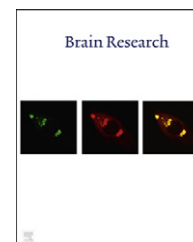


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## Research Report

# Intra-paragigantocellularis lateralis injection of orexin-A has an antinociceptive effect on hot plate and formalin tests in rat

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### ABSTRACT

In the present study, the effect of orexin-A (ORXA) microinjection into the paragigantocellularis lateralis (LPGi) on nociceptive behaviors, using hot-plate and formalin tests as thermal and chemical models of pain in rat, was examined. Also, we determined whether the pretreatment with SB-334867, a selective OX1-receptor antagonist, would prevent the antinociceptive effect of orexin-A. ORXA (0.1–100 nM/0.5  $\mu$ L) microinjected into the LPGi nucleus, dose-dependently decreased the formalin induced nociceptive behaviors and also produced a dose-dependent antinociceptive effect in the hot-plate test. Pretreatment with a selective orexin receptor 1 (OX1R) antagonist, SB-334867, also inhibited the effect of ORXA on formalin induced nociceptive behaviors while the SB-334867 (100  $\mu$ M) alone had no effect on formalin test. These data demonstrated that the ORXA-induced antinociception in formalin test is mainly mediated through the OX1R in LPGi which might play a potential role in processing the pain information associated with descending pain modulation.

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## 1. Introduction

Orexin-A (ORXA, a cyclic 33-amino acid peptide also known as hypocretin-1) and orexin-B (ORXB, or hypocretin-2, a linear, 28-amino acid peptide) are produced from the precursor protein prepro-orexin in neurons and restricted to a few regions of the lateral hypothalamus (LH) (Peyron et al., 1998; Sakurai et al., 1998). ORXA and ORXB activate the two G-protein coupled receptors, orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R) (Sakurai et al., 1998). Several lines of

evidence imply that the orexin is involved in reward, addiction (Aston-Jones et al., 2009, 2010; Harris et al., 2005), and nociceptive sensory processes (Azhdari Zarmehri et al., 2008, 2011; Bingham et al., 2001; Mobarakeh et al., 2005; Yamamoto et al., 2002). ICV injection of ORXA (hypocretin-1) has been shown to elicit analgesic responses processes (Bingham et al., 2001; Mobarakeh et al., 2005; Yamamoto et al., 2002) and OX1R is involved in responsiveness to both pain and stressful stimuli (Sofi-Abadi et al., 2011; Watanabe et al., 2005). However, the locations of central sites that may mediate

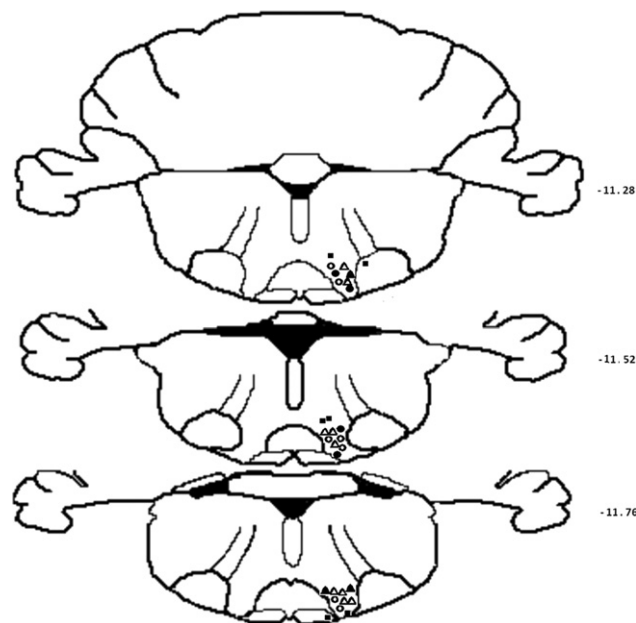
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these effects have not been clearly elucidated. The orexin receptors and orexinergic projections are localized in regions previously shown to play a role in pain modulation such as periaqueductal gray (PAG), locus coeruleus, and paraventricular nucleus (PVN) (Mondal et al., 1999; Peyron et al., 1998). The LPGi in the medulla is implicated in several functions including cardiovascular control, respiration, pain, and analgesia (Van Bockstaele et al., 1989; Van Bockstaele and Ston-Jones, 1995; Zhou et al., 1993, 1995). Furthermore, the LH appears to modify nociception, in part, through the brain stem (Behbehani et al., 1988; Holden and Pizzi, 2008) and there are even several studies in which the interaction of LH with LPGi is documented (Andrezik et al., 1981; Li and Lovick, 1985). Nevertheless, the role of ORXA within LPGi in pain control has not been determined yet. In our previous study, we have shown that the ORXA has a moderate effect on PAG in formalin induced nociceptive behaviors (Azhdari Zarmehri et al., 2011). There is widespread projections of orexinergic pathway and orexin receptors expression in LPGi (Ciriello et al., 2003; Mondal et al., 1999; Peyron et al., 1998). Therefore, in this study, the effect of ORXA microinjection into LPGi on formalin induced nociceptive behaviors and hot-plate test was investigated. To further verify the role of OX1Rs in the LPGi, the effect of the selective OX1R antagonist on antinociceptive effect of ORXA was also examined in formalin test.

