Pharmacology, Biochemistry and Behavior 185 (2019) 172761

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Intra-accumbal orexin-1 receptor inhibition prevents the anxiolytic-like effect of ethanol and leads to increases in orexin-A content and receptor expression

Morales-Mulia Marcela*

Bases Moleculares de las Adicciones, Subdirección de Investigaciones Clínicas, INPRFM, México D.F., Mexico

ARTICLE INFO

Keywords:
Orexin-A
Orexin-1-receptors
Ethanol
Anxiety
SB-334867
Nucleus accumbens

ABSTRACT

Alcohol use is frequently associated with mood disorders. Similarly, individuals suffering from these disorders have a higher risk of developing alcoholism. Several reports have implicated orexin signaling in different behaviors related to alcohol consumption, whereas antagonists block these actions. However, the involvement of orexin-1-receptor (Orx_1R) in ethanol-induced anxiolysis remains relatively unexplored. The purpose of this study was to investigate whether intra-accumbal inhibition of Orx_1R blocks the anxiolytic-like effect of ethanol and to determine if ethanol administration modifies orexin-A content and Orx_1R expression in the nucleus accumbens (NAc). The elevated-plus-maze test (EPM-test) was used to measure anxiety; orexin-A content and Orx_1R expression were determined by enzyme-immunoassay and western blot, respectively. The results showed that the pretreatment with a selective antagonist of Orx_1R, SB-334867 (SB, 3 µg/side), prevents the anxiolytic-like behavior induced by acute ethanol (2.5 g/kg). SB-334867 per se had no effect on anxiety levels. Pretreatment with SB-334867 followed by ethanol (SB + Et) increased orexin-A content and Orx_1R levels in the NAc in comparison to the groups that only received ethanol (V + Et) or SB-334867 (SB + S). Ethanol treatment significantly augmented Orx_1R expression but not the peptide content. The increase in orexin-A observed in SB + Et animals could be due in part to the inhibition of Orx_1R, since SB-334867 prevents the binding of orexin-A to the receptor. This increase in orexin-A may, in turn, induce an up-regulation of receptor.

Other possible explanations were discussed. In general, these findings suggest that orexin-A contributes largely to expression of ethanol-induced anxiolytic-like effect through the signaling of Orx_1R in the NAc.

1. Introduction

Stress, anxiety, and mood disorders can increase susceptibility to prolonged alcohol consumption. In fact, subjects with stress-related disorders may be more vulnerable to the anxiolytic effects of ethanol in an attempt to attenuate the negative affective symptoms than subjects who show little stress or low levels of anxiety. Therefore, motivation plays a critical role in the initiation and escalation of ethanol intake. It is known that orexin peptides modulate neuronal-activity and arousal-related processes of both negative and positive emotional valence. Besides, they regulate motivated and reward-seeking behaviors (España, 2012; Mahler et al., 2012; Mieda and Sakurai, 2012), such as ethanol seeking and ethanol self-administration (Brown and Lawrence, 2013).

The orexin-A and orexin-B peptides (or hypocretin-1 and hypocretin-2, respectively) are synthesized exclusively in the lateral hypothalamus and adjacent perifornical area (LH/PFA) (de Lecea et al., 1998; Sakurai et al., 1998). The orexins act through two G-protein-coupled receptors, the orexin-1-receptor (Orx_1R) and orexin-2-receptor (Orx_2R) (de Lecea et al., 1998; Sakurai et al., 1998; Zhu et al., 2003). Orx_2R binds both forms with equal affinity, while Orx_1R binds orexin-A with a tenfold greater affinity relative to orexin-B (Sakurai et al., 1998). Both receptors are widely expressed in the meso-accumbens system (Carelli, 2002; Koob and Bloom, 1988; Peyron et al., 1998; Sutcliffe and de Lecea, 2002; Wise and Rompre, 1989) and have been associated with drug craving, promote alcohol drinking, and relapse (Mahler et al., 2012).

Systematic administration of a selective Orx_1R antagonist, SB-334867, reduces cue-induced reinstatement of alcohol seeking behavior in alcohol-preferring rats (iP) (Lawrence et al., 2006) and stress induced

* Corresponding author at: Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Calzada México-Xochimilco 101, San Lorenzo Huipulco, Tlalpan, CP. 14370 México, D.F., Mexico.
E-mail address: mmulia@imp.edu.mx.

https://doi.org/10.1016/j.pbb.2019.172761
Received 10 May 2019; Received in revised form 14 August 2019; Accepted 15 August 2019
Available online 16 August 2019

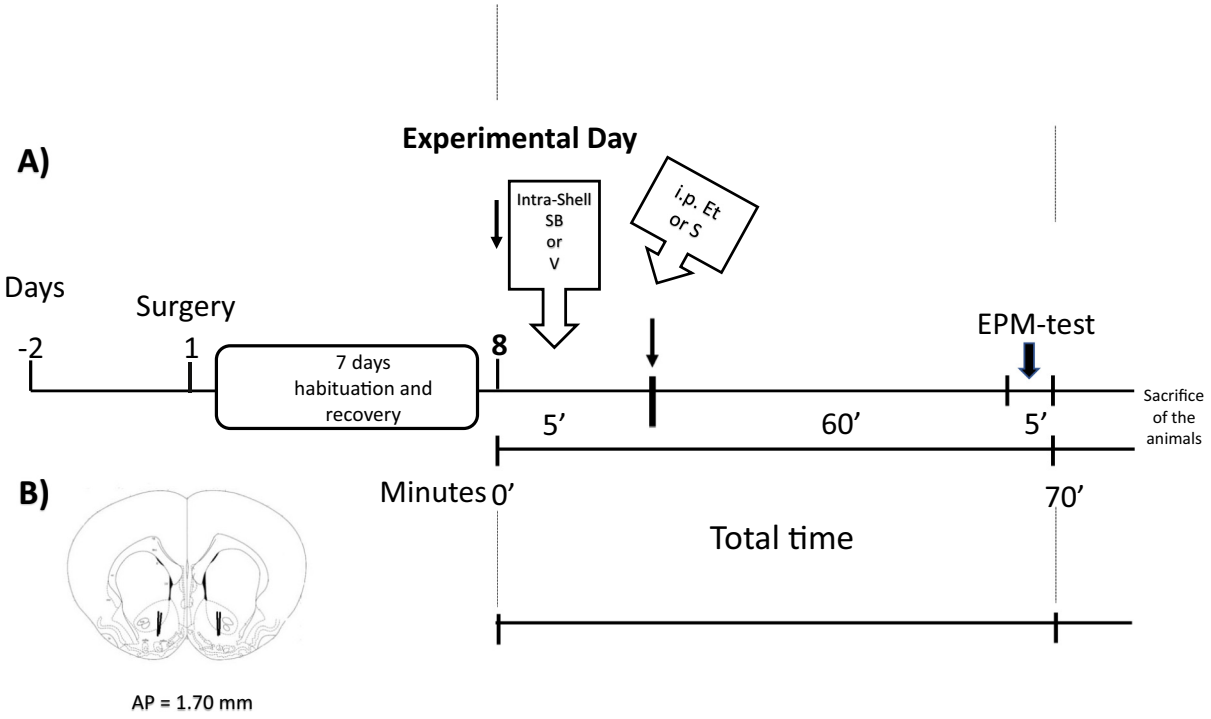

Fig. 1. Experimental protocol. (A) Timeline of experimental design. The animals underwent surgery for the implantation of the cannulae. After 7 days of surgical recovery, the animals received bilaterally the infusion of SB-334867 (SB) or saline (S) in the subregion shell of the nucleus accumbens (NAc). Five minutes later, animals received intraperitoneal (i.p.) injection of ethanol or S. Sixty minutes later, animals were exposed to the EPM-test for 5 min. At the end of the behavioral test, the animals were sacrificed and their brains were obtained. (B) Schematic representation of coronal sections from the NAc shell, coordinates: AP = +1.7 mm respective to Bregman, L = ± 1.6 mm with respect to the midline and DV = −7.8 mm from the skull surface (Paxinos and Watson, 2007).

reinstatement of ethanol self-administration in Wistar rats (Martin-Fardon and Weiss, 2012) and Long-Evans rat (Richardson et al., 2008). In addition, intraperitoneal (i.p.) injection of SB-334867 reduces progressive ratio responding for alcohol in inbred alcohol-preferring (iP) rats (Jupp et al., 2011) and ethanol self-administration in outbred rats and alcohol preferring P rats (Dhaher et al., 2010). Interestingly, SB-334867 effectively reduces the voluntary ethanol consumption in rats exposed to the paradigm of free 2-bottle choice, but only in rats that show a high preference for ethanol intake (Moorman and Aston-Jones, 2009). Overall, these data suggest that Orx_1R signaling modulates emotions associated with stress-related and addiction-related psychiatry disorders.

