Title: Role Of The Orexinergic System Within The Ventral Tegmental Area In The Development Of Sensitization To Morphine Induced By Lateral Hypothalamus Stimulation

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Abstract

The lateral hypothalamus (LH) has long been known to implicate in the addictive behaviors of drugs of abuse. The ventral tegmental area (VTA) is a major area of the mesolimbic system that is strongly involved in the development of morphine sensitization. The current study aimed to examine the role of intra-VTA orexin receptors in the LH stimulation-induced sensitization to the antinociceptive response of morphine. One hundred fourteen adult male Wistar rats underwent unilateral implantation of two separate cannulae into the LH and VTA using the stereotaxic apparatus. Intra-VTA administration of the orexin-1 (OX1) and orexin-2 (OX2) receptor antagonists, SB334867 and TCS OX2 29 (1, 3, and 10nM/0.3μl DMSO), respectively, was performed five minutes before concurrent microinjection of carbachol (250nM/0.5μl saline) into the LH and an ineffective dose of morphine (0.5 mg/kg; sc) during 3-day sensitization period. After a 5-day free drug period, on the 9th day, for assessing the morphine sensitization, the nociceptive response was measured before and after morphine injection (1 mg/kg; sc) using the tail-flick test. The results revealed that the concurrent administration of carbachol (250 nM) and an ineffective dose of morphine significantly induced morphine sensitization. Besides, the blockade of OX1 and OX2 receptors within the VTA before intra-LH carbachol injection attenuated morphine sensitization. These findings suggest that LH stimulation potentiates the sensitization to morphine antinociceptive responses via affecting orexin receptors located in the VTA. However, the contribution of OX1Rs in the VTA was more predominant than that of OX2Rs to morphine sensitization in the rat.

Keywords: Morphine sensitization; Orexin system; Lateral hypothalamus; Ventral tegmental area; Tail-flick test; Rat
1. Introduction

Morphine is a valuable drug in the clinic for its' analgesic properties. The morphine use is, however, limited because of its' addictive nature. Development of behavioral sensitization, which defines as an enhanced systemic reaction to the same dose of morphine or any other addictive substance, occurs in response to continuous and intermittent administration of these drugs in rodents (Lv et al., 2019; Reisi et al., 2014; Vezina & Leyton, 2009). Several neurotransmitters and neuromodulators involve in opioid-induced behavioral sensitization, including dopamine (Charmchi et al., 2016), glutamate (Sepehrizadeh et al., 2008), serotonin (Pang et al., 2016), as well as orexin (Łupina et al., 2018; Razavi et al., 2014). Ventral tegmental area (VTA) and nucleus accumbens (NAc) are thought to play a predominant role in the development of morphine sensitization via dopamine receptor activation (Reisi et al., 2014). Orexinergic neurons located in the lateral hypothalamus (LH) evoke their effects via two metabotropic receptors: orexin receptor type 1 (OX1) and orexin receptor type 2 (OX2), which are widely distributed in the various brain areas (Marcus & Elmquist, 2006; Sakurai et al., 1998). The lateral hypothalamus sends orexinergic projections all over the mesolimbic dopaminergic pathway such as VTA (Fadel & Deutch, 2002), a cerebral region which is highly involved in behavioral sensitization (Borgland et al., 2006) and chemical stimulation of LH has been shown to involve in pain modulation via acting on orexin receptors located in this area (Ezzatpanah et al., 2016). Generally, the mesocorticolimbic pathway is referred to as a dopaminergic projection derived from VTA into the NAc and the prefrontal cortex, which participates in addictive behaviors (Stott & Ang, 2013). Our laboratory's prior work revealed that chemical stimulation of LH potentiated morphine sensitization in the conditioned place preference (CPP) paradigm through the OX1 receptors located in the VTA region in rats' brain (Razavi et al., 2014). Although plenty of studies have been performed in the context of
morphine sensitization, few studies have indicated the alterations of antinociceptive effects of morphine in morphine-sensitized animals (Zarrindast et al., 2007). So considering that mesolimbic dopamine system has a crucial role in induction of morphine sensitization and given the distribution of orexinergic projections all over the mesolimbic system including the VTA, the necessity for the study based on the role of LH in the induction of sensitization to morphine antinociceptive responses and the probable role of intra-VTA OX1 and OX2 receptors in this phenomenon seems essential.

