Orexin 2 receptor stimulation enhances resilience, while orexin 2 inhibition promotes susceptibility, to social stress, anxiety and depression

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HIGHLIGHTS

\begin{itemize}
  \item Stress Alternatives Model (SAM) produces resilient (Escape) and susceptible (Stay) phenotypes.
  \item icv Orx\textsubscript{2} agonist reduces anxious and depressive behaviors in Stay mice.
  \item icv Orx\textsubscript{2} antagonist increases anxious and depressive behavior plus plasma corticosterone.
  \item Stay mice exhibit fear conditioning, reversed by an Orx\textsubscript{2} agonist.
  \item icv Orx\textsubscript{2} agonist increases immediate-early gene EGR\textsubscript{1} in BLA parvalbumin GABA interneurons.
\end{itemize}

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[Ala\textsubscript{11}, d-Leu\textsubscript{15}]-Orx\textsubscript{b}, a modified Orx\textsubscript{b} peptide used as an Orx\textsubscript{2} receptor agonist
MK-1064, 5′-chloro-N-[(5,6-dimethoxy-2-pyridinyl)methyl](2,2′:5′,3″-terpyridine)-3′-carboxamide - an Orx\textsubscript{2} antagonist

ABSTRACT

Knockdown of orexin/hypocretin 2 receptor (Orx\textsubscript{2}) in the basolateral amygdala (BLA) affects anxious and depressive behavior. We use a new behavioral paradigm, the Stress Alternatives Model (SAM), designed to improve translational impact. The SAM induces social stress in adult male mice by aggression from larger mice, allowing for adaptive decision-making regarding escape. In this model, mice remain (Stay) in the oval SAM arena or escape from social aggression (Escape) via routes only large enough for the smaller mouse. We hypothesized intracerebroventricular (icv) stimulation of Orx\textsubscript{2} receptors would be anxiolytic and antidepressive in SAM-related social behavior and the Social Interaction/Preference (SIP) test. Conversely, we predicted that icv antagonism of Orx\textsubscript{2} receptors would promote anxious and depressive behavior in these same tests. Anxious behaviors such as freezing (both cued and conflict) and startle are exhibited more often in Stay compared with Escape phenotype mice. Time spent attentive to the escape route is more frequent in Escape mice. In Stay mice, stimulation of Orx\textsubscript{2} receptors reduces fear conditioning, conflict freezing and startle, and promotes greater attention to the escape hole. This anxiolysis was accompanied by activation of a cluster of inhibitory neurons in the amygdala. A small percentage of those Stay mice also begin escaping; whereas Escape is reversed by the Orx\textsubscript{2} antagonist. Escape mice were also Resilient, and Stay mice Susceptible to stress (SIP), with both conditions reversed by Orx\textsubscript{2} antagonism or stimulation respectively. Together, these results suggest that the Orx\textsubscript{2} receptor may be a useful potential target for anxiolytic or antidepressive therapeutics.

1. Introduction

Anxious behavior is widely expressed across vertebrate species (Kandel, 1983), and while anxiety is an evolutionarily conserved and adaptive suite of emotions (Bergstrom and Meacham, 2016; Smith et al., 2016; Trimmer et al., 2015), these emotions in their pathological
forms also constitute the most common human psychological disorders (Kessler et al., 2010). It is highly comorbid with numerous other conditions such as depression (Spinhoven et al., 2011) and Post-Traumatic Stress Disorder (PTSD) (Zlotnick et al., 1999). Evidence is mounting that a relatively newly identified pair of neuropeptides, called the orexins or hypocretins (Broberger et al., 1998; Peyron et al., 1998; Sakurai et al., 1998), are intimately involved in stress, motivation, and affective disorders (Allard et al., 2004; Giardino and de Lecea, 2014; Sakurai, 2005; Trivedi et al., 1998; Yoshida et al., 2006; Zhang et al., 2005) and that choice to remain with the larger aggressor (Stay) exhibit much lower elevated plasma corticosterone, behavioral Pavlovian fear conditioning, elevated anxiolytic neuropeptide S (NPS) gene expression in the central amygdala (CeA), and enhanced gene expression of cannabinoid 2 (CB2) receptors in the hippocampus (Robertson et al., 2017; Smith et al., 2014, 2016). While Escape and Stay behavioral phenotypes
Fig. 1. Experimental design, Stress Alternatives Model (SAM), and Social Interaction/Preference test (SIP). A) Experimental timeline: 5-day acclimation (not shown), icv surgeries, recovery for 7–10 days, followed by aggressive social interaction (SAM). Days 1–4 included the pairing of the CS⁺ (tone) with US⁺ (aggressive social interaction) prior to social interaction. Drug treatments were given prior to social interaction on day 3. On day 5, test mice underwent an SIP test followed by a final SAM exposure that included the CS⁺ but not the US⁺, and sampled thereafter. B) Design of the SAM apparatus: a clear rectangular (91 × 22 × 26 cm) apparatus, containing an oval escapable open field (OF) arena, divided into three sections by curved opaque barriers with L-shaped tunnel escape routes at the apex (only large enough for the test mouse to pass through) that lead to safe areas. The test mouse is placed in an opaque cylinder (16.75 × 20 cm) in the middle of the OF and the aggressor is placed outside this area. The cylinder is removed just prior to social interaction. C) Design of SIP apparatus; an opaque square arena (40 × 40 × 40 cm) with a clear perforated container set at the middle of one wall. Test mice are first placed in the apparatus at the opposite end from an empty container (left) for 2.5 min before being removed. The empty container is then replaced with a container that has a novel CD1 mouse strain/size matched to mice used as aggressors in SAM (right). The test mouse is placed back into the apparatus in the same manner as previously described for another 2.5 min before being removed.
are relatively stable, both reserve the requisite behavioral plasticity to reverse phenotype under the influence of experience or pharmacological treatments. For example, the anxiolytic corticotropin releasing factor type 1 receptor (CRF₁) antagonist, antalarmin allows escape in mice that were previously only submissive (Stay). Alternatively, the Escape phenotype can be reversed through the anxiogenic α₂-receptor antagonist, yohimbine (Smith et al., 2016). This model (Fig. 1B) produces a gradient of anxiety, where familiar escape is least stressful, such that animals which have escaped more than once exhibit reduced apparatus and escape tunnel-related novelty stress that was expressed during the initial escape. As such, the latency to escape is dramatically reduced, which is another reliable measure of diminished anxious behavior in the SAM (Robertson et al., 2015; Smith et al., 2014). Other anxiolytic factors, such as wheel-running exercise and icv NPS, reduce the initial latency to escape (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014, 2016; Summers et al., 2017; Yaeger et al., 2018). Alternatively, remaining submissively (Stay) leads to social defeat, which is the most stressful condition (Koolhaas et al., 1997) in our model. The pharmacological results of anxiogenic and anxiolytic agents clearly demonstrate that the stress of socially aggressive interaction is the mechanism that inhibits escape for the Stay phenotype. Recent work from our lab and others suggests that the orexin (Orx; hypocretin, Hcrt) system interacts with the circuitry producing this social stress-induced anxiety gradient (Giardino et al., 2018; Winsky-Sommerer et al., 2004, 2005), with type 1 receptors (Orx₁) creating anxiogenic and depressive responses, and type 2 receptors (Orx₂) producing contrasting anxiolytic or antidepressive results (Arendt et al., 2013, 2014).

