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ORIGINAL PAPER

Spinal Orexin-2 Receptors are Involved in Modulation of the Lateral Hypothalamic Stimulation-Induced Analgesia

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Abstract

Role of the orexinergic system in pain modulation is well studied and involvement of the spinal orexin-1 receptors is well documented. In this study, we examined role of the spinal orexin-2 receptors in modulation of inflammatory pain in rat. Fifty-one adult male Wistar rats were implanted unilaterally with a guide cannula into the LH and intrathecal tubing in the lumbar space between L4 and L5. Chemical stimulation of LH by carbachol (250 nM/0.5 µL saline) induced remarkable analgesia during the two phases of formalin test and Intrathecal administration of different doses of TCS OX2 29 (10, 30 and 100 µM/ 0.5 µL DMSO) prior to LH stimulation, dose-dependently antagonized the antinociceptive effect of the LH-stimulation during the two phases of formalin test. The effect size of the TCS OX2 29 was $\eta^2 = 0.47$ and $\eta^2 = 0.87$ for the early and late phases of the test, respectively. Also, intrathecal administration of TCS OX2 29 alone (without stimulation of the LH) had no significant effect on formalin induced pain-related behaviors. Our results showed that spinal orexin-2 receptors are involved in modulation of the LH-stimulation induced analgesia in a persistent inflammatory pain model. These findings may suggest spinal orexin-2 receptors in particular and the orexin system in general as a useful therapeutic target for treatment of chronic pains.

Keywords Pain · Orexin-2 receptor · Lateral hypothalamus · Spinal cord · Intrathecal administration · Formalin test

Introduction

The orexinergic system is made of two G-protein coupled receptors, orexin-1 (OX1) and orexin-2 (OX2) receptors plus two neuropeptides, orexin-A and B that are produced in the cell bodies of orexinergic neurons located in the lateral hypothalamus (LH) [1]. These cells extend their axons widely throughout the central nervous system [2]. Orexin receptors are expressed in many brain and spinal cord structures, hence the wide range of physiological roles of orexin [1, 3–8]. Among many physiological roles of orexins such as arousal, reward seeking behavior, energy homeostasis, feeding and sleep regulation, stress processing, etc., its involvement in modulation of pain has recently gained increasing attention [1].

Involvement of the orexinergic system in different models of pain at spinal and supra-spinal levels is well documented. Descending projections from the LH that arrive at spinal cord [3, 9, 10] bring Orexin A and B peptides where both type 1 and 2 orexin receptors are expressed [10, 11], causing wide range of physiological effects at the spinal level. In this regard, several studies have shown that administration of orexin-A in different loci and nuclei within the brain can produce analgesia [1]. For instance, microinjection of Orexin-A into the rostral ventromedial medulla generated antinociception in a mouse model of inflammatory pain and this effect was reversed by microinjection of the OX1 receptor antagonist in the site [12]. Another study by Azhadri-Zarmehri [13] and colleagues suggested similar role for orexin-A in the periaqueductal gray matter of the rat. Besides the numerous investigations on the role of Orexin-A and OX1 receptors in pain modulation, a few studies have focused on the modulatory role of orexin-2 receptors in different pain models. It has been shown that blockade of the

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OX2 receptors in the ventral tegmental area (VTA) and the nucleus accumbens reverses the analgesic effect of chemical stimulation of the LH [14]. Also, a previous study in our group reached similar results when OX2 receptors were blocked in the CA1 region of the hippocampus in a rat model of inflammatory orofacial pain [15].

Presence of the orexin receptors in the spinal cord suggests a potential role for orexin in pain modulation at spinal level [16, 17]. Few studies have already examined involvement of the OX1 receptors of the spinal cord in pain modulation using different pain models [18–21]. Our team recently published a study on the involvement of the spinal OX1Rs in modulation of inflammatory pain in the rat and concluded that blockade of these receptors can reverse the analgesic effect of chemical stimulation of the LH but not under tonic release of orexins [22]. To best of our knowledge however, we are—for the first time—studying role of the spinal OX2 receptors in a rat model of inflammatory pain (formalin test). We designed this experiment to test the hypothesis that blockade of the spinal OX2 receptors can reduce the LH-stimulation induced analgesia in formalin test.

