The role of orexin type-1 receptors in the development of morphine tolerance in locus coeruleus neurons: An electrophysiological perspective

Hakime Abdollahi¹, Masoumeh Ghaemi-Jandabi¹, Hossein Azizi*, Saeed Semnanian

Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

¹ These authors contributed equally to this study

***Corresponding author:**

Hossein Azizi, PhD

Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, Tel: +98-21-82884587, E-mail address: azizih@modares.ac.ir

Acknowledgement:

This study is under auspices of the Iran National Science Foundation (INSF, Grant No. 93046826) and Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

Abstract:

Long-term exposure to opioid agonists results in tolerance to their analgesic effects, so the effectiveness of opioid agonists in the management of pain becomes limited. The locus coeruleus (LC) nucleus has been involved in the development of tolerance to opiates. Orexin type-1 receptors (OX1Rs) are highly expressed in LC nucleus. Orexin plays a noteworthy role in the occurrence of morphine tolerance. The purpose of the present study is to investigate the role of orexin type-1 receptors in the development of morphine tolerance in LC neurons.

In this study, adult male Wistar rats weighing 250-300 g were utilized. Induction of morphine tolerance was obtained by single injection of morphine per day for 6 successive days. An orexin typ-1 receptor antagonist (SB-334867) was injected into the lateral ventricle instantly prior to morphine injection. On day 7, the effect of morphine on the electrical activity of LC neurons was studied using in vivo extracellular single unit recording.

The results demonstrate that morphine injection for 6 consecutive days led to the development of morphine-induced tolerance in LC neurons. In other words, there was a significant decrease in LC neuronal responsiveness to morphine injection. Inhibitory responses of LC neurons to intraperitoneally applied morphine can be observed with the treatment of the SB-334867 prior to morphine injection.

This study showed that OX1R blockade by SB-334867 prevents the development of morphine tolerance in LC neurons. We hope that further studies will lead to considerable progress in understanding the molecular adaptations that contribute to morphine tolerance.

Keywords: Orexin, SB-334867, Morphine, Tolerance, Extracellular in vivo single unit recording, LC neurons

1. Introduction

Morphine is one of the most commonly consumed compounds for severe pain treatment (Davis et al., 2005). Prolonged utilization of morphine results in tolerance to its analgesic effects. The cellular mechanisms involved in morphine tolerance are still poorly comprehended, though many mechanisms have been proposed (Dang and Christie, 2012).

Locus coeruleus (LC) nucleus located on the base of the fourth ventricle in the rostral pons, is the major noradrenergic nucleus of the brain that innervates the entire neuraxis (Aston-Jones and Cohen, 2005; Foote et al., 1983). A great deal of evidence confirms the idea that LC is involved in some aspects of tolerance and physical dependence, induced by long-term exposure to opiates (Aghajanian, 1978; Bird and Kuhar, 1977; Duman et al., 1988; Korf et al., 1974; North and Williams, 1985). Acute opiate administration inhibits the spontaneous activity of LC neurons via activation of an inwardly rectifying K^+ channel and the inhibition of a slow voltage-activated non-selective cation channel (Aghajanian and Wang, 1987). Chronic exposure to these drugs leads to the development of tolerance and dependence i.e. depressed firing rate gradually recovers towards the normal level and the administration of an opioid receptor antagonist causes a dramatic increase in the firing rates above normal levels (Aghajanian, 1978; Akaoka and Aston-Jones, 1991; Rasmussen and Aghajanian, 1989; Rasmussen et al., 1990). It has been reported that long-lasting administration of opiates leads to a dramatic up-regulation of the cAMP system (Nestler, 1990). In addition, chronic opiate treatment augments the levels of adenylate cyclase (Duman et al., 1988) and cAMP-dependent protein kinase (Nestler and Tallman, 1988) in rat LC. Opiate up-regulation of the cAMP pathway increases the intrinsic excitability of LC neurons by which is accounted for the development of opiate tolerance (Nestler, 1990). It has been demonstrated that prolonged exposure of LC neurons to morphine induces extensive μ -opioid receptor (MOR) desensitization (Bailey et al., 2009) and this phenomenon is considered as the first step in the development of opioid tolerance.