Not surprisingly, stress-reactive orexins play important roles in anxiety, fear, and depression through activation of both Orx receptor subtypes (Abbas et al., 2015; Arendt et al., 2013, 2014; Eacret et al., 2018; Giardino et al., 2018; Johnson et al., 2010, 2015; Khalil and Fendt, 2017; Lungwitz et al., 2012; Wang et al., 2017). Intracranial or icv injection of Orx₁ promotes anxious behavior (Lungwitz et al., 2012; Suzuki et al., 2005), and similar types of anxiogenic (including panic) physiological and behavioral responses can be reversed by blocking Orx₁ receptors (Johnson et al., 2010, 2015; Staples and Cornish, 2014; Vanderhaven et al., 2015). Designer receptor activation of Orx neurons plus antagonism of Orx₂ receptors (Graf et al., 2017) potentially increases activity only at Orx₁ receptors, or at receptors for Orx-colocalized factors such as glутamат, dynorphin, galanin or nitric oxide (Cheng et al., 2003; Chou et al., 2001; Hakansson et al., 1999; Torrealla et al., 2003), and results in increased anxiety, as well as decreased social interaction and latency to defeat, similar to Orx neuron stimulation alone (Eacret et al., 2018). We have previously suggested that Orx₁ function is diametrically opposed to that of Orx₂ (Arendt et al., 2013). We also demonstrated that knockdown of Orx₂ receptors in the basolateral amygdala (BLA) produces anxiogenic responses, suggesting that the natural function of Orx₂ is anxiolytic (Arendt et al., 2014). Amygdalar inhibition of anxious behavior is thought to be regulated through GABAergic interneurons in the BLA or intercalated region of the amygdala (IC; Busi et al., 2011; Lee et al., 2013; Toyote et al., 2015, 2016). Recent experimentation with orexin-deficient mice resulted in increased anxiety (Abbas et al., 2015; Khalil and Fendt, 2017), which we speculate may be due to lack of Orx₂ activation.

Therefore, we posited that modulation of Orx₂ function would result in modification of adaptive emotional and defensive behavior in mice. As Orx has been shown to be functionally effective following intra-nasal delivery (Baier et al., 2008; Dhuria et al., 2009; Modi et al., 2017; Van de Bittner et al., 2018), a putatively systemic brain Orx₂-induced anxiolysis could be of therapeutic value. We hypothesized that icv delivery of an Orx₂ agonist would promote resilience, result in a reversal of submissive behavior, and decrease anxious and depressive behaviors. In relation to this agonist effect, we hypothesized that specific parvalbumin positive GABAergic neurons in the BLA or IC would be activated by Orx₂ stimulation in submissive Stay mice. We also hypothesized that intracerebroventricular (icv) delivery of an Orx₂ antagonist would inhibit escape behavior, promote stress susceptibility, and increase anxious and depressive behaviors, such as startle responses and freezing time.

2. Methods

2.1. Subjects and housing

Adult (6–8 weeks) male C57BL/6N mice weighing ~22–26 g (Envigo, Indianapolis, IN; N = 56) were group housed (4–5 per cage) for 5 days of acclimation (Fig. 1A). They were singly housed (including cage controls) following icv cannulation for the remainder of the experiment (12 days), on a 12:12 light-dark cycle (lights off at 6 p.m.) at 22°C, with ad libitum food and water. A separate cohort of male Hsd:ICR (CD1) retired breeder mice weighing ~50 g (Envigo, Indianapolis, IN; N = 37) were similarly housed, and used to provide aggression during social interaction in the Stress Alternatives Model (SAM; Fig. 1B) and a target for the Social Interaction/Preference (SIP) tests. During the dark active, phase mice (C57BL/6N) were exposed to daily handling starting 2 days after icv cannulation, followed by behavioral aggression and testing. Handling included loosening and re-tightening the cannula insert for ~1 min. All surgical and behavioral procedures were performed in a manner that minimized suffering and the number of animals used, and were 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. Experimental design

Experimental groups included: home cage control (N = 10; used to derive baseline hormone and protein [immunohistochemistry, IHC] expression levels), vehicle control (N = 20), Orx₂ receptor antagonist (N = 17), and Orx₂ receptor agonist (N = 9). All treatment groups, except cage control, underwent 4 days of aggressive social interaction in the Stress Alternatives Model apparatus (SAM; Fig. 1A and B), which was preceded by fear conditioning on each day (described below; Fig. 1A). On day 5, treated animals underwent a Social Interaction/ Preference (SIP; Fig. 1C) test, followed by measurement of freezing response in the SAM to the conditioned stimulus in the absence of social interaction (CR = conditioned response to tone; Fig. 1A). Drug treatments were administered on day 3. After testing on day 5, mice were briefly anesthetized with isoflurane (5% at 1.0 min/L for ~1.5 min) and rapidly decapitated. Whole brains and trunk blood plasma were collected and stored at −80°C for further analysis.

2.2.1. Stress Alternatives Model (SAM)

The SAM apparatus is constructed of a clear rectangular box (91 cm long, 22 cm wide, and 26 cm high) with curved opaque dividers (r = 10.25 cm) that partition it into 3 sections: two enclosed areas (10 × 22 × 26 cm) at each end of the oval-shaped open field (OF = 71 × 22 × 26 cm) arena for social interaction (Fig. 1B). All behavioral interactions were conducted during scotophase (dark; active period). The two enclosed areas are accessible from the OF arena through escape routes (one each at the distal ends of the oval) only large enough for a smaller C57BL/6N test mouse to pass through (Fig. 1B).

A short time before social interaction began, a novel large retired breeder CD1 mouse (aggressor) was placed into the SAM OF arena outside of a centered opaque cylindrical divider (15 cm diameter and 20 cm tall). A smaller C57BL/6N mouse (test mouse) was then placed inside the opaque cylinder (Fig. 1B). Our lab has previously shown that fear conditioning influences gene expression of Orx receptors, endocannabinoid receptors (CB₁), and brain-derived neurotropic factor (BDNF) in the amygdala and hippocampus (Robertson et al., 2017;
Smith et al., 2014); therefore, to incorporate a SAM-inclusive fear conditioning paradigm, after a 30 s acclimation period inside the cylindrical divider, a 5 s tone (2500 Hz at 75 dB, CS ‚) was presented. Following the tone there was a 10 s trace period, after which the divider was lifted allowing test and aggressor (US ‚) mice to freely interact within the SAM arena for 5 min.