Materials and Methods

Animals

In total, 59 male Wistar rats weighing (130–270 g) were obtained from Pasteur Institute (Tehran, Iran). Animals were caged in groups of 2–3 rats per cage and kept under standard constant condition (T = 22 °C, 12/12 h light/dark cycle) and had access to standard rodent food and water ad libitum. Prior to the surgeries, animals were given 1 week for habituation in which they received handling training by the experimenters. All experiments and surgeries were conducted and performed in accordance with the guidelines for use and care of laboratory animals (National Institutes of Health Publication, No. 80-23, revised 1996) and were approved by the ethics committee of Shahid Beheshti University of Medical sciences (IR.SBMU.PHNS.REC.1397.026), Tehran, Iran.

Stereotaxic Surgery

Animals were anesthetized using a mixture of Ketamine (10%) and Xylazine (2%) and the head was fixed in the stereotaxic apparatus (Stoelting, USA) after shaving and disinfecting the scalp. Stereotaxic coordinates of the lateral hypothalamus were calculated as follows: 2.5–2.8 mm caudal to bregma, Lat = ± 1.3 mm lateral to midline and DV = 8.6 mm ventral to skull surface [23] and guide cannula was fabricated from hypodermic needles (21 G) by cutting it to length (10 mm) and was unilaterally implanted 1 mm above the actual injection site. The microinjections were performed using a hypodermic needle (27 G) which was cut to extend 1 mm below the tip of the guide cannula inside the brain, and was connected to a Hamilton syringe (1 µL) using a PE tubing. During the recovery period, guide cannulae were protected using stainless steel stylet.

Intrathecal catheter implantation

Intrathecal catheter implantation was performed 2 days after the brain surgery. Polyethylene catheters (PE-10) were implanted with the tip positioned at the level of lumbar enlargement (L4/L5 segments) [24]. Each intrathecal catheter was externalized to the back of the neck and sealed with a steel wire, and the musculature and skin were ligated with 3-0 silk sutures. Animals were given one week of recovery. Animals showing any motor deficit or other neurological deficits (n = 6) and generally weak conditions were excluded and euthanized.

Drug Administration

Chemical stimulation of the LH was achieved by 0.5 µL of 250 nM solution of carbachol (Carbamylcholine chloride; Sigma-Aldrich, USA) in normal saline (0.9%). In order to evaluate effects of blockade of the OX2 receptors at the spinal level on nociceptive behaviors, TCS OX2 29 (Tocris, Bristol, UK) was dissolved in Dimethyl Sulfoxide (DMSO, 12%) in 10, 30 and 100 µM and 10 µL concentrations and microinjected intrathecally between L4–L5 lumbar segment of the spinal cord using a 10 µL Hamilton syringe. The formalin 2.5% solution was prepared from formaldehyde 37% (Merck, Germany) diluted in normal saline.

Formalin Test

In all experiments, microinjection of carbachol into the LH was performed 5 min before the intrathecal microinjections and 5 min after the intrathecal injection, animals received a subcutaneous (S.C.) injection of 50 µL formalin 2.5% into the plantar surface of the hind paw contralateral to the surgery side (Left/right LH). Rats were immediately placed in a transparent Plexiglas chamber (35 × 35 × 35 cm) which featured a mirror underneath it, angled at 45° to provide a better view to the observer during the formalin test. Injection of diluted formalin solution into the plantar surface of the hind paw induces a very painful stimulus and generates a biphasic persistent inflammatory pain in rodents [25]. The first 5-min block after formalin injection is considered as early phase (0–5 min), and the late phase takes more time and is defined from 15 to 60 min after formalin injection [26].