The meso-accumbens pathway is constituted by dopaminergic neurons that project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). The NAc consists of two anatomically, biochemically, and behaviorally distinct subregions referred as shell and core (Meredith et al., 1992). The dopaminergic pathway is critically involved in the neuronal mechanisms underlying reward-seeking and motivation through the activation of the dopamine-1-receptors (D_1R) and dopamine-2-receptors (D_2R) (Ikemoto and Panksepp, 1999). Orexin neurons send dense projections to both the VTA and the medial NAc shell (Baldo et al., 2003; Peyron et al., 1998), where both, Orx_1R and Orx_2R, are expressed (Cluderay et al., 2002; Peyron et al., 1998). The NAc shell is implicated in locomotor activity (Thorpe and Kotz, 2005) and emotional responses (Imperato et al., 1992). Central administration of orexin-A increases the firing rate of dopaminergic neurons in the VTA (Aston-Jones et al., 2009; Avolio et al., 2011; Borgland et al., 2006; Korotkova et al., 2003), as well as DA release and its metabolites in PFC, NAc shell and core (España et al., 2011; Narita et al., 2006; Vittoz and Berridge, 2006). The infusion of orexin-A also induces neuronal activation evaluated by immunohistochemical visualization of Fos, the protein product of the immediate gene c-fos, in the VTA, PFC, and NAc shell (Vittoz et al., 2008). Similarly, systemic administration of the selective D_1R-type agonist, A-77636, and the D_2R-type agonist, quinpirole, induce Fos immunoreactivity in orexin cells (Bubser et al., 2005; Estabrooke et al., 2001; Fadel et al., 2002). These effects were abolished by the co-administration of SCH23390- (D_1R-antagonist) -haloperidol (D_2R-antagonist) (Bubser et al., 2005; Estabrooke et al., 2001; Fadel et al., 2002), suggesting that the stimulation of either D_1R or D_2R receptor is enough for the activation of hypothalamic orexin neurons (Bubser et al., 2005). These data support the hypothesis that orexins exert a positive regulatory effect on the mesolimbic pathway and that DA also modulates the activity of orexin neurons.

Although there exists extensive literature on the contribution of hypothalamic orexin neurons and Orx_1R signaling in stress-induced reinstatement of ethanol-seeking behavior, little is known about their contribution to the acute effects of ethanol, specifically on its anxiolytic-like effect. Therefore, the purpose of this study was to study if Orx_1R activation in the NAc shell is required in anxiolysis induced by acute ethanol administration and to determine if ethanol treatment alters orexin-A content and expression of Orx_1R in this region.

2. Material and methods

2.1. Animals

Three-month-old male Wistar rats were single-housed on a 12 h light/dark cycle (7:00–19:00 h). Food and water were available ad libitum. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize the suffering and number of animals used (n = 7–9 animals per/group).

2.2. Drugs and treatment

The Orx_1R antagonist, SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridine-4-ylurea hydrochloride, Tocris Bioscience), in doses of 3 μg/0.5 μl/side were dissolved in 1.5% dimethyl sulfoxide (DMSO, Sigma) in 0.9% saline (vehicle). The dose of SB-334867 was chosen based on previous studies (Brown et al., 2016; Lei et al., 2016b). Ethanol (2.5 g/kg b.w.) or saline were i.p. administrated. This dose of ethanol was used according to our previous study in which we characterized the anxiolytic effect of ethanol; this effect is observed 60 min after drug-treatment (Morales-Mulia et al., 2012). Ethanol solution (31.5%, v/v) was prepared by diluting 2.5 g ethanol in distilled water to a final volume of 10 ml. Five minutes prior to ethanol (Et) or saline (S) administration, SB-334867 (SB) or vehicle (V) were injected bilaterally in the NAc/shell (Fig. 1A, B). Animals were randomly assigned to four groups: (1) V + S, (2) V + Et, (3) SB + S, and (4) SB + Et.

2.3. Elevated plus maze-test (EPM-test)

Briefly, the EPM-test consists of an elevated (40 cm above the floor) plus-shaped maze placed in a room illuminated by a 40-W red light bulb. The maze comprises four perpendicular arms, 50 cm long and 10 cm wide. Two opposing arms are surrounded by 40 cm high, white, opaque, plastic walls (closed arms), while the open arms lack walls. The animal was placed in the center of the maze facing an open arm. An entry into an arm was determined when the animal placed all four paws inside this part. The cumulative time spent in the open arms, the number of entries made into the open arms, and the total number of entries were video-recorded over one 5-min session with a digital camera. Data are expressed as the percentage of the total time spent in the open arms, total number of entries into the open arms (these two parameters reflect anxiety levels), percentage of the total time spent in the closed arms, and total number of entries. The latter is a measure from the exploratory behavior of animals. After testing each animal, the apparatus was cleaned to prevent olfactory cues from affecting the behavior of the subsequently tested rats. All experiments were performed between 9:00–13:00 h, when the animals show low levels of waking (light-period) since wake-promoting actions of orexin-A do not differ substantially across the circadian-cycle (España et al., 2002; Magdaleno-Madrigal et al., 2019).

2.4. Stereotaxic surgery

The rats were anesthetized with ketamine (0.45 ml/kg) and xylazine (0.2 ml/kg) and placed in a stereotaxic instrument (TSE, Systems Germany). Coordinates for NAc shell were: AP = 1.7 mm from Bregman, L = ± 1.6 mm with respect to the midline, and DV = −7.8 mm from the skull surface (Paxinos and Watson, 2007) (Fig. 1B). Vehicle or SB-334867 infusion was applied through a cannula attached to a Hamilton syringe (0.1 μl/min), the cannula was left for 2 min before removal to prevent drug or vehicle to be drawn back by capillarity. The drill hole was covered with bone wax after the administration of antibiotic powder (sulfatiazol®), and the skin was sutured.

2.5. Tissue collection and sample preparation

Frozen brains were coronally sliced by hand between 2.3 and 1.3 mm from Bregman, according to Paxinos and Watson (2007). Subsequently, a square of tissue (1 mm^2) containing the anterior commissure was obtained from the right hemisphere for the determination of orexin-A content by immunoassay and from the left hemisphere for the Orx_1R expression by western blot. Briefly, tissues were sonicated for 10–15 s in 250 μl of homogenization buffer (HB) containing 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5% Triton x-100, 1% NP40, 0.1% β-mercaptoethanol, proteinase (complete mini; Roche Indianapolis, IN, USA), and phosphatase inhibitors (PhosphosStop; Roche Indianapolis, IN, USA). The homogenate was centrifuged at 12,000 rpm for 30 min at 4 °C; the pellet containing mainly nuclei and large debris was discarded. The supernatant was again centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatant was used to measure the protein for western blot.

2.6. Orx_1R expression

Protein concentration was determined with the BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific) and finally quantified to a concentration of 1 μg/μl. Protein samples of 20 μg were resolved by 10% sodium duodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio RAD). The membranes were blocked with 3% skimmed milk in Tris-buffered saline (TBS) for 90 min at room temperature (RT) and then incubated with primary antibody to Orx_1R (E-9: sc-166111, 1:500; Santa Cruz Biotecnology) or β-actin (C4: sc-47778, 1:800; Santa Cruz Biotecnology). After three washes, membranes were incubated with goat anti-mouse IgG-HRP conjugated antibody (sc-2005, 1:1000; Santa Cruz Biotecnology) for 90 min at RT. Blots were washed again. Immunoreactive bands were detected using the Western blotting Chemiluminiscence Luminol Reagent (Santa Cruz Biotechnology). Immunoblots were scanned and quantification was performed with Molecular Image® ChemiDoc™ XRS + with Image Lab™ Software. The amount of protein blotted onto each lane was normalized to levels of β-actin and its corresponding total protein (Marino-Crespo et al., 2017; Zhang et al., 2016).

2.7. Orexin-A content

Orexin-A content was determined with an extraction free EIA kit Protocol for rat (Phoenix Pharmaceuticals, Inc.); sensitivity: 0.13 ng/ml, inter- and intra-assay variation: < 15% and < 10%, respectively. The homogenates were diluted 1:1 with diluent buffer prior to performing the assay.

2.8. Data analysis

All data were analyzed with SPSS Statistics 21 and expressed as the mean ± SEM. A factorial ANOVA was used, followed by the Bonferroni post hoc test to analyze the behavior, orexin-A content, and Orx_1R expression. The level of significance was established at $p < 0.05$.

3. Results

3.1. Orx_1R antagonist blocks the anxiolytic-like behavior induced by acute ethanol administration

The anxiolytic effect was analyzed 1 h after ethanol administration in animals that performed the EPM-test (Morales-Mulia et al., 2012). To determine if Orx_1R plays a key role in this behavior, a selective Orx_1R antagonist, SB-334867 (SB, 3 μl/side) was infused 5 min before ethanol injection (2.5 g/kg, i.p.) (Fig. 1A, B). Factorial ANOVA analysis data revealed a significant increase (194%, $p < 0.0001$) in the time spent in open arms in the ethanol group (V + Et) compared to the control group (V + S) (Fig. 2A). No anxiolytic-like effect was observed in the other experimental groups (Fig. 2A). In contrast, SB-334867 pretreatment prevented the increase (50%, $p < 0.005$) in the time spent in open arms induced by ethanol in the SB + Et group (Fig. 2A). In addition, a significant reduction (47%, $p < 0.012$) was observed between the V + E and SB + S groups (Fig. 2A). Statistical analysis of the time spent in closed arms showed that the treatment with ethanol (V + Et) reduced the time in closed arms by 52% ($p < 0.0001$), compared to the V + S group (Fig. 2B). On the other hand, significant reductions were observed between the SB + S vs. V + Et (106.7%, $p < 0.0001$) and SB + Et vs. V + Et (90.9%, $p < 0.001$) groups (Fig. 2B). Factorial

