Acetaminophen and pregabalin attenuate central sensitization in rodent models of nociceplastic widespread pain

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ABSTRACT

The “nociplastic pain,” a recently proposed novel mechanistic pain descriptor, is defined as pain occurring through altered nociception without nociceptor activation and nerve injury. Nociplastic pain is often characterized by widespread pain sensitization (WSP) in multiple body regions (Fitzcharles et al., 2021). As many patients with primary chronic pain would have nociplastic backgrounds, developing appropriate methods to evaluate drug effects against nociplastic pain in animal models is in great demand. Using two rat models with the WSP involving central amygdala (CeA) activation by orofacial inflammation or direct chemogenetic activation (Sugimoto et al., 2021), we examined whether widely used analgesics, acetaminophen (AcAph), pregabalin (PGB), and duloxetine (DLX) could attenuate the WSP. AcAph (100 or 200 mg/kg, i.p.) significantly elevated 50%-paw withdrawal threshold (PWT\textsubscript{50}), which had been lowered significantly by upper lip injection of formalin, or systemic injection of clozapine-N-oxide in the rats with excitatory designer receptors (hM3Dq) expressed in the right CeA. This effect lasted for >~4 h. PGB (30 mg/kg, i.p.) also significantly counteracted the lowered PWT\textsubscript{50} in rats with orofacial formalin injection for >~6 h. DLX was ineffective on the WSP. Based on these results, we propose that these preclinical models could be used to evaluate drug effects for primary chronic pain. We conclude that the widely used pain killers, AcAph and PGB, also relieve nociplastic widespread sensitization in the absence of ongoing nociceptor activation and nerve injury.

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1. Introduction

Widespread ectopic sensitization is a hallmark symptom of chronic pain that is characterized by aberrantly enhanced pain sensitivity at multiple body regions remote from the original injury or inflammation (Arendt-Nielsen et al., 2010; Bernstein and Burstein, 2012; Bigal et al., 2008; Campi et al., 2017; Fingleton et al., 2015; Greenspan et al., 2013; Peng and May 2019; Sarlani and Greenspan, 2003). It is generally accepted that nociplastic changes and altered activity in the brain network playing roles in nociceptive signal processing and the control of descending pain modulation underlie these reported cases of widespread sensitization. Indeed, we have recently demonstrated that formalin-induced latent inflammatory pain in the face activates neurons and synapses in the lateral parabrachial nucleus (LPB) and, predominantly, the right central amygdala (CeA) at 6 h post-inflammation (Miyazawa et al., 2018) and results in mechanical sensitization in the bilateral hind paws that lasts for 13 days post-injection (Sugimoto et al., 2021). In addition, we have already demonstrated that selective activation of the GABAergic neurons in the right CeA using chemogenetically activated pro-excitatory receptors gives rise to bilateral sensitization of the hind paw in non-inflamed naïve rats and that the bilateral hind paw sensitization after orofacial latent inflammatory pain is significantly attenuated by inhibition of the neuronal activities of the right CeA by activation of pro-inhibitory receptors (Sugimoto et al., 2021). These results indicate that the CeA, particularly the right CeA, is responsible for ectopic sensitization by latent inflammatory pain.

Therefore, it is expected that drugs affecting the pain network underlying such central sensitization would be promising candidates for pharmacotherapy for primary chronic pain with widespread sensitization. In many clinical cases, gabapentinoids and selective serotonin-and-noradrenaline reuptake inhibitors (SNRIs) are used, but their efficacy remains limited (Percie Du Sert and Rice, 2014). Non-steroidal anti-inflammatory drugs (NSAIDs) have also been used in the clinics, but it

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remains unexplored how inhibition of prostaglandin synthesis could lead to antinociception. Of particular interest to mechanisms of action of acetaminophen (AcAph), an analgesic frequently used in patients with gastrointestinal damage to whom NSAIDs cannot be prescribed, remain only poorly understood. Lines of evidence support the notion that AcAph exerts analgesic effects by affecting endocannabinoid signaling in the central pain network (Klinger-Gratz et al., 2018) and in peripheral/central transient receptor potential (TRP) V1 channels (Ohashi and Kohno, 2020), making it likely that AcAph would affect the widespread sensitization in ectopic inflammation, as in central network activation without injury or inflammation. However, the effects of these drugs have been exclusively analyzed in rodent models of inflammatory pain or neuropathic pain, in which the tactile sensitivity and nocifensive movements are evaluated only at the site of injury or inflammation. Until today, there has been no ideal animal model that enabled analysis of the effects of analgesics on the nocifensive behaviors appearing at a site remote from the injury or even in the absence of primary inflammation or injury. As such sensitization at a remote or distant site, as well as at widespread sites, should involve activation of central mechanisms, establishment of models that allow evaluation of widespread sensitization are necessary for the development of novel drugs that effectively mitigate primary pain of central origin.

This study aimed to examine whether analgesics clinically used for chronic pain of various etiologies could attenuate the sensitization observed in our animal model of inflammation-induced widespread pain (iWSP), which shows manifest mechanical sensitization at remote body sites where there is no injury or tissue damage (Sugimoto et al., 2021). For this purpose, we examined the effects of the following three drugs: 1) AcAph, an analgesic with a limited anti-inflammatory profile and recently proposed central site of action (Barrière et al., 2020); 2) pregabalin (PGB), a ligand for α2δ subunits of voltage-dependent calcium channels, which are expressed in the superficial layer of the dorsal horn but also in the nuclei belonging to the central pain network, such as the LPB and periaqueductal gray (PAG)(Allen Mouse Brain Atlas, 2022); and 3) duloxetine (DLX), an inhibitor of noradrenaline and 5-HT reuptake, which may function in the spinal cord (Kinosita et al., 2013) but also in the brain limbic network (van Marle et al., 2011). If any of these drugs attenuate the widespread sensitization in our iWSP model, it would be interpreted that the drug exerts an analgesic effect not only by affecting peripheral or spinal mechanisms, but also through its effect on the central pain network underlying widespread pain. Secondly, we also examined the effect of AcAph on the nocicplastic pain in our model of amygdala activity-dependent widespread pain (aWSP) to examine whether this nocicplastic pain model without activation of nociceptors or in the absence of neuropathy at the site of sensitization can reveal the analgesic effect of drugs that might not have a site of action in the periphery to the spinal cord. Based on the results presented here, we propose that these animal models of widespread sensitization involving central mechanisms could be used as gold standards for evaluating the effects of drugs potentially used for primary chronic pain with central sensitization.

2. Materials and Methods

Ethical statement

The manipulation of the rats followed the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan (2006), and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Jikei University (2015-008; 2016-066; 2020-062).

2.1. Animals

The animals in all experiments were housed in isolated ventilation cages at 2 to 3 rats/cage with free access to food and water and placed in a temperature/humidity-controlled room with a light/dark cycle (7:00–19:00, white light; 19:00–0:00, red light; 0:00–7:00 dark). The floor of this home cage was covered with conventional soft animal floor bedding (Alpha-dri, EP Trading, Tokyo, Japan). The following two rat strains were used: 1) Wistar/ST rats (Slc: Wistar/ST; Japan SLC, Inc., Shizuoka, Japan; called Wistar rats hereafter); and 2) VGAT-cre BAC transgenic rats (called VGAT-cre rats) (Sugimoto et al., 2021). The VGAT-cre rats were of Wistar strain origin (Crlj:WI; Charles River Laboratories Japan, Inc.), and heterozygote VGAT-cre rats were used. Only male rats were used in this study to minimize the influence of sex hormone variations (see discussion in Sugimoto et al. (2021)).

For experiments using the iWSP model, we used 128 Wistar/ST rats (96, 16, and 16) to test the effects of AcAph, PGB, and DLX, respectively. The rat body weight ranged from 199 g to 417 g, 259 g–282 g, and 246 g–278 g at the start of the AcAph, PGB, and DLX experiments, respectively. For experiments using the aWSP model, 52 VGAT-cre rats were injected with adeno-associated virus (AAV) solution into the right CeA for designer receptors exclusively activated by designer drug (DREADD) experiments (7–15 weeks old; 265 g–494 g on the day of the microinjection). The behavioral data from 28 of these rats were included in the results. The data from the other 24 rats were not included after careful exclusion according to the verification of the injection site (Materials and Methods 2.6.).
2.2. Experimental protocols

We evaluated the effects of AcAph, PGB, and DLX on the estimated 50% threshold for the paw withdrawal responses (50% paw withdrawal threshold, PWT$_{50}$) in 1) the iWSP model (AcAph, PGB, and DLX), and 2) the aWSP model (AcAph).