Nociceptive behaviors were quantified as following: 0, the injected paw was not favored; 1, the injected paw had little or no weight placed on it; 2, the injected paw was elevated and

did not touch the floor; and 3, the injected paw was licked or bitten [25]. The time spent in each type of aforementioned behaviors (each score) was recorded in 5-min blocks for a 60-min test period. Then, a "weighted nociceptive score", ranging from 0 to 3, was calculated by multiplying the time spent in each category by the category weight, summing these products and dividing by the total time (300 s) for each 5-min block of time:

$$Nociceptive\ \ score = \frac{(t_0 \times 0) + (t_1 \times 1) + (t_2 \times 2) + (t_3 \times 3)}{t_0 + t_1 + t_2 + t_3}$$

Experimental Design

In total, we had six experimental groups and one control group which only received vehicles saline and DMSO 12% as the vehicle for carbachol and TCS OX2 29, respectively. The "carbachol + DMSO" group was intended to study effects of chemical stimulation of the LH on the nociceptive behavior and also served as the control group for possible effects of the DMSO as the TCS XO2 29 vehicle, and received carbachol into the LH followed by injection of formalin 2.5% in the contralateral paw. Stimulation of the LH increases release of orexin and generates strong analgesia in formalin test [22]. In order to evaluate potentially reversing effect of blockade of the spinal OX2 receptors on carbachol induced analgesia, TCS OX2 29 was intrathecally microinjected in three doses 10 ("TCS OX2 29 10 µM + Car" group), 30 ("TCS OX2 29 30 µM + Car" group) and 100 µM ("TCS OX2 29 30 µM + Car" group), five minutes before administration of carbachol in the LH. Additionally, in order to study possible effect of blockade of spinal OX2 receptors on natural release of orexins under the noxious stimulation of formalin, the "TCS OX2 20 30 µM + Saline" and "TCS OX2 29 100 µM + Saline" groups received 30 and 100 µM of TCS OX2 29, five minutes before administration of saline into the LH, respectively. Each group consisted of 6–7 animals.

Histological Verification

Upon completion of the formalin test, animals were euthanized and their brains were fixed in a 4% formalin solution. Coronal sections (100 µm thickness) were made and studied for correct placement of the injector cannula in the lateral hypothalamus. The included data in the analyses are only from animals with verified correct placement of the injection site (Fig. 1).

Statistics

All data are expressed as mean either + or − SEM (standard error of mean), and p-value < 0.05 was considered

