acute inflammatory pain model (Supplemental Figure 1). In the acetic acid-induced visceral pain model, 24 h fasting also did not affect the mRNA expression of c-Fos at the thoracolumbar spinal cord, whereas the mRNA expression of cnr1 and NAPE-PLD tended to increase slightly at thoracolumbar spinal cord (Supplemental Figure 2). We recorded *in vivo* spinal neuronal firings in response to cutaneous formalin injection. Formalin injection into the paw elicited neuronal firings in the spinal dorsal horn in free-fed group. As shown in Figure 3(Ca) and 3 (Cb), the time course of the unit firing number showed a biphasic response. In 24 fasted group, formalin injection also showed a similar biphasic response, and the firing unit number in the 2nd phase was not different between free-fed and 24 fasted groups (Figure 3(Cc)). That in 24

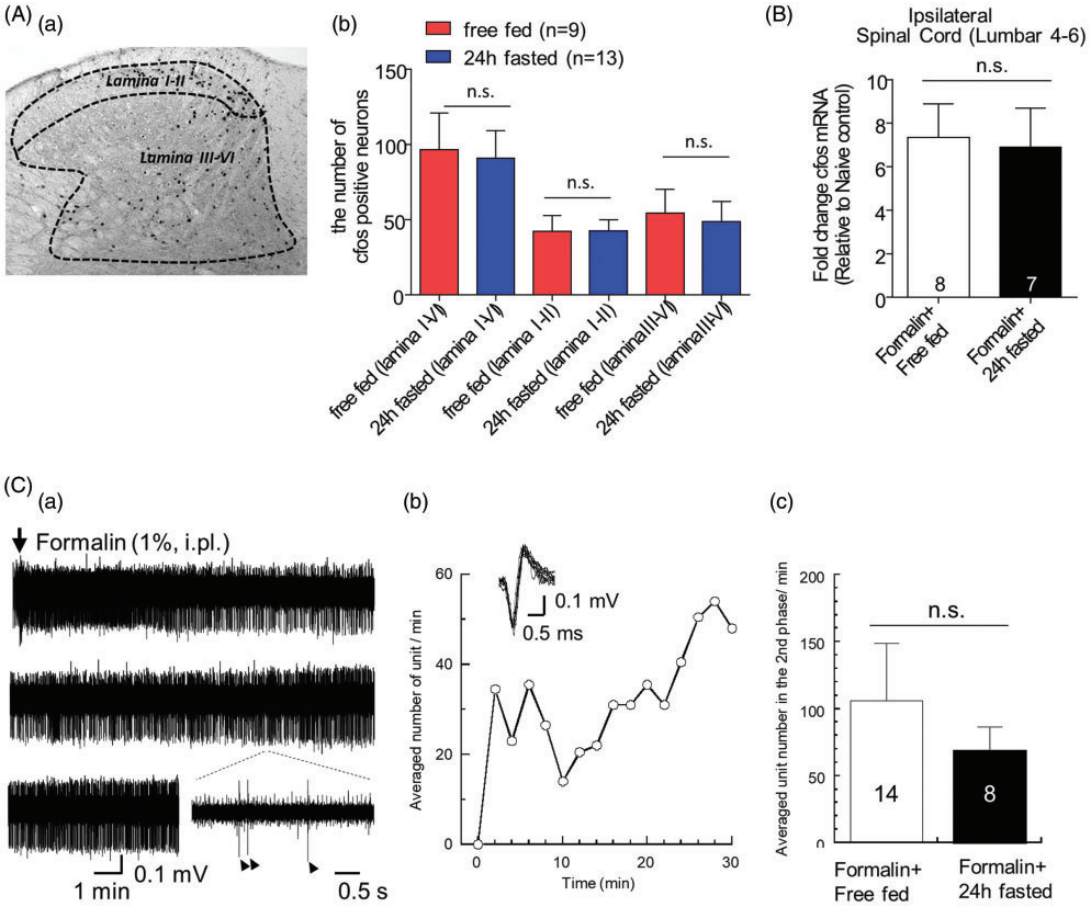

Figure 3. The effect of fasting on nociceptive signaling in the spinal cord in the formalin-induced acute inflammatory pain model. (A) Formalin-induced c-Fos protein expression in the spinal cord of lumbar (L4-L5) segments. Representative expression of c-Fos protein in L4-L5 (a). The quantification of c-Fos protein expression (b). The total number of c-Fos positive neuron from lamina I to VI and the number of c-Fos positive neuron from lamina I to II (superficial dorsal horn) were counted. (B) Formalin-induced c-Fos mRNA expression in the spinal cord of lumbar segments. As compared with the free-fed group, formalin-induced c-Fos expression was not different in the 24 h fasted group. (C) An example of continuous chart recording showing spinal neuronal unit firings in response to cutaneous formalin injection in free-fed mice (a). The lowest trace in the right are shown in an expanded timescale. Arrowheads indicate unit neuronal firings during the action (in the 2nd phase) of formalin. The time-course of the average number of unit firing shown in (*Ca)* showing a formalin-induced biphasic response (b). Insets show ten superimposed single unit firings indicated by arrowheads in *a*. 24 fasted mice also showed a similar formalin-induced biphasic response in the spinal dorsal horn (see *Results*). The number of unit firings in the 2nd phase between free-fed and 24 fasted groups was not different (c) ((A), (B), (C) unpaired t-test).

fasted groups treated with AM 6545 was not also different between free-fed and 24 fasted groups (Supplemental Figure 3).

Collectively, our results showed that the CB1R expressed in DRG and its central inputs to the spinal cord might not play a critical role in fasting-induced analgesia.

SR 141716, but not AM 6545, reversed fasting-induced analgesia after subdiaphragmatic vagotomy

Peripheral CB1Rs are known to regulate feeding behavior via the vagus nerve.[11,28] Therefore, we investigated whether the activation of CB1R from the gastrointestinal (GI) system could modulate fasting-induced analgesia via the vagus nerve. A formalin test was performed at least 1 week after vagotomy (Figure 4(A)). In consistent with a previous study,[29] subdiaphragmatic vagotomy itself significantly suppressed formalin-induced pain behavior only in the second phase (Figure 4(Ba) and (Ca)). As compared to the sham group, fasting-induced analgesia did not differ after subdiaphragmatic vagotomy (Figure 4(Ba) and (Ca)). Following subdiaphragmatic vagotomy, AM 6545 tended to reverse the fasting-induced analgesia, although the effect was not significant (Figure 4(Bb) and (Cb)). On the other hand, SR 141716 significantly reversed fasting-induced analgesia (Figure 4(Bb) and (Cb)). These results suggest that the CB1Rs in the GI system, rather than sensory neurons, may contribute to fasting-