Orexin A and orexin B (hypocretin 1 and hypocretin 2) are produced in the lateral hypothalamus (LH) and they bind to two receptors that are G protein-linked: orexin type 1 and 2 receptors (OX1R and OX2R) (de Lecea et al., 1998; Sakurai et al., 1998). Orexin neurons project panoramically throughout the adult rat brain. Among the major recipients of orexinergic projections is the LC (Nambu et al., 1999) that profusely expresses orexin type-1 receptors (Marcus et al., 2001). Several observations imply that the orexinergic system has been involved

in the development of morphine-induced tolerance (Erami et al., 2012; Ranjbar-Slamloo et al., 2012).

The present study aims to elucidate the role of orexin type-1 receptors (OX1Rs) in the development of morphine tolerance in LC neurons.

2. Results

2.1. LC Spontaneous discharge

Putative LC neurons were identified based on their low-frequency spontaneous discharge (0.5–6.0 Hz) and long-duration action potentials (1.5 to 2 ms). In anesthetized rats (n=6), the mean firing rate of LC neurons was 2.44 ± 0.56 spikes/s. Besides, these neurons exhibited a short-latency excitation followed by a prolonged quiescent period (> 0.5 s) in response to contralateral hindpaw pinch (Fig. 1).

2.2. LC neuronal response to acute morphine injection

In acute morphine administration group, rats received morphine (10 mg/kg, i.p.) after 10 minutes of baseline recording. Fig. 2 shows that LC neurons exhibited a decrease in firing rate following acute morphine injection (baseline: 2.45 ± 0.56 spikes/s; after morphine administration: 1.13 ± 0.30 spikes/s, $t=3.88$, $df: 5$, $*P<0.05$).

2.3. LC neuronal response to morphine injection in morphine treated rats

In this group, morphine was injected (10 mg/kg, i.p.) once a day for 6 consecutive days. On day 7, the effect of morphine (10 mg/kg, i.p.) on the electrical activity of LC neurons was investigated to confirm morphine tolerance. Morphine injection did not induce any change in the activity of LC neurons in morphine-dependent rats (baseline: 3.44 ± 0.64 spikes/s; after morphine administration: 3.44 ± 0.61 spikes/s, $t=0.15$, $df: 5$, P value=0.88) (Fig. 3). None of the neurons showed significant response to injection of morphine.

2.4. LC neuronal response to morphine injection in SB-334867-morphine treated rats

In this group, SB-334867 (a selective OX1R antagonist) was microinjected into the lateral ventricle (10 μ g/10 μ l, i.c.v.) instantly prior to morphine injection (10 mg/kg, i.p.). On day 7, the effect of morphine injection (10 mg/kg, i.p.) on LC neuronal activity was investigated.

Administration of morphine (10 mg/kg, i.p.) caused a decrease in the impulse activity of LC neurons in SB-morphine treated animals (baseline: 2.76 ± 0.43 spikes/s; after morphine administration: 1.85 ± 0.24 spikes/s, $t = 3.59$, $df: 5$, $*P < 0.05$) (Fig. 4).

2.5. LC neuronal response to morphine injection in vehicle-morphine treated rats

In this group, the vehicle was microinjected into the lateral ventricle (10 μ l, i.c.v.) instantly prior to morphine injection (10 mg/kg, i.p.). On day 7, the effect of morphine injection (10 mg/kg, i.p.) on LC neuronal activity was assessed. Morphine injection had no substantial effect on single-unit activity of LC neurons (baseline: 2.11 ± 0.73 spikes/s; after morphine administration: 2.08 ± 0.75 spikes/s, $t = 0.62$, $df: 5$, $P \text{ value} = 0.57$) (Fig. 5). None of the neurons showed significant response to the injection of morphine.