2.2.1. iWSP model

First, in all animals, we measured the PWT$_{50}$ immediately before starting the following protocols for the “pre” control values. We created an orofacial inflammatory pain model by subcutaneous injection of 50 μL of 5% formalin solution (prepared by diluting 37% formaldehyde solution with 0.9% saline; Nacalai Tesque Inc., Kyoto, Japan) into the left upper lip of the rats, just lateral to the nose, using a syringe with a 30-gauge needle (Becton, Dickinson and Company, Fukushima, Japan) under brief 5% isoflurane anesthesia. An equal volume of saline was injected into some rats (called the “saline” group). All animals injected with formalin, but not those with saline, displayed typical face rubbing behavior lasting for <60 min, a sign of acute nociceptive and inflammatory responses (Miyazawa et al., 2018), which was not analyzed in this study, and the post-formalin measurements of hind paw sensitization were started 1 h after the formalin injection.

2.2.2. aWSP model

A total of 52 VGAT-cre rats received intracranial injection of AAV vector for the expression of DREADDs. The intracranial injection of viral vectors was performed according to a published protocol (Sugimoto et al., 2021). In brief, VGAT-cre rats were initially anesthetized with isoflurane (5% in 100% O$_2$) and given a mixture of medetomidine hydrochloride (0.15 mg/kg, i.p.; Zeneoa, Orion Corporation, Espoo, Finland), midazolam (2.0 mg/kg, i.p.; Astellas, Tokyo, Japan), and butorphanol tartrate (2.5 mg/kg, i.p.; Meiji Seika Pharma, Tokyo, Japan). Under topical infiltration of lidocaine (1%, 0.3 mL; AstraZeneca, Osaka, Japan) into the scalp, an incision was made to expose the skull surface. A small hole was drilled in the skull, and the underlying dura was removed. Using a 2-μL Hamilton microsyringe with a 30-gauge needle (7002 KH, NeuroSyringe), a 0.7-μL virus suspension containing an AAV (serotype 5) encoding one of the following two constructs was delivered into the right CeA: 1) AAV-hSyn-DIO-hM3Dq-mCherry (Addgene), and 2) AAV-hSyn-DIO-mCherry (Addgene). The hM3Dq is a G protein-coupled receptor (GPCR) linked with a Gq-type trimeric G protein. In some experiments, a 1-μL suspension (FluoSpheres, 0.02 μm, yellow-green 505/515; Life Technologies) was mixed with 10 μL AAV for post-experimental confirmation of the injection sites. The stereotaxic coordinates for this injection were 1.9–2.3 mm caudal to the bregma, 4.1–5.0 mm lateral to the midline, and 8.0–8.5 mm ventral to the dorsal brain surface, which was empirically adjusted according to the size and age of the rat. In these VGAT-cre rats, a 2-week daily acclimation to the observation chamber and von Frey test maneuvers (see next section) was started 1 week after the AAV vector microinjection. At 3 weeks after AAV injection, we started repeated measurements of the hind paw sensitization on 3 days: day 1, day 2, and day 3. Immediately after the first measurement, clozapine-N-oxide (CNO) 3 mg/kg i.p. was administered (time 0), and measurements were repeated at 20 min, 40 min, and 60 min for day 1 and day 3 and with an additional measurement at 90 min for day 2. The test drug or vehicle was injected at the same time as CNO on day 2.

2.3. Evaluation of the PWT$_{50}$ (von Frey test)

2.3.1. iWSP model

Beginning a week before the day of the upper lip injection experiments, we removed one rat from the home cage and habituated it in the observation chamber (25 cm × 25 cm, mesh-floor) for about 30 min. The rat was habituated for von Frey test maneuvers every 2–3 days. For the von Frey test, a calibrated series of von Frey filaments (0.4 g–15 g; North Coast Medical, Inc., Gilroy, CA, USA) was applied to the hind paw plantar from underneath the mesh floor, and the withdrawal response was recorded to evaluate the 50% response threshold with the up-and-down method (Chaplan et al., 1994) in a semi-blinded manner to calculate the PWT$_{50}$. The evaluators were semi-blinded to the presence of formalin injection-associated facial swelling by putting the chamber on a dark shelf with minimal light to observe the hind limb movement (in all von Frey filament tests). Because of the dark shelf and minimal light, the experimenter could not see the faces of the rats. However, if the evaluators were to see the rat face, they would be able to guess whether the rat had swelling on the cheek, even under dim light. Although they tried not to do so, we thus describe the evaluators as being semi-blinded. The PWT$_{50}$ values at the left and right hind paw were always measured in pairs. Their mean values are indicated as “mean±S PWT$_{50}$” in the text and figures. After the von Frey test, the rat was immediately returned to the home cage, unless otherwise stated.

On the day of the orofacial formalin or saline injection, we evaluated the PWT$_{50}$ (this value is called “Pre” hereafter), as in the previous days. Then, the rat was placed in a box filled with 5% isoflurane and formalin or saline solution was injected into the subcutaneous tissue of the left upper lip under light anesthesia. The experimenter was blinded to the injected solution. After injection, the rat was immediately brought back to the home cage with free access to food and water. In cases where the von Frey test was done at 30-min intervals, the rats were not returned to the home cage after every von Frey test measurement, and freely accessible water was provided in the observation chamber. The exact time points at which the measurements were made relative to the drug injection timing are indicated in each figure.

2.3.2. aWSP model

Following a week of rest after AAV vector injection and a subsequent 2 weeks of daily acclimation to the observation chamber and von Frey test maneuvers, as in the iWSP model, a rat with intra-amygadal AAV injection was removed from the home cage and placed in the observation chamber for around 30 min. The experiment was done over 3 days. On day 1 and day 3, after we evaluated the PWT$_{50}$ (Pre1,3), we intraperitoneally injected the rat with a 3-mg/mL solution of CNO. At 20, 40, and 60 min after injection, we evaluated the PWT$_{50}$. On day 2, after evaluating the PWT$_{50}$ (Pre2), we intraperitoneally injected AcAph (200 mg/kg, i.p.) with CNO. We evaluated the PWT$_{50}$ at 20, 40, 60, and 90 min after injection. The von Frey evaluation was repeated according to the timing shown in each figure until day 3. The PWT$_{50}$ evaluators were blinded to the type of the injected AAV.

2.4. Preparation and injections of drugs

2.4.1. Preparation of drugs

AcAph (Sigma-Aldrich, Tokyo, Japan) was dissolved in 0.9% saline at 15 mg/mL immediately before use. This solution was used for intraperitoneal injections (dose, 100 or 200 mg/kg body weight, i.e., 6.7–13.3 mL/kg). The 0.9% saline solution was used as the control and called “vehicle” to avoid confusion with the saline used as the control for the formalin. PGB C–V (Sigma-Aldrich) was dissolved in 0.9% saline at 10 mg/mL and used immediately or frozen overnight until use. This solution was used for intraperitoneal injections (dose, 30 mg/kg body weight, i.e., 3 mL/kg). DLX hydrochloride (Santa Cruz Biotechnology) was dissolved in 0.9% saline at 1 mg/mL and used immediately or frozen overnight until use. This solution was used for intraperitoneal injections (dose, 10 mg/kg body weight, i.e., 10 mL/kg). For the vehicle group, the same volume of 0.9% saline solution was administered. These injection solutions were administered using a 1-mL or 5-mL syringe, depending on the injected volume, with a 23-gauge needle. CNO was dissolved at a concentration of 3 mg/mL with 0.9% saline, dispensed into stock solutions, and kept frozen at −30 °C until the day of use. The solutions were thawed immediately before administration, and a solution of 3 mg/mL concentration was used for intraperitoneal injections (dose, 3 mg/kg

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body weight, i.e., 1 mL/kg). Freeze/thaw cycles were limited to three times but mostly comprised one overnight thaw. Each rat received an injection of either drug or saline solution, which was masked to the injector at injection time.