Retired breeder CD1 mice are naturally aggressive toward other male mice, including smaller C57BL/6N, and interactions led to high intensity social aggression and defeat (Golden et al., 2011). Different from traditional social defeat models (Golden et al., 2011), test mice in the SAM are provided the option to either exit the arena (Escape) through one of the two escape routes or remain in the arena (Stay) with the larger aggressive mouse. Mice that escape the arena (Escape) have been shown to produce lower physiological and behavioral measures of stress when compared to their submissive non-escaping (Stay) counterparts (Robertson et al., 2015; Smith et al., 2014, 2016). It is important to note, that both Escape and Stay mice experience high levels of aggression from the novel CD1 mouse, and have elevated hormonal, behavioral, and gene expression stress responses, though these measures are significantly higher in the Stay mice. Results from this and previous experiments have demonstrated that test mice generally choose a behavioral phenotype (Escape or Stay) by the second day of SAM interaction.

During interactions in which an aggressor mouse threatened the life of a test mouse, a clear, perforated divider (15 cm wide and 20 cm high) was used to briefly interrupt (5–10 s) these intensely aggressive bouts. Life threatening attacks included repeated bites to the head or neck of the test mouse. After 5 min of interaction, both mice were removed from the arena and placed back into their home cages. If test mice escaped, they were left in the enclosed area of the SAM until the end of the 5 min interaction period.

Test mice were exposed to 4 consecutive days of the SAM social aggressive interaction, following fear conditioning. Drug infusions were given prior to SAM interaction on day 3 (described below). A novel aggressor mouse was used on each day. On day 5 the Social Interaction/ Preference test (SIP) was performed (described below), followed by a final SAM exposure: placement in SAM cylinder, 5 s CS ‚ (tone), and 10 s trace period, testing for a conditioned response (CR). However, on this day the cylinder was not lifted and there was no large mouse present, and therefore, no social aggression. All interactions were recorded manually and digitally using GoPro Hero3+ cameras under red light. Digitally recorded interactions were scored by individuals naive to the treatment of each test mouse.

2.2.1.1. Fear conditioning behavioral analysis. Freezing behavior, commonly used to assess fear conditioning (Blanchard and Blanchard, 1988), was measured for 4 days prior to aggressive social interaction (US), and on the 5th day in the absence of the US. Freezing is the amount of time (>1 s) that mice remained motionless, except for movements related to respiration. Freezing was measured during the time that a test mouse spent inside the opaque dividing cylinder; which included time prior to the tone (CS), during the tone, and after the tone (ending when the cylinder was removed). Percent fear freezing ratio was calculated as: [(freezing time after tone/15 s) / (freezing time before tone/30 s)] × 100; this ratio has the advantage of revealing fear conditioned freezing, as opposed to spontaneous freezing, as a number greater than 100. Significant fear conditioning should always be represented by a mean > 100.

2.2.1.2. SAM behavioral analysis. Behavioral analysis for SAM socially aggressive interaction began following the removal of the cylinder and ended when the test mouse escaped, or at the end of the 5 min interaction time. Mice were designated as escapers (Escape) if they left the arena through an available escape tunnel on one or more of the two test days prior to drug treatment. Mice that did not escape, remained submissively (Stay) with the aggressor CD1. Interaction time was measured as the time from the removal of the cylinder until the end of the interaction (when the test mouse was removed from the arena or escaped). Freezing, startle, and time attentive to the escape route measurements were normalized by the total time spent interacting with an aggressor. Latency to escape was measured as the time starting from the removal of the cylinder and ending when the torso of the test mouse was through the escape hole. Locomotor activity was measured following SAM interactions on the day of drug treatment, in the home cage by counting line crossings of a grid with 10 cm between lines.

Freezing behavior during SAM interactions (conflict freezing) was defined in the same way as freezing during assessment of fear conditioning (before the interaction begins). Conflict freezing always occurs during social interaction, and includes freezing in anticipation of aggression, in response to aggression, and contextual freezing in response to the arena in which aggression is experienced (especially on days 2–4 of SAM interactions). Freezing time was converted into percent freezing time ((time s frozen/total interaction time) × 100).

Startle responses (whole-body flinching) measure an animal’s reaction to sudden environmental stimuli, often acoustic (Hess ink et al., 2017; Sallinen et al., 1998). We surmised, that as the intensity and amplitude of the acoustic-startle response are affected by stress-related events (Belzung and Griebel, 2001; Risbrough et al., 2004), the frequency of socially-induced startle might also be enhanced by increasing stress during aggressive interactions. With this in mind, we pirated the concept of startle for use in the SAM, because we noticed that active approach of an aggressor mouse also elicits a similar startle response. Therefore, we made use of a stimulus that exists as a part of aggressive social interaction, the threat of attack, and measured the number of flinches or recoils that were induced by the approach of the larger CD1 mouse during each entire social interaction.

The amount of time the test mouse spent in contact with the escape tunnel is an indicator of stress-sensitive novelty exploration (Eagle et al., 2013; Jacinto et al., 2013). Although the entire SAM arena is novel initially, the escape route is a unique attribute, distinctly different from the rest of the apparatus, as it clearly presents a physically different path for movement. The chamber at the other end of the escape route is also unknown, until the first passage. Thus, this indicator of novelty assessment is also specifically associated with the stress-sensitive act by which the Escape and Stay Phenotypes are demarcated. We defined the amount of time spent attentive to the hole as only including time when the test mouse was actively interested in and investigating the escape hole and tunnel directly (sniffing and placing head in the hole). This measure is a novel indicator of anxious behavior and decision-making unique to this model, which has been effectively used in SAM experiments on rainbow trout (Summers et al., 2017). We consider Escape and Stay behavioral outcomes to be the result of decision-making because early responses are variable, become stable with experience, and are modifiable by learning as well as anxiogenic or anxiolytic drugs (Carpenter and Summers, 2009; Smith et al., 2014, 2016; Summers et al., 2017; Yaeger et al., 2018).

2.2.2. Social Interaction/Preference Test (SIP)

The Social Interaction/Preference (SIP) test was performed on day 5 as a reliable indicator of whether mice are resilient or susceptible to social stress following the SAM interactions and drug treatments (Arendt et al., 2014; Krishnan et al., 2007). The SIP test was conducted in a square box (40 cm²) with a perforated clear container (12 cm diameter and 20 cm high) placed alongside the middle of one wall (Fig. 1C). To begin, test mice were placed into the arena at the opposite end from the empty container and allowed to explore for 2.5 min, before being removed. The empty vessel was then replaced with an identical container holding a novel CD1 mouse. Test mice were then placed back into the arena for another 2.5 min.

2.2.2.1. SIP behavioral analysis. The amount of time test mice spent in
close proximity to (within 3 cm) the empty vessel, and the one containing a novel CD1 mouse was recorded. To determine whether animals were resilient or susceptible to social stress, the amount of time near the jar containing the aggressor mouse was divided by the time spent near the empty jar ([(Time 3 cm or less from aggressor’s jar/Time 3 cm or less near empty jar) x 100]. Animals with values of 100% or greater were interpreted as being resilient, and those less than 100% were construed to be susceptible to social stress (Arendt et al., 2014; Krishnan et al., 2007).

