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The pronociceptive role of 5-HT$_6$ receptors in ventrolateral orbital cortex in a rat formalin test model

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ABSTRACT

Recent studies have shown the 5-HT$_6$ receptors are expressed in regions which are important in pain processing such as the cortex, amygdala, thalamus, PAG, spinal cord and dorsal root ganglia (DRG), suggesting a putative role of 5-HT$_6$ receptors in pain modulation. The ventrolateral orbital cortex (VLO) is part of an endogenous analgesic system, consisting of the spinal cord – thalamic nucleus submedius (Sm) – VLO – periaqueductal gray (PAG) – spinal cord loop. The present study assessed the possible role of 5-HT$_6$ receptors in the VLO in formalin-induced inflammatory pain model. Firstly we found that microinjection of selective 5-HT$_6$ receptor agonists EMD-386088 (5 μg in 0.5 μl) and WAY-208466 (8 μg in 0.5 μl) both augmented 5% formalin-induced nociceptive behavior. Microinjection of selective 5-HT$_6$ receptor antagonist SB-258585 (1,2 and 4 μg in 0.5 μl) significantly reduced formalin-induced flinching. Besides, the pronociceptive effects of EMD-386088 and WAY-208466 were dramatically reduced by SB-258585, implicating 5-HT$_6$ receptor mechanisms in mediating these responses. In addition, the pronociceptive effect of EMD-386088 was also prevented by the adenylate cyclase (AC) inhibitor SQ-22536 (2 nmol in 0.5 μl) and the protein kinase A (PKA) inhibitor H89 (10 nmol in 0.5 μl), respectively. We further confirmed the above results with quantification of spinal *c-fos* expression. Taken together, our results suggested that 5-HT$_6$ receptors play a pronociceptive role in the VLO in the rat formalin test due to its activation of AC - PKA pathway. Therefore, cerebral cortical 5-HT$_6$ receptors could be a new target to develop analgesic drugs.

1. Introduction

The serotonin 6 receptor (5-HT$_6$ receptor) is one of the latest 5-HT receptors to be discovered and does not yet have a well-defined functional role in the brain (Brouard et al., 2015). Given its predominant expression in the central nervous system (CNS), the 5-HT$_6$ receptor has been considered as a valuable target for the development of CNS drugs with limited side effects (Karila et al., 2015).

The VLO is a pivotal part of prefrontal cortex. Previous studies have shown that the VLO is part of an endogenous analgesic system, consisting of the spinal cord – thalamic nucleus submedius (Sm) –VLO – periaqueductal gray (PAG) – spinal cord loop (Tang et al., 2009; Wei et al., 2016). Furthermore, the localization of 5-HT$_6$ receptors in regions which are important in pain processing such as the cortex, amygdala, thalamus, PAG, spinal cord and dorsal root ganglia (DRG) suggests that this receptor is well-placed to modulate the neural substrates underlying nociception (Finn et al., 2007). However, the role of 5-HT$_6$ receptors in pain modulation has never been characterized at the cortex level.

Under agonist stimulation, the 5-HT$_6$ receptors activate cAMP formation via Gs-coupled receptor. In addition to its coupling to G proteins, the 5-HT$_6$ receptors can also interact with the Src family tyrosine kinase Fyn, the Jun activation domain-binding protein 1, and the microtubule-associated protein Map1b as well as the mammalian Target of

Abbreviations: AI, agranular insular cortex; Cl, claustrum; fmi, forceps minor corpos callosum; VLO, ventrolateral orbital cortex; DMSO, dimethyl sulfoxide; i.p., intraperitoneally; KYNA, kynurenic acid; vlPAG, ventrolateral periaqueductal gray; AC, adenylate cyclase; PKA, protein kinase A; Sm, thalamic nucleus submedius

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Rapamycin (mTOR) Complex 1 under different circumstances (Deraredj Nadim et al., 2016). However, how 5-HT_6 receptors navigate under pain conditions and the underlying mechanism remain to be established.

Formalin test has been widely used to study persistent inflammatory pain and the efficacy of analgesic drugs (Barkai et al., 2019; Cha et al., 2019; Dang et al., 2011; Kumar et al., 2019). Formalin injection to the rat unilateral hindpaw pad evokes biphasic nociceptive behavior; the flinching response is considered to be a flexor reflex mediated at the spinal level, thus the paw flinching responses reveal the descending modulation actions of the cerebral higher centers on spinal nociception (Qu et al., 2015). Spinal neurons which are responsive to chemical noxious stimuli send ascending projections to the brainstem/cortex (Harris, 1998). C-fos expression is considered as a neuron activation marker and therefore the quantification of Fos-positive neurons provides a reliable approach to compare the effects of various manipulations on nociceptive processing (Cha et al., 2019).

The present study first examined the role of 5-HT_6 receptors in the VLO in formalin-induced nociceptive behavior by microinjecting its selective agonists EMD-386088 and WAY-208466, as well as its selective antagonist SB-258585. Second, the cellular mechanisms underlying their effect were analyzed using selective Gs protein downstream inhibitors, e.g. adenylate cyclase (AC)/protein kinase A (PKA) inhibitors. Last, we analyzed the number of Fos-positive neurons in the dorsal horn of the lumbar spinal cord to further confirm the effects of agonists, antagonist as well as AC/PKA inhibitors. Overall, our current study aims to reveal the role of 5-HT_6 receptors in the VLO in modulating inflammatory pain as well its underlying mechanisms.

2. Materials and methods

2.1. Animals

Experiments were performed on male Sprague–Dawley rats (220–280 g), which were provided by the Experimental Animal Center of Shaanxi Province, China. Experimental protocols were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University and were in accordance with ethical guidelines from the International Association for the Study of Pain (Zimmermann, 1983). All efforts were made to minimize the number of animals used, as well as distress to the animals.

2.2. Intracerebral guide cannula placement

The rats were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg), and the head was immobilized in a stereotaxic frame. Pain modulation is mostly unilateral, i.e., the higher order pain processing (such as VLO) is in charge of contralateral formalin-injected hindpaw. Therefore, the cannula was placed unilaterally in the VLO. A small craniotomy was performed just above the VLO. A stainless steel guide cannula (0.8 mm in diameter) was stereotaxically inserted, with the tip 2.0 mm dorsal to the VLO, at the following coordinates: 3.2 mm anterior to bregma, 2.0 mm lateral, and 2.6 mm below cortical surface (Paxinos and Watson, 1997). The cannula was then attached to the skull with three microscrews and dental cement. Once the animals recovered from anesthesia, sodium penicillin was administered (0.2 million units/day for 4 days, intraperitoneally) to prevent wounds and intracerebral infections. The animals were carefully nursed and fed in clean cages.

2.3. Formalin test

The formalin test was performed as previously described (Huo et al., 2010). Briefly, rats were placed in the plastic chamber with a mirror positioned below the chamber at a 45° angle to allow unobstructed observation of the rats' injected paw. Ten minutes after intracerebral injection, rats received a 50 μl subcutaneous injection of diluted (5%) formalin into the hind paw pad, contralateral to the intracerebral injection. Rats were then immediately returned to the chamber. Formalin-induced nociceptive behaviors were observed, and the number of times the injected paw flinched was counted every 5 min during a 60-min observation period, including early phase (0–10 min) and late phase (15–60 min) (Godinez-Chaparro et al., 2011). The observer was trained and blind to the treatment conditions.

2.4. Intracerebral drug microinjection

On the day of testing, the rats were acclimated to the experimental arena for 30 min prior to testing. Then a 1.0 μl microsyringe, with the tip extending 2 mm beyond the end of the guide cannula, was inserted to the VLO through the guide cannula. The drugs were dissolved in saline or 10% dimethyl sulfoxide (DMSO) and slowly infused (0.5 μl) through the microsyringe at a constant speed over a 60-s period to observe the effect on formalin-induced nociceptive behavior.

Drugs used in the present study, including the selective 5-HT_6 receptor agonists EMD-386088 and WAY-208466, selective 5-HT_6 receptor antagonist SB-258585, were purchased from RBI/Sigma (St. Louis, MO, USA). WAY-208466 (4.0 and 8.0 μg in 0.5 μl) was freshly prepared in saline (Liu et al., 2016; Monti et al., 2013; Zhang et al., 2016), while EMD-386088 (2.5 and 5.0 μg in 0.5 μl) and SB-258585 (1.0, 2.0 and 4.0 μg in 0.5 μl) were dissolved in 10% DMSO (Nikiforuk et al., 2011; Pratt et al., 2009; Pratt et al., 2012). SQ-22536 and N-[2-(p-bromocinnamylami-no)ethyl]-5-isoquinolinesulfonamide (H89) were obtained from Tocris Cookson (Bristol, UK) and dissolved in 10% DMSO, with their final concentration of 2 nmol/0.5 μl (Li et al., 2013) and 10 nmol/0.5 μl (Tang et al., 2008), respectively. Agonists were injected into the VLO contralateral to the affected hindpaw 10 min prior to formalin injection, and antagonist was administered 5 min prior to the agonist injection. Drug doses were chosen according to previous studies, where they were reported to be effective. Equal volumes of saline or 10% DMSO were injected into the VLO as vehicle controls.

2.5. Histology, immunohistochemistry and quantification of fos immunoreactive neurons

At the end of the experiment, the drug injection sites were marked by injection of Pontamine Sky Blue dye (0.5 μl, 2% in 0.5 M sodium acetate solution). Under deep anesthesia, the rats were transcardially perfused with 0.9% normal saline, followed by 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The brain or spinal cord was immediately removed and fixed in 4% PFA and then 30% sucrose solution/0.1 M PB (pH 7.4). The brains were cut into 40 μm thick sections using a freezing microtome, and then the slices were stained with Cresyl Violet. The injecting sites were histologically identified to be within the VLO for data analysis (Qu et al., 2006).