2.4.2. Injection timing

For iWSP experiments, the PWT<sub>50</sub> was evaluated immediately before formalin injection (“Pr”), and analgesic drugs were intraoperatively injected at 3.5 h (AcAph, PGB, and DLX) or 6.5 h (AcAph) after orofacial formalin injection. For aWSP experiments, CNO alone (on days 1 and 3) or together with AcAph (on day 2) was injected. The PWT<sub>50</sub> was evaluated immediately before the CNO injections (days 1, 2, and 3; “Pr”) and repeated until 60 min (days 1 and 3) or 90 min (day 2). The details of the timing of the paw withdrawal threshold measurements are described in the schema in the corresponding figures. During repeated von Frey tests, the rats were kept in the allodynia observation chamber, in which water was freely available.

2.5. Post-experimental histological confirmation of the injection sites

At the end of the behavioral experiments, the rats were sacrificed under 5% isoflurane. The brains were removed and immersed in 4% paraformaldehyde for 1 or 2 nights. Then, they were sectioned into 150-μm slices and coverslipped. The fluorescence of mCherry and microspheres in the CeA was visualized by a fluorescence microscope (BX-63; Olympus, Tokyo, Japan) and visually confirmed after registration of the slice to the Paxinos and Watson atlas (Paxinos and Watson, 2007) using PowerPoint, and the border of the fluorescence signal was drawn on the atlas of the corresponding brain levels. Three experienced authors, blind to the behavioral results, observed the microscope pictures taken using the abovementioned methods. We examined the slices from 54 rats, but 2 of these rats died unexpectedly during the injection operation and were thus not included in the results; thus the total number of rats used for the behavior and histological analyses was 52. The criteria for “successful injection and gene expression” were as follows. 1) Fluosphere bead fluorescence was found in the CeA, showing that the tip of the injection needle reached the CeA. However, the Fluosphere beads were not always evident after weeks of preservation, presumably due to loss of the tissue around the tip location during the tissue sectioning. 2) The whole CeA region (CeC, CeL, and CeM) showed consistent mCherry fluorescence. This was often accompanied by extra-CeA expression (dorsally in the globus pallidus and laterally in the lateral amygdala). 3) Successful injection and gene expression for the behavior and histological analyses was 52. The criteria for the behavior and histological analyses was 52. The criteria for 2 of these rats died unexpectedly during the injection operation and were thus not included in the results; thus the total number of rats used for the behavior and histological analyses was 52. The criteria for “successful injection and gene expression” were as follows. 1) Fluosphere bead fluorescence was found in the CeA, showing that the tip of the injection needle reached the CeA. However, the Fluosphere beads were not always evident after weeks of preservation, presumably due to loss of the tissue around the tip location during the tissue sectioning. 2) The whole CeA region (CeC, CeL, and CeM) showed consistent mCherry fluorescence. This was often accompanied by extra-CeA expression (dorsally in the globus pallidus and laterally in the lateral amygdala). 3) Successful injection and gene expression for the behavior and histological analyses was 52. The criteria for the behavior and histological analyses was 52. The criteria for 2 of these rats died unexpectedly during the injection operation and were thus not included in the results; thus the total number of rats used for the behavior and histological analyses was 52. The criteria for “successful injection and gene expression” were as follows. 1) Fluosphere bead fluorescence was found in the CeA, showing that the tip of the injection needle reached the CeA. However, the Fluosphere beads were not always evident after weeks of preservation, presumably due to loss of the tissue around the tip location during the tissue sectioning. 2) The whole CeA region (CeC, CeL, and CeM) showed consistent mCherry fluorescence. This was often accompanied by extra-CeA expression (dorsally in the globus pallidus and laterally in the lateral amygdala). 4) Finally, if the fluorescence signal was weaker than in extra-CeA regions or undetectable in the CeA, it was also judged a “failure”. After evaluation based on these criteria, 24 cases were considered injection failures and the remaining 28 “success” cases were used for the statistical analysis of the behavioral data. We did not attempt to use the data from these “failure” cases as “placement controls” because of the considerable heterogeneity of the mCherry expression pattern in these failure cases, which gave inconsistent responses to CNO administration, ranging from no effect to effects similar to those of “success” cases.

2.6. Data and statistical analysis

Statistical comparisons were performed using SPSS 23 (IBM, Tokyo, Japan). We used the following approaches to test null hypotheses for each independent comparison: 1) four-group comparison with time course (AcAph vs. vehicle, and formalin vs. saline); 2) two-group comparison with time course (AcAph, PGB, or DLX vs. vehicle), and 3) four-group comparison with time course (hM3Dq vs. mCherry, and AcAph vs. vehicle). The procedures for these statistical analyses are schematically described in Table 1. The details of the statistical tests are described in each figure and its legend. All graphs were made with Igor Pro 8 and 9 (WaveMetrics, Lake Oswego, OR, USA) using procedures written by FK.

### Table 1

| Case                                      | First test | Post-hoc test |
|------------------------------------------|------------|---------------|
| Four-group comparison with time course   | Two-way ANOVA (time and treatment) | 1) Multiple pairwise between-group comparison with Gabriel test for each of four pairs of comparisons at each time point. |
| (AcAph vs. vehicle, and formalin vs saline) | Two-way ANOVA (time and treatment) | 2) Multiple with-control comparisons (vs. pre-formalin and vs. pre-analgesics) with Dunnett test for each of four treatment groups. |
| Two-group comparison with time course    | Two-way ANOVA (time and treatment) | 1) Between-group comparison with Mann-Whitney U test at each time point. |
| (AcAph, PGB, or DLX vs. vehicle)         | Two-way ANOVA (time and treatment) | 2) Multiple with-control comparison (vs. pre-formalin and vs. pre-analgesics) with Dunnett test for each of analgesics and vehicle groups. |
| Four-group comparison with time course   | Two-way ANOVA (time and treatment) | 1) Multiple pairwise between-group comparison with Gabriel test for each of four pairs of comparisons (vector type and AcAph). |
| (hM3Dq vs eGFP, and AcAph vs. vehicle)   | Two-way ANOVA (time and treatment) | 2) Multiple with-control comparisons (vs. pre-CNO and vs. pre-AcAph) with Dunnett test for each of four treatment groups. |

Values are expressed as the mean values ± standard error of the mean (SEM). Differences with a P value less than 0.05 were considered significant.

3. Results

3.1. Effect of acetaminophen on widespread ectopic sensitization in rats with orofacial inflammatory pain

We have already reported that a single injection of formalin into the upper lip results in a long-lasting (>13-day) decrease in the PWT<sub>50</sub> at the bilateral hind paw (Sugimoto et al., 2021). Therefore, this is a model for nociceplastic pain in which inflammation in the face gives rise to a mechanical sensitization at the hind paw where there is no injury or inflammation. We confirmed this phenomenon by injecting formalin into the upper lip and evaluating the PWT<sub>50</sub> at the bilateral hind paws.

3.1.1. Effect of acetaminophen on widespread ectopic sensitization

Fig. 1A shows the experimental protocol. Formalin/saline was injected into the left upper lip at time 0 (thin arrow), and PWT<sub>50</sub> measurements were performed at the arrowheads. AcAph (100 mg/kg, i.p.)/vehicle was injected 3.5 h after the formalin injection (thick arrow). Fig. 1B shows the results of the formalin and subsequent AcAph injections. The colored marker plots in Fig. 1B indicate the results of statistical comparisons for the mean right and left PWT<sub>50</sub> at each measurement. The details for the comparisons are described in the graph. The line and marker plots in Figs. 1B1 and 1B2 show the time course of the mean<sub>L</sub> PWT<sub>50</sub> in each of the four groups for a combination of formalin (Fig. 1B2) or saline (Fig. 1B3) injection and subsequent AcAph or vehicle injection.

