The blockade of GABA_A receptors attenuates the inhibitory effect of orexin type 1 receptors antagonist on morphine withdrawal syndrome in rats

Mahnaz Davoudi, Hossein Azizi, Javad Mirnajafi-Zadeh, Saeed Semnanian*

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

***Corresponding author:**

Saeed Semnanian

Department of Physiology, School of Medical Sciences, Tarbiat Modares University, Jalal Al Ahmad Highway, Tehran, Iran

Tel. & Fax: +98-21-82884520

E-mail address: ssemnan@modares.ac.ir

Acknowledgments

This work is supported by the Iran National Science Foundation (INSF) and Faculty of Medical Sciences, Tarbiat Modares University.

Abstract

The aim of present study was to investigate the involvement of orexin-A neuropeptide in naloxone-induced morphine withdrawal syndrome via modulating neurons bearing GABA_A receptors. The locus coeruleus (LC) is a sensitive site for expression of the somatic aspects of morphine withdrawal. Intra-LC microinjection of GABA_A receptor agonist attenuates morphine withdrawal signs in rats. Here we studied the influence of LC orexin type 1 receptors blockade by SB-334867 in presence of bicuculline, a GABA_A receptor antagonist, on naloxone-induced morphine withdrawal syndrome.

Adult male Wistar rats, weighing 250–300 g, were rendered dependent on morphine by subcutaneous (s.c.) injection of increasing morphine doses (6, 16, 26, 36, 46, 56 and 66 mg/kg, 2 ml/kg) at set intervals of 24 h for 7 days. On 8th day, naloxone (3 mg/kg, s.c.) was injected and the somatic signs of morphine withdrawal were evaluated. Intra-LC microinjections (0.2 µl) of either bicuculline (15 µM) or SB-334867 (3 mM) or a combination of both chemicals were done immediately before naloxone injection. Intra-LC microinjection of bicuculline (15 µM) had no significant effect on morphine withdrawal signs, whereas intra-LC microinjection of SB-334867 considerably attenuated morphine withdrawal signs. However, the effect of SB-334867 in attenuating naloxone-induced morphine withdrawal signs was blocked in presence of bicuculline. This finding, for the first time, indicated that orexin-A may participate in expression of naloxone-induced morphine withdrawal syndrome partly through decreasing the activity of neurons bearing GABA_A receptors.

Keywords: Orexin-A; orexin type 1 receptor; GABA_A receptor; locus coeruleus; morphine withdrawal syndrome

1. Introduction

Prolonged exposure to opioid receptor agonists, like morphine, leads to the development of physical dependence that is characterized by withdrawal signs [1-3]. Opiate withdrawal syndrome is a multifaceted phenomenon involving various areas of the brain, such as the locus coeruleus, periaqueductal gray and ventral tegmental area [4-8]. The locus coeruleus (LC) is a sensitive site for expression of opiate withdrawal signs [6]. Previous studies have demonstrated that the LC neuronal excitability contributes to the physical aspects of opiate addiction, namely, physical dependence and opiate withdrawal syndrome [7-9]. LC nucleus is potently inhibited by the GABAergic system [10], most likely via selective GABA_A receptors

[11]. It is shown that the fast inhibitory effect of GABAergic system on LC neurons is primarily mediated via GABA_A receptor [12-14]. Previous studies have shown that infusion of muscimol, a GABA_A receptor agonist, attenuates naloxone-induced withdrawal signs [15-17].

It has been implicated that orexin neuropeptides are neurotransmitters playing an important role in addiction [7,18,19]. Orexin neuropeptides, (orexin-A and -B) [20,21], activate two distinct G-protein-coupled receptors, orexin type 1 (OX1R) and orexin type 2 (OX2R) receptors [20]. Orexin neuropeptides are exclusively expressed in the lateral hypothalamus (LH), and send dense projections to various parts of the brain such as LC nucleus [22,23]. There are some evidence suggesting that the blockade of OX1Rs in LC attenuates somatic morphine withdrawal signs in rats [2,17,24]. Overall, these data point to a major role of orexinergic system in modulating responses to morphine.