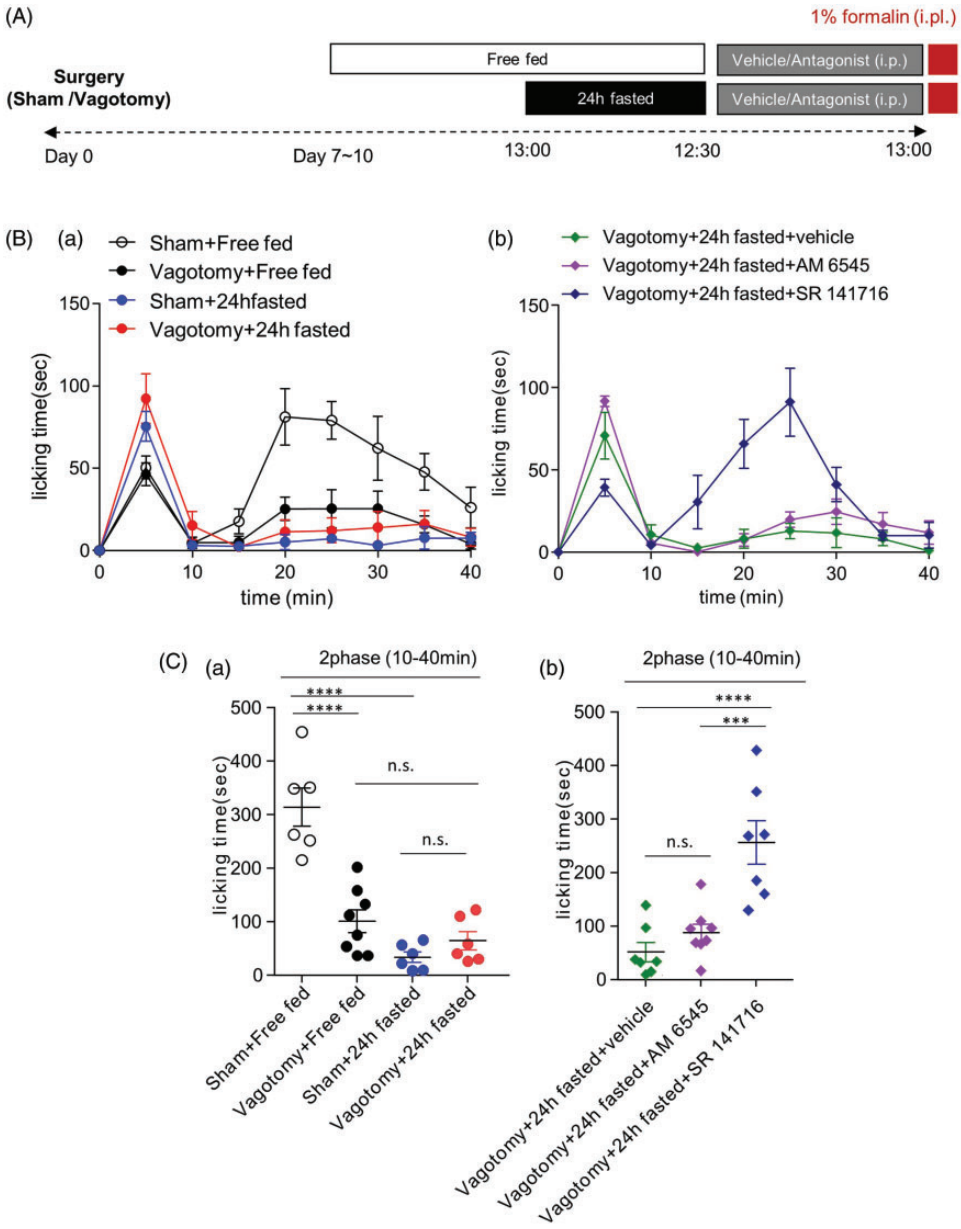

Figure 4. The effect of subdiaphragmatic vagotomy on fasting-induced analgesia in formalin-induced acute inflammatory pain model. (A) Time course of spontaneous pain behavior following intraplantar injection of formalin. (B) Formalin-induced pain behavior was divided into two phases, and the second phase was analyzed. (C) As compared to the sham free-fed group, subdiaphragmatic vagotomy significantly reduced formalin-induced pain behavior. Following subdiaphragmatic vagotomy, AM 6545 did not affect fasting-induced analgesia. SR 141716 reversed the effect of fasting/vagotomy-induced analgesia ***$p<0.001$, ****$p<0.0001$ ((B) one-way ANOVA followed by Tukey test).

induced analgesic effects by transmitting fasting signals to the brain which also expresses CB1Rs.

Discussion

In this study, we found that the analgesic effect of 24 h fasting was reversed by systemic treatment of peripherally restricted CB1R antagonist (AM 6545, i.p.), but not by local injection of CB1R antagonist (SR 141716) into the hind paw in formalin-induced acute inflammatory pain model. Furthermore, there is no difference in formalin-induced c-Fos expression at the spinal cord between the free-fed group and fasted group, and 24 h fasting did not affect formalin-induced neural activity in the superficial dorsal horn of the spinal cord. These results indicate that CB1R expressed in DRG and its

central inputs to the spinal cord are less likely to be involved in the analgesic effect of fasting. On the other hand, the reversal effect of AM 6545 on fasting-induced analgesia was not observed after subdiaphragmatic vagotomy, which suggests a possible critical role of CB1Rs in the GI system for the fasting-induced analgesia.