## 2. Results

### 2.1. Intra-LPGi application of ORXA decreased formalin-induced nociceptive behaviors

Formalin injection into the plantar surface of the right hind paw produced typical biphasic nociceptive responses (Abbott et al., 1995; Dubuisson and Dennis, 1977). The first and second phases were separated by a brief inter-phase where the nociceptive behaviors were sporadically observed in the vehicle group. The columns represent the mean nociceptive score at each phase: phase 1 (minutes 1–7), inter-phase (minutes 8–14), and phase 2 (minutes 15–60) for all figures. Only those rats with microinjection site and diffusion located within the nucleus were included in the results (Fig. 1, data from 100 nM orexin-A are shown). To determine the appropriate dose of ORXA for microinjection into the LPGi and showing the antinociceptive effect in formalin test, the orexin microinjection into the LPGi started from 0.1 nM concentration (Fig. 2C). In part B of the figure, the average pain scores of different doses of ORXA 0.1 nM ( $n=4$ ), 0.05 nM ( $n=5$ ), 10 nM ( $n=4$ ), 50 nM ( $n=6$ ), and 100 nM in 0.5  $\mu$ l at different phases of formalin test is shown. The antinociceptive effect of ORXA was observed from 10 nM in phase 1 and 1 nM in phase 2 of the formalin test. Intra-LPGi microinjection of 10, 50, and 100 nM of ORXA caused a decrease in formalin-induced nociceptive behaviors in phase 1 as compared to saline-treated rats (Fig. 2C,  $P<0.05$  by ANOVA), and the effect was dose-dependent. Similarly, the intra-LPGi microinjection of ORXA (1, 10, 50, and 100 nM) also caused a decrease in formalin-induced nociceptive behaviors in phase 2 as compared to saline-treated rats (Fig. 2C,  $P<0.05$  by ANOVA), and again the effect occurred in a dose-dependent

