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Jazmine D W Yaeger

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**MODULATION OF STRESS-INDUCED BEHAVIORS THROUGH OREXINERGIC  
SIGNALING IN THE BASOLATERAL AMYGDALA**

By

Jazmine DW Yaeger

M.S., University of South Dakota, 2017


A Dissertation Submitted in Partial Fulfillment  
of the Requirements for the Degree of Doctor of Philosophy

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Department of Biology

Neuroscience Program  
In the Graduate School  
The University of South Dakota  
December 2021

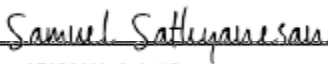
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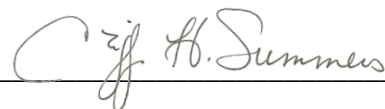
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## ABSTRACT

Stress initiates behavioral disturbances, which are often seen as symptoms of psychiatric disorders, like post-traumatic stress disorder (PTSD), depression, and anxiety. While stress is involved in the formation of disordered states, only certain individuals are vulnerable to, and therefore experience, these outcomes. Further, females are more likely to be diagnosed with stress-induced psychiatric disorders. Elements within stress neurocircuitry offer insight into differential behavioral outcomes associated with stressful experiences; and the basolateral amygdala (BLA), where pro- and anti-stress signals are integrated, is likely an important mediator in phenotype development. The orexin system, too, while being strongly associated with sleep, motivation, and arousal, is critical for directing stress-induced responses. Produced in the hypothalamus, orexins ( $Orx_A$  and  $Orx_B$ ) are released into the BLA where they target and activate two receptor subtypes:  $Orx_1R$  and  $Orx_2R$ . These receptors are found on different cells within BLA microcircuits, with  $Orx_1R$  predominantly being localized to glutamatergic neurons and  $Orx_2R$  having slightly higher expression in GABAergic cells. Pharmacological inhibition of  $Orx_1R$  in the BLA rescues resilient behavior in stress vulnerable mice, while reducing fear freezing behavior, and promoting social learning. Alternatively,  $Orx_2R$  inhibition in the BLA upsets fear learning in resilient populations, but enhances social avoidance. Alternatively, activation of  $Orx_2R$  in BLA cells reduces fear freezing and increases social preference. Female mice exhibit unique behavioral patterns as a result of social stress compared to males, but phenotypic responses are observed when females are administered an  $Orx_2R$  antagonist. While females have higher  $Orx_2R$  expression in the BLA compared to males, pharmacological intervention with an  $Orx_2R$  antagonist reveals even further distinctions within female behavioral phenotypes. Together, these results suggest the orexin system is important for defining behavioral outcomes after stress, and while sexual dimorphism exists in behavior and physiology, orexin receptor activity in the BLA appears to be a critical gating mechanism in both male and female stress-induced phenotype development.

Dissertation Advisor



Cliff H Summers, PhD  
Nolop Distinguished Professor



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This dissertation is dedicated with love to Delores & Harold Ward.

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## **Chapter 1: Counterbalanced microcircuits for Orx<sub>1</sub>R and Orx<sub>2</sub>R regulation of stress reactivity**

### **ABSTRACT**

Orexins are hypothalamic neuropeptides regulating a range of behaviors broadly associated with sleep, motivation, and feeding. These responses highlight the importance of orexins in maintaining foundational biological processes, but also indicate a connection to stress-related dysfunction, which results in aberrations to normal states of sleep, motivation, and feeding. As such, we predict, based on clinical and preclinical evidence, that irregularities in orexin signaling contribute to changes in affect and the formation of psychological disorders. In support of this, orexin-producing neurons innervate several brain areas important for mediating stress responses, including the prefrontal cortex and amygdala, where intracellular signaling results from activation of orexin receptors (Orx<sub>1</sub>R and Orx<sub>2</sub>R). While stimulation of Orx<sub>1</sub>R and Orx<sub>2</sub>R initiate similar intracellular pathways, signaling dynamics may be modified through receptor location, dimerization, or genetic regulation. We further note, based on evidence from our lab, that Orx<sub>1</sub>R and Orx<sub>2</sub>R elicit opposing stress responses after activation, suggesting the existence of a counterbalanced mechanism for inducing physiological and behavioral stress states. Our research and others' demonstrate that antagonistic neurocircuits promoting either pro- or anti-stress responses, may be bidirectionally shifted with activation of Orx<sub>1</sub>R or Orx<sub>2</sub>R. Although clinically approved drugs that target the orexin system, like dual orexin receptor antagonists (DORAs), are moderately effective for the treatment of sleep- and, perhaps, addiction-related disorders, they may inadvertently disrupt mood or exaggerate existing affective dysfunction. We propose a novel idea for pharmacological intervention that accounts for the counterbalanced influence of orexin receptor activity on stress-induced behaviors: selective orexin receptor

crossover drugs (SORCOs). These SORCOs are hypothesized to potently shift signaling biases and restore balance in stress neurocircuitry.

## **OREXINS – HYPOCRETINS**

A novel pair of related neuropeptides/hormones was discovered in 1998 by the de Lecea, Kilduff, Sakurai, Sutcliffe, and Yanagisawa labs [1, 2] and given different names – hypocretins (Hcrt) or orexins (Orx) – based on what each lab was investigating. The name “orexin” refers to the molecule’s role in feeding and appetitive behavior (from the Greek orexis, “appetite”); “hypocretin” comes from its site of synthesis (hypothalamus) and from a molecule with which it shares homology (secretin). Much of the early work by these labs focused on excitatory function in feeding, arousal, and sleep-wake regulation [3-13]. The two orexins, or two hypocretins, Or<sub>X<sub>A</sub></sub> (Hcrt1) and Or<sub>X<sub>B</sub></sub> (Hcrt2), are cleaved equally from a single prepro-orexin peptide [2], and bind to two receptor subtypes derived from separate genes, Or<sub>X<sub>1</sub></sub>R (gene: HcrtR1) and Or<sub>X<sub>2</sub></sub>R (HcrtR2) [14]. However, binding affinity of Or<sub>X<sub>A</sub></sub> is high for both Or<sub>X<sub>1</sub></sub>R and Or<sub>X<sub>2</sub></sub>R, but is 5-100 times greater than Or<sub>X<sub>B</sub></sub> for the Or<sub>X<sub>1</sub></sub>R [14, 15]. The Or<sub>X<sub>2</sub></sub>R is bound and activated equally well by both Or<sub>X<sub>A</sub></sub> and Or<sub>X<sub>B</sub></sub> [15].

Synthesis of the Or<sub>X<sub>A</sub></sub>/Or<sub>X<sub>B</sub></sub> neuropeptides is limited to the perifornical region of the hypothalamus [16, 17], and is functionally divided into lateral (LH) and dorsomedial-perifornical (DMH/PeF) subgroups [18]. Importantly, synthesis of Orx, and activation of LH-DMH/PeF Orx neurons is greater in females [19, 20]. Most orexinergic neurons colocalize the excitatory amino acid transmitter, glutamate (Glu), the inhibitory opiate, dynorphin (Dyn), and the neuropeptides, neurotensin and neuronal activity-regulated pentraxin (NARP), which modify and increase the potential actions of these neurons [21-28]. Additionally, orexin-producing

neurons are commonly found in close proximity to cells that synthesize melanin-concentrating hormone (MCH), which often has opposing actions [16, 29] and those that serve a similar function, but express pyroglutamylated arginine-phenylalanine-amide peptide (QRFP) [30]. Although orexin perikarya are located exclusively in the hypothalamus, orexinergic projections and receptors are distributed throughout the brain [31, 32]. The LH-DMH/PeF orexinergic system has broadly projecting axons and terminals, innervating numerous limbic and cortical structures, such as the prefrontal cortex (PFC), nucleus accumbens (NAc) shell, septum, hypothalamus (paraventricular [PVN], anterior [AH], arcuate [ARC], dorsomedial [DMH], and ventromedial [VMH] nuclei), bed nucleus of the stria terminalis (BNST), thalamus, and amygdala [17, 33, 34]. These projections are reciprocated, with limbic and cortical regions sending numerous afferents to orexinergic neurons [35, 36]. Reciprocal connections with such a comprehensive assortment of brain regions may explain how orexins are implicated in such a wide variety of physiological and behavioral functions [3, 14].

Orexin projections, functioning by means of Orx<sub>1</sub>R and Orx<sub>2</sub>R, promote stimulatory (for the most part, see Receptor intracellular signaling and dimerization) influence [37] via activation of coupled G proteins [14], which in turn promote downstream signaling pathways like cyclic adenosine monophosphate (cAMP), phospholipase C (PLC), and extracellular signal-regulated kinase (ERK) [14, 38, 39]. Orexin receptors have also been demonstrated to modulate molecular systems that control neural plasticity, including Protein Kinase B (Akt), mammalian target of rapamycin (mTOR) intracellular signaling [40, 41], and brain-derived neurotrophic factor (BDNF) expression [39, 42, 43]. Additionally, excitatory functions of Orx may be enhanced by Glu release as a co-transmitter, or inhibited by Dyn co-transmission [24-26].

While much of the early work on Orx focused on excitatory roles in arousal, sleep-wake

regulation, and feeding [3-13], Or<sub>XA</sub> and/or Or<sub>XB</sub> have also been implicated in a wide variety of physiological and behavioral functions [3, 14], which include learning [44-51], reward [18, 52-56], and stress [46, 57-61]. As stress has significance in many other orexin-linked actions such as food seeking [62, 63], reward [18, 52-56], biorhythms [64-67], and arousal [5, 55, 59, 68], it may be that orexin's most important roles involve "stress responsiveness." Given that chronic stress is a reliable predictor of depression and may be necessary for its establishment [69-71], manipulation of the Orx system via Or<sub>x1R</sub> or Or<sub>x2R</sub> [14] could prove to hold therapeutic value in treating depression and similarly pernicious affective disorders [72], as they have pro-depressive and anti-depressive effects, respectively [73, 74]. It is important to note that while Or<sub>x1R</sub> and Or<sub>x2R</sub> may have opposing actions relative to stress-regulation and affect in some regions of the brain (see sections below), in other regions they may work in concordance relative to these functions [75, 76].

## **QUALITIES OF OREXIN RECEPTORS**

Difficult to consider, and often ignored when discussing influence over the signaling balance of neurocircuits, are certain receptor features like location, intracellular signaling, dimerization, and genetic regulation. While not easily evaluated for their functional contributions to microcircuit tone, these receptor capacities support changes in neural signaling and behavioral output. Some of these receptor attributes will go unacknowledged in our own predicted description of Or<sub>x1R</sub>-Or<sub>x2R</sub> interplay (see COUNTERBALANCED Or<sub>x1R</sub> VS Or<sub>x2R</sub> MODULATION), but here we recognize Orx receptor dynamics as multifaceted and important instruments for managing stress responsiveness.

## Receptor location

Unlike Orx signaling peptides (Orx<sub>A</sub> & Orx<sub>B</sub>), which are processed from a single prepro-orexin molecule [2], Orx receptors are not bound by shared transcriptional and translational processes. As such, Orx<sub>1</sub>R and Orx<sub>2</sub>R are not always found together, with mRNA [31, 33, 77] and protein [77, 78] distributions varying based on brain location. This differential expression suggests distinct physiological functions of the Orx receptor subtypes.

Even more specifically, Orx receptors are found on different neuronal populations. Examples of Orx receptors concentrating on glutamatergic [79-83] and GABAergic [82, 84, 85] neurons have been reported. Guiding system tone, Orx receptors also function directly on serotonergic cells [86, 87], adrenergic neurons [88, 89], histamine-producing cells [90], dopaminergic neurons [91], microglia [92], and astrocytes [93]. In hypothalamic cell populations, Orx<sub>1</sub>R is co-localized with numerous peptides, including Orx, corticotropin releasing factor (CRF), and MCH [94]. Furthermore, orexin-producing neurons express Orx<sub>2</sub>R, which may initiate a positive feedback mechanism for enhancing broadly dispersed orexinergic tone [95]. However, more recent evidence suggests that Orx neurons do not express autoreceptors [96], suggesting that feedback requires indirect mechanisms. These examples demonstrate a selective influence of Orx signaling over specific neural systems and cell types that may collectively work to balance physiological and behavioral responses.

Still more precise, Orx receptors can function on various locations on neurons. For example, in the central amygdala (CeA) [79] and PFC [80, 81], Orx receptors have been suggested to mediate glutamatergic signaling presynaptically. However, Orx receptors appear to also regulate cell activity from dendrites and cell bodies [87, 95, 97]. Together, these results demonstrate a diverse profile of Orx receptors to control neurophysiological responses through

regional specificity at both the tissue and cellular levels. Signaling characteristics of Orx<sub>1</sub>R and Orx<sub>2</sub>R may further expand influence over intracellular mechanisms.

### **Receptor intracellular signaling and dimerization**

Receptor signaling cascades are intricate and while we will not go into immense detail (see Kukkonen & Leonard, 2014 and Leonard & Kukkonen, 2014 for reviews), it is possible that some features associated with orexin's intracellular signaling dynamics derive from unique qualities of the Orx<sub>1</sub>R and Orx<sub>2</sub>R subtypes, and potentially from cellular mechanisms that modify the cascade, such as dimerization. Noteworthy, Orx receptor signaling cascades, as well as those from other G protein-coupled receptors (GPCR), are not fully understood, in part, due to limitations in methodologies [98]. However, we will describe some broad qualities of Orx receptor signaling (Fig. 1) with the caveat that a complete understanding of the underlying signaling mechanisms remains unknown.

As stimulation of Orx receptors results in elevated Ca<sup>2+</sup> [2, 39] and PLC activation [99], it is presumed that they work as GPCRs of the G<sub>q</sub> variety. In support of this, Orx<sub>1</sub>R stimulation in the prelimbic region of the PFC (PrL) leads to activation of phosphokinase C (PKC) and the suppression of hyperpolarization-activated/cyclic nucleotide (HCN) currents [97]. However, in human adrenal tissue, Orx<sub>2</sub>R may couple with G<sub>q</sub>, G<sub>s</sub>, or G<sub>i</sub> [100, 101]. In the dorsal raphe (DRN), locus coeruleus (LC), and pontine reticular nuclei, Orx<sub>A</sub> promotes coupling of G<sub>i</sub>; an effect that is suppressed with an Orx<sub>1</sub>R antagonist [102]. Further, hypothalamic Orx receptors can couple with G<sub>q</sub>, G<sub>s</sub>, G<sub>o</sub>, or G<sub>i</sub>; and during food deprivation, coupling preference shifts to favor G<sub>q</sub>, G<sub>s</sub>, or G<sub>o</sub>, over G<sub>i</sub> [103]. Specificity and coupling of G proteins may be dependent on ligand concentration, with G<sub>i</sub> and G<sub>q</sub> pathways being preferred and G<sub>s</sub> contributing only when Orx levels are elevated [104]. Interestingly, bound Orx<sub>1</sub>R is also capable of interacting with β-



arrestins, prompting receptor internalization [105, 106]. Also, activation of Orx<sub>1</sub>R or Orx<sub>2</sub>R in mouse hypothalamic neurons triggers signaling cascades through the mTOR pathway [40, 41], which may stimulate cell growth and neuroplasticity.

Cell signaling initiated by Orx receptor activation, while plastic in the ability to trigger multiple downstream effectors, is further diversified during dimerization with other GPCRs where signaling cascades may be altered (Fig. 1). Orexin receptor subtypes can form homodimers/oligomers [107]. Curiously, Orx<sub>1</sub>R homodimers/oligomers may be abundant at stable cellular states, where receptor activation leads to more dimerization and inhibition promotes separation into the monomer formation [108]. Splice variants of Orx<sub>2</sub>R (Orx<sub>2</sub>R $\alpha$  & Orx<sub>2</sub>R $\beta$ ), with distinct C-terminus regions, also dimerize, resulting in enhanced Ca<sup>2+</sup> signaling [109].

It is important to note that most therapeutic drugs have not been assessed for binding affinity to receptor dimers. Understanding the potential dimerization of these receptors is critical for pharmacotherapeutics since these dimers may either modify the desired actions of drugs or be required for their actions. Several heterodimers form as a result of Orx<sub>1</sub>R interacting with other GPCRs, including Orx<sub>2</sub>R [107], Cb<sub>1</sub> [107, 110, 111], pyroglutamylated RFamide (QRFP; an orexin-like peptide) receptor [112], CRF1 [113], CRF2 [114], kappa opioid receptor (KOR) [115], cholecystinin type 1 (CCK1) [116], and ghrelin receptors, GHSR1a [117] and GHSR1b [118]. Though not as heavily explored, Orx<sub>2</sub>R has been demonstrated to form heterodimers with Cb<sub>1</sub> [107], QRFP receptor [112], and 5-HT<sub>1A</sub> [119]. While the neurophysiological significance of GPCR dimers remains uncertain, they are, perhaps, functioning in a way that mediates cell signaling and downstream transcriptional changes. Further, it is not clear what effect genetic variation imposes on receptor dimerization capabilities [120].

## Receptor genetics

In humans, genetic variants of both Orx<sub>1</sub>R and Orx<sub>2</sub>R have been linked to stress-related dysfunction. For example, the Orx<sub>1</sub>R gene (HcrtR1) variant that leads to an amino acid substitution at the 408th position (Ile408Val) has been linked to increased mood disorders [121, 122], elevated stress-induced aggressive behaviors [123], and polydipsic schizophrenia [124]. Similarly, the HcrtR2 variant that results in an amino acid substitution at the 308th position (Val308Ile) is associated with panic disorders in females [125] and nicotine dependence [126]. Other HcrtR2 variants were discovered in patients experiencing daytime sleepiness (Pro10Ser) or Tourette's syndrome (Pro11Thr) symptoms [127]. Although it is unknown whether variants of Orx receptors are functional [120], it is clear when the Orx system is disrupted, stress-induced disorders become more prevalent.

Changes in transcriptional control of Orx receptors has been demonstrated in many systems, but we focus on just a few examples related to stress and stress-provoked behavioral abnormalities. Unpredictable chronic mild stress (UCMS) in female rodents increases Orx<sub>1</sub>R mRNA expression [128], while chronic alcohol reduces Orx<sub>1</sub>R mRNA in the PFC [129]. Our own lab demonstrated that Orx<sub>1</sub>R mRNA in the PrL does not change with ten days of social defeat [130]. Single prolonged stress induces upregulation of Orx<sub>1</sub>R in both the hypothalamus and hippocampus [131]. Further, we have demonstrated that social stress enhances Orx<sub>1</sub>R and reduces Orx<sub>2</sub>R mRNA in the basolateral amygdala (BLA) of (susceptible) mice that demonstrate social avoidance behavior in the Social Preference/Interaction (SIP) test [130], or animals that demonstrate social avoidance behavior in the Social Interaction/Preference (SIP) test. In humans, males that have committed suicide exhibit elevated Orx<sub>2</sub>R mRNA in the anterior cingulate cortex (ACC) [128]. Interestingly, HcrtR2 expression undergoes diurnal fluctuations

in both the hypothalamus and cortex (Hcrtr1 shows this expression pattern in only the cortex), and is correlated with expression patterns of Bmal1, a gene important in establishing circadian rhythms [132]. Together, these examples highlight the intricacies of homeostatic and physiological balance during periods of stress; they identify Orx receptors as important contributors for establishing neural and behavioral normalcy.

