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Intra-periaqueductal gray matter administration of orexin-A exaggerates pulpitis-induced anxiogenic responses and c-fos expression mainly through the interaction with orexin 1 and cannabinoid 1 receptors in rats

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ABSTRACT

Different types of trigeminal pains are frequently associated with psychophysiological concerns. Orexin-A and orexin 1 receptor (OX1R) are involved in modulation of both trigeminal pain and anxiety responses. Ventrolateral periaqueductal gray matter (vlPAG), a controlling site for nociception and emotion, receives orexinergic inputs. Here, the role of vlPAG OX1Rs and their interaction with cannabinoid 1 (CB1) receptor was evaluated in anxiety-like behavior following capsaicin-induced dental pulp pain. Rats were cannulated in the vlPAG and orexin-A was injected at the doses of 0.17, 0.35 and 0.51 μg/rat prior to the induction of pain. The elevated plus maze (EPM) and open field (OF) tests were used for assessing the anxiety responses. In addition, the induction of c-fos, in the vlPAG, was investigated using immunofluorescence microscopy. Capsaicin-treated rats displayed significantly higher anxiogenic behavior on EPM and OF tests. Pretreatment with orexin-A (0.51 μg/rat) attenuated capsaicin-mediated nociception, while exaggerated anxiogenic responses ($p < 0.05$). In addition, orexin-A effects were diminished by the administration of OX1R (SB-334867, 12 μg/rat) and cannabinoid 1 (AM251, 4 μg/rat) receptor antagonists. Intradental capsaicin induced a significant increase in c-fos expression in the vlPAG that was exaggerated by orexin-A (0.51 μg/rat). Blockage of OX1R and CB1 receptors attenuated the effect of orexin-A on c-fos expression in capsaicin-treated rats. In conclusion, the data suggest that manipulation of OX1R and CB1 receptors in the vlPAG alters capsaicin-evoked anxiety like behaviors and c-fos induction in rats.

1. Introduction

Toothache is primarily induced by injury to the sensory nerves of the dental pulp (Holland, 2013). Besides sensory features, it often produces considerable neurological problems such as sleep disruption, mental deficiencies and increasing vulnerability to emotional and anxiety disorders (Armfield and Heaton, 2013; Lavigne and Sessle, 2016; Raoof et al., 2015). In particular, a positive correlation has been observed between odontalgia and anxiety responses in both clinical and preclinical trials (Armfield and Heaton, 2013; Newton and Buck, 2000; Raoof et al., 2016).

After primarily processing in the spinal trigeminal nuclei, sensory signals from orofacial structures including dental pulp are carried to the thalamus, mainly to the ventral posterior medial nucleus; and projected to the primary somatosensory cortex (Merrill, 2007; Schulte et al., 2016). Brain imaging studies have indicated that the stimulation of trigeminal nerve can activate midbrain areas such as periaqueductal gray matter (PAG) that are involved in the modulation of nociceptive signals (Knight and Goadsby, 2001; May, 2009). The PAG is subdivided into distinct functional and anatomical parts including dorsomedial, lateral, dorsolateral, and ventrolateral columns. The dorsal portions coordinate autonomic system and activate defensive behaviors, whereas the ventrolateral part of the PAG (vlPAG) elicits passive-coping responses including anxiety and nociception (Behbehani, 1995; Carrive,

Abbreviations: PAG, periaqueductal gray matter; vlPAG, ventrolateral periaqueductal gray matter; OX1R, orexin 1 receptor; CB1, cannabinoid 1 receptor; EPM, elevated plus maze; OF, open field; Vc, trigeminal nucleus caudalis; CREB, the cAMP response element binding protein; MAPK, mitogen-activated protein kinases

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1993; Mendes-Gomes et al., 2011). Interestingly, it has been demonstrated that sensory spinal trigeminal nuclei receive direct projections from ventral and dorsal PAG regions (Li et al., 1993). It has been also indicated that trigeminovascular pain alters the functional and structural connectivity of the PAG circuits (Knight and Goadsby, 2001).

Neuropeptides orexin-A and -B are exclusively expressed by hypothalamic neurons and activate their target cells via two G-protein coupled receptors, orexin 1 and 2 receptors (OX1R and OX2R, respectively) (Sakurai et al., 1998). Orexin receptors are expressed in many brain regions including amygdala, brain stem nuclei, hippocampus, hypothalamus and PAG, the areas that are involved in emotional and nociceptive responses. It has been reported that orexin-A treatment can induce anxiety-like responses in rodents (Li et al., 2010; Lungwitz et al., 2012; Suzuki et al., 2005). Additionally, previous data confirmed that orexin-A is a potential analgesic agent (Inutsuka et al., 2016; Razavi and Hosseinzadeh, 2017). Based on pharmacological and electrophysiological studies, orexin-A plays a beneficial role in modulation of trigeminal nociceptive signals in laboratory animals (Bartsch et al., 2004; Holland et al., 2006; Kooshki et al., 2016). Interestingly, administration of orexin-A into the trigeminal nucleus caudalis (Vc) decreases capsaicin-induced orofacial nociception, whereas it increases anxiety responses in rats (Bahaaddini et al., 2016; Kooshki et al., 2016).

To exert physiological responses, the orexinergic system interacts with a wide spectrum of other neurotransmitters (Liu et al., 2002; Selbach et al., 2004). Specially, OXIRs are co-expressed with cannabinoid 1 (CB1) receptors in the PAG. Pharmacological data also support such interpolation for modulating nociceptive responses (Cristino et al., 2016; Esmaeili et al., 2016). CB1 receptors are activated by arachidonic acid derivatives including anandamide and 2-arachidonoylglycerol (Howlett, 2005). It has been indicated that activation of CB1 receptors in the PAG can modulate both nociceptive and emotional responses (Campos and Guimarães, 2009; Finn et al., 2003).