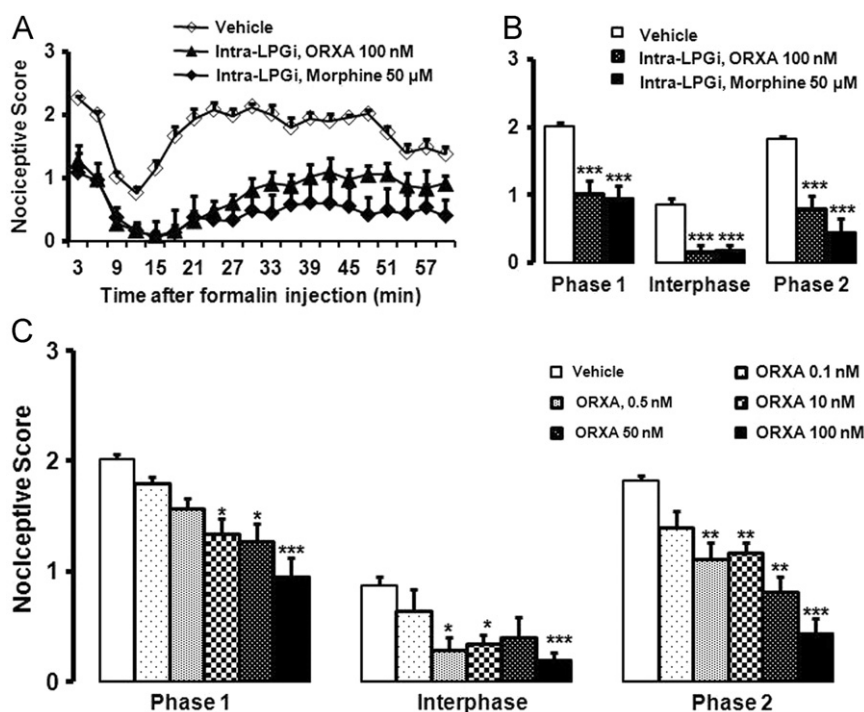


**Fig. 1** – Location of injections as percentage of maximum possible effect (%MPE) on the LPGi nucleus: black square for 0–10%, black circle for 10–25%, white circle for 25–50%, black triangle for 50–75%, and white triangle for over 75% effect. The plane is modified to a series of three sections for the brain area from the atlas of Paxinos and Watson (The Rat Brain in Stereotaxic Coordinates, fourth ed. Academic Press, New York; Paxinos and Watson, 2005) the approximate coordinate indicated is in millimeters posterior to the bregma (data shown are only from 100 nM ORXA in formalin test).

manner (Fig. 2C). To compare the antinociceptive effects of ORXA with morphine, the latter was microinjected (50 nM/0.5  $\mu$ l,  $n=6$ ) into the LPGi and the outcome was a vigorous decrease in formalin-induced nociceptive behaviors in phase 1, inter-phase, as well as the phase 2 of the formalin test (Fig. 2A and B). The antinociceptive effect of intra-LPGi injection of ORXA (100 nM) was similar to that of morphine in the formalin test. All microinjections were made into the right side of the LPGi and the results presented here only include the animals for which the confinement of injection to the LPGi region was confirmed and the data from all dubious injections were rejected. When the peptide was microinjected into the reticular formation surrounding the LPGi, it failed to produce any obvious effect on formalin induced nociceptive behaviors, indicating that the site for peptide action was within the LPGi rather than the adjacent medullary reticular formation.

### 2.2. Effects of ORXA injection into the LPGi nucleus on hot-plate test

According to the formalin test experiments, after LPGi microinjection of ORXA, different doses of ORXA (1–100 nM), to compare the antinociceptive effect of this drug on hot-plate test, was also administered. Intra-LPGi microinjection of ORXA at 50 and 100 nM concentrations significantly increased the latency in the hot-plate test at 15 ( $P<0.01$ ), 30 ( $P<0.01$ ) and 60



**Fig. 2** – Time course of formalin-induced nociceptive behaviors score ( $\pm$  S.E.M.) following intra-LPGi infusion of ORXA (100 nM in 0.5  $\mu$ l,  $n=7$ ) and morphine, measured every 3 min for 60 min (A). Bar chart shows the average pain scores of ORXA (100 nM in 0.5  $\mu$ l) and morphine at each phase of the formalin test (B). Average pain scores of different doses of ORXA 0.1 nM ( $n=4$ ), 0.05 nM ( $n=5$ ), 10 nM ( $n=4$ ), 50 nM ( $n=6$ ), and 100 nM in 0.5  $\mu$ l at different phases of formalin test (C). For clarity, only ORXA (100 nM) and morphine (50 nM,  $n=6$ ) is plotted in separate graphs, with the same vehicle group. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$  in comparison with saline (the number of animals for different doses of orexin 0.1, 0.5, 10 and 50 nM was 6).