2. Material and Methods

2.1. Animals

One hundred fourteen adult male Wistar rats (Pasteur Institute, Tehran, Iran; 220-250 gr) were randomly chosen and assigned into 18 groups (n= 6-8 in each group). Animals were maintained in a 12/12 h light/dark cycle with food and water *ad libitum*. All experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and were confirmed by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.PHNS.REC.1398.133), Tehran, Iran.

2.2. Surgical preparation

The animals were anesthetized with intraperitoneal (ip) administration of xylazine 2% (10 mg/kg) and ketamine 10% (100 mg/kg) mixture and placed in a stereotaxic instrument (Stoelting, USA). Two stainless steel guide cannulae (23-gauge, 11 mm in length) were implanted unilaterally (right or left side) into the LH and VTA injection sites and anchored with steel screw. The incision was closed with dental cement. The coordinate for VTA region
according to the Paxinos and Watson rat brain atlas (Paxinos & Watson, 1982) was as follows: anteroposterior (AP) = 4.8 mm caudal to bregma, Lateral to midline (Lat) =±0.9 mm, Dorsoventral (DV) = 8.3 mm ventral from the skull surface and the coordinate for LH was: AP = -2.92 mm, Lat = ± 1 mm and DV = 8.1 mm ventral to the skull surface. Rats were then allowed to recover for one week before the beginning of experiments.

2.3. Drugs and drug administration

In this study, the following drugs were used: morphine sulfate dissolved in sterile saline 0.9 % (Temad, Tehran Iran), different solutions (62.5, 125 and 250 nM) of carbachol (Sigma-Aldrich, USA), which were dissolved in 0.5 μl saline. Different doses of SB334867 (1, 3, and 10 nM) as an OX1 receptor antagonist or TCS OX2 29 (1, 3, and 10 nM) as an OX2 receptor antagonist (Tocris Bioscience, Bristol, UK) which were dissolved in a volume of 0.3 μl dimethyl sulfoxide 12% (DMSO; Sigma-Aldrich, Germany) as a vehicle of both orexin receptor antagonists. All drugs or vehicle solutions were infused slowly over 60 seconds. All of the microinjections were conducted in animals via a stainless-steel injector (30-gauge needle) connected to a 1-μl Hamilton syringe via a polyethylene tube (PE-20).

2.4. Tail-flick test

In this study, morphine sensitization has been assessed by the antinociceptive response of morphine. The nociceptive response was measured using the tail-flick apparatus (Harvard Apparatus, USA). A thermal stimulus was applied in succession after the 3, 5, or 7 cm from the caudal tip of the tail. An automatic sensor detected the tail-flick response and reaction time between the onset of the thermal stimulus and tail-flick response recorded as tail-flick latency (TFL). The rats were tested before and 5, 15, and 30 min after morphine injection (1 mg/kg; sc). The obtained value of each TFL time was calculated on the average of two consecutive tail-flick tests at each time point. The radiant heat intensity was manually set at 45% of the
maximum intensity that yields baseline TFL values in the range of 3-4 s. A cut-off time of 10 s was applied to avoid tissue damage.

The TFL value was expressed as a percentage of maximal possible effect (%MPE), which was calculated as follows:

\[
\%MPE = \frac{\text{Post–drug administration latency (s)} - \text{Baseline latency (s)}}{\text{Cut–off value (s)} - \text{Baseline latency (s)}} \times 100
\]

2.5. Experimental design

Briefly, animals received intra-LH carbachol after intra-VTA OX1/OX2 receptor antagonists, followed by subcutaneous injection of an ineffective dose of morphine (0.5 mg/kg; sc) for 3 consecutive days as sensitization period and then, after five days of free drug administration, on the 9th day, the tail-flick test was performed before and after the morphine (1 mg/kg; sc) injection (Fig. 1).

2.5.1. The effect of morphine injection during sensitization period on the induction of morphine sensitization

To understand the effect of morphine (5 mg/kg; sc) administration for three consecutive days (sensitization period) on the development of morphine sensitization, animals received saline (1 ml/kg; sc) or morphine (5 mg/kg; sc) during sensitization period. After five days of free drug administration, on the 9th day, tail-flick test was performed before and after morphine injection. To find out the appropriate dose of morphine for induction of morphine sensitization, different doses of morphine (0.1, 0.5, and 1 mg/kg; sc) were injected before the tail-flick test.