As described above, there is some evidence indicating the functional and anatomical associations between PAG afferents and sensory trigeminal nerves. Considering the important roles of vlPAG in modulating nociceptive and emotional responses, in the present study we sought to investigate the possible involvement of vlPAG OX1R and its interaction with CB1 receptors in modulation of nociceptive responses and anxiety-like behaviors in an animal model of capsaicin-evoked pulpal nociception. In addition, in order to assess the effects of such treatment on vlPAG neuronal activity, the expression of c-fos was also evaluated.

2. Materials and methods

2.1. Animals

The present study was done on adult male Wistar rats (230–260 g). Animals were kept in a temperature-controlled environment (23 ± 1 °C) under a regular light/dark cycle (12:12 h). Food and water were available ad libitum. All experiments were approved by the ethical committee of Kerman Neuroscience Research Center, University of Medical Sciences, Kerman, Iran (EC 96). The rats were acclimatized to the laboratory conditions at least one week prior to the tests.

2.2. Drugs

The drugs including capsaicin, orexin-A, SB-334867 and AM251 were purchased from Sigma -Aldrich (USA). Capsaicin was dissolved in a vehicle comprising of tween 80, ethanol and distilled water (1:1:8). Orexin-A was dissolved in normal saline. SB-334867 and AM251 (as OX1R and CB1 receptor antagonists, respectively) were dissolved in dimethylsulfoxide (DMSO) and further diluted with artificial cerebrospinal fluid (aCSF) to make the working concentration. The total concentration of DMSO was < 0.1%.

2.3. Surgical procedure

The animals were anesthetized intraperitoneally (i.p.) with ketamine (100 mg/kg) and xylazine (10 mg/kg). Then they were mounted on a stereotaxic frame. A 23-gauge stainless steel guide cannula was implanted into the right vlPAG according to the following coordinates adapted from the atlas of (Paxinos and Watson, 2007): 7.8 mm posterior to the bregma, 0.6 mm lateral from the midline and 5.9 mm ventral to the cortical surface. Rats were allowed to recover for a week before the start of the experiments. After the experiments, methylene blue was injected via the guide cannula to confirm the correct placement of the cannula. If the cannula was not fixed in the correct place, the rat's data were discarded from the analysis.

2.4. Microinjection

Drugs and their vehicles were microinjected into the vlPAG using a 30-gauge stainless steel cannula connected through a polyethylene tube to a 5 µl Hamilton syringe. To reach the vlPAG, the injection needle was extended 1 mm beyond the tip of the guide cannula. All injections were delivered over 60 s in a volume of 1 µl. The needle was left in situ for at least 30 s to allow the solution to diffuse.

2.5. Experimental procedure

After one week recovery period, the rats were randomly assigned to the following groups (n = 6/group): capsaicin-vehicle group that intradentally received a small cotton pellet moistened with capsaicin vehicle (distilled water: ethanol: tween 80), capsaicin group that received intradental application of capsaicin (100 µg), orexin-A treated groups that received intra-vlPAG injection of orexin-A (0.17, 0.35 and 0.51 µg/rat) prior to capsaicin application, orexin-A vehicle group that received intra-vlPAG normal saline as an orexin-A vehicle before pain induction, two groups of rats received intra-vlPAG microinjection of orexin-A (0.51 µg/rat) in combination with either OX1R-antagonist SB-334867 (12 µg/rat) or CB1 receptor antagonist AM251 (4 µg/rat) prior to the administration of capsaicin, and another group of rats which were microinjected with DMSO (0.1%) as the vehicle of both SB-334867 and AM251. Intradental capsaicin was delivered 10 min after intra-vlPAG drug administrations. Immediately after the administration of capsaicin, nociceptive scores were recorded for a forty-minute period. Anxiety-related behaviors were then assessed in the elevated plus-maze (EPM) and the open field (OF) tests. The same rats were used in both behavioral tests and a ten-minute interval time was considered between the experiments.

2.6. Nociceptive procedure

On the test days, the rats (n = 6/group) were placed in the test room for a 30-minute habituation period. Ten minutes after drugs administration, dental pulp was stimulated by intradental application of capsaicin as reported previously (Raoof et al., 2018). Briefly, after a short-duration anesthesia with low concentration of carbon dioxide (CO2), a small cavity (2 mm^3) was prepared in the gingival third of distal aspect of left mandibular incisors' crowns using a small fissure bur in a high-speed handpiece with water coolant. With the help of magnification (2.5 ×), pulp exposure was prevented. A small cotton pellet moistened with capsaicin solution (100 µg) was left in the cavity under a light-cured glass-ionomer (Fuji II, GC, Japan) restoration. Upon capsaicin administration, each rat was placed in the transparent box (30 cm^3) with a mirror set at an angle of 45° to detect unbarred observation of the animals. The rats' behaviors were continually recorded for 40 min and nociceptive scores were calculated as previously described (Chidiac et al., 2002): 0: normal behavior such as grooming; 1: atypical head shaking or continuous placement of the jaw on the floor the box; 2: abnormal continuous shaking of the lower jaw and 3:

excessive and continuous rubbing of the mouth near the injection site.

2.7. Assessment of anxiety-like behavior

2.7.1. EPM test

EPM test is a valid experimental method to assess anxiety-like behaviors in rodents (Dawson and Tricklebank, 1995). The maze was elevated 50 cm above the ground and embraces of two open arms walled by Plexiglas ledges with 0.5 cm tall and two close arms bordered by 40 cm high wooden walls. Four arms had an equal size (60 cm) and attached at a central square (5 cm × 5 cm). Each rat (n = 6/group) was placed in the central area of the maze facing an open arm and permitted to explore the maze for 5 min. The time spent in each arm and the number of entries into each arm were recorded by video tracking system and calculated using the ANY-maze software.