The rats were sacrificed and the spinal cord were dissected 1.5 h after the formalin injection. The Fos immunohistochemistry and quantification were performed as previously described (Huo et al., 2010). Briefly, the lumbar L4-5 spinal cords were cut in 30 mm serial sections on a freezing microtome (Kryostat 1720, Leitz, Mannheim, Germany). The free-floating sections were used for immunohistochemistry staining of Fos using the avidin–biotin-peroxidase (ABC) method. Then the sections were incubated sequentially with: (1) rabbit anti-serum against Fos polyclonal antibody (ab7963, 1:500 dilution; Abcam, Cambridge, MA, USA) in 0.01 M PBS containing 5% normal goat serum (NGS), 0.3% Triton X-100, 0.05% NaN_3, and 0.25% carrageenan (pH 7.4) for 48–72 h at 4 °C; (2) biotinylated goat anti-rabbit IgG (1:200 dilution; Vector, Burlingame, CA) in PBS-NGS overnight at 4 °C; and (3) ABC Elite complex (Vector: 1:100) in 0.01 M PBS containing 0.3% Triton X-100 for 2 h at room temperature. Bound peroxidase was visualized by incubation with 0.05% 3, 3-diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto, Japan) and 0.003% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.6) for 20–30 min. The

sections were mounted onto gelatin-coated glass slides and observed under light microscope. Fos-labeled nuclei were quantified by an examiner who was blind to treatment of each animal. For each animal, two counts were made in the sections: (1) the mean number of Fos-labeled nuclei in the entire spinal dorsal horn, and (2) the mean number of Fos-labeled nuclei in laminae I–II and V–VI.

2.6. Data analysis

All data were expressed as mean ± SEM. One-way ANOVA or two-way ANOVA analyses were performed using IBM SPSS statistics 19.0 (SPSS Inc, Chicago, IL). For results with significant interaction effects in two-way ANOVA, simple effect test was conducted for further analysis. For results without significant interaction effects, Bonferroni's post-hoc test or Student's t-test was further conducted as needed. To determine differences between two groups, a Student's t-test was performed. The significance level was set at $P < 0.05$.

3. Results

Subcutaneous injection of 5% formalin into the right hind paw produced a typical pattern of flinching behavior characterized by a biphasic time course. Phase 1 of the nociceptive response began immediately after formalin administration and then declined gradually in approximately 10 min. Phase 2 began about 15 min after formalin administration and lasted about 45 min as previously described (Godinez-Chaparro et al., 2011).

3.1. Pronociceptive effect of microinjection of EMD-386088 and WAY-208466 into the VLO on formalin-induced nociception

EMD-386088 (2.5 and 5.0 μg in 0.5 μl, respectively), a selective 5-HT_6 receptor agonist, was administered into the VLO, contralateral to the affected paw. Microinjection of EMD-386088 (5.0 μg) into the VLO, 10 min prior to formalin injection significantly augmented the number of flinches. As shown in Fig. 1A, time course curves of number of flinches for 10% DMSO and different doses of EMD-386088 treated groups were different between treatments ($F_{(2,\ 372)} = 28.61$, $P < 0.0001$), across times ($F_{(11,372)} = 10.37$, $P < 0.0001$) and treatment × time interaction ($F_{(22,372)} = 1.474$, $P = 0.0786$). Further analyses indicated that the mean number of flinches in the EMD-386088 (5.0 μg) treated group was significantly more than the 10% DMSO group at 5 of 12 time points ($P < 0.05$), as well as the EMD-386088 (5.0 μg) group at 2 of 12 time points compared with EMD-386088 (2.5 μg) group ($P < 0.05$). However, no significant difference ($P > 0.05$) was measured between the EMD-386088 (2.5 μg) group and the 10% DMSO group ($P > 0.05$), as shown in Fig. 1A. Significant differences between EMD-386088 (5.0 μg) group and the 10% DMSO group in the late phase were shown in Fig. 1B. EMD-386088 (2.5 μg) into the VLO did not influence the nociceptive behavior, the number of flinches in this group was not significantly different from the 10% DMSO group, in entire observation period or at any time point (Fig. 1A and B).

To substantiate the indication that 5-HT_6 receptor agonist facilitated the inflammatory pain in the VLO, we microinjected another selective 5-HT_6 receptor agonist WAY-208466 into this region. WAY-208466 (4.0 and 8.0 μg in 0.5 μl, respectively) was administered into the VLO and WAY-208466 (8.0 μg in 0.5 μl) significantly augmented formalin-induced flinch behavior. As shown in Fig. 1C, time course curves of number of flinches for Saline and different doses of WAY-208466 treated groups were different between treatments ($F_{(2,\ 420)} = 16.82$, $P < 0.0001$), across times ($F_{(11,420)} = 23.22$, $P < 0.0001$) and treatment × time interaction ($F_{(22,420)} = 1.224$, $P = 0.2220$). Further analyses indicated that the mean number of flinches in the WAY-208466 (8.0 μg) treated group was significantly more than the Saline group at 5 of 12 time points ($P < 0.05$), as well as than WAY-208466 (4.0 μg) group at 3 of 12 time points ($P < 0.05$). Significant differences between WAY-208466 (8.0 μg) group and Saline group in the late phase were shown in Fig. 1D.

Subcutaneous injection of 5% formalin resulted in a massive spinal Fos expression ipsilaterally to the formalin-injected hindpaw, and the Fos-labeled neurons were mainly located in the superficial dorsal horn

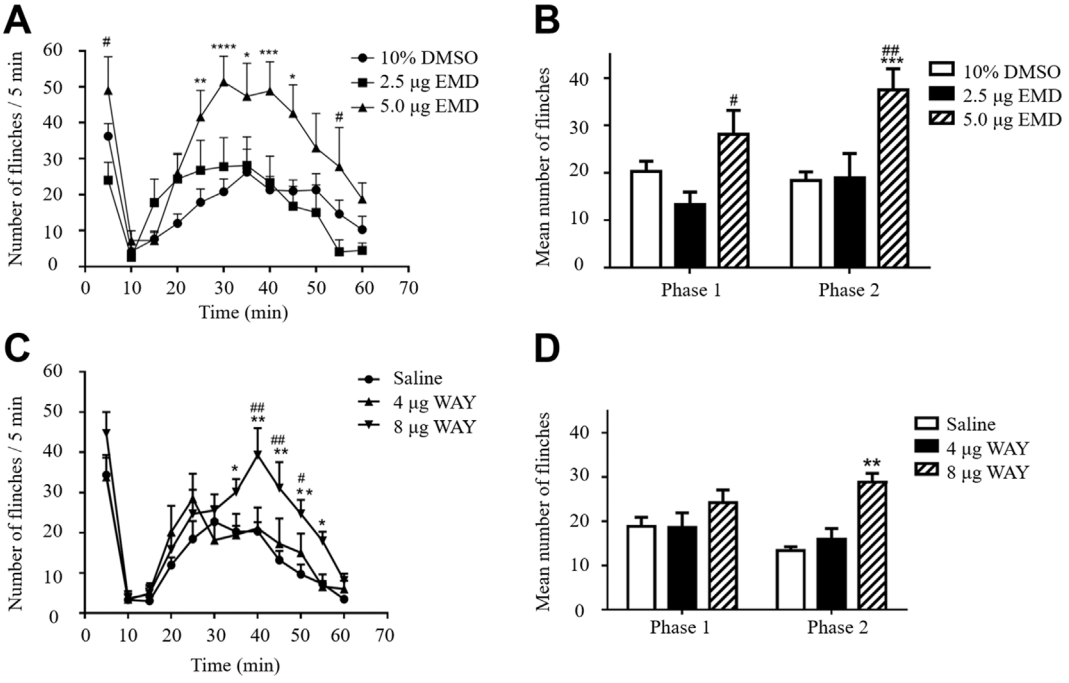

Fig. 1. The pronociceptive effects of microinjection of 5-HT_6 receptor agonists EMD-386088 (2.5 μg and 5.0 μg) and WAY-208466 (4.0 μg and 8.0 μg) into the VLO on the formalin-evoked flinching behavior. (A) and (C) Time course curves of flinching number at different time points. (B) and (D) Bar graphs showing mean number of flinches per 5 min during early phase and late phase. $^{*}P < 0.05$ compared with 10% DMSO group or Saline group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with 2.5 μg EMD-386088 or 4.0 μg WAY-208466 group at their corresponding time points or phases. n = 8–15 rats/group.

