HHS Public Access

Author manuscript

Psychoneuroendocrinology. Author manuscript; available in PMC 2015 March 17.

Published in final edited form as:

Psychoneuroendocrinology. 2014 February ; 40: 17–26. doi:10.1016/j.psyneuen.2013.10.010.

Anxiolytic function of the orexin 2/hypocretin A receptor in the basolateral amygdala

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Summary

The orexin/hypocretin system interacts with many of the same circuitries contributing to stress-associated disorders like depression and anxiety. These include potentially reciprocal connections with corticotropin releasing factor (CRF) neurons which drive the hypothalamic–pituitary–adrenal (HPA) endocrine response in addition to having an anxiogenic effect in the central amygdala (CeA). Antagonism of the orexin type 1 receptor (Orx_1) in the hypothalamus has also been shown to block panic attacks. However, few studies have investigated the effect of orexinergic signaling in the basolateral amygdala (BLA) which is responsible for contextual fear, and modulates the activity of the CeA. To this end, we chronically stressed c57bl/6 mice with social defeat and examined the gene expression of the orexin receptors in the BLA. We found that the transcripts for the Orx_1 and Orx_2 receptors diverged in the BLA with Orx_1 increasing and Orx_2 decreasing in animals that were susceptible to the chronic defeat. These changes were not seen in the prelimbic cortex (PrL) which sends efferents to the BLA. We then tried to recapitulate these expression patterns in the BLA using short hairpin interfering sequences delivered by adeno-associated viruses to knock down the orexin receptors. While the Orx_1 knockdown did reduce locomotor activity, it did not decrease depressive or anxious behaviors. Knocking down the Orx_2 receptors in

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Conflict of interest statement

None declared.

The contents of this paper do not represent the views of the Department of Veterans Affairs or the United States Government

the BLA increased anxious behavior as measured by reduced social preference and reduced time spent in the center of an open field. Due to the divergent expression patterns of the two receptors in response to chronic stress, orexinergic activity in the BLA may be responsible for bidirectional modulation of anxious behavior. Furthermore, these data raise the possibility that an Orx_2 agonist may serve as an effective means to treat anxiety disorders.

Keywords

Orexin; Hypocretin; Anxiety; Amygdala; Orexin 2 receptor; shRNA; Knockdown; Basolateral amygdala

1. Introduction

The orexin (hypocretin) system is involved in regulating motivated behavior and arousal (de Lecea et al., 1998; Deadwyler et al., 2007; Lin et al., 1999; Sakurai et al., 1998). The neural systems associated with these behaviors are also often affected by disorders such as anxiety and depression (Krishnan and Nestler, 2010; Pittenger and Duman, 2008; Ressler and Mayberg, 2007; Yehuda et al., 1996). Our work and that of others has shown that orexin may play a role in depression due to its involvement in states such as chronic stress and learned helplessness which appear to be part of the etiology of the disorder (Arendt et al., 2013; Lutter et al., 2008; Nollet et al., 2011; Nollet and Leman, 2013; Scott et al., 2011). In addition, orexin is involved in anxiety disorders which are highly comorbid with depression (Avolio et al., 2011; Johnson et al., 2012a,b; Kessler et al., 2003; Li et al., 2010). Most of this work examines the activity of the two heterotrimeric G protein coupled receptors of the orexin system, type 1 and type 2 (Orx_1, Orx_2; de Lecea et al., 1998; Sakurai et al., 1998). Two endogenous ligands bind to these receptors, orexin A and B (Orx_A, Orx_B). While the Orx_2 receptor binds both forms with equal affinity, the Orx_1 receptor binds Orx_A with a tenfold greater affinity relative to Orx_B (Sakurai et al., 1998).

The central (CeA) and basolateral (BLA) subnuclei of the amygdala contain orexin projections and express the transcript for orexin receptors (Marcus et al., 2001; Peyron et al., 1998). There may also be cross talk between the two systems as amygdalar subregions project to orexin neurons (Sakurai et al., 2005). Application of either Orx_A or Orx_B excites CeA neurons (Bisetti et al., 2006) and produces increased behavioral anxiety (Avolio et al., 2011). The BLA is involved in fear/anxiety as well (Barot et al., 2009; Orsini and Maren, 2012; Tye et al., 2011). Inhibition of glutamatergic signaling in the BLA blocks anxiety responses as measured by social preference (Sajdyk and Shekhar, 1997). Furthermore, upregulating CREB in the BLA, a transcription factor positively associated with LTP and memory formation (Kida, 2012), increases behavioral anxiety (Wallace et al., 2004). In addition to its role in anxiety, the BLA processes contextual information about fear stimuli which is then conveyed to the CeA (Barot et al., 2009; Orsini and Maren, 2012). Despite the BLA's role in these behaviors, little is known about orexinergic signaling in this area and its impact on anxiety. In the past, we found a correlation between higher expression of learned helplessness, as measured by the forced swim test, and amounts of Orx_A and the Orx_1 gene transcript in the amygdala (Arendt et al., 2013). In the study reported herein we examine the effect of chronic social defeat on the gene expression of Orx_1 and Orx_2 receptors. Our

hypothesis, that anxiety and depression are mediated through activation of the Orx_1 receptor in the BLA, was tested by determining if its knockdown would produce anxiolytic and antidepressive effects. We further hypothesize that the Orx_2 receptor in the BLA functionally counter-balances the Orx_1 receptor, with anxiolytic and antidepressive effects. We hypothesize that the knockdown of the Orx_2 receptor will be anxiogenic and depressive. We tested these using adeno-associated viruses to insert stably expressing short hairpin sequences to knockdown the mRNA transcripts for each of the orexin receptors.