2.3. icv stereotaxic surgeries

Following a 5 day acclimation period, but prior to exposure to behavioral paradigms, stereotaxic surgery for intracerebroventricular (icv) cannula implantation was performed on C57BL/6N mice that were to undergo vehicle, Orx receptor agonist, or antagonist treatment (N = 46). Mice were anesthetized with isoflurane (2% at 1.0 L/min flow rate) and a guide cannula (26 ga cut to 1.5 mm) was inserted into the right lateral ventricle (Bregma; AP: −0.50 and ML: −1.0). Animals were allowed 7–10 days to recover before aggressive social interaction began.

2.4. Drugs & infusion

Drug treatments were given icv, to mice on day 3, either 1 (agonist or vehicle) or 30 min (agonist; to allow for longer peptide diffusion) prior to being placed into the SAM arena. Drug plus placement in the SAM was followed by the CS and then social aggression interaction, similar to all SAM interaction days (Fig. 1A and B). The SAM was designed to include both within subject and across treatment group comparisons. Injection on day 3 allows comparison of each animal to itself on days 2 and 3, before and after injection, as well as comparisons with the vehicle control. The design was intended to measure acute drug effects on day 3, just after injection. However, three kinds of longer-term effects are possible: 1) carryover effects for drugs that degrade slowly, 2) effects associated with persistent modification of stress responsiveness that yields longer-term changes in anxious or depressive behaviors, and 3) drug effects that promote neural plasticity and yield gene expression or learning.

The Orx receptor antagonist, MK-1064 (0.3 nmol, MCE, Monmouth Junction, NJ), and Orx receptor agonist, which is a modified Orx peptide, [Ala<sup>11</sup>, D-Leu<sup>15</sup>]-OrxB (0.3 nmol, TOCRIS, Minneapolis, MN), were dissolved in 0.9% saline plus 5% DMSO (= vehicle). The doses for both drugs were chosen to avoid enhanced locomotor activity or induction of sleep. Of the two drugs used, only [Ala<sup>11</sup>, D-Leu<sup>15</sup>]-OrxB has been administered icv previously, at a dose that increased locomotion (Samson et al., 2010), and we chose a dose that was ten-fold smaller (Fig. 3D). For MK-1064, we chose a dose that was 10,000 fold smaller than that given orally, avoiding induction of sleep or acute HPA axis activity (Gottet et al., 2016; Grafe et al., 2017), but equivalent to the icv dose chosen for [Ala<sup>11</sup>, D-Leu<sup>15</sup>]-OrxB, because the IC<sub>50</sub> (half-maximal inhibitory) and EC<sub>50</sub> (half-maximal effective) concentrations are similar for these two drugs. All treatments were directed into the lateral ventricle via micro-syringe (extended 0.2 mm below the guide cannulae) and delivered at a rate of 0.5 μL/min, with a total injection volume of 1 μL. All icv injections (1 or 30 min prior) were followed by placement into the SAM arena, plus an additional 45 s (for fear conditioning) before initiation of the aggressive interaction. As MK-1064 crosses the blood-brain barrier, and substances delivered to the cerebrospinal fluid via icv injections are transferred to the cerebral microvasculature via the choroid plexus (Partridge, 2016), we assume this drug is delivered throughout the brain. In contrast, similar sized peptides that include [Ala<sup>11</sup>, D-Leu<sup>15</sup>]-OrxB, OrxA, and OrxB have been administered icv previously (Flores et al., 2014; Gottet et al., 2016), producing behavioral results such as enhanced locomotion and influencing consolidation of fear memories, the latter function dependent on the BLA (Flores et al., 2014; Gottet et al., 2016).

Fig. 2. Escape behavior is promoted by Orx<sub>2</sub> agonism, and limited by Orx<sub>2</sub> antagonism, while latency to escape is unchanged. A) Escape behavior was significantly reduced, following icv injection of Orx<sub>2</sub> receptor antagonist (MK-1064) on day 3 (light gray hatched bar) in mice that were previously Escape phenotype (light gray hatched bar; # indicates significance in antagonist-treated animals from Days 1&2), but remained unchanged in vehicle-treated animals (light gray bar). B) Escape behavior was promoted following day 3 icv injection of Orx<sub>2</sub> receptor agonist ([Ala<sup>11</sup>, D-Leu<sup>15</sup>]-Orexin B), on day 4 (dark gray hatched bar; not significant) in mice that were previously Stay phenotype. C) Escape latency was significantly reduced (*) on day 4 of SAM interaction compared to day 1. There was no change seen between Escape animals treated with vehicle and animals treated with Orx<sub>2</sub> antagonist.
et al., 2017). We followed this precedent, suggesting that the Orx peptides are functionally available following icv injections to brain regions associated with fear, anxiety and depression.

### 2.5. Hormone analysis

Following testing on day 5, trunk blood was taken and centrifuged for ~5 min to separate plasma. Plasma was frozen at −80 °C for further analysis. Plasma corticosterone concentrations [B] were quantified in duplicate, in a single run, using a corticosterone enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Farmingdale, NY).

### 2.6. Immunohistochemistry

Frozen coronal sections (16 μm) from AP -0.94 to −1.70 relative to bregma were incubated with EGR1 (1:500; rabbit monoclonal from Cell Signaling 4154S) and parvalbumin (Pv, 1:100; goat polyclonal from Abcam ab32895) primary antibody combination in solution, 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), at 4 °C overnight (Sathyanesan et al., 2017). Antibodies were used as per manufacturer's instructions and specificity was tested using incubation in antibody solutions lacking primary antibody. Following primary antibody incubation, slides were washed in 1×PBS three times for 5 min each at room temperature. Slides were then incubated in fluorescent secondary antibody (1:500, Alexa-488 and 594: AF488 donkey anti-goat - A11055 for Pvc and AF594 chicken anti-rabbit - AF21442 for EGR1; Life Technologies) in 2.5% BSA in PBS for 2 h at room temperature. The slides were then rinsed in 1× PBS three times for 5 min each and coverslipped with VECTASHIELD HardSet mounting medium with 4’,6-diamidino-2-phenylindole (DAPI). Three sections from each mouse were analyzed, from which an area (210×240 μm) was defined at AP -1.06, centered on ML + or - 2.65 and DV 4.50 relative to bregma, in which all DAPI labeled neurons were counted and scored for EGR1 and Pv labeling. Sections were reviewed by an unbiased observer to evaluate differences and images were captured using a Nikon Eclipse Ni microscope equipped with a DS-Qi1 monochrome, cooled digital camera and NIS-AR4.20 Elements imaging software, using 20× and 40× objective magnifications. Sections from stressed and control mice were captured using identical exposure settings.