3. Discussion

A great deal of interest has been devoted to the locus coeruleus (LC) as a prominent model for studying the mechanisms underlying the acute and chronic effects of opioid drugs (Christie, 1991; de Carvalho and de Azevedo, 2000). In this study, we examined the effect of acute injection of morphine on single-unit activity of LC neurons. The results indicated that acute morphine administration causes a diminution in the LC neuronal activity. This finding is in tandem with the results of the previous studies indicating that the acute administration of opioids inhibits the electrical activity of rat LC neurons (Aghajanian, 1978; Bird and Kuhar, 1977; Korf et al., 1974).

Several studies suggested that chronic exposure to morphine could lead to the development of tolerance in LC neurons (Aghajanian, 1978; Christie et al., 1987). Many protocols for inducing morphine tolerance have been identified so far. In the present study, the induction of morphine tolerance was attained by single injection of morphine per day for 6 successive days. Then on day 7, the effect of intraperitoneally applied morphine on the impulse activity of LC neurons was studied. Morphine application did not cause any alteration in the firing rate of LC neurons in the morphine treated rats. This observation confirmed that the protocol which was used has been appropriate to produce robust morphine tolerance in LC neurons.

Some behavioral studies have reported that orexin type-1 receptor antagonist SB-334867 inhibits the development of morphine tolerance in rats (Erami et al., 2012; Ranjbar-Slamloo et al., 2012).

As mentioned in the previous studies, because SB-334867 has been applied intracerebroventricularly (i.c.v.), the involvement of orexin type-1 receptors in morphine tolerance could not be ascribed to a specified brain region. However, based on some evidence, we would like to propose LC nucleus as a dependable target for mediating the effect of SB-334867 on the development of morphine tolerance: First, the LC is one of the locale with a high density of orexinergic fibers that express mainly the OX1R (Marcus et al., 2001; Nambu et al., 1999; Sakurai et al., 1998) and second, μ -opioid receptors (MOR) in LC neurons are involved in the development of tolerance and physical dependence to morphine (Bailey et al., 2009; Mj et al., 1985).

In order to investigate the effect of SB-334867 on morphine tolerance, SB-334867 was injected into the lateral ventricle instantly prior to morphine administration. Then on day 7, the effect of morphine application on electrical activity of LC neurons was examined. In comparison to animals unresponsive to morphine following a 6-day morphine treatment, the co-injection of SB-334867 with morphine resulted in the recovery of inhibitory responses to morphine. Co-administration of the SB-vehicle with morphine for 6 days in a row did not modify the responsiveness to intraperitoneally administered morphine in comparison to the group treated with morphine. Based on these results, it can be concluded that orexin might have a pivotal function in the development of morphine tolerance in LC neurons and the role of orexin type-1 receptor may be essential for this phenomenon.

Many G-protein-coupled receptors are potentially capable of modulating voltage-dependent calcium channels. Activation of G_q by the binding of orexin to the orexin type-1 receptor can lead to an augmentation in the intracellular calcium and inositol triphosphate (IP3) (Johansson et al., 2008; Muroya et al., 2004; Sakurai et al., 1998). The augmentation in the intracellular calcium causes the activation of protein kinases, including protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPKs) and calmodulin kinase II (CaMKII) (Ammoun et al., 2006; Ekholm et al., 2007; Holmqvist et al., 2005; Selbach et al., 2010). Also, pharmacological studies have introduced a number of signaling proteins involved in morphine-induced tolerance that includes the N-methyl-D-aspartate acid glutamate receptor (NMDAR) (Manning et al., 1996; Mendez and Trujillo, 2008), nitric oxide synthase (NOS) (Liu et al., 2006; Romero et al., 2010), PKC, PKA (Gabra et al., 2008; Mayer et al., 1995) and CaMKII (Fan et al., 1999; Tang et al., 2006). MOR- and NMDAR-mediated signals work together in a successive