2. Materials and methods

2.1. Animals

Adult male c57bl/6 mice (Harlan, Indianapolis; $N = 59$) were housed in pairs during the first half of the study prior to the social defeat ($N = 28$), and housed singly in the second half ($N = 22$). A separate cohort of animals was used to validate the viral vectors ($N = 9$). Mice were on a 12:12 reversed light–dark cycle (lights off at 9 AM) with food and water provided *ad libitum*. Retired CD1 breeder mice were used for social defeat and the social preference tests. All testing took place between 10 AM and 2 PM. All experiments were executed in a manner that minimized suffering and the number of animals used, in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and approved by the Institutional Animal Care and Use Committee of the University of South Dakota.

2.2. Behavioral measures/experimental design

The first half of this study examines the effects of chronic social stress on the expression of Orx_1 and Orx_2 receptor transcripts in the BLA and PrL. After ten days of chronic social defeat, an animal's susceptibility or resilience to social defeat was assessed on day eleven by a social preference test (Fig. 1). Orexin receptor expression in susceptible and resilient animals in the appropriate brain regions was examined using qPCR.

The second half of the study was carried out in a separate cohort of mice, for which orexin receptors were chronically knocked down in the BLA of unstressed animals. After orexin receptor knockdown, behavioral endpoints of depression and anxiety were assessed using measures intended to quantify behavior associated with depression and anxiety, listed below (Fig. 4C). Testing took place between 10 AM and 2 PM under red light unless otherwise listed.

2.2.1. 10 Day social defeat—Cages were divided longitudinally by perforated Plexiglas to allow olfactory and minimal tactile contact. The CD1 mice were housed singly on one side of this enclosure for one week to establish territorial attachment to the home cage. For the following ten days social defeat was established in c57bl/6 test mice by placing one on the side of the divider where the territorial CD1 resided, after which aggression rapidly ensued. The c57bl/6 was left with the CD1 for up to 5 min, or until 5 attacks on the intruder were observed. Once removed, the c57bl/6 was placed on the opposite side of the divider where it remained in visual and olfactory (but not physical) contact with the CD1 for the next 24 h. This sequence took place for 10 days, each day with a novel CD1; no additional

defeat followed on day 11 when the social preference test was administered. To control for social housing over the 10 days, two c57bl/6 mice were housed on opposite sides of a similar divider, but not allowed to fight.

2.2.2. Social preference—Testing of social avoidance or preference took place in a square box (40 cm^3) with a transparent perforated cylinder (diameter 12 cm) set against the middle of one wall. This test was used for both socially defeated and AAV-treated Orx receptor knockdown mice. After ten days of chronic social defeat, on day eleven c57bl/6 mice were moved directly into the social preference test from the opponent's home cage. In AAV-treated mice, with injection on day one, the social preference test followed on day 22. The c57bl/6 mouse was allowed to explore the box with the empty cylinder for 2.5 min before being removed, and then the empty cylinder was exchanged for one containing a non-aggressive CD1 mouse. Test animals were not defeated on the day of the social preference test (days 11 or 22). The c57bl/6 was then put back into the box and allowed to explore the cylinder with a social target for an additional 2.5 min. All interactions were monitored with Ethovision software (Noldus; Leesburg VA) to measure the amount of time the c57bl/6 mouse spent in close proximity (3 cm) to the cylinder, as well as the total distance (cm) traveled in the apparatus. The test animal's final social preference score was determined by using the proportion of time the c57bl/6 mouse spent in close proximity (3 cm) to the cylinder when it was occupied vs when it was empty ([time within close proximity when occupied]/ [time within close proximity when empty] × 100 = social preference score). Typically, undefeated control mice spent more time in close proximity to the social target giving them a social preference score of greater than 100%. A portion of the defeated mice displayed social preference greater than 100% and were thus termed "resilient", as these preference scores were similar to control animals. In contrast, on the day after defeat "susceptible" mice spent less time in close proximity to the social target producing a social preference score less than 100%.

2.2.3. Sucrose preference test—This test quantifies an animal's preference for a naturally rewarding substance, a sweet sucrose solution, and was carried out over 6 days beginning on day 15 after AAV injection. On the first and second days (15 and 16 post-AAV) animals were presented with 2 bottles of water to acclimate them to a choice of bottles. One of the water bottles was replaced with a sucrose solution (1%) for the third through the sixth days (17–20 post-AAV). Sucrose and water bottles were alternated each day to prevent the influence of location bias. The third day (17 post-AAV) was not recorded, but served as a means to acclimate the mice to the taste of sucrose. Weight of bottles containing sucrose solution and water were recorded on the fourth-sixth days (18–20 post-AAV) to determine the percentage of fluid intake that was sucrose solution ([sucrose volume]\[sucrose + water volume] × 100 = % sucrose intake).

2.2.4. Open field test—This test measures an animal's exploratory behavior of a novel environment, and was administered on day 21 after AAV injection. Animals are less likely to explore the center of the apparatus if they are more anxious. Mice were placed in a square box (40 cm × 40 cm × 40 cm) for 5 min. Trials were recorded and examined using

Ethovision software (Noldus, Leesburg, VA) for the amount of time animals spent in the center (28 cm) and outer walls (6 cm) of the box as well as the total distance traveled.

2.2.5. Elevated plus maze—Similar to the open field, this test was administered on day 21 after AAV injection, and measures the amount of time an animal spends exploring an exposed area vs one that is more protected. Animals were placed on the center of the Elevated Plus Maze (EPM) facing the same closed arm for every trial. Movement was recorded using Ethovision software (Noldus, Leesburg, VA) for the total amount of time the animal spent in the open and closed arms and total distance traveled. This test was performed on the same day as the open field test with a 5 min interval separating the two tests.