At 1 h after the formalin injection, the rats started to show sensitization, that is, lowering of the PWT<sub>50</sub> and, at 3 h post-formalin, the PWT<sub>50</sub> was significantly decreased in all groups receiving formalin.
(Fig. 1B2, red- and orange-filled circles; “Dunnett vs. Pre”). This did not occur in the group receiving saline (Fig. 1B3, light blue- and green-filled circles), indicating that orofacial inflammation-induced widespread sensitization is established at 3 h post-formalin. We administered AcAph (100 mg/kg, i.p.) to these rats at 3.5 h post-formalin. AcAph significantly re-elevated the lowered PWT$^{50}$ at 4.5–6 h post-formalin (i.e., 1–2.5 h post-AcAph), compared to the value measured at 3 h post-formalin (Fig. 1B, “Dunnett vs. 3 h”). These values of the re-elevated mean PWT$^{50}$ after AcAph administration did not differ significantly from the “Pre” value in the rats receiving AcAph (“Dunnett vs. Pre”) from 4 to 6 h post-formalin (i.e., 0.5–2.5 h post-AcAph). At 5.5 h post-formalin (i.e., 2 h post-AcAph), the mean PWT$^{50}$ was significantly higher in the group receiving AcAph than in that receiving vehicle (“Gabriel” post-hoc comparison in Fig. 1B1). At 9 h post-formalin, there

Fig. 1. The effect of AcAph (100 mg/kg, i.p.) on the mechanical withdrawal threshold in the hind paws of rats with orofacial inflammation. (A) Summary of the experimental procedure. Rats received orofacial formalin/saline injection (thin arrow at 0 h) and repeatedly underwent von Frey filament testing (vF) (Pre, 1, 3, 4, 4.5, 5, 5.5, 6, 9, 24, and 28 h after formalin/saline injection, arrowheads). “Pre” denotes just before the formalin/saline injection. At 3.5 h after the formalin/saline injection, they were injected with i.p. AcAph/vehicle (thick arrow). (B) PWT$^{50}$ of the hind paws after upper lip injection of formalin (B2)/saline (B3). Vertical axes, the mean PWT$^{50}$ at the bilateral hind paws. Horizontal axes, time after formalin (thin arrow at 0 h in B2)/saline (thin arrow at 0 h in B3) injection. Mean ± SEM. The treatment group and number of rats are shown in the boxes next to each graph. Colored circles above B2 and B3 (B1) show the statistical results (P values). The compared pair is shown on the left side for each line of the statistical results. The differences in the PWT$^{50}$ between the AcAph- and vehicle-treatment groups at each time point were analyzed by the Gabriel test (top four lines of comparisons, “Gabriel”). The differences in the PWT$^{50}$ between before and after formalin/saline injection (Pre vs. 1–28 h) were analyzed by Dunnett’s test (bottom four lines of comparisons, “Dunnett (vs. Pre)”). The differences in the PWT$^{50}$ between before and after AcAph/vehicle i.p (3 h vs. 4–28 h) were analyzed by Dunnett’s test (middle four lines of comparisons, “Dunnett (vs. 3 h)” ). The P values are indicated by the color scale on the right side of the statistical results. (C) The right-left ratio of the PWT$^{50}$ at each time point examined (the right PWT$^{50}$ divided by the left). Large circles with thick lines and small circles with thin lines indicate the average and individual values of the right-left ratio of the PWT$^{50}$, respectively. From the top: Formalin-AcAph, Formalin-Vehicle, Saline-AcAph, Saline-Vehicle. (D) Summary of the effect of formalin/saline and AcAph/vehicle on the PWT$^{50}$ at the right (right graph-set) and left (left graph-set) hind paw. From the left side of each graph set: the PWT$^{50}$ at three time points in the Formalin-AcAph, Formalin-Vehicle, Saline-AcAph, and Saline-Vehicle groups, respectively. Pre (white bars), formalin (red bars), and saline (green bars), the PWT$^{50}$ at 3 h after formalin/saline injection; +AcAph (pink bars) and +vehicle (yellow bars), the PWT$^{50}$ at 1.5 h after AcAph/vehicle i.p. (i.e., 5 h after formalin/saline injection). Dark red and dark blue lines with circles show the PWT$^{50}$ of individual formalin/saline-injected rats, respectively. The differences in the PWT$^{50}$ between each time point were compared by Dunnett’s test. **, P < 0.01; NS, P ≥ 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
was no longer a significant difference from the PWT\textsubscript{50} at 3 h post-formalin in both the AcAph and vehicle groups. However, the values were significantly lower than the Pre values, indicating that the mitigation of sensitization by AcAph observed at 4–6 h post-formalin (0.5–2.5 h post-AcAph) had almost faded at 9 h post-formalin (5.5 h post-AcAph). The lowered PWT\textsubscript{50} observed at 3 h post-formalin remained in both the vehicle and AcAph groups at 9 h and beyond. The rats receiving formalin showed a significantly lower PWT\textsubscript{50} from 9

Fig. 2. The effect of AcAph (200 mg/kg, i.p.) on the mechanical withdrawal threshold at the hind paws of rats with orofacial inflammation. Refer to the legend of Fig. 1, except for the dose of AcAph. In Fig. 2D, the differences in the PWT\textsubscript{50} between each time point were compared by Dunnett’s test. **, \( P < 0.01 \); *, \( P < 0.05 \); NS, \( P \geq 0.05 \).
to 28 h post-formalin than before formalin injection, regardless of AcAph injection (‘Dunnett vs. Pre’ in Fig. 1B1). In contrast to the formalin-treated rats, there were no significant post-injection effects of saline (Fig. 1B3, dark green and light blue markers; “Dunnett vs. Pre”) and no significant post-injection effects of AcAph or vehicle (Fig. 1B3, dark green and light blue makers; “Dunnett vs. 3 h” for AcAph and vehicle).

In this series of latent inflammatory pain model experiments, formalin was injected into the left upper lip. The values shown in Fig. 1B are the mean values of the PWT$_{50}$ at the right and left hind paws. However, we have already reported that mechanical sensitization in this model occurs symmetrically in the bilateral hind paws (Sugimoto et al., 2021). Therefore, to examine whether the changes in the PWT$_{50}$ after formalin injection and AcAph administration occurred bilaterally, we calculated the ratio of the PWT$_{50}$ at the right to left hind paw (Fig. 1C). Despite slight variations in individual values, the right-to-left ratio of the PWT$_{50}$ was consistently around 1.0 throughout the course of the experiments, suggesting that the sensitization and its recovery by AcAph occurred bilaterally. Fig. 1D confirms these bilateral changes with separate statistical analyses for the PWT$_{50}$ values from the left and right hind paws for “Pre” values obtained at 3 h post-formalin (“formalin”) and at 1.5 h post-AcAph (“+AcAph 100”).

These results suggest that AcAph (100 mg/kg, i.p.) attenuates the ectopic sensitization after upper lip formalin injection from 4 to 6 h post-formalin (i.e., 0.5–2.5 h post-AcAph).

**3.1.2. Effect of acetaminophen at a later phase of orofacial formalin inflammation**

Our previous study indicates that a widespread lowering of the PWT$_{50}$ started in some animals at 1 h post-formalin (Fig. 1) and was stably observed at 3 h post-formalin (Sugimoto et al., 2021). In the experiments in Figs. 1 and 2, AcAph was injected at 3.5 h post-formalin, a time point with a consistent appearance of stably lowered PWT$_{50}$ in all animals examined. To examine whether such an early pharmacological intervention and repeated touches with the von Frey filament influence the effects of AcAph on the PWT$_{50}$, we injected AcAph 200 mg/kg at 6.5 h post-formalin, where the orofacial formalin injection-induced sensitization was observed stably in the bilateral hind limb. Fig. 3A shows the effect of AcAph (200 mg/kg, i.p.) at the subacute phase of the inflammation (6 h after formalin injection) on the mechanical withdrawal threshold at the hind paws of the rats. (A) Summary of the experimental procedure. Rats received an orofacial formalin injection (thin arrow at 0 h) and repeatedly underwent von Frey filament testing (vF) (Pre, 3, 6, 9, 10, 11, 24, and 28 h after formalin injection, arrowheads). Pre denotes just before the formalin injection. At 6.5 h after the formalin injection, they were injected with i.p. AcAph/vehicle (thick arrow). (B) PWT$_{50}$ of the hind paws after upper lip injection of formalin (B2). Vertical axes, the mean PWT$_{50}$ at the bilateral hind paws. Horizontal axes, time after formalin injection (thin arrow at 0 h). Mean ± SEM. The treatment group and number of rats are shown in the box on the left of the graph. The colored circles above B2 (B1) show the statistical results (P values). The compared pair is shown on the left side for each line of the statistical results. The differences in the PWT$_{50}$ between the For + AcAph and For + Veh groups at each time point were analyzed by the Mann-Whitney U test (the top line of comparisons, “Mann-Whitney U test”). The differences in the PWT$_{50}$ between before and after formalin injection (Pre vs. 3–28 h) were analyzed by Dunnett’s test (bottom two lines of comparisons, “Dunnett (vs. Pre)”). The differences in the PWT$_{50}$ between the different conditions were compared by Dunnett’s test (bottom two lines of comparisons, “Dunnett (vs. Pre)”).