2.7.2. OF test

The OF test was performed in a square wooden box (70 cm × 70 cm) that was bordered by 30 cm high walls. The rats (n = 6/group) were allowed to freely explore the apparatus for 5 min. Time spent and the number of entries into the center zone (covering 40 cm × 40 cm) were used as the measures of the anxiety-like behavior (Prut and Belzung, 2003).

2.8. Immunohistochemistry

After behavioral assessments, the rats (n = 4/group) were anesthetized by injection of ketamine and xylazine (100 and 10 mg/kg i.p., respectively) and perfused transcardially with 100 ml of 0.9% saline followed by 500 ml of 4% paraformaldehyde. The brain was fixed overnight in 4% paraformaldehyde. The slices were made from the PAG at a thickness of 200 μm according to the Paxinos and Watson (1998) rat brain atlas (0.5 mm lateral to the midline and 7.8 mm posterior to the bregma). The samples were embedded in paraffin and then 2-μm serial sections (three sections for each rat) were prepared from the PAG containing paraffin blocks and deparaffinized before immunostaining. The sections then were treated for antigen retrieval by microwave treatment for 30 min in citrate buffer (pH = 6) and washed for 3 min in phosphate-buffered saline solution (PBS). The slides were dipped in hydrogen peroxide for 10 min and then exposed to anti-c-fos protein primary antibody diluted at 1:500 (Santa cruz, USA) overnight at a humidity chamber. The slides were washed in PBS and incubated for 90 min with goat anti-rabbit IgG-CFL 488 secondary antibody diluted at 1:1000 (Santa cruz, USA) and washed again by PBS. Then, they were exposed to propidium iodide (PI) at room temperature, and immediately washed by PBS. Finally, the images were captured using a fluorescence microscope (Olympus) with 40× magnification. In the vlPAG area, the number of c-fos positive cells (white box, Fig. 8, Panel A 2) was counted by an experienced examiner who was blinded to the experimental conditions. The size of containing frame (60 × 90 μm) has been determined by Image J software.

2.9. Statistical analysis

All data are presented as mean ± SEM and the differences among the groups were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Anxiety-like behaviors

3.1.1. EPM test

As shown in Fig. 1A and B, the time spent ($p < 0.01$) and the number of entries into the open arms ($p < 0.01$) were significantly decreased in capsaicin-treated rats as compared to capsaicin-vehicle group. A significant decrease in the time spent on the open arms and in the number of entries into the open arms were observed in the animals that received orexin-A (0.51 μg/rat) prior to capsaicin ($p < 0.05$) (Fig. 1A and B). However, the mentioned anxiogenic effects of orexin-A (0.51 μg/rat) were significantly diminished by either SB-334867 (12 μg/rat) or AM 251 (4 μg/rat) (Fig. 2A and B). In addition, as shown in Fig. 5, there are no significant differences in total distance travelled by rats [$F(7, 47) = 0.656$, $P = 0.706$] (Fig. 5A). The examples for paths of representative rats in the EPM test are shown in Fig. 6B.

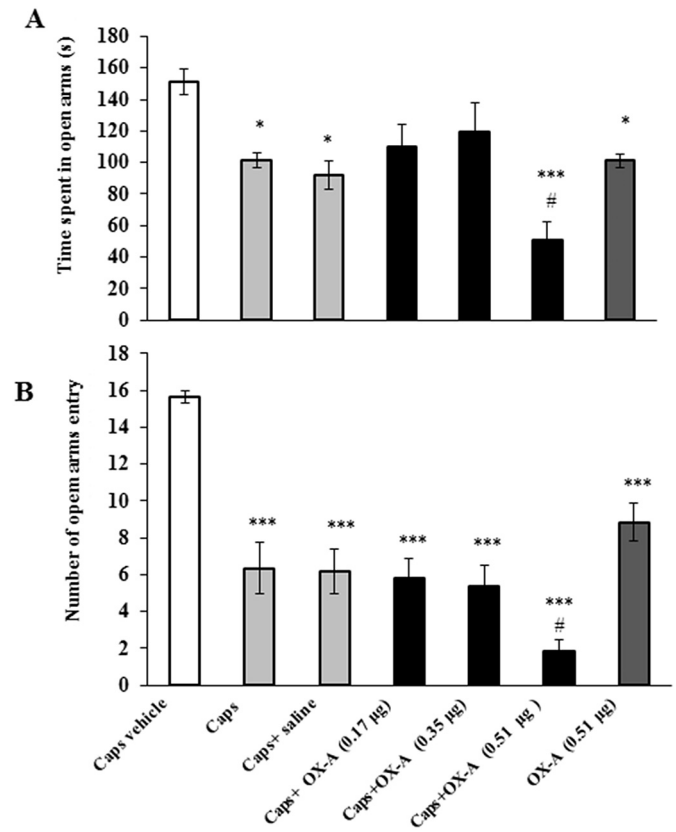

Fig. 1. The effect of intra-vlPAG administration of orexin-A on the total mean time (in seconds) spent in the open arms (A) and the total mean numbers of open arm entries (B) during a 5-min observation period on the elevated plus-maze in capsaicin-treated rats and capsaicin groups that had intra-vlPAG pretreatment with orexin-A (0.17, 0.35 and 0.51 μg/rat) (n = 6). Bar graphs illustrate mean ± SEM values. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs intact group, # $P < 0.05$ vs Caps group. Caps: capsaicin, OX-A: orexin-A.

3.1.2. OF test

In capsaicin-treated rats, the amount of time spent ($p < 0.001$) and the number of visits ($p < 0.01$) to the center zone of the OF apparatus were significantly decreased as compared to capsaicin-vehicle group. Microinjection of orexin-A (0.51 μg/rat) into the vlPAG could enhance the effects of capsaicin on those parameters (both $p < 0.05$) (Fig. 3). However, the anxiogenic effects of orexin-A were diminished by either SB-334867 or AM251 (Fig. 4A and B). There were no significant differences among experimental groups in total distance travelled in the OF test [$F(7, 47) = 1.168$, $P = 0.343$] (Fig. 5B). The examples for paths of representative rats in the test are shown in Fig. 6A.