## **OREXINS AND PSYCHIATRIC DISORDERS**

The critical element in the relationship between Orx activity and psychiatric disorders is stress [72]. Stress responsiveness is a crucial factor in the promotion of dysfunctional affect and maladaptive behavior in numerous psychological conditions including attention deficit disorder (ADD), anxiety disorders, autism spectrum disorders, bipolar disorder, major depressive disorder (MDD), drug addiction, eating disorders, obsessive-compulsive disorder (OCD), panic, post-traumatic stress disorder (PTSD), reactive attachment disorders, schizophrenia, and sleep disorders [133, 134]. It is significant that females and males respond differently to stress [135], with women reported to have twice the rates of affective disorder diagnoses [136, 137]. In addition to significantly more women being diagnosed with stress-promoted behavioral disorders [138] such as major depression, high comorbidity with anxiety exacerbates this problem [139, 140]. What is more, human neuroimaging and animal studies suggest that neural atrophy and other structural deficits, which play key roles in these disorders, are exacerbated by stress [141-149]. The most intense stressors experienced by humans are social [150-153]. As social stress is the most intensive [154] and unpredictable [155] of stressors, it specifically promotes maladaptive behavior, including depression, anxiety, PTSD, sociophobia, loss of self-esteem, and other behavioral symptoms in humans [151, 153]. The incidence rates of adult affective

disorders steeply rise during adolescence in parallel with a structural and functional reorganization of the neural circuitry underlying stress reactivity [156-160]. Further, stress circuits and neuromodulatory factors are predisposing factors for human depression [161-163] and anxiety [161, 162]. Orexin fibers heavily innervate stress-related brain regions important for stress-induced affective disorders, including the amygdala [62, 164, 165]. This structure's connectome [34, 58, 164] implicates roles for Orx in arousal [1, 18, 55, 58, 166-168], food regulation [2-4, 8, 10, 47, 62], and reward [18, 56]; but because these functions are relevant to affective disorders, Orx is also likely to be involved in fear, anxiety [57, 130, 169-172], and depression [73, 173-178]. Social stress is strongly influenced by Orx and Orx receptor actions, and these data are strongly suggestive of their direct involvement in psychological disorders [72, 179].

### **Clinical studies and trials**

Orexins appear to play a role in many psychological disorders [180-183]. Orexinergic cell function is reliably dysregulated by depression [184]. In depressed patients, mean cerebrospinal fluid (CSF) Orx levels are elevated, but also show reduced diurnal fluctuation [182]. Depressed patient CSF orexin levels, however, are diminished after attempted suicide [180, 181]. Human brain, blood, and CSF levels of orexins fluctuate with steady state disruptions occurring as a result of childhood mistreatment [185], anxiety [172, 185], sleep disorders [186-194] panic [172], schizophrenia [195, 196], traumatic brain injury [197, 198], Alzheimer's Disease [186, 199, 200], and heart disease [201], as well as by depression and suicide [180-182, 185]. These reports together suggest that reduced Orx is critically associated with depression and preclinical depressive behavior [184, 202, 203]. This interpretation of these data is consistent with reported effects in animal models, in which, orexins prevent depressive behavior by promoting stress

resilience (see section Preclinical evidence) [84, 177, 204-207].

There appear to be clinically relevant sex differences in Orx function with respect to stress and affect [208]. While there are no significant differences in plasma Orx<sub>A</sub> in healthy younger women and men [209], among older subjects, women have higher levels of Orx<sub>A</sub> in CSF than men [210]. Interestingly, narcolepsy which is associated with profound reductions in neural orexinergic function, does not distinguish patients' plasma levels of Orx<sub>A</sub>, which are more commonly male, from those without narcolepsy [211]. Sleep disturbance appears to be divided by sex along lines associated with Orx function. The probability of reduced Orx function and greater narcolepsy in males, and potentially increased Orx function leading to insomnia in females [212, 213], are suggestive of brain Orx systems differentiated by sex. Female patients with major depression exhibit elevated Orx<sub>A</sub> in PFC [128], which is not seen in healthy females or males [128, 209]. Interestingly, these female patients were postmenopausal, suggesting that any sex differences were not due directly to sex hormone actions, although indirect long-term effects may have contributed the differences. The relationship between Orx function and affective disorders seems to be worth further investigation.

However, while Orx receptor antagonist drugs have been approved for treatment of insomnia [214, 215], virtually no large-scale clinical examinations of Orx<sub>1</sub>R- or Orx<sub>2</sub>R-related drug effectiveness specifically for psychiatric disorder treatment has been undertaken. The dual orexin receptor antagonist (DORA) sleep-promoting drug, suvorexant (Belsomra), is clinically approved as a treatment to encourage somnolence by reducing arousal and wakefulness [216]. It will be important to clinically examine its effects on stress and affect. Suvorexant lowers stress hormone levels of cortisol and norepinephrine, and reduces the severity of anxiety and depression in psychiatric patients with insomnia [217], while effectively reducing sleep onset

times and increasing sleep duration, without rebound or withdrawal [218]. These results are consistent with the Orx<sub>1</sub>R antagonist activities of the drug, but different from those expected for its Orx<sub>2</sub>R antagonist actions [179]. Interestingly, in a preclinical trial of the competitive Orx<sub>1</sub>R + Orx<sub>2</sub>R antagonist, almorexant, a DORA that had been in phase II clinical trials, anxious and depressive behavior were reduced to the same extent as with the antidepressant, fluoxetine [219]. As chronic insomnia is highly comorbid with affective disorders, and is associated with up to a 4-fold increased risk of developing major depression [220-222], it seems appropriate to test interactions between drugs, such as DORAs, taken for insomnia and antidepressants [223]. Drug interactions between desipramine, a tricyclic antidepressant (TCA), and almorexant, revealed that the enzyme primarily responsible for metabolism of the antidepressant is inhibited by almorexant. Not surprisingly, almorexant increased exposure to desipramine by nearly four times, whereas the antidepressant had no relevant pharmacokinetic effects on almorexant. There was, however, a slight increase in calmness in patients using almorexant. Regrettably, this DORA with possible capacity for lowering affective symptoms was removed from clinical trials based on its safety profile [224]. Filorexant (MK-6096) is another dual Orx receptor antagonist in phase II trials for patients with major depression, but these were terminated early, without showing a significant difference in depression rating [225]. One of the two most common adverse events for filorexant was suicidal ideation, which is common in this type of patient.

Recent development and clinical trials for two new single orexin receptor antagonist (SORA) drugs have presented potentially promising treatments for anxiety and depression through Orx<sub>1</sub>R or Orx<sub>2</sub>R inhibition, respectively. The clinical potential for limiting Orx<sub>1</sub>R activity has been successfully examined in phase I trials, with the Orx<sub>1</sub>R antagonist, ACT-539313, developed specifically for the purpose of reducing anxiety [226, 227]. The other SORA drug is an Orx<sub>2</sub>R

antagonist, seltorexant JNJ-42847922/MIN-202, designed to reduce insomnia [178, 228-230], which was demonstrated to be safe and effective in clinical phase 1, 1b and 2 trials for that purpose. Importantly, this Orx<sub>2</sub>R antagonist was also demonstrated to reduce self-reported depression in some of these small sample trials. The potential antidepressant action of this Orx<sub>2</sub>R antagonist runs counter to the primary hypothesis of this paper, that Orx<sub>2</sub>R stimulation inhibits stress-responsive neurocircuitry to limit anxious and depressive behavior. A different Orx<sub>2</sub>R antagonist, MK-1064, delivered icv or directly into the BLA of the mouse brain, increases stress responsiveness and behavior [179]. We are excited by the development of these new SORA drugs, and are anxious to test their activity on stress-related neurocircuitry with objective measures of behavior in our animal model (see COUNTERBALANCED Orx<sub>1</sub>R VS Orx<sub>2</sub>R MODULATION). This kind of comparison will be necessary to help determine the value of our suggestion of a clever new type of drug or combination of drugs awaiting drug discovery, selective orexin receptor crossover (SORCO), which makes use of the opposing functional effects of Orx<sub>1</sub>R and Orx<sub>2</sub>R actions [72], which was derived solely from pre-clinical studies.

### **Preclinical evidence**

The effects of Orx<sub>A</sub> peptide action in emotion-related regions of the brain (sometimes via systemic delivery) promote anxious behavior or anxiety [204, 231-234]. The actions of Orx<sub>A</sub> often shows similar effects to those of Orx<sub>1</sub>R agonists, suggesting that the more common Orx<sub>1</sub>R binding is the primary transducer for Orx<sub>A</sub>. Effects of whole brain (intracerebroventricular [icv]) infusion of Orx<sub>A</sub> in mice increases anxiogenic behaviors in the Light-Dark Test (LDT) and Elevated Plus Maze (EPM) [235]; while effective pharmacological reversal (using antagonists selective for dopamine or serotonin receptors - haloperidol, ritanserin, or metergoline) of the anxious behaviors stimulated by icv injection of Orx<sub>A</sub> or Orx<sub>B</sub> is highly dependent on the drug's

specificity for monoaminergic receptors [236]. Stress induced through corticosterone administration in mice promotes anxious responses in EPM, commensurate with increased Orx<sub>A</sub> [237]. Furthermore, orexin-deficient mice exhibit reduced reactivity to foot shocks, heightened anxious behavior in open field (OF) and LDT paradigms, and an increased fear response to predatory odors [204]. In contrast, optogenetic excitation of Orx cells in rats increases anxiogenic aversion for a social target, enhances exploratory behaviors, and results in the internalization of Orx<sub>1</sub>R in the paraventricular thalamus (PVT) and locus coeruleus (LC) [233]. Optogenetic stimulation of mouse hypothalamic Orx neurons or terminals activates the noradrenergic LC, which when those Orx terminals in LC are optogenetically stimulated results in enhanced fearful threat learning [238] and fear generalization [89, 239]. Interestingly, optogenetic studies reveal the LC is one of the structures that Orx stimulates to promote wakefulness [88, 240]. A combination of optogenetic and chemogenetic tools helped identify another circuit associated with anxious behavior from GABAergic CRF and cholecystokinin neurons in the BNST to hypothalamic Orx neurons [241]. In this circuit, optogenetic and chemogenetic stimulation of CRF or CCK BNST neurons projecting on to Orx neurons increased anxious behavior. Additionally, optogenetic activation of Orx neurons in the LH that target the PVN is sufficient to stimulate hormone secretion from the HPA axis [242]. These data suggest the hypothalamic orexinergic system is a hub for arousal of stress responses and motivation [243, 244].

Chronic corticosterone-induced stress also promotes depressive reactions in Tail Suspension Test (TST) trials in mice, but further is associated with increased Orx<sub>A</sub>-containing cells in the hypothalamus [237]. Wistar-Kyoto rats, a strain demonstrating depressive behaviors and disrupted sleep patterns, possess fewer Orx<sub>A</sub>-expressing neurons, and these cells have a reduced



size in comparison to Wistar control rats [205]. Contrastingly, in a genetic animal model of depression, the Flinders Sensitive Line, female mice exhibit an elevated number of Orx neurons in the hypothalamus [173]. Following traumatic stress, in a predator-odor model of PTSD, hypothalamic Or<sub>XA</sub> and Or<sub>XB</sub> levels are significantly lower in individuals with extreme behavioral disruption (PTSD-phenotype) compared to those with minimal disruption, who display up-regulated Or<sub>XA</sub> and Or<sub>XB</sub> [245]. Similarly, social defeat-induced depressive behavior in rats is accompanied by a reduction of Or<sub>XA</sub> and Or<sub>XB</sub> in tissue samples taken from the ventral tegmental area (VTA), medial prefrontal cortex (mPFC), and hypothalamus [246]. Levels of Or<sub>XA</sub> and Or<sub>XB</sub> may be influenced by age as well as affective state. In the clomipramine-induced depression model, juvenile Long-Evans rats exhibit reduced Orx levels, but in adults they are significantly enhanced [247]. Administered into distinct areas of the stress circuit, such as the BNST [234], CeA [169], and BLA [82], Orx produces anxious and depressive behavior. In addition, chronic social defeat epigenetically reduces prepro-orexin mRNA, but calorie restriction thereafter enhances activation of Orx cells, which results in an antidepressive response [177]. Similarly, early life stress dampens restraint-stimulated Orx cell activity, and produces a depressive behavioral phenotype, all of which can be reversed by exercise in adolescent male rats [248]. These reports together suggest that reduced Orx is critically associated with depression and preclinical depressive behavior [203, 249, 250]. What is more, in genetic models of depression, reduced Orx levels have also been measured [205, 206].

Downstream of Orx neurons, and Or<sub>XA</sub> + Or<sub>XB</sub> release, most work in animal models has focused on the effects of Or<sub>X1R</sub> actions, which influence emotion-related regions of the brain (sometimes via systemic delivery) to promote anxious behavior or panic [57, 171, 172, 204, 231-234, 251]. In some regions of the brain, like the CeA, the effect of Or<sub>XA</sub> to produce anxious or

conditioned fear behavior appears to depend only on the Orx<sub>1</sub>R [79, 252]. Rodents bred with the Orx<sub>1</sub>R gene knocked out exhibit increased anxious responses [207], reduced depressive behaviors [74, 207], and impaired fear conditioning in reaction to cued and contextual stimuli [89, 253]. Injections of SB-334867 (intraperitoneal [ip]), a potent selective antagonist of Orx<sub>1</sub>R, can mimic the response of Orx<sub>1</sub>R null mice, showing reduced depressive reactions in tests of behavioral despair (forced swim test [FST] and TST) [74] and fear conditioning paradigms [253]. Intra-amygdalar injections of Orx<sub>1</sub>R antagonist diminishes anxiety and fear conditioning, but also decreases memory acquisition in a rodent model of PTSD [254]. In contrast, SB-334867 (ip) inhibits the reported antidepressive actions of whole brain (icv) Orx<sub>A</sub> in the FST, and prevents orexin-induced proliferation in the dentate gyrus (DG) [176].

Interestingly, oral delivery of a dual Orx<sub>1</sub>R/Orx<sub>2</sub>R antagonist (SB-649868) limits elevation of the pituitary stress hormone adrenocorticotrophic hormone (ACTH) stimulated by mild cage exchange stress, but not as effectively as a selective Orx<sub>2</sub>R antagonist (JNJ-42847922), or in Orx<sub>2</sub>R knockout animals [255]. The authors suggest that the results are consistent with predominant Orx<sub>2</sub>R expression in the PVN [31, 33], however, the systemic delivery makes it unclear whether the effects were neural or hormonal.

Again, it is likely that animal models will reveal sex differences in Orx function as an important component of stress-related responses [208]. While plasma levels of estrogens appear to correlate positively with Orx neural activation, those of testosterone do not [208]. Further, there appears to be a relationship between estrous cycle and expression (both mRNA and protein) of orexins and their receptors [256-258], but neither orchidectomy nor estrogen or testosterone replacement has an influence on Orx neuronal transcripts of prepro-orexin or Orx receptors [20], perhaps explaining why other studies have not measured a relationship between

estrous cycling and hypothalamic Orx mRNA [259]. These data suggest that direct influences on Orx synthesis due to reproductive cycling may be extra-hormonal, but Orx neuron activity is enhanced by estrogens. Nevertheless, prepro-orexin mRNA is twice as high in female compared to male rats [20], plus elevated hypothalamic Orx neuron activation along with higher CSF Orx<sub>A</sub> concentrations in females [19]. Additionally, Orx<sub>1</sub>R and Orx<sub>2</sub>R mRNA have been shown to be higher in female PVN and whole hypothalamus compared to males [20, 260]. Therefore, there is ample evidence to suggest that there are sex differences in Orx function, that Orx plays a role in affective disorders, and that those psychological disorders are influenced, even driven, by stress.

## **OREXINS IN STRESS**

Orexins are important for arousal and for the reactive transition to coping with stressful provocations because they play a critical role in modulating the neural systems that respond to stressful stimuli [208, 261]. Stressful conditions potently modify Orx and Orx receptor expression in the brain [254]. Beginning in the hypothalamus, where orexins are made in the LH-DMH/PeF, these neurons are activated by acute stress [73, 262, 263], producing elevated plasma and CSF levels of Orx<sub>A</sub> [262, 264]. Additionally, orexins have a cross-connected interactive relationship with the primary stress neuropeptide CRF [58, 59, 265-267]. Specific GABAergic CRF and CCK neurocircuitries from the BNST activate hypothalamic orexinergic neurons, and influence emotional and stressful outcomes [241]. In addition to CRF, Orx neurons link emotional stress to autonomic responses [268]. What is more, persistent pain and stress activate Orx pathways [269]. Stressful behavioral paradigms, such as fear conditioning, increase Orx<sub>1</sub>R in the amygdala [254]. Stress-activated Orx neurons exhibit upregulated expression of BDNF, and another stress regulatory neuromodulator, neuropeptide Y (NPY) [245].

The chief output region of the amygdala is the CeA, which regulates hypothalamic neuroendocrine (hypothalamo-pituitary-adrenal [HPA] axis) function, and extrahypothalamic stress-related functions such as anxiety. In the CeA, Orx<sub>1</sub>R, but not Orx<sub>2</sub>R, activity modifies neuronal depolarization and firing rate [79, 252]. The initial hormone in the HPA cascade, CRF (produced and secreted as a hormone from the PVN and as a neuromodulator in the CeA and BNST) increases Orx neuronal activity [58, 241]. Downstream, Orx<sub>2</sub>R is implicated in stress-induced secretion of adrenocorticotrophic hormone (ACTH) release from the pituitary [263]. Taken together, the evidence from recent experiments suggests that Orx plays an important role in most, if not all, stress-induced responses [58, 59, 68, 265, 270].

## **OREXINS AND STRESS NEUROCIRCUITRY**

Orexinergic innervation of stress-related neurocircuitry is evident in both behaviorally relevant limbic structures and, with significant overlap, limbic plus hypothalamic structures regulating neuroendocrine stress hormone output (see previous section). Applicable to the specific neurocircuitry necessary to produce affective behavior and fear learning, Orx neurons innervate the thalamic terminals in mPFC. They also innervate BLA neurons receiving inputs from mPFC, CeA neurons receiving input from BLA, and periaqueductal gray (PAG) neurons, which receives input from the CeA. Additionally, as the regions of extended amygdala play important roles in anxiety and depression, orexinergic projections to the BNST and the dopaminergic reward circuitry from VTA to NAc and the surrounding ventral striatum may be important for those affective responses. This latter relationship is evident in work linking Orx and stress to addiction [271-276]. Recent evidence points to another specific Orx stress circuit that promotes addiction and relapse, beginning in the Neuropeptide S (NPS) neurons of the Kölliker-Fuse nucleus of the

parabrachial nucleus (PBN) and the region around the LC, which directly stimulate hypothalamic Orx neurons. These Orx neurons project to the VTA, and through binding of Orx<sub>1</sub>R and PLC 2nd messenger, stimulate production of the endocannabinoid 2-AG, which disinhibits VTA dopaminergic neurons by way of Cb1 receptors [277-281]. However, there are specific additional neurocircuits for stress-related activity, from BNST, via GABAergic CRF and CCK neurons, to hypothalamic Orx neurons [241], and then to noradrenergic neurons in the LC [89, 238, 239]. The interactive relationship between stress neurocircuitry and Orx also importantly connects the food-seeking function of the peptide, with both reward and arousal [282-286]. These cross-linked functional roles have suggested that the primary role for Orx is motivational [287, 288]. Further, recent work from our lab suggests Orx acts in decision-making neurocircuitry (which also overlaps stress neurocircuitry) with definitive effects on behavioral choice outcomes [72, 179]. As stress, arousal, reward, anhedonia, and modified decision-making are all critical elements related to the onset of anxiety and/or depression, it seems likely that Orx also plays a role in regulating the neurocircuitry involved in affective disorders [72, 73, 130, 179].