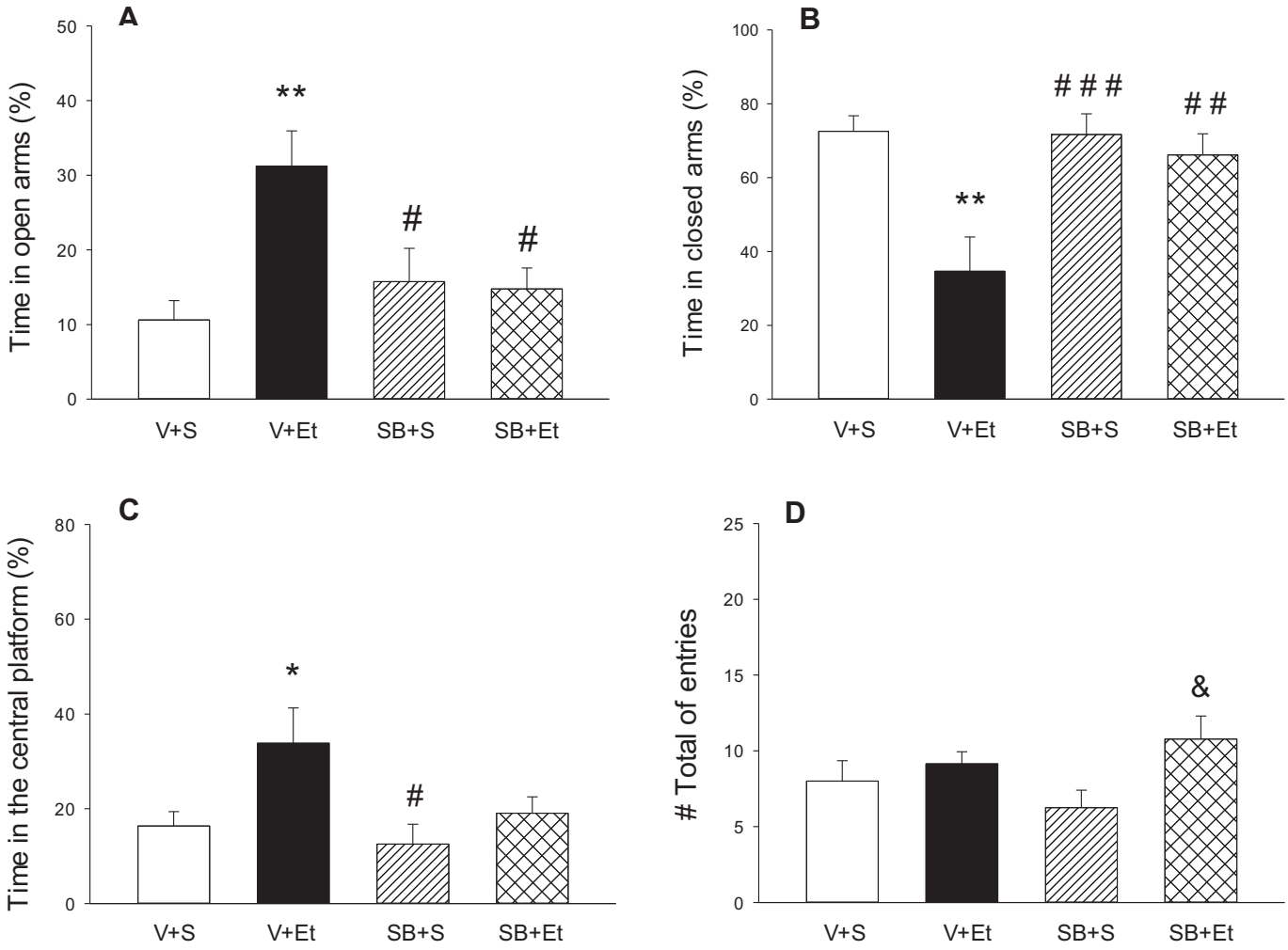

Fig. 2. Bilateral infusion of SB-334867 into the nucleus accumbens blocked the anxiolytic-like behavior induced by ethanol. Animals were subjected to the elevated plus maze test (EPM-test) 60 min after receiving a single dose of ethanol (2.5 g/kg b.w., i.p.) or saline. (A) Percentage of time spent in the open arms, (B) percentage of time spent in the closed arms, (C) percentage of time in the central platform, (D) number total of entries/5 min. Data are expressed as the percentage of time spent in the open arms, in the closed arms or in the central platform vs. control (total time spent in the three compartments = 100%); data are the mean ± SEM of 7–9 animals. *$p < 0.050$, **$p < 0.0001$ vs. V + S; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, $^{\#\#\#}p < 0.0001$ vs. V + Et; $^{\&}p < 0.050$ vs. SB + S.

ANOVA analysis data revealed a significant increase (107%, $p < 0.026$) in the time spent in central platform between the V + S and V + Et groups (Fig. 2C). In contrast, a significant reduction (63%, $p < 0.004$) in the time spent in central platform was observed between the V + Et and SB + S groups (Fig. 2C). SB-334867 followed by ethanol injection (SB + Et) increased by 72.4% ($p < 0.044$) the number of total entries to the arms in comparison to the SB + S group (Fig. 2D). However, ethanol treatment did not alter the total number of entries (Fig. 2D). These results indicate that the increase in the time spent in open arms by animals treated with ethanol was not due to an increase in locomotor activity. Moreover, SB-334867 per se (SB + S group) did not alter the total number of entrances to the arms (Fig. 2D).

3.2. Pretreatment with SB-334867 followed by ethanol administration increased orexin-A content in NAc

Factorial ANOVA analysis data showed a significant increase (202%, $p < 0.001$) in orexin-A content in the NAc in animals that received pretreatment with SB-334867 followed by the ethanol group (SB + Et) in comparison to saline group (V + S) (Fig. 3). Animals pretreated with SB-334867 and ethanol (SB + Et) also exhibited a significant increase (135%, $p < 0.005$) in orexin-A compared to the ethanol group (V + Et) (Fig. 3). In contrast, ethanol administration (V + Et) was insufficient to significantly increase (29%, p < n.s.) the orexin-A content in the NAc in relation to the V + S group. On the other hand, SB per se (SB + S group) did not significantly augment the concentration of orexin-A in comparison to the control (V + S, 92.5%, p < n.s.) or ethanol (V + Et, 49.5%, p < n.s.) or SB + Et (36.4%, p < n.s.) groups (Fig. 3).

3.3. Pretreatment with SB-334867 followed by ethanol administration increased Orx_1R expression in NAc

Factorial ANOVA analysis data reveled a significant increase (2.08-fold, $p < 0.050$) in Orx_1R expression in animals treated with ethanol (V + Et) in comparison to the control (V + S) (Fig. 4A, B). SB-334867 infusion followed by acute ethanol administration (SB + Et) also significantly increased Orx_1R levels by 3.6-fold over control group (SB + Et vs. V + S, $p < 0.0001$) (Fig. 4A, B). In addition, significant rises were observed between V + Et in relation to SB + Et (1.7-fold, $p < 0.001$), as well as between SB + S and SB + Et (3.4-fold, $p < 0.0001$) (Fig. 4A, B). On the other hand, SB-334867 per se (SB + S group) had no effect on protein levels compared to the control (V + S, p < n.s.) or ethanol (V + Et, p < n.s.) groups. (Fig. 4A, B).

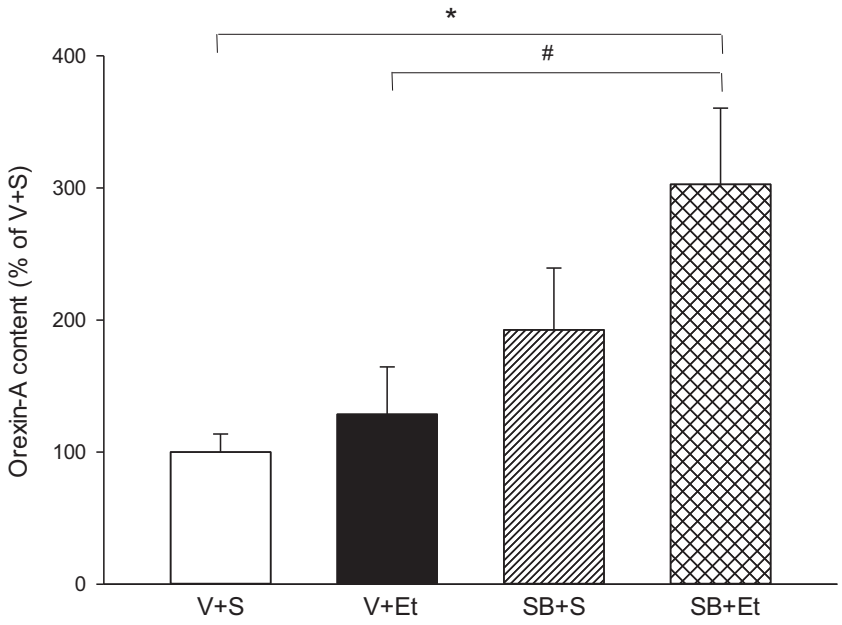

Fig. 3. Pretreatment with SB-334867 followed by ethanol administration increased orexin-A content in the NAc. Animals were subjected to EPM-test 60 min after receiving a single dose of ethanol (2.5 g/kg b.w., i.p.) or saline. Orexin-A content was determined by EIA. Data are expressed as the percentage of control values (V + S = 100%); data are the mean ± SEM. *p < 0.001 vs. V + S; #p < 0.005 vs. V + Et. Orexin-A content: V + S = 0.532 ± 0.07 ng/ml; V + Et = 0.684 ± 0.19 ng/ml; SB + S = 1.025 ± 0.24 ng/ml; SB + Et = 1.610 ± 0.30 ng/ml.