2.2.6. Forced swim test—The forced swim test was administered on day 23 after AAV injection, and measures immobility in an inescapable cylinder of water, which is correlated with learned helplessness. Exposure to stressful stimuli *apriori* increases immobility times while antidepressants decrease immobility. Mice were placed in a 1 L cylinder of 25 °C water and recorded for 8 min. The last 5 min of the video were scored for the total amount of time that the animals spent in an immobile state. Immobility was defined as either no limb movement, or the minimal amount of limb movement required to keep the animal's nose above the water.

2.3. qPCR

Defeated mice were sedated with 5% isoflurane and decapitated with brains frozen on dry ice. The majority of each brain region of interest was micro-dissected from frozen coronal sections (200 μm) on a Peltier cold plate (T = −25 °C; Physitemp, Clifton, NJ) using a blunt 24 gauge needle. Prelimbic cortex was taken from approximately 1.54–1.98 mm anterior to bregma while the BLA was sampled from −1.22 to −2.06 posterior to bregma. Regions were immediately injected into lysis buffer for subsequent RNA purification (Life Technologies, Grand Island, NY). Final RNA samples were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). Purified RNA was used for complementary DNA (cDNA) synthesis in 20 μl reactions using the High Capacity cDNA Archive Kit (Life Technologies, Grand Island, NY). The cDNA product was used in the qPCR reactions on a Step One Plus Real-Time PCR System (Life Technologies, Grand Island, NY). Samples were examined using Taqman Assay On Demand primer/probe sets (Life Technologies, Grand Island, NY) for Orx_1 (mm 01185776_m1) and Orx_2 (mm 01179312_m1). Each sample was run in duplicate and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; mm03302249q1). The qPCR was performed at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Changes in gene expression were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), comparing all samples to the average ΔC_T value of the control animals. Values were expressed as mean fold change ± standard error of the mean (SEM).

2.4. AAV shRNA injections

We used a similar protocol as Hommel et al. (2003) for the viral vectors designed to knock down the expression of the orexin receptors. These AAV vectors were generated by the DiLeone lab. Mice were sedated with 5% isoflurane before being placed in a Kopf stereotaxic frame. During the surgery, sedation was maintained with 3% isoflurane. Holes were drilled into the skull and bilateral injections were made targeting the BLA (AP −1.5, ML ±3.25, DV 4.75) according to bregma. Viral delivery was done manually with a stereotaxically mounted Hamilton syringe over ten minutes. The syringe was left in place for an additional 5 min to minimize reflux migration of the viral vector up the injection track. We injected 400 nL of a serotype II adeno-associated virus which delivered a plasmid that coded for shRNA knockdown sequences and an EGFP reporter. Behavioral testing took place 18 days after viral injections to allow expression of the shRNA product.

Separate hairpin RNA structures were designed to target specific regions of the Orx_1 and Orx_2 mRNAs. Critical to this structure was a 24-nucleotide sequence that had a consensus sequence on each orexin receptor mRNA. Each designed shRNA contains a 24-nucleotide antisense sequence followed by the sense sequence (matched to a consensus sequence on each orexin receptor mRNA), with sense and antisense sequences separated by a loop sequence (CTTCCTGTCA). Three sequences were used for experiments with sense target sequences:

Orx_1: CCAAAGGTCCCCACAGACATATTC

Orx_2: AGAAACCCTTCAGTGGGACTTAAC

Scrambled control: CGGAATTTAGAAACCCGGCTCCAC

The oligonucleotides had *Sap*I and *Xba*I overhangs to allow for ligation downstream of the mU6pro region of a modified pAAV-MCS vector, pAAV-shRNA. This vector was designed to coexpress hairpin RNAs, under the control of a mouse U6 promoter and an SV40 polyadenylation site, as well as EGFP controlled by an independent CMV promoter and hGH poly-adenylation sequence (Hommel et al., 2003; Sharf et al., 2010).

2.5. Statistics

Group comparisons of different brain regions were performed using one way ANOVA, followed by post hoc comparisons of control, susceptible, and resilient groups using the Newman–Keuls test. Correlative effects between social preference and gene effects were performed with regression analysis. Following AAV injections, animals were included in the final analysis if they unilaterally or bilaterally expressed GFP in the BLA as behavioral results were not statistically different (scramble: $F = 7.6$, $p > 0.1$; Orx_1: $F = 2.9$, $p > 0.16$; Orx_2: $F = 2.2$, $p > 0.2$) for left, right or bilaterally accurate injections. All differences for AAV treated animals were compared using *t*-tests between animals that received the control virus and those receiving the Orx_1 or Orx_2 viral treatment.

3. Results

3.1. Chronic defeat produces susceptible and resilient populations

Mice that underwent the 10 day defeat protocol had a wider range of social preference times relative to controls that were not defeated (Fig. 1). Defeated animals were divided according to whether they exhibited social preference ("resilient", social preference score > 100%) or social avoidance ("susceptible", social preference score < 100%; Krishnan et al., 2007). All but one of the control mice not previously exposed to an aggressive CD1 had social preference scores greater than 100%. Normally, rodents display a social preference for novel conspecifics. However, it may be possible that social preference scores in control animals were also influenced by the novelty of the strain of mouse (CD1) used to assess affiliation or avoidance during the test.

3.2. Chronic defeat decreases Orx_2 receptor mRNA in the BLA

There were no significant differences in orexin receptor gene expression between control and resilient groups for either of the brain areas (BLA, PrL) we examined. In the BLA, susceptible animals showed an increase in Orx_1 transcription ($F_{2,13} = 7.753$, $p < 0.006$; Fig. 2A) and a decrease for Orx_2 ($F_{2,11} = 7.142$, $p < 0.010$; Fig. 2B). These changes were not seen in the PrL cortex for either the Orx_1 ($F_{2,25} = 0.764$, $p < 0.476$; Fig. 2C) or Orx_2 ($F_{2,25} = 0.556$, $p < 0.581$; Fig. 2D) transcripts.