**Fig. 3.** The effect of AcAph (200 mg/kg, i.p.) at the subacute phase of the inflammation (6 h after formalin injection) on the mechanical withdrawal threshold at the hind paws of the rats (A) Summary of the experimental procedure. Rats received an orofacial formalin injection (thin arrow at 0 h) and repeatedly underwent von Frey filament testing (vF) (Pre, 3, 6, 9, 10, 11, 24, and 28 h after formalin injection, arrowheads). Pre denotes just before the formalin injection. At 6.5 h after the formalin injection, they were injected with i.p. AcAph/vehicle (thick arrow). (B) PWT$_{50}$ of the hind paws after upper lip injection of formalin (B2). Vertical axes, the mean PWT$_{50}$ at the bilateral hind paws. Horizontal axes, time after formalin injection (thin arrow at 0 h). Mean ± SEM. The treatment group and number of rats are shown in the box on the left of the graph. The colored circles above B2 (B1) show the statistical results (P values). The compared pair is shown on the left side for each line of the statistical results. The differences in the PWT$_{50}$ between the For + AcAph and For + Veh groups at each time point were analyzed by the Mann-Whitney U test (the top line of comparisons, “Mann-Whitney U test”). The differences in the PWT$_{50}$ between before and after formalin injection (Pre vs. 3–28 h) were analyzed by Dunnett’s test (bottom two lines of comparisons, “Dunnett (vs. Pre)”). The differences in the PWT$_{50}$ between the different conditions were compared by Dunnett’s test (bottom two lines of comparisons, “Dunnett (vs. Pre)”).

**Fig. 2** indicates the effects of i.p. AcAph at 200 mg/kg on formalin-induced widespread sensitization in the hind limb. The experimental protocol was the same as that for the results for AcAph shown in Fig. 1A, except for the dose of AcAph (100 mg/kg in Figs. 1 and 200 mg/kg in Fig. 2). AcAph administered at 3.5 h after the formalin injection elevated the mean$_{50}$ PWT$_{50}$ at as early as 0.5 h post-AcAph and to a significantly higher value than that in the vehicle-injected group at 5 h post-formalin (1.5 h post-AcAph injection; Fig. 2B2). After 6 h post-formalin (2.5 h post-AcAph), there was no longer a significant difference between the PWT$_{50}$ in AcAph- and vehicle-injected rats treated with formalin. These changes were almost consistently observed in the bilateral hind paws (Fig. 2C and D).
experimental protocol. Formalin was injected into the left upper lip at time 0 (thin arrow), and PWT50 measurements were performed at the arrowheads. AcAph (200 mg/kg, i.p.) was injected at 6.5 h after the formalin injection (thick arrow).

Fig. 3B shows the results of the formalin and AcAph injections. AcAph 200 mg/kg administered at 6.5 h post-formalin drastically elevated the mean±SE PWT50 to a value significantly larger than the PWT50 at 6 h post-formalin and not significantly different from that before formalin injection (Fig. 3B). At 10 h post-formalin (3.5 h post-AcAph), the mean±SE PWT50 in the AcAph group no longer differed from that in the vehicle group.

Again, the changes in the PWT50 after formalin injections and those after AcAph occurred bilaterally, as evidenced by the only slight deviation of the right-to-left ratio from unity, despite significant changes in the PWT50 (Fig. 3C). The significant decrease in the PWT50 with formalin and the significant recovery with AcAph 200 mg/kg, but not with vehicle, was confirmed both in the left and right hind paws (Fig. 3D).

Altogether, these results show that AcAph attenuates widespread sensitization in the hind limb in the latent orofacial inflammatory pain model, indicating that AcAph would affect the central mechanisms mediating latent inflammatory pain-induced central sensitization.

3.2. Pregabalin attenuates the mechanical hypersensitivity of the bilateral hind limb in the orofacial formalin model

The results shown above clearly indicate that AcAph mitigates the widespread sensitization at the bilateral hind paws caused by latent inflammatory pain at the trigeminally innervated regions, strongly supporting the notion that this drug at least in part intervenes in the central mechanisms underlying the widespread regulation of pain sensitivity. However, the molecular target of AcAph remains equivocal, with several possibilities, including molecules involved in endocannabinoid signaling and TRPV1-mediated neuronal excitability in the brain regions playing roles in descending pain regulation, such as the PAG and rostral ventromedial medulla (RVM) (Barriere et al., 2020; Klinger-Gratz et al., 2018). In contrast to AcAph, the targets of the analgesic effects of gabapentinoids are unequivocally identified: the α5 Ca2+ channel subunits.

Pregabalin (PGB) (30 mg/kg, i.p.) on the mechanical withdrawal threshold at the hind paws of rats with orofacial inflammation. (A) Summary of the experimental procedure. Rats received an orofacial formalin injection (thin arrow at 0 h) and repeatedly underwent von Frey filament testing (vF) (Pre, 1, 3, 4, 4.5, 5, 5.5, 6, 9, 24, and 28 h after formalin injection, arrowheads). Pre denotes just before the formalin injection. At 3.5 h after the formalin injection, they were injected with i.p. pregabalin (PGB) (thick arrow). (B) PWT50 of the hind paws after upper lip injection of formalin (B2). Vertical axes, the mean PWT50 at the bilateral hind paws. Horizontal axes, time after formalin injection (thin arrow at 0 h). Mean ± SEM. The treatment group and number of rats are shown in the box on the left of the graph. Colored circles above B2 (B1) show the statistical results (P values). The compared pair is shown on the left side for each line of the statistical results. The differences in the PWT50 between the For + PGB and For + Veh groups at each time point were analyzed by the Mann-Whitney U test (the top line of comparisons, “Mann-Whitney U test”). The differences in the PWT50 between before and after formalin injection (Pre vs. 3–28 h) were analyzed by Dunnett’s test (bottom two lines of comparisons, “Dunnett (vs. Pre)”).

The differences in the PWT50 between before and after PGB/Veh i.p. (3 h vs. 4–28 h) were analyzed by Dunnett’s test (middle two lines of comparisons, “Dunnett vs. 3 h”). The P values are indicated by the color scale on the right side of the statistical results. (C) The right-left ratio of the PWT50 at each time point examined (the right PWT50 divided by the left). Large circles with thick lines and small circles with thin lines indicate the average and individual values of the right-left ratio of the PWT50, respectively. Top and bottom are Formalin-PGB and Formalin-Vehicle, respectively. (D) Summary of the effect of formalin and PGB/Vehicle on the PWT50 at the right (upper graph-set) and left (lower graph-set) hind paw. From the left side of each graph-set: the PWT50 at three time points in the Formalin-PGB and Formalin-Vehicle group, respectively. Pre (white bars); formalin (red bars), the PWT50 at 3 h after formalin injection; + PGB 30 (blue bars) and - vehicle (yellow bars), the PWT50 at 1.5 h after PGB/vehicle i.p. (i.e., 5 h after formalin injection). Dark red lines with circles show the PWT50 of individual formalin-injected rats. The differences in the PWT50 between each time point were compared by Dunnett’s test. **, P < 0.01; NS, P ≥ 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
nociceptive signaling is enhanced (Miyazawa et al., 2018).

Fig. 4A shows the experimental protocol. Formalin injection and PWT<sub>50</sub> measurements were performed as in previous experiments. PGB (30 mg/kg, i.p.) was injected at 3.5 h after the formalin injection (thick arrow). Fig. 4B shows the results of the formalin and PGB injections. The line and marker plots at the bottom (Fig. 4B1) show the time course of the mean PWT<sub>50</sub> in each of the two groups (combination of formalin injection and subsequent PGB or vehicle injection).