3.4. Correlations

We performed a correlation followed by a linear regression analysis of receptor expression and the time spent in open arms in the EPM test in the SB + Et and V + Et groups. An inverse relationship was found between the up-regulation of Orx_1R and time in open arms in the SB + Et group ($p < 0.0455$, $r = -0.676$) (Fig. 4C), which indicates dependence between the up-regulation of the receptor and the lack of anxiolytic effect induced by ethanol. In contrast, there was no significant correlation between Orx_1R levels and the time spent in closed arms in SB + Et animals. In addition, no significant correlations were found between Orx_1R up-regulation and the time spent in the open or closed arms in the V + Et group.

4. Discussion

Several studies have revealed the contribution of orexins in stress-induced ethanol intake behaviors (Amodeo et al., 2018; Barson and Leibowitz, 2017; Greenwald, 2018; James et al., 2017; Lawrence et al., 2006; Martin-Fardon and Weiss, 2012; Mendoza-Ruiz et al., 2018; Moorman, 2018; Olney et al., 2017; Richardson et al., 2008; Summers et al., 2018). However, there is no evidence supporting the involvement of orexins in the behavioral effects elicited by acute ethanol administration. Given that a dysfunction in orexins signaling through Orx_1R could represent an important and interesting mechanism that promotes the initiation of ethanol intake, mainly in subjects suffering from disorders related to stress and anxiety, the proposal of this study was to study, on one side, if the activation of Orx_1R in the NAc shell is required for the expression of the anxiolysis induced by acute ethanol administration, and, on the other, to determine if ethanol exposure alters orexin-A content and Orx_1R expression in this region. These results are the first to demonstrate that the blocking of Orx_1R in the NAc shell prevented the ethanol-induced anxiolysis, suggesting that accumbal-orexins system plays a dynamical role in acute rewarding aspects of ethanol. Recent studies have implicated orexins and Orx_1R in stress, anxiety, and fear (Arendt et al., 2014; Flores et al., 2014; Johnson et al., 2012, 2015; Li et al., 2010a, 2010b; Staton et al., 2018; Steiner et al., 2012; Summers et al., 2018). The role of these peptides in anxiety is not entirely clear, and there are, in fact, inconsistencies and/or negative findings. Biochemical evidences have demonstrated that the i.c.v. injection of orexins activates the hypothalamic-pituitary-adrenal axis (HPA) and produces sympathetic stimulation (Hagan et al., 1999; Ida et al., 1999; Kayaba et al., 2003; Kuru et al., 2000; Sakamoto et al., 2004; Shirasaka et al., 1999). At a behavioral level, orexins stimulate behaviors related to acute stress, such as grooming, face washing, and burrowing (Ida et al., 1999), and anxiogenic-like effects (Avolio et al., 2011; Li et al., 2010a, 2010b; Suzuki et al., 2005); however, orexin-A infusion does not always induce anxiogenic-like responses (Magdaleno-Madrigal et al., 2019; Suzuki et al., 2005). We previously showed that the i.c.v. administration of orexin-A only keeps the animals awake and alert without changes in anxiety levels, simultaneously evaluated by EPM-test and electroencephalographic-activity (EEG) (Magdaleno-Madrigal et al., 2019). On the other hand, increases in orexins mRNA levels have been observed in animals exposed to different stressors, such as immobilization (Ida et al., 2000), cold stress (Ida et al., 2000), or hypoglycemia (Griffond et al., 1999). In general, these data suggest that orexins respond mainly to conditions that require a high degree of alertness and arousal and only to certain stressful conditions.

The results of this study revealed that the Orx_1R activity is an essential component in the mechanisms underlying the positive effects of acute ethanol consumption. In addition, the effect of SB was specific on the ethanol group, since animals treated only with antagonists did not show changes in their anxiety baseline levels, as it has been reported by other authors (Rodgers et al., 2013; Scott et al., 2011; Staples and Cornish, 2014). Future studies are required to explorer if a dysregulation in Orx_1R signaling in the NAc shell could be a risk factor that generates resistance to the positive reinforcement effects of alcohol intake and if it subsequently promotes the continuous, prolonged, and excessive alcohol drinking in subjects suffering from mood disorders. Supporting this proposal is the work conducted in mice lacking the orexin-1-receptor ($Orx_1R-/-$ mice) (Abbas et al., 2015). These mice showed altered depression-like behavior, increased anxiety-like behavior, impairment of sensorimotor gating, abnormal social behavior, and decreased locomotor activity compared with the wild-type control mice (Abbas et al., 2015). Therefore, an altered activity of Orx_1R in subjects suffering from mood disorders may be a trigger that stimulates the consumption of ethanol with the purpose of counteracting the negative affective symptoms.

Moreover, we also evaluated whether acute ethanol treatment changes the orexin-A content in the NAc. Our results indicated that a dose of ethanol was insufficient to significantly alter the orexin-A concentration after 1 h of the ethanol injection. This finding contrasts

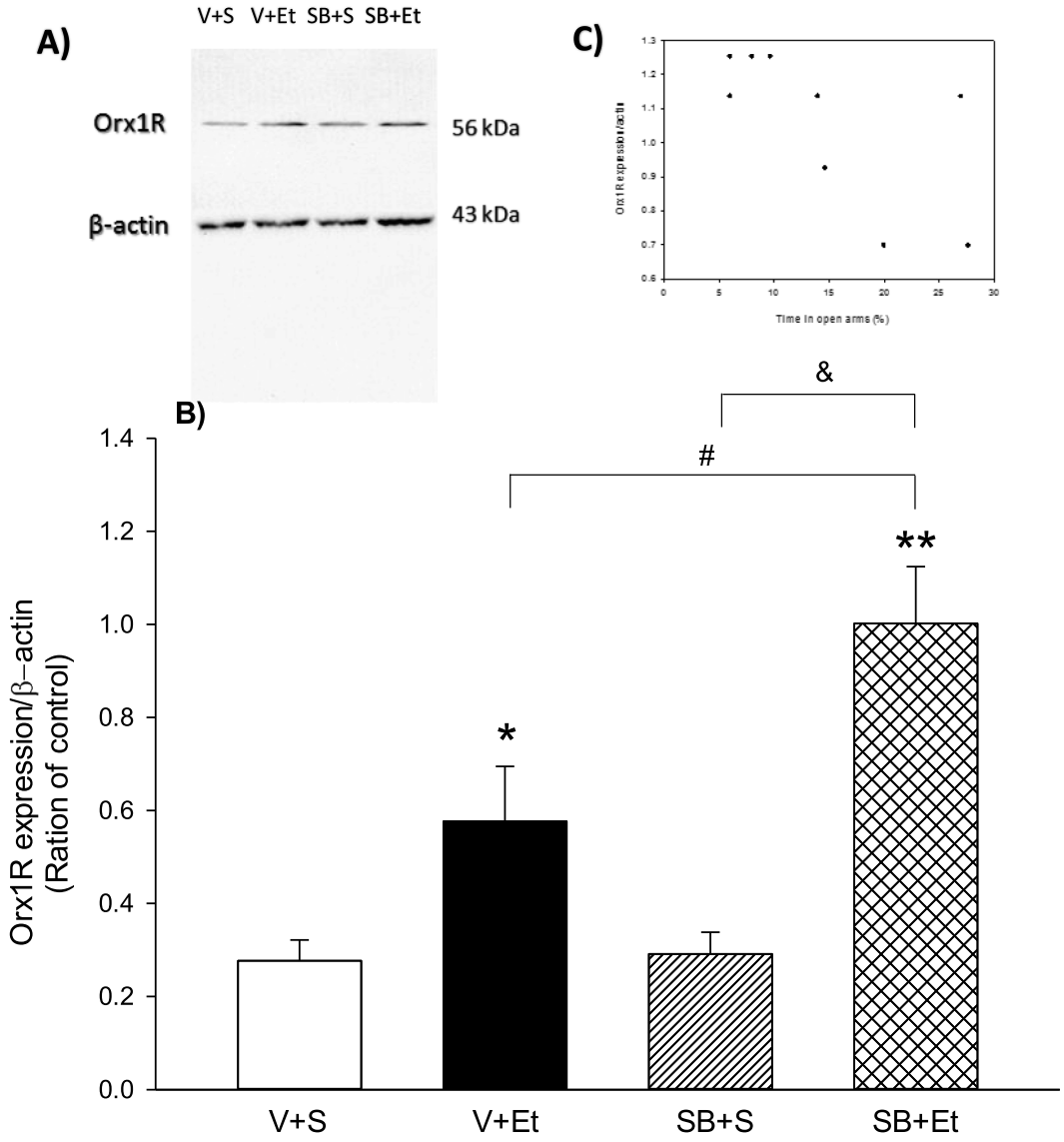

Fig. 4. Pretreatment with SB-334867 followed by ethanol administration increased the Orx_1R expression in the NAc. Animals were subjected to EPM-test 60 min after receiving a single dose of ethanol (2.5 g/kg b.w., i.p.) or saline. Orx_1R expressions were determined by western blot. (A) Immunodetection of Orx_1R and β-actin levels. (B) Increase in Orx_1R expression with respect to V + S group. (C) Pearson correlation between the time spent in open arms vs. Orx_1R expression in SB + Et group. Data are the mean ± SEM. $^{*}p < 0.001$ vs. V + S. $^{\#}p < 0.001$, $^{\#\#}p < 0.05$ vs. SB + Et.

with that reported by Morganstern et al. (2010), who showed that the same dose of ethanol (2.5 g/kg) increased orexin levels in the LH 2 h after drug treatment without changes in orexin mRNA expression. They also found that ethanol exposure at a low dose (0.75 g/kg) stimulated orexin expression, but not peptide levels in the LH. In general, all results suggest that the stimulating effect of ethanol on orexins content seems to be dose- and time-dependent. Discrepancies observed between the study by Morganstern et al. (2010) and this work may be mainly due to three reasons. First, because orexin measurements were carried out in different brain areas (LH vs. NAc). Second, the techniques used to analyze the content of peptide were not the same (immunofluorescence histochemistry vs. immunoassay). Third, the times at which orexin were quantified were different (2 vs. 1 h). Surprisingly, pretreatment with SB followed by ethanol injection revealed a higher increase in orexin-A content in comparison to animals that received vehicle (V + S and V + E) in the NAc. A possible explanation could involve blocking Orx_1R, since the antagonist prevents the binding of orexin-A to the receptor, thus maintaining a high concentration of the peptide. In support of this, SB per se also showed a tendency to increase the orexin-A content but this was not significant in comparison with the other experimental groups.