3.2. Assessment of nociceptive behavior

Nociceptive scores induced by intradental administration of capsaicin are presented in Fig. 7. Intradental capsaicin elicited a significant nociceptive response that was significantly diminished following intra-

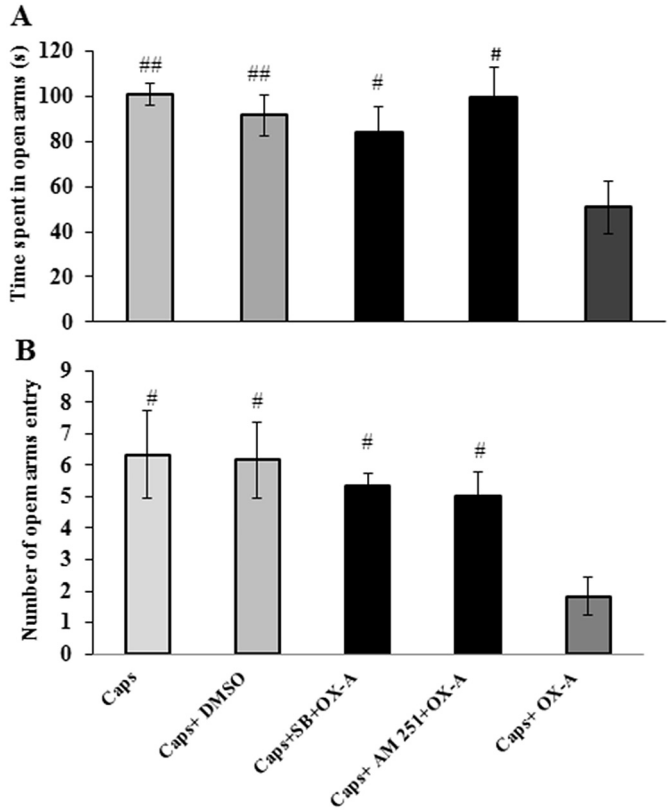

Fig. 2. The total time spent (in seconds) in the open arms (A) and the total numbers of open arm entries (B) during a 5-minute observation period on the elevated plus-maze in rats treated by orexin-A (0.51 μg/rat) alone, or in combination with either SB-334768 (12 μg/rat) or AM251 (4 μg/rat) prior to administration of capsaicin (n = 6). Bar graphs illustrate mean ± SEM values. ## P < 0.01, # P < 0.05 vs Caps + OXA group. Caps: capsaicin, OX-A: orexin-A.

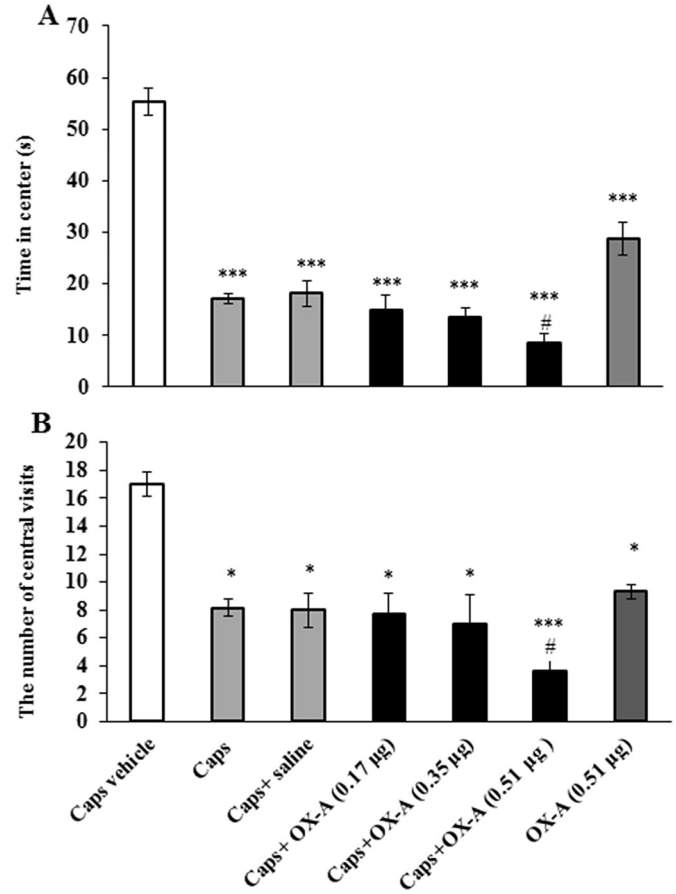

Fig. 3. The total mean time spent in the center (A) and the total mean numbers of entries into the center (B) of the open field test during a 5-minute observation period in capsaicin-treated rats and capsaicin groups that had intra-vlPAG orexin-A (0.17, 0.35 and 0.51 μg/rat) (n = 6). Bar graphs illustrate mean ± SEM values. *** P < 0.001, * P < 0.05 vs intact group, # P < 0.05 vs Caps group. Caps: capsaicin, OX-A: orexin-A.

vlPAG administration of orexin-A at doses of 0.35 ($p < 0.01$) and 0.51 μg/rat ($p < 0.001$). However, the analgesic effect of orexin-A (0.51 μg/rat) was inhibited following preadministration of both SB-334867 (12 μg/rat) ($p < 0.01$) and AM251 (4 μg/rat) ($p < 0.05$) (Fig. 7).