## **MEDIAL PREFRONTAL CORTEX (mPFC) AND AMYGDALA MICROCIRCUITS**

The mPFC has a critical role in cognition, learning, executive control of emotional states, stress-coping strategies, decision-making, and social interaction. Similarly, the amygdala is involved in determining and learning emotional behavior, stress-coping strategies, and behavioral responses to aversive and rewarding stimuli. Even though mPFC and amygdala are involved in many overlapping functions related to emotional learning, the sub-nuclei of these regions display unique patterns of connectivity, suggesting distinct parallel circuits between the

mPFC and amygdala (Fig. 2) that preferentially drive behaviors and emotion-related learning.

### **Prelimbic (PrL) and infralimbic (IL) cortices**

In the rodent brain, the mPFC is composed of three main subregions: anterior cingulate (ACC; Brodmann Area 24 in humans), prelimbic (PrL; BA 32 in humans), and infralimbic (IL; BA 25 in humans) cortices. In these regions, layer V pyramidal neurons provide output to subcortical structures like amygdala, hippocampus, and striatum [289-291]. Additionally, inhibitory interneurons containing gamma-aminobutyric acid (GABA) in the mPFC regulate the outgoing signal of pyramidal neurons, or other interneurons.

Activity in the PrL and IL is predominantly involved in opposing aspects of emotional learning [292]. Together, activation of terminals in the PrL and IL projecting from the BLA causes anxiogenic behaviors and a reduction in social preference; while inhibition of these terminals produces the opposite effect [293]. However, individual activation of the PrL and IL promotes unique responses, suggesting each region serves distinctive functions [294-299]. These response-specific features, perhaps, derive from differential and reciprocal projection patterns with the BLA, NAc, VTA, BNST, dorsal raphe (DRN), LC, hippocampus, hypothalamus, and thalamus [300]. While the PrL primarily mediates cognitive-limbic functions like decision-making, goal-directed behavior, and working memory, IL activity tends to influence visceral/autonomic functions like heart rate, gastrointestinal functions, blood pressure, and respiration [300].

Activity in the PrL is critical for the expression of fear, as well as forming and maintaining fear memories, which can be enhanced or diminished upon PrL stimulation or inhibition respectively [294-297]. Conversely, learning and maintaining behaviors related to reward, including those associated with fear extinction, are increased with stimulation of IL and

suppressed during IL inhibition [294-297]. Important for its role in fear extinction, IL activation can simulate inhibitory interneurons in the ventral intercalated region of the amygdala (vITC), which suppress medial CeA (mCeA) neurons that become disinhibited to trigger fear responses [297, 298].

### **Anterior and posterior basolateral amygdala**

The BLA receives input from brain structures relaying information about external and internal sensory information, memory, and decision-making. This cortex-like structure consists of about ~80-85% glutamatergic pyramidal projection neurons (PNs) and ~15-20% GABAergic inhibitory interneurons [301, 302], which act together to regulate BLA signaling. In the BLA, PNs are the main source of output, exciting cells in numerous downstream brain regions. Interneurons act by inhibiting PNs and/or other interneurons, thereby modifying the outgoing signal. Acquisition of a fear or reward memory occurs from the convergence of sensory information projecting to the lateral (LA) portion of the BLA [301], which drives appropriate projections and activity in the more basal area. Interneurons (specifically those expressing parvalbumin [PV<sup>+</sup>]) supply inhibitory tone to pyramidal neurons in the LA, but dampen their suppressive effect during fear conditioning [303]. The resulting excitatory signal from BLA activates the main fear output of the amygdala, the CeA.

The CeA is a striatal-like structure almost entirely composed of medium spiny GABAergic interneurons and inhibitory projection neurons [302]. Expression of fear is ultimately the result of activating inhibitory neurons in the mCeA, which are usually under inhibitory control of neurons originating in the lateral CeA (lCeA) [304]. Projections from interconnected inhibitory circuits of the lateral capsular CeA (lcCeA), lCeA, and mCeA regulate inhibitory tone over stress-induced behaviors, like freezing.

Distinct populations of BLA pyramidal neurons become activated in response to either aversive or appetitive unconditioned stimuli. Selectively activating these cells produces innate fear or reward behaviors and can reinforce learning of either fear or reward [305]. Additionally, “fear” neurons, projecting from the BLA to the PrL, increase their firing rate in response to fear conditioning and decrease their activity following extinction learning; alternatively, “extinction” neurons of the BLA become more activated in response to extinction learning, and innervate the IL and CeA [306, 307]. During extinction learning, previously formed fear memories are not erased (or unlearned), but rather, suppressed to allow the new extinction memory to become expressed. A potential mechanism underlying extinction memory expression is the silencing of BLA “fear” neurons through inhibitory signaling of PV<sup>+</sup> interneurons [308]. These studies identify separate BLA neuronal populations acting within microcircuits to mediate behavioral and emotional output.

Two genetically unique populations of excitatory PNs in the BLA preferentially process positive (rewarding) or negative (aversive) valence, as well as guide behavioral responses to seek and avoid these types of stimuli respectively [299]. These nearly non-overlapping populations along the anterior-posterior axis make up almost all the glutamatergic neurons within the BLA. The anterior BLA (aBLA) contains magnocellular glutamatergic PNs that express the genetic marker R-spondin 2 (Rspo2<sup>+</sup>), which are preferentially activated by aversive stimuli, and, upon stimulation, decrease motivation to seek reward and increase freezing behavior [299]. These aBLA neurons send dense projections to the capsular CeA (cCeA) and PrL, and send ~30% of the projections from the BLA to NAc [299]. In contrast, the posterior BLA (pBLA) preferentially contains parvocellular glutamatergic PNs that express the genetic marker Ppp1r1b, encoding for the dopamine- and cAMP-regulated neuronal phosphoprotein



(DARPP-32) [299]. These pBLA neurons become activated by exposure to rewarding stimuli, and when optogenetically stimulated, disrupt aversive behavioral response [299]. Connections from Ppp1r1b<sup>+</sup> neurons target the lCeA, mCeA, and IL, and make up ~70% of the connections from the BLA to the NAc [299]. Furthermore, Rspo2<sup>+</sup> and Ppp1r1b<sup>+</sup> neurons reciprocally suppress each other's activity; however, ~25% of connections from Ppp1r1b<sup>+</sup> to Rspo2<sup>+</sup> and ~17% from Rspo2<sup>+</sup> to Ppp1r1b<sup>+</sup> neurons produce excitatory effects [299].

### **Pro-stress microcircuits**

Through reciprocal connectivity, the PrL and aBLA promote learning and expression of fear-related behaviors and therefore describe the “pro-stress” microcircuits (Fig. 2A) [307, 309]. Projections from the BLA to the PrL are triggered during high fear states [310], providing bottom-up negative valence processing that requires excitation of Rspo2<sup>+</sup> PNs [299]. Furthermore, BLA neurons that target the CeA, presumably Rspo2<sup>+</sup> cells innervating the cCeA [299], are activated during cue-induced fear responses [307]. Interestingly, while activation of the PrL reduces social preference, this motivational social learning is driven through PrL to NAc connections and not PrL innervations of the amygdala or VTA [311]. Therefore, bottom-up signaling to the PrL from specific aBLA neurons may prompt social aversion [293] through downstream projection pathways. Collectively, these findings illustrate a pro-stress microcircuit centered around aBLA neurons that send/receive signals to the PrL, as well as relay behavioral output information to the CeA.

### **Anti-stress microcircuits**

The IL and pBLA Ppp1r1b<sup>+</sup> neurons, through reciprocal connections, are important for consolidating fear extinction memories and driving reward-related behaviors. Although only ~8% of neurons in the IL project to the BLA in rats [312], enhanced activity in the IL and pBLA

circuits are considered “anti-stress” microcircuits (Fig. 2B) that become activated upon exposure to safety and rewarding cues. While BLA neurons innervating the IL display enhanced excitation during fear extinction learning, and BLA-activated ventral PAG-projecting IL neurons are also essential for fear extinction [313-315]. Further, activation of pyramidal neurons in the IL results in inhibition of pro-stress PrL pyramidal neurons [316], perhaps, as a result of IL-originating NPY-positive (NPY<sup>+</sup>) GABAergic neurons that project to the PrL [317]. In the BLA, neurons innervating the IL are more susceptible to activity-dependent suppression than those connecting with PrL, an effect that is mediated through endocannabinoid signaling dynamics onto BLA interneurons (specifically cholecystinin-positive [CCK<sup>+</sup>] cells) [310]. Additionally, social behaviors, perhaps associated with Ppp1r1b<sup>+</sup> neurons projecting to the NAc [299], are modulated by endocannabinoid interaction with BLA cells [318]. In sum, these results indicate that anti-stress microcircuits are distinct from pro-stress circuits (Fig. 2), and primarily involve signaling between the IL and pBLA.

### **The concept of counterbalanced microcircuits**

While it is easier to consider stress reactivity in the guise of “stress on” and “stress off” conditions, microcircuits exist in dynamic relationships where stressful events skew response signaling to favor one behavioral state over another. When equilibrium is shifted, the permanence associated with the manifestation of phenotypic display is largely dependent on the degree to which the neural signaling is altered. This counterbalance theory of stress responsiveness predicts that the realignment of pro- and anti-stress signaling to a more balanced state may serve to correct affective dysfunction.

Amygdala hyperactivity in response to threat has been observed in clinical anxiety disorders [319], and may serve to predict future risk of developing anxious or depressive disorders [320].

Brain imaging and post-mortem studies show hypoactivity and reduced volumes in the PFC of humans with depression [321]. Individuals that are better able to suppress negative emotions display greater attenuation of amygdalar activity and higher inverse coupling between the ventromedial PFC (vmPFC; human IL homologue) and amygdala [322]. This suggests mood disorders may manifest due to changes in connectivity and functionality of the mPFC and amygdala, with heightened amygdalar and reduced PFC activity upsetting executive control over emotions. However, distinguishing specific mechanistic frameworks in BLA-mPFC pro- and anti-stress microcircuits that work in a counterbalanced fashion may provide insight as to how stress-related dysfunctions arise and, perhaps more importantly, how to correct them. One such system, potent and dualistic in its physiological and behavioral provocations during periods of stress, includes the targets of orexins: the Orx receptors.

## **OREXIN RECEPTORS AND STRESS RESPONSIVENESS**

Stress-induced behavioral and physiological responses are mediated through complex interactions of genetic and environmental influences that direct signaling biases in pro- and anti-stress microcircuits. Within these circuits, Orx receptors (Orx<sub>1</sub>R and Orx<sub>2</sub>R) gate the emergence of stress-related behaviors. While evidence for Orx receptor modulation of stress responses exists outside the microcircuits highlighted above (see Summers et al., 2020 for review), in this section we will focus on findings that specifically incorporate the PFC and amygdala.

### **Gating stress responses through the prefrontal cortex (PFC)**

The PrL and IL divisions of the PFC are heavily innervated by glutamatergic neurons originating in the thalamus, particularly mediodorsal (MDT) and PVT nuclei [323]. Orexin-producing neurons of the hypothalamus densely project to the PVT [34] where Orx<sub>A</sub>, and Orx<sub>B</sub>

to a greater extent, activate glutamatergic cells [324]. Direct infusions of Or<sub>XA</sub> or Or<sub>XB</sub> into the PVT enhance anxious responses in the EPM; and EPM-related anxious behaviors can be alleviated by blocking Or<sub>X1R</sub> [75] or Or<sub>X2R</sub> [76] in the PVT. A homeostatic consequence of activating orexin-producing neurons is internalization of PVT-expressing Or<sub>X1R</sub> [233]; an effect that is reversed with Or<sub>X1R</sub> antagonism in the PVT [75]. Although the Orx system can alter PFC activity indirectly through thalamic nuclei, orexinergic projections also supply direct signaling to the PFC [34].

Transcriptional expression of Or<sub>X1R</sub> is moderate in both the IL and PrL; however, Or<sub>X2R</sub> is expressed minimally in the IL and appears to be absent in the PrL [33]. In the PrL, Or<sub>X1R</sub> is localized to cell bodies and neuronal processes of pyramidal neurons of layer V [97]. Further, Or<sub>X2R</sub> activation increases Ca<sup>2+</sup> in the presynaptic terminals of axons projecting from the thalamus to layer V PFC neurons [81]. Interestingly, in layer V of frontal area 2 (FA2), a large PFC region lateral to the PrL, Or<sub>X1R</sub> is localized on intracortical glutamatergic presynaptic terminals, but not on thalamocortical terminals [80]. Together, these findings suggest that within the PFC, there is a regional dependence of Orx system control over signaling tone; and this is initiated through direct activation of PFC neurons or regulation of Glu release from thalamocortical inputs. However, social stress leads to a reduction in hypothalamic release of Or<sub>XA</sub> and Or<sub>XB</sub> to the PFC [246], diminishing orexin-induced activation of PFC neurons. The significance of Or<sub>X2R</sub> signaling in the PFC with respect to stress responses can only be hypothesized (see section COUNTERBALANCED Or<sub>X1R</sub> VS Or<sub>X2R</sub> MODULATION); however, few studies have considered the role of Or<sub>X1R</sub>-containing PFC neurons in behaviors relevant to stress circuitry. For example, chronic alcohol exposure reduces Or<sub>X1R</sub> mRNA in the PFC [129], which possibly contributes to alcohol- and withdrawal-induced changes in signaling

dynamics of the PFC [325] and amygdala [326] that lead to hyperreactive stress states [327]. Also, blocking Orx<sub>1</sub>R in the PFC, specifically the ACC and orbitofrontal cortex (OFC), interferes with stable decision-making strategies [328], a characteristic applicable to stress-induced affective disorders [329]. Further, blocking Orx<sub>1</sub>R in the PFC abolishes cue-induced feeding behavior [330], a response associated with stress-provoked eating [331, 332]. Systemic administration of an Orx<sub>1</sub>R antagonist also reduces cue-induced feeding behavior and increases neuronal activity of the IL, PrL, and PVT [333].

### **Gating stress responses through the amygdala**

In the BLA, Orx receptors are localized to both glutamatergic and GABAergic neurons [82, 83]. Optogenetic activation of glutamatergic neurons in the perifornical region (PeF) of the hypothalamus that project to the amygdala exaggerates the fear expression profile in rats [334]. Blocking intra-amygdalar Orx<sub>1</sub>R suppresses anxiety and fear responses in stressed rodents [83, 254]. Similarly, systemic (ip) administration of an Orx<sub>1</sub>R antagonist reduces contextual and cued fear learning responses [253] and is associated with activation of IL-projecting BLA cells and calbindin-expressing BLA interneurons [335]. Perhaps these effects, in part, are related to impairments in spatial learning that are associated with intra-BLA Orx receptor antagonism [336], which can reduce long-term potentiation (LTP) in DG granular cells [337]; however, recent preliminary results from our lab suggest that small doses of an Orx<sub>1</sub>R antagonist into the BLA enhances spatial learning during periods of stress [83]. This finding remains consistent with the report that intra-BLA antagonism of Orx<sub>1</sub>R or Orx<sub>2</sub>R does not impair memory retrieval, but does impact consolidation [336]. Systemic (ip) Orx<sub>2</sub>R inhibition has also been shown to diminish contextual fear responses but has no effect on cued fear expression [253]. In our lab, we demonstrated that cued fear associated with social stress can be reduced through icv infusion

of a small dose of an Orx<sub>2</sub>R agonist [179]. Additionally, repeated restraint stress in mice increases Orx release to the BLA, and this increase BLA Orx is associated with depressive behaviors [82].

Orexin-producing neurons send projections to the CeA where they regulate release of Glu by targeting presynaptic Orx<sub>1</sub>R [79]. As the CeA houses predominantly GABAergic neurons [338,339], some of which are CRF-producing [340, 341], presynaptic Orx<sub>1</sub>R may be localized on the terminals of BLA projection cells. Microinfusions of either Orx<sub>A</sub> or Orx<sub>B</sub> into the CeA increases anxious behaviors in the LDT and EPM [169]. Inhibition of CeA Orx<sub>1</sub>R can reduce conditioned fear [79].

Collectively, these examples demonstrate the ability of Orx receptor activity in PFC and amygdala microcircuits to bias stress reactivity. Further, these data indicate that Orx<sub>1</sub>R and Orx<sub>2</sub>R opposition is not a fundamental element of Orx functioning everywhere in the brain, and that these two receptors can regulate stress and affect concordantly. While a complete understanding of the cellular and molecular mechanisms employed to shift stress responses remains an ambitious and long-term goal of ours, in the following section we will predict, based on evidence from our lab, a counterbalanced mechanism for mediating stress-induced behaviors via Orx<sub>1</sub>R and Orx<sub>2</sub>R interaction.

## **COUNTERBALANCED Orx<sub>1</sub>R VS Orx<sub>2</sub>R MODULATION**

The idea of the Orx system contributing to bidirectional stress responses is not novel. Discrete and parallel circuits, incorporating the lateral hypothalamus and orexin-producing neurons, initiate opposing behavioral responses to emotionally relevant stimuli [241]. Furthermore, motivated behavior promoted through Orx release is antagonistic to the action of

Dyn, an endogenous opioid that is co-expressed with Orx, in the VTA [27]. At the level of synapses, Orx<sub>1</sub>R activation through G<sub>q</sub> signaling can lead to the production and release of 2-arachidonoylglycerol (2-AG) (Fig. 4C) [342], which may suppress the initial Orx system stimulation in circuits tied to food-seeking and pain [343]. While these examples demonstrate orexin's involvement in opposing systems, we propose that dualistic Orx<sub>1</sub>R versus Orx<sub>2</sub>R signaling within stress microcircuits shifts behavioral responses to favor maladaptive or adaptive outcomes.

### **Inhibition of Orx<sub>1</sub>R in stress microcircuits**

Using a test of behavioral despair, we observed a positive relationship between despair (immobility) and amygdalar Orx<sub>1</sub>R mRNA levels [73]. Additionally, we detected elevated Orx<sub>1</sub>R mRNA in the BLA of individuals displaying susceptibility after 10 days of social defeat [130]. These transcription levels of Orx<sub>1</sub>R were further negatively correlated with social preference behavior [130]. Together, these results suggest that Orx<sub>1</sub>R activity in the BLA is important for one side of a system that balances stress reactivity.

Using a preclinical social stress paradigm designed by our lab, called the Stress Alternatives Model (SAM), we set out to determine if pharmacological manipulation of Orx<sub>1</sub>R in the BLA (intra-BLA) could shift stress responsivity (Fig. 3). The SAM takes advantage of the fact that when mice are exposed to intense stress (like social stress), they diverge into behavioral phenotypes, adopting either active or passive coping strategies [344-346]. We have demonstrated that mice displaying the active strategy (called Escape mice) are behaviorally and physiologically resilient to stress, while those of the passive phenotype (called Stay mice) are susceptible to stress [179, 347]. Following phenotype commitment in the SAM, antagonism of Orx<sub>1</sub>R in the BLA shifted behaviorally susceptible (Stay) individuals toward the resilient

(Escape) phenotype (Fig. 3E), suggesting a positive influence of drug delivery on decision-making during stress [83]. Inhibition of Orx<sub>1</sub>R also reduced fear learning responses (Fig. 3A) in mice conditioned to associate a tone with social aggression [83]. In a similar way, blocking intra-BLA Orx<sub>1</sub>R decreased aggression-induced conflict-freezing responses, but had no effect on fear-related startle response (Fig. 3B, C) [83]. It is noteworthy that stimulation of Orx<sub>1</sub>R in the BLA produced opposing effects [83].