2.5.2. The effect of co-administration of carbachol and ineffective dose of morphine during the sensitization period on the induction of morphine sensitization
To elucidate the role of intra-LH administration of carbachol in the development of morphine sensitization, during 3-day sensitization period, different doses of carbachol (62.5, 125, and 250 nM/0.5 µl saline) were microinjected just before the injection of saline (1 ml/kg; sc) or the ineffective dose of morphine (0.5 mg/kg; sc) during sensitization period, and after five days of free drug administration, on the 9th day, the tail-flick test was performed before and after the morphine (1 mg/kg; sc) injection.

2.5.3. The role of OX1 and OX2 receptors within the VTA in the LH stimulation-induced morphine sensitization

In this set of experiments, different doses of SB334867 (1, 3, and 10 nM) as an OX1 receptor antagonist or TCS OX2 29 (1, 3, and 10 nM/0.3 µl DMSO) as an OX2 receptor antagonist were microinjected five minutes before concurrent administration of the ineffective dose of morphine (0.5 mg/kg; sc) and intra-LH administration of the highest dose of carbachol (250 nM/0.5 µl saline) during 3-day sensitization period to evaluate the role OX1 and OX2 receptors within the VTA in the LH stimulation-induced morphine sensitization. After five days of free drug administration, the 9th day, a tail-flick test was performed before and after the morphine (1 mg/kg; sc) injection.

2.6. Histological verification

After completion of the experiments, animals were anesthetized with a ketamine and xylazine mixture. Animals were then transcardially perfused with 0.9% normal saline and formaldehyde solution (10%). After removing the rat brains, 50-µm transverse brain sections were prepared, and the location of the guide cannula tips was compared with the VTA coordinates in the rat
brain atlas (Paxinos & Watson, 1982). The animals with the wrong cannulae placements were excluded from the data analysis.

2.7. Statistics

All statistical analyses were performed using commercially available software GraphPad Prism® 6.0 (GraphPad Software, CA, USA). Data were expressed as mean ± SEM (standard error of mean). The obtained %MPE at any time set intervals in all groups were subjected to a one-way analysis of variance (ANOVA) followed by post-hoc Dunnett or Newman-Keuls multiple comparisons test. P values less than 0.05 were considered significant statistically.

3. Results

3.1. The effect of morphine injection during sensitization period on the induction of morphine sensitization

One-way ANOVA followed by Dunnett post-hoc test [F (6, 48) = 21.51, P<0.0001; Fig. 2] showed that morphine administration (5 mg/kg; sc) for three consecutive days (sensitization period) enhanced sensitization to the antinociceptive response of morphine in animals which received morphine 1 mg/kg but not 0.1 or 0.5 mg/kg before the tail-flick test. Thus, the dose of 1 mg/kg of morphine was selected as the appropriate dose for the rest of the experiments. The mean percentage of maximal possible effect (%MPE) was considered as an antinociceptive index. As it has been shown in Fig. 2, saline (1 ml/kg; sc) administration instead of morphine (5 mg/kg; sc) during the sensitization period could not induce morphine sensitization in animals which received different doses of morphine (0.1, 0.5, or 1 mg/kg; sc) before the nociceptive test.
3.2. The effect of concomitant administration of carbachol and morphine during the sensitization period on the induction of morphine sensitization

One-way ANOVA followed by Newman-Keuls multiple comparisons test indicated that intra-LH microinjection of carbachol (125 and 250 nM/ 0.5 μl saline) just before injection of an ineffective dose of morphine (0.5 mg/kg; sc) during the sensitization period enhanced sensitization to the antinociceptive response of morphine [F (3, 23) = 16.63, P<0.0001; Fig. 3, right panel], while, 62.5 nM of carbachol could not induce morphine sensitization. As shown in Fig. 3, one-way ANOVA followed by Dunnett post-hoc test [F (3, 26) = 0.1277, P=0.9427; Left panel] indicated that concurrent administration of saline (1 ml/kg; sc) and different doses of intra- LH carbachol (62.5, 125, and 250 nM/ 0.5 μl saline) for three consecutive days could not induce sensitization to the antinociceptive response of morphine (1 mg/kg; sc) measured by the tail-flick test.