Changes in BLA Orx receptor gene expression also translated to significant correlations between social preference score and expression of Orx_1 ($F_{1,14} = 24.322$, $p < 0.001$, $r^2 = 0.635$; negative regression; Fig. 3A) and Orx_2 ($F_{1,12} = 23.365$, $p < 0.001$, $r^2 = 0.661$; positive regression; Fig. 3B) receptor transcripts. These relationships were not significant for Orx_1 ($F_{1,26} = 0.021$, $p < 0.887$, $r^2 < 0.001$; Fig. 3C) or Orx_2 ($F_{1,26} = 0.001$, $p < 0.981$, $r^2 < 0.001$; Fig. 3D) mRNA in the PrL.

3.3. Viral shRNA knockdown of Orx_2 in the BLA induces anxious behavior

The shRNA constructs reduced the amount of endogenous mRNA to 40.2% of control expression for the Orx_1 sequence ($t_7 = 3.458$, $p < 0.011$; Fig. 4A) and 51.9% for the Orx_2 sequence ($t_5 = 2.852$, $p < 0.036$; Fig. 4A). As behavioral outcomes were comparable, animals with both unilateral and bilateral delivery of shRNA to the basolateral complex were included in the final behavioral analysis (Fig. 4B). Neither of the AAV shRNA constructs targeted at the Orx_1 or Orx_2 receptor mRNA sequences significantly affected behavioral measures of depression for anhedonia in the sucrose preference test (Orx_1, $t_{11} = 0.736$, $p < 0.477$; Orx_2, $t_{12} = 0.382$, $p < 0.709$; Fig. 5A), or immobility in the forced swim test (Orx_1, $t_{10} = 0.529$, $p < 0.608$; Orx_2, $t_{11} = -0.738$, $p < 0.476$; Fig. 5B).

With regard to anxious behavior, knocking down orexin receptor expression in the BLA did not have any effect on EPM performance for either Orx_1 ($t_{12} = -0.159$ $p < 0.877$) or Orx_2 ($t_{12} = -0.835$, $p < 0.420$; Fig. 6A). However, knocking down Orx_2 receptor expression, but not Orx_1, significantly decreased social preference (Orx_2, $t_{11} = 2.373$, $p < 0.037$; Orx_1, $t_{10} = -0.898$ $p < 0.390$, Fig. 6B). Similarly, the Orx_2 receptor AAV knockdown had an anxiogenic effect, decreasing the amount of time spent in the center of the arena of the open

field test compared to controls ($t_{11} = 2.704$, $p < 0.021$; Fig. 6C). The same effect in the open field was not seen for the Orx_1 receptor ($t_{12} = 1.014$, $p < 0.331$). Total locomotor activity in the social preference test was significantly reduced for the Orx_1 AAV treatment group ($t_{10} = 2.468$, $p < 0.033$) but not for mice receiving the Orx_2 treatment ($t_{12} = 1.510$, $p < 0.159$; Fig. 6B). There were no differences in open field locomotor activity between the control and experimental groups (Orx_1, $t_{12} = 0.242$, $p < 0.813$; Orx_2, $t_{11} = -1.541$, $p < 0.152$; Fig. 6C).

4. Discussion

This study provides evidence that Orx_2 receptor signaling in the BLA constrains anxious responses in mice, which may include general and social anxiety. The results also suggest that a more inclusive orexin-derived response may ensue following social defeat, since Orx_1 and Orx_2 receptor gene expression is changed in susceptible mice. What is more, the gene expression in orexin receptor subtypes appears to be regulated in opposite directions following social defeat, suggesting opposing functional responses of Orx_1 and Orx_2 receptors. While both the BLA and PrL play a role in anxiety/ fear learning (Orsini and Maren, 2012; Sajdyk and Shekhar, 1997; Vidal-Gonzalez et al., 2006), chronic social defeat only changed mRNA expression of orexin receptors in the BLA. A decrease in Orx_2 mRNA was seen in susceptible animals, and localized BLA Orx_2 receptor knockdown led to the expression of anxiety-like behaviors in social preference and open field tests in defeat-naïve mice. Taken together, these results suggest that Orx_2 receptors in the BLA are functionally associated with alleviating anxiety.

After 10 days of defeat, clearly distinctive social preference scores varied across our experimental population, producing three easily definable groups based on treatment and social response (control, resilient, and susceptible; Fig. 1) similar to that of a study by Krishnan and colleagues who used a similar protocol in a much larger study (Krishnan et al., 2007). While the prelimbic region (PrL) of the prefrontal cortex is a potential site for orexin to regulate anxious behavior since this region is associated with anxiety states (Miller et al., 2012; Wall et al., 2012), we did not see any changes in Orx_1 or Orx_2 receptor transcription in this area. In the BLA, susceptible animals expressed a divergent pattern of orexin receptor transcript where Orx_1 was increased, and Orx_2 was decreased (Fig. 2). What is more, regressions comparing Orx_1 and Orx_2 gene expression in the BLA with social preference scores strongly suggest that the action of each of the two receptors within the BLA are functionally opposing that of the other with respect to social anxiety (Fig. 3). This pattern of opposing receptor function is similar to another study that examined knockout mice in the forced swim test where Orx_1 null animals displayed less learned helplessness while Orx_2 knockouts exhibited more (Scott et al., 2011). While our study employed a different experimental protocol, the contrasting effects of the orexin receptors in both studies reinforce the possibility that the two receptors could ultimately have different, if not opposite, influences on stress-related disorders.