Another possible explanation for the increase in orexin-A observed in the SB + Et group implies a positive feedback mechanism of dopaminergic projections to hypothalamic orexin neurons, since the activities of both systems are strongly influenced by each other. In support to this hypothesis, it has been shown that the systemic administration of DA receptor agonists stimulates orexin neurons, as measured by induction of Fos immunoreactivity (Bubser et al., 2005; Estabrooke et al., 2001; Fadel et al., 2002). Likewise, apomorphine-elicited Fos expression in orexin cells was blocked by the concurrent administration of D_1R and D_2R antagonists, but not by pretreatment with D_1R or D_2R antagonists administrated separately, which suggests that both receptors contribute to the activation of orexin neurons in the LH. However, the mechanism by which DA modulates hypothalamic orexin neurons is unknown. It has been proposed that DA receptor agonists indirectly activate orexin neurons, due to the almost complete lack of DA receptors expression by LH/PFA cells (Bubser et al., 2005). Therefore, afferent projections to the LH/PFA that come from different brain

regions that express DA receptors, such as NAc and VTA among others, could transsynaptically activate orexin neurons. It is known that acute ethanol induces DA release in the NAc in a dose-dependent manner by increasing the firing rate of dopaminergic neurons in the VTA (Brodie et al., 1990; Gessa et al., 1985; Yim et al., 2000). In addition, it has been proposed that amphetamine-induced activation of orexin neurons may occur secondarily to increases in extracellular DA (Fadel et al., 2002). Thus, ethanol-induced DA release might be enough to excite the hypothalamic orexin cells and subsequently increase the peptide content in the NAc.

Another probable explanation is based on the results of Morganstern et al. (2010) mentioned above, assuming that ethanol activates orexin neurons in the LH where orexin cells not only send projections to remote areas of the brain but also contribute to the local network, including the LH/PFA, where orexin neurons establish synaptic contacts with each other, in such a way that the administration of ethanol could indirectly stimulate the release of orexin in the NAc. In fact, an injection of orexin-A in LH/PFA results in an increase in Fos expression in the shell of NAc and other hypothalamic areas, suggesting that endogenous orexin release within LH/PFA has an excitatory effect, which may lead to an amplification of excitation by further activating other orexin neurons whose projections reach the shell of NAc (Mullett et al., 2000). However, future studies are necessary to elucidate the responsible mechanisms that explains this finding.

We observed increases in the Orx_1R expression in the NAc of animals treated with ethanol in presence (SB + Et) or absence of SB (V + Et). The increase in Orx_1R levels in the SB + Et group appears to be a synergistic effect between ethanol and antagonist. Orx_1R upregulation can reflect a feedback mechanism in response to the persistent increase in orexin-A content in the SB + Et group, since SB inhibits the negative regulation of the receptor by binding to its ligand. In addition, it has been reported that ethanol indirectly increases opioid receptor expression in different cell lines and this seems to be associated with the activation or inhibition of signaling cascades (Charness et al., 1983, 1986, 1993; Jenab and Inturrisi, 1997). Similarly, ethanol-induced DA release in the NAc could also contribute to the up-regulation of Orx_1R by increasing the number of receptors on the surface of the membrane, as it occurs with Na-K-ATPase (Ridge et al., 2002), where DA induces translocation of Na-K-ATPase from the cytosol to the membrane by activating protein kinase C-ε and -δ (Ridge et al., 2002). These data suggest that the amount of Orx_1R present in the surface of membrane might be regulated by different events involving the activation and/or inhibition of intracellular messengers.

Another explanation is based on the activation of Orx_2R by orexin-A, since orexin-A can bind to both Orx_1R and Orx_2R with the same affinity (Sakurai et al., Sakurai et al., 1998). Previous studies have shown that orexins can modulate the expression and content of Orx_1R and Orx_2R (Cataldi et al., 2014; Liu et al., 2015). Cataldi et al. (2014) showed that orexin-A and orexin-B treatment decreased the mRNA expression of Orx_1R and this effect was not observed in the presence specific antagonists (SB-334867, Orx_1R-antagonist and JNJ-10397049, Orx_2R-antagonist) of each receptor when administered alone, but it was abolished when both antagonists were present, indicating that both receptors are involved. In contrast, the mRNA expression of Orx_2R was significantly increased by orexin-B but not by orexin-A, an effect that was inhibited only by Orx_2R antagonist (Cataldi et al., 20,014). These results suggest that both neuropeptides can modify the expression of their cognate receptors, Orx_1R and Orx_2R. In addition, orexin-A can induce apoptosis in CHO and AR42J cells through Orx_2R (Voisin et al., 2006), it stimulates the release of amylase in AR42J cells (Harris et al., 2002) and inhibits the synthesis of cAMP in primary neuronal cultures (Urbańska et al., 2012). Therefore, orexin-A via Orx_2R may induce the up-regulation of Orx_1R in the NAc, though the precise mechanism is unknown.

Considerations. Although the role of Orx_2R in the anxiolytic-like effect of ethanol was not explored in this study, it has been shown that Orx_2R is also part of the cellular and molecular mechanisms through which orexins regulate reward processes and induce neuronal activation in the NAc shell (Mukai et al., 2009; Patyal et al., 2012), both types of orexin receptors are expressed in the NAc shell, where Orx_1R level is relatively lower than that of Orx_2R (Marcus et al., 2001; Cluderay et al., 2002; Martin et al., 2002). It has been demonstrated that orexin signaling in the NAc shell, including Orx_1R and Orx_2R, is also a part of the neural mechanism of which the NAc shell participates in stress-related behaviors. Qi et al. (2013) found that the infusion of Orx_1R or Orx_2R antagonists into the NAc shell could prevent the foot shock-induced morphine CPP reinstatement. However, they did not find a functional difference between Orx_1R and Orx_2R in foot shock-induced reinstatement of opioid-seeking at the NAc shell level since both contribute to this behavior. These data indicate that both, Orx_1R and Orx_2R, are targets for the acute effects of ethanol. Although a role for Orx_1R has been established in both ethanol reinforcement and ethanol-seeking behavior, the role of Orx_2R in these behaviors is relatively less-studied. The blockade of Orx_2R reduced (Brown et al., 2013) or not (Lei et al., 2016a) ethanol consumption, additionally, the microinjection of a specific antagonist of Orx2R, the TCS-OX2-29 into core, but not shell, decreased the response to ethanol, but not cue-conditioned ethanol-seeking (Brown et al., 2013). Therefore, it is speculated that both receptors differentially modulate the mechanisms related to the reinforcing properties of ethanol consumption. For example, Barson et al. (2014) found that the injection of orexin-A and orexin-B in the anterior thalamic paraventricular nucleus (aPVT) increased ethanol consumption and this was blocked only by injection of the Orx_2R antagonist (TCS OX2 29) but not the Orx_1R antagonist (SB-334867). In addition, ethanol drinking increased orexin precursor mRNA levels in the PF/LH and Orx_2R mRNA expression but not Orx_1R in the aPVT (Barson et al., 2014). Similarly, ethanol gavage increased double-labeling of c-Fos with Orx_2R but not Orx_1R in the aPVT (Barson et al., 2014). Future studies are needed to determine the differential modulation exerted by ethanol on the orexins transmission through the signaling pathways of their receptors in the NAc and aPVT.

In conclusion, the present study suggests that the anxiolytic-like effect induced by acute ethanol administration requires the entire activation of Orx_1R in the NAc. In addition, a decrease in the signaling pathway of Orx_1R can cause a large increase in orexin-A levels that could later lead to excessive alcohol consumption. These data suggest that the orexin system remains an excellent target to create new drugs for clinical treatment in addiction to alcohol.

Declaration of competing interest

The author declares that she has no conflicts of interest.

Acknowledgements

This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT 241216) and the Instituto Nacional de Psiquiatría Ramón de la Fuente Muñíz (IC16033.0).

References

Abbas, M., Shoji, H., Soya, S., Hondo, M., Miyakawa, T., Sakurai, T., 2015. Comprehensive behavioral analysis of male Ox1r−/− mice showed implication of orexin receptor-1 in mood, anxiety, and social behavior. Front. Behav. Neurosci. 9, 1–10. https://doi.org/10.3389/fnbeh.2015.00324.