3.3. The effect of intra-VTA injection of OX1 receptor antagonist, SB334867 on the LH stimulation-induced morphine sensitization

One-way ANOVA followed by Dunnett post-hoc test indicated that the blockade of OX1 receptors within the VTA by SB334867 before intra-LH microinjection of carbachol could decrease the morphine sensitization-induced by co-administration of carbachol (250 nM/0.5 μl saline) and ineffective dose of morphine (0.5 mg/kg; sc) [F (5, 41) = 14.37, P<0.0001; Fig. 4]. However, intra-VTA administration of the highest dose of SB334867 (10 nM/0.3 μl DMSO) alone did not induce morphine sensitization measured by the tail-flick test. Moreover, statistical analysis showed that intra-VTA administration of SB334867 (3, and 10 nM/0.3 μl DMSO) could block the morphine sensitization-induced by carbachol microinjection compared to the group which received intra-VTA DMSO instead of SB334867 (Fig. 4).
3.4. The effect of intra-VTA injection of OX2 receptor antagonist, TCS OX2 29 on the LH stimulation-induced morphine sensitization

As it has been shown in Fig. 5, one-way ANOVA followed by Dunnett post-hoc test indicated that intra-VTA microinjection of OX2 receptor antagonist, TCS OX2 29 before co-administration of carbachol (250 nM/0.5 µl saline) and ineffective dose of morphine could decrease the morphine sensitization [F (5, 40) = 16.5, P<0.0001; Fig. 5]. However, intra-VTA administration of the highest dose of TCS OX2 29 (10 nM/0.3 µl DMSO) alone did not induce morphine sensitization. Besides, one-way ANOVA showed that only the highest dose of TCS OX2 29 (10 nM/0.3 µl DMSO) could block the morphine sensitization induced by carbachol microinjection compared to the group which received intra-VTA DMSO instead of TCS OX2 29 in this set of experiments (Fig. 5).

4. Discussion

The current study demonstrated the contribution of OX1- and OX2 receptors within the VTA in morphine sensitization induced by LH’s chemical stimulation prior to the subcutaneous injection of the ineffective dose of morphine. The significant findings of this study were as follows: (1) Concurrent microinjection of carbachol into the LH and subcutaneous injection of morphine for three consecutive days as the sensitization period, enhanced sensitivity to the antinociceptive effects of morphine; (2) Blockade of the OX1 and OX2 receptors within the VTA during sensitization period, significantly reduced the morphine sensitization induced by co-administration of carbachol and morphine and (3) The contribution of OX1 receptors in the VTA was more predominant than that of OX2 receptors to morphine sensitization.

The present study indicated that subcutaneous injection of 5 mg/ kg morphine for three consecutive days followed by five days free-morphine administration induced sensitization to
the antinociceptive response of morphine 1 mg/kg but not morphine 0.1 or 0.5 mg/kg. In this respect, certain similar studies indicated that repeated administration of morphine (5 mg/kg; sc) for three consecutive days followed by five days washout increased antinociceptive responses of morphine (1 mg/ kg) in sensitized animals (Charmchi et al., 2016; Molaei et al., 2014; Reisi et al., 2014). On the other hand, it has been reported that repeated morphine administration (20 mg/kg; ip) for seven days (Roeckel et al., 2017) or a regimen of three days morphine (20 mg/kg; ip) followed by a five days washout led to opioid-induced hyperalgesia in mice (Ahmadi et al., 2014). It seems that this discrepancy stems from the dosage and duration of morphine administration. The obtained results also showed that intra-LH microinjection of carbachol for three consecutive days followed by five days no drug administration could not potentiate morphine sensitization, while co-administration of intra-LH carbachol and ineffective dose of morphine (0.5 mg/ kg; sc) during the sensitization period enhanced sensitivity to the antinociceptive response of morphine. Previously it has been shown that chemical stimulation of LH potentiated morphine sensitization in the CPP paradigm (Razavi et al., 2014), and orexinergic neurons of the LH, and not nearby melanin-concentrating hormone (MCH) neurons, have µ-opioid receptors and implicate in the addictive behaviors in response to chronic morphine administration (Georgescu et al., 2003). Intracerebroventricular (ICV) injection of SB-334867 as selective OX1 receptor antagonist prior to subcutaneous injection of morphine has been reported to decrease morphine-induced antinociceptive response in formalin test (Azhdari-Zarmehri et al., 2013). Steiner et al. demonstrated that blockade of both orexin receptors by oral gavage of almorexant, decreased morphine-induced sensitization to the locomotor activity in sensitized rats (Steiner et al., 2013). Therefore, it seems that some effects of morphine administration mediate by orexin receptors. The results of the present study revealed that chemical stimulation of LH induced morphine sensitization through affecting OX1 and OX2 receptors within the VTA. There is a dense projection of orexin neurons from
the LH to the dopaminergic and nondopaminergic neurons in the VTA. Accordingly, single-unit extracellular and whole-cell patch-clamp recordings indicated that orexin depolarizes these neurons and increase firing frequency of either group of neurons (Korotkova et al., 2003). Functional interplay between orexin and dopamine neurons of mesolimbic system has been reported to implicate in rewarding effect and hyperlocomotion-induced by morphine (Narita et al., 2006).