Several lines of evidence suggest that orexinergic system effect on GABAergic system in some of functions such as motor activity [25], reward [26], feeding [27,28], sleep/wakefulness [29-31] and antinociception [32]. Numerous convincing evidence have also implicated that orexin-A modulates GABA_A receptors activity in certain brain areas. Orexin-A decreases GABAergic inhibitory postsynaptic currents (IPSCs) by inhibiting evoked GABA release in periaqueductal gray [33]. The effect of OX1R on GABAergic system has not been studied yet in morphine withdrawal syndrome within the LC region [34-35].

However the activity of the orexinergic system increases in naloxone-induced withdrawal syndrome [2,18]. As aforementioned, intra-LC infusion of GABA_A receptor agonist attenuates naloxone-induced withdrawal signs [15,17]. Thus, it is probable that orexinergic system excites LC neurons during naloxone-induced morphine withdrawal through inhibiting GABAergic system. Therefore, it was hypothesized that OX1R activation may play an important role in naloxone-induced morphine withdrawal syndrome by reducing GABAergic transmission. To verify this hypothesis, we investigated the effect of OX1R blockade using SB-334867 in presence of bicuculline, a GABA_A receptor antagonist, on the expression of naloxone-induced morphine withdrawal signs in LC.

2. Materials and methods

2.1. Animals

62 male Wistar rats weighing between 250–300 g were used (Pasteur Institute of Tehran). Animals received water and regular rat chow ad libitum and were housed under climate-

controlled conditions with a 12 h light/dark cycle (the light period started at 7 a.m.). In order to reduce animals' stress, rats were gently handled for several days prior to the experiments. Attempts were made to diminish the number of animals used and any possible discomfort. All procedures were approved by the "Ethical Committee of Faculty of Medical Sciences, Tarbiat Modares University", which are based on the "NIH Guide for the Care and Use of Laboratory Animals". In order to avoid the bias of circadian rhythms, all experiments were done at 10:00 a.m. to 2:00 p.m. The same experimenter performed all experiments.

2.2. Drugs

The chemicals used in the experiments were morphine sulfate (Temad, Tehran, Iran), naloxone hydrochloride (Sigma-Aldrich, St. Louis, USA), SB-334867 (Tocris, Bristol, UK), SB-334867 vehicle (2% β -cyclodextrin and 7% DMSO), ketamine (Trittau, Germany), xylazine (Alfasan, The Netherlands), bicuculline methiodide (Sigma Chemicals, St. Louis, USA) and bicuculline vehicle (aCSF; artificial cerebrospinal fluid).

2.3. Experimental groups

Animals were randomly assigned to 7 groups as follows (n=7 per group):

Group 1: morphine treated rats which received naloxone injection; Group 2: morphine treated rats which received SB-334867 (3 mM, 0.2 μ l) immediately before naloxone injection; Group 3: morphine treated rats which received SB-334867 vehicle (2% β -cyclodextrin and 7% DMSO, 0.2 μ l) immediately before naloxone injection; Group 4: morphine treated rats which received bicuculline (15 μ M, 0.2 μ l) immediately before naloxone injection; Group 5: morphine treated rats which received bicuculline vehicle (aCSF) immediately before naloxone injection; Group 6: morphine treated rats which received bicuculline and SB-334867 immediately before naloxone injection and Group 7: morphine treated rats which received bicuculline vehicle and SB-334867 immediately before naloxone injection.

2.4. Surgical procedure and induction of morphine withdrawal syndrome

Rats were anesthetized with ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg) intraperitoneally. Animals were secured in stereotaxic apparatus using blunt rodent ear bars. The incisor bar was set at -3.3 mm. Briefly, under local anesthesia with 2% lidocaine, the

skull was surgically exposed and a stainless-steel guide cannula (13 mm long, 0.65 mm outer diameter, 23 gauge needle) with a fitted stylet of equal length inside, was unilaterally (right side) implanted 1 mm above the LC to reduce cellular damage at the injection site. The cannula was stabilized using two small stainless-steel screws anchored to the skull and dental acrylic cement. The stereotaxic coordinates for LC region, obtained from the atlas of Paxinos and Watson [36], were as follows: 9.8 mm caudal to bregma, 1.3 mm lateral from the medial suture, and 7.2 mm below the skull surface. After surgery, the rats were allowed to recover for one week. Afterwards, increasing doses of morphine (6, 16, 26, 36, 46, 56 and 66 mg/kg, 2 ml/kg, s.c.) were injected during 7 days. Morphine withdrawal syndrome was induced by naloxone (3 mg/kg, s.c.) 24 h after the last morphine injection on the 8th day.