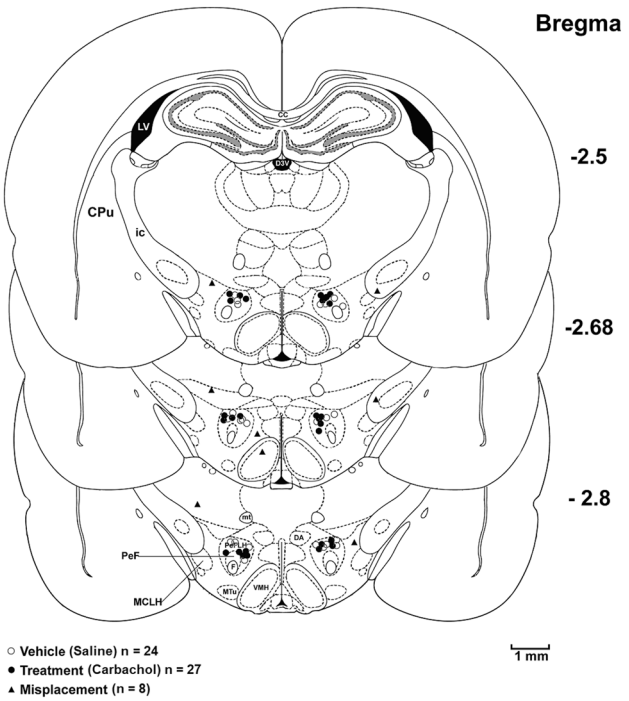

Fig. 1 Microinjection sites into the lateral hypothalamus. Circles and dark circles represent vehicle (saline, 0.5 µL; n = 24) and carbachol (n = 27) microinjection locations, respectively and triangles show the misplaced injections (n = 8). *cc* corpus callosum, *CPu* caudate putamen, *D3V* dorsal 3rd ventricle, *DA* dorsal hypothalamic area, *f* fornix, *LH* lateral hypothalamus, *ic* internal capsule, *MCLH* magnocellular nucleus of lateral hypothalamus, *mt* mammillothalamic tract, *MTu* medial tuberal nucleus, *PeF* perifornical nucleus, *PeFLH* perifornical part of lateral hypothalamus, *VMH* ventromedial hypothalamus. Scale: 1 mm

statistically significant. To specify the treatment and time effects on the pain behaviors, the mean nociceptive scores of control and experimental groups were compared by two-way analysis of variance (ANOVA) followed by Tukey's test.

In order to evaluate the effect of drugs on early and/or late phases of the formalin test, the area under the curve (AUC) and/or normalized percentages were analyzed by randomized block design one-way ANOVA followed by Tukey's tests for multiple comparisons, as appropriate. The AUC was calculated as raw pain scores × time using the linear trapezoidal method. Paired/unpaired student t-test was also used to compare two corresponding groups in the early and late phases as needed.

Results

Effect of Different Doses of Intrathecal Administration of the OX2 Receptor Antagonist TCS OX2 29 on the LH-Stimulation Induced Analgesia

Figure 2 shows the bi-phasic pain score profile for all experimental and control groups, that received carbachol into the LH. Two-way ANOVA followed by Tukey's post-*hoc* showed that formalin injection induced significant bi-phasic pain in the injected paw (Fig. 2 white circles) and that chemical stimulation of the LH significantly reduced pain score in both phases of the formalin test (Fig. 2, gray circles) [treatment effect: $F(4, 308) = 63.07$, $p < 0.0001$, time effect: $F(11, 308) = 9.892$, $p < 0.0001$ and treatment × time interaction effect: $F(44, 308) = 2.339$, $p < 0.0001$]. We used three different doses of TCS OX2 29 (10, 30 and 100 µM) in order to assess effect of blocking spinal OX2 receptors on LH-stimulation induced analgesia. The lowest dose (10 µM) failed to show any significant effect on the LH-stimulation induced analgesia in either phases of the formalin test (Fig. 2).

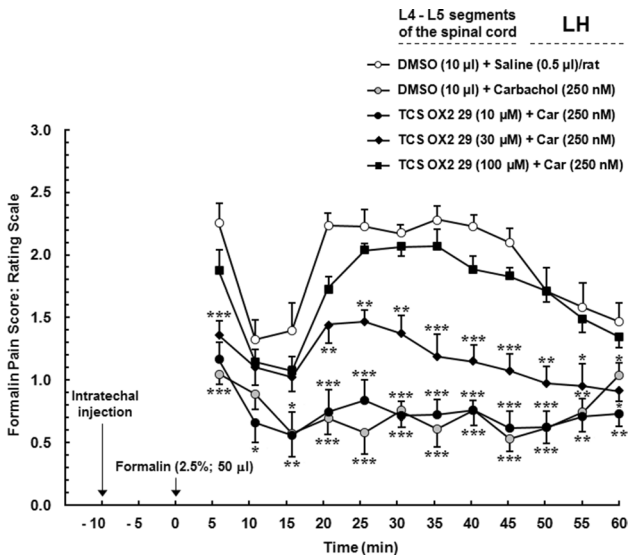

Fig. 2 An overview of all experimental and control groups that received carbachol into the LH. The white circles indicate the vehicle control group (DMSO-Saline) and a represent a typical pain score profile in formalin test. In contrast, the gray circles show the significant suppression of pain (microinjection of 250 µM/0.5 µL of carbachol into the LH) at all points throughout the test compared with the control group. Intrathecal administration of 10 µM dose of TCS OX2 29 failed to reduce analgesia while two higher doses (30 and 100 µM) did significantly reverse effects of carbachol microinjection. Each point shows the mean ± SEM for 6–7 rats in each group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to the DMSO-Saline control group