Our previous study found that 10 mg/kg of SR 141716 completely blocked the effect of 24 h fasting on formalin-induced pain behavior. Since CB1Rs are expressed throughout the central nervous system and peripheral organs, AM 6545, as a peripheral restricted CB1R antagonist, is likely to have a partial effect on fasting-induced analgesia. However, comparing the sum of formalin-induced pain behavior from 10 min to 40 min, the reversal effect of AM 6545 is comparable to that of SR 141716 (Figure 1(C)). These results suggest that peripheral CB1R has an important role in the analgesic effect of fasting.

It has been suggested that short-term fasting suppresses the expression of pain-related protein such as p-ERK, p-CREB, and mTOR in both DRG and spinal cord.[30] Long-term calorie restriction has protective effects against apoptosis, oxidative stress, and increased calcium signaling in DRG by inhibiting the TRPV1 channel.[31] It is also well demonstrated that dietary restriction (intermittent fasting or calorie restriction) has neuroprotective effects in the peripheral nervous system as well as the central nervous system by inhibiting nerve damage.[32–35] Thus, fasting might produce an analgesic effect by modulating DRG neurons. However, i.pl injection of SR 141716 had no effect on fasting-induced analgesia in this study (Figure 2(A)). Besides, the expression of ATF3, a reliable marker of nerve injury, in DRG, which is known to increase by i.pl administration of formalin in a dose-dependent manner,[36,37] was not affected by 24 h fasting (Figure 2(Bb)). Our results also showed that 24 h short-term fasting did not affect the formalin-induced nociceptive signals at the superficial dorsal horn of the lumbar spinal cord (Figure 3). In addition, the mRNA expression of c-Fos was not affected by 24 h short-term fasting at the thoracic spinal cord in the acetic acid-induced visceral pain model (Supplemental Figure 2). Thus, 24 h short-term fasting is less likely to inhibit pain signals via peripheral sensory neurons.