Our results support the prediction that Orx<sub>1</sub>R activity in the BLA drives pro-stress responses and blocking these receptors biases anti-stress microcircuit signaling. Several other studies provide complimentary findings that bolster our claim that inhibition of Orx<sub>1</sub>R promotes positive behavioral outcomes to stressful events [74, 75, 170-172, 251, 253, 254, 330, 348-350]. We propose that selective Orx<sub>1</sub>R inhibition may provide a potential therapeutic quality for affective disturbances, like those observed in MDD, by correcting signaling imbalances within stress neurocircuitry. However, our results establish an equally viable pharmacological target in the Orx<sub>2</sub>R (Fig. 3).

### **Orx<sub>2</sub>R stimulation in stress microcircuits**

In response to social defeat, susceptible individuals express lower levels of Orx<sub>2</sub>R mRNA in the BLA and the level of expression is positively correlated with social preference [130]. In the SAM, we demonstrated that inhibition of whole brain (icv) Orx<sub>2</sub>R enhanced susceptible behaviors in previously resilient mice, including the blockade of escape behavior, enhanced freezing, increased startle, and diminished social preference [179]. We observed similar results with intra-BLA infusion of an Orx<sub>2</sub>R antagonist [83].

In contrast, rescue of escape behavior in susceptible (Stay) mice was possible using whole brain [179] and intra-BLA [83] administration of an Orx<sub>2</sub>R agonist (Fig. 3E). Further, we



observed a reduction in fear-related conditioned/conflict freezing and startle (Fig. 3B, C) [83, 179]. The SAM paradigm incorporates decision-making [344-346] and allows for measurements of motivated behaviors that are sometimes blunted during periods of stress, like escape-seeking behavior (called Attention Toward Escape) [83, 179]. Stimulation of Orx<sub>2</sub>R [83, 179] and inhibition of Orx<sub>1</sub>R [83] activate this adaptive response in susceptible mice (Fig. 3D). Although few studies have directly addressed Orx<sub>2</sub>R activity in response to stress-related behaviors, there are those that complement our findings [74, 84]. Collectively, these observations expose a mechanism by which Orx<sub>1</sub>R and Orx<sub>2</sub>R activity bidirectionally balances stress responsivity. Importantly, our preclinical results illustrate that Orx receptor signaling in stress microcircuits mediates stress reactivity by altering behavioral output related to fear, decision-making, and motivation (Fig. 3). We posit that simultaneous antagonism of Orx<sub>1</sub>R and stimulation of Orx<sub>2</sub>R (see section THE POTENTIAL FOR SELECTIVE OREXIN RECEPTOR CROSSOVER DRUGS (SORCOs)) would provide a potent effect on biasing resilient behaviors in response to stress.

### **Competing Orx<sub>1</sub>R and Orx<sub>2</sub>R signaling to balance pro- and anti-stress microcircuits**

Focusing on a small piece of the overall stress neurocircuitry, namely distinct areas of the mPFC (IL & PrL) and the amygdala, we can offer simplistic predictions as to how signaling from Orx<sub>1</sub>R and Orx<sub>2</sub>R work to oppose biases in stress responsivity (Fig. 4). Expression of Orx<sub>1</sub>R in both the IL and PrL is limited in deep cortical layers (layers V & VI) but more abundant in layer II [33], suggesting a regional dependence on signaling that may more effectively drive signals from interneurons within layer II/III. However, in the PrL Orx<sub>1</sub>R is found on pyramidal cell bodies and neuronal processes [97], indicating a potentially potent pro-stress response upon activation (Fig. 4A).

In the PrL, Orx<sub>2</sub>R mRNA is possibly absent; however, some expression exists within the IL [33]. In addition, Orx<sub>2</sub>R enhances thalamocortical signaling from thalamus-projecting presynaptic terminals [81], perhaps further potentiating IL activity (Fig. 4B). These observations advocate a preference for anti-stress signaling upon Orx<sub>2</sub>R activation.

Amygdalar Orx receptors are less characterized; however, Orx<sub>1</sub>R signaling from presynaptic terminals in the mCeA [79], may originate from glutamatergic projection neurons in the BLA (Fig. 4D). This prediction is not baseless as glutamate-producing neurons in the BLA have been shown to express Orx<sub>1</sub>R [82, 83, 351]. As antagonism of Orx<sub>1</sub>R in the BLA results in activation of IL-projecting neurons as well as calbindin-positive (Calb<sup>+</sup>) GABA cells [335], we predict blockade of Orx<sub>1</sub>R on CeA-projecting pyramidal neurons deactivates endocannabinoid-mediated suppression of Calb<sup>+</sup> GABA neurons (Fig. 4C; similar to mechanisms proposed by Berrendero, Flores, & Robledo, 2018) and allows for Orx<sub>2</sub>R signaling bias of IL-targeting fear extinction circuits.

Expression of Orx receptors have also been identified in GABA neurons [82] and with whole brain Orx<sub>2</sub>R stimulation, PV<sup>+</sup> GABA cells in intercalated/BLA regions of the amygdala (Fig. 4C) become activated [179]. Intercalated GABA cells that suppress pro-stress responses [352] are activated by inputs from the IL [353], so activation of Orx<sub>2</sub>R within the IL may induce anti-stress reactivity (Fig. 4B). It is also possible that Orx<sub>2</sub>R activation of IL-projecting neurons results in a positive feedforward of circuit control that directs adaptive stress responses.

The Orx<sub>1</sub>R-Orx<sub>2</sub>R signal balance may also be achieved through regional expression in anterior and posterior BLA neurons. The configuration of non-overlapping aBLA and pBLA cells that antagonistically regulate emotional responsivity [299] suggests there may be an organizational structure from which balance of the Orx system stabilizes reactions to stress. In

this model, Orx<sub>1</sub>R activation may selectively activate aBLA circuits, while Orx<sub>2</sub>R stimulation shows bias towards the pBLA connections (Fig. 4C, E).

All predictions reported here may work individually or in combination to support the opposing actions of Orx<sub>1</sub>R and Orx<sub>2</sub>R regulation of stress neurocircuitry (Fig. 4).

Counterbalance theory of stress responsiveness suggests that upsetting stable states in PFC and BLA results in imbalances that may be corrected through opposing systems. During stress-induced disorders, including affective disorders, we argue that pro-stress microcircuits become favorable leading to behavioral disturbances. We hypothesize, based on supporting evidence presented here, that Orx<sub>1</sub>R-Orx<sub>2</sub>R counterbalanced systems are responsible, in part, for establishing equilibrium during periods of stress. As such, we propose a novel idea for therapeutic intervention of stress-related disorders through simultaneous inhibition of Orx<sub>1</sub>R and stimulation of Orx<sub>2</sub>R.

## **THE POTENTIAL FOR SELECTIVE OREXIN RECEPTOR CROSSOVER DRUGS**

### **(SORCOs)**

Our proposal for a hypothetical class of drugs or combined drug treatment is based on two important factors. The first is that the mPFC-BLA microcircuits play an important role, perhaps even a dominant role, in the regulation of stress responsiveness and emotional behavior. To be sure, however, mPFC-BLA are not the only stress-related specific microcircuits in the brain. Other circuits, including those emanating from GABAergic CRF and CCK neurons in the BNST, stimulating hypothalamic Orx neurons, which innervate important stress/arousal regions of the brain, such as the LC [89, 238, 239, 241], and perhaps also influence the mPFC-BLA microcircuits.

In the mPFC-BLA reciprocal set of microcircuitries, the functions of Orx<sub>1</sub>R and Orx<sub>2</sub>R appear to be opposing, primarily because their expression is likely segregated to functionally distinct cell types. The critical role of the mPFC-BLA systems regulating stress-related functions and behaviors remains to be more carefully demonstrated. The evidence thus far for its importance, comes from experiments using icv injection of Orx<sub>2</sub>R agonist and antagonist drugs, with a potentially pan-brain exposure suggesting that Orx<sub>2</sub>R function is primarily anti-stress. Similarly, systemic delivery of Orx<sub>1</sub>R antagonists reduces stress-related functions and behavior, suggesting a pro-stress function for Orx<sub>1</sub>R. It is clear however, that in some regions of the brain, the Orx<sub>1</sub>R and Orx<sub>2</sub>R appear to function concordantly, and this may be the prevalent disposition of these two receptors in the brain. Further clarification will be necessary to determine if the kind of drug or drug combination that we propose below is appropriate.

The currently available Orx drugs (and those in clinical trials) are antagonists of both Orx<sub>1</sub>R and Orx<sub>2</sub>R called DORAs as well as selective receptor antagonists (SORAs) for Orx<sub>1</sub>R or Orx<sub>2</sub>R, which were developed for use in treatment of insomnia [178, 228-230, 354] or anxiety [226, 227], and with some effectively yielding sleep-promoting results [178, 216, 218, 228-230], have been suggested to be potentially useful in treatment of addiction [355] and depression [178, 228] as well. These drugs, though not selective for the Orx<sub>1</sub>R alone, have also displayed some promise as therapeutic tools for relieving signs of affect. Orally administered DORA-12 enhances social interaction time in rats subjected to high levels of CO<sub>2</sub> to promote a panic state [251]. Almorexant, a competitive Orx receptor antagonist designed to treat insomnia, lowers blood pressure (BP) in hypertensive rats, while having no impact on the resting BP of wildtype animals [356]. Further, almorexant, in a dose-dependent fashion, reduces fear-potentiated startle in conditioned rats, while having no myorelaxant effects [51]. When administered almorexant

daily, mice experience decreased anxious and depressive behaviors in an UCMS model for depression that are comparable to fluoxetine-treated animals [219]. The effectiveness of DORAs suggests the Orx receptors function in a similar fashion, which is often true relative to sleep-wake cycles, insomnia, and narcolepsy, but not always, as Orx<sub>1</sub>R may oppose the sleep-inducing effects of Orx<sub>2</sub>R [357], and SORA Orx<sub>2</sub>R antagonist drugs differentially increase NREM sleep [358, 359] over REM-induction by DORAs. We make the point that it is also not true for stress neurocircuitry and responsiveness or behavioral affect [72, 73, 83, 130, 179, 351]. Consequently, sometimes the typical, presumably Orx<sub>1</sub>R-mediated, effect is not manifest, such as the case with the predator odor PTSD model, in which rats treated with almorexant display a higher prevalence of the PTSD phenotype [245]. Therefore, to achieve an effective, efficient anxiolytic or anti-depressant drug based on specific Orx<sub>1</sub>R and Orx<sub>2</sub>R functions [72, 83, 130, 179, 351] in the stress neurocircuitry that regulates enhanced responsiveness and affect, a distinctively new kind of drug, or drug combination is necessary. The need for a singular or dual drug with the ability to modify both pro-stress and anti-stress circuitries seems to be critical for the treatment anxiety and/or depression [72, 73, 83, 130, 179, 351]. This new drug should limit the output of pro-stress circuitries through inhibition of Orx<sub>1</sub>R and promote anti-stress circuitries and actions by means of stimulation of Orx<sub>2</sub>R. A selective orexin receptor crossover (SORCO) drug or combination of drugs would, we believe, produce a complete treatment related to specific causes of anxiety and depression, and not just to symptomology. In the process, SORCO actions could positively modify behavior associated with stress, arousal, reward, anhedonia, and modified decision-making thereby limiting significant elements related to the etiologies of those affective disorders.

We make one additional point regarding the clinical implications for DORAs and SORAs

used for treatment of insomnia in comparison with the potential actions of a SORCO designed to limit anxiety or depression. However, comparisons may be difficult, since the dosages used for rodent experiments are on the order of ten-fold higher (compared only within a treatment type, such as icv injection) in sleep- or wake-promoting treatments, than for our studies related to stress responsiveness [72, 179]. This difference in dosage is significant for several reasons, the first of which is that cross effects, and side effects, may be avoided, although significant further testing would be necessary to determine this. Suggesting that some Orx system-targeting drugs may have a wide range of efficacy, anxious, panic, and depressive behavior can be inhibited by doses of Orx<sub>1</sub>R and Orx<sub>2</sub>R antagonists SB334867, ACT-539313 and JNJ-42847922 (seltorexant) drugs similar to those used for sleep induction [72, 170-172, 251]. The low doses necessary to produce anxiolytic or antidepressive actions from Orx<sub>2</sub>R agonist treatment may prove to be a therapeutic advantage [72]. Thus, inhibition of Orx<sub>1</sub>R, and stimulation of Orx<sub>2</sub>R, both appear to have the potential for anxiolytic and antidepressive actions. While DORA treatment confounds the opposing actions of Orx<sub>1</sub>R and Orx<sub>2</sub>R in the mPFC-BLA microcircuits, it seems likely that anti-panic effects derive mostly from antagonizing Orx<sub>1</sub>R actions [251], given the evidence that blocking Orx<sub>2</sub>R is anxiogenic and pro-depressive (for icv and intra-BLA injections) [179]. Additionally, at first blush, these promising results based on the new Orx<sub>2</sub>R antagonist seltorexant (JNJ-42847922) suggest the opposite action for Orx<sub>2</sub>R than the anti-stress function that we suggest. It may be that our suggestion for Orx<sub>2</sub>R anti-stress function is limited to a specific neurocircuitry (see sections above). However, results from icv injection of both Orx<sub>2</sub>R agonist and antagonist [179] suggest that the anti-stress/anxiolytic/antidepressant role for Orx<sub>2</sub>R is more systemic, at least within the brain. While early clinical trials with limited sample sizes support antidepressant actions for seltorexant, which are potentially very important, there are

also precautions that should be taken based on subjective (self-report) data that also may be reliant on codependent physiological actions, such as sleep. As the authors of those studies note, insomnia or sleep dysregulation powerfully predisposes subjects to depressive affect, and resolving dysfunctional sleep, may have a potent antidepressive effect, without actually engaging or chemically realigning the neurocircuitry that is primarily responsible for anxiety or depression. At doses that do not affect activity or sleep, Orx<sub>2</sub>R-targeting drugs may influence stress responsiveness and affective behavior in a way that suggests that these receptors promote resilience in pre-clinical studies.

## **SUMMARY**

Parallel pro-stress and anti-stress neurocircuitries exist between the prefrontal cortices and the basolateral amygdala, and these reciprocal and interacting circuits are critical for the development and expression of affective behaviors and disorders. Projecting neurons from the orexin-producing region of the hypothalamus modulate both pro-stress and anti-stress elements by means of Orx<sub>1</sub>R and Orx<sub>2</sub>R. The affect-related functions of Orx<sub>1</sub>R and Orx<sub>2</sub>R are opposing, with Orx<sub>1</sub>R promoting anxious, panic, and despair-related behaviors, and Orx<sub>2</sub>R limiting those responses as well as reinforcing behavior associated with stress resilience. The evidence suggests that DORAs work at counter purposes related to affective behavior, and that a new selective crossover drug (or combination of drugs) that inhibits type 1 Orx receptors while stimulating type 2 receptors (SORCO) is a potentially effective method to reorient mood and behavior associated with affective disorders.

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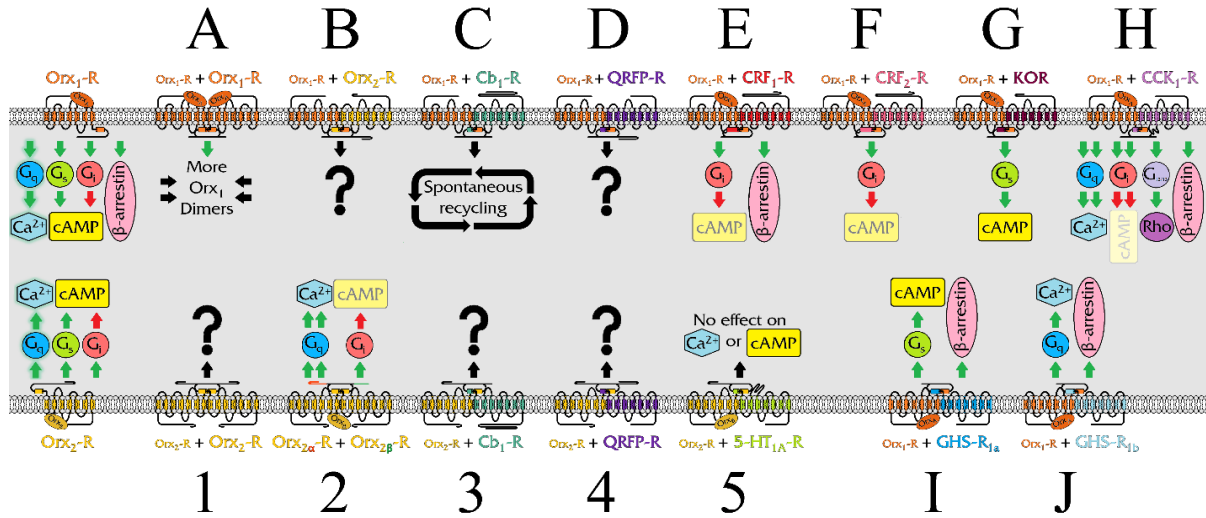
**Table 1. Clinical Assessment of Anxious and Depressive Symptomology following Administration of Orx Receptor-Targeting Drugs**

Drug	Subject(s)	Duration	Single Dose (Oral)	Behavioral/Physiological Effect	Citation
<b>DORA</b>					
Suvorexant (MK4305)	Psychiatric Inpatients (Men & Women)	8 weeks Every night	15 or 20 mg	↓ Severity of anxiety and depression ↓ Plasma cortisol & WBC count ↓ Pulse rate	Nakamura & Nagamine, 2017
Suvorexant (MK4305)	59-Year-Old Outpatient (Woman)	2 days Each night	10 mg & 15 mg	↑ Depressive symptoms Onset of suicidal thoughts	Petrous & Femanga, 2018
Suvorexant (MK4305)	Elderly Patients (Men & Women)	3 days Every night	15 mg	↓ Delirium	Hatta et al., 2017
Almorexant	Healthy Men	10 days (w/ desipramine)	200 mg	↑ Calmness (slightly) ↓ Attention	Cruz et al., 2014
Filorexant (MK-6096)	Patients w/ Recurrent MDD (Men & Women)	6 weeks Every night	10 mg	No change in anxious/ depressive behavior	Connor et al., 2017
<b>SORA (Orx<sub>1</sub>R)</b>					
ACT-539313	Healthy Men	Single ascending doses 10 days	10, 30, 100, 200, 400 mg	↓ Saccadic peak velocity at 200 & 400 mg	Kaufmann et al., 2016
ACT-539313	Healthy Men	Single & Multiple doses (w/ midazolam)	200 mg	↓ Urinary cortisol & 6β-OH cortisol	Berger et al., 2020
<b>SORA (Orx<sub>2</sub>R)</b>					
Seltorexant (JNJ-42847922) (MIN-202)	Men, WOCBP, & WONCBP	28 days Every night	20 mg	↓ Clinician reported depression	Recourt et al., 2019
Seltorexant (JNJ-42847922) (MIN-202)	Men & Women	10 days Every day	5-60 mg	↑ (mild) Attention 4 hours post-administration	van der Ark et al., 2018
Seltorexant (JNJ-42847922) (MIN-202)	Men & Women	Single dose w/ 7-day washout	10, 20 or 40 mg	Trend toward subjective ↑ in mood	Brooks et al., 2019