### 2.7. Statistical analysis

All experimental designs and statistical analyses were based on a priori hypotheses. For conditions that changed over 4 days of SAM social interaction, we compared outcomes using a two-way repeated measures ANOVA (Orx2 drug x behavioral phenotype x day of SAM interaction design), where phenotype was either Stay or Escape. In addition, two-way ANOVA (Orx2 drug x Phenotype design) was utilized to determine the influence of activating Orx2 receptors (Orx2 Effects) relative to the expression of behavioral phenotypes (Stay vs Escape; Phenotype Effects), Phenotype by Conditioning (Interaction Effects), and analysis of BLA EGR1, Pv, and DAPI triple-labeling. To compare changes occurring within a treatment group across SAM interaction days, a one-way repeated measures ANOVA (Orx2 drug x day of SAM interaction design) was performed. While the statistics for behavior did not include cage controls; these controls were necessary for interpretation of hormonal corticosterone levels, because samples from SAM treatments were compared to baseline levels determined by the mean.
(B) value of home-cage control animals. Therefore home-cage controls were added for specific one-way ANOVA comparisons. Comparison of locomotion in the home cage after drug treatment was also accomplished by one-way ANOVA. Comparisons between two treatments (Vehicle, Orx2 receptor antagonist, or Orx2 receptor agonist) within a given phenotype (Escape or Stay) were analyzed by Student’s t-tests. To determine differences in percentage of escape, a chi-square statistical analysis was performed, where results from previous days were utilized as expected values.

Each animal provided only a singular datum for all analyses. Five assumptions of parametric statistics were applied to the data, which were transformed when necessary, but graphed in their raw form. Analyses with both non-parametric and parametric statistics (previously mentioned) were performed along with examination for multiple comparisons using the Holm-Sidak method, and when the statistical analyses match, as they do for the data herein, we report the parametric results without α adjustment (Feise, 2002; Jennions and Moller, 2003; Moran, 2003; Nakagawa, 2004; Perneger, 1998; Rothman, 1990). Significant effects between groups for one-way analyses were examined with Student–Newman–Keuls post hoc analyses (to minimize Type I error) and Duncan’s Multiple Range Test (to minimize Type II error).

3. Results

3.1. Stress Alternatives Model

In previous experiments using the SAM arena for mice, rats, and trout, the proportion of animals exhibiting Escape and Stay phenotypes have been approximately 50:50 (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014, 2016). Recent work in rainbow trout suggested that these phenotypes were fugible, even without drug treatment, and that the proportions of the two phenotypes may be altered by the degree of stress effects from the captive environment (Summers et al., 2017). As occasionally happens there was not an even distribution of phenotypes in these experiments, such that prior to any treatment the proportion of escaping mice was only 28.26%, leaving 71.74% submissive (Stay) mice. This ratio of phenotype distribution required non-random division of phenotypes into groups. Since the Orx2 receptor antagonist (MK-1064) was expected to reduce escape behavior, a relatively higher proportion of Escape mice (40%) were used for this treatment. In contrast, since application of the Orx2 receptor agonist ([Ala11, D-Leu15]-OrxB) was hypothesized to promote escape behavior, and the availability of this phenotype was low, we allotted 0% of the individuals displaying Escape behavior to this treatment group (100% Stay).

3.1.1. Escape vs. stay behavior; escape latency

As predicted, in Escape mice, escaping behavior did not change following injection of vehicle (Fig. 2A), suggesting that the additional stress of icv injection was not enough to modify behavioral phenotype (Smith et al., 2014). Escape behavior was significantly reduced (within subject comparison, \(\chi^2: F_1 = 4.5, P \leq 0.034\)) by injection of the Orx2 receptor antagonist (MK-1064) on day 3 (50% Escape), in comparison with the first two days of interaction (100% Escape; Fig. 2A). In contrast, day 3 injection of Orx2 receptor agonist in Stay animals slightly increased escape behavior one day after icv administration (11% Escape on day 4), in comparison with the first three days of interaction (0% Escape; Fig. 2B).

Escape latency was progressively reduced (Repeated measures one-way ANOVA: F3,30 = 6.18, P \leq 0.002; Fig. 2C) by experience in all icv injected mice taken together. As is typical of results previously reported for untreated animals (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014) the latency for days 2–4 was significantly reduced compared to day 1. However, comparison to previous studies also reveals that all latency times in icv injected animals were greater than those of untreated mice, even though the stressful icv injection occurred only on day 3. Since the availability of escaping mice was limiting, the Orx2 agonist was not given to Escape mice. The Orx2 receptor antagonist MK-1064 blocked escape in some animals, but did not increase the latency to escape for those that continued to do so (Fig. 2C), suggesting that future experiments should also include Orx2 agonist treatment in Escape mice to determine if latency would be reduced.

3.1.2. Fear conditioning response prior to the SAM interaction

As in previous studies (Smith et al., 2014), only Stay mice reacted to fear conditioning stimuli (CS + US pairing = tone + aggression from a larger CD1 mouse) during isolation in the opaque cylinder. The Stay phenotype froze significantly (t13 = 2.7, P \leq 0.018) more than Escape mice on test day 5 in the absence of the US (Fig. 3A). The data are presented as a ratio of (freezing during and after the tone/time during and after tone)/(freezing prior to the tone/time prior to tone) x 100, such that significant conditioned freezing [ = CR] is greater than 100%. Importantly, the distinction between the significant CR in Stay mice and not in Escape mice was extant despite the additional stress of icv vehicle injection on day 3. Administration of the Orx2 agonist (MK-1064) did not change the fear freezing ratio (Two-Way ANOVA, treatment effect: \(F_{1,19} = 0.06, P \leq 0.817\); phenotypic effect: \(F_{1,19} = 3.28, P \leq 0.086\); interaction effect: \(F_{1,19} = 0.86, P \geq 0.37\) when compared to vehicle treated Escape or Stay mice (Fig. 3B). However, freezing (and the fear freezing ratio) was significantly reduced in Stay mice (t10 = 2.92, P \leq 0.015) that received icv injection of the Orx2 agonist ([Ala11, D-Leu15]-Orx3) two days before testing (Fig. 3C). For this or any behavior reported, activity changed by Orx2 agonist or antagonist treatment is unlikely to be the cause, as home cage locomotion is unchanged (One-way ANOVA: F2,35 = 0.391, P \geq 0.679) immediately after icv injection and SAM social interaction on day 3 (Fig. 3D).

3.1.3. Freezing during SAM interaction

Conflict freezing behavior in C57BL/6N test mice during the SAM social interaction with larger CD1 aggressors, increases significantly (One-Way Repeated Measures ANOVA: F3,30 = 5.08, P \leq 0.006) in mice adopting the Stay phenotype after 1 day of interaction (Fig. 4A and B). This increase includes continued longer freezing time on the day of vehicle treatment (day 3, Fig. 4A and B), suggesting that the additional stress of icv injection did not increase the total duration of freezing. In addition, greater freezing time was exhibited by vehicle-treated Stay compared to Escape mice on all days (Two-Way Repeated Measures ANOVA, phenotype effect: \(F_{1,4 } = 5.17, P \leq 0.039\); time effect: \(F_{1,42} = 4.43, P \leq 0.009\); Fig. 4A), including injection day (day 3; \(t_{16} = 2.38, P \leq 0.03\); Fig. 4A), but not day 4. Importantly, there were no significant differences between Stay mice in different icv injection groups on days 1 & 2 prior to icv injection or day 4 after treatment (Fig. 4B).