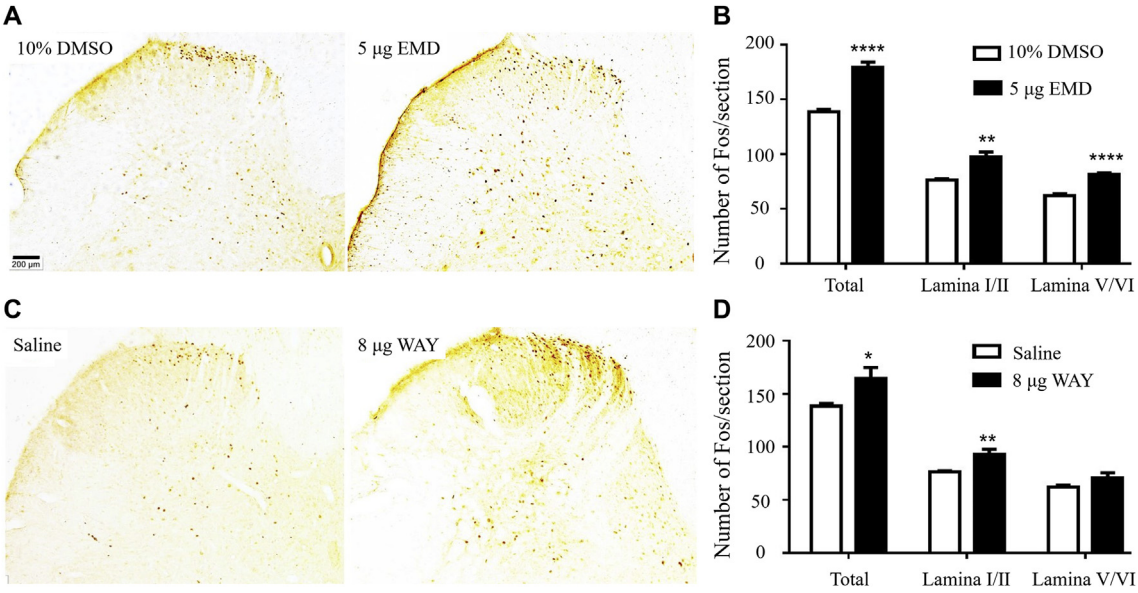

Fig. 2. The enhancing effect of microinjection of 5-HT$_6$ receptor agonist EMD-386088 into the VLO of rats on formalin-induced Fos expression. (A) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in 10% DMSO group and EMD-386088 group. (C) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in Saline group and WAY-208466 group. (B and D) Bar graphs showing mean number of the expression of Fos in the total, superficial (laminae I-II) and deep (laminae V-VI) dorsal horn neurons. *$P < 0.05$,**$P < 0.01$,***$P < 0.001$ and ****$P < 0.0001$,compared with their corresponding 10% DMSO group or Saline group. n = 4–6 rats/group.

(laminae I–II) and in the deep dorsal horn (laminae V–VI). Microinjection of 5 μg EMD-386088 into the VLO significantly elevated the formalin-evoked Fos expression in the entire spinal dorsal horn ($t = 7.419$, $P < 0.0001$), the superficial dorsal horn (laminae I–II, $t = 4.432$, $P = 0.0013$) as well as in the deep dorsal horn (laminae V–VI, $t = 9.13$, $P < 0.0001$), compared with 10% DMSO group (Fig. 2B). Similarly, microinjection of 4.0 μg WAY-208466 into the VLO significantly elevated the formalin-evoked Fos expression in the entire spinal dorsal horn ($t = 2.48$, $P = 0.0325$) and the superficial dorsal horn (laminae I–II, $t = 3.338$, $P = 0.0075$) but not in the deep dorsal horn (laminae V–VI, $t = 1.527$, $P = 0.1557$), compared with Saline group (Fig. 2D). These results suggested that the 5-HT$_6$ receptor agonists exerted pronociceptive effect in the VLO.

3.2. Inhibitory effect of microinjection of 5-HT$_6$ receptor antagonist SB-258585 into the VLO on formalin-induced nociception

Microinjection (−15 min) of the 5-HT$_6$ receptor antagonist SB-258585 (1.0, 2.0 and 4.0 μg in 0.5 μl, respectively) reduced 5% formalin-induced nociceptive behavior expressed as both time course curves and mean number of flinches/min during both phases of the formalin test. As shown in Fig. 3A, time course curves of number of flinches for 10% DMSO- and different doses of SB-258585- treated groups were different between treatments ($F_{(3, 468)} = 48.66$, $P < 0.0001$), across times ($F_{(11,468)} = 7.991$, $P < 0.0001$) and treatment × time interaction ($F_{(33,468)} = 1.232$, $P = 0.1800$).

Microinjection of 1.0, 2.0 and 4.0 μg SB-258585 into the VLO significantly prevented the formalin-evoked Fos expression in the entire spinal dorsal horn ($F_{(3, 20)} = 70.49$;$P < 0.0001$), the superficial dorsal horn ($F_{(3, 20)} = 61.47$;$P < 0.0001$) as well as in the deep dorsal horn ($F_{(3, 20)} = 47.68$;$P < 0.0001$), compared with 10% DMSO group, as shown in Fig. 4B. These results further suggested that the 5-HT$_6$ receptor antagonists exerted analgesic effect in the VLO.

3.3. Antagonizing effect of microinjection of SB-258585 on EMD-386088 and WAY-208466 -induced pronociception

To further assess the cortical participation of the 5-HT$_6$ receptors on the pronociceptive activity of EMD-386088 in the 5% formalin test, 5-HT$_6$ receptor antagonist SB-258585 were injected before (−5 min) the 5-HT$_6$ receptor agonist. Cortical pronociceptive effects of the 5-HT$_6$ receptor agonist EMD-386088 (10 μg in 0.5 μl) were significantly prevented by intra-VLO injection of the 5-HT$_6$ receptor antagonist SB-258585 (2.0 μg in 0.5 μl) in the 5% formalin test (Fig. 5). As shown in Fig. 5A, time course curves of number of flinches for 10% DMSO +10% DMSO, 10% DMSO +5 μg EMD-386088 and 2 μg SB-258585 + 5 μg EMD-386088 treated groups were different between treatments ($F_{(2,372)} = 31.04$, $P < 0.0001$), across times ($F_{(11,372)} = 10.67$, $P < 0.0001$) and treatment × time interaction ($F_{(22,372)} = 1.879$, $P = 0.0101$). Significant differences among 3 groups in either the early phase or the late phase were shown in Fig. 5B. EMD-386088 (5 μg in 0.5 μl) significantly increased flinch number in the late phase, and intra-VLO injection of the 5-HT$_6$ receptor antagonist SB-258585 (2.0 μg in 0.5 μl) dramatically blocked this increase in the late phase ($P < 0.001$).

The pronociceptive effect of another selective 5-HT$_6$ receptor agonist was also blocked by SB-258585. As shown in Fig. 5C, time course curves of number of flinches for 10% DMSO +10% DMSO, 10% DMSO +8.0 μg WAY-208466 and 2.0 μg SB-258585 + 8.0 μg WAY-208466 treated groups were different between treatments ($F_{(2,324)} = 25.87$, $P < 0.0001$), across times ($F_{(11,324)} = 18.74$, $P < 0.0001$) and treatment × time interaction ($F_{(22,324)} = 2.229$, $P = 0.0014$). These results suggested that 5-HT$_6$ receptors in the VLO modulated formalin-induced inflammatory pain behaviors. Similarly, significant differences among 3 groups in either the early phase or the late phase were shown in Fig. 5D. WAY-208466 (8 μg in 0.5 μl) significantly increased flinch number in the late phase, while intra-VLO injection of the 5-HT$_6$ receptor antagonist SB-258585 (2.0 μg in 0.5 μl) dramatically blocked this increase in the late phase ($P < 0.01$).

Quantitative analyses showed significant differences of formalin-evoked Fos expression among 10% DMSO +10% DMSO group, 10%

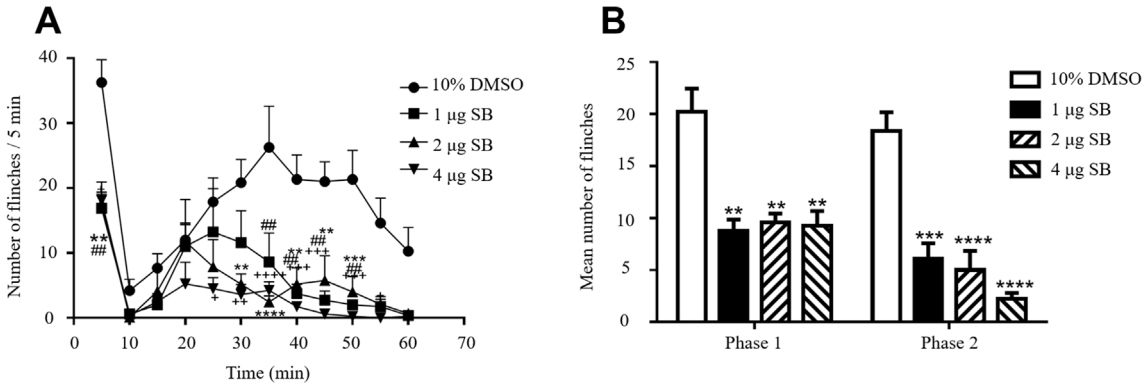

Fig. 3. The antinociceptive effects of microinjection of 5-HT$_6$ receptor antagonist SB-258585 into the VLO on the formalin-evoked flinching behavior. (A) Time course curves of flinching number at different time points. (B) Bar graphs showing mean number of flinches per 5 min during early phase and late phase. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared with 10% DMSO group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with 1.0 μg SB-258585 group, $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with 2.0 μg SB-258585 group at their corresponding time points or phases. n = 8–15 rats/group.

DMSO +5 μg EMD-386088 group and 2.0 μg SB-258585 + 5.0 μg EMD-386088 group in the entire spinal dorsal horn ($F_{(2, 15)} = 36.38; P < 0.0001$), the superficial dorsal horn ($F_{(2, 15)} = 14.18; P = 0.0003$) as well as in the deep dorsal horn ($F_{(2, 15)} = 57.36; P < 0.0001$). Microinjection of 5.0 μg EMD-386088 into the VLO significantly elevated the formalin-evoked Fos expression, while pre-treatment with 2.0 μg SB-258585 reversed this effect (Fig. 6B). In the entire spinal dorsal horn, or laminae I–II and laminae V–VI, the number of Fos-positive neurons was significantly less in the 2.0 μg SB-258585 + 5.0 μg EMD-386088 group compared with 10% DMSO +5.0 μg EMD-386088 group ($P < 0.01$ and $P < 0.0001$), as shown in Fig. 6.