At 1 h after the formalin injection, the rats receiving formalin started to show symptoms of sensitization (i.e., a lower PWT<sub>50</sub>) and, at 3 h post-formalin, the PWT<sub>50</sub> was significantly decreased in all groups receiving formalin (Fig. 4B2, blue- and orange-filled circles; “Dunnett vs. Pre”), indicating that orofacial inflammation-induced widespread sensitization was established. PGB (30 mg/kg) injected at 3.5 h post-formalin increased the PWT<sub>50</sub> significantly at 4–9 h post-formalin (i.e., 0.5–5.5 h post-PGB), compared to the value measured at 3 h post-formalin (Fig. 4B, “Dunnett vs. 3 h”). From 4 to 9 h post-formalin (i.e., 0.5–5.5 h post-PGB), the mean PWT<sub>50</sub> for the rats receiving PGB did not differ significantly from the “Pre” value (“Dunnett vs. Pre”). From 4 to 9 h post-formalin (i.e., 0.5–5.5 h post-PGB), the mean PWT<sub>50</sub> was significantly higher in the group receiving PGB than in that receiving vehicle (“Mann-Whitney U test”). At 24 h post-formalin, there were no longer significant differences from the PWT<sub>50</sub> at 3 h post-formalin in both the PGB and vehicle groups, though the values were significantly lower than the Pre values, indicating that the mitigation of sensitization by PGB observed at 4–9 h post-formalin (0.5–5.5 h post-PGB) had almost faded. The rats receiving formalin showed a lower PWT<sub>50</sub> from 24 to 28 h post-formalin regardless of the PGB injection. The changes in the PWT<sub>50</sub> with formalin and PGB were essentially symmetrical (Fig. 4C) and apparent in both the left and right hind paws (Fig. 4D), supporting the belief that the PGB targeted brain circuits underlying widespread sensitization and not the peripheral to second-order signaling. These results indicate that PGB exerts potent and long-lasting mitigation of widespread sensitization, which was observed at body sites where there is no nociception or neuropathy. This might indicate that PGB is also effective in patients with nociceplastic pain (Fitzcharles et al., 2021).

3.3. Duloxetine exerts limited effects on the bilateral ectopic sensitization in the latent inflammatory pain model

We then tried to examine another central-acting analgesic, DLX. Fig. 5A shows the experimental protocol. DLX (10 mg/kg, i.p.) was injected at 3.5 h after the formalin injection (thick arrow). Fig. 5B shows the results of the formalin and DLX injections. The line and marker plots in Fig. 5B2 show the time course of the mean PWT<sub>50</sub> in each of the two groups (combination of formalin injection and subsequent DLX or vehicle injection).

DLX (10 mg/kg, i.p.), injected at 3.5 h post-formalin, did not significantly affect the PWT<sub>50</sub>, compared to the value measured at 3 h post-formalin (Fig. 5B, “Dunnett vs. 3 h”). From 1 to 28 h post-formalin, the mean<sub>Pre</sub> PWT<sub>50</sub> of formalin-injected rats was significantly lower than that at Pre before DLX administration and after DLX or vehicle administration (“Dunnett vs. Pre”). At 5 h and 5.5 h post-formalin (i.e., 1.5–2 h post-DLX), the mean PWT<sub>50</sub> was significantly higher in the group receiving vehicle than in rats receiving DLX (Mann-Whitney U test).

3.4. Effects of acetaminophen on the widespread sensitization induced by chemogenic excitation of the CoA neurons in non-inflamed rats

To date, there has been no established experimental model in which...
the activation of the brain circuit underlying pain-associated plasticity alone causes pain-resembling behaviors in the absence of tissue or nerve injury/inflammation. Establishing such a model is in demand for developing pharmacotherapies against pain with nociplastic mechanisms. We have demonstrated that chemogenetic activation of the GABAergic neurons in the CeA results in widespread sensitization even in the absence of nociceptor activation and neuropathy (Sugimoto et al., 2021). Using this model, we examined whether AcAph can attenuate the increased sensitivity at the hind paw in rats with artificial activation of the right CeA in the absence of any tissue damage. We prepared rats in which the GABAergic neurons in the right CeA expressed hM3Dq, a Gq-coupled GPCR, which excites CeA neurons when activated by the

Fig. 6. The effect of AcAph on the mechanical withdrawal threshold at the hind paws of the amygdala-activated widespread pain (aWSP) model rats. (A) Experimental procedure. AAV for the expression of hM3Dq-Cherry ("hM3Dq" in Fig. 6) or mCherry ("mCherry" in Fig. 6) in the CeA GABAergic neurons was injected into the right CeA. Three weeks after the AAV injection, von Frey filament testing ("vF" with arrowheads) was repeatedly performed before (Pre1, Pre2, Pre3) and 20, 40, and 60 min after CNO injection (3 mg/kg, i.p.) over 3 days. On day 2, AcAph or vehicle was administered as a mixed solution with CNO. (B) PWT50 of the hind paws after CNO i.p. (B1). Vertical axes, the mean PWT50 at the bilateral hind paws. Horizontal axes, time after CNO injection (thick arrowhead at 0 min of each day). Mean ± SEM. The AAV-drug combination and number of rats are shown in the box on the left of the graphs. Colored circles above B2 (B1) show the statistical results (P value). The compared pair is shown on the left side for each line of the statistical results. The differences in the PWT50 among all four groups at each time point were analyzed by the Gabriel test (the top four lines of comparisons, “Gabriel”). The differences in the PWT50 between before and after CNO injection (Pre vs. 20–60 min on Day 1 and 3, Pre2 vs. 20–90 min on Day 2) were analyzed by Dunnett’s test (bottom four lines of comparisons, “Dunnett (vs. Pre)”). The P values are indicated by the color scale on the right side of the statistical results. (C) The right-left ratio of the PWT50 at each time point examined (the right PWT50 divided by the left). Large circles with thick lines and small circles with thin lines indicate the average and individual values of the right-left ratio of the PWT50, respectively. From the top: hM3Dq + AcAph, hM3Dq + Vehicle, mCherry + AcAph, and mCherry + Vehicle groups, respectively. (D) Summary of the effect of the CeA activation by DREADD and application of ApAph/Vehicle on the PWT50 at the right (right graph-set) and left (left graph-set) hind paw on Day 2. From the left side of each graph-set: the PWT50 at three time points in the hM3Dq + AcAph, hM3Dq + Vehicle, mCherry + AcAph, and mCherry + Vehicle groups, respectively. Pre (white bars); CNO + drugs (middle bars and right bars indicate 60 and 90 min after i.p. injection, respectively), Dark red and dark blue lines with circles show the PWT50 of individual hM3Dq- and mCherry-expressing rats, respectively. The differences in the PWT50 between each time point were compared by Dunnett’s test. **, P < 0.01; *, P < 0.05; NS, P > 0.1. (E) Schema of the mCherry fluorescence spread around the injection site. Histological representation of the mCherry signals around the CeA was performed with the coronal section of the brain slice and adapted to the Paxinos and Watson atlas (2007). The numbers of rats are 4 (mCherry + AcAph), 5 (mCherry + Vehicle), 9 (hM3Dq + AcAph), and 10 (hM3Dq + Vehicle). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
artificial ligand CNO. The control group expressed mCherry instead of hM3Dq.

Fig. 6A shows the schedule for the AAV injections and behavioral tests with CNO injection. We injected CNO (3 mg/kg, i.p.) on days 1, 2 and 3 into all rats. We repeated the PWT50 measurements at timings indicated with downward arrows in Fig. 6A. Pre1, Pre2, and Pre3 indicate PWT50 measurements made immediately (<15 min) before the CNO injections. We divided the rats into hM3Dq and mCherry groups, and each of these groups was divided into those receiving AcAph (200 mg/kg, i.p.) or vehicle only on day 2 with CNO. Rats did not receive AcAph or vehicle injection on day 1 and day 3, regardless of the group.