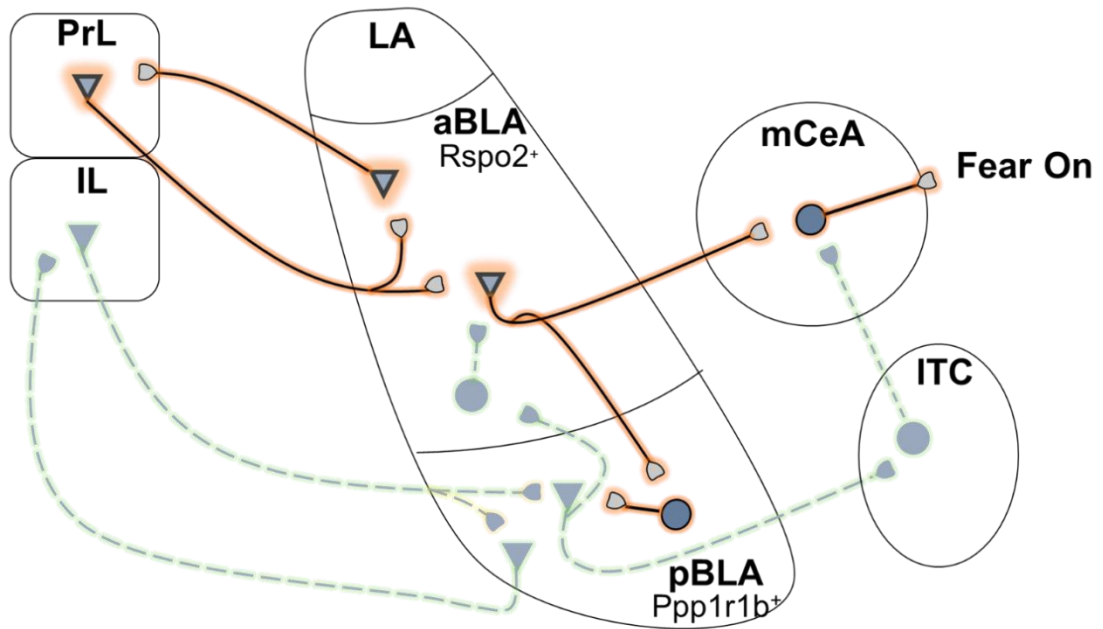
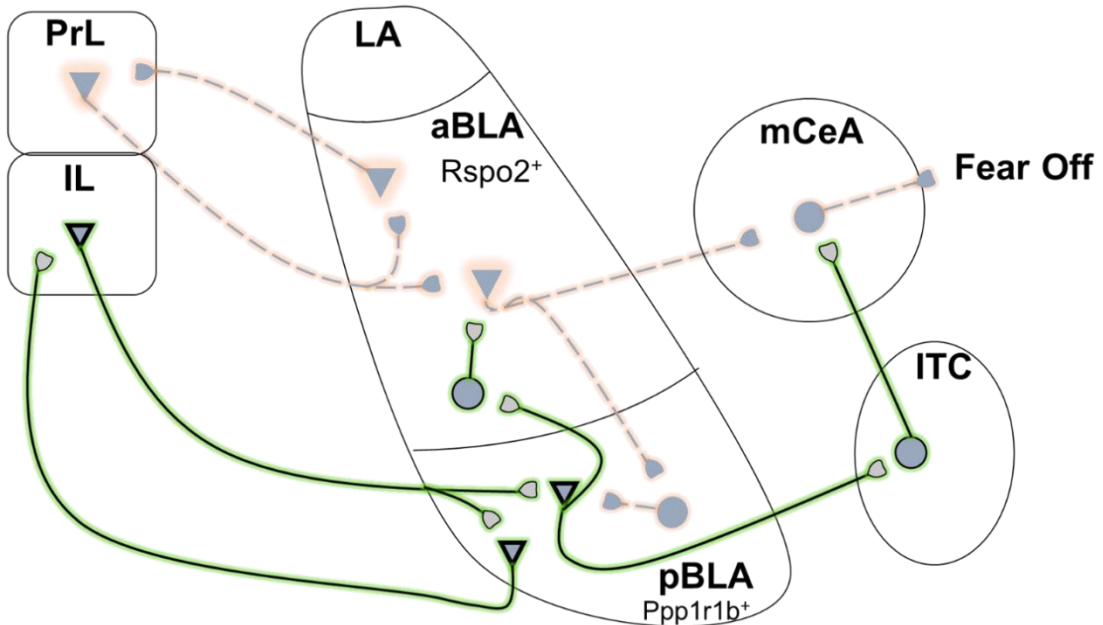
Abbreviations: DORA = Dual Orexin Receptor Antagonist; SORA = Single Orexin Receptor Antagonist; WBC = white blood cell; WOCBP = women of child-bearing potential; WONCBP = women of non-child-bearing potential

Note – Only results pertinent to affective dysfunction are reported. For additional results, see referenced studies.

**Table 1.** Listing of clinical trials and studies using receptor antagonists for the Orx<sub>1</sub>R or Orx<sub>2</sub>R (SORAs) or both (DORAs). For a table of preclinical results please refer to Summers et al., 2020.



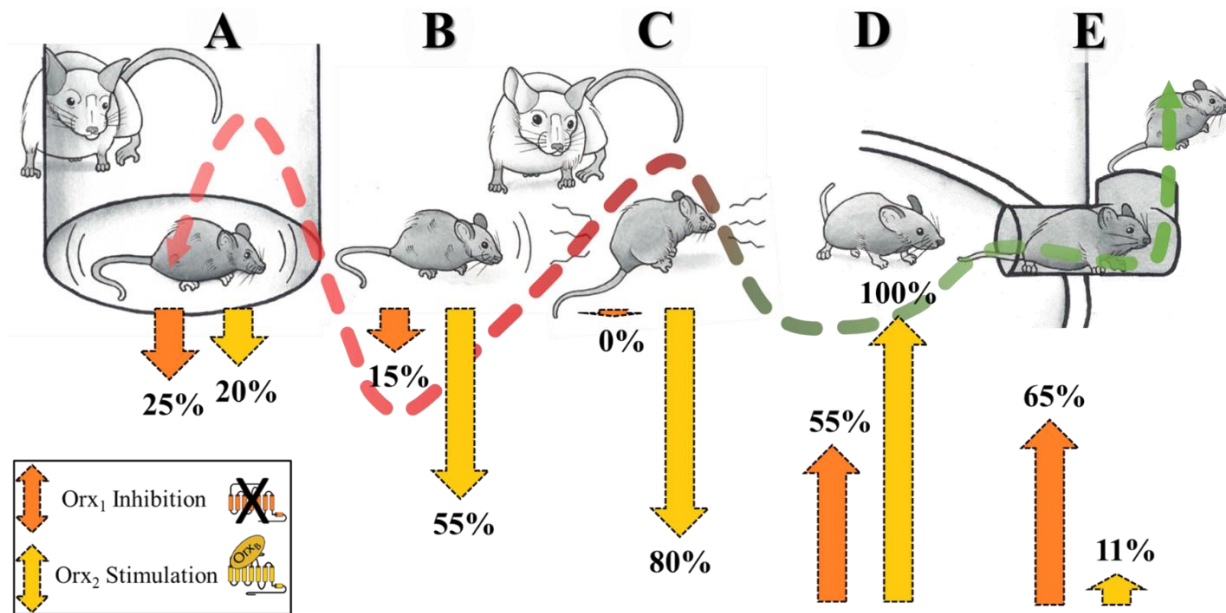
**Figure 1.** Dimerization of Orx receptors changes signaling dynamics. Activation of Orx<sub>1</sub>R (orange; top left) may result in G<sub>q</sub> (blue), G<sub>s</sub> (green), G<sub>i</sub> (red), or β-arrestin (pink) signaling cascades. When stimulated, dimers of Orx<sub>1</sub>R initiate intracellular pathways that are unique from the monomers: (A) homodimers may recruit more dimerization [108]; (B) Orx<sub>1</sub>R+Orx<sub>2</sub>R has unknown properties [107]; (C) Orx<sub>1</sub>R+Cb<sub>1</sub> allows for spontaneous receptor recycling [110, 111]; (D) Orx<sub>1</sub>R+QRFP-R has unknown signaling properties but may be neuroprotective through the activation of ERK pathways [112]; (E) Orx<sub>1</sub>R+CRF<sub>1</sub> favors G<sub>i</sub> and the recruitment of β-arrestin [113]; (F) Orx<sub>1</sub>R+CRF<sub>2</sub> signals through G<sub>i</sub> pathways [114]; (G) Orx<sub>1</sub>R+KOR uses the G<sub>s</sub> pathway [115]; (H) Orx<sub>1</sub>R+CCK<sub>1</sub> enhances G<sub>q</sub> and G<sub>i</sub> signaling, but also recruits signaling from G<sub>12/13</sub> and β-arrestin pathways [116]; (I) Orx<sub>1</sub>R+GHSR<sub>1a</sub> utilizes G<sub>s</sub> and β-arrestin signaling [117]; and (J) GHSR<sub>1b</sub> favors G<sub>q</sub> and β-arrestin intracellular pathways [118]. As a monomer, Orx<sub>2</sub>R (yellow; bottom left) can signal via G<sub>q</sub>, G<sub>s</sub>, and G<sub>i</sub> pathways. Dimerization results in differential signaling patterns: (1) homodimers have unknown signaling qualities [107]; (2) Orx<sub>2</sub>R variant dimers (Orx<sub>2α</sub>+Orx<sub>2β</sub>) displays exaggerated G<sub>q</sub> and normal G<sub>i</sub> signaling [109]; (3) Orx<sub>2</sub>R+Cb<sub>1</sub> has unknown signaling properties [107]; (4) Orx<sub>2</sub>R+QRFP-R may be neuroprotective [112], but has not been heavily investigated; (5) Orx<sub>2</sub>R+5-HT<sub>1A</sub> returns signaling to basal conditions [119]. Note, referenced studies rely on *in vitro* techniques to explore signaling cascades, and results reported here are only pathways observed when Orx peptides (Orx<sub>A</sub> or Orx<sub>B</sub>) are present. Other pathways may be evident when the partner receptor's ligand is present.

**A****B**

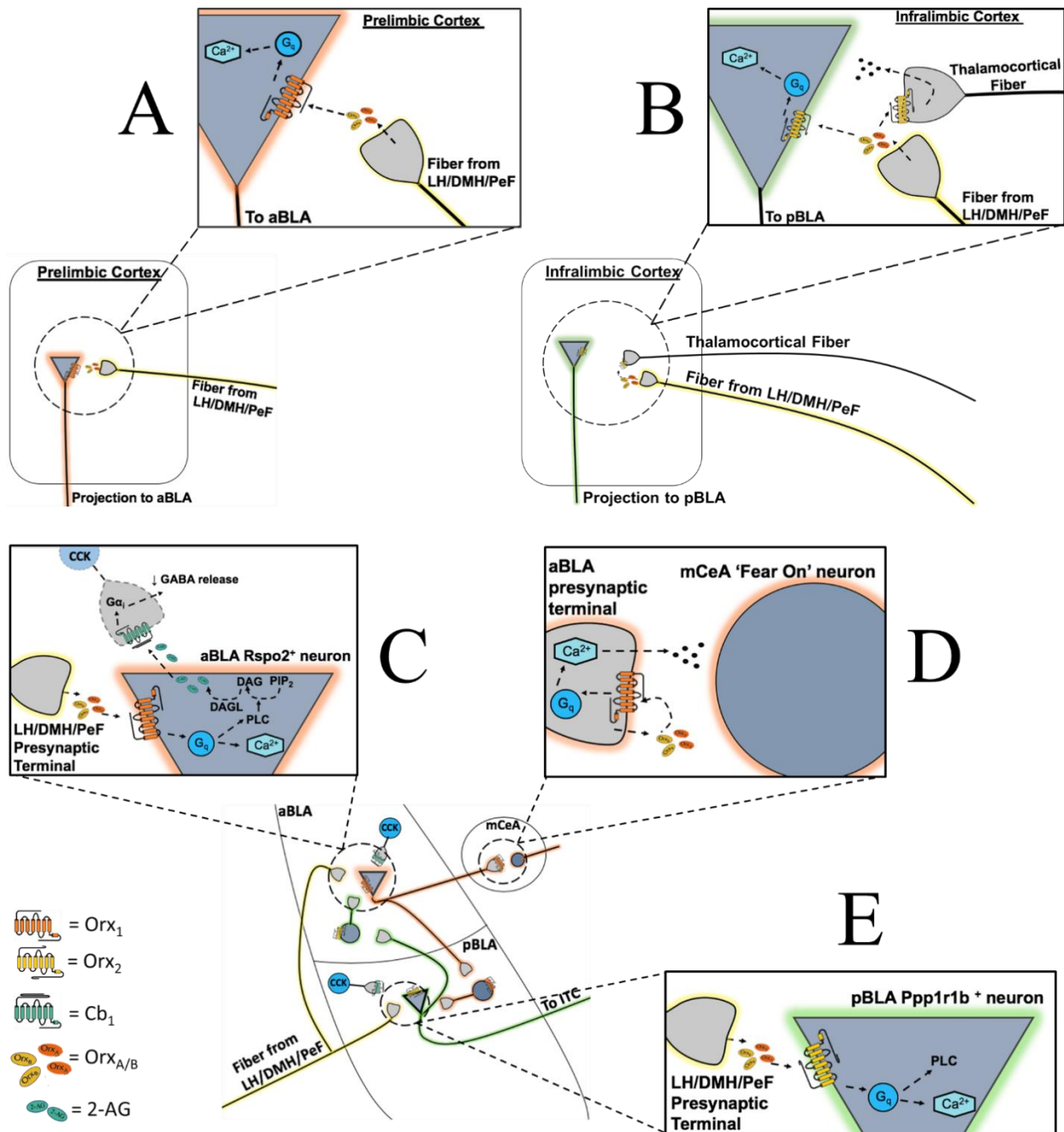
**Figure 2.** Stress responses are initiated through counterbalanced parallel neurocircuits. (A) Pro-stress behaviors are initiated through activation of reciprocal PrL and aBLA (*Rspo2*<sup>+</sup> cells) connections that lead to inhibition of pBLA projection neurons and activate mCeA outputs to promote “Fear On”

signaling. (B) Anti-stress responses are promoted through reciprocally innervated IL and pBLA (*Ppp1r1b*<sup>+</sup>) neurons, which suppress projection neurons in the aBLA and indirectly in the mCeA through ITC GABAergic cells. Importantly, this represents a simplistic model of a highly complicated system; and does not include many components that are also necessary for modulation of stress reactivity.





**Figure 3.** Pro-stress behaviors are alleviated, and anti-stress responses are promoted through Orx<sub>1</sub>R inhibition (orange arrows) or Orx<sub>2</sub>R stimulation (yellow arrows). In the SAM, mice display behaviors consistent with a gradient of stress-induced responses [346] (dashed line), where fear-associated behaviors (far left, red dashed line) are consistent with vulnerability, and active avoidance through escape (far right, green dashed line) relating to stress resilience. Mice exposed to the SAM show diminished pro-stress behaviors with treatments, including (A) fear conditioned freezing, (B) conflict freezing, and (C) startle response. Conversely, with specific Orx receptor-targeted treatments, anti-stress responses are increased, including (D) motivational behaviors (Attention Toward Escape) and (E) resiliency (Escape behavior). All values are approximations combined from icv [179] and intra-BLA [83, 351] studies and are represented as percent from control (vehicle-treated mice exposed to the social stress paradigm).



**Figure 4.** Pro- and anti-stress circuits are bidirectionally mediated through Orx<sub>1</sub>R and Orx<sub>2</sub>R activity.

(A) In the PrL, Orx<sub>1</sub>R stimulation activates (increased Ca<sup>2+</sup>) aBLA-projecting neurons, and biasing counterbalanced microcircuits to favor pro-stress behavioral responses. (B) The IL expresses Orx<sub>2</sub>R on presynaptic thalamocortical terminals that, when activated, promote increased Glu release onto pBLA-projecting neurons. These pBLA-connecting IL neurons also express Orx<sub>2</sub>R, which, upon stimulation, increases signaling to the pBLA and favoring anti-stress microcircuit activation. (C) The aBLA houses

mCeA-innervating neurons that express Orx<sub>1</sub>R. When activated, Orx<sub>1</sub>R promotes excitation of projection neurons, but also stimulates the production of 2-AG, which inhibits GABA release from interneurons (CCK<sup>+</sup>) and leads to disinhibition (and hyper-excitability) of aBLA projection neurons, biasing pro-stress responses. Note that Orx<sub>2</sub>R may be located on interneurons within the aBLA to help suppress pro-stress signaling. (D) Presynaptic terminals express Orx<sub>1</sub>R in the mCeA. When activated, these receptors increase glutamate release onto mCeA neurons and promote pro-stress responsiveness. (E) The pBLA contains Orx<sub>2</sub>R on pyramidal projection neurons that signal in support of anti-stress pathways. Interneurons in the pBLA may also express Orx<sub>1</sub>R, which would suppress anti-stress signaling if stimulated. Noteworthy, these are predicted interactions of Orx receptors in pro- and anti-stress microcircuits. The precise locations and functions of these receptors within these areas of stress neurocircuitry remains largely unknown.

## **Chapter 2: Orexin 1 receptor antagonism in the basolateral amygdala shifts the balance from pro- to anti-stress signaling and behavior**

### **ABSTRACT**

**BACKGROUND:** Stress produces differential behavioral responses through select molecular modifications to specific neurocircuitry elements. The orexin system targets key components of this neurocircuitry in the basolateral amygdala (BLA).

**METHODS:** We assessed the contribution of BLA Orexin 1 receptors (Orx<sub>1</sub>R) in the expression of stress-induced phenotypes. Using the Stress Alternatives Model (SAM), a social stress paradigm that produces two behavioral phenotypes, we characterized the role of BLA Orx<sub>1</sub>R using acute pharmacological inhibition (SB-674042) and genetic knockdown (AAV-U6-Orx<sub>1</sub>R-shRNA) strategies.

**RESULTS:** In the BLA, we observed that Orx<sub>1</sub>R (*HCRTR1*) mRNA is predominantly expressed in CamKII $\alpha$ <sup>+</sup> glutamatergic neurons and rarely in GABAergic cells. While there is a slight overlap in Orx<sub>1</sub>R and Orexin 2 receptor (Orx<sub>2</sub>R; *HCRTR2*) mRNA expression in the BLA, we find that these receptors are most often expressed in separate cells. Antagonism of intra-BLA Orx<sub>1</sub>R after phenotype formation shifted behavioral expression from stress sensitive (Stay) to resilient (Escape) responses, an effect that was mimicked by genetic knockdown. Acute inhibition of Orx<sub>1</sub>R in the BLA also reduced contextual and cued fear freezing responses in Stay animals. This phenotype-specific behavioral change was accompanied by biased molecular transcription favoring *HCRTR2* over *HCRTR1*, and *MAPK3* over *PLCB1* cell signaling cascades and enhanced *BDNF* mRNA.

**CONCLUSIONS:** The functional reorganization of intra-BLA gene expression after Orx<sub>1</sub>R antagonism promotes elevated *HCRTR2*, to greater *MAPK3*, yielding increased *BDNF*

expression. Together, these results provide evidence for a receptor-driven mechanism that balances pro- and anti-stress responses within the BLA.

## **INTRODUCTION**

Stress-induced alterations in neurocircuitry result in divergent behavioral responses. Enhanced stress reactivity (pro-stress) in rodent models is similar to human affective dysfunction in mood disorders like depression, fear-/anxiety-related disorders, or post-traumatic stress disorder (PTSD) [1]. Current pharmacotherapies for affective disorders have limited success, and a mechanistic understanding remains elusive.

Balance within key stress circuits may be disrupted during periods of intense or prolonged stress to shift signaling dynamics in pro- or anti-stress pathways [2-4]. Stressful stimuli are interpreted, in part, through converging signals in the basolateral amygdala (BLA), where glutamatergic projection neurons are influenced by distinctive GABAergic interneurons, to direct behavioral responses [5]. Additionally, activity in the BLA is modified by hypothalamic orexinergic neurons, which are critical for panic [6, 7] and motivation [8, 9].