Figure 3 sums up normalized average pain score of all control and experimental groups for the early (Fig. 3a) and late phase (Fig. 3b) separately. One-way ANOVA followed by Tukey's post-*hoc* showed significant effect of chemical stimulation of the LH and the reversing effect of blockade of spinal orexin-2 receptors in the early [compared to the vehicle control group: $F(4, 32) = 11.96$, $p < 0.0001$, Fig. 4a, compared to the LH-stimulation group (DMSO): $F(3, 26) = 6.663$, $p = 0.0021$] and late phases [compared to the vehicle control group: $F(4,32) = 74.22$, $p < 0.0001$; DMSO: $F(3,26) = 52.85$, $p < 0.0001$, Fig. 3b] of the formalin test.

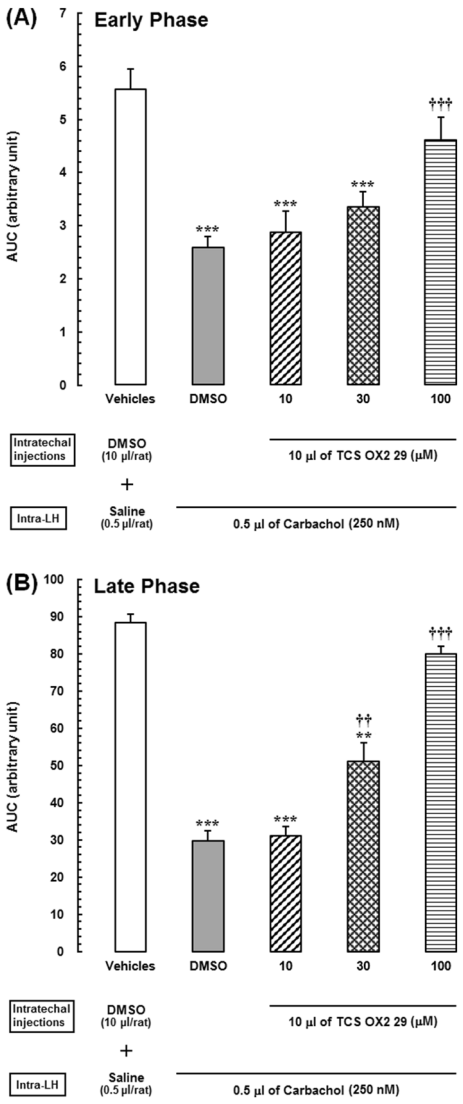

Fig. 3 AUCs calculated for pain scores in the early (**a**) and late (**b**) phases of formalin test. AUC values decreased during both phases of formalin test in the carbachol group (Carbachol-control group). Saline-DMSO (Vehicles) and carbachol-DMSO as control groups showed the most and least AUC values. Each bar represents mean ± SEM AUC value for 6–7 rats. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to the Vehicles-control group. $^{\dagger}p < 0.05$ and $^{\dagger\dagger\dagger}p < 0.001$ compared with the Carbachol-control group