( $P<0.05$  for 50 nM and  $P<0.01$  for 100 nM;  $n=6$  for each group) minutes following drug administration (Fig. 3). Similarly, the microinjection of ORXA at 10 nM ( $n=6$ ) concentration significantly increased the latency in the hot-plate test at 15 ( $P<0.05$ ), 30 ( $P<0.05$ ) and 60 ( $P<0.05$ ) minutes after administration of drug (Fig. 3). However, the microinjection of ORXA at 1 nM concentration ( $n=6$ ) only caused an increase in latency following hot-plate test at 15 ( $P<0.05$ ) and 30 ( $P<0.05$ ) minutes after drug administration (Fig. 3) but not at 60 min.

### 2.3. Effects of SB-334867 (OX1R antagonist) microinjection into the LPGi on ORXA-induced attenuation of nociceptive behaviors in formalin test

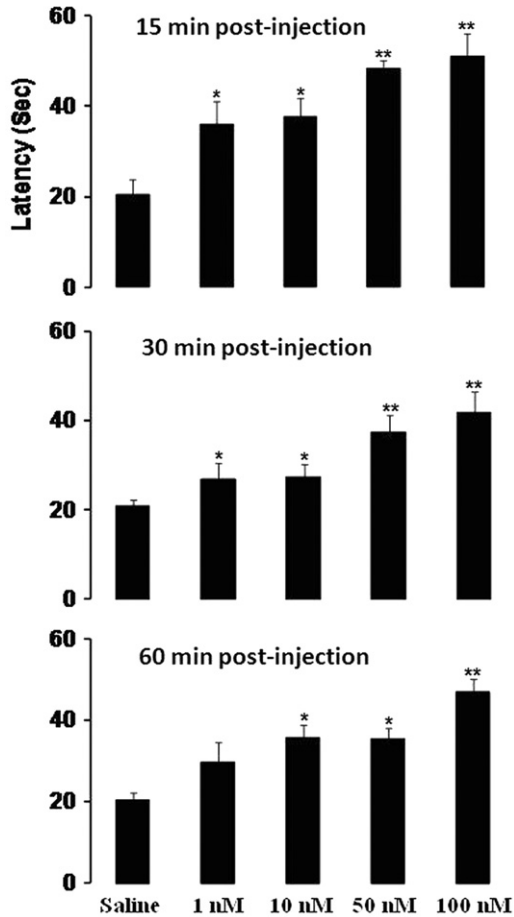
Pre-treatment with SB-334867 (100  $\mu$ M/0.5  $\mu$ l,  $n=6$ ) antagonized the effect of 100 nM ORXA ( $n=5$ ) induced antinociceptive behaviors in LPGi (Fig. 4A and B). Intra-LPGi microinjection of SB-334867 alone ( $n=7$ ) produced no effect on phase 1, inter-phase, and phase 2 in formalin test compared with the LPGi vehicle-injected rats ( $1.78\pm0.11$ :  $1.73\pm0.06$ ,  $P>0.05$  for phase 1;  $1.10\pm0.08$ :  $0.95\pm0.16$ ,  $P>0.05$  for inter-phase;  $1.81\pm0.08$ :  $1.74\pm0.08$ ,  $P>0.05$  for phase 2). SB-334867 was administered 10 min before ORXA injection.

## 3. Discussion

Orexin-A is produced from the precursor protein prepro-orexin in neurons which are restricted to a few regions of