On day 3, injection of icv Orx2 receptor antagonist MK-1064 in mice that Stay produces significantly greater time freezing compared to days without treatment (1, 2, and 4; One-Way Repeated Measures ANOVA: F3,30 = 3.3, P \leq 0.035). Escaping animals also exhibit a nearly significant (One-Way Repeated Measures ANOVA: F3,15 = 2.94, P \leq 0.067) increase in percent freezing time on the day of the Orx2 antagonist treatment (data not shown). Conversely, Stay animals treated with the Orx2 agonist ([Ala11, D-Leu15]-Orx3) exhibit reduced freezing on day 3 compared to vehicle-treated mice (One-Way Repeated Measures ANOVA: F3,30 = 4.68, P \leq 0.031), despite being elevated similar to vehicle-contrls on days without treatment (2 and 4; Fig. 4B). Additionally, when comparing injection treatment groups in Stay mice based on a priori hypotheses regarding each Orx receptor treatment (Two-way repeated measures ANOVAs 1. Vehicle vs antagonist: treatment effect: \(F_{1,60} = 6.92, P \leq 0.016\); time effect: \(F_{3,60} = 6.23, P \leq 0.001\); 2. Vehicle vs agonist: time effect: \(F_{2,39} = 6.06, P \leq 0.002\); 3. Agonist vs antagonist: treatment effect: \(F_{2,39} = 6.28, P \leq 0.026\),
3.1.4. Startle responses during SAM interactions

Startle responses during SAM social interactions steadily increased over time in vehicle-injected Stay mice, significantly so by day four (One-Way Repeated Measures ANOVA: \( F_{3,24} = 4.12, P \leq 0.017 \); Fig. 5A and B). Notably, vehicle-injected Stay and Escape mice did not exhibit a significant difference in startle responses on injection day (3) compared to the first two days of interaction (1 & 2), suggesting that the additional stress from icv injection had minimal effects on startle responses (Fig. 5A). Additionally, within the vehicle-injected cohort, Stay animals exhibit a significant increase in startle responses compared to Escape animals (Two-way repeated measures ANOVA 1. phenotype effect: \( F_{1,35} = 11.46, P \leq 0.004 \); 2. time effect: \( F_{3,35} = 3.41, P \leq 0.028 \); Fig. 5A), including on the day of treatment (day 3; \( t_{14} = 2.66, P \leq 0.019 \)).

Mice (Stay) to be injected icv with Orx\(_2\) receptor antagonist MK-1064 and agonist \([\text{Ala}^{11}, \text{d-Leu}^{15}]\)-OrxB exhibited similar changes in startle response on days 1 and 2 prior to injection on day 3 (Fig. 5B), and were not significantly different over time. However, when comparing injection treatment groups in Stay mice based on a priori hypotheses regarding each Orx receptor treatment (Two-way repeated measures ANOVA 1. Vehicle vs antagonist: interaction effect: \( F_{3,50} = 2.67, P \leq 0.05 \); 2. Vehicle vs agonist: treatment effect: \( F_{1,38} = 12.14, P \leq 0.004 \); 3. Agonist vs antagonist: treatment effect: \( F_{1,30} = 5.67, P \leq 0.033 \)), startle responses after treatment in Orx\(_2\) agonist-injected Stay mice are reduced compared to vehicle (\( t_{13} = 2.02, P \leq 0.064 \); Fig. 5B). This reduction was not observed after Orx2 antagonist (\( t_{14} = 1.29, P \leq 0.217 \)) treatment in mice on injection day (Fig. 5C and D), nor on day 4 (Fig. 5B). Furthermore, there was not a significant difference between Stay animals given vehicle and those given Orx\(_2\) antagonist (Fig. 5B).
3.1.5. Time attentive to hole

Time attentive to the escape hole did not change across days in Stay mice injected with vehicle (Fig. 6A and B). However, prior to (days 1 & 2) and on the day (day 3) of treatment, vehicle-treated Escape mice spent significantly more time attentive to the escape hole compared to Stay vehicle-treated mice (Two-Way Repeated Measures ANOVA, treatment effect: $F_{1,42} = 31.99, P < 0.001$, time effect: $F_{3,42} = 4.37, P = 0.009$, interaction effect: $F_{3,42} = 4.2, P = 0.011$; Fig. 6A), including treatment day (3; $t_{16} = 4.59, P = 0.001$; Fig. 6 A). Stay mice injected with Orx2 receptor antagonist MK-1064 on day 3 of SAM social interactions exhibited no differences in time spent attentive to the escape hole on any day (Fig. 6B).

On the other hand, Stay mice injected with Orx2 receptor agonist [Ala$^{11}$, d-Leu$^{15}$]-Orx2 display a significant and robust increase in time spent attentive to the escape hole (days 3 & 4) compared to the first and second day of SAM interaction prior to treatment (day 1 & 2; One-Way Repeated Measures ANOVA: $F_{3,9} = 4.23, P = 0.04$; Fig. 6B). When, based on a priori hypotheses, Stay mice treated icv with Orx2 receptor agonist were compared to vehicle (Two-Way Repeated Measures ANOVA 1. Vehicle treated: treatment effect: $F_{1,34} = 14.29, P = 0.003$, time effect: $F_{3,34} = 36.74, P = 0.001$, interaction effect: $F_{3,34} = 36.01, P = 0.001$) and Orx2 antagonist-treated (Two-Way Repeated Measures ANOVA 1. Vehicle treated: treatment effect: $F_{1,34} = 14.29, P = 0.003$, time effect: $F_{3,34} = 36.74, P = 0.001$, interaction effect: $F_{3,34} = 36.01, P = 0.001$), a significant increase in time spent attentive to hole on injection day (3; vehicle: $t_{14} = 2.64, P = 0.02$, Orx2 antagonist: $t_{14} = 2.64, P = 0.02$) and after (day 4; vehicle: $t_{14} = 2.64, P = 0.02$, Orx2 antagonist: $t_{14} = 2.64, P = 0.02$; Fig. 6B) was evident.

3.1.6. Social Interaction/Preference Test

During the SIP test on day 5, after SAM social interactions were completed, significant differences in time spent near the novel container with a social target/time near the container alone x 100 (often used as a measure of social stress resilience/susceptibility [Arendt et al., 2014; Krishnan et al., 2007]) emerged between phenotypes. Among vehicle injected mice, the Stay phenotype were significantly less resilient than mice that escape ($t_{3,8} = 3.82, P = 0.002$; Fig. 7A and B). Importantly, this indicates susceptibility to stress in Stay mice that is not seen in Escape mice (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014, 2016). However, in Escaping mice, time spent near the target was significantly decreased by treatment with Orx2 receptor antagonist MK-1064, when compared with vehicle-injected Escape mice ($t_{11} = 2.6, P = 0.025$; Fig. 7A and B). Conversely, icv injection of Orx2 receptor agonist [Ala$^{11}$, d-Leu$^{15}$]-Orx2 to Stay mice resulted in a significant increase in social novelty seeking when compared to vehicle-treated Stay animals ($t_{16} = 3.71, P = 0.002$; Fig. 7C and D).