Knocking down the Orx_1 transcript in the BLA did not appear to have a significant effect in any of the behaviors we observed that are indicative of depression or anxiety, although the treatment did appear to inhibit locomotion during the social preference test (Fig. 6B). Altering locomotor activity via orexin signaling is not entirely without precedent; as orexin

knockout mice display reduced movement during resident intruder testing (Kayaba et al., 2003). In contrast, injecting Orx_A or Orx_B into the CeA can increase locomotor activity (Avolio et al., 2011). Furthermore, the locomotor change is not surprising as the amygdala is part of a larger circuitry responsible for defensive flight responses (Canteras et al., 2010; Tannure et al., 2009).

Since chronic defeat increased the Orx_1 gene transcript, we expected that knocking it down in healthy animals would have antidepressive or anxiolytic effects. Others have shown that icv injections of Orx_A, which preferentially binds to the Orx_1 receptor, increases anxious behavior (Suzuki et al., 2005). Furthermore, Johnson and colleagues have repeatedly shown that systemic antagonism of the Orx_1 receptor (SB-334867) has a panicolytic effect in rats (Johnson et al., 2010, 2012b). Potentially, the differential results obtained in socially defeated and defeat-naïve mice suggest that the additional stress associated with social defeat may activate a more complete orexin receptor response in the basolateral amygdala. More experimentation is necessary to discern whether Orx_1 receptors in BLA have the potential to counteract the anxiolytic responses mediated by BLA Orx_2 receptors.

The AAV shRNA targeted destruction of the Orx_2 transcript in the BLA had an anxiogenic effect (Fig. 6). Specifically, this group of animals exhibited decreased preference for a novel CD1 mouse, and spent less time in the center of an open field. While we did not see a significant effect on the EPM, this may be expected as a previous study that systemically blocked both Orx_1 and Orx_2 receptors also did not appear to alter EPM performance (Steiner et al., 2012). Similarly, systemic delivery of an Orx_1 antagonist, SB-334867, did not affect anxiety behavior on the EPM (Rodgers et al., 2013). Despite the lack of anxiety-like behavior on the EPM, our results suggest that Orx_2 receptor activity in the BLA is associated with reduction of both general and social anxiety. This effect with the Orx_2 receptor knockdown complements the decrease of Orx_2 transcript from chronic defeat, given that both are associated with decreased social preference. However, the combination of ten day social defeat and AAV knockdown of Orx_2 receptor (and potentially Orx_1) may increase our knowledge of the relationship between anxiety and depression, which are highly comorbid disorders, both of which may be influenced by orexin (Arendt et al., 2013; Kessler et al., 2005; Nollet and Leman, 2013). Ultimately, the two parts of this experiment make a compelling argument for the Orx_2 receptor having an anxiolytic function in the BLA.

While we conclude that Orx_2 receptors in the BLA have anxiolytic properties, we are unable to distinguish the exact mechanism at this point. This is because neither the micro-dissection technique used on the chronic defeat animals, nor the AAV shRNA vectors distinguishes between neuronal subtypes in the BLA (pyramidal projection neurons vs interneurons). The medial nucleus of the CeA (mCeA) is the main output projection of the amygdala and its activation increases anxious behavior (Krettek and Price, 1978; Tye et al., 2011). The lateral nucleus of the central amygdala (lCeA) and medial paracapsular intercalated cells (ITC) both forward GABAergic projections to the mCeA effectively inhibiting anxiety (Avolio et al., 2011; Orsini and Maren, 2012). The BLA projects to all three of these areas amplifying the number of potential mechanisms that could explain why Orx_2 receptor signaling would ultimately inhibit anxiety as our data suggest. Orexin system receptors, Orx_1 and Orx_2, are associated with G_q and G_s proteins, with previous work implicating a primarily stimulatory

role for both, with the caveat that G_i association has also been noted (Akbari et al., 2011; Bernard et al., 2003; de Lecea et al., 1998; Hoang et al., 2003; Karteris et al., 2005; Kukkonen, 2013; Sakurai et al., 1998). If the Orx_2 receptor has stimulatory actions in the BLA pyramidal neurons (Bisetti et al., 2006), then those neurons would have to make connections with either the lCeA or ITCs to ultimately inhibit the anxiety producing activities of the mCeA. Alternatively, if we assume stimulatory Orx_2 receptors reside on BLA GABAergic interneurons, inhibition of BLA pyramidal neurons that directly project to the mCeA should limit amygdalar anxiogenic output (Avolio et al., 2011). These two proposed circuitries would be switched in the event that the Orx_2 receptor is coupled instead to a G_i protein, such that Orx_2 G_i receptors in the BLA inhibited GABA interneurons which would disinhibit pyramidal output to ITC and/or lCeA. The alternative is for Orx_2 G_i output to directly inhibit BLA pyramidal neurons projecting to mCeA. If there is an anxiogenic role for Orx_1 receptors to influence the BLA after stress, as our preliminary evidence suggests, any hypothetical circuitry would have to balance the two receptor outputs.

Summary

In conclusion, Orx_2 receptors in the BLA have the potential to alleviate general and social anxieties. There is also a possibility that the two receptors associated with this system may functionally counteract each other, providing a mechanism to bi-directionally control anxiety via Orx_1 and Orx_2 receptors in the BLA. An Orx_2 receptor specific agonist may provide a means to subvert the complex interconnectivity of the amygdala and act as a novel treatment for anxiety disorders. The efficacy of intranasal Orx_A application in rats and primates (Deadwyler et al., 2007; Dhuria et al., 2009a,b) raises the possibility that non-invasive therapeutic activation of orexin receptors may be applicable for use in human clinical trials and eventual treatments for general and social anxiety.