and interconnected manner to ultimately induce MOR desensitization (Garzón et al., 2012). MOR desensitization is considered as the first step in the development of opioid tolerance and has a remarkable PKC-dependent element. PKC-induced MOR desensitization plays a substantial role in the morphine tolerance that develops in mature neurons following prolonged exposure to the drug in vitro and in vivo (Bailey et al., 2009). With this regard, some studies have indicated that tolerance to the antinociceptive effects of morphine can be abrogated by either co-administration of PKC inhibitors (Bohn et al., 2002; Granados-Soto et al., 2000; Hua et al., 2002; Inoue and Ueda, 2000; Smith et al., 2003) or by the blockade of various Gq-coupled receptors (Dourish et al., 1990; Popik et al., 2000; Ranjbar-Slamloo et al., 2012; Tortorici et al., 2003). These findings support the view that orexin neuropeptides through affecting Gq-coupled receptor OX1 and a PKC-dependent mechanism contribute to morphine tolerance in LC neurons. Also, It has been reported that almost 50% of orexinergic neurons express μ opioid receptors, and most of these cells respond to morphine either by induction of cAMP response element (CRE) activity or c-Fos. The induction of CRE activity and c-Fos in orexinergic cells suggests that regulation of the cells by chronic morphine is mediated via direct actions of morphine on these cells (Georgescu et al., 2003). According to these points, it is probable that the endogenous release of orexin would increase following chronic morphine challenge and the blockade of orexin receptors by SB-334867 could inhibit the modulatory effect of orexin in responses to morphine. Moreover, we should bear in mind that since OX1R exist in the LC nucleus, we have two hypotheses for the effect of orexin in developing morphine tolerance: in the first hypothesis, orexin directly acts on its receptors in the LC nucleus and plays its role in the development of tolerance to morphine. In the second hypothesis, orexin indirectly leads to developing morphine tolerance in this nucleus via influencing other brain structures.

The study by Borgland et al. (2006) indicated that orexin A induces long-term potentiation in the ventral tegmental area (VTA) by means of the recruitment of NMDA receptors (Borgland et al., 2006). Kodama et al. have declared that intravenous administration of orexin A increases glutamate levels in the LC nucleus (Kodama and Kimura, 2002). The converging lines of evidence (Bisaga and Popik, 2000; Garzón et al., 2012) demonstrated an ostensible participation of glutamate receptors in phenomena in relation to opioid tolerance and dependence. For example, the NMDA receptor antagonists attenuate the development of morphine tolerance and dependence (Kozela et al., 2003; Marek et al., 1991; Trujillo and Akil, 1991).

Therefore, we can suppose that neuropeptide orexin-A might accelerate the procedure of developing morphine tolerance through potentiation of NMDAR-mediated signals and increasing glutamate level in LC nucleus. Altogether, our results suggest that the blockade of signal transduction of orexin type-1 receptor with SB-334867 prevents morphine tolerance in LC neurons.

4. Experimental procedure

4.1. Animals

Adult male Wistar rats weighing 250-300 g (Pasteur Institute, Tehran, Iran) were employed for this study. They were housed three per cage within a temperature-controlled animal facility with food and water available *ad libitum*. Animals were exposed to a 12-h light/dark cycle (lights on at 07:00 am). All efforts were made to conduct the experiment in a way that it utilizes the minimum number of animals and to cause the least degree of suffering. All experiments were done in conformity with the ethical guidelines of the Faculty of Medical Sciences Ethics Committee, Tarbiat Modares University, which are established on the NIH Guide for the Care and Use of Laboratory Animals.

4.2. Drugs

Morphine sulfate (Temad, Tehran, Iran) was dissolved in 0.9% injectable saline. Selective OX1R antagonist, SB-334867 (Tocris, Bristol, UK) was dissolved in pure DMSO and further diluted with 93% artificial cerebrospinal fluid (aCSF), thus the final fraction of DMSO was 7%. SB-334867 aliquots were kept frozen in -20°C until the day of injection.