3.3. Immunohistochemistry

The amount of c-fos immunoreactivity was determined in the vlPAG sections in different experimental groups. As shown in Fig. 8 (panel B), c-fos -positive cells are observed in the vlPAG section of all experimental groups. The expression of c-fos was significantly increased following intradental application of capsaicin ($p < 0.05$). Moreover, intra-vlPAG administration of orexin-A (0.51 μg/rat) prior to intradental capsaicin could significantly increase the capsaicin-induced c-fos expression in the vlPAG ($p < 0.001$) (Fig. 8, panel C). However, the promoting effect of orexin-A on c-fos expression was prevented by SB-334867 and AM251 ($p < 0.01$). Besides, intra-vlPAG administration of orexin-A (without capsaicin) could significantly increase c-fos positive cells in the vlPAG sections as compared to the control ($p < 0.001$) and capsaicin + orexin-A ($p < 0.01$) groups.

4. Discussion

In the present study, capsaicin-induced pulpal pain increased behavioral indices as factors to explain anxiety-related behaviors of animals. Moreover, intra-vlPAG administration of orexin-A could exaggerate (dose dependently) the anxiogenic effects of capsaicin. However, it decreases capsaicin-induced pulpal pain. Orexin-A-induced behavioral changes were inhibited by either OX1R or CB1 antagonists. In addition, intradental administration of capsaicin increased c-fos expression in the vlPAG which was more pronounced by orexin-A at dose of 0.51 μg/rat, an effective dose for inducing a prompt anxiety response.

Capsaicin is a principal agonist of transient receptor potential vanilloid 1 channels on trigeminal types A and C sensory fibers and is often used as a tool to examine sensory nerve responses (Bae et al., 2004; Pelissier et al., 2002). Some emotional and psychological abnormalities have been also associated with the application of capsaicin in rodents. These behaviors might increase the possibility of anxiogenic responses. In particular, previous studies showed anxiety-like behaviors following oral and intradental administrations of capsaicin in rats (Choi et al., 2013; Raoof et al., 2016). In addition, oral capsaicin exposure increased c-fos expression in the nucleus tractus of solitarius and paraventricular hypothalamic nucleus that are involved in the affective components as well as modulation of nociceptive signals (Choi et al., 2013; Condés-Lara et al., 2015; Hsu et al., 2014; Venkatraman et al., 2017). A hyperactivity of the hypothalamic-pituitary-adrenal axis, a component of the stress response system, has been also reported following the administration of capsaicin (Choi et al., 2013).

Because of the massive distribution of neural signals in the pathways involved in emotions and anxiety, orexin-A has a pivotal modulatory role in psychophysiological responses such as anxiety-like behaviors (Johnson et al., 2012a; Trivedi et al., 1998). The behavioral data indicated that the orexin enhances the anxiety responses

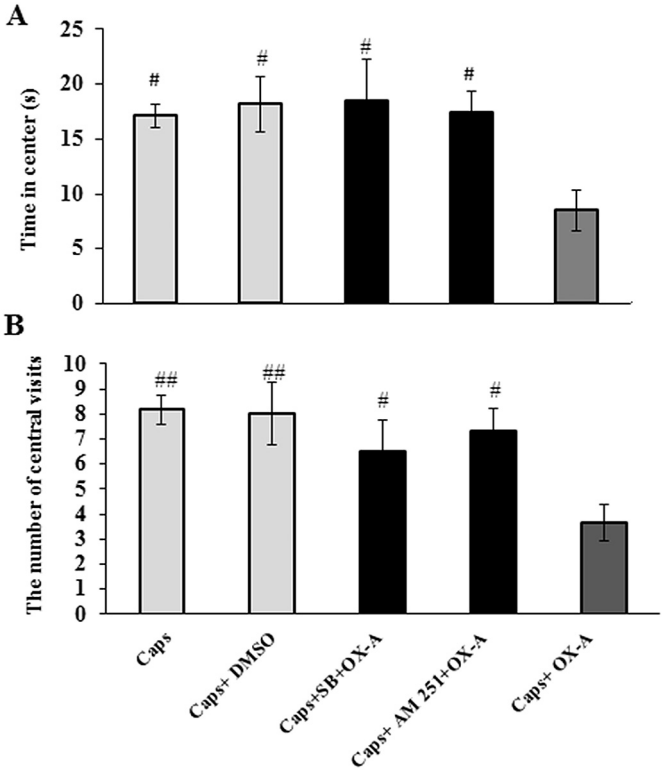

Fig. 4. The total mean time spent in the center (A) and the total mean numbers of entries into the center (B) of the open field test during a 5-minute observation period in rats treated with orexin-A (0.51 μg/rat) alone, or in combination with either SB-334768 (12 μg/rat) or AM251 (4 μg/rat) prior to the administration of capsaicin (n = 6). Bar graphs illustrate mean ± SEM values. $^{\#\#}$ $P < 0.01$, $^{\#}$ $P < 0.05$ vs Caps + OXA group. Caps: capsaicin, OX-A: orexin-A.