Next we used another 5-HT$_6$ receptor agonist WAY-208466 to test the role of 5-HT$_6$ receptor in formalin-induced Fos expression. Quantitative analyses showed a significant difference of formalin-evoked Fos expression among 10% DMSO + Saline group, 10% DMSO +8.0 μg WAY-208466 group and 2.0 μg SB-258585 + 8.0 μg WAY-208466 group in the entire spinal dorsal horn ($F_{(2, 18)} = 28.39; P < 0.05$), the superficial dorsal horn ($F_{(2, 18)} = 5.421; P < 0.05$), but not in the deep dorsal horn ($F_{(2, 15)} = 26.521; P > 0.05$). Microinjection of 5.0 μg WAY-208466 into the VLO significantly elevated the formalin-evoked Fos expression, while pre-treatment with 2.0 μg SB-258585 reversed this effect (Fig. 7B). The total number of Fos-positive neurons and the number of Fos-positive neurons in the laminae I–II were significantly less in the 2.0 μg SB-258585 + 8.0 μg WAY-208466 group compared with 10% DMSO +8.0 μg WAY-208466 group ($P < 0.05$), as shown in Fig. 7B.

3.4. Blocking effect of AC inhibitor SQ-22536 and PKA inhibitor H89 on EMD-386088 -induced pronociception

Pretreatment with AC inhibitor SQ-22536 (2 nmol) or PKA inhibitor H89 (10 nmol), 5 min prior to EMD-386088 (5.0 μg) injection, significantly blocked the EMD-386088- evoked pronociception in the VLO as the number of flinching was reduced to the saline control level. The same dose of SQ-22536 or H89 applied alone to the VLO had no effect on formalin-induced nociception as the number of flinching was the same with the saline control level (Fig. 8A and C). The detailed comparisons at individual time points are shown in Fig. 8A and C.

As shown in Fig. 8A, time course curves of number of flinches for 10% DMSO, SQ-22536, 5.0 μg EMD-386088 and SQ-22536 + EMD-386088 treated groups were different between treatments ($F_{(3,440)} = 13.18$, $P < 0.0001$), across times ($F_{(11,440)} = 21.40$, $P < 0.0001$) and treatment × time interaction ($F_{(33,440)} = 2.822$, $P < 0.0001$). The flinch number of the SQ-22536 + EMD-386088 group during the 60 min observation period was significantly smaller than that of the EMD-386088 (5.0 μg) group ($P < 0.001$, Fig. 8B). Furthermore, there was no significant difference between SQ-22536 + EMD-386088 and SQ-22536 groups compared with the 10% DMSO control group ($P > 0.05$), as shown in Fig. 8B.

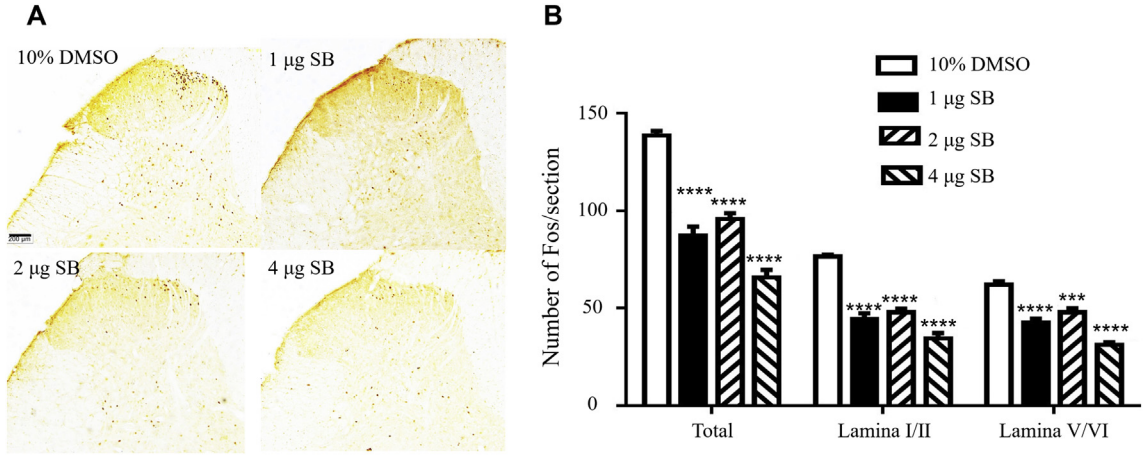

Fig. 4. The inhibitory effect of microinjection of 5-HT$_6$ receptor antagonist into the VLO of rats on formalin-induced Fos expression. (A) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in 10% DMSO group, 1.0 μg SB-258585 group, 2.0 μg SB-258585 group and 4.0 μg SB-258585 group. (E) Bar graphs showing mean number of the expression of Fos in the total, superficial (laminae I-II) and deep (laminae V-VI) dorsal horn neurons. $^{***}P < 0.001$ and $^{****}P < 0.0001$,compared with 10% DMSO group. n = 4–6 rats/group.

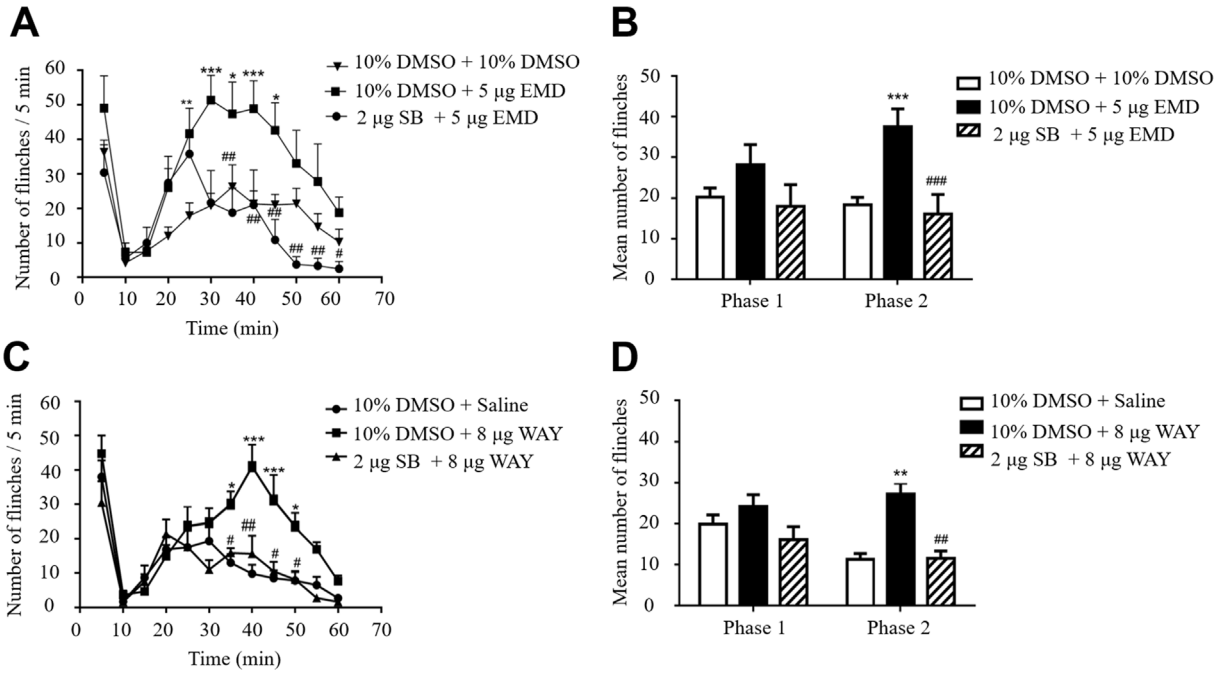

Fig. 5. The blocking effect of microinjection of the 5-HT$_6$ receptor antagonist SB-258585 on the pronociception produced by agonists (EMD-386088 or WAY-208466) in the VLO of rats submitted to the 5% formalin test. Note that intra-VLO EMD-386088 increased formalin-induced nociception during Phase 2 whereas that pretreatment of SB-258585 prevented the pronociceptive effect induced by EMD-386088 and WAY-208466. (A) and (C) Time course curves of flinching number at different time points. (B) and (D) Bar graphs showing mean number of flinches per 5 min during early phase and late phase. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with 10% DMSO + Saline group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with 10% DMSO +5.0 μg EMD-386088 or 10% DMSO +8.0 μg WAY-208466 group at those time points or phases. n = 8–15 rats/group.

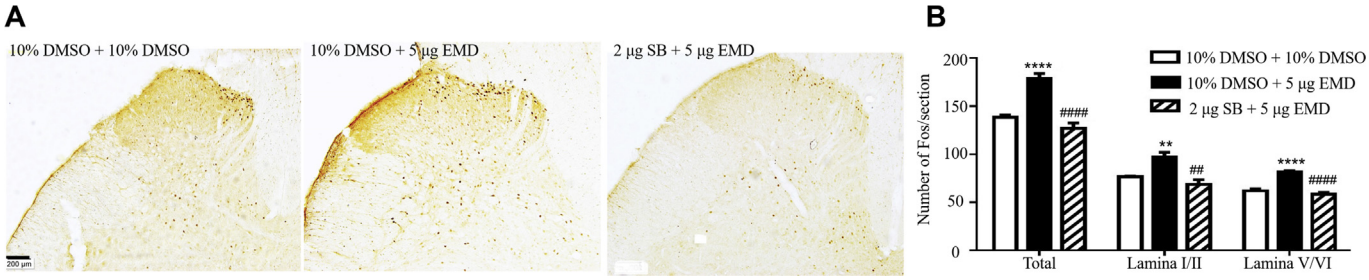

Fig. 6. The blocking effect of intra-VLO microinjection of 5-HT$_6$ receptor antagonist SB-258585 on EMD-386088- induced increase of formalin-induced Fos expression. (A) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in 3 different groups. (B) Bar graphs showing mean number of the expression of Fos in the total, superficial (laminae I-II) and deep (laminae V-VI) dorsal horn neurons in rats. $^{**}P < 0.01$ and $^{****}P < 0.0001$,compared with their corresponding 10% DMSO group +10% DMSO group; $^{\#\#}P < 0.01$ and $^{\#\#\#\#}P < 0.0001$,compared with their corresponding 10% DMSO group +5.0 μg EMD group. n = 4–6 rats/group.