Orexin A (Or<sub>XA</sub>) and orexin B (Or<sub>XB</sub>), neuromodulators derived from a single pre-propeptide, activate two G-coupled protein receptors: orexin 1 receptors (Or<sub>X1R</sub>), having greater affinity for Or<sub>XA</sub>, and orexin 2 receptors (Or<sub>X2R</sub>), which binds equally well to Or<sub>XA</sub> and Or<sub>XB</sub> [10]. These receptors stimulate heterotrimeric G<sub>q</sub> proteins which increase intracellular Ca<sup>2+</sup> [11] to activate phospholipase C (PLC) pathways [12]. The PLC<sub>β1</sub> isozyme variant is transcribed in the amygdala [13], and its dysfunction is linked to psychopathologies like depression [14], bipolar disorder [15], addiction [16], and schizophrenia [17, 18].

Stimulation of Orx<sub>1</sub>R can also activate extracellular signal-regulated protein kinase (ERK). In the amygdala, recruitment of ERKs is important for consolidation, reconsolidation, and extinction of fear memories [19, 20]. While Orx<sub>1</sub>R in the BLA are important in regulating fear [21, 22], depression [23, 24], and anxiety [25], it is unclear how shifts in molecular signaling cascades mediate such responses and initiate stress-induced phenotype development.

Utilizing the Stress Alternatives Model (SAM), a behavioral paradigm that separates individuals into social stress resilient (Escape) and vulnerable (Stay) populations [26], we explored how Orx<sub>1</sub>R activity in the BLA is involved in the formation of stress-related phenotypes. As a social interaction and avoidance paradigm in which smaller subjects encounter intense attacks from larger novel aggressors over a four-day period, the SAM produces two separate subsets of animals, exhibiting social avoidance or enhanced fear conditioned responses [27, 28]. Unlike a traditional social defeat outcome, the SAM provides mice an opportunity to avoid social aggression by exiting the arena through one of two escape tunnels only large enough for the smaller mouse. By the end of the second day of social interaction, test subjects stably commit to a phenotype: Escape or Stay. These stable phenotypes may be altered through pharmacological manipulations administered on the third day of the SAM [28-30]. Thus, the SAM is a useful tool for studying the development of stress-induced phenotypes, while providing an opportunity to explore physiological and clinically relevant molecular mechanisms.

We investigated if inhibition of intra-BLA Orx<sub>1</sub>R, alters the formation of social stress-induced behavioral phenotypes. We predict that pharmacological inhibition or genetic knockdown will shift behavioral patterns in vulnerable (Stay) populations toward resilience (Escape). Further, we explored if Orx<sub>1</sub>R inhibition affects conditioned fear responses and alters the expression of genes responsible for balancing signaling in pro- and anti-stress

neurocircuitries. Together, these results allow us to propose a neurocircuit model that defines the role of intra-BLA Orx<sub>1</sub>R signaling in the balance of pro- and anti-stress states.

## **METHODS & MATERIALS** (*see also* COMPLETE METHODS & MATERIALS)

### **Social Stress and Decision-Making Paradigm**

Aggressive social interactions between larger novel CD1 and smaller male C57BL/6NHsd mice dyads in the SAM apparatus (Fig. 1) involve four trials, lasting up to five minutes each, allowing test animals the opportunity to shorten stressful encounters by making use of size-restricted tunnels at the apical end of the oval open field interaction arena. A tone given during isolation in the SAM apparatus prior to social interaction permits comparisons between cued and contextual fear conditioning. The escape routes provide a decision-making opportunity, producing two stable phenotypes: active avoidance (Escape) and enhanced fear conditioning (Stay), which may be modified by drug treatment on Day 3. The treatment regimen allows for statistical comparisons between groups, and within subjects, by comparing responses to SAM interactions before and after treatment. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the University of South Dakota Institutional Animal Care and Use Committee.

### **Experimental Overview** (*see also Supplemental Information*)

The primary treatment for these experiments is inhibition of BLA Orx<sub>1</sub>R, via the antagonist SB-674042 (0.3 nmol/0.3  $\mu$ L delivered bilaterally intra-BLA, 1h prior to interaction on Day 3) or short-hairpin knockdown (bilateral intra-BLA transduction beginning 30 days prior to SAM interaction). Considering the difference in timing of delivery, these treatments were done and analyzed separately, with unique hypotheses. All behavioral measures were performed during the dark cycle, and included Escape (use of the apical tunnels), Stay (remaining in the SAM

arena with the novel aggressor), time spent attentive to the escape hole, latency to escape (for Escape mice), fear conditioned freezing (measured in response to the tone and context, prior to the social interaction unconditioned stimulus [US], and as a conditioned response [CR on Day 5] in the absence of the US), and food intake. Thus, treatment groups included home cage controls, intra-BLA vehicle injection, and intra-BLA SB-674042 injection of Escape and Stay mice. In addition, local knockdown treatment groups included home cage controls, intra-BLA AAV-Orx<sub>1</sub>R-shRNA injection, and intra-BLA AAV-scramble-shRNA injection. Brains and blood were collected for visual representations of gene expression (using RNAscope) of *HCRTR1*, *HCRTR2*, calbindin (*CALB1*), Ca<sup>++</sup>/Calmodulin Kinase type 2 alpha (*CAMKIIα*), Glutamate Decarboxylase (*GADI*), and parvalbumin (*PVALB*) in the BLA, as well as to measure plasma concentrations of the stress hormone corticosterone (by enzyme linked immunosorbent assay). Gene expression (using qRT-PCR) of *HCRTR1*, *HCRTR2*, *PLCB1*, *MAPK1*, *MAPK3*, *BDNF*, and *GAPDH* (housekeeping gene) were measured in BLA tissue. All experimental designs and statistical analyses were based on *a priori* hypotheses, using two-way repeated measures ANOVA, two-way ANOVA, one-way ANOVA, regression analyses, and t-test, followed (where appropriate) by *post hoc* analyses.

## **RESULTS** (*see also* COMPLETE RESULTS)

### **Orx<sub>1</sub>R are expressed in BLA glutamatergic neurons**

The glutamatergic marker, CamKIIα, was identified in the vast majority of BLA neurons (~80%; Fig. S2) as well as those expressing *HCRTR1* [31, 32], though the signal was also found in some calbindin-expressing GABAergic neurons (Fig. 2). Few (<20%) BLA *HCRTR1*-possessing cells express *GADI* (GABAergic marker) and co-express parvalbumin (PV, ~10%; Figs. 2I-J). While



Orx<sub>1</sub>R are localized predominantly on glutamatergic neurons in the BLA (Fig. 2P), our results suggest *HCRTR1* is expressed in 10-15% of BLA glutamatergic neurons and ~5% of GABA cells (Fig. 2K).

### **Intra-BLA Orx<sub>1</sub>R inhibition and knockdown increase motivation for active avoidance**

#### **(Escape)**

In the SAM, typically animals evenly self-select one of two stable [27-29, 33] behavioral phenotypes, Escape or Stay (Figs. 1A) [26, 27, 29, 33, 34], 44.7% Escape and 55.3% Stay (Figs. S1B, C).

We wished to determine if inhibition of Orx<sub>1</sub>R (SB-674042) in BLA [2] stimulated motivation for active avoidance. Time spent investigating the escape route is both a predictor of active avoidance and an indicator of motivation to escape [28]. Time spent attentive to the hole was significantly greater in vehicle-treated Escape mice (Fig. 3A), but intra-BLA infusion of the Orx<sub>1</sub>R antagonist (Escape: Figs. 3B, C; Stay: Figs. 3B, D) or AAV-U6-Orx<sub>1</sub>R-shRNA (Fig. 3E) increases attention to the escape route. Further, receptor activation with Orx<sub>A</sub> reduced the time Escape mice spent investigating the escape route (Fig. S3). Together these results characterize an important function of BLA Orx<sub>1</sub>R in modulating adaptive motivation in a social stress environment (Fig. 3F), where inhibition or knockdown promote increased motivation to Escape.

#### **Inhibition and knockdown of BLA Orx<sub>1</sub>R promote active avoidance (Escape)**

As motivation to escape was enhanced, we wished to determine if inhibition of Orx<sub>1</sub>R in BLA also resulted in more proactive anxiolytic behavior (Escape). Upon intra-BLA injections of an Orx<sub>1</sub>R antagonist on SAM Day 3, a substantial number of Stay mice exhibited Escape behavior (Fig. 4A), with a 30% shift that day, and a significant increase the day after (Day 4 = 70% increase). Interestingly, intra-BLA activation of both Orx receptors with Orx<sub>A</sub> or biased

activation of Orx<sub>1</sub>R with dual delivery of Orx<sub>A</sub> and an Orx<sub>2</sub>R antagonist blocked Escape behavior in a small proportion of mice on Days 3 and 4 (Fig. S4), and though not statistically significant, does support the pro-stress role of Orx<sub>1</sub>R.

As knockdown reduced Orx<sub>1</sub>R expression prior to stressful interactions experiments, we did not expect a dramatic change in behavior over the course of SAM trials, but while scramble control mice remained stable, those treated with AAV-U6-Orx<sub>1</sub>R-shRNA selected to Escape incrementally (though not significantly) more on the last two days of SAM exposure (Fig. 4B). By the end of Day 4, 72.7% of AAV-U6-Orx<sub>1</sub>R-shRNA-treated mice displayed the Escape phenotype compared to 54.5% of those that received the scramble control.

Escape mice spent significantly less time in the SAM arena with the CD1 mouse on Days 2 through 4 [26, 27, 29, 33, 35], thus escape latency was reduced (Fig. 4C). Stay mice remain submissively for the entire 5 min period, unless treated with the Orx<sub>1</sub>R antagonist, significantly reducing the time spent with the aggressive CD1 mouse on Day 4 (Fig. 4D). Neither Orx<sub>1</sub>R inhibition nor knockdown influenced escape latency in Escape animals (Figs. 4D, E). These results, in combination, suggest that intra-BLA Orx<sub>1</sub>R promote coping strategies associated with responses to increased stress, and acute inhibition of these receptors allows for greater expression of behavior derived from reduced output of pro-stress neurocircuitry (Escape; Fig. 4F).

Importantly neither of the Orx<sub>1</sub>R manipulations, antagonist or knockdown treatments, influenced arousal/locomotion (Figs. S5), but did result in small but significant decreases in food intake and body weight (Figs. S6).

### **BLA Orx<sub>1</sub>R inhibition reduces cued and contextual fear conditioning**

As motivation for and actual active stress-avoidance were promoted by intra-BLA Orx<sub>1</sub>R inhibition, we probed whether SAM social stress exposure (US<sup>+</sup>) could be associated with fear

conditioning (tone = CS; freezing behavior =CR; absence of social aggressor = US<sup>-</sup>; Fig. 1A). Cued fear responses significantly enhanced freezing in both Escape and Stay phenotypes (Figs. 5A, B), and Stay mice displayed heightened freezing behavior to context (CS<sup>-</sup>, opaque cylinder divider) as well (Fig. 5B). Although inhibition of intra-BLA Orx<sub>1</sub>R did not affect the fear freezing profile in Escape mice (Figs. 5A, D), antagonist-treated Stay mice exhibited significantly reduced contextual (CS<sup>-</sup>) and cued (CS<sup>+</sup>) fear responses (Figs. 5A, H). Like mice of the Escape phenotype, knockdown of BLA Orx<sub>1</sub>R (AAV-U6-Orx<sub>1</sub>R-shRNA) did not affect conditioned freezing behavior (Fig. S7). Importantly, activation of intra-BLA Orx receptors with Orx<sub>A</sub> did not change the fear freezing profile in Escape or Stay mice compared to Vehicle control (Figs. 5A, E, I). However, biased stimulation of Orx<sub>1</sub>R in the BLA with a combination of Orx<sub>A</sub> and an Orx<sub>2</sub>R antagonist eliminated the conditioned response in Escape (Figs. 5A, F), but not Stay mice (Figs. 5A, J).

### **Corticosterone levels are reduced with intra-BLA Orx<sub>1</sub>R antagonism**

As intra-BLA Orx<sub>1</sub>R inhibition increased motivation for, and promoted, stress-avoidance, as well as reducing conditioned fear, we hypothesized that this treatment would also reduce plasma concentrations of the stress hormone, corticosterone (Fig. 5C). Social stress in SAM interactions increases corticosterone concentrations in both Escape and Stay animals [27, 28, 33], although Stay mice have higher levels of corticosterone compared to Escape. Inhibition of BLA Orx<sub>1</sub>R decreased Stay corticosterone concentrations compared to vehicle-treated Stay animals; and did not differ significantly from non-stressed mice (Fig. 5C). Treatments with Orx<sub>A</sub> or the combination of Orx<sub>A</sub> and an Orx<sub>2</sub>R antagonist did not change corticosterone levels relative to vehicle-treated controls, however, the differences between Escape and Stay were eliminated and

levels were elevated compared to Orx<sub>1</sub>R antagonist-treated mice (Fig. 5C). Inhibition of BLA Orx<sub>1</sub>R not only reduces social fear responses, but also reverses social stress responsiveness.

### **Antagonism of intra-BLA Orx<sub>1</sub>R recruits alternative signaling**

With Orx<sub>1</sub>R antagonism in the BLA, we predicted orexin receptor gene (*HCRTR1* & *HCRTR2*) expression may be influenced by phenotype and treatment (Figs. 6). Although *HCRTR1* expression was unaltered following vehicle treatment, Orx<sub>1</sub>R antagonism reduced intra-BLA *HCRTR1* in Escape mice compared to non-stressed cage controls (Fig. 6A), and simultaneously elevated *HCRTR2* expression in Stay mice compared to Escape and vehicle-treated Stay mice (Fig. 6B). In vehicle controls *HCRTR2* expression was higher in Escape mice compared to both Stay and Orx<sub>1</sub>R antagonist-treated Escape mice (Fig. 6B). Changes in both *HCRTR1* and *HCRTR2* expression due to Orx<sub>1</sub>R inhibition appear to occur in a phenotype-dependent way in the BLA.

Transcription of BLA PLC<sub>β1</sub> (*PLCB1*) mRNA [13] is likely important for Orx<sub>1</sub>R signaling [36] in the BLA, so we predicted Orx<sub>1</sub>R antagonist might limit *PLCB1* expression levels (Fig. 6C). Interestingly, Escape mice in both vehicle and Orx<sub>1</sub>R antagonist groups expressed lower amounts of *PLCB1* compared to Stay animals (Fig. 6C). Escape mice in both vehicle- and Orx<sub>1</sub>R antagonist-treated groups had lower *PLCB1* mRNA compared to cage control animals (Fig. 6C). These data suggest adaptive physiological shifts in intra-BLA *PLCB1* expression may play a role in, or result from, phenotype development, without identifying how Orx<sub>1</sub>R antagonism is involved.

Alternative molecular pathways recruited during G<sub>q</sub> activation are driven by ERK genes (*MAPK1* & *MAPK3*). Although ERK<sub>2</sub> (*MAPK1*) mRNA was unaffected (Fig. S8), Orx<sub>1</sub>R antagonism in Stay mice resulted in a significant increase in *MAPK3* expression compared to similarly treated Escape, vehicle-treated Stay, and non-stressed cage control mice (Fig. 6D). These results suggest potential links amongst Orx<sub>1</sub>R inhibition, phenotype plasticity, and PLC<sub>β1</sub> and ERK<sub>1</sub> signaling recruitment in behaviorally distinctive groups, which also differ in stress sensitivity.

The transcription of brain-derived neurotrophic factor (BDNF) is tied to neuroplasticity [37, 38] and behavioral changes like extinction of fear memories [39], so we predicted an increase in *BDNF* might be associated with intra-BLA Orx<sub>1</sub>R inhibition (Fig. 6E). As hypothesized, intra-BLA Orx<sub>1</sub>R antagonism resulted in elevated *BDNF* in Stay compared to Escape mice and vehicle-treated Stay mice (Fig. 6E). As Stay mice treated with an Orx<sub>1</sub>R antagonist experienced shifts from stress-vulnerable to resilient behavioral responses, the alterations in gene expression reported here (Fig. 6F) may be implicit in this behavioral plasticity.

### **Molecular restructuring following intra-BLA Orx<sub>1</sub>R inhibition is related to fear responsiveness**

As altered transcription coincided with behavioral change produced by intra-BLA Orx<sub>1</sub>R antagonist treatment, we hypothesized that correlations would exist between them. Expression levels of *HCRTR2* in both vehicle- and Orx<sub>1</sub>R antagonist-treated mice are negatively correlated with cued freezing (Figs. 7A, B). Relative expression levels of *PLCBI* were positively correlated with cued freezing behavior in vehicle-treated mice (Fig. 7C); however, this relationship is not observed after intra-BLA Orx<sub>1</sub>R inhibition (Fig. 7D). Contextual freezing behavior was associated with *MAPK3* expression in only vehicle-treated mice (Figs. S9I). By contrast, intra-BLA antagonism of Orx<sub>1</sub>R cued freezing behavior was negatively correlated to *MAPK3* expression (Fig. 7F), but not in vehicle-treated mice (Fig. 7E). The lack of gene expression correlations with cued fear freezing when phenotypes were assessed independently (Figs. S10), indicates that behavioral and transcriptional relationships exist within collective operational adaptations that link behavioral change to molecular modification. Together, these results suggest a functional connection between Orx<sub>1</sub>R antagonist-induced shifts in gene expression and fear-related behaviors.

### **Cells expressing Orx<sub>1</sub>R in the BLA do not co-express Orx<sub>2</sub>R**

Given that Orx<sub>1</sub>R antagonism within the BLA alters expression of Orx<sub>2</sub>R (*HCRTR2*) mRNA, and is related to cued fear responses, we decided to investigate whether both orexin receptor subtypes

are expressed within the same amygdalar neurons (Fig. 2). In BLA cells, mRNA for *HCRT1* and *HCRT2* largely do not overlap, as ~80% of *HCRT1*<sup>+</sup> cells do not co-express *HCRT2* (Figs. 2L-O). Importantly, even when BLA Orx<sub>1</sub>R are inhibited, native Orx<sub>A</sub> and Orx<sub>B</sub> will bind Orx<sub>2</sub>R. Previous research from our lab suggests Orx<sub>2</sub>R may be predominantly localized to specific GABAergic neurons within the BLA [28].

### **Fear response after Orx<sub>2</sub>R inhibition is phenotypically different from Orx<sub>1</sub>R antagonism**

As blocking Orx<sub>1</sub>R in the BLA produced major effects on conditioned fear freezing in Stay mice (Fig. 5H), and increased *HCRT2* gene expression (Figs. 6B, 7B), we predicted antagonism of Orx<sub>2</sub>R (MK-1064) might affect fear behavior in Escape mice. Acute inhibition of Orx<sub>2</sub>R in the BLA eliminated the cued (CS<sup>+</sup>) freezing response in Escape mice observed in vehicle control animals and significantly reduced freezing during the post-tone (CS<sup>+</sup>) period (Figs. 5A, G). Stay mice treated with an Orx<sub>2</sub>R antagonist displayed no statistical differences in the levels of contextual (CS<sup>-</sup>) and cued (CS<sup>+</sup>) freezing compared to animals in the vehicle control group (Figs. 5A, K). These results suggest Orx receptor activity in the BLA influences social stress-induced fear behavior in receptor type and phenotype dependent fashions.