Acknowledgments

We would like to thank Dr. Diane Lagace and Mirela Hasu for their invaluable advice regarding the social preference test. This work was supported by NIH RR15567, and by anonymous donors to the Summers' lab via the USD Foundation.

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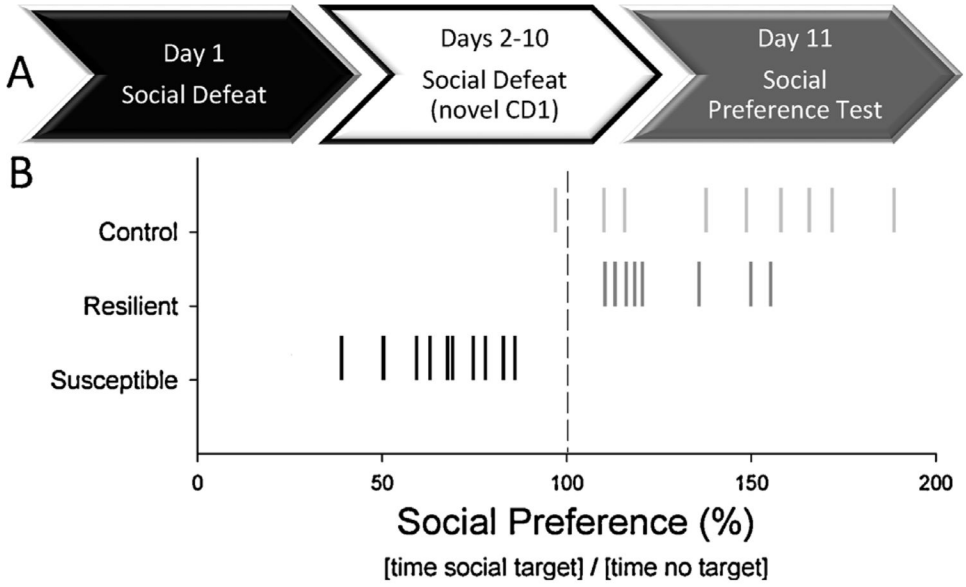

Figure 1.

(A) Sequence and timing of social defeats (by a novel CD1 mouse once/day for 10 days) and following social preference test (day 11). (B) Social preference scores based on the amount of time test mice spent near and away from a social target delineate animals that were resilient and susceptible to social defeat. “Resilient” animals were defeated, but still showed a social preference (>100% time spent with social target/time spent away from the social target ×100) similar to “Controls”. Defeated animals that displayed a social aversion (<100%) relative to controls and were defined as “Susceptible”.

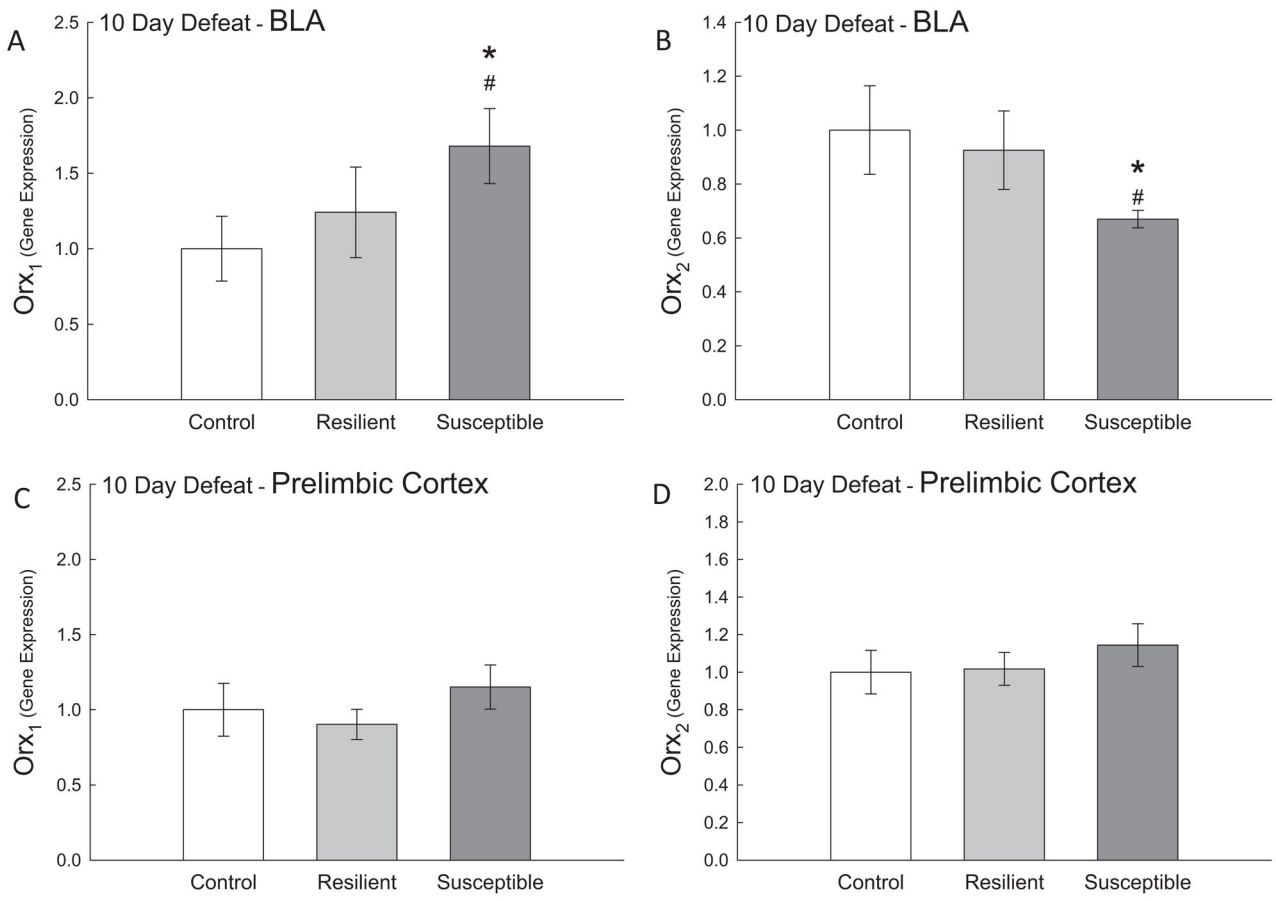

Figure 2.