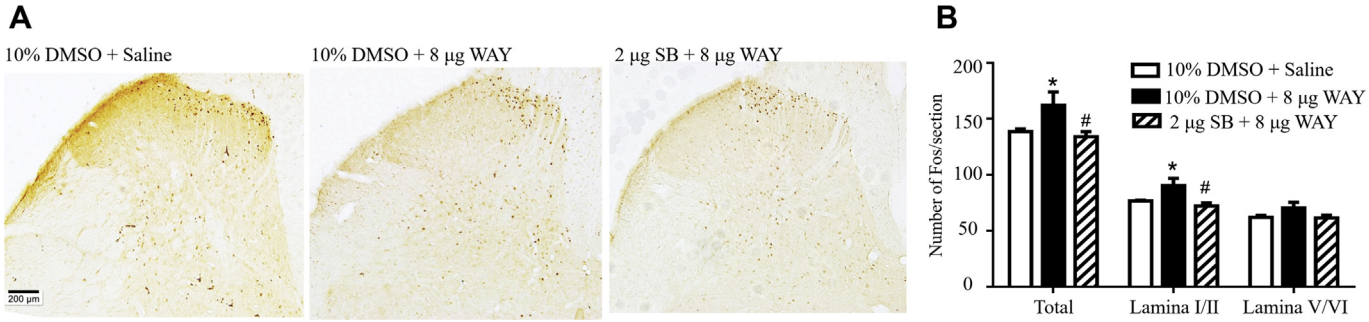

Fig. 7. The blocking effect of intra-VLO microinjection of 5-HT$_6$ receptor antagonist SB-258585 on WAY-208466- induced increase of formalin-induced Fos expression. (A) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in 3 different groups. (B) Bar graphs showing mean number of the expression of Fos in the total, superficial (laminae I-II) and deep (laminae V-VI) dorsal horn neurons. $^{*}P < 0.05$ compared with their corresponding 10% DMSO group + Saline group; $^{\#}P < 0.05$ compared with their corresponding 10% DMSO group +8 μg WAY group. n = 4–6 rats/group.

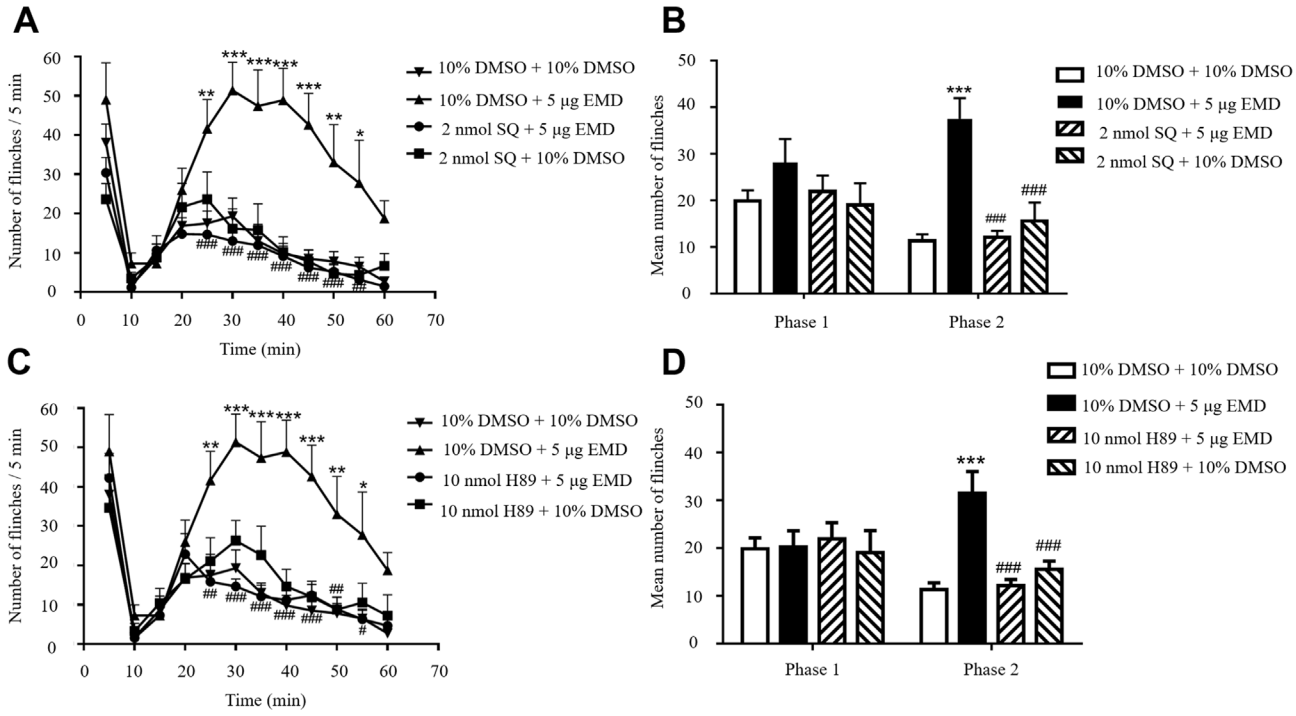

Fig. 8. The blocking effect of microinjection of the AC inhibitor SQ-22536 and PKA inhibitor H89 on the pronociception produced by agonist EMD-386088 in rats submitted to the 5% formalin test. (A) Time course curves of flinching number at different time points. (B) Bar graphs showing mean number of flinches per 5 min during early phase and late phase. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with 10% DMSO group +10% DMSO group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with 10% DMSO group +5 μg EMD group. n = 8–15 rats/group.

As shown in Fig. 8C, time course curves of number of flinches for 10% DMSO, H89, 5.0 μg EMD-386088 and H89 + EMD-386088 treated groups were different between treatments ($F_{(3,352)} = 13.02$, $P < 0.0001$), across times ($F_{(11,352)} = 22.57$, $P < 0.0001$) and treatment × time interaction ($F_{(33,352)} = 2.261$, $P < 0.0001$). Similarly, the flinch number of the H89 + EMD-386088 group during the 60 min observation period was significantly reduced than that of the EMD-386088 (5.0 μg) group ($P < 0.001$, Fig. 8D). Furthermore, there was no significant difference between H89 + EMD-386088 and H89 groups compared with the 10% DMSO control group ($P > 0.05$), as shown in Fig. 8D.

Similarly, next we assessed the Fos expression after the drug treatment. Two-way ANOVA comparing the total number of Fos-positive neurons among the 4 groups revealed significant effects of both EMD-386088 and SQ-22536 but not interaction between agonist and inhibitor effect ($F_{(1, 24)} = 21.576$, $P = 0.0170$, agonist factor; $F_{(1, 24)} = 12.16$, $P = 0.0321$, inhibitor factor; $F_{(1, 24)} = 3.578$, $P = 0.0609$, interaction). T-test showed that the number of Fos-positive neurons was significantly increased after agonist injection ($P < 0.001$). However, microinjection of SQ-22536 dramatically blocked the EMD-386088-induced augmentation of Fos expression ($P < 0.001$) (Fig. 9B). This inhibitory effect of SQ-22536 was unlikely the general effect because microinjection of SQ-22536 alone did not alter basal Fos levels in vehicle-treated rats ($P > 0.05$) (Fig. 9B). The enhancing effect of EMD-386088 and the inhibitory effect of SQ-22536 were also observed in the superficial dorsal horn as well as in the deep dorsal horn. These findings suggested that EMD-386088 may regulate the formalin-induced Fos expression via adenylate cyclase.

Two-way ANOVA comparing the total number of Fos expression among the 4 groups revealed significant effects of both EMD-386088 and H89 but not interaction between agonist and inhibitor effect ($F_{(1, 33)} = 21.34$, $P = 0.009$, agonist factor; $F_{(1, 33)} = 22.23$, $P = 0.031$, inhibitor factor; $F_{(1, 33)} = 1.518$, $P = 0.149$, interaction). T-test showed that Fos number was significantly increased after 5-HT_6 receptor agonist injection ($P < 0.001$). However, microinjection of H89 dramatically blocked the EMD-386088 -induced increase ($P < 0.001$), while microinjection of SQ-22536 alone did not alter basal Fos levels in vehicle-treated rats ($P > 0.05$) (Fig. 10B). The enhancing effect of EMD-386088 and the inhibitory effect of H89 were also observed in the superficial dorsal horn as well as in the deep dorsal horn. These findings suggested that EMD-386088 may regulate the formalin-induced Fos expression via protein kinase A.

4. Discussion

In the past decade, 5-HT_6 receptor has drawn more and more attention due to its important role in memory, cognition and depression (Aparicio-Nava et al., 2019; Barkai et al., 2019; Liu et al., 2019; Shahidi et al., 2019). Although a fair amount of data has implicated a significant role of 5-HT_6 receptors in neuropsychiatric disorders (Jastrzebska-Wiesek et al., 2018; Li et al., 2017; Partyka et al., 2019; Suarez-Santiago et al., 2017), much less is known about the modulation function of 5-HT_6 receptor on pain. To the best of our knowledge, the present study is the first to demonstrate a potential role for 5-HT_6 receptors modulating nociceptive behavior at the cerebral cortex level.