4.3. Stereotaxic surgery and cannula implantation

Rats were anesthetized with intraperitoneal (i.p.) injection of ketamine 10% and xylazine 2% (100 mg/kg and 10 mg/kg, respectively) and then were fixed in a stereotaxic instrument (Narishige, Japan) with two blunt ear bars. After surgical exposing of the skull, stainless steel guide cannulae (23-gauge) were implanted unilaterally in 1 mm above the injection site in lateral ventricle. The stereotaxic coordinates for lateral ventricle were -0.9 mm posterior to bregma, 1.3 mm lateral to the midline and 2.5 mm ventral to bregma. Microinjections were delivered through 30-gauge dental needles as infusion cannulae that extended 1 mm below the implanted guide

cannulae (reaching the lateral ventricle area). The guide cannulae were secured to the skull surface using two tiny stainless-steel screws embedded in dental acrylic cement. Animals were allowed to convalesce for 6 days postoperatively.

4.4. i.c.v. injection

Drugs were infused in freely moving rats through infusion cannulae attached to a 10 µl Hamilton syringe by a polyethylene tube (PE-20). Drugs were manually injected over a 60 s period and the infusion cannula was kept in place for an additional 60 s to permit the dispersal of the drug. Then, the infusion cannula was withdrawn and the guide cannula was capped by equal length stainless steel stylet to ascertain that the cannula would remain patent during the post-surgical recovery period.

4.5. Induction of morphine tolerance and experimental design

Animals were rendered dependent on morphine by administration of morphine (10 mg/kg, i.p.) once a day for 6 days. SB-334867 (10 µg/10 µl, i.c.v.) or its vehicle was microinjected into the lateral ventricle instantly prior to morphine injection. Animals were divided into 4 groups with 6 individuals in each: group 1 (control), animals received saline (1 ml/kg, i.p.) for 6 days and then on the seventh day, the effect of morphine injection (10 mg/kg, i.p.) on LC neuronal activity was investigated; group 2, animals received morphine (10 mg/kg, i.p.) for 6 days so as to demonstrate the development of morphine tolerance in LC neurons; group 3, animals received SB-334867 (10 µg/10 µl, i.c.v.) instantly prior to morphine injection (10 mg/kg, i.p.) for 6 days to show whether SB-334867 can inhibit the development of morphine tolerance in LC neurons and in group 4, animals received SB-vehicle (10 µl, i.c.v.) instantly prior to morphine injection (10 mg/kg, i.p.) for 6 days to prove that the effect of SB-334867 is by virtue of the SB-334867 per se and neither the solvent nor the volume of the injection.

4.6. LC recordings and data collection

Rats were anesthetized with urethane (1.2 g/kg, i.p.), and supplemental doses (0.15 g/kg, i.p.) were given as necessary to have a profound level of anesthesia. Rats were tracheostomized to reduce noises caused by respiratory movements of animals. Body temperature was maintained at 37 °C by means of an electrically controlled heating pad. Rats were positioned in a stereotaxic

apparatus with the head oriented at a 15° angle to the horizontal plane (nose down). The skull was uncovered and a hole (roughly 3 mm diameter) was made for the insertion of the recording electrode at 1.3 mm lateral to the midline and 3.7–3.9 mm caudal to the lambdoid fontanelle over the cerebellum. The coordinates were taken according to the atlas of Paxinos and Watson (Paxinos G and Watson C, 1998). The dura over the cerebellum was carefully taken out. From each rat, only the extracellular electrical activity of single neuron was recorded.

LC neurons were identified according to well-defined electrophysiological features, including a biphasic positive-negative extracellular spike of long duration (1.5–2 ms), a tonic and slow spontaneous discharge (0.5–6.0 Hz), and a phasic excitation followed immediately by long-duration inhibition (> 0.5 s) in response to noxious stimuli (for example, tail or hindpaw pinch). The extracellular electrical activity of LC neurons was recorded by using glass micropipettes (3–12 MΩ resistance) which were filled with 2% pontamine sky blue in 0.5 M sodium acetate. Micropipettes were lowered into the LC nucleus using a micromanipulator (MO-8, One-axis Oil Hydraulic Micromanipulator, Narishige, Japan). Recorded extracellular signals were amplified (×1000) by an AC differential amplifier (DAM 80, WPI, UK), and filtered signals (0.3–3 kHz bandpass) were continually displayed on an oscilloscope. Also signals were monitored simultaneously using an audio analyzer (Fredrick Haer, FHC, Inc., USA). Signals were then digitized by a commercial analog to digital multi-channel data acquisition hardware system (PowerLab 4/30, ADInstruments Pty Ltd, Australia). At the end, data were analyzed by Chart 5 software with the Spike Histogram Module (ADInstruments). Recorded signals were displayed as a rate histogram (1-min bin size). After 10 minutes of baseline recording, 10 mg/kg morphine was injected intraperitoneally. An alteration in the activity was determined as an increase or decrease in firing rate by the mean baseline activity $\pm$ 2 SD, respectively.