### **Transcriptional changes after Orx<sub>2</sub>R antagonism contrast those observed after Orx<sub>1</sub>R inhibition**

Since blocking Orx<sub>1</sub>R produced changes in mRNA expression relevant to BLA cell signaling dynamics, we predicted Orx<sub>2</sub>R antagonism to induce opposing changes to these transcriptional relationships (Fig. 6). While Orx<sub>1</sub>R inhibition resulted in a reduction in *HCRT1* gene expression in Escape mice, Orx<sub>2</sub>R antagonism presented a similar decrease, but only in Stay animals (Fig. 6A). Expression of *HCRT2* in the BLA was reduced in both Escape and Stay phenotypes after blocking Orx<sub>2</sub>R, contrasting with Orx<sub>1</sub>R antagonism, which enhanced *HCRT2*

mRNA levels in Stay mice (Fig. 6B). Further, intra-BLA Orx<sub>2</sub>R inhibition muted the reduction in *PLCB1* observed in Escape mice under Vehicle treatment conditions (Fig. 6C) while having no effect on *MAPK3* gene expression (Fig. 6D). Finally, Orx<sub>2</sub>R antagonist treatment enhanced *BDNF* expression in Escape mice, while diminishing transcription in Stay animals, an effect that is phenotypically opposite to that observed after Orx<sub>1</sub>R inhibition (Fig. 6E). Importantly, no relationships between gene expression and conditioned fear freezing were observed for any of the tested cell signaling markers after Orx<sub>2</sub>R antagonism except for *BDNF*, in which a significant negative correlation was revealed (Fig. S12E).

### **Gene expression uncovers a potential molecular mechanism behind intra-BLA Orx<sub>1</sub>R antagonism**

To help generate a *theoretical* mechanism to explain the physiological basis surrounding the observed behavioral (and phenotypic) shifts resulting from intra-BLA inhibition of Orx<sub>1</sub>R, we explored transcriptional relationships in systems that exhibited similar regression patterns (Fig. 8). With antagonism of Orx<sub>1</sub>R, there is a steeply positive relationship between *HCRTR2* and *MAPK3* expression (Fig. 8A). Importantly, this association does not exist after vehicle or Orx<sub>2</sub>R antagonist treatment (Fig. S11A). While there are no observed relationships between *BDNF* and *HCRTR2* expression levels (Figs. 8B, S13), *BDNF* expression is positively correlated to *MAPK3* expression in animals treated with an Orx<sub>1</sub>R antagonist (Fig. 8C). Notably, no relationships exist between *HCRTR1* expression and the other genes of interest (Figs. S13D-I). These data allowed us to predict a working model to explain how BLA Orx<sub>1</sub>R may function to establish behavioral patterns consistent with stress-induced phenotype development (Fig. 9).

## **DISCUSSION**

Antagonism of Orx<sub>1</sub>R in the BLA can reverse or diminish expression of stress-related behavior. Our results suggest BLA Orx<sub>1</sub>R play a central role in stress responsiveness [40, 41] and related behavioral,

physiological, and molecular outcomes that are important components of affective disorders [42, 43], such as anxiety [7], depression, and PTSD. Acute inhibition of intra-BLA Orx<sub>1</sub>R promotes Escape over Stay responses and limits freezing during fear conditioning in a phenotype-dependent way. Further, inhibition of Orx<sub>1</sub>R alters gene expression associated with critical signaling cascades. Following intra-BLA Orx<sub>1</sub>R antagonism, transcription for receptors and intracellular signaling becomes biased toward Orx<sub>2</sub>R (*HCRTR2*) over Orx<sub>1</sub>R (*HCRTR1*), and ERK<sub>1</sub> (*MAPK3*) over PLC<sub>β1</sub> (*PLCBI*) pathways. The relationship of these behavioral and molecular changes to enhanced expression of *HCRTR2* mRNA, largely in BLA neurons that do not contain Orx<sub>1</sub>R (Figs. 2L-O), suggests receptor-mediated mechanisms that balance pro- and anti-stress responses in BLA microcircuits.

Aggressive social interactions in the SAM produced two behavioral phenotypes that represent risk assessment and decision-making: Escape and Stay. These phenotypes, like those exposed to social defeat paradigms [44, 45], exhibit resilience (tightly linked to Escape) and susceptibility (highly correlated with Stay) in the Social Interaction/Preference (SIP) test [28]. However, unlike traditional social defeat, SAM-separated phenotypes are expressed early in the behavioral paradigm, providing insight into the development and progression of stress-induced behavior and pathophysiology. Anxiolytic drugs (such as CRF<sub>1</sub> receptor antagonist antalarmin and the Orx<sub>2</sub>R agonist [Ala<sup>11</sup>, d-Leu<sup>15</sup>]-Orx<sub>B</sub>) promote escape, while anxiogenic drugs (such as the α<sub>2</sub> antagonist yohimbine and the Orx<sub>2</sub>R antagonist MK-1064) delay and/or block escape behavior [28, 29]. Surprisingly, neither the Orx<sub>1</sub>R antagonist (Fig. 4D) nor knockdown (Fig. 4E) influenced escape latency, although it is reduced by anxiolytic factors such as exercise, Neuropeptide S, antalarmin, and increased by anxiogenic factors like yohimbine [29]. We posit that enhanced escape on Day 4, following BLA Orx<sub>1</sub>R inhibition (on Day 3, drug treatment), is a reflection of the shift toward anti-stress signaling indicated by downregulation in pro-stress signaling (*HCRTR1*), and upregulation of anti-stress systems (*HCRTR2*, *MAPK3*, *BDNF*). These stress-induced effects are paired with important learning and motivational components during SAM interactions [27, 29, 33, 35], and in human affective disorders [46].



In addition to species-specific anxious behavior and learning, social stress promotes behavioral inhibition, depressed behavioral drive and motivation in some individuals [47], plus a lower rate of adaptive behavior [48]. Behavioral depression reveals two distinctive phenotypes related to stress responsiveness in humans and other animals [45, 49, 50]. In SAM social interaction trials, Stay animals exhibit significantly less interest in exploring/investigating the escape route (Fig. 3A) and indecisiveness relative to escape [35]. Measuring motivation in the SAM is derived from a simple decision-making process, Escape or Stay [26, 27]. Antagonism and knockdown of Orx<sub>1</sub>R increases interest in the escape route for both Stay and Escape mice (Figs. 3C, D). Thus, BLA Orx<sub>1</sub>R regulate stress-induced motivational behaviors; greatest in Escape mice, but marking a dramatic behavioral reversal in Stay mice that typically avoid the escape route (Figs. 3B-C). The complementary results of intra-BLA knockdown of Orx<sub>1</sub>R (Fig. 3E) supports the notion that during periods of stress, intra-BLA Orx<sub>1</sub>R activity may provoke behavioral depression. Attention to the escape route happens prior to escape, and is thus the first evidence of phenotypic differentiation in the SAM [28, 35]. Latency to escape, and escape behavior also are influenced by motivation, although as previously demonstrated, these behaviors are strongly affected by stress and fearfulness associated with familiarity of the SAM or social interaction [27-29, 33, 35]. Our results, like those of others, suggest Orx activity plays a fundamental role in motivation [8, 51], and in this case, specifically in the BLA for behaviors associated with stress-related motivation and decision-making.

Understanding the development of decision-making and motivation in the SAM is enhanced by pairing aversive aggression (US) with a non-threatening stimulus (tone CS) prior to interaction, promoting potent cued and contextual conditioned responses (CR) similar to standard fear conditioning approaches that utilize foot shock as a US [52]. While the CRs elicited are similar, e.g. freezing [53], the ethological and ecological relevance of the US to the subject are not. By associating naturally aversive US with a benign stimulus [54], the SAM allows views into development of fear learning as it relates to the etiology of stress-provoked neurocircuitry changes, and demonstrates a connection between stress-induced fear expression and phenotype (Fig. 5). While early work suggested only Stay animals exhibited

cued fear learning [27, 33], it is now clear both Stay and Escape respond to auditory cues with enhanced freezing compared to pre-tone freezing, and Stay mice also show contextual (prior to the cue) fear conditioning (Fig. 5B). Additionally, BLA Orx activity modulates associative fear learning [22], with Orx<sub>1</sub>R, but not Orx<sub>2</sub>R, inhibition reducing both contextual and cued conditioned fear responses in Stay animals (Figs. 5H, K). Antagonizing Orx<sub>1</sub>R reduces fear/panic-induced freezing [7, 55, 56], with Orx<sub>2</sub>R antagonism appearing to eliminate all fear learning in Escape mice; and our results demonstrate a phenotype-dependent effect (Figs. 5D, H). Stimulation of intra-BLA Orx<sub>1</sub>R and Orx<sub>2</sub>R receptors using Orx<sub>A</sub> in Stay mice produces no reduction in contextual or cued fear conditioning (Fig. 5I), suggesting that the inhibition of both types of learned fear responses result specifically from Orx<sub>1</sub>R inhibition in Stay mice. To clarify the roles of Orx<sub>1</sub>R and Orx<sub>2</sub>R, we administered Orx<sub>A</sub> while concurrently inhibiting Orx<sub>2</sub>R (MK1064), leaving Orx<sub>1</sub>R stimulated, and again there was no statistically significant reduction in either type of fear conditioning response (Fig. 5J). Interestingly, knockdown of Orx<sub>1</sub>R did not affect the fear freezing profile (Fig. S7). As knockdown occurred before the introduction of social stress, activity levels of Orx<sub>1</sub>R after SAM exposure allowed for fear learning (higher freezing after CS), but did not diminish freezing as observed with acute antagonism after stress and phenotype development (Fig. 5H).

Molecular gene expression during SAM fear conditioning and phenotype development indicated potential shifts in receptor-linked intracellular signaling cascades (Fig. 6). Acute inhibition of intra-BLA Orx<sub>1</sub>R lowered *HCRTR1* expression in Escape mice while enhancing *HCRTR2* in Stay animals (Figs. 6A, B). Antagonism of Orx<sub>2</sub>R in BLA did the opposite, reducing *HCRTR1* only in Stay mice, and reducing *HCRTR2* in both phenotypes (Figs. 6A, B). Mice exhibiting escape and reduced fear freezing, expressed lower *PLCBI* compared to the Stay phenotype; an effect unaltered by SB-674042 treatment, but reversed by Orx<sub>2</sub>R antagonism (Fig. 6C). However, intra-BLA Orx<sub>1</sub>R antagonism increased *MAPK3* and *BDNF* expression in Stay animals only, with Orx<sub>2</sub>R inhibition having no effect on expression of *MAPK3*, and enhancing *BDNF*, but only in Escape mice, while reducing *BDNF* in Stay mice (Figs. 6D-G). These results suggest social stress disrupts gene expression, and potentially alters BLA signaling pathways depending on an individual's stress state. Therefore, pharmacological interventions (like acute Orx<sub>1</sub>R

antagonism) may functionally amend behavior through signaling adaptations that are phenotype dependent.

Fear conditioning responses appear to be related to specific transcriptional reorganization taking place during/after intra-BLA Orx<sub>1</sub>R inhibition (Fig. 7). In treated animals, negative regressions exist between cued fear freezing behavior and *HCRTR2* as well as *MAPK3* [57] transcriptional changes (Figs. 7B, F). Without treatment (vehicle), cued freezing was positively linked to *PLCB1* gene expression (Fig. 7C), an effect not observed with Orx<sub>1</sub>R antagonism (Fig. 7D). These associations provide evidence for potential mechanistic remodeling (Fig. 9) in the BLA during periods of stress that is tied to phenotype formation and involves Orx receptor activity. This balancing act between Orx<sub>1</sub>R and Orx<sub>2</sub>R creates an influence over BLA microcircuits, which further defines downstream signaling dynamics, in a way that can modify stress-induced behavior [2]. Since changes in *HCRTR2* expression after intra-BLA Orx<sub>1</sub>R inhibition are positively associated with *MAPK3* but not *BDNF* transcription levels (Figs. 8A, B), it appears the adjusted bias of Orx<sub>2</sub>R over Orx<sub>1</sub>R activity favors ERK<sub>1</sub> signaling (Fig. 9). Amplification of ERK<sub>1</sub>, in turn, may lead to enhanced *BDNF* expression (Fig. 8C) and plastic changes within BLA microcircuits (Fig. 9) [57, 58]. Importantly, these findings highlight a role of intra-BLA Orx<sub>1</sub>R in establishing pro-stress behavioral states; but exposes a receptor-driven balance that takes part in the fluid, not static, appearance of phenotype-specific behavior.

## Conclusions

Modulation of BLA stress-regulatory pathways via Orx<sub>1</sub> receptors found predominantly on glutamatergic pyramidal neurons modifies gene expression and behavior. Modulation of pro-stress BLA microcircuits via Orx<sub>1</sub>R inhibition reduces stress-induced behavior. In the process, Orx<sub>1</sub>R BLA inhibition modifies gene expression of *HCRTR2* which impedes pro-stress responses. Concurrently, transcription levels for downstream molecular signaling systems associated with Orx receptor signaling are also tilted toward increased ERK<sub>1</sub> (*MAPK3*), rather than PLC<sub>β1</sub> (*PLCB1*) signaling pathways, potentially altering behavior.

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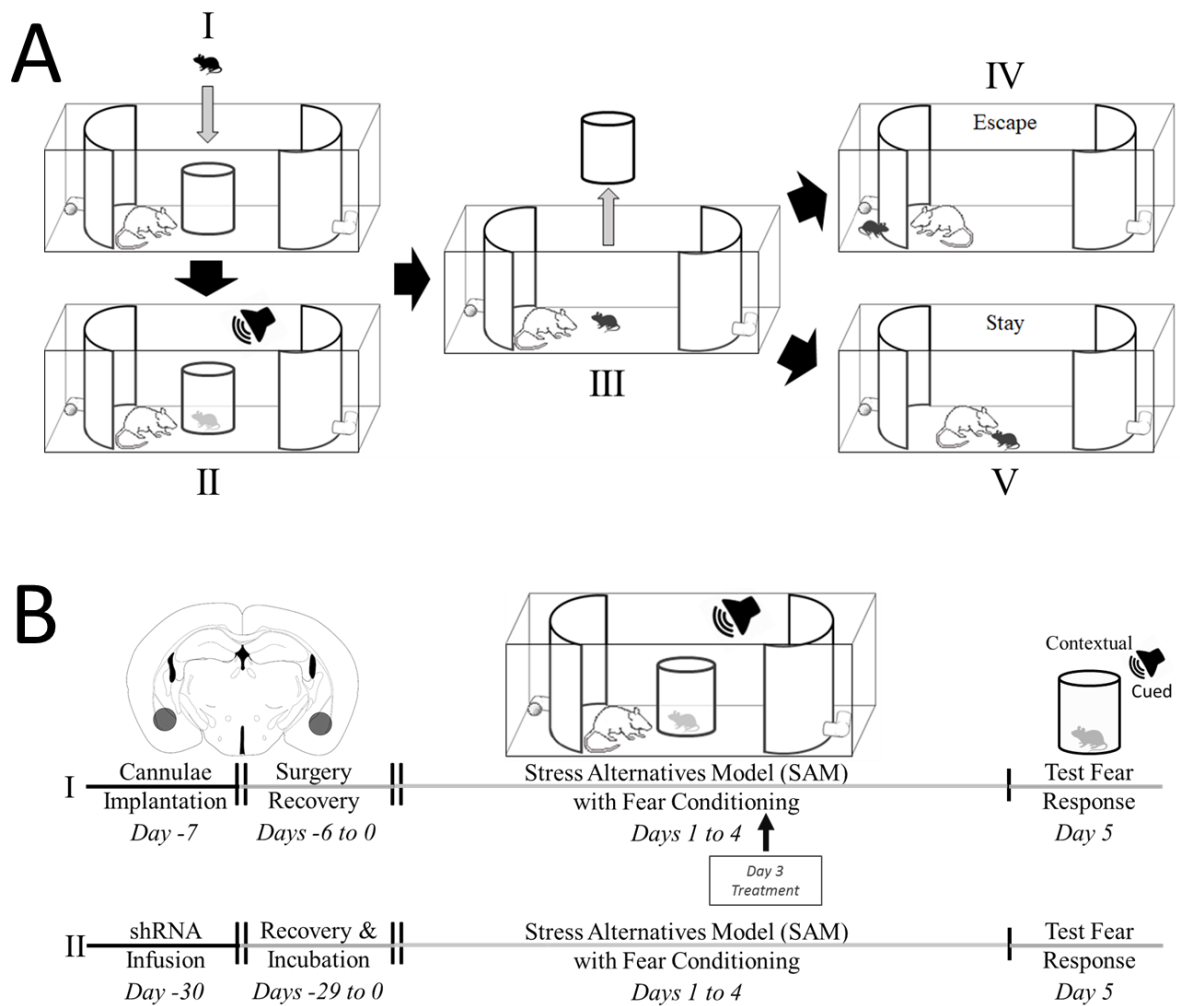
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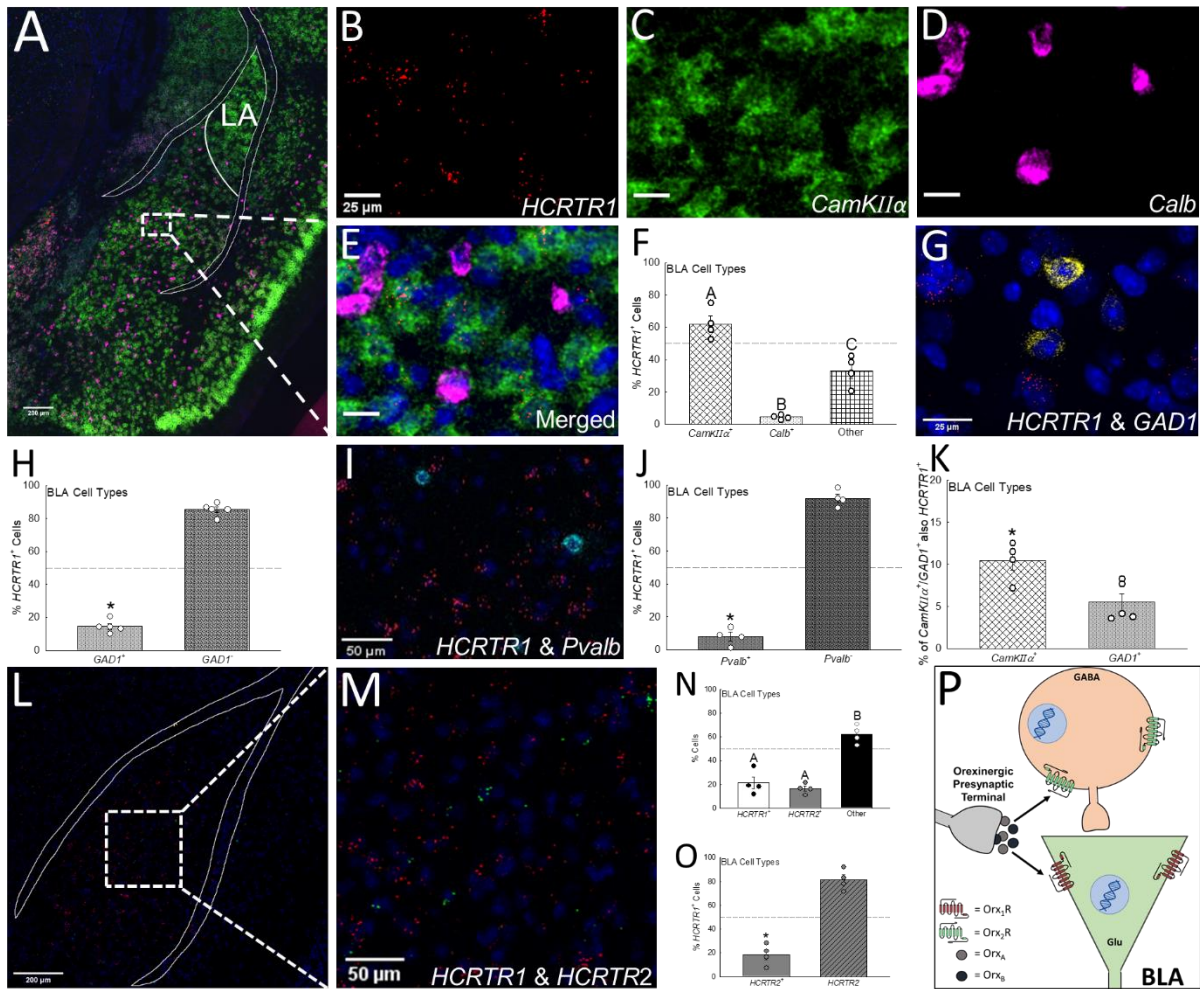
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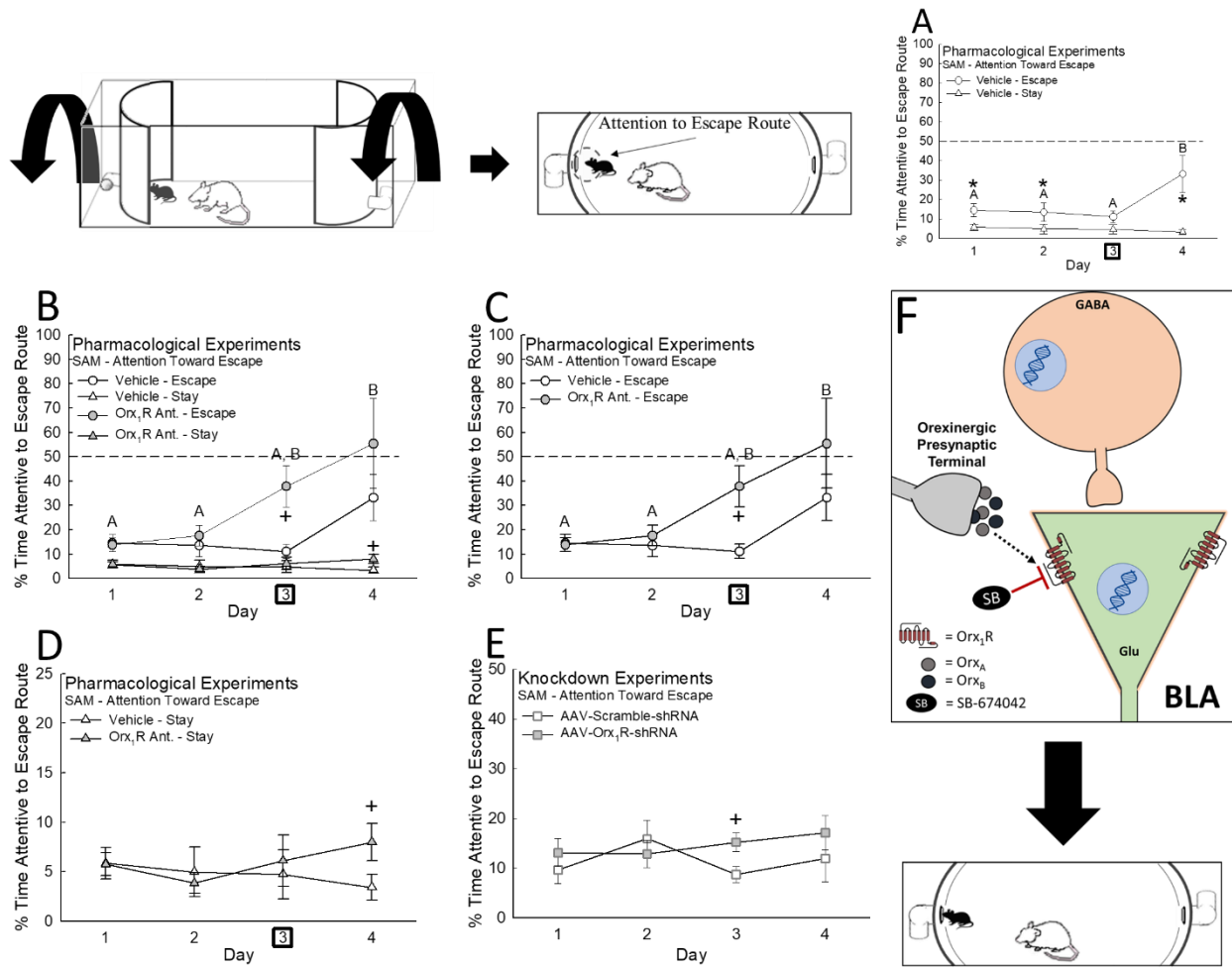