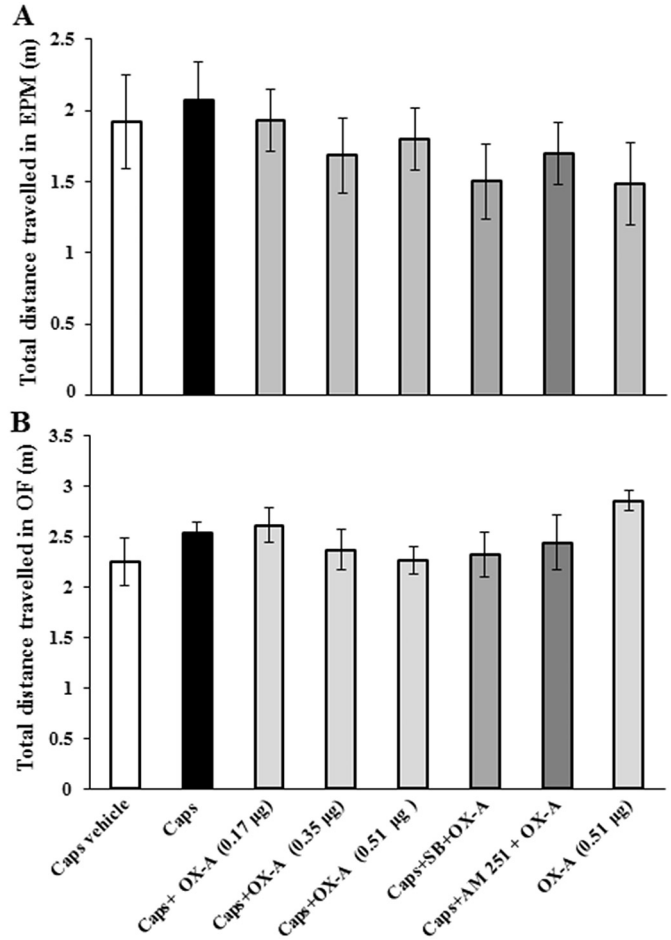

Fig. 5. Comparison the animals' locomotor behaviors of different experimental groups in the elevated plus-maze (A) and the open field (B) tests. Bar graphs illustrate mean ± SEM values. Caps: capsaicin, OX-A: orexin-A, SB: SB-334867.

(Lungwitz et al., 2012; Palotai et al., 2014; Suzuki et al., 2005). It has also been reported that neuronal activity and gene expression of orexin systems are increased in panic-prone state developed rats (Johnson et al., 2010). Moreover, orexin-A microinjection into the Vc, the key nucleus to relay orofacial noxious inputs to the higher central nervous system, exaggerates anxiety-like behaviors in capsaicin-treated rats (Bahaaddini et al., 2016). Despite robust evidence illustrating the anxiogenic roles of orexin-A, the underlying mechanisms of this effect have been poorly understood.

Here, orexin-A anxiogenic effects were attenuated following the blockade of OX1Rs in the vlPAG. Orexin-A binding to the OX1Rs is generally related to elevated calcium influx that may result in activation of diverse signaling pathways such as the mitogen-activated protein kinases (MAPK) pathway especially extracellular signal-regulated kinases (ERK). Orexin receptors can easily couple to the G-protein families and possibly other proteins, through which they regulate the activation of non-selective cation channels, adenylyl cyclase, phospholipases, and protein kinases (Kukkonen and Leonard, 2014). Orexin-A downstream signaling molecules are also able to modulate neuronal excitation and emotional responses (Rupprecht and Di Benedetto, 2017; Zamponi, 2016). It has been reported that the activation of protein kinase A in mice that were exposed to social defeat stress can modulate anxiety responses via increases in the expression of cAMP response element binding protein (CREB) and c-fos protein in the basolateral amygdala (Yang et al., 2016). Moreover, predator stress can induce CREB phosphorylation in the periaqueductal gray of rats (Adamec et al., 2003). So, in the present study, orexin-A-provoking effects on capsaicin-induced anxiety-like behaviors might be mediated by, at last in part, OX1R downstream signaling events.

The data also showed that CB1 receptors blockade could suppress orexin-A-mediated anxiety responses. In various brain areas such as the PAG, OX1Rs and CB1 receptors are often in close proximity (Cota et al., 2003; Thompson et al., 2017). Pharmacological manipulation of CB1 receptors exerts significant and also bimodal effects on anxiety-like behaviors depending on dosage, time course and injection site (Rey et al., 2012; Viveros et al., 2005). Here, AM251 as a CB1 receptor antagonist, was used to evaluate the possible role of CB1 receptors signaling on orexin-A-anxiogenic effects.

The data showed that CB1 receptor antagonist diminishes orexin-A effects. Although the mechanisms underlying the interaction between orexin and cannabinoid receptors have not been fully understood, the existence of receptor heteromers and activation of common intracellular signaling pathways (cross-talk and cross-modulation) can be considered as the basis for such interplay (Berrendero et al., 2018). It has been demonstrated that most GPCRs exist as dimers or, potentially, as high order oligomers and therefore their ligands with high pharmacological selectivity would be expected to target different types of receptors (Gomes et al., 2016). Anatomical, biochemical and behavioral studies show that there are bidirectional interactions between OX1Rs and CB1 receptors (Berrendero et al., 2018; Flores et al., 2013). For example, it has been reported that orexin-A infusion into the vlPAG induces analgesia via endocannabinoid system in rats (Ho et al., 2011). In addition, central administration of a selective CB1 antagonist can reduce orexin-A expression in the lateral hypothalamus (Merroun et al., 2015). Specially, in CHO cells, co-expression of both receptors shows a functional cross-talk which is associated with 100-fold increase in orexin-A activity and also inactivation of CB1 receptors, could suppress

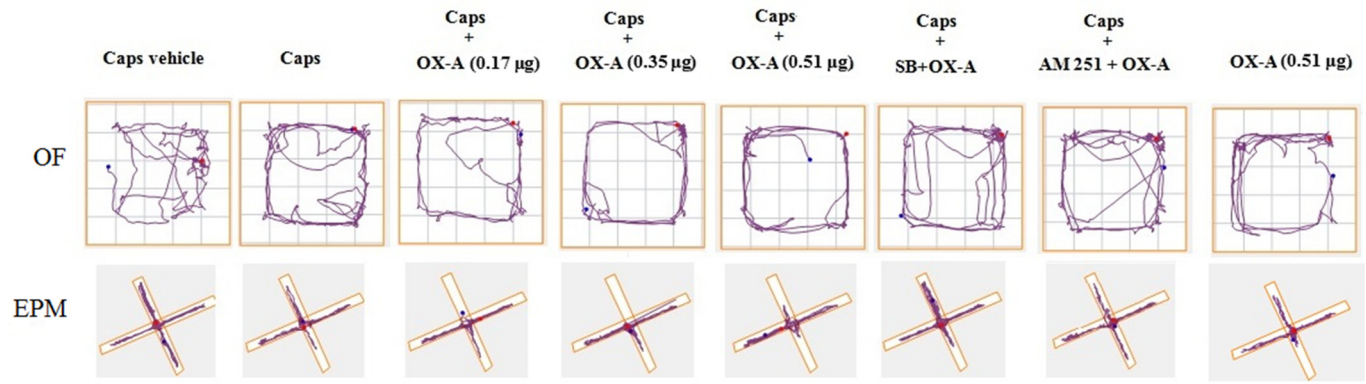

Fig. 6. An illustrative example of a rat's travel pathway in the open field (A) and the elevated plus-maze (B) tests. Caps: capsaicin, OX-A: orexin-A, SB: SB-334867.