the LH (Peyron et al., 1998; Sakurai et al., 1998). The LH appears to modify nociception, in part, through the brain stem in particular the PAG and rostral ventromedial medulla (RVM) which includes the nucleus raphe magnus (NRM), the adjacent gigantocellularis pars alpha (NGC $\alpha$ ) and LPGi (Behbehani et al., 1988; Holden and Pizzi, 2008). Moreover, the interaction of LH with LPGi is documented in several studies (Andrejik et al., 1981; Li and Lovick, 1985). The injection of orexin-A into the nuclei of the descending antinociceptive pathway may provide an opportunity to investigate the role of this neurotransmitter in the up-down modulating pain system. In our previous study, the authors investigated the role of midbrain PAG, as a common substrate in orexin-A antinociception, in formalin and tail flick tests (Azhdari Zarmehri et al., 2011). We demonstrated that the microinjection of orexin-A into the PAG reduced the formalin evoked-nociceptive behaviors in the inter-phase and the late phase but not the initial phase. Orexin-A failed to produce any effect on tail flick up to a dose that was effective in formalin test although the effect of orexin was lower than that observed with morphine. Recently, Ho et al. (2011) also reported the intra-PAG microinjection of orexin-A into rats produced analgesic effect in hot-plate test. The finding that intra-PAG microinjection of orexin-A has analgesic effect in the rat confirms the PAG to be an important site of action for orexin-induced supraspinal antinociception (Azhdari Zarmehri et al., 2011; Ho et al., 2011); however, it does not rule out the involvement of other supraspinal areas in the orexin-A induced analgesia. There is widespread projections of orexinergic pathway and expression of orexin receptors in the brain stem including gigantocellularis

and LPGi (Ciriello et al., 2003; Date et al., 1999; Mondal et al., 1999; Peyron et al., 1998). In this study, we investigated the role of LPGi as a common substrate in orexin-A antinociception in formalin and hot-plate tests.



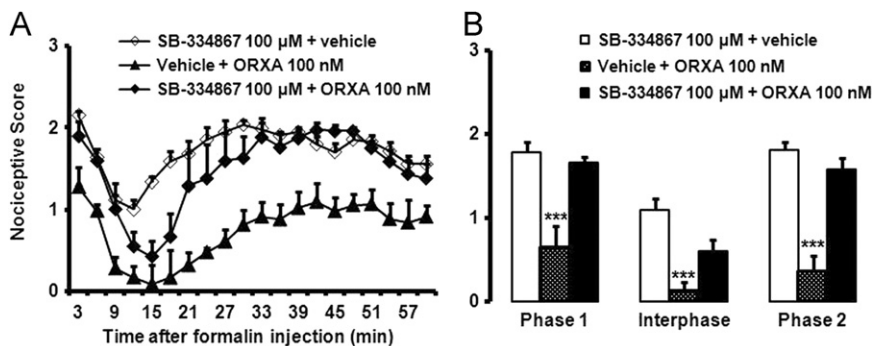
**Fig. 3** – Effect on 52 °C hot plate latencies of various doses of ORXA (A–C columns), at 15 min, 30 min, and 60 min after intra-paragigantocellularis injection. Values given are means  $\pm$  S.E.M., and  $n=6$  in each group. \* $p < 0.05$ ; \*\* $p < 0.01$ ; as compared to saline group.

Our experiments clearly showed that the microinjection of ORXA into the LPGi has a dose-dependent antinociceptive effect on acute and tonic phases of formalin test and also produced an antinociceptive effect on hot-plate test, again in a dose-dependent manner. Pretreatment with selective OX1R (SB-334867) partially antagonized the ORXA-induced antinociceptive behaviors after intra-LPGi injection of ORXA, indicating that the antinociceptive effect of ORXA is mediated through OX1R.

Formalin test is a widely used animal model of unremitting pain (Abbott et al., 1995; Dubuisson and Dennis, 1977). This model is used because formalin induces an adequate painful stimulus leading to spontaneous nociceptive behaviors in animals. Moreover, formalin as a noxious chemical stimulus, induces distinct nociceptive behaviors with two distinctive phases, possibly reflecting the presence of different types of pain mechanisms in which the spinal or supraspinal pain modulating networks are involved (Hunskar et al., 1986; Hunskar and Hole, 1987).