3.1.7. Corticosterone concentrations

Plasma corticosterone levels taken on test day in both Escape and Stay animals treated with vehicle are significantly increased (One-way ANOVA: $F_{2,14} = 23.27, P < 0.001$) when compared to home cage controls (Fig. 8A). There was no significant change observed between phenotypes (Escape vs Stay vehicle treated mice; Fig. 8A). However, Stay mice that were treated with Orx2 antagonist had significantly higher corticosterone levels (Two-Way ANOVA 1. Treatment effect: $F_{1,22} = 4.27, P = 0.05$; 2. Phenotype effect: $F_{1,22} = 5.02, P = 0.035$) compared to Stay mice treated with vehicle ($t_{13} = 2.85, P = 0.014$) and to Escape mice also treated with MK-1064 ($t_{13} = 3.03, P = 0.01$; Fig. 8B). Comparing only Stay mice, plasma corticosterone concentrations in mice treated with Orx2 agonist [Ala$^{11}$, d-Leu$^{15}$]-Orx2 were significantly lower (One-way ANOVA: $F_{2,24} = 27.9, P < 0.001$) than those treated with Orx2 antagonist MK-1064 (Fig. 8C).

3.1.8. EGR1/Pv immunohistochemistry

After immunolabeling four sets of brains from each group (Vehicle- Escape, Vehicle-Stay, Orx2 agonist-Stay, Orx2 antagonist-Escape, Orx2 antagonist-Stay), taken on day 5 after CR testing, for the immediate–early gene product early growth response protein 1 (EGR1), Zif268, NGF1a, Krox24, or TIS8, the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI), and the GABA neuron calcium-binding protein Parvalbumin (Pv), the images (Fig. 9) clearly indicate that a distinct cluster of cells in the BLA, or intercalated region of the amygdala (ItC) (Busti et al., 2011; Lee et al., 2013), are activated by Orx2 agonist binding. A small tight cluster of cells located in the region AP -1.06, centered on ML + or - 2.65 and DV 4.50 relative to bregma were triple labeled, but only in the Orx2 agonist-treated Stay animals (Fig. 9). This group of cells had clear boundaries, when viewed with DAPI and/or Pv labeling, even though significant DAPI and Pv labeling was evident throughout the BLA, which were not significantly different in number based on treatment group (One-way ANOVA: $F_{3,21} = 1.38, P = 0.28$). For all vehicle and antagonist (MK-1064) treated groups, there was no significant effect on triple-labeled neuron count (Two-way ANOVA: 1.
Treatment effect: \( F_{1,12} = 1.75, P \geq 0.21 \); 2. Phenotype effect: \( F_{1,12} = 1.59, P \geq 0.23 \); 3. Interaction effect: \( F_{1,12} = 2.25, P \geq 0.16 \) of \( \text{Pv-positive neurons, or double-labeled count of \text{Pv-negative cells}} \). (Two-way ANOVA: 1. Treatment effect: \( F_{1,12} = 2.96, P \geq 0.11 \); 2. Phenotype effect: \( F_{1,12} = 0.52, P \geq 0.48 \); 3. Interaction effect: \( F_{1,12} = 0.89, P \geq 0.38 \)). However, one group, Stay mice treated with the Orx2 agonist \([\text{Ala}^{11}, \text{D-Leu}^{15}\text{-Orx}^6]\), displayed significantly increased EGR1 labeling in \text{Pv-positive cells} (One-way ANOVA: \( F_{3,13} = 8.4, P \leq 0.002, \text{power} = 0.944 \)) and in \text{Pv-negative neurons} (One-way ANOVA: \( F_{3,13} = 3.9, P \leq 0.034, \text{power} = 0.551 \)) although in a much smaller percentage of cells, with no other treatment or phenotype having more than nominal EGR1, \text{Pv}, and DAPI labeling. Importantly, while evidence of EGR1 labeling in the BLA is suggestive of the Orx2 agonist stimulation of these cells; we cannot be sure that icv delivery reaches these neurons or that the activation is direct. There were significantly more neurons that exhibited triple labeling in Stay mice treated with the Orx2 agonist (T-test: \( t_7 = 3.88, P < 0.006 \)), than in Stay mice treated with Orx2 antagonist. Additionally, the \text{Pv} labeling was unique in this area, with a smaller region labeled in each cell, than in the surrounding BLA neurons. At this time point after icv injection, this tight cluster of cells was uniquely triple labeled only in Stay mice receiving Orx2 agonist treatment (Fig. 9).

4. Discussion

The primary thrust of experiments on the orexinergic system relative to affective disorders, has been to describe anxiogenic and antidepressive actions of Orx1 (Eacret et al., 2018; Ito et al., 2009; Lungwitz et al., 2012; Nollet et al., 2011), through actions of Orx1 receptors (Johnson et al., 2010; Lu et al., 2017; Ozsoy et al., 2017; Staples and Cornish, 2014), although dual Orx receptor antagonists have been shown to be antidepressive (Nollet et al., 2011, 2012). There have also been hints that Orx1 and Orx2 receptors have opposing actions (Flores et al., 2016; Lu et al., 2017), including stress-reduced thalamic and hypothalamic Orx2 expression that was reversed by the antidepressant fluoxetine (Nollet et al., 2011). Our recent work suggests that activation of Orx2 receptors may be anxiolytic and antidepressive, at least in the amygdala or hippocampus, and may have opposing actions to those of Orx1 (Arendt et al., 2013, 2014). Here we describe, for the first time, that intracerebroventricularly activated Orx2 receptors are anxiolytic and antidepressive. This icv Orx2 agonist injection also produced activation, measured by labeling of the immediate-early gene EGR1, of a small cluster of GABAergic cells, co-expressing the calcium binding protein \text{Pv}, in the BLA or ITG, suggesting that these inhibitory cells are at least partially responsible for the anxiolysis (Fig. 9A–E). A small group of non-Pv containing neurons may also play a role (Fig. 9F). Activation of these inhibitory neurons coincident with reduced anxious...
behavior is supported by experiments using Orx₂ KD in the BLA (Arendt et al., 2014). This conclusion is confirmed additionally by the contrast of anxiogenic responses plus increased plasma corticosterone triggered by Orx₂ antagonist treatment. This conclusion is not based on a singular behavioral result, but includes several preclinical measures in a social stress protocol (SAM), addressing recent concerns that anxiety measures that do not include social dynamics also do not translate well to human clinical treatments (Haller and Alicki, 2012; Haller et al., 2013).

Our work suggests that promoting Orx₂ receptor activity (via the Orx₂ agonist [Ala₁ⁿ¹, D-Leu₁⁵]⁻Orx₆) decreases anxious and/or depressive behaviors such as fear conditioned-2, as well as conflict-freezing and startle responses (Figs. 3C, 4B and 5B). Stimulation of Orx₂ receptors also increases such adaptive, anxiolytic, and antidepressive behaviors as the amount of time the test mouse spends attentive to the escape hole in the SAM arena and to a novel social target during the SIP test (resilience/susceptibility to stress). Together, these results are suggestive of an anxiolytic and antidepressive profile which stems from whole brain pro-Orx₂ treatments.