In the basolateral amygdala (BLA), (A) susceptible animals had significantly elevated amounts of Orx_1 mRNA relative to control (*) and resilient (#) animals. (B) This effect in susceptible animals occurred with a concomitant decrease in BLA Orx_2 mRNA. In the prelimbic cortex (PrL), there were no differences between the groups for the (C) Orx_1 or (D) Orx_2 receptor transcripts. *,#$p < 0.05$.

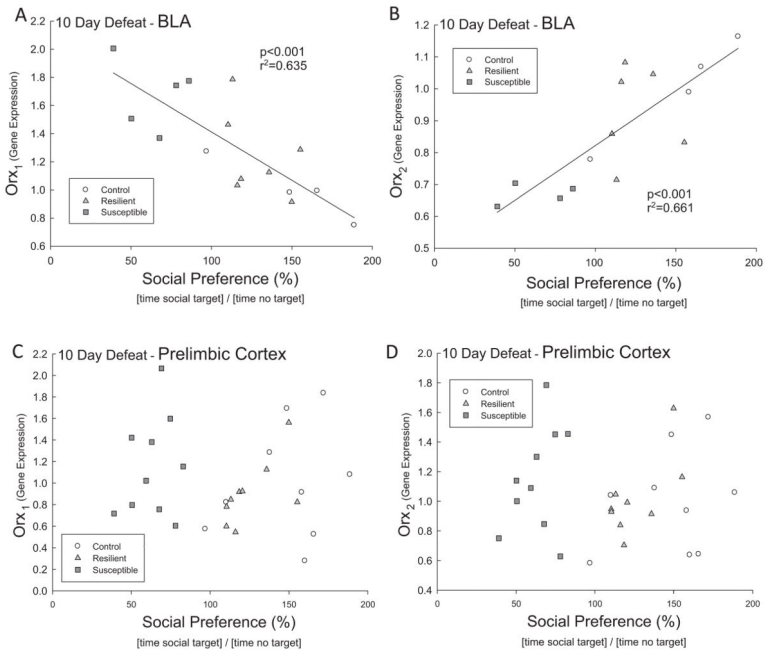

Figure 3.

Social preference scores were significantly correlated with BLA expression of (A) Orx_1 and (B) Orx_2 mRNA. In the BLA there was a significant ($p < 0.001$, $r^2 = 0.635$) negative regression between the Orx_1 mRNA and social preference, and a significant ($p < 0.001$, $r^2 = 0.661$) positive regression between the Orx_2 mRNA and social preference. These correlations were absent in the PrL for (C) Orx_1 and (D) Orx_2 receptor transcripts.

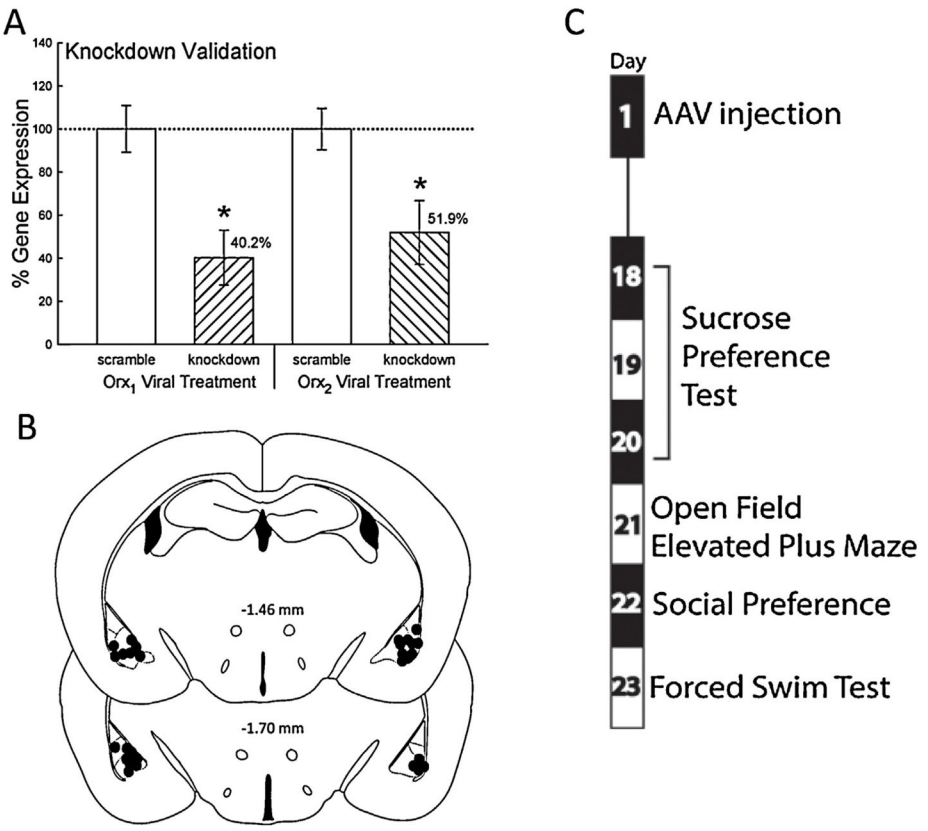

Figure 4.