Amodeo, L.R., Wills, D.N., Sanchez-Alavez, M., Nguyen, W., Conti, B., Ehlers, C.L., 2018. Intermittent voluntary ethanol consumption combined with ethanol vapor exposure during adolescence increases drinking and alters other behaviors in adulthood in female and male rats. Alcohol 73, 57–66. https://doi.org/10.1016/j.alcohol.2018.04.003.

Arendt, D.H., Hassell, J., Li, H.2., Achua, J.K., Guarnieri, D.J., Dileone, R.J., Ronan, P.J., Summers, C.H., 2014. Anxiolytic function of the orexin 2/hypocretin A receptor in the basolateral amygdala. Psychoneuroendocrinology 40, 17–26. https://doi.org/10.1016/j.psyneuen.2013.10.010.

Aston-Jones, G., Smith, R.J., Moorman, D.E., Richardson, K.A., 2009. Role of lateral

hypothalamic orexin neurons in reward processing and addiction. Neuropharmacology 56, 112–121. https://doi.org/10.1016/j.brainres.2009.09.106.
Avolio, E., Alò, R., Carelli, A., Canonaco, M., 2011. Amygdalar orexinergic–GABAergic interactions regulate anxiety behaviors of the Syrian golden hamster. Behav. Brain Res. 218, 288–295. https://doi.org/10.1016/j.bbr.2010.11.014.
Baldo, B.A., Daniel, R.A., Berridge, C.W., Kelley, A.E., 2003. Overlapping distributions of orexin/ hypocretin- and dopamine-ß-hydroxylase immunoreactive fibers in rat brain regions mediating arousal, motivation, and stress. J. Comp. Neurol. 464, 220–237. https://doi.org/10.1002/cne.10783.
Barson, J.R., Leibowitz, S.F., 2017. Orexin/hypocretin system: role in food and drug overconsumption. Int. Rev. Neurobiol. 136, 199–237. https://doi.org/10.1016/bs.irn.2017.06.006.
Barson, J.R., Ho, H.T., Leibowitz, S.F., 2014. Anterior thalamic paraventricular nucleus is involved in intermittent access ethanol drinking: role of orexin receptor 2. Addict. Biol. 2, 469–481. https://doi.org/10.1111/adb.12139.
Borgland, S.L., Taha, S.A., Sarti, F., Fields, H.L., Bonci1, A., 2006. Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. Neuron 49, 589–601. https://doi.org/10.1016/j.neuron.2006.01.016.
Brodie, M.S., Shefner, S.A., Dunwiddie, T.V., 1990. Ethanol increases the firing rate of dopamine neurons of the rat ventral tegmental area in vitro. Brain Res. 508, 65–69. https://doi.org/10.1016/0006-8993(90)91118-z.
Brown, R.M., Lawrence, A.J., 2013. Ascending orexinergic pathways and alcohol-seeking. Curr. Opin. Neurobiol. 2, 467–472. https://doi.org/10.1016/j.conb.2013.02.014.
Brown, R.M., Khoo, S.Y., Lawrence, A.J., 2013. Central orexin (hypocretin) 2 receptor antagonism reduces ethanol self-administration, but not cue-conditioned ethanol-seeking, in ethanol-preferring rats. Int. J. Neuropsychopharmacol. 16, 2067–2079. https://doi.org/10.1017/S1461145713000333.
Brown, R.M., Kim, A.K., Khoo, S.Y., Kim, J.H., Jupp, B., Lawrence, A.J., 2016. Orexin-1 receptor signaling in the prelimbic cortex and ventral tegmental area regulates cue-induced reinstatement of ethanol-seeking in iP rats. Addict. Biol. 21, 603–612. https://doi.org/10.1111/adb.12251.
Bubser, M., Fadel, J.R., Jackson, L.L., Meador-Woodruff, J.H., Jing, D., Deutch, A.Y., 2005. Dopaminergic regulation of orexin neurons. Eur. J. Neurosci. 21, 2993–3001. https://doi.org/10.1111/j.1460-9568.2005.04121.x.
Carelli, R.M., 2002. Nucleus accumbens cell firing during goal-directed behaviors for cocaine vs. 'natural' reinforcement. Physiol. Behav. 76, 379–387. https://doi.org/10.1016/S0031-9384(02)00760-6.
Cataldi, N.I., Lux Lantos, V.A., Libertun, C., 2014. Orexin A and B in vitro modify orexins receptors expression and gonadotropins secretion of anterior pituitary cells of proestrous rats. Regul. Pept. 188, 25–30. https://doi.org/10.1016/j.regpep.2013.12.002.
Charness, M.E., Gordon, A.S., Diamond, I., 1983. Ethanol modulation of opiate receptors in cultured neural cells. Science 222, 1246–1248. https://doi.org/10.1126/science.6316506.
Charness, M.E., Querimit, L.A., Diamond, I., 1986. Ethanol increases the expression of functional delta-opioid receptors in neuroblastoma x glioma NG108-15 hybrid cells. J. Biol. Chem. 261, 3164–3169.
Charness, M.E., Hu, G., Edwards, R.H., Querimit, L.A., 1993. Ethanol increases delta-opioid receptor gene expression in neuronal cell lines. Mol. Pharmacol. 44, 1119–1127.
Cluderay, J.E., Harrison, D.C., Hervieu, G.J., 2002. Protein distribution of the orexin-2 receptor in the rat central nervous system. Regul. Pept. 104, 131–144. https://doi.org/10.1016/S0167-0115(01)00357-3.
de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L., Gautvik, V.T., Bartlett 2nd., F.S., Frankel, W.N., van den Pol, A.N., Bloom, F.E., Gautvik, K.M., Sutcliffe, J.G., 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. U. S. A. 95, 322–327.
Dhaher, R., Hauser, S.R., Getachew, B., Bell, R.L., McBride, W.J., McKinzie, D.L., Rodd, Z.A., 2010. The Orexin-1 receptor antagonist SB-334867 reduces alcohol relapse drinking, but not alcohol-seeking, in alcohol-preferring (P) Rats. J. Addict. Med. 4, 153–159. https://doi.org/10.1097/ADM.0b013e3181bd893f.
España, R.A., 2012. Hypocretin/orexin involvement in reward and reinforcement. Vitam. Horm. 89, 185–208. https://doi.org/10.1016/B978-0-12-394623-2.00010-X.
España, R.A., Plahn, S., Berridge, C.W., 2002. Circadian-dependent and circadian-independent behavioral actions of hypocretin/orexin. Brain Res. 943, 224–236. https://doi.org/10.1016/s0006-8993(02)02653-7.
España, R.A., Melchior, J.R., Roberts, D.C.S., Jones, S.R., 2011. Hypocretin 1/orexin A in the ventral tegmental area enhances dopamine responses to cocaine and promotes cocaine self-administration. Psychopharmacology 214, 415–426. https://doi.org/10.1007/s00213-010-2048-8.
Estabrooke, I.V., McCarthy, M.T., Ko, E., Chou, T.C., Chemelli, R.M., Yanagisawa, M., Saper, C.B., Scammell, T.E., 2001. Fos expression in orexin neurons varies with behavioral state. J. Neurosci. 2, 1656–1662. https://doi.org/10.1523/JNEUROSCI.21-05-01656.2001.
Fadel, J., Bubser, M., Deutch, A.Y. 2002. Differential activation of orexin neurons by antipsychotic drugs associated with weight gain. J. Neurosci. 22, 6742–6746. doi.org/https://doi.org/10.1523/JNEUROSCI.22-15-06742.2002.
Flores, Á., Valls-Comamala, V., Costa, G., Saravia, R., Maldonado, R., Berrendero, F., 2014. The hypocretin/orexin system mediates the extinction of fear memories. Neuropsychopharmacology 39, 2732–2741. https://doi.org/10.1038/npp.2014.146.