4.7. Histological verification

At the end of each recording, the location of micropipettes tips was stained by iontophoretic ejection of pontamine sky blue (DC current for 10 min). Rats were then euthanized with an overdose of urethane and perfused transcardially with saline followed by 10% buffered formalin. Then brains were removed and immersed in phosphate-buffered formalin for 24 h. The fixed tissues were sliced into 300 μm-thick coronal sections and recording sites were histologically verified and schematized on sections derived from the atlas of Paxinos and Watson (Fig. 6).

4.8. Data analysis

The values were presented as the mean $\pm$ standard error of mean (SEM). The effects of morphine injection on the mean firing rates were analyzed by paired Student's t-test (counts were normally distributed).

Acknowledgement:

This study is under auspicious of the Iran National Science Foundation (INSF, Grant No. 93046826) and Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

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Figure legends:

Fig.1- LC neurons identification. Left: LC neurons exhibited a broad (1.5–2 ms) biphasic waveform. Right: Reactivity of LC neurons to contralateral hindpaw pinch. Application of pinch stimulus is depicted by an arrow. Noxious stimulus induces a phase excitation followed by an extended inhibition period in the activity of LC neurons.

Fig.2- Response of LC neurons to acute morphine injection in control group. Morphine (10 mg/kg, i.p.) was injected at time 10. Bars represent the mean firing rate of LC neurons before (baseline) and after morphine injection. Error bars represent SEM and the asterisk indicates statistically significant difference (* $P < 0.05$, Student's paired t-test, $n=6$).

Fig.3- Response of LC neurons to morphine injection in morphine treated group. Morphine (10 mg/kg, i.p.) was injected once a day for 6 days. Morphine (10 mg/kg, i.p.) was injected at time 10. Bars represent the mean firing rate of LC neurons before (baseline) and after morphine injection. Error bars represent SEM ($n=6$).

Fig.4- Response of LC neurons to morphine injection in SB-334867-morphine treated group. SB-334867 (10 $\mu\text{g}/10 \mu\text{l}$, i.c.v.) was administered instantly prior to morphine injection (10 mg/kg, i.p.) once a day for 6 days. Morphine (10 mg/kg, i.p.) was injected at time 10. Bars represent the mean firing rate of LC neurons before (baseline) and after morphine injection. Error bars represent SEM and the asterisk indicates statistically significant difference (* $P < 0.05$, Student's paired t-test, $n=6$).

Fig.5- Response of LC neurons to morphine injection in the vehicle-morphine treated group. SB vehicle (10 μl , i.c.v.) was administered instantly prior to morphine injection (10 mg/kg, i.p.) once a day for 6 days. Morphine (10 mg/kg, i.p.) was injected at time 10. Bars represent the mean firing rate of LC neurons before (baseline) and after morphine injection. Error bars represent SEM ($n=6$).

Fig.6- Histological verification of the recording sites by pontamine sky blue 2% in LC nucleus. Figures are redrawn from the atlas of Paxinos and Watson.

Drugs treatment protocol

(10 mg/kg, i.p.) Mor Mor Mor Mor Mor Mor
1 2 3 4 5 6
Day

(10 µg/10 µl, i.c.v.) SB SB SB SB SB SB
+ + + + + +
(10 mg/kg, i.p.) Mor Mor Mor Mor Mor Mor
1 2 3 4 5 6
Day

1

Response to morphine (10 mg/kg, i.p.) on day 7

2

Induction of morphine tolerance

Prevention of morphine tolerance by SB

3