Although 24 h fasting might not be likely to change the transmission of pain signals from DRG to the spinal cord, AM 6545 reversed fasting-induced analgesia in the formalin model (Figure 1). Accumulating evidence suggests that the vagal gut-to-brain axis plays a critical role in modulating cognitive function and behavior. The vagal sensory neurons innervating the GI tract are the primary neuroanatomic substrate that synapses with enteroendocrine cells and rapidly sends signals to the brain.[38,39] Interestingly, peripheral CB1R is known to regulate feeding behavior via the vagus nerve.[11,28] Peripherally restricted pharmacological inhibition of CB1R suppresses alcohol preference behavior by reducing ghrelin production in the stomach cell, which was abolished by chemical or surgical vagotomy.[28] CB1R expressed in upper small-intestinal epithelium regulate secretion of the satiation peptide, cholecystokinin (CCK), and anorexic effect of AM 6545 was blocked by inhibition of CCK_A receptors known to be abundant on the peripheral vagal afferent neuron.[40] Furthermore, fasting-induced hyperphagia was prevented by CB1R antagonist, which was abolished by chemical vagotomy.[11] Thus, vagus nerve transmits feeding signals from the GI system to the brain, which is involved in peripheral CB1R-mediated feeding behavior. In the present study, we found that AM 6545 had no effect on fasting-induced analgesia after subdiaphragmatic vagotomy (Figure 4), whereas AM 6545 significantly inhibited the analgesic effect of fasting in naïve mice (Figure 1). It is known that although CB1R is localized in the vagus nerve as well as the GI tract, CB1R in the vagus nerve did not affect fasting-induced hyperphagia and anorexic effect of SR 141716.[41] Therefore, these results suggest that the CB1Rs expressed in the GI tract transmit fasting signals to the brain, which may induce fasting-induced analgesic effects. It is also interesting to note that although AM 6545 failed to reverse the analgesic effect induced by fasting and vagotomy in the formalin test, SR 141716 blocked these analgesic effects (Figure 4). Thus, the CB1Rs expressed in the brain might be also involved in the analgesic effects of fasting and vagotomy.

The modulation of pain by subdiaphragmatic vagus nerve is known to exhibit bidirectional effect and gender dimorphism. In the previous studies, the bradykinin-induced hyperalgesia was enhanced by subdiaphragmatic vagotomy in both male and female rats.[42,43] However, gonadectomy and adrenal medullectomy completely reverse the effect of subdiaphragmatic vagotomy only in male rats but not in female rats.[43] Moreover, subdiaphragmatic vagotomy significantly reduced formalin-induced pain behavior in male rats but not in female rats.[29] In our present study, the second phase of the formalin test was also significantly decreased by subdiaphragmatic vagotomy in male mice (Figure 4). Besides, 48 h fasting enhances formalin-induced pain behavior in female rats but not in male rats and the effect of 48 h fasting was blocked by subdiaphragmatic vagotomy.[44] However, subdiaphragmatic vagotomy did not affect the effect of 24 h fasting in male mice (Figure 4). In male mice, 48 h fasting enhanced the first phase of formalin-induced pain behavior while suppressing the second phase (Supplemental Figure 4). Therefore, the relationship between the vagus nerve and the effect of fasting on pain varies according to gender, species, and

fasting time, which remains to be elucidated in future studies.

From the present study, our findings suggest that fasting activates peripheral ECS in the GI tract and transmits a hunger signal to the brain via the vagus nerve, which may produce an analgesic effect. Therefore, peripheral ECS and the vagus nerve can be an important factor in the analgesic effect of fasting. Further research is needed to determine the brain circuits that mediate fasting-induced analgesia via the vagus nerve.

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Author Contributions

SBO conceived the idea, obtained funding for the study and guided the project; JYL and CHW performed formalin test; JYL and YJK performed c-Fos staining and histological data analysis; PRL and JYL performed PCR; GJL and JYL performed vagotomy; AN and HF performed *in vivo* recording; JYL and SBO wrote the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental material

Supplemental material for this article is available online.

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