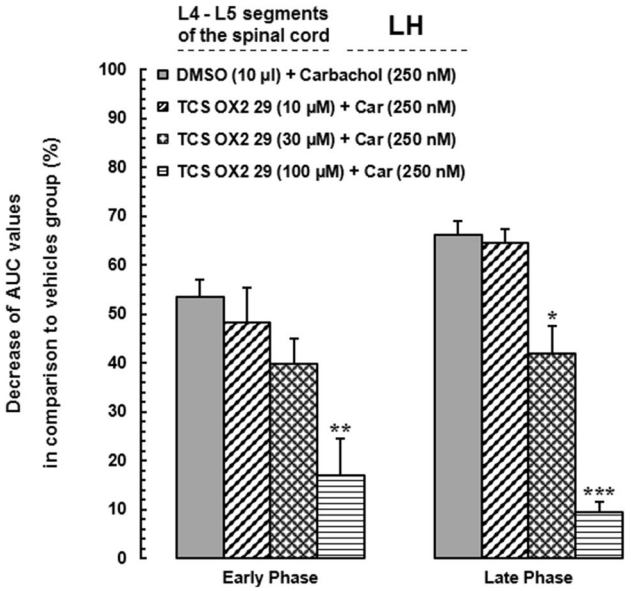

Fig. 4 Comparison of the decrease in AUC values of different experimental groups with the carbachol control group. In the early phase of the formalin test only highest dose (100 µM) of TCS OX2 29 showed significant decrease in analgesia induced by carbachol microinjection into the LH while during the late phase, 30 µM dose also had significant reversing effect on the carbachol induced analgesia. *$p<0.05$, **$p<0.01$ and *** $p<0.001$ compared with the Carbachol-control group

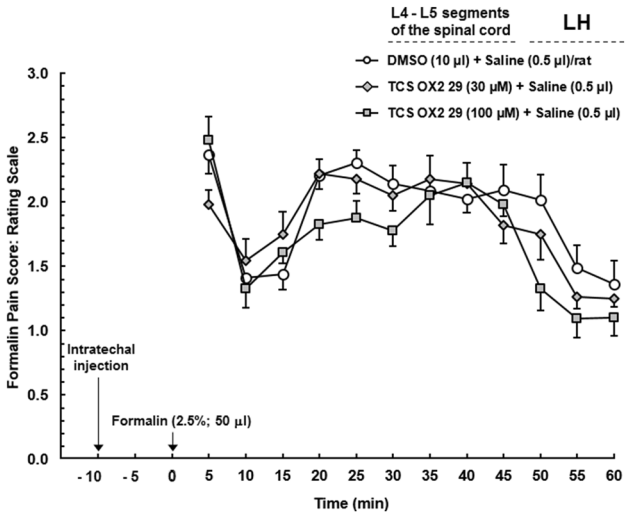

Fig. 5 Comparison of the effects of intrathecal administration of TCS OX2 29 on pain related behaviors in absence of stimulation of the LH. The OX2 receptor antagonist alone has no effect on the pain score during the two phases of formalin test. Each point shows the mean ± SEM for six rats in each group

We compared the decrease of AUCs of different doses of TCS OX2 29 for first (Right panel) and second (Left panel) phases of formalin test separately using One-way ANOVA followed by Tukey's (Fig. 4). During the early phase of the formalin test only highest dose of TCS OX2 29 significantly reversed LH-stimulation induced analgesia [$F(3, 26)=3.417$, $p=0.0263$;] and in the late phase the 30 µM dose of the drug was able to significantly reduce the antinociceptive effect of carbachol microinjection along with the 100 µM dose (albeit to a lower extent) [$F(3, 26)=52.85$; $p<0.0001$].

Effect of Different Doses of Intrathecal Administration of the OX2 Receptor Antagonist TCS OX2 29 on Formalin Induced Nociception

We injected 30 and 100 µM doses of the OX2 receptor antagonist, TCS OX2 29 intrathecally without administration of carbachol into the LH in order to test possible effect of blockade of the spinal OX2 receptors on formalin induced nociception in the rat. We compared effects of this treatment with the vehicle control group using Two-Way ANOVA followed by Tukey's post-*hoc* [treatment effect, $F(2,165)=1.095$, $p=0.3597$; time effect, $F(11,165)=9.795$ $p<0.0001$; interaction effect, $F(22,165)=1.052$, $p=0.4046$ Fig. 5]. Clearly, TCS OX2 29 cannot significantly alter pain-related behaviors in formalin test under tonic release of orexins. One can also conclude that injection of formalin itself does not stimulate release of orexins to the extent sufficient for a significant effect at spinal level.