Ciriello et al. (2003) have shown that the distribution of ORXA-labeled axons varies within the specific regions of the magnocellular reticular area. ORXA axon labeling was observed mainly within the GiA and LPGi and to lesser extent within the Gi and RVM (Ciriello et al., 2003). The finding of a relatively dense ORXA fiber labeling within the GiA and LPGi is of some interest, as these areas of the brain stem have previously been demonstrated to play important roles in pain modulation (Erami et al., 2011; Azami et al., 1982; Heinricher and Rosenfeld, 1983; Lovick, 1986). However, there are significant differences among the sites mediating the ORXA-induced analgesia in formalin test. As previously reported, injections of ORXA directly into the GiA and not the LPGi, elicit a decrease in muscle tone and cardioacceleration (Ciriello et al., 2003). Therefore, the orexinergic projections to Gi might be involved in cardioacceleration and muscle tonicity and that the ORXA terminals in LPGi are critical for pain modulation (Ciriello et al., 2003).

Since the LH is considered as the exclusive area for ORXA expression and also implicated as part of a descending system associated with the modulation of nociceptive transmission in the first synapse in dorsal horn neuron of spinal cord, thus, it seems that this effect of LH to be mediated in



**Fig. 4** – Time course of formalin-induced nociceptive behaviors score ( $\pm$  S.E.M.) following infusions of the vehicle (0.5  $\mu$ l)+ORXA (100 nM/0.5  $\mu$ l) ( $n=5$ ), SB-334867 (100 nM/0.5  $\mu$ l)+vehicle (0.5  $\mu$ l) ( $n=7$ ), and SB-334867 (100 nM/0.5  $\mu$ l)+ORXA (100 nM/0.5  $\mu$ l) ( $n=6$ ) into the LPGi, measured every 3 min for 60 min (A). All microinjections were made into the right side of the LPGi. \* $P < 0.05$ ; \*\*\* $P < 0.001$  in comparison with vehicle group.

part by the brain stem nuclei (Behbehani et al., 1988; Dafny et al., 1996). The present findings showed that the ORXA microinjection into the LPGi produced an antinociceptive effect on formalin and hot-plate tests, highlighting the possibility that the origin of the orexinergic inputs to these structures is involved in top-down pain modulation. The LPGi nucleus may influence the neurons in dorsal horn of spinal cord which are involved in both the neurotransmission of noxious stimuli and modulating the activity of this synapse as the first gate for ascending nociceptive pathway (Vanegas and Schaible, 2004; Zhuo and Gebhart, 1997). We suggest that ORXA might act as a mediator from LH to LPGi to modulate the pain.

Microinjection of morphine into LPGi produced persistent and profound analgesia in all phases of formalin test which was comparable to orexin. This finding is consistent with an earlier report by Azami et al. (1982) in which morphine was injected into the LPGi. Akaike et al. (1978) found that the reticular region consisting the LPGi was 20 times more sensitive than Gi in demonstrating analgesia by morphine microinjection and that the electrical stimulation of LPGi caused analgesia whereas such effect was not present by Gi stimulation. In the present study, we used formalin test as a pain model different from those used in previous studies performed on this region (Akaike et al., 1978; Azami et al., 1982). It seems likely that the ORXA produces analgesia through activation of a circuit that is required for opioid analgesia in the LPGi region, an assumption which needs further studies.

Consistent with other reports on formalin and hot plate tests (Azhdari Zarmehri et al., 2011; Ho et al., 2011; Yamamoto et al., 2002), pretreatment with selective OX1R (SB-334867) antagonized the antinociceptive behaviors of ORXA in LPGi, indicating the presence and potential importance of OX1R in the modulation of pain. It has been reported that the selective OX1R antagonist is pro-hyperalgesic in the mouse carrageenan assay and therefore, suggesting that during a carrageenan-induced thermal hyperalgesia test in mice, a tonically activated OX1R mediated inhibitory system is present (Bingham et al., 2001). However, SB-334867, at 100  $\mu$ M concentration alone had no effect on formalin evoked-behaviors, indicating that a tonic OX1R mediated inhibitory system does not exist in the LPGi during the formalin test. This may reflect the presence of strain differences, route of SB-334867 administration (systemic against intra-nucleus injection), application of different models (as seen in hyperalgesia induced by carrageenan and formalin tests), and also different methodologies (such as stress handling or anesthesia prior to formalin injection, both known to influence the test). Our data confirm the previous studies in which it was revealed that the SB-334867, a selective antagonist of OX1R, restores the analgesic effect of ORXA after ICV administration in mice and rats (Bingham et al., 2001; Yamamoto et al., 2002). Also, it has been suggested that the antinociceptive effects of ORXA are mediated both via the spinal and supraspinal mechanisms.