2.5. Microinjection of drugs

Morphine and naloxone were dissolved in physiological saline and injected subcutaneously. The selective OX1R antagonist, SB-334867, was dissolved in aCSF containing 2% β -cyclodextrin and 7% DMSO and then the solution was aliquoted and kept frozen (-20 °C). Bicuculline was dissolved in aCSF. The intra-LC microinjection of SB-334867 (3 mM, 0.2 μ l) and/or bicuculline (15 μ M, 0.2 μ l) were performed immediately after removing the stylet from guide cannula and lowering a stainless-steel injecting cannula down (30 gauge needle, 1 mm below the guide cannula). In the microinjection method, we used the injection cannula, which was connected to a 1 μ l Hamilton micro-syringe through polyethylene-20 tubing (PE-20) and 0.2 μ l of drug solution or vehicle was microinjected over a period of 60 s. The injection cannula was carefully removed after injection to prevent the backflow of the drugs into the injection pathway, and then the stylet was replaced. In all injections, the movement of a small air bubble into the PE-20 tube was observed to ensure the movement of fluid during the microinjection procedure. Injection intervals and drug doses were selected according to earlier studies [24, 37].

2.6. Monitoring the morphine withdrawal signs

On the 8th day, rats were kept in a plexiglass chamber for 1 h in order to get acclimatized to the new environment. After the 1 h habituation period, morphine withdrawal syndrome was induced by naloxone (3 mg/kg, s.c.). Immediately after naloxone administration, behavioral signs were monitored for 25 minutes in a transparent Plexiglas test chamber (30 cm diameter,

50 cm height). The main behavioral manifestations were defecation, head tremor, genital licking, rearing, sniffing, teeth chattering, wet-dog shake, writhing and scratching [24, 38].

2.7. Histological verification

Upon completion of experimental protocols and for confirming the localization of the implanted cannula, 0.2 μ l of pontamine sky blue (2%) [39, 40] was applied to the injection site using the same injector. Rats were profoundly anesthetized with an overdose of ketamine and xylazine and their brains were removed and fixed by immersing in 10% buffered formalin for 24 h. Under the light microscope, serial coronal sections were prepared and location of the cannula tips was confirmed by the Paxinos and Watson atlas (Fig. 1). Rats with misplaced cannula were excluded from the study.

2.8. Data analysis

All data are presented as mean $\pm$ standard error of the mean (SEM) and statistical analysis was performed using GraphPad Prism Software, version 6.0. Normality of the data distributions was evaluated by the Kolmogorov–Smirnov or the Shapiro–Wilk test. All behavioral signs apart from defecation (which was not normally distributed) were analyzed by ANOVA followed by post hoc Bonferroni test. For defecation, Kruskal–Wallis and Dunn’s test were used. The asterisks in the figures indicate the following: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

In order to precipitate morphine withdrawal syndrome, morphine dependent rats received naloxone (3 mg/kg, s.c.). Results indicates that intra-LC microinjection of bicuculline, attenuated the inhibitory effect of OX1Rs antagonist on expression of withdrawal signs in rats. Expression of withdrawal signs were statistically significant in morphine dependent rats, which had received naloxone compared to the saline treated animals (data not shown).

3.1. Effect of intra-LC microinjection of SB-334867 on naloxone-induced morphine withdrawal syndrome

To further evaluate the role of OX1R in naloxone-induced morphine withdrawal syndrome, OX1R antagonist, SB-334867 (3 mM, 0.2 μ l), was directly injected into LC immediately before induction of withdrawal by naloxone administration. As depicted in figure 2, intra-LC microinjection of SB-334867 remarkably attenuated naloxone-induced morphine withdrawal

signs. With respect to the attenuation seen overall in checked signs, rearing, sniffing and genital licking alone were lowered significantly ($p < 0.05$). Defecation, head tremor and scratching also were reduced by SB-334867 ($P < 0.01$). At least teeth chattering, wet-dog shake, writhing were significantly decreased ($P < 0.001$). Intra-LC microinjection of SB-334867 vehicle had no significant effect on naloxone-induced morphine withdrawal signs (Fig. 2).