Several lines of the study revealed that the induction of drug sensitization is not only associated with the release of dopamine (Lv et al., 2019; Stout et al., 2016) but are also pertinent to the alterations in the sensitivity of dopamine receptors in the mesolimbic structures, including the NAc, VTA, striatum, and hippocampus (for review, see (Listos et al., 2019)). Besides, the blockade of dopamine receptors within the VTA and NAc prevents sensitization to the antinociceptive response of morphine (Reisi et al., 2014). It has been reported that the levels of dopamine and its major metabolites in the NAc markedly increase by intra-VTA administration of both orexin A and orexin B (Narita et al., 2006). Moreover, orexin A can induce its' antinociceptive response by activating intra-VTA orexinergic receptors, which activate the dopaminergic inputs to the NAc in rats (Yazdi-Ravandi et al., 2014). It has been demonstrated that microglial activation following chronic administration of morphine leads to disruption of the VTA dopaminergic circuitry (Taylor et al., 2016). Besides, Glial fibrillary acidic protein (GFAP), an important marker of astrocyte activation, showed an increase in the striatum and in the prefrontal cortex in morphine sensitized rats. However, intraperitoneal administration of SB-334867 reversed these alterations (Łupina et al., 2018). So it seems that the interaction of orexin and mesolimbic dopamine system plays a crucial role in the induction of sensitization to the antinociceptive response of morphine. Summing up, this study confirms and extends the contribution of OX1/ OX2- receptors within the VTA in sensitization to the
antinociceptive response of morphine-induced by concomitant intra-LH administration of carbachol and subcutaneous injection of morphine.

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References


1120–1130. https://doi.org/10.17877/DE290R-7236


**Figure Legends**

**Fig. 1.** A schematic timeline of the experimental protocol. Carbachol was dissolved in 0.5 µl Saline and was microinjected into the lateral hypothalamus region in rats' brain. SB334867 and TCS OX2 29 were dissolved in 0.3 µl DMSO (12%) and were microinjected into the ventral tegmental area region in rats' brain. TF, Tail-flick; sc, subcutaneous.

**Fig. 2.** Effect of morphine administration during the sensitization period on the induction of morphine sensitization measured by the tail-flick test. Morphine administration (5 mg/kg; sc) for three consecutive days (sensitization period) followed by five days no drug administration, enhanced sensitization to the antinociceptive response of morphine (1 mg/kg; sc). The percentage mean of maximal possible effect (%MPE) was considered as an antinociceptive index. Injection of saline instead of morphine did not induce morphine sensitization at any doses of morphine. Each point shows the mean ± SEM for seven rats in each group.

*** P<0.001 compared to the saline-control group

**Fig. 3.** Effect of chemical stimulation of the lateral hypothalamus (LH) by carbachol prior to morphine injection during sensitization period on the induction of morphine sensitization measured by the tail-flick test. Intra-LH microinjection of carbachol just before injection of an ineffective dose of morphine (0.5 mg/kg; sc) during the sensitization period (right panel) enhanced sensitization to the antinociceptive response of morphine, while concurrent administration of saline (1 ml/kg; sc) and different doses of intra- LH carbachol during sensitization period (left panel) could not induce morphine sensitization. Each point shows the mean ± SEM for 6-7 rats in each group.