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## 4. Conclusion

The present study supports a role for OX1Rs in modulation of pain caused by LPGi. In addition, the antinociceptive activity

of ORXA on formalin-induced behaviors within the LPGi demonstrated a significant difference. However, the LPGi was found to be sensitive to antinociceptive effect of ORXA in both formalin and hot-plate tests.

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## 5. Experimental procedures

### 5.1. Subjects

All experiments involving the study animals were conducted according to the policy of Iranian Convention for the Protection of Vertebrate Animals used for Experimental Purposes, and the protocol was approved by the Ethics Committee of the Medical School, Qazvin University of Medical Sciences, Qazvin, Iran. Adult Sprague-Dawley rats (220–300 g) were purchased from Razi institute (Karaj, Iran). Animals were housed in groups of three rats per cage and kept at temperature-controlled room, under a 12 h light–dark cycle with lights on at 7:00–19:00. Food and water were provided ad libitum. In all experiments, strict attention was paid to the regulations of local authorities for handling of laboratory animals.

### 5.2. Surgical preparation for intra-LPGi microinjections

To perform direct intra-LPGi or ICV administrations of drugs or the respective vehicle (saline), rats were anaesthetized with Ketamine (100 mg/kg)/ Xylazine (10 mg/kg), placed in a stereotaxic apparatus (Narishige, Japan). Later, 2 mm holes at 90° angles were drilled in the skull over the LPGi or right ventricle, and the dura was removed to allow the placement of a guide cannula. A 23-gauge, 8.6 mm-long stainless steel guide cannula was stereotaxically (Narishige, Japan) lowered into the brain at an angle of 90° from the longitudinal axis of the brain, until the tip was 2 mm above the LPGi by applying coordinates from the atlas of Paxinos and Watson (2005) with the bregma serving as the reference for each plane (LPGi; AP, –11.2 –11.8 from bregma; L, 1.5 mm to right; V, 10.6 mm ventral to the bregma). The cannula was anchored using dental cement and 2 stainless steel screws in the skull bone. Immediately after waking up from the surgery, rats were returned to their home cages to await the formalin test procedure. One week after the recovery from surgery, rats were moved to the test room at least an hour before the commencement of the experiment. Direct intra-nucleus administration of drugs, or respective vehicle was conducted with a stainless steel cannula (30 G, 0.3 mm outer diameter) connected with a polyethylene tube to a Hamilton syringe. For microinjection procedure, the animal was simply held in one hand while the microinjection cannula tip was lowered through the guide cannula (the microinjector tip extended 2 mm below the tip of the guide cannula) and the drug solutions, or vehicles, in volumes of 0.5  $\mu$ l, were injected into the nucleus over a period of 60 s and the injection cannula was gently removed 1.5 min later. During microinjections, animals were free to move around the cage with the cannulae in place. Formalin was injected into the plantar surface of the right hind paw using a disposable insulin syringe with a fixed 30 G needle.