3.2. The effect of OX1R and GABA_A receptor blockade on naloxone-induced morphine withdrawal syndrome

The effect of intra-LC microinjection of bicuculline (15 μ M, 0.2 μ l) on morphine withdrawal signs was examined. Intra-LC microinjection of bicuculline (Fig. 3) or its vehicle (graph is not shown) before naloxone had no effect on morphine withdrawal signs. As shown in figure 3, the effect of orexin-A receptor antagonist, SB-334867 (3 mM, 0.2 μ l), in presence of bicuculline (15 μ M, 0.2 μ l) on morphine withdrawal signs was examined. Intra-LC microinjection of SB-334867 in presence of bicuculline not only did not decrease the naloxone-induced morphine withdrawal signs but also increased somewhat of naloxone-induced morphine withdrawal signs. There was no significant difference between animals which received bicuculline vehicle and SB-334867 prior to naloxone injection (group 7) and animals which received SB-334867 alone (Fig. 3).

4. Discussion

In this study, it was demonstrated that orexin-A may participate in expression of naloxone-induced morphine withdrawal syndrome partly through decreasing the activity of neurons bearing GABA_A receptors. Intra-LC microinjection of SB-334867, an OX1R antagonist, significantly attenuated somatic signs of naloxone-induced morphine withdrawal syndrome. These results are fully consistent with behavioral experiments, which are in progress by our research group. In a recent study conducted by Azizi et al. [24], it was shown that OX1R blockade in LC attenuates naloxone-induced morphine withdrawal behaviors in rats. Another group of researchers such as Sharf et al. [2] demonstrated that systemic inhibition of OX1R induced by intraperitoneal injection of SB-334867 reduces morphine withdrawal syndrome. In addition, the attenuation of morphine withdrawal in orexin knockout mice showed a major functional role of orexinergic system in modulating responses to morphine [18].

We found that intra-LC microinjection of bicuculline (15 μ M), GABA_A receptor antagonist, before naloxone injection has no significant effect on morphine withdrawal signs. Aston-Jones et al. [41] have also shown that the hyperactivity of LC neurons could not be prevented by 15 μ M bicuculline after naloxone injection in morphine dependent rats. Finally, in evaluating the OX1R blockade effect with SB-334867 in presence of bicuculline, we have shown that bicuculline may prevent the orexin-A effect on naloxone-induced morphine withdrawal syndrome in LC. These results lead us to conclude that GABAergic system is involved in attenuation of naloxone-induced morphine withdrawal signs by intra-LC administration of SB-334867. Dergacheva et al. [42] have shown that the effect of orexin-A on GABA release is probably complex in different brain region. Orexin-A effect depends on the involved pathways and activated receptors.

The main role of GABA_A receptors in orexin-A signaling is speculative in the LC, because almost no research work has addressed this question. It seems that the imbalance in excitatory and inhibitory neurotransmitter levels in the LC may play an important role in the expression of naloxone-induced morphine withdrawal signs [17]. Although changes in glutamatergic receptors activity may have an important role in expression of morphine withdrawal syndrome, mediation of inhibitory neurotransmitters such as GABA_A should not be ignored in expression of morphine withdrawal syndrome. However, Borgland and colleagues showed the role of orexin-A to increase glutamate transmission is in parallel with a decrease in GABA transmission. They also have shown that morphine induces an OX1R-dependent decrease in the probability of GABA release in dopamine neurons [43].

Altogether, our results provide a new insight for understanding the involved mechanisms by which orexin-A affect the expression of morphine withdrawal signs in LC region. Based on this report, it is tempting to suggest that the orexinergic system effect on neurons bearing GABA_A receptors probably play a major role in morphine withdrawal syndrome in locus coeruleus.

5. Conclusion

This study indicates that the effect of SB-334867 in attenuating naloxone-induced morphine withdrawal signs is blocked in presence of bicuculline. This finding, for the first time, suggests that orexin-A might have an effect on GABAergic systems within the LC region and this contribution affects the expression of naloxone-induced morphine withdrawal syndrome.

Acknowledgments

This work is supported by the Iran National Science Foundation (INSF) and Faculty of Medical Sciences, Tarbiat Modares University.