On day 1, CNO injection in the hM3Dq group significantly lowered the PWT50 at 20–60 min in the hM3Dq + Vehicle group (light red markers in Fig. 6B) and at 40–60 min in the hM3Dq + AcAph group (red markers in Fig. 6B; note neither AcAph nor vehicle was administered on day 1). On day 2, on which AcAph or vehicle was administered with CNO, the PWT50 was not significantly altered by CNO at 20–90 min in the hM3Dq group receiving AcAph, while it was significantly and markedly lowered at 40–90 min in the hM3Dq group receiving vehicle. On day 3, the PWT50 of all rats of the hM3Dq group was significantly lowered at 20–60 min after CNO injection, regardless of whether they had received AcAph or vehicle on day 2. Between hM3Dq rats receiving AcAph or vehicle, the PWT50 significantly differed at 40 and 60 min post-CNO, but not at 90 min (Fig. 6B). No rat belonging to the mCherry group showed significant changes in the PWT50 after CNO, AcAph, or vehicle injection (blue and light blue markers in Fig. 6B). The right-to-left ratio of the PWT50 was consistently almost equal, despite large changes in the PWT50 (Fig. 6C), suggesting that the activation of hM3Dq in the right CeA changes the PWT50 bilaterally. These results indicate that AcAph significantly mitigates the lowered threshold by CNO only in the rats with hM3Dq expressed in the right CeA.

The values in Fig. 6B are the mean PWT50 measured at the right and left hind paws. Fig. 6D shows the individual data from the right and left hind paw at Pre2 (control for the CNO and AcAph or vehicle effects on day 2), 60 min, and 90 min after CNO injection. Only the rats expressing hM3Dq and receiving CNO and vehicle showed a significant decrease in the PWT50 at 60 min post-CNO, while such a reduction in the PWT50 was not observed in other groups. Thus, the results of these experiments using aWSP, a model with widespread sensitization in the complete absence of peripheral injury or inflammation, indicate that AcAph attenuates the widespread sensitization caused purely by non-pathological mechanisms but only with aberrant central network activity underlying nociceptive pain.

4. Discussion

4.1. Novel model for evaluating the effects of analgesics on nociceptive sensitization

The recently proposed third mechanistic descriptor of pain is nociceptive pain, a pain caused in the absence of activated nociceptors and neuropathy (Kosek et al., 2021). Many patients worldwide suffer from pain left undiagnosed due to the lack of identifiable biological causes that could account for the ongoing pain symptoms (Fitzcharles et al., 2021). Nociceptive pain is expected to provide a novel therapeutic framework for these patients with primary chronic pain, which allows this type of pain to be acknowledged and treated appropriately, even in the absence of evidence for the tissue or nerve injury. However, at this moment, it remains challenging to design an appropriate pharmacological strategy to mitigate nociceptive pain, as there is not yet an established model that enables the evaluation of analgesic effects in preclinical setups. In the past, the effects of analgesics have been exclusively analyzed in rodent models of acute inflammatory pain or neuropathic pain, in which the tactile sensitivity and nociceptive behaviors are evaluated at the site of injury or inflammation or sites innervated by nerves with injury (Percie Du Sert and Rice, 2014). Until today, there has been no ideal animal model that enabled analysis of the effects of analgesics on the pain-like behaviors or sensitization at remote and widespread body sites, resulting from central mechanisms.

In this study, we have demonstrated that the widely used analgesics AcAph and PGB attenuate widespread sensitization in novel models of nociceptive pain. The first one, the iWSP model, involves bilateral hind paw sensitization after transient inflammation at a trigeminally innervated region, accompanied by robust and specific activation of the right CeA neurons and enhanced synaptic transmission in the CeA (Miyazawa et al., 2018). The other one, the aWSP model, is a model with bilateral hind paw sensitization in response to the artificial activation of the CeA without any injury/inflammation in the body. We have described the possible mechanisms for these two models in detail in our previous reports (Miyazawa et al., 2018; Sugimoto et al., 2021). We examined the effects of AcAph, PGB, and DLX because 1) these drugs are widely used for patients with various types of chronic pain (Chou et al., 2017; Freo et al., 2021; Maffei, 2020) and 2) these drugs affect brain functions, which might play roles in the analgesic effects of these drugs (Barriere et al., 2020; Wang et al., 2019; Yamamoto et al., 2021). Based on the results presented here, we propose that these animal models for widespread sensitization involving central plastic mechanisms could be used to evaluate drug effects in primary chronic pain with underlying nociceptive mechanisms. The plausible mechanisms underlying the effect of each drug are described below.

4.2. The mitigation of widespread sensitization by AcAph

4.2.1. The TRPV1–DAGL–mGluR5–PLC–CB1 cascade for the AcAph effect in the periaqueductal gray

AcAph (also called paracetamol) is one of the most popular analgesics. It has been widely used as an over-the-counter drug and as a prescribed drug against acute and chronic pain for more than 70 years. The most crucial finding in this study was that AcAph attenuated widespread sensitization resulting from chemogenetic activation of the GABAergic neurons in the CeA (aWSP model). This aWSP model is purely nociceptive with central sensitization in the complete absence of peripheral nociceptor activation and neuropathy in the somatosensory system. AcAph also effectively mitigated ectopic sensitization occurring at sites where there was neither injury nor inflammation in iWSP. These results strongly support the notion that AcAph attenuates widespread sensitization occurring through central nociceptive mechanisms.

The mechanism of the analgesic effect of AcAph has long been a subject of debate (Ohashi and Kohno, 2020). It has been established that AcAph has a weak anti-inflammatory effect and thus likely exerts its analgesic effects by affecting central mechanisms. Recently, Barriere et al. (2020) convincingly demonstrated that activation of an intracellular multi-molecule cascade composed of TRPV1 channels, diacylglycerol lipase (DAGL), type 5 metabotropic glutamate receptors (mGluR5), phospholipase C (PLC), and CB1 receptors is the essential process contributing to the analgesic effect of AcAph. Importantly, all of these cascade members are localized in the PAG, which is an essential part of the central mechanism for the descending pain modulation, making it the best candidate for the target of the central analgesic effect of AcAph (Barriere et al., 2020). On the other hand, accumulated lines of evidence indicate that CeA neurons regulate the excitability of the PAG, which might play roles in the analgesic effects of these drugs (Barriere et al., 2020; Wang et al., 2019; Yamamoto et al., 2021). Based on the detailed brain mechanism underlying the widespread sensitization in the iWSP and aWSP models that we used in this study remains to be demonstrated, a plausible interpretation is that the activation of the CeA network in these models modulated PAG network activity to cause widespread sensitization, which AcAph mitigated through its modulatory effect on the PAG activity.

4.2.2. Other possible brain targets for AcAph effects

In addition to the PAG, there could be other possible targets of AcAph.
for exerting its anti-sensitization effect in iWSP and aWSP models. A possible site is the RVM. Local injection of the CB1 receptor antagonist rimonabant into the RVM blocks the analgesic effect of AcAph on tactile hyperalgesia caused by intraplantar injection of zymosan (Klinger-Gratz et al., 2018). Likewise, RVM injection of AM404 mitigates this hyperalgesia in wild-type mice but not in mice lacking CB1 receptors (Klinger-Gratz et al., 2018). There are lines of evidence supporting the idea that the bilateral sensitization following CeA activation that we observed would involve PAG-RVM projections (Li et al., 2017; Palazzo et al., 2011). As such, the analgesic effect of AcAph likely involves its direct action on RVM neurons mediating PAG-RVM-dorsal horn connections.

It is also possible that the most upstream elements of this amygdala-PAG-RVM-dorsal horn descending pathway are directly affected by AcAph. The amygdala (basolateral amygdala (BLA) and CeA) is rich in molecules involved in endocannabinoid signaling (Ramikie and Patel, 2012). The BLA, the upstream sub-amygdala nucleus to the CeA, highly expresses CB1 receptor protein (Katona et al., 2001), and the neuronal activity in the BLA is potently modified by endocannabinoids (Ramikie and Patel, 2012). Using the same orofacial inflammatory latent pain model as the iWSP model, we demonstrated that the activation pattern of the BLA neurons was significantly correlated with that of the right CeA, suggesting that the BLA neurons control the excitability of CeA neurons in this particular model (Miyazawa et al., 2018).

Despite the lower level of expression of CB1 receptor proteins in the CeA than in the BLA (Katona et al., 2001), lines of evidence indicate that enzymes involved in this process and CB1 receptors are functional in the CeA and that the endocannabinoid system plays roles in the regulation of synaptic transmission therein (Ramikie et al., 2014; Roberto et al., 2010). Interestingly, the anti-nociceptive effects of systemic injection of a synthetic CB1 agonist, WIN55212-2, are reduced by local injection of muscimol into the CeA but not into the BLA, suggesting a pivotal role of the CeA in the expression of endocannabinoid-mediated analgesia (Manning et al., 2003). Therefore, one of the possible mechanisms of the attenuated sensitization by AcAph is mediated by inhibition of CeA neuron excitability by CB1 receptor activation following AcAph administration (Ramikie and Patel, 2012). Furthermore, the CeA neurons have direct and indirect projections to the PAG and RVM and regulate activities of pronociceptive “ON-cells” and anti-nociceptive “OFF-cells” in the RVM (Ansaah et al., 2009; McGaraughty and Heinricher, 2002), indicating that changes in CeA neuronal activities can modulate descending RVM control of the spinal nociceptive circuits.