4.1. Activation of 5-HT_6 receptors in the VLO produced pronociception

Numerous animal studies using *in situ* hybridization histochemistry assays, immunohistochemisty and autoradiography, have demonstrated that the 5-HT_6 receptors were exclusively expressed at the CNS, while faint or negligible expression was also detected outside of CNS (Ruat et al., 1993; Stefulj et al., 2000; Wesolowska, 2010; Yang et al., 2006). In the peripheral, local ipsilateral pre-treatment with the selective serotonin reuptake inhibitor fluoxetine (0.3–3 nmol/paw) increased 0.5% formalin-induced nociception; and this peripheral pronociceptive effect of fluoxetine was prevented by the 5-HT_6 receptor antagonist (Cervantes-Duran et al., 2013), suggesting the involvement of peripheral 5-HT_6 receptors on inflammatory pain modulation. Further, local peripheral or intraperitoneal administration of selective 5-HT_6 receptor

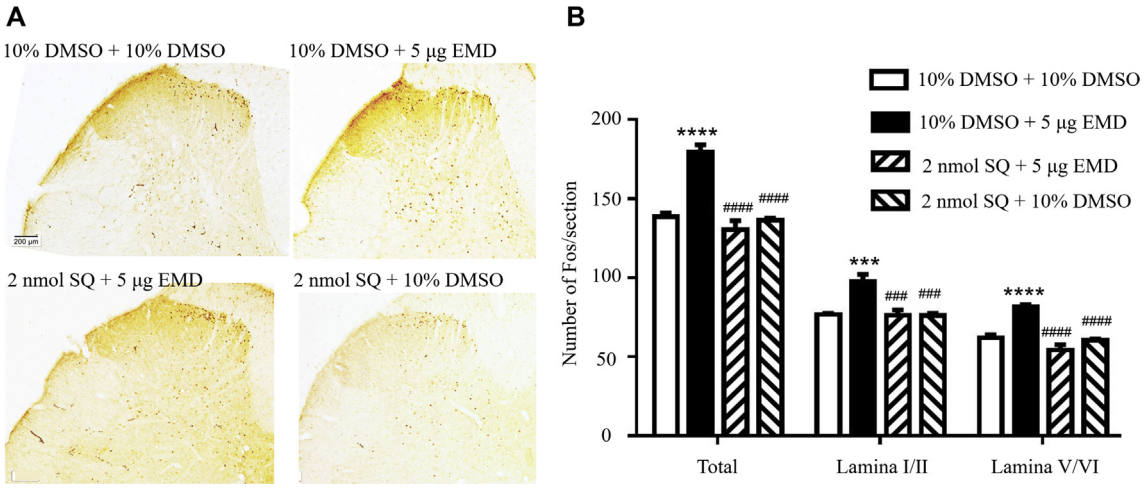

Fig. 9. The blocking effect of intra-VLO microinjection of the AC inhibitor SQ-22536 on EMD-386088- induced increase of formalin-induced Fos expression. (A) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in 4 different groups. (B) Bar graphs showing mean number of the expression of Fos in the total, superficial (laminae I-II) and deep (laminae V-VI) dorsal horn neurons in the lumbar spinal cord. $^{***}P < 0.001$ and $^{****}P < 0.0001$,compared with their corresponding 10% DMSO +10% DMSO group; $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$,compared with their corresponding 10% DMSO +5 μg EMD-386088 group. n = 4–6 rats/group.

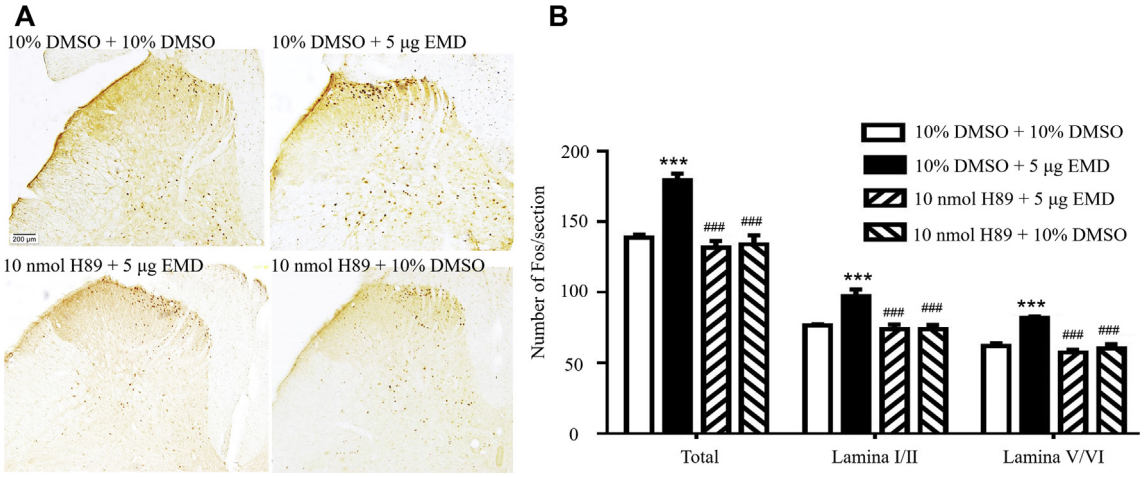

Fig. 10. The blocking effect of intra-VLO microinjection of the AC inhibitor SQ-22536 on WAY-208466- induced increase of formalin-induced Fos expression. (A) Sections processed for immunohistochemistry show the expression of Fos protein in the lumbar spinal cord after s.c. 5% formalin injection in 4 different groups. (B) Bar graphs showing mean number of the expression of Fos in the total, superficial (laminae I-II) and deep (laminae V-VI) dorsal horn neurons. $^{***}P < 0.001$ compared with their corresponding 10% DMSO +10% DMSO group; $^{\#\#\#}P < 0.001$ compared with their corresponding 10% DMSO +5 μg EMD group. n = 4–6 rats/group.

antagonists significantly reduced formalin-induced flinching (Finn et al., 2007), while 5-HT_6 receptor agonist augmented this nociceptive behavior. Besides, the local pronociceptive effect of EMD-386088 could be reduced by 5-HT_6 receptor antagonists. In the DRG, a remarkable increase of 5-HT_6 receptor mRNA expression has been demonstrated at 4 h after subcutaneous injection of bee venom (BV), which indicates that 5-HT_6 receptor might exert some functional influence on BV-induced nociceptive behavior (Liu et al., 2005). In the spinal cord, spinal injection of EMD-386088 enhanced formalin-induced nociception and this pronociceptive effect was completely prevented by the intrathecal (i.t.) administration of 5-HT_6 receptor antagonists, implicating that activation of spinal 5-HT_6 receptors promote nociception (Castaneda-Corral et al., 2009). These results consistently suggested that 5-HT_6 receptor in the peripheral, DRG, or spinal cord could exhibit pronociception. However, its pain modulating function was never identified at cortex level although 5-HT_6 receptors were almost exclusively expressed at central nervous system.

Numerous studies in our laboratory have demonstrated that the VLO is part of an endogenous analgesic system consisting of an ascending pathway from the spinal cord to the VLO via the nucleus submedius (Sm) and a descending pathway to the spinal cord relaying in the PAG (Taati and Tamaddonfard, 2018; Tamaddonfard et al., 2017; Tang et al., 2009). The current study investigated the possible contribution of 5-HT_6 receptor and its selective agonists/antagonist in the VLO in the formalin-induced nociceptive behaviors in rats. Firstly, our findings demonstrated that administration of EMD-386088 (5.0 μg/0.5 μl) or WAY-208466 (8.0 μg/0.5 μl) into the VLO increased formalin-induced nociceptive behaviors. This finding suggested that application of 5-HT_6 receptor agonist EMD-386088 into the VLO would produce pronociceptive effects in rats. As EMD-386088 also possessed moderate affinity for the 5-HT_3 receptors, next we used another selective 5-HT_6 receptor agonist WAY-208466 to confirm its effect. Consistently, microinjection of WAY-208466 into the VLO also increased the number formalin-induced flinch; besides, pretreatment with SB-258585 into the VLO almost completely blocked EMD-386088 and WAY-208466 induced pronociception, respectively.

Secondly, application of SB-258585 (1.0, 2.0 and 4.0 μg/0.5 μl) into the VLO significantly inhibited nociceptive behaviors. Application of

SB-258585 alone into the VLO might potentially activate the descending pain modulation pathway, thus producing antinociceptive effects, and these effects are mediated by 5-HT_6 receptors in the VLO. The current data provide novel behavioral evidence in antinociceptive effects on the involvement of 5-HT_6 receptors in the VLO in the descending modulation in persistent inflammatory nociception.