References

- [1] G.F. Koob, P.P. Sanna, F.E. Bloom, Neuroscience of addiction, *Neuron* 21 (1998) 467-476.
- [2] R. Sharf, M. Sarhan, R.J. Dileone, Orexin mediates the expression of precipitated morphine withdrawal and concurrent activation of the nucleus accumbens shell, *Biol. Psychiatry* 64 (2008) 175-183.
- [3] G.K. Aghajanian, Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal response by clonidine, *Nature* 276 (1978) 186-188.
- [4] K. Rasmussen, D.B. Beitner-Johnson, J.H. Krystal, G.K. Aghajanian, E.J. Nestler, Opiate withdrawal and the rat locus coeruleus: behavioral, electrophysiological, and biochemical correlates, *J. Neurosci.* 10 (1990) 2308-2317.
- [5] M.J. Christie, J.T. Williams, R.A. North, Cellular mechanisms of opioid tolerance: studies in single brain neurons, *Mol. Pharmacol.* 32 (1987) 633-8.
- [6] R. Maldonado, L. Stinus, L.H. Gold, G.F. Koob, Role of different brain structures in the expression of the physical morphine withdrawal syndrome, *J. Pharmacol. Exp. Ther.* 261 (1992) 669-677.
- [7] S.J. Grant, Y.H. Huang, D.E.Jr. Redmond, Behavior of monkeys during opiate withdrawal and locus coeruleus stimulation, *Pharmacol. Biochem. Behav.* 30 (1988) 13-19.
- [8] R. Andrade, C.P. Vandermaelen, G.K. Aghajanian, Morphine tolerance and dependence in the locus coeruleus: single cell studies in brain slices, *Eur. J. Pharmacol.* 22 (1983) 161-9.
- [9] D.E.Jr. Redmond, J.H. Krystal, Multiple mechanisms of withdrawal from opioid drugs, *Annu. Rev. Neurosci.* 7 (1984) 443-478.
- [10] M. Ennis, G. Aston-Jones, Potent inhibitory input to locus coeruleus from the nucleus prepositus hypoglossi, *Brain Res. Bull.* 22 (1989) 793-803.
- [11] V.A. Maubecin, J.T. Williams, Developmental Changes that Regulate the Activity of Locus Coeruleus Neurons, *Tokai. J. Exp. Clin. Med.* 24 (1999) 41-51.

- [12] K. Iijima, K. Ohtomo, Immunocytochemical study using a GABA antiserum for the demonstration of inhibitory neurons in the rat locus coeruleus, *Am. J. Anat.* 181 (1988) 43–52.
- [13] M. Ennis, G. Aston-Jones, GABA-mediated inhibition of locus coeruleus from the dorsomedial rostral medulla, *J. Neurosci.* 9 (1989) 2973–2981.
- [14] I.K. Ho, H.H. Loh, E.L. Way, Pharmacological manipulation of gamma-aminobutyric acid (GABA) in morphine analgesia, tolerance and physical dependence, *Life Sci.* 18 (1976) 1111-1123.
- [15] A.O. El-Kadi, S.I. Sharif, Modification by muscimol of naloxone-precipitated withdrawal symptoms in morphine-dependent mice, *Gen. Pharmacol.* 26 (1995) 1579-1581.
- [16] M.R. Zarrindast, E. Mousa-Ahmadi, Effects of GABAergic system on naloxone induced jumping in morphine-dependent mice, *Eur. J. Pharmacol.* 381 (1999) 129-133.
- [17] I. Mirzaii-Dizgah, S.M. Karimian, Z. Hajimashhadi, E. Riahi, T. Ghasemi, Attenuation of morphine withdrawal signs by muscimol in the locus coeruleus of rats, *Behav. Pharmacol.* 19 (2008) 171-175.
- [18] D. Georgescu, V. Zachariou, M. Barrot, M. Mieda, J.T. Willie, A.J. Eisch, M. Yanagisawa, E.J. Nestler, R.J. DiLeone, Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal, *J. Neurosci.* 23 (2003) 3106-3111.
- [19] K.S. Eriksson, O.A. Sergeeva, O. Selbach, H.L. Haas, Orexin (hypocretin)/dynorphin neurons control GABAergic inputs to tuberomammillary neurons, *Eur. J. Neurosci.* 19 (2004) 1278-84.
- [20] T. Sakurai, A. Amemiya, M. Ishii, I. Matsuzaki, R.M. Chemelli, H. Tanaka, S.C. Williams, J.A. Richardson, G.P. Kozlowski, S. Wilson, J.R. Arch, R.E. Buckingham, A.C. Haynes, S.A. Carr, R.S. Annan, D.E. McNulty, W.S. Liu, J.A. Terrett, N.A. Elshourbagy, D.J. Bergsma, M. Yanagisawa, Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior, *Cell* 92 (1998) 573-585.
- [21] L. de Lecea, T.S. Kilduff, C. Peyron, X. Gao, P.E. Foye, P.E. Danielson, C. Fukuhara, E.L. Battenberg, V.T. Gautvik, F.S. Bartlett, W.N. Frankel, A.N. van del Pol, F.E. Bloom, K.M. Gautvik, J.G. Sutcliffe, The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity, *Proc. Nat. Acad. Sci. U.S.A.* 95 (1998) 322-3277.
- [22] C. Peyron, D.K. Tighe, A.N. van den Pol, L. de Lecea, H.C. Heller, J.G. Sutcliffe, T.S. Kilduff, Neurons containing hypocretin (orexin) project to multiple neuronal systems, *J. Neurosci.* 18 (1998) 9996-10015.