A Log Dose–Response Curve of the Effect of Intrathecal Administration of TCS OX2 29 on Carbachol-Induced Antinociception in Both Phases of Formalin Test

In order to calculate the ED_{50} of TCS OX2 29 in both phases of formalin test, we used percent of changes in the AUC values of the carbachol control group compared with the AUC values of saline control group. Figure 6 shows the comparison between the AUC values of experimental (TCS OX2 29-treated) groups were compared to those of carbachol-control group in both phase of formalin test. The AUC values of the lowest dose (10 µM) for both early and late phases of the test show more than 96% similarity with the carbachol control group and 100 µM dose of TCS OX2 29 showed close to 16% and 30% similarity for the early and late phases of formalin test, respectively.

Based on this log dose–response curve, the 50% effective dose (ED_{50}) of TCS OX2 29 for the early phase was 71.77 while this value for the late phase was 42.41 which shows that a lower dose of TCS OX2 29 is needed for reversing the antinociceptive effect of carbachol during the late phase. This difference is particularly remarkable during the last quarter of the second phase and is possibly due to the natural washout of the painful stimulus (formalin) after 45 min from the injection (Fig. 2).

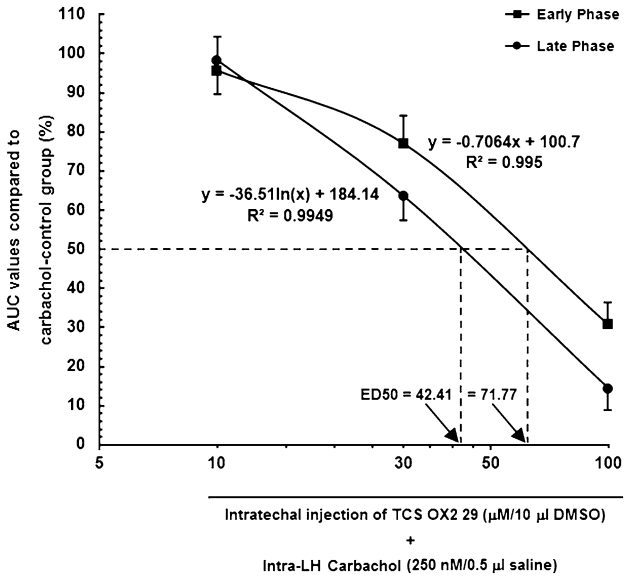

Fig. 6 A log dose–response curve of the effect of intrathecal administration of TCS OX2 29 on carbachol-induced antinociception (compared to DMSO-control; Carbachol group) during the early and late phases of formalin test. The effective dose (ED_{50}) of TCS OX2 29 in the early phase (71.77 nM) was greater than that of it in the late phase (42.41 nM). This shows that less amount of the OX2 receptor antagonist is required to reverse the effect of carbachol in the late phase of formalin test

We calculated effects size (Eta-squared, η^2) of the drug for the early and late phases of formalin test separately by dividing the SS value of between groups by total SS. The effect size of TCS OX2 29 for the early was $\eta^2 = 0.47$ and for the late phase was $\eta^2 = 0.87$ proving that the drug was nearly two times more effective during the late phase compared with the early phase.