Viral shRNA constructs significantly (*) reduced the expression of the endogenous mRNA sequence for the respective orexin receptor. (A) Gene expression comparisons were made between the control group that received a nonspecific “scramble” virus and the experimental group which received the “knockdown” virus targeting the Orx_1 or Orx_2 receptor. (B) Behavioral data were analyzed for animals with expression of GFP (●) in the basolateral complex. (C) Sequence and timing of a series of tests commonly used to detect depression and anxiety related behaviors to which transfected animals were exposed. *$p < 0.05$.

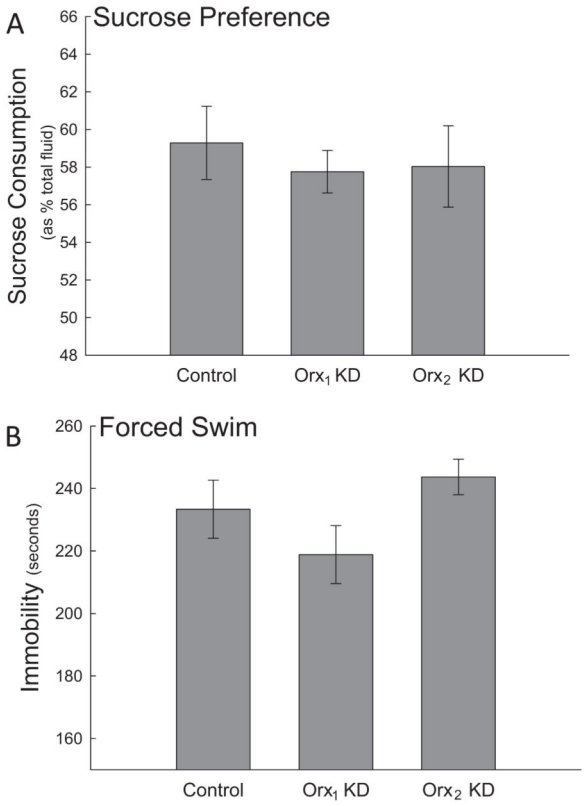

Figure 5.

Knockdown of either orexin receptor in the BLA produced no significant differences in behavioral measures of depression for either the (A) sucrose preference test or (B) forced swim test relative to the control treatment.

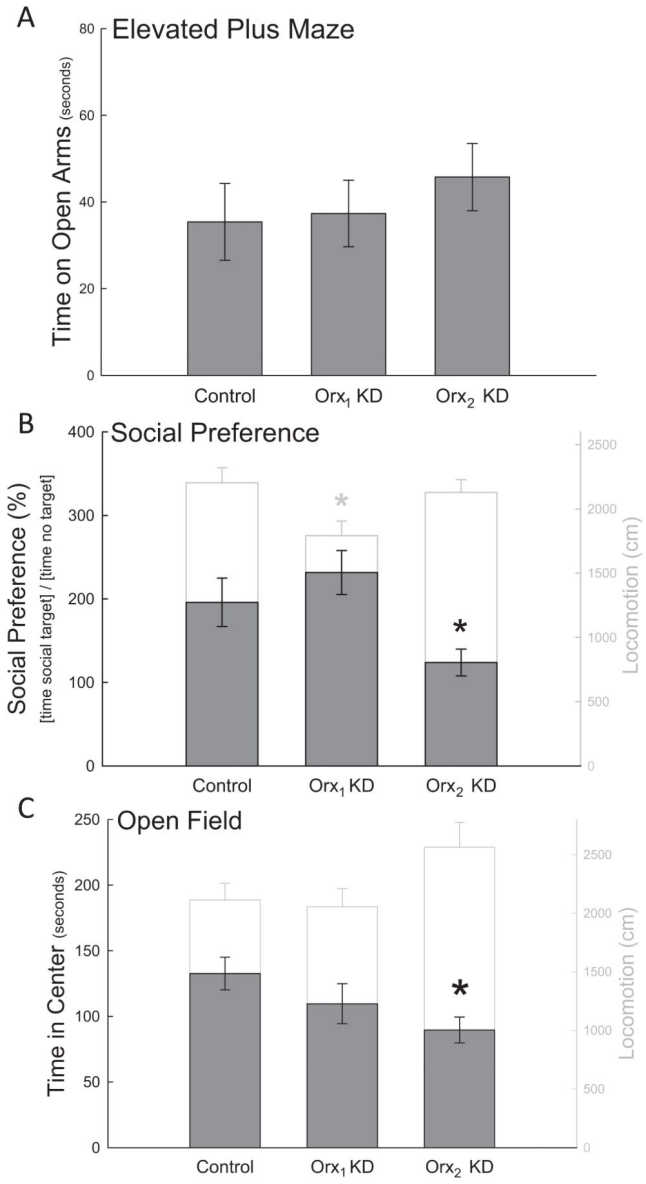

Figure 6.

Knocking down the Orx_2 receptor in the BLA increased anxiety-like behaviors. (A) While none of the treatment groups exhibited altered EPM performance, (B) the viral shRNA Orx_2 knockdown significantly decreased (*) social preference relative to controls. Locomotion was unaffected in the social preference test with Orx_2 knockdown. There was no significant change in social preference for animals that received the Orx_1 knockdown despite a significant decrease in the total amount of locomotor activity. (C) Orx_2 knockdown also decreased time spent in the center of an open field apparatus. There were no significant changes in locomotion due to Orx_1 or Orx_2 knockdown in the open field. $*p < 0.05$.