- [23] Z. Arihara, K. Takahashi, O. Murakami, K. Totsune, M. Sone, F. Satoh, S. Ito, Y. Hayashi, H. Sasano, T. Mouri, Orexin-A in the human brain and tumor tissues of ganglioneuroblastoma and neuroblastoma, *Peptides* 21 (2000) 565-570.
- [24] H. Azizi, J. Mirnajafi-Zadeh, K. Rohampour, S. Semnanian, Antagonism of orexin type 1 receptors in the locus coeruleus attenuates signs of naloxone-precipitated morphine withdrawal in rats, *Neurosci. Lett.* 482 (2010) 255-259.
- [25] C.M. Kotz, C. Wang, J.A. Teske, A.J. Thorpe, C.M. Novak, K. Kiwaki, Orexin-A mediation of time spent moving in rats: neural mechanisms, *Neurosci.* 142 (2006) 29-36.
- [26] G.C. Harris, M. Wimmer, G. Aston-Jones, A role for lateral hypothalamic orexin neurons in reward seeking, *Nature* 437 (2005) 556-55.
- [27] A.J. Thorpe, D.F. Doane, D.C. Sweet, J.L. Beverly, C.M. Kotz, Orexin-A in the rostromedial hypothalamic area induces feeding by modulating GABAergic transmission, *Brain Res.* 1125 (2006) 60-6.
- [28] D.M. Kokare, A.M. Patole, A. Carta, C.T. Chopde, N.K. Subhedar. GABA (A) receptors mediate orexin-A induced stimulation of food intake, *Neuropharmacol.* 50 (2006) 16–24.
- [29] M.N. Alam, S. Kumar, T. Bashir, N. Suntsova, M.M. Methippara, R. Szymusiak, D.J. McGinty, GABA-mediated control of hypocretin- but not melanin-concentrating hormone-immunoreactive neurones during sleep in rats, *J. Physiol.* 563 (2005) 569-582.
- [30] P. Henny, B.E. Jones, Innervation of orexin/hypocretin neurons by GABAergic, glutamatergic or cholinergic basal forebrain terminals evidenced by Immunostaining for presynaptic vesicular transporter and postsynaptic scaffolding proteins, *J. Comp. Neurol.* 499 (2006) 645-661.
- [31] D.C. Piper, N. Upton, M.I. Smith, A.J. Hunter, The novel brain neuropeptide, orexin-A, modulates the sleep–wake cycle of rats, *Eur. J. Neurosci.* 12 (2000) 726-730.
- [32] Y.C. Ho, H.J. Lee, L.W. Tung, Y.Y. Liao, S.Y. Fu, S.F. Teng, H.T. Liao, K. Mackie, L.C. Chiou, Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition, *J. Neurosci.* 31 (2011) 14600-10.
- [33] O. Dergacheva, K. Philbin, R. Bateman, D. Mendelowitz, Hypocretin-1 (orexin-A) prevents the effects of hypoxia/hypercapnia and enhances the GABAergic pathway from the lateral parabrachial nucleus to cardiac vagal neurons in the nucleus ambiguus, *Neurosci.* 175 (2011) 18-23.