Gessa, G.L., Muntoni, F., Collu, M., Vargiu, L., Mereu, G., 1985. Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. Brain Res. 348, 201–203. https://doi.org/10.1016/0006-8993(85)90381-6.
Greenwald, M.K., 2018. Anti-stress neuropharmacological mechanisms and targets for addiction treatment: a translational framework. Neurobiol. Stress 11 (9), 84–104. https://doi.org/10.1016/j.ynstr.2018.08.003.
Griffond, B., Risold, P.Y., Jacquemard, C., Colard, C., Fellmann, D., 1999. Insulin-induced hypoglycemia increases preprohypocretin (orexin) mRNA in the rat lateral hypothalamic area. Neurosci. Lett. 262, 77–80. https://doi.org/10.1016/s0304-3940(98)00976-8.
Hagan, J.J., Leslie, R.A., Patel, S., Evans, M.L., Wattam, T.A., Holmes, S., Benham, C.D., Taylor, S.D., Routledge, C., Hemmati, P., Munton, R.P., Ashmeade, T.E., Shah, A.S., Hatcher, J.P., Hatcher, P.D., Jones, D.N., Smith, M.I., Piper, D.C., Hunter, A.J., Porter, R.A., Upon, N., 1999. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc. Natl. Acad. Sci. U. S. A. 96, 10911–10916. https://doi.org/10.1073/pnas.96.19.10911.
Harris, D.M., Go, V.L., Reeve Jr., J.R., Wu, S.V., 2002. Stimulation of amylase release by orexin is mediated by orexin 2 receptor in AR42J cells. Pancreas 25, 405–410.
Ida, T., Nakahara, K., Katayama, T., Murakami, N., Nakazato, M., 1999. Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioral activities of rats. Brain Res. 821, 526–529. https://doi.org/10.1016/s0006-8993(99)01131-2.
Ida, T., Nakahara, K., Kuroiwa, T., Fukuia, K., Nakazato, M., Murakami, T., Murakami, N., 2000. Both corticotropin releasing factor and neuropeptide Y are involved in the effect of orexin (hypocretin) on the food intake in rats. Neurosci. Lett. 293, 119–122. https://doi.org/10.1016/s0304-3940(00)01498-1.
Ikemoto, S., Panksepp, J., 1999. The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking. Brain Res. Brain Res. Rev. 31, 6–41. doi.org/https://doi.org/10.1016/S0165-0173(99)00023-5.
Imperato, A., Angelucci, L., Casolini, P., Zocchi, A., Puglisi-Allegra, S., 1992. Repeated stressful experiences differently affect limbic dopamine release during and following stress. Brain Res. 577, 194–199. https://doi.org/10.1016/0006-8993(92)90274-d.
James, M.H., Campbell, E.J., Dayas, C.V., 2017. Role of the orexin/hypocretin system in stress-related psychiatric disorders. Curr. Top. Behav. Neurosci. 33, 197–219. https://doi.org/10.1007/7854_2016_56.
Jenab, S.1., Inturrisi, C.E., 1997. Activation of protein kinase A prevents the ethanol-induced up-regulation of delta-opioid receptor mRNA in NG108-15 cells. Brain Res. Mol. Brain Res. 47, 44–48. https://doi.org/10.1016/S0169-328X(97)00061-2.
Johnson, P.L., Samuels, B.C., Fitz, S.D., Federici, L.M., Hammes, N., Early, M.C., Truitt, W., Lowry, C.A., Shekhar, A., 2012. Orexin 1 receptors are a novel target to modulate panic responses and the panic brain network. Physiol. Behav. 107, 733–742. https://doi.org/10.1016/j.physbeh.2012.04.016.
Johnson, P.L., Federici, L.M., Fitz, S.D., Renger, J.J., Shireman, B., Winrow, C.J., Bonaventure, P., Shekhar, A., 2015. Orexin a and 2 receptor involvements in CO2-induced panic-associated behavior and autonomic responses. Depress Anxiety 32, 671–683. https://doi.org/10.1002/da.22403.
Jupp, B., Krivdic, B., Krstew, E., Lawrence, A.J., 2011. The orexin1 receptor antagonist SB-334867 dissociates the motivational properties of alcohol and sucrose in rats. Brain Res. Arch. 1391, 54–59. https://doi.org/10.1016/j.brainres.2011.03.045.
Kayaba, Y., Nakamura, A., Kasuya, Y., Ohuchi, T., Yanagisawa, M., Komuro, I., Fukuda, Y., Kuwaki, T., 2003. Attenuated defense response and low basal blood pressure in orexin knockout mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285, R581–R593. https://doi.org/10.1152/ajpregu.00671.2002.
Koob, G.F., Bloom, F.E., 1988. Cellular and molecular mechanisms of drug dependence. Science 242, 715–723. https://doi.org/10.1126/science.2903550.
Korotkova, T.M., Sergeeva, O.A., Eriksson, K.S., Haas, H.L., Brown, R.E., 2003. Excitation of Ventral Tegmental Area Dopaminergic and Nondopaminergic Neurons by Orexins/Hypocretins. (f).
Kuru, M., Ueta, Y., Serino, R., Nakazato, M., Yamamoto, Y., Shibuya, I., Yamashita, H., 2000. Centrally administered orexin/hipocretins activates HPA axis in rats. Neuroreport 11, 1977–1980.
Lawrence, A.J., Cowen, M.S., Yang, H.-J., Chen, F., Oldfield, B., 2006. The orexin system regulates alcohol-seeking in rats. Brit J. Pharmacol. 148, 752–759. https://doi.org/10.1038/sj.bjp.0706789.
Lei, K., Wegner, S.A., Yu, J.H., Hopf, F.W., 2016a. Orexin-1 receptor blockade suppresses compulsive-like alcohol drinking in mice. Neuropharmacology 110 (Pt A), 431–437. https://doi.org/10.1016/j.neuropharm.2016.08.008.
Lei, K., Wegner, S.A., Yu, J.H., Mototake, A., Hu, B., Hopf, F.W., 2016b. Nucleus accumbens shell and mPFC but not insula orexin-1 receptors promote excessive alcohol drinking. Front. Neurosci. 10, 400. https://doi.org/10.3389/fnins.2016.00400.
Li, Y., Li, S., Wei, C., Wang, H., Sui, N., Kirouac, G.J., 2010a. Orexins in the paraventricular nucleus of the thalamus mediate anxiety-like responses in rats. Psychopharmacology 212, 251–265. https://doi.org/10.1007/s00213-010-1948-y.
Li, Y., Li, S., Wei, C., Wang, H., Sui, N., Kirouac, G.J., 2010b. Changes in emotional behavior produced by orexin microinjections in the paraventricular nucleus of the thalamus. Pharmacol. Biochem. Behav. 95, 121–128. https://doi.org/10.1016/j.pbb.2009.12.016.
Liu, Y., Zhao, Y., Ju, S., Guo, L., 2015. Orexin A upregulates the protein expression of OX1R and enhances the proliferation of SGC-7901 gastric cancer cells through the ERK signaling pathway. Int. J. Mol. Med. 35, 539–545. https://doi.org/10.3892/ijmm.2014.2038.
Magdaleno-Madrigal, V.M., Morales-Mulia, S., Nicolinic, H., Genis-Mendoza, A., Cázares-Martínez, Claudia E., Pérez-Luna, José M., Morales-Mulia, M., 2019. Orexin-A promotes EEG changes but fails to induce anxiety in rats. Behav. Brain Res. 361, 26–31. https://doi.org/10.1016/j.bbr.2018.12.037.
Mahler, S.V., Smith, R.J., Moorman, D.E., Sartor, G.C., Aston-Jones, G., 2012. Multiple roles for orexin/hypocretin in addiction. Prog. Brain Res. 198, 79–121. https://doi.org/10.1016/B978-0-444-59489-1.00007-0.
Marcus, J.N., Aschkenasi, C.J., Lee, C.E., Chemelli, R.M., Saper, C.B., Yanagisawa, M., Elmquist, J.K., 2001. Differential expression of orexin receptor 1 and 2 in the rat