** P<0.01 and *** P<0.001 compared to the saline-control group
†† P<0.01 and ††† P<0.001 compared to the respective vehicle group

**Fig. 4.** The effect of intra-VTA injection of SB334867 on the morphine sensitization-induced by co-administration of carbachol and morphine. Intra-VTA administration of OX1 receptor antagonist, SB334867 before co-administration of intra-LH carbachol (250 nM/0.5 µl saline) and ineffective dose of morphine (0.5 mg/kg; sc) during the sensitization period could decrease the morphine sensitization-induced by concurrent administration of carbachol and morphine. Morphine sensitization was measured by the tail-flick test. Each point shows the mean ± SEM for seven rats in each group.

* P<0.05 and *** P<0.001 compared to the saline-control group
† P<0.05 and ††† P<0.001 compared to the DMSO group

**Fig. 5.** The effect of intra-VTA injection of TCS OX2 29 on the morphine sensitization-induced by co-administration of carbachol and morphine. Intra-VTA administration of OX2 receptor antagonist, TCS OX2 29 (10 nM/0.3 µl DMSO) before co-administration of intra-LH carbachol (250 nM/0.5 µl saline) and ineffective dose of morphine (0.5 mg/kg; sc) during the sensitization period could decrease the morphine sensitization by concurrent administration of carbachol and morphine. Morphine sensitization was measured by the tail-flick test. Each point shows the mean ± SEM for 6-8 rats in each group.

* P<0.05 and *** P<0.001 compared to the saline-control group
† P<0.05 compared to the DMSO group
Figure (1)

Days

<table>
<thead>
<tr>
<th>1-3</th>
<th>4-8</th>
<th>9</th>
</tr>
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<tbody>
<tr>
<td>Treatment Period</td>
<td>Rest</td>
<td>Test day</td>
</tr>
</tbody>
</table>

Saline (1 ml/kg; sc)  
Morphine (5 mg/kg; sc)  
Carbachol (62.5, 125, or 250 nM/0.5 μl Saline) + Saline (1 ml/kg; sc)  
Carbachol (62.5, 125, or 250 nM) + Morphine (0.5 mg/kg; sc)  
SB334867 (1, 3, or 10 nM) + Carbachol (250 nM) + Morphine (0.5 mg/kg; sc)  
SB334867 (10 nM) + Morphine (0.5 mg/kg; sc)  
TCS OX2 29 (1, 3, or 10 nM) + Carbachol (250 nM) + Morphine (0.5 mg/kg; sc)  
TCS OX2 29 (10 nM) + Morphine (0.5 mg/kg; sc)  

Morphine (0.1, 0.5, or 1 mg/kg; sc) + Tail-Flick test  
No drug administration  
Morphine (1 mg/kg; sc) + Tail-Flick test

Figure (2)

Morphine (5 mg/kg; sc)  
during sensitization period

Saline (1 ml/kg; sc)  
during sensitization period

Maximal Possible Effect (%)  

Saline  | 0.1  | 0.5  | 1    |
|--------|------|------|------|
Figure (3)

Maximal Possible Effect (%)

Saline (1 ml/kg; sc) for sensitization

Morphine (0.5 mg/kg; sc) for sensitization

Intra-LH administration of Carbachol (nM/0.5 μl saline) during sensitization period

Morphine (1 mg/kg; sc) for nociceptive test
Figure (4)

Morphine (0.5 mg/kg; sc) for sensitization

Maximal Possible Effect (%)

<table>
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<tr>
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<th>1</th>
<th>3</th>
<th>10</th>
<th>10</th>
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<td>Intra-LH</td>
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<tr>
<td>Intra-VTA SB334867 (nM/0.3 µl DMSO)</td>
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<tr>
<td>Intra-LH administration of Carbachol (250 nM/0.5 µl saline)</td>
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</table>
Figure (5)

Morphine (0.5 mg/kg; sc) for sensitization

Maximal Possible Effect (%)

Saline
DMSO 1 3 10
Intra-VTA TCS OX2 29 (nM/0.3 µl DMSO)

Intra-LH administration of Carbachol (250 nM/0.5 µl saline)