It is interesting that activation of 5-HT_6 receptors in the VLO produced pronociception, as the VLO is an analgesic brain region (Tang et al., 2009; Wei et al., 2016). Previous studies showed that WAY-208466 (10 mg/kg, s.c.) preferentially elevated cortical GABA levels following both acute and chronic (14 days) administration, indicating an enhanced GABAergic activity following 5-HT_6 receptor stimulation (Schechter et al., 2008). Microdialysis studies actually showed that systemic administration of a 5-HT_6 receptor antagonist produced a two- and three-fold increase in basal glutamate levels in the rat hippocampus and frontal cortex, respectively (Dawson et al., 2001; L.A. Dawson et al., 2000). Previous studies in our lab have demonstrated that the $GABA_A$ receptor may exert a tonic inhibitory influence on VLO neurons projecting to PAG, and its blockage results in enhancement of the activity of the VLO – PAG brainstem descending inhibitory system and depression of the nociceptive inputs at the spinal cord level. Therefore, it is not difficult to propose that, the pronociceptive effect of two 5-HT_6 receptor agonists in the present study could be attributed to the activation of 5-HT_6 receptor on the GABAergic neurons, which are suggested by previous studies (Cole et al., 2007; Schechter et al., 2005), while the GABAergic neurons are widely present throughout all layers of the VLO, especially in layer II and V (Huo et al., 2005). On the contrary, directly inhibiting the GABAergic inhibitory interneurons by the 5-HT_6 receptor antagonist SB-258585 could lead to indirect activation of the descending antinociceptive pathway through a disinhibitory effect on the VLO output neurons and depression of the nociceptive inputs at the spinal cord level. Of course, we cannot exclude the possibility that 5-HT_6 receptor antagonist SB-258585 may also increase glutamate level, thereby exciting the VLO output neurons to exert antinociception. Future study will focus on addressing and disentangling the interaction between 5-HT_6 receptors and glutamatergic/GABAergic neurons.

Subcutaneous formalin injection into the hindpaw pad of rats induces dramatic Fos expression in the lumbar spinal cord dorsal horn ipsilateral to the injected hindpaw and a significant paw flinching response. The Fos were densely distributed in the L4–5 spinal dorsal horn, which have been suggested to contain numerous nociceptive primary afferents and nociceptive neurons (Jinks et al., 2002). Mounting evidence suggested that Fos expression in the spinal cord not only reveals the intensity and duration of the noxious stimulus (Abbadie et al., 1997), but also represents nociceptive transmission as well as antinociceptive modulation occurring in the spinal cord following noxious stimuli such as subcutaneous formalin injection (Huo et al., 2010). In this way, activation of 5-HT_6 receptors on the GABAergic neurons disinhibits the VLO output neurons, resulting in activation of the descending periaqueductal gray (PAG) – spinal cord pathway. Therefore, the upregulated information transmission in the spinal cord increased activation of spinal neurons.

4.2. The AC/PKA signaling pathway might be responsible for the pronociception of 5-HT_6 receptors

The 5-HT_6 receptor is a typical G-protein-coupled receptor (GPCR) that positively stimulates AC activity, resulting in an increase in cyclic adenosine monophosphate and causing PKA activation. However, 5-HT_6 receptor has also been reportedly linked to several other cellular signaling pathways, including Fyn-tyrosine kinase or K^+ channels. More recently, three Gs-protein-independent pathways were found to be involved in 5-HT_6 receptor signaling: (i) the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, which plays an important role in cell proliferation, cell survival and cell death; (ii) the pathway which leads to Jun activation; (iii) recently, the mTOR pathway, which plays an important role in neurodevelopment (Karila et al., 2015); (iv) most recently, human 5-HT_6 receptor could also directly binds to neuro-oncological ventral antigen 1 (Nova-1), a brain-enriched splicing regulator, and this interaction contributes to the proteasomal degradation of 5-HT_6 receptor (Kim et al., 2019). To the best of our knowledge, however, there is no direct evidence shedding light on which signaling pathway underlies pain regulation up to now. In this study, the AC inhibitor SQ-22536 or the PKA inhibitor H89 were microinjected into the VLO prior to EMD-386088 injection at the same site. The pronociceptive effect of EMD-386088 was substantially reduced by either inhibitors. These results suggested that the sensitizing effect of 5-HT_6 receptors in inflammatory pain is a consequence of activation of Gs-protein-coupled AC/PKA cascade. Its activation produces an excitatory effect on target neurons by depolarizing cell membrane (Lesiak et al., 2018). In this way, activation of 5-HT_6 receptors on the GABAergic neurons disinhibits the VLO output neurons, resulting in activation of the descending antinociceptive pathway.

Taken together, the present results provided novel evidence that 5-HT_6 receptors in the VLO would produce pronociception in persistent inflammatory pain processing. The pronociceptive role in the rat formalin test was due to its activation of AC/PKA pathway, potentially via the PAG-brainstem descending inhibitory system. It is the first information about its pronociceptive effect in the higher cortex level. Future investigation using morphological and electrophysiological approaches will be necessary to elucidate the underlying molecular mechanisms of 5-HT_6 receptors biasing GABAergic rather than glutamatergic neurons in the VLO under inflammatory pain conditions.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104562.

References

Abbadie, C., Taylor, B.K., Peterson, M.A., Basbaum, A.I., 1997. Differential contribution of the two phases of the formalin test to the pattern of c-fos expression in the rat spinal cord: studies with remifentanil and lidocaine. Pain 69, 101–110.

Aparicio-Nava, L., Tellez, R., Gonzalez, R., Liy-Salmeron, G., Meneses, A., 2019. Intrahippocampal administration of 5-HT6 receptor drugs on memory consolidation and amnesia protocols. Behav. Brain Res. 359, 378–385.

Barkai, O., Puig, S., Lev, S., Title, B., Katz, B., Eli-Berchoer, L., Gutstein, H.B., Binshtok, A.M., 2019. Platelet-derived growth factor activates nociceptive neurons by inhibiting M-current and contributes to inflammatory pain. Pain 160, 1281–1296.

Brouard, J.T., Schweimer, J.V., Houlton, R., Burnham, K.E., Queree, P., Sharp, T., 2015. Pharmacological evidence for 5-HT6 receptor modulation of 5-HT neuron firing in vivo. ACS Chem. Neurosci. 6, 1241–1247.

Castaneda-Corral, G., Rocha-González, H., Araiza-Saldana, C., Ambriz-Tututi, M., Vidal-Cantú, G., Granados-Soto, V., 2009. Role of peripheral and spinal 5-HT 6 receptors according to the rat formalin test. Neuroscience 162, 444–452.

Cervantes-Duran, C., Rocha-Gonzalez, H.I., Granados-Soto, V., 2013. Peripheral and spinal 5-HT receptors participate in the pronociceptive and antinociceptive effects of fluoxetine in rats. Neuroscience 252, 396–409.

Cha, M., Lee, K., Won, J.S., Lee, B.H., 2019. Manganese-enhanced magnetic resonance