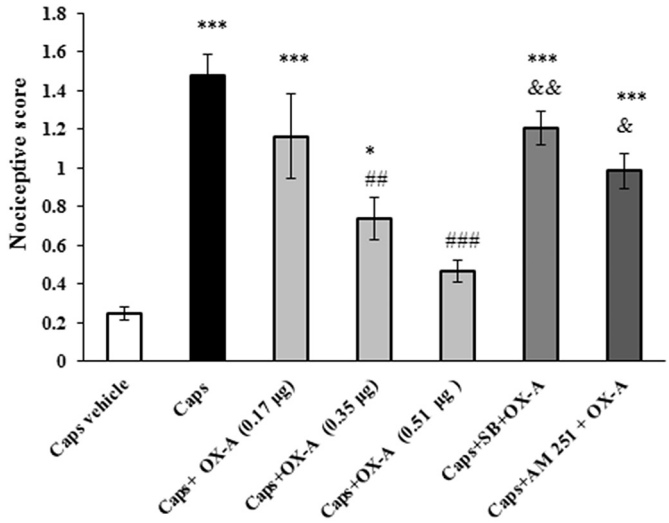

Fig. 7. Comparison of intradental capsaicin-induced nociceptive behaviors in the experimental groups (n = 6). Data represent mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs intact animals; $^{###}p < 0.001$, $^{##}p < 0.01$ vs Caps group; $^{\&\&}p < 0.01$, $^{\&}p < 0.05$ vs Caps + OX-A (0.51 µg/rat). Caps: capsaicin, OX-A: orexin-A, SB: SB-334867.

the potency of orexin-A to activate the MAP kinases (Hilairet et al., 2003). It has also been reported that blockage of CB1 receptors could suppress the potency of orexin-A to activate the MAP kinases in HEK293 cells co-expressing both receptors (Ellis et al., 2006). The interplay of OX1R and CB1 receptor systems in the PAG has been reported in previous molecular and behavioral studies (Ellis et al., 2006; Kargar et al., 2015). It means that the co-localization and heterodimerization of OX1Rs and CB1 receptors permit the receptors to modulate the functions of each other especially when one of them is blocked or activated. However, additional studies are still required to clarify the detail mechanisms.

The data showed that the intra-PAG injection of sole orexin-A resulted in enhanced anxiety states and c-fos induction in the vlPAG of control rats. Consistently, it has been previously reported that orexin-A has anxiogenic properties in both physiological and pathological situations. Specifically, orexin-A administration into the brain regions involved in controlling anxiety and panic, such as the bed nucleus of the stria terminalis and amygdala, increases anxiety (Johnson et al., 2012a). Interestingly, it has been indicated that central blockage of OX1Rs attenuates FG-7142 (a benzodiazepine inverse agonist)-induced anxiety behaviors and c-fos induction in neuronal network including the dlPAG and vlPAG subdivisions of PAG" (Johnson et al., 2012b; Reddy and Kulkarni, 1997).

Here, the orexin-A exaggerating effect on anxiety-like behavior was associated with the suppression of capsaicin-evoked dental pulp nociception. This was also accompanied by significant increases in vlPAG c-fos expression. The detail mechanisms underlying such dual effect of orexin-A on pain and anxiety has not been completely known. However, these discrepant effects can be explained in the following ways. Although orexin-A is almost anxiogenic, it also increases consolidation of fear aversive memory and c-fos induction in some brain areas involved in negative emotion, specially basolateral amygdala (Flores et al., 2014). In addition to fundamental association with the brainstem as well as highly brain regions involved in controlling nociceptive signals, the PAG links with multiple centers involved in emotional, affective and cognitive functions (Benarroch, 2012). So, bimodal effect of orexin-A may be mediated by simultaneous activation of PAG projections to special brain areas for controlling nociceptive and emotional information.

Experimental studies have indicated that nociception has a positive correlation with anxiety (Armfield and Heaton, 2013; Voog, 2000). However, there is not always a direct relationship between pain severity and increased anxiety-related behaviors. For example, brain chemistry demonstrates dual states of pain and anxiety during chronic low back pain (Grachev et al., 2002). Moreover, orexin-A is not the only agent that shows concurrent analgesia and anxiogenic effects. It has been indicated that corticotropin-releasing factor as well as caffeine exert such contradictory effects on nociceptive and anxiety related behaviors (Lariviere and Melzack, 2000; Nawrot et al., 2003; Risbrough and Stein, 2006; Sztainberg and Chen, 2012). However, the biological aspects and mechanisms of such relationships remain to be elucidated.

The expression of c-fos is a useful tool for identifying activated neurons (Hoffman et al., 1993). In particular, there is a positive correlation between anxiety level and c-fos expression (Duncan et al., 1996; Spiga et al., 2006; Tye et al., 2011). It has been indicated that the administration of nitric oxide donors' into the PAG can induce flight behaviors and c-fos expression in the PAG of rats (de Oliveira et al., 2000). Following noxious stimulation of nociceptive fibers, c-fos is overexpressed in the spinal and supra-spinal pathways of pain (De Felipe et al., 1998; Kalynovska et al., 2017). However, it has been reported that bacterial lipopolysaccharide-induced hyperalgesia attenuates c-fos induction in the PAG columns including vlPAG (Zouikr et al., 2014). The PAG substructures are innervated by different populations of neurons which modulate emotional and nociceptive responses (Coulombe et al., 2016). In almost all of the previous studies, chemical or electrical activation of PAG neuronal matrix elicits anxiety-like states, while induces analgesic responses (Batista et al., 2015; Fardin et al., 1984; Graeff et al., 1993). In the present study, it seems that the distinct behavioral responses evoked by orexin-A might be mediated by different signaling and modulating pathways of the vlPAG terminals. However, more supplementary experiments are necessary to elucidate these contradictory functions of orexin-A in the vlPAG.