Altogether, in addition to drugs that promote AM404 production, such as AcAph, the substances that could modulate the function of the cascade composed of TRPV1, CB1, and mGluR5 would be promising candidates for the effective treatment of such widespread pain through modulation of the amygdala (BLA/CeA)-PAG-RVM network.

4.3. Mitigation of widespread sensitization by PGB

The robust and long-lasting attenuation of the widespread sensitization in the iWSP model by PGB was as expected. First, PGB has already been approved and prescribed in many countries for the treatment of fibromyalgia, a disease with typical “nociceptive” pain with the following three major hallmarks of nociceptive pain (Kosek et al., 2021): 1) pain occurs without injury or inflammation at the site of sensitization, 2) pain is widespread, and 3) pain is accompanied by central sensitization. The iWSP model that we used shares all of these characteristics. We have confirmed that PGB significantly attenuates the bilateral sensitization in the aWSP model (data not shown and the manuscript is in preparation), indicating that PGB is effective in a nociceptive pain model entirely without any injury, but with only altered central activity. Second, the α5 subunit of the voltage-dependent Ca2+ channel, the primary target of PGB, is densely expressed in the regions involved in the sensitization of this particular model, such as the LPB, BLA, and, mostly, the PAG (Cole et al., 2005; Stahl et al., 2013), suggesting that PGB could effectively attenuate aberrant activity at these nuclei caused by orofacial inflammation. This finding also supports the notion that the spinal cord dorsal horn, which is also rich in α5 subunit expression, is not the only target of the analgesic action of PGB, particularly in the models or in patients without identifiable peripheral activation of nociceptors (Stahl et al., 2013). The well-established potent effects of PGB in the rodent models of neuropathic pain and patients with neuropathy should be reevaluated in light of the effect of PGB on these limbic and brainstem networks.

4.4. DLX does not exert detectable effects on widespread sensitization in the latent inflammation model

DLX is an inhibitor of the membrane transporters for noradrenaline and 5-HT. Thereby, it increases the extracellular concentration of these monoamines. DLX is approved as an antidepressant and also for the treatment of chronic pain in many countries. Because these monoamines regulate synaptic transmission in the dorsal horn, it is generally believed that attenuation of synaptic transmission in the dorsal horn resulting from increased extracellular noradrenaline and 5-HT gives rise to anti-nociceptive effects. Unexpectedly, we failed to observe any significant effect of DLX on the bilateral allodynia in the iWSP model (Fig. 5). It is not likely that the dose that we used (10 mg/kg, i.p.) was too low to attenuate the hind limb sensitization because we have reported a significant increase in the PWT50 with the same dose of DLX used in this study in a streptozotocin-induced painful diabetic neuropathy model (Kinoshita et al., 2013). Munro (2009) reported that 10 mg/kg, but not 3 mg/kg, i.p. DLX significantly reduced the second-phase flinching responses caused by intraplantar injection of 5% formalin into rats, suggesting that the widespread pain caused by formalin injection in this study is less sensitive to DLX. These results are in agreement with a report by Zhang et al. (2018), who found that the intraplantar formalin-induced decrease in the withdrawal threshold at the injected paw at 24 h post-formalin was significantly attenuated with 30 mg/kg, but not with 10 mg/kg of DLX, while 10 mg/kg i.p. DLX could significantly ameliorate the earlier sensitization (1-3 h post-formalin) and pERK and Fos expression in the CeA at 24 h post-formalin. These results suggest that the sensitivity of the mechanical allodynia to DLX is dependent on the course of pain chronification and that a dose of 10 mg/kg could be ineffective, depending on the model and the duration (Kinoshita et al., 2013; Kremer et al., 2018; Zhang et al., 2018). Although these authors did not evaluate any ectopic sensitization, it seems that DLX exerts only a weaker effect on the central sensitization occurring at latent stages. One of the possible interpretations is that the changes (reduction) in the extracellular levels of noradrenaline and 5-HT play only a minor role in the augmented spinal nociceptive response in these latent inflammatory pain models. As we have not tested in this study the effect of DLX 30 mg/kg, which is much higher than the clinical doses (Kremer et al., 2018), whether this high-dose DLX can attenuate the ectopic sensitization in our models remains an open question. In addition, the effects of repeated administration of DLX should be examined in a more clinically relevant way in the future (Kremer et al., 2018).

It should be noted that it remains equivocal whether DLX exerts its analgesic effect by affecting the extracellular concentration of noradrenaline and 5-HT at the dorsal horn, as in our previous study (Kinoshita et al., 2013), or by affecting the intra-brain mechanisms involved in descending pain modulation, such as the CeA, as in the study by Zhang et al. (2018). For example, Maire et al. (2016) demonstrated that the hypoalgesic effect of clonidine, an α2 adrenoceptor agonist, injected into the CeA requires intact spinal noradrenergic neurotransmission but does not require an intact PAG-RVM system (Maire et al., 2016). They also found that this property is in marked contrast to the effects of opioid and endocannabinoid system activation in the CeA or BLA on the hind limb nociceptive responses, mediated by PAG-RVM descending modulatory system (Maire et al., 2016). Therefore, it is
likely that noradrenergic modulation of the nociceptive sensitivity is examined and clarified in the future. Because another main effect of (Viisanen and Pertovaara, 2007) in widespread sensitization should be CeA-to-LC (including pericoerulear regions of the LC) complicated (Kohro et al., 2020). In this regard, the role played by the opposed regulations by noradrenaline due to distinct roles of distinct addition, even in the dorsal horn, there are multiple and mutually not mediated by the increased monoamine concentration in the spinal study indicated that the slow-onset long-lasting analgesic effect of clinical doses of DLX in a long-term chronic pain model with nerve cuff is not mediated by the increased monoamine concentration in the spinal cord (Kremer et al., 2018). They found, instead, that the anti-neuromimmune effects involving peripheral α2 receptor activation and downregulation of the TNFα-NFκB signaling pathway mediate the effect of DLX (Kremer et al., 2018). Altogether, the effect of DLX on chronic pain would have multiple and diverse mechanisms, each of which is recruited separately depending on the modality and duration of the chronic pain. Though the iWSP model was not effective in detecting the effect of DLX on semi-acute latent nociplastic pain in the present study, this result would imply the importance of understanding various mechanisms underlying individual pain to advance toward precision/individual pain medicine.

4.5. Limitations of this study

4.5.1. The sex of the experimental animals

In this study, we used only male rats. Sex-dependent differences in the mechanism underlying sustained pain in animals have been repeatedly shown and acknowledged as one of the most influential factors of pain behaviors (Mogil, 2020; Smith, 2019). Fibromyalgia, for example, a disease possibly involving nociceplastic mechanisms, is more prevalent in female than in male patients (Fitzcharles et al., 2021). Our unpublished observations in female Wistar rats with upper lip inflammation and female VGAT-cre rats with hM3Dq-expressing vector in the CeA both show potent hind limb sensitization and support the notion that the involvement of sex-dependent factors in this CeA-mediated sensitization, if any, would be limited. This issue should be directly tested in the near future, particularly before this model is applied to the development of therapeutics.

5. Conclusions

We have shown that PGB and AcAph, two of the most popular and widely used analgesics for neuropathic and nociceptive pain, significantly attenuate widespread ectopic sensitization of central mechanisms. Therefore, there is hope that these two drugs would also be effective in mitigating chronic pain with nociceplastic mechanisms in human patients. In contrast, DLX would likely affect a distinct mechanism. These models of nociceplastic pain with central sensitization would be helpful to develop and evaluate novel chemicals for patients with chronic pain of nociceplastic mechanisms.

Declaration of competing interest

Fusao Kato is a recipient of the collaborative study on the gaba-pentinooid effects with Daiichi-Sankyo Co. Ltd.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2022.109029.

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