- [34] A. Viggiano, M. Monda, A. Viggiano, F. Fuccio, B. De Luca, Extracellular GABA in the medial hypothalamus is increased following hypocretin-1 administration, *Acta. Physiol. Scand.* 182 (2004) 89-94.
- [35] T.M. Korotkova, K.S. Eriksson, H.L. Haas, R.E. Brown, Selective excitation of GABAergic neurons in the substantia nigra of the rat by orexin/hypocretin in vitro, *Regul. Pept.* 104 (2002) 83-9.
- [36] G. Paxinos, C. Watson, *The Rat Brain in Stereotaxic Coordinates*, fourth ed., Academic Press, San Diego, 1998.
- [37] Y. Mousavi , H. Azizi , J. Mirnajafi-Zadeh , M. Javan , S. Semnanian, Blockade of orexin type-1 receptors in locus coeruleus nucleus attenuates the development of morphine dependency in rats, *Neurosci. Lett.* 578 (2014) 90-4.
- [38] A. Pinelli, S. Trivulzio, Quantitative evaluation of opioid withdrawal signs in rats repeatedly treated with morphine and injected with naloxone, in the absence or presence of the antiabstinence agent clonidine, *J. Pharmacol. Toxicol. Methods.* 38 (1997) 117-31.
- [39] S.G. Walling, C.W. Harley, Locus coeruleus activation initiates delayed synaptic potentiation of perforant path input to the dentate gyrus in awake rats: a novel beta-adrenergic- and protein synthesis-dependent mammalian plasticity mechanism, *J. Neurosci.* 24 (2004) 598-604.
- [40] K. Snyder, W.W. Wang, R. Han, K. McFadden, R.J. Valentino, Corticotropin-releasing factor in the norepinephrine nucleus, locus coeruleus, facilitates behavioral flexibility, *Neuropsychopharmacol.* 2 (2012) 520-30.
- [41] A. Ivanov, G. Aston-Jones, Local opiate withdrawal in locus coeruleus neurons in vitro, *J. Neurophysiol.* 85 (2001) 2388-97.
- [42] O. Dergacheva, R. Bateman, P. Byrne, D. Mendelowitz, Orexinergic modulation of GABAergic neurotransmission to cardiac vagal neurons in the brain stem nucleus ambiguus changes during development, *Neurosci.* 209 (2012) 12-20.
- [43] C. Baimel, S.L. Borgland, Orexin Signaling in the VTA Gates Morphine-Induced Synaptic Plasticity, *J. Neurosci.* 35 (2015) 7295-303.

Figure legends:

Figure 1: Histological verification of injection site. The figure represents a photomicrograph Scan of animal's brain (300 μ m, coronal section) indicating the site of intra-LC microinjection of 0.2 μ l pontamine sky blue 2%. The stereotaxic coordinates for LC region were 9.8 mm posterior to bregma, 1.3 mm lateral to midline and 7.2 mm vertical from the Dura. LC, locus coeruleus; 4V, fourth ventricle; 1Cb, 1st cerebellar lobe; and 7n, facial nerve. Scale represents 1 mm.

Figure 2: Effect of unilateral intra-LC microinjection of SB-334867 (3 mM, 0.2 μ l) in morphine treated rats (6, 16, 26, 36, 46, 56 and 66 mg/kg/day, s.c.) on naloxone (3 mg/kg, s.c.)-induced morphine withdrawal signs (n=7 per group). Data are expressed as mean $\pm$ SEM, *P<0.05, **P<0.01 and ***P<0.001. Rats which received SB-334867 before naloxone were compared with the ones pretreated with SB-334867 vehicle (SB Veh) and naloxone (Nal).

Figure 3: Effect of co-administration of SB-334867 (3 mM, 0.2 μ l) with bicuculline (15 μ M, 0.2 μ l), in morphine treated rats (6, 16, 26, 36, 46, 56 and 66 mg/kg/day, s.c.) on naloxone (3 mg/kg, s.c.) induced morphine withdrawal signs (n=7 per group). Data are expressed as mean $\pm$ SEM, *P<0.05 and **P<0.01. Rats were pretreated with SB-334867 vehicle (SB Veh) prior to naloxone (Nal). #P<0.05, ##P<0.01 and ###P<0.001 represent differences from ones receiving bicuculline vehicle along with SB-334867 (Bic Veh + SB) prior to naloxone.

Figure 1

Figure 2

Figure 3