The proportion of Escape to Stay mice in these experiments was particularly low (28:72) compared with the more typical (50:50) self-imposed division of phenotype from previous studies (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014, 2016). Among researchers using various social defeat models it is anecdotally noted that cohorts of fish, mice or rats are not equivalently partitioned into stress-susceptible or resilient animals, which may explain our skewed distribution. However, we have recently noticed in fish and mice, that the proportions of the two phenotypes may be altered by stress effects from the captive environment (Summers et al., 2017; Yaeger et al., 2018). This recent work also suggests that these phenotypes are plastic, and may be affected by prior stress, exercise, or experience (Robertson et al., 2017; Smith et al., 2016; Summers et al., 2017; Yaeger et al., 2018). Our evidence suggests that this skewed distribution of Escape and Stay phenotypes devolve from prior non-experimental stressors in the captive environment. This allowed greater emphasis to be placed on anxiolytic and antidepressive treatment of Stay animals. It is important to note that the mutable status of these phenotypes is most common in the first two days of the SAM, but that a proportion of the mice in either phenotype is susceptible to reversal of phenotype, if the neuromodulatory elements of the stress circuitry are altered, through pharmacological treatments or environmental conditions, such as exercise or familiarity, to reduce stress (Robertson et al., 2017; Smith et al., 2016; Summers et al., 2017; Yaeger et al., 2018). The result is, for example in Stay mice, that a subset of that phenotype is disposed to amelioration (Yaeger et al., 2018), which produces some variability in the treatment results (Fig. 7B, D).

Freezing in response to conditioned stimuli (CS) or contexts associated with fearful environments represents adaptive behavior that reduces the exposure of the quiescent animal to predators and competitors (Demuth et al., 2017). This conditioned-fear response (Blanchard and Blanchard, 1988; Iwata and LeDoux, 1988; Iwata et al., 1986) is intensity dependent, such that anxious animals develop a higher freezing response rate to a CS as the severity of the fearful conditions increase (Robertson et al., 2015; Smith et al., 2014, 2016). Consistent with our previous findings (Robertson et al., 2015; Smith et al., 2014, 2016), these results demonstrate that phenotype plays an important role in the development of classical conditioning, as Escape mice did not exhibit freezing in response to the CS, but Stay mice did. This test day result is suggestive of increased anxiety in Stay mice, which develops as a result of decision-making in the SAM arena (Smith et al., 2014). Therefore, escape is an adaptive behavior that results in reduced anxiety and stress reactivity (Robertson et al., 2015; Smith et al., 2014, 2016). However, when Stay mice were injected with Orx₂ agonist, freezing in response to the CS decreased to the level of vehicle-treated escape mice, suggesting that the treatment reduced anxiety. Fear conditioning has been shown to be regulated by activation of GABAergic neurons in the lIC and BLA (Busti et al., 2011; Lee et al., 2013; Toyote
impaired extinction of fear memories, which is reversed by Orx1 treatment (Flores et al., 2014, 2016), comparable to those stimulated (EGR1 labeling) in this study by Orx2 agonist [Ala15, d-Leu11]-OrxB. In PV-positive neurons (E) only in Stay mice treated with Orx2 agonist [Ala15, d-Leu11]-OrxB was the EGR1 labeling significantly increased (dark gray right-hatched bar) compared to cage control (clear bar), vehicle (dark gray bar) and Orx2 antagonist (MK-1064)-treated mice (dark gray left-hatched bar). F) In PV-negative neurons, a much smaller, but similar increase in EGR1 labeling occurred. G) Labeling of PV was similar in all treatment groups.

Fig. 9. Anxiolysis in Stay mice is accompanied by activation of BLA or ITC inhibitory interneurons. Photomicrographs (magnification 40×) of the amygdala in the AP -1.06, centered on ML + or - 2.65 and DV 4.50 relative to bregma were triple labeled with A) the immediate-early gene product early growth response protein 1 (EGR1, Zif268, NGFI-A, Krox20, or TIS2), B) the GABA neuron calcium-binding protein Parvalbumin (PV), and C) the nuclear marker 4′-diamidino-2-phenylindole (DAPI). D) The EGR1/PV/DAPI triple labeling is visible in the merged image; scale bar equals 50 μm. This confluence of labeling, particularly the presence of EGR1, is only evident in Stay mice that have been injected icv with the Orx2 agonist [Ala15, d-Leu11]-OrxB. In PV-positive neurons E) only in Stay mice treated with Orx2 agonist [Ala15, d-Leu11]-OrxB was the EGR1 labeling significantly increased (dark gray right-hatched bar) compared to cage control (clear bar), vehicle (dark gray bar) and Orx2 antagonist (MK-1064)-treated mice (dark gray left-hatched bar). F) In PV-negative neurons, a much smaller, but similar increase in EGR1 labeling occurred. G) Labeling of PV was similar in all treatment groups.
significantly greater than that of Stay phenotype mice. In mice with the Stay phenotype, more time was spent near the empty container than near the social target (Fig. 7A), suggesting that Stay mice are susceptible to stress, anxious and depressive behavior. Approach and avoidance behaviors are regulated by parallel reciprocal orexinergic circuits to limbic regions such as the bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN) and amygdala (Giardino et al., 2018). Elevated plasma corticosterone from samples taken on the day of SIP to test stress, anxious and depressive behavior. Approach and avoidance (Stay) phenotype, more time was spent near the empty container than significantly greater than that of Stay phenotype mice (Fig. 7C).

5. Conclusions

Consistent with our previous work, the results reported here indicate that Orx2 receptor activity, in contrast with Orx1 receptor actions, are anxiolytic and antidepressive (Arendt et al., 2013). Results that devolve specifically from social interactions in the SAM arena, including Orx2 activation that reduced cued and conflict fear conditioned freezing, reduced startle responses, increased attentiveness to the escape route, and promoted stress resilience, as well as recruitment of a discrete cluster of Ic or BLA GABAergic Pv-containing inhibitory interneurons, suggest that Orx2 receptors, putatively expressed in these cells, are well placed to promote anxiolysis and reduce depressive behavior. Corroborating evidence comes from Orx2 antagonist which reversed the Escape phenotype and increased plasma corticosterone in Stay mice. Taken together, data from agonist and antagonist give credence in multiple formats for this assertion. What is more, the SAM-specific results are supported and validated by the classic social defeat model measure of susceptibility and resilience (SIP) test. As such, we suggest that the use of the SAM can be applied to evaluate social susceptibility and resilience in a shorter period of time with more behavioral valuations to verify the outcome. Finally, given that measurements from both SAM and SIP suggest that Orx2 receptors mediate anxiolytic and antidepressive actions, targeting these receptors may lead to new potential pharmacotherapies.

Author contributions

C.D.S., J.D.W.Y., T.R.S. and C.H.S. designed research; C.D.S., J.D.W.Y., D.K., F.H., B.K.S., M.S., S.S.N., and C.H.S. performed research; C.D.S., J.D.W.Y., D.K., F.H., B.K.S., J.S.F., T.R.S., S.S.N., and C.H.S. analyzed data; C.D.S., J.D.W.Y., T.R.S., S.S.N. and C.H.S. wrote and edited the paper. All authors approved the final version for publication.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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