imaging of the spinal cord in rats with formalin-induced pain. Neurosci. Res. https://doi.org/10.1016/j.neures.2019.01.007. pii: S0168-0102(18)30521-2.
Cole, D.C., Stock, J.R., Lennox, W.J., Bernotas, R.C., Ellingboe, J.W., Boikess, S., Coupet, J., Smith, D.L., Leung, L., Zhang, G.M., Feng, X., Kelly, M.F., Galante, R., Huang, P., Dawson, L.A., Marquis, K., Rosenzweig-Lipson, S., Beyer, C.E., Schechter, L.E., 2007. Discovery of N1-(6-chloroimidazo[2,1-b][1,3]thiazole-5-sulfonyl)tryptamine as a potent, selective, and orally active 5-HT(6) receptor agonist. J. Med. Chem. 50, 5535–5538.
Dang, Y.H., Xing, B., Zhao, Y., Zhao, X.J., Huo, F.Q., Tang, J.S., Qu, C.L., Chen, T., 2011. The role of dopamine receptors in ventrolateral orbital cortex-evoked antinociception in a rat formalin test model. Eur. J. Pharmacol. 657, 97–103.
Dawson, L.A., N, H.Q., Li, P., 2000. In vivo effects of the 5-HT6 antagonist SB-271046 on striatal and frontal cortex extracellular concentrations of noradrenaline, dopamine, 5-HT, glutamate and aspartate. Br. J. Pharmacol. 130, 23–26.
Dawson, L.A., Nguyen, H.Q., Li, P., 2001. The 5-HT(6) receptor antagonist SB-271046 selectively enhances excitatory neurotransmission in the rat frontal cortex and hippocampus. Neuropsychopharmacology 25, 662–668.
Deraredj Nadim, W., Chaumont-Dubel, S., Madouri, F., Cobret, L., De Tauzia, M.L., Zajdel, P., Benedetti, H., Marin, P., Morisset-Lopez, S., 2016. Physical interaction between neurofibromin and serotonin 5-HT6 receptor promotes receptor constitutive activity. Proc. Natl. Acad. Sci. U. S. A. 113, 12310–12315.
Finn, D.P., Fone, K.C., Beckett, S.R., Baxter, J.A., Ansell, L., Marsden, C.A., Chapman, V., 2007. The effects of pharmacological blockade of the 5-HT 6 receptor on formalin-evoked nociceptive behaviour, locomotor activity and hypothalamo–pituitary–adrenal axis activity in rats. Eur. J. Pharmacol. 569, 59–63.
Godinez-Chaparro, B., Barragan-Iglesias, P., Castaneda-Corral, G., Rocha-Gonzalez, H.I., Granados-Soto, V., 2011. Role of peripheral 5-HT(4), 5-HT(6), and 5-HT(7) receptors in development and maintenance of secondary mechanical allodynia and hyperalgesia. Pain 152, 687–697.
Harris, J.A., 1998. Using c-fos as a neural marker of pain. Brain Res. Bull. 45, 1–8.
Huo, F.Q., Wang, J., Li, Y.Q., Chen, T., Han, F., Tang, J.S., 2005. GABAergic neurons express mu-opioid receptors in the ventrolateral orbital cortex of the rat. Neurosci. Lett. 382, 265–268.
Huo, F.Q., Huang, F.S., Lv, B.C., Chen, T., Feng, J., Qu, C.L., Tang, J.S., Li, Y.Q., 2010. Activation of serotonin 1A receptors in ventrolateral orbital cortex depresses persistent nociception: a presynaptic inhibition mechanism. Neurochem. Int. 57, 749–755.
Jastrzebska-Wiesek, M., Siwek, A., Partyka, A., Kolaczkowski, M., Walczak, M., Smolik, M., Latacz, G., Kiec-Kononowicz, K., Wesolowska, A., 2018. Study on the effect of EMD386088, a 5-HT6 receptor partial agonist, in enhancing the anti-immobility action of some antidepressants in rats. Naunyn Schmiedeberg's Arch. Pharmacol. 391, 37–49.
Jinks, S.L., Simons, C.T., Dessirier, J.M., Carstens, M.I., Antognini, J.F., Carstens, E., 2002. C-fos induction in rat superficial dorsal horn following cutaneous application of noxious chemical or mechanical stimuli. Exp. Brain Res. 145, 261–269.
Karila, D., Freret, T., Bouet, V., Boulouard, M., Dallemagne, P., Rochais, C., 2015. Therapeutic potential of 5-HT6 receptor agonists. J. Med. Chem. 58, 7901–7912.
Kim, S.H., Seo, M., Hwang, H., Moon, D.M., Son, G.H., Kim, K., Rhim, H., 2019. Physical and functional interaction between 5-HT6 receptor and nova-1. Exp. Neurobiol. 28, 17–29.
Kumar, S., Singh, A.K., Vinayak, M., 2019. ML171, a specific inhibitor of NOX1 attenuates formalin induced nociceptive sensitization by inhibition of ROS mediated ERK1/2 signaling. Neurochem. Int. 129, 104466.
Lesiak, A.J., Brodsky, M., Cohenca, N., Croicu, A.G., Neumaier, J.F., 2018. Restoration of physiological expression of 5-HT6 receptor into the primary cilia of null mutant neurons lengthens both primary cilia and dendrites. Mol. Pharmacol. 94, 731–742.
Li, P., Sun, H.J., Han, Y., Wang, J.J., Zhang, F., Tang, C.S., Zhou, Y.B., 2013. Intermedin enhances sympathetic outflow via receptor-mediated cAMP/PKA signaling pathway in nucleus tractus solitarii of rats. Peptides 47, 1–6.
Li, X.H., Wang, Q.Y., Hu, T.T., Wang, Y., Zhao, J., Lu, J., Pei, G., 2017. A tricyclic antidepressant, amoxapine, reduces amyloid- ss generation through multiple serotonin receptor 6-mediated targets. Sci. Rep. 7, 10.
Liu, X.Y., Wu, S.X., Wang, Y.Y., Wang, W., Zhou, L., Li, Y.Q., 2005. Changes of 5-HT receptor subtype mRNAs in rat dorsal root ganglion by bee venom-induced inflammatory pain. Neurosci. Lett. 375, 42–46.
Liu, K.C., Li, J.Y., Xie, W., Li, L.B., Zhang, J., Du, C.X., Zhang, Y.M., Tan, H.H., Wang, H.S., Zhang, L., 2016. Activation and blockade of serotonin6 receptors in the dorsal hippocampus enhance T maze and hole-board performance in a unilateral 6-hydroxydopamine rat model of Parkinson's disease. Brain Res. 1650, 184–195.
Liu, C., Wen, Y., Huang, H., Lin, W., Huang, M., Lin, R., Ma, Y., 2019. Over-expression of 5-HT6 receptor and activated jab-1/p-c-jun play important roles in pilocarpine-induced seizures and learning-memory impairment. J. Mol. Neurosci. 67, 388–399.
Monti, J.M., Jantos, H., Schechter, L.E., 2013. The effects of systemic and local microinjection into the central nervous system of the selective serotonin 5-HT6 receptor agonist WAY-208466 on sleep and wakefulness in the rat. Behav. Brain Res. 249, 65–74.
Nikiforuk, A., Kos, T., Wesolowska, A., 2011. The 5-HT6 receptor agonist EMD 386088 produces antidepressant and anxiolytic effects in rats after intrahippocampal administration. Psychopharmacology (Berlin) 217, 411–418.
Partyka, A., Jastrzebska-Wiesek, M., Antkiewicz-Michaluk, L., Michaluk, J., Wasik, A., Canale, V., Zajdel, P., Kolaczkowski, M., Wesolowska, A., 2019. Novel antagonists of 5-HT6 and/or 5-HT7 receptors affect the brain monoamines metabolism and enhance the anti-immobility activity of different antidepressants in rats. Behav. Brain Res. 359, 9–16.
Paxinos, G., Watson, C., 1997. Rat Brain in Stereotaxic Coordinates.
Pratt, W.E., Blackstone, K., Connolly, M., Skelly, M.J., 2009. Selective serotonin receptor stimulation of the medial nucleus accumbens causes differential effects on food intake and locomotion. Behav. Neurosci. 123, 1046–1057.
Pratt, W.E., Schall, M.A., Choi, E., 2012. Selective serotonin receptor stimulation of the medial nucleus accumbens differentially affects appetitive motivation for food on a progressive ratio schedule of reinforcement. Neurosci. Lett. 511, 84–88.
Qu, C.-L., Tang, J.-S., Jia, H., 2006. Involvement of GABAergic modulation of antinociception induced by morphine microinjected into the ventrolateral orbital cortex. Brain Res. 1073, 281–289.
Qu, C.L., Dang, Y.H., Tang, J.S., 2015. Administration of somatostatin analog octreotide in the ventrolateral orbital cortex produces sex-related antinociceptive effects on acute and formalin-induced nociceptive behavior in rats. Neurochem. Int. 87, 77–84.
Ruat, M., Traiffort, E., Arrang, J.M., Tardivel-Lacombe, J., Diaz, J., Leurs, R., Schwartz, J.C., 1993. A novel rat serotonin (5-HT6) receptor: molecular cloning, localization and stimulation of cAMP accumulation. Biochem. Biophys. Res. Commun. 193, 268–276.
Schechter, L.E., Ring, R.H., Beyer, C.E., Hughes, Z.A., Khawaja, X., Malberg, J.E., Rosenzweig-Lipson, S., 2005. Innovative approaches for the development of antidepressant drugs: current and future strategies. NeuroRx 2, 590–611.
Schechter, L.E., Lin, Q., Smith, D.L., Zhang, G., Shan, Q., Platt, B., Brandt, M.R., Dawson, L.A., Cole, D., Bernotas, R., Robichaud, A., Rosenzweig-Lipson, S., Beyer, C.E., 2008. Neuropharmacological profile of novel and selective 5-HT6 receptor agonists: WAY-181187 and WAY-208466. Neuropsychopharmacology 33, 1323–1335.
Shahidi, S., Hashemi-Firouzi, N., Asl, S.S., Komaki, A., 2019. Serotonin type 6 receptor antagonist attenuates the impairment of long-term potentiation and memory induced by Abeta. Behav. Brain Res. 364, 205–212.
Stefulj, J., Jernej, B., Cicin-Sain, L., Rinner, I., Schauenstein, K., 2000. mRNA expression of serotonin receptors in cells of the immune tissues of the rat. Brain Behav. Immun. 14, 219–224.
Suarez-Santiago, J.E., Briones-Aranda, A., Espinosa-Raya, J., Picazo, O., 2017. Agonist E-6837 and antagonist SB-271046 of 5-HT6 receptors both reverse the depressive-like effect induced in mice by subchronic ketamine administration. Behav. Pharmacol. 28, 582–585.
Taati, M., Tamaddonfard, E., 2018. Ventrolateral orbital cortex oxytocin attenuates neuropathic pain through periaqueductal gray opioid receptor. Pharmacol. Rep. 70, 577–583.
Tamaddonfard, E., Erfanparast, A., Farshid, A.A., Delkhosh-Kasmaie, F., 2017. Role of ventrolateral orbital cortex muscarinic and nicotinic receptors in modulation of capsaicin-induced orofacial pain-related behaviors in rats. Eur. J. Pharmacol. 815, 399–404.
Tang, B., Ji, Y., Traub, R.J., 2008. Estrogen alters spinal NMDA receptor activity via a PKA signaling pathway in a visceral pain model in the rat. Pain 137, 540–549.
Tang, J.S., Qu, C.L., Huo, F.Q., 2009. The thalamic nucleus submedius and ventrolateral orbital cortex are involved in nociceptive modulation: a novel pain modulation pathway. Prog. Neurobiol. 89, 383–389.
Wei, L., Zhu, Y.M., Zhang, Y.X., Liang, F., Jia, H., Qu, C.L., Wang, J., Tang, J.S., Lu, S.M., Huo, F.Q., Yan, C.X., 2016. Activation of alpha1 adrenoceptors in ventrolateral orbital cortex attenuates allodynia induced by spared nerve injury in rats. Neurochem. Int. 99, 85–93.
Wesolowska, A., 2010. Potential role of the 5-HT6 receptor in depression and anxiety: an overview of preclinical data. Pharmacol. Rep. 62, 564–577.
Yang, G.B., Qiu, C.L., Zhao, H., Liu, Q., Shao, Y., 2006. Expression of mRNA for multiple serotonin (5-HT) receptor types/subtypes by the peripheral blood mononuclear cells of rhesus macaques. J. Neuroimmunol. 178, 24–29.
Zhang, Y.M., Zhang, L., Wang, Y., Sun, Y.N., Guo, Y., Du, C.X., Zhang, J., Yao, L., Yu, S.Q., Liu, J., 2016. Activation and blockade of prelimbic 5-HT6 receptors produce different effects on depressive-like behaviors in unilateral 6-hydroxydopamine-induced Parkinson's rats. Neuropharmacology 110, 25–36.
Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.