brain. J. Comp. Neurol. 435, 6–25. https://doi.org/10.1002/cne.1190.
Marino-Crespo, O., Fernández, A., Martín, G.E., 2017. Normalización de señal en Western blotting cuantitativo. Uso de la proteína total mediante la tinción con azul brillante de Coomassie. Investigación, Cultura, Ciencia y Tecnología (ICCT) 9, 59–66.
Martin, G., Fabre, W., Siggins, G.R., and de Lecea, L., 2002. Interaction of the hypocretins with neurotransmitters in the nucleus accumbens. Regul. Pept. 104, 111–117. doi.org/https://doi.org/10.1016/S0167-0115(01)00354-8.
Martin-Fardon, R., Weiss, F., 2012. N-(2-methyl-6-benzoxazolyl)-N-1,5-naphthyridin-4-yl urea (SB334867), a hypocretin receptor-1 antagonist, preferentially prevents ethanol seeking: comparison with natural reward seeking. Addict. Biol. 19, 233–236. https://doi.org/10.1111/j.1369-1600.2012.00480.x.
Mendoza-Ruiz, L.G., Vázquez-León, P., Martínez-Mota, L., Juan, E.R.S., Miranda-Páez, A., 2018. Forced ethanol ingestion by Wistar rats from a juvenile age increased voluntary alcohol consumption in adulthood, with the involvement of orexin-A. Alcohol 70, 73–80. https://doi.org/10.1016/j.alcohol.2018.01.008.
Meredith, G.E., Agolia, R., Arts, M.P., Groenewegen, H.J., Zahm, D.S., 1992. Morphological differences between projection neurons of the core and shell in the nucleus accumbens of the rat. Neuroscience 50, 149–162. doi.org/https://doi.org/10.1016/0306-4522(92)90389-J.
Mieda, M., Sakurai, T., 2012. Overview of orexin/hypocretin system. Prog. Brain Res. 198, 5–14. https://doi.org/10.1016/B978-0-444-59489-1.00002-1.
Moorman, D.E., 2018. The hypocretin/orexin system as a target for excessive motivation in alcohol use disorders. Psychopharmacology 235, 1663–1680. https://doi.org/10.1007/s00213-018-4871-2.
Moorman, D.E., Aston-Jones, G., 2009. Orexin-1 receptor antagonism decreases ethanol consumption and preference selectively in high-ethanol-preferring Sprague Dawley rats. Alcohol 43, 379–386. https://doi.org/10.1016/j.alcohol.2009.07.002.
Morales-Mulia, M., Estrada-Camarena, E., Amaya, M.I., Mejía-Mauríes, S., Sollozo-Dupont, I., Mengod, G., de Gortari, P., 2012. Anxiolytic effects of ethanol are partially related to a reduced expression of adenylyl cyclase 5 but not to μ-opioid receptor activation in rat nucleus accumbens. Behav. Brain Res. 235, 189–194. https://doi.org/10.1016/j.bbr.2012.07.036.
Morganstern, I., Chang, G.-Q., Barson, J.R., Ye, Z., Karatayev, O., Leibowitz, S.F., 2010. Differential effects of acute and chronic ethanol exposure on orexin expression in the perifornical lateral hypothalamus. Alcohol. Clin. Exp. Res. 34, 886–896. https://doi.org/10.1111/j.1530-0277.2010.01161.x.
Mukai, K., Kim, J., Nakajima, K., Oomura, Y., Wayner, M.J., Sasaki, K., 2009. Electrophysiological effects of orexin/hypocretin on nucleus accumbens shell neurons in rats: an in vitro study. Peptides 30, 1487–1496. https://doi.org/10.1016/j.peptides.2009.04.018.
Mullett, M.A., Billington, C.J., Levine, A.S., Kotz, C.M., 2000. Hypocretin I in the lateral hypothalamus activates key feeding-regulatory brain sites. Neuroreport 11, 103–108.
Narita, M., Nagumo, Y., Hashimoto, S., Narita, M., Khotib, J., Miyatake, M., Sakurai, T., Yanagisawa, M., Nakamachi, T., Shioda, S., Suzuki, T., 2006. Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. J. Neurosci. 26, 398–405. https://doi.org/10.1523/JNEUROSCI.2761-05.2006.
Olney, J.J., Navarro, M., Thiele, T.E., 2017. The role of orexin signaling in the ventral tegmental area and central amygdala in modulating binge-like ethanol drinking behavior. Alcohol. Clin. Exp. Res. 41, 551–561. https://doi.org/10.1111/acer.13336. (Epub 2017 Feb 9).
Patyal, R., Woo, E.Y., Borgland, S.L., 2012. Local hypocretin-1 modulates terminal dopamine concentration in the nucleus accumbens shell. Front. Behav. Neurosci. 6, 82. https://doi.org/10.3389/fnbeh.2012.00082.
Paxinos, G., Watson, C., 2007. The Rat Brain in Stereotaxic Coordinates, 6th Ed. Academic Press, San Diego.
Peyron, C., Tighe, D.K., van den Pol, A.N., de Lecea, L., Heller, H.C., Sutcliffe, J.G., Kilduff, T.S., 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J. Neurosci. 18, 9996–10015. https://doi.org/10.1523/JNEUROSCI.18-23-09996.1998.
Qi, K., Wei, C., Li, Y., Sui, N., 2013. Orexin receptors within the nucleus accumbens shell mediate the stress but not drug priming-induced reinstatement of morphine conditioned place preference. Front. Behav. Neurosci. 7, 144–152. https://doi.org/10.3389/fnbeh.2013.00144.
Richardson, H.N., Lee, S.Y., O'Dell, L.E., Koob, G.F., Rivier, C.L., 2008. Alcohol self-administration acutely stimulates the hypothalamic-pituitary- adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state. Eur. J. Neurosci. 28, 1641–1653.
Ridge, K.M., Dada, L., Lecuona, E., Bertorello, A.M., 2002. Dopamine-induced exocytosis of Na,K-ATPase is dependent on activation of protein kinase C-ε and –δ. Mol. Biol. Cell 13, 1381–1389. https://doi.org/10.1091/mbc.01-07-0323.
Rodgers, R.J., Wright, F.L., Snow, N.F., Taylor, L.J., 2013. Orexin-1 receptor antagonism fails to reduce anxiety-like behaviour in either plus-maze-naïve or plus-maze-experienced mice. Behav. Brain Res. 243, 213–219. https://doi.org/10.1016/j.bbr.2012.12.064.
Sakamoto, F., Yamada, S., Ueta, Y., 2004. Centrally administered orexin-A activates corticotropin-releasing factor-containing neurons in the hypothalamic paraventricular nucleus and central amygdaloid nucleus of rats: possible involvement of central orexins on stress-activated central CRF neurons. Regul. Pept. 118, 183–191. https://doi.org/10.1016/j.regpep.2003.12.014.
Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.-M., Tanaka, H., Williams, S.C., Richarson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., Yanagisawa, 1998. Orexins and orexin receptors: a family of hypothalamic neuropéptidos and G protein-coupled receptors that regulate feeding behavior. Cell 92, 573–585. doi:https://doi.org/10.1016/s0092-8674(02)09256-5.
Scott, M.M., Marcus, J.N., Pettersen, A., Birnbaum, S.G., Mochizuki, T., Scammell, T.E., Nestler, E.J., Elmquist, J.K., Lutter, M., 2011. Hcrtr1 and 2 signaling differentially regulates depression-like behaviors. Behav. Brain Res. 222, 289–294. https://doi.org/10.1016/j.bbr.2011.02.044.
Shirasaka, T., Nakazato, M., Matsukura, S., Takasaki, M., Kannan, H., 1999. Sympathetic and cardiovascular actions of orexins in conscious rats. Am. J. Phys. 277, R1780–R1785. https://doi.org/10.1152/ajpregu.1999.277.6.R1780.
Staples, L.G., Cornish, J.L., 2014. The orexin-1 receptor antagonist SB-334867 attenuates anxiety in rats exposed to cat odor but not the elevated plus maze: an investigation of Trial 1 and Trial 2 effects. Horm. Behav. 65, 294–300. https://doi.org/10.1016/j.yhbeh.2013.12.014.
Staton, C.D., Yaeger, J.D.W., Khalid, D., Haroun, F., Fernandez, B.S., Fernandez, J.S., Summers, B.K., Summers, T.R., Sathyanesan, M., Newton, S.S., Summers, C.H., 2018. Orexin 2 receptor stimulation enhances resilience, while orexin 2 inhibition promotes susceptibility, to social stress, anxiety and depression. Neuropharmacol 143, 79–94. https://doi.org/10.1016/j.neuropharm.2018.09.016.
Steiner, M.A., Lecourt, H., Jenck, F., 2012. The brain orexin system and almorexant in fear-conditioned startle reactions in the rat. Psychopharmacology 224, 465–475. https://doi.org/10.1007/s00213-012-2736-7.
Summers, C.H., Yaeger, J.D.W., Staton, C.D., Arendt, D.H., Summers, T.R., 2018. Orexin/hypocretin receptor modulation of anxiolytic and antidepressive responses during social stress and decision-making: potential for therapy. Brain Res. https://doi.org/10.1016/j.brainres.2018.12.036. pii: S0006-8993 (18)30662-0.
Sutcliffe, J.G., de Lecea, L., 2002. The hypocretins: setting the arousal threshold. Nat. Rev. Neurosci. 3, 339–349. https://doi.org/10.1038/nrn808.
Suzuki, M., Beuckmann, C.T., Shikata, K., Ogura, H., Sawai, T., 2005. Orexin-A (hipocretins-1) is possibly involved in generation of anxiety-like behavior. Brain Res. 1044, 116–121. https://doi.org/10.1016/j.brainres.2005.03.002.
Thorpe, A.J., Kotz, C.M., 2005. Orexin A in the nucleus accumbens stimulates feeding and locomotor activity. Brain Res. 1050, 156–162. https://doi.org/10.1016/j.brainres.2005.05.045.
Urbańska, A., Sokołowska, P., Woldan-Tambor, A., Biegańska, K., Brix, B., Jöhren, O., Namiecińska, M., Zawilska, J.B., 2012. Orexins/hypocretins acting at Gi protein-coupled OX 2 receptors inhibit cyclic AMP synthesis in the primary neuronal cultures. J. Mol. Neurosci. 46, 10–17. https://doi.org/10.1007/s12031-011-9526-2.
Vittoz, N.M., Berridge, C.W., 2006. Hypocretin/orexin selectively increases dopamine efflux within the prefrontal cortex: involvement of the ventral tegmental area. Neuropsychopharmacology 31, 384–395. https://doi.org/10.1038/sj.npp.1300807.
Vittoz, N.M., Schmeichel, B., Berridge, C.W., 2008. Hypocretin/orexin preferentially activates caudomedial ventral tegmental area dopamine neurons. Eur. J. Neurosci. 28, 1629–1640. https://doi.org/10.1111/j.1460-9568.2008.06453.x.
Voisin, T., Firar, A.E., Avondo, V., Laburthe, M., 2006. Orexin-induced apoptosis: the key role of the seven-transmembrane domain orexin type 2 receptor. Endocrinology 147, 4977–4984. https://doi.org/10.1210/en.2006-0201.
Wise, R.A., Rompre, P.P., 1989. Brain dopamine and reward. Annu. Rev. Psychol. 40, 191–225. https://doi.org/10.1146/annurev.ps.40.020189.001203.
Yim, H.J., Robinson, D.L., White, M.L., Jaworski, J.N., Randall, P.K., Francine E. Lancaster, F.E., Gonzales, R.A., 2000. Dissociation between the time course of ethanol and extracellular dopamine concentrations in the nucleus accumbens after a single intraperitoneal injection. Alcohol. Clin. Exp. Res. 24, 781–788. doi.org/https://doi.org/10.1111/j.1530-0277.2000.tb02056.x.
Zhang, J., Wang, N., Chena, B., Wang, Y., He, J., Cai, X., Zhang, H., Wei, S., Li, S., 2016. Blockade of Cannabinoid CB1 receptor attenuates the acquisition of morphine-induced conditioned place preference along with a downregulation of ERK, CREB phosphorylation, and BDNF expression in the nucleus accumbens and hippocampus. Neurosci. Lett. 630, 70–76. https://doi.org/10.1016/j.neulet.2016.07.047.
Zhu, Y., Miwa, Y., Yamanaka, A., Yada, T., Shibahara, M., Abe, Y., Sakurai, T., Goto, K., 2003. Orexin receptor type-1 couples exclusively to pertussis toxin-insensitive G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and –insensitive G-proteins. J. Pharmacol. Sci. 92, 259–266. doi.org/10.1254/jphs.92.259.