Discussion

We tested the effect of blockade of spinal OX2 receptors on LH-stimulation induced analgesia in the formalin test as a model of prolonged inflammatory pain in the rat. Our results showed that (1) Intrathecal administration of TCS OX2 29 as orexin-2 receptor antagonist reduces LH stimulation-induced antinociception during the early and late phases of the formalin test in a dose dependent manner and this effect was stronger in the late phase of the formalin test, (2) Intrathecal administration of TCS OX2 29 alone had no effect on pain-related behaviors. Similar results were obtained in our previous study in which we showed that under the exact same experimental paradigm and conditions, OX1Rs can significantly alter, LH-stimulation induced analgesia [22].

Shono and Yamamoto in 2008 examined the effect of spinally applied orexin-A and orexin-B on the primary afferent fiber-evoked nociceptive reflex in the isolated spinal cord of the neonatal rat [27]. They showed that both application of orexin-A and orexin-B on isolated spinal cord slices inhibits the slow ventral root potential (VRP) and that application of a selective OX2 receptor antagonist but not OX1R antagonist depressed the slow VRP. Therefore, they concluded that spinal OX2 receptors may play an inhibitory role in nociceptive transmission in the spinal cord of the juvenile rat. Although this may partially explain role of the spinal OX2 receptors in pain modulation in neonatal rats, it is in contrast with behavioral findings in adult animals that seemingly activation of either receptor types suppress pain related behaviors.

Although majority of studies have been done on the orexin-A and its interaction with type 1 receptors, there are a few molecular mechanisms that may explain our observation of which inhibition of Ca^{2+} influx into the dorsal root ganglion (DRG) neurons and activation of the glycinergic inhibitory interneurons are supported with numerous evidences.

Orexin-A can exert its actions in pain modulation via inhibition of high K^+-induced Ca^{2+} increase in DRG neurons [28]. It was then concluded that orexin-A blocked Ca^{2+} influx through L-type Ca^{2+} channel. This study however did not apply any orexin receptor antagonist but because orexin-A has strong affinity for both types of the orexin receptor, one can conclude that activation of either types of orexin receptors can lead to similar consequences in terms of ionic transmission in DRG neurons that play remarkable role in signaling of pain at spinal level.

Evidence for involvement of OX2 receptors of the Lamina II of spinal cord in adult rats comes from a research by [29] in which they showed that orexin B affects spontaneous synaptic transmission in lamina II neurons, which play a pivotal role in regulation of nociceptive transmission. They demonstrated that application of orexin-B on spinal slices enhances glycinergic spontaneous inhibitory transmission in majority of the lamina II neurons, whereas GABAergic transmission was unaffected and these activities were inhibited by an orexin-2 receptor antagonist (JNJ10397049) but not an orexin-1 receptor antagonist (SB334867).

Additionally, orexin neurons contain several other neurotransmitters like dynorphin [30] or neurotensin [31] that can contribute to suppression of pain related behavior.

We did not observe any significant effect of OX2 receptor antagonist on pain related behaviors without stimulation of the LH. It seems that activation of nociceptors does not trigger release of orexin to the extent sufficient for a prominent analgesic effect. Similar to our previous findings [22], it seems that orexin receptors at the spinal level cannot play a significant role in absence of the LH stimulation making their physiological role in pain modulation probably non-primary. Finally, we observed enhanced effectiveness of TCS XO2 29 in the second phase compared with the early

phase of the formalin test. This is possibly due to natural wash-out and decay of the painful stimulus particularly the last 15 min of the test, as generally pain score profile decays in this last potion of the test.

Overall, we showed that the analgesic effects of stimulated release of orexins from the LH can be reversed by blockade of the spinal OX2 receptors in a model of inflammatory pain. Our results provide additional supporting evidence for involvement of the orexin system in pain modulation. We believe that selective agonists of the orexin-1 and 2 receptors provide potential complementary treatment for pain management in addition to (or as a continuation of) conventional opiates such as morphine with the benefit of reducing the risk of development of tolerance and dependence to these drugs in the patient and a new therapeutic perspective for clinical use of these peptides in management of chronic pains.

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