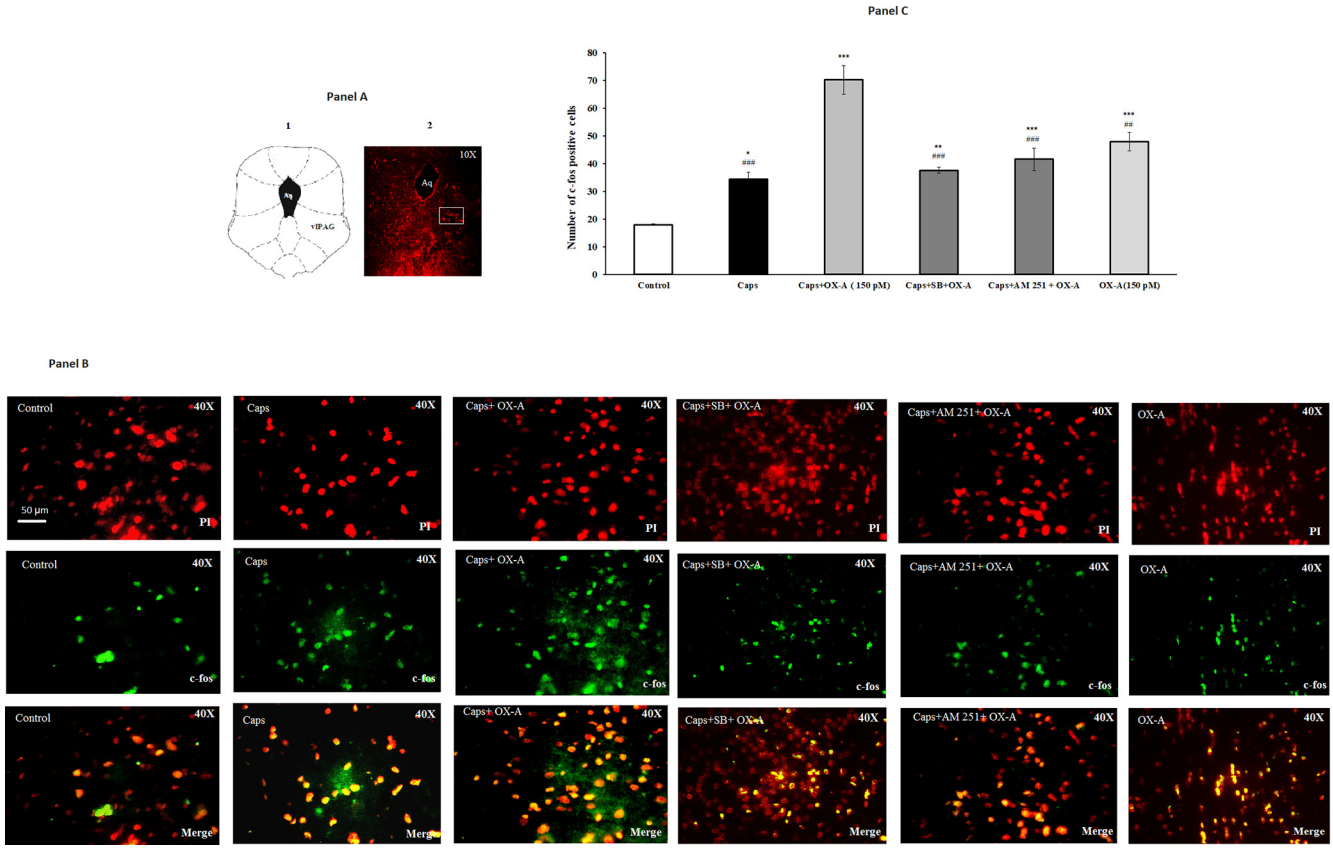

Fig. 8. Immunofluorescence detection of c-fos protein in the ventrolateral periaqueductal gray matter (vlPAG) sections of control, Caps, Caps + OX-A (0.51 µg/rat), Caps + SB (12 µg/rat) + OX-A (0.51 µg/rat), Caps + AM251 (4 µg/rat) + OX-A (0.51 µg/rat) and OX-A (0.51 µg/rat) treated rats (n = 4 per each group). Panel A shows a schematic coronal plane through vlPAG amended from the atlas of Paxinos and Watson (1) and a representative vlPAG section used for immunofluorescence assessment (2). Panel B displays c-fos staining (green), Propidium Iodide (PI) staining to indicate the nucleus (red) and the merged image of c-fos protein and PI (yellow). Panel C, statistical comparison of the number of c-fos positive cells in the vlPAG sections of the experimental groups (n = 4). Data are presented as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs intact rats, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ vs Caps + OX-A (0.51 µg/rat) treated rats. Caps: capsaicin, OX-A: orexin-A, SB: SB-334768. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusion

The data suggest that intradental capsaicin elicited a significant nociceptive response that was significantly diminished by intra-vlPAG administration of orexin-A. However, intra-vlPAG orexin-A was associated with the exaggeration of capsaicin-evoked anxiety-like behaviors. Those effects were mediated, at least in part, through OX1R and CB1 receptors activation and increases of c-fos expression in the vlPAG.

Conflict of interest

All authors declared that they have no conflict of interest.

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