### 5.3. Formalin test

The formalin test was performed in a clear plastic box ( $30 \times 30 \times 30 \text{ cm}^3$ ) with a mirror placed underneath at  $45^\circ$  angle to allow an unimpeded view of the animal's paws. In the present study, rats were first acclimatized for 30 min in an acrylic observation chamber followed by injecting  $50 \mu\text{l}$  of 2% formalin into the plantar surface of the right hind paw with a 30 gauge needle, subcutaneously. To ensure stable scores from the formalin test, it was necessary to make certain that the needle was inserted through the skin and run for 5 mm under the skin. The OX1R antagonist, SB-334867, was given 5 min before the administration of ORXA, whereas the ORXA was given 5 min before the formalin test. Following formalin injection, each rat was immediately returned to the observation box and the behavioral recording commenced. Pain behaviors were scored as follows: 0, the injected paw was not favoured; 1, the injected paw had little or no weight placed on; 2, the injected paw was elevated and not in contact with any surface; and 3, the injected paw was licked or bit. Recording of the nociceptive behaviors began immediately after the formalin injection (time 0) and was continued for 60 min. The scores of nociceptive behaviors for each 3 min interval was calculated as the weighted average of the number of seconds spent on each nociceptive behavior from the start of the experiment. Scores were recorded for vehicle injected rats as well as those given ORXA.

### 5.4. Hot-plate test

The hot-plate test was used to measure the response latencies according to the method previously described (Ferreira et al., 2002; Rhodes and Liebeskind, 1978; Woolfe and MacDonald, 1944) with minor modifications. In these experiments, the hot-plate (Analgesia meter IITC, Life Science, USA) apparatus was maintained at  $52 \pm 0.1^\circ\text{C}$ . The animals, presenting training latencies higher than 30 s, were excluded. Animals were placed into an acrylic box ( $22.5 \times 22.5 \text{ cm}^2$  in diameter) on a heated surface, and the time (in seconds) between the placement and licking of their hind paws or jumping (whichever occurred first), was recorded as the response latency. A 50 s cut-off was used to prevent tissue damage. Two days before the experiment, all rats were habituated to the experimental procedure (measurement of latency) in order to minimize the novelty-induced antinociception (Siegfried et al., 1987). On the day of experiment, the animals were injected intra-LPGi with vehicle or drug and subjected to the hot-plate test 15, 30, and 60 min thereafter. The dose of orexin-A was selected according to the formalin test experiments. Different rats were used for formalin test and hot plate experiments.

### 5.5. Drugs

Two percent formalin was diluted (Temad, Iran) with sterile physiological saline solution (Soha, Iran). Orexin-A (MW: 3561, Sigma) and morphine sulfate (Temad Co., Tehran, Iran) was dissolved in saline, and OX1R antagonist; SB-334867 [N-(2-methyl-6-benzORXA zoly)-N''-1,5-naphthyridin-4-yl urea], (MW weight: 356, Tocris), dissolved in dimethyl sulfoxide (DMSO) and diluted in saline (1:100 in 0.9% w/v saline

solution). All drugs were diluted in saline immediately before use on the morning of an experiment.

### 5.6. Histology

At the end of the experiments, rats were deeply anaesthetized with Ketamine overdose; a volume of  $0.5 \mu\text{l}$  of pontamine sky blue (%0.2) was also injected into the site 10–20 min before sacrificing the animals. Rats were then perfused intracardially with 100 ml of 4% formalin solution and the brain was removed and sectioned. Only those rats with microinjection site and diffusion located within the nucleus were included in the results. In all experiments, ORXA was microinjected into the right LPGi while the formalin injected into the plantar surface of the right hind paw (Fig. 1, data from 100 nM orexin-A is shown).

In the formalin test, the percentage of maximum possible effect (%MPE) was calculated as the percentage difference between the measured nociceptive behavior during the late phase (15–60 min) for a group of animals that had been injected with drug (post-saline) or saline (post-saline). The following formula was used:

$$\%MPE = \frac{\text{post-saline} - \text{post-drug}}{\text{post-saline}} \times 100$$

### 5.7. Data analysis

Data were presented as mean of 3 min blocks ( $\pm$  S.E.M.) the total time points of 60 min duration was divided into three phases marked as phase 1 (from time 0 to minute 7), inter-phase (from minutes 8 to 14), and phase 2 (from minutes 15 to 60). Data were analyzed by one-way analysis of variance between the groups. Also, Tukey's test was employed to determine whether a significant difference occurred. The defined level for statistical significance was  $P < 0.05$ .

## Acknowledgments

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## Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.brainres.2012.08.013>.

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