**Figure 1.** The Stress Alternatives Model (SAM) is used to assess the development of stress-induced phenotypes. (A) The SAM is a 4-day behavioral paradigm in which (I) a test mouse is placed into an opaque cylinder, (II) presented a tone, (III) exposed to social aggression, and commits to a phenotype: (IV) Escape or (V) Stay. (B) The behavioral timelines for (I) pharmacology and (II) genetic knockdown experiments (mice are the same age at testing) include surgeries targeting the BLA, SAM exposure (Days 1-4), and the testing of contextual and cued fear responses (Day 5).



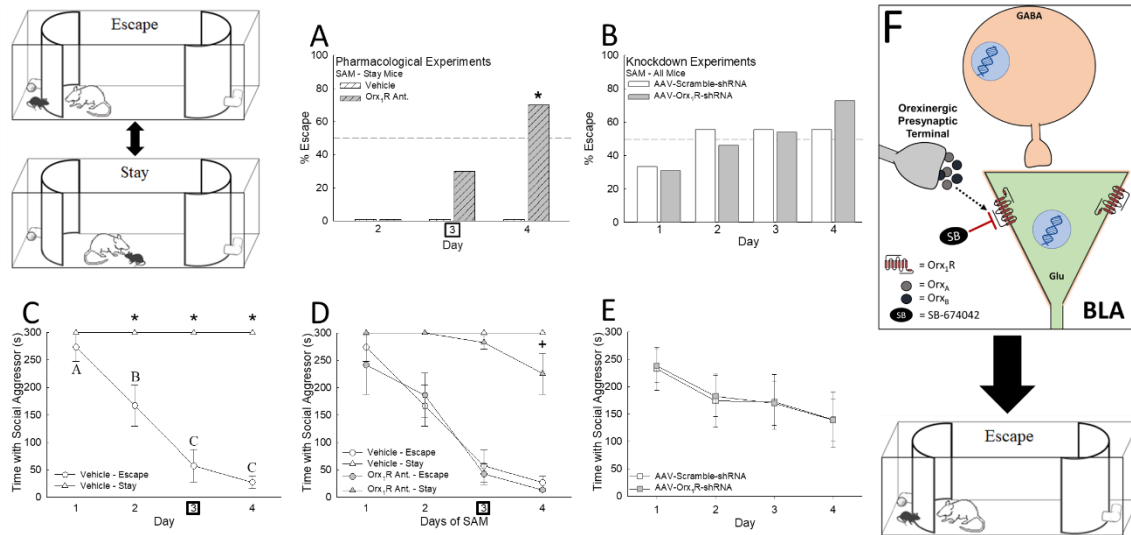
**Figure 2.** In the untreated BLA, Orx<sub>1</sub>R are expressed predominantly in glutamatergic neurons and are rarely co-expressed with Orx<sub>2</sub>R. (A) Imaged sections containing BLA cells (LA = lateral amygdala) stained with probes targeting mRNA of (B) *HCRTR1* (red), (C) *CamKIIα* (green), and (D) *Calb* (Magenta) revealed when (E) merged (with DAPI) that (F) Orx<sub>1</sub>R<sup>+</sup> cells mostly co-express the glutamatergic cell marker, *CamKIIα* (N = 4,  $F_{2,9} = 54.4$ ,  $p < 0.001$ ;  $CamKIIα^+$  vs  $Calb^+$ :  $t_6 = 10.4$ ,  $p < 0.001$ ;  $CamKIIα^+$  vs Other:  $t_6 = 5.2$ ,  $p < 0.001$ ;  $Calb^+$  vs Other:  $t_6 = 5.2$ ,  $p < 0.001$ ; bars are statistically different from one another as illustrated with unique letters, e.g. A is significantly different from B and C;  $p < 0.001$ ). (G) Expression of *HCRTR1* (red) *GAD67* (*GAD1*) mRNA (yellow) infrequently overlap with (H) most *HCRTR1*<sup>+</sup> cells being absent of the GABAergic marker (N = 5,  $t_8 = 29.5$ ,  $*p < 0.001$ ). (I) While a subset of BLA GABAergic neurons produce the calcium-binding protein parvalbumin (*Pvalb*<sup>+</sup>),

(J) *HCRTR1*<sup>+</sup> (red) cells are mostly absent of *Pvalb* expression (light blue) with less than 10% being both *HCRTR1*<sup>+</sup> and *Pvalb*<sup>+</sup> (N = 4,  $t_6 = 23.1$ , \* $p < 0.001$ ). (K) Further, more BLA glutamatergic (*CamKII $\alpha$* <sup>+</sup>) neurons (compared to GABAergic  $\rightarrow$  *GAD1*<sup>+</sup>) also express *HCRTR1* (N = 9,  $t_7 = 3.2$ , \* $p \leq 0.015$ ). (L) Images of BLA cells with fluorescent markers labeling (M) *HCRTR1* mRNA (red) and *HCRTR2* mRNA (green) demonstrate (N) most BLA cells express neither *HCRTR1* nor *HCRTR2* (N = 4,  $F_{2,9} = 42.1$ ,  $p < 0.001$ ; *HCRTR1*<sup>+</sup> vs Other,  $t_6 = 7.5$ ,  $p < 0.001$ ; *HCRTR2*<sup>+</sup> vs Other,  $t_6 = 8.4$ ,  $p < 0.001$ ; bars are statistically different from one another as illustrated with unique letters, e.g. A is significantly different from B). (O) Most *HCRTR1*<sup>+</sup> cells in the BLA do not express *HCRTR2* (N = 4,  $t_6 = 10.1$ , \* $p < 0.001$ ), as depicted in (P) showing Orx<sub>1</sub>R on glutamatergic neurons.

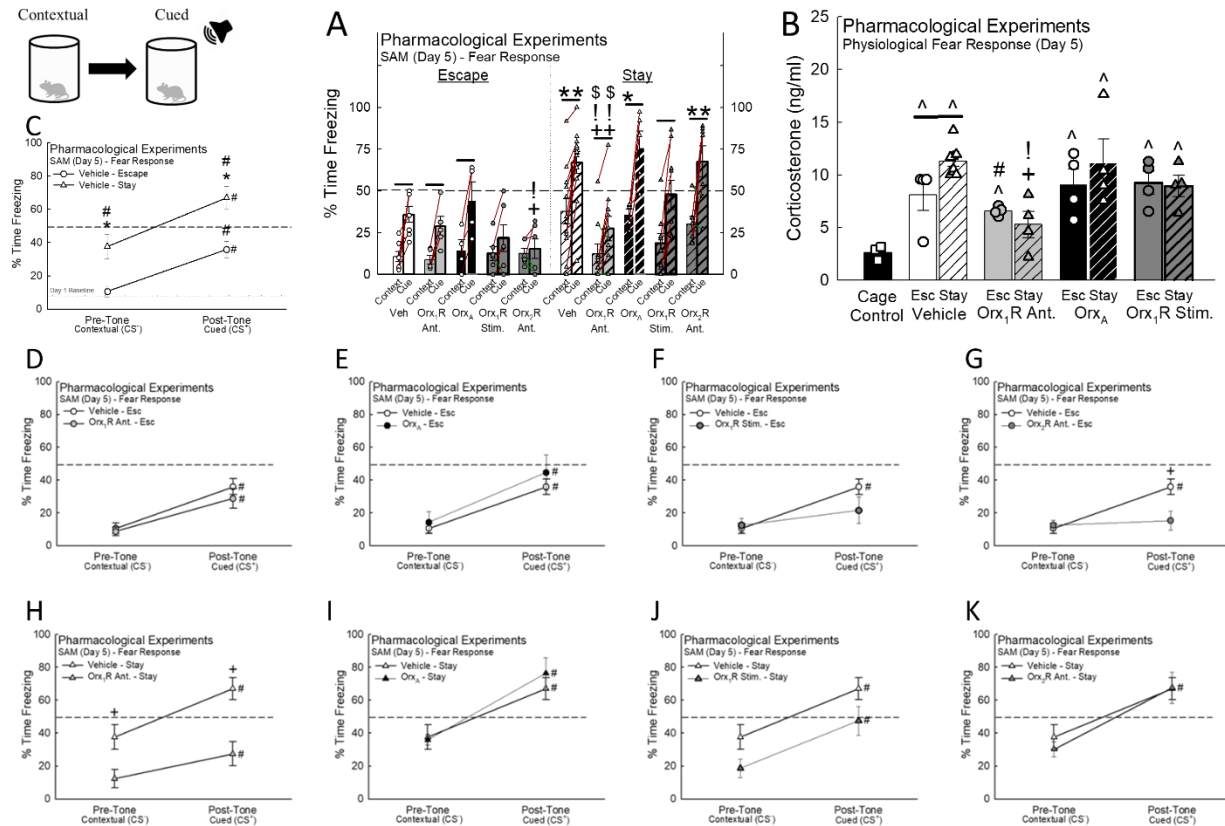


**Figure 3.** Motivation toward Escape behavior is impacted through inhibition of intra-BLA Orx<sub>1</sub>R. (A) Escape mice, as compared to those expressing the Stay phenotype, spend a greater % of time investigating the SAM escape routes (N = 19, Phenotype Effect:  $F_{1,51} = 16.4$ ,  $p < 0.001$ ; Escape vs Stay: Day 1,  $t_{17} = 2.6$ ,  $*p \leq 0.018$ ; Day 2,  $t_{17} = 2.5$ ,  $*p \leq 0.017$ ; Day 4,  $t_{17} = 4.2$ ,  $*p < 0.001$ ). (B) While Escape mice, in general, explore the escape routes more often, (C) inhibition of intra-BLA Orx<sub>1</sub>R promotes even more attention toward the escape tunnels (N = 34, Treatment Effect:  $F_{1,30} = 7.7$ ,  $p \leq 0.019$ ; Day 3 Vehicle Escape vs Orx<sub>1</sub>R Ant. Escape,  $t_{10} = 2.5$ ,  $+p \leq 0.018$ ). (D) Antagonism of intra-BLA Orx<sub>1</sub>R only slightly stimulates escape route exploration in Stay mice (Day 4 Vehicle x Orx<sub>1</sub>R Ant.,  $t_{20} = 2.1$ ,  $+p \leq 0.05$ ). (E) Knockdown of intra-BLA Orx<sub>1</sub>R temporarily and minimally increases attention toward escape on Day 3 of the SAM (N = 22, Day 3 Scramble vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{20} = 2.4$ ,  $+p \leq 0.024$ ). (F) Illustration

demonstrating inhibition of intra-BLA  $Orx_1R$  on predominantly on glutamatergic neurons promotes attention toward the escape route in the SAM arena. In Pharmacological Experiments, drug treatment is administered on Day 3 as designated by the bold square.



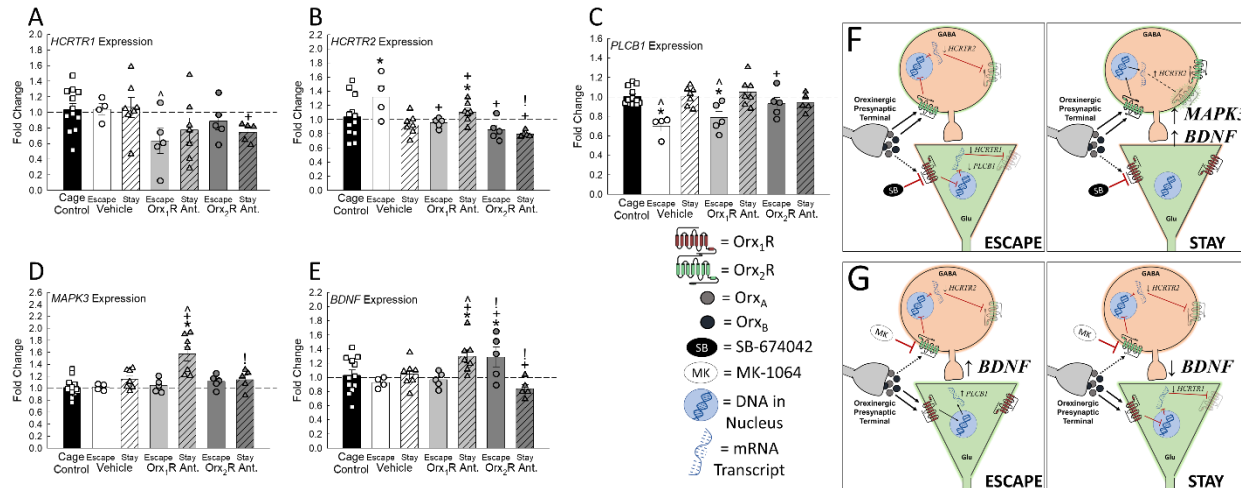
**Figure 4.** Intra-BLA Orx<sub>1</sub>R mediates stress-related behavioral phenotype development. (A) Infusion of an Orx<sub>1</sub>R antagonist (SB-674042) into the BLA promotes Escape behavior in Stay mice (N = 22, Day 4,  $\chi^2$ :  $F_1 = 9.3$ ,  $*p < 0.001$ ). (B) Knockdown of Orx<sub>1</sub>R (AAV-Orx<sub>1</sub>R-shRNA) upsets normal Day 2 phenotype commitment behavior (as observed with AAV-Scramble-shRNA controls), inducing more Escape behavior on Days 3 and 4 (N = 22). (C) Escape animals learn to efficiently utilize the escape route to avoid social aggression over the course of 4 days while Stay mice submit to the aggressor (N = 19, Phenotype Effect:  $F_{1,45} = 175.3$ ,  $p < 0.001$ ; Time Effect:  $F_{3,45} = 26.1$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,45} = 26.1$ ,  $p < 0.001$ ; Escape vs Stay: Day 2,  $t_{17} = 5.8$ ,  $*p < 0.001$ ; Day 3,  $t_{17} = 10.6$ ,  $*p < 0.001$ ; Day 4,  $t_{17} = 11.9$ ,  $*p < 0.001$ ; Within Escape phenotype comparison,  $F_{3,18} = 17.8$ ,  $p < 0.001$ , Day 1 vs Day 3,  $t_6 = 5.7$ ,  $p < 0.001$ ; Day 1 vs Day 4,  $t_6 = 6.5$ ,  $p < 0.001$ ; Day 2 vs Day 3,  $t_6 = 2.9$ ,  $p \leq 0.009$ ; Day 2 vs Day 4,  $t_6 = 3.7$ ,  $p \leq 0.002$ ;  $p < 0.05$  for Days marked with unique lettering, e.g. A is different from B and C). (D) Antagonizing intra-BLA Orx<sub>1</sub>R promotes aggressor avoidance in Stay mice (N = 34, Time Effect:  $F_{3,54} = 2.9$ ,  $p \leq 0.043$ ; Interaction Effect:  $F_{3,54} = 2.9$ ,  $p \leq 0.043$ ; Day 4 Vehicle Stay vs Orx<sub>1</sub>R Ant. Stay,  $t_{20} = 3.4$ ,  $*p < 0.001$ ), but has no effect on those animals exhibiting the Escape phenotype. (E) Knockdown of intra-BLA Orx<sub>1</sub>R does not impact the overall latency of aggressor avoidance (N = 22). Overall, (F) inhibition of Orx<sub>1</sub>R in the BLA appears to prompt Escape behavior. In Pharmacological Experiments, drug treatment is administered on Day 3 as designated by the bold square.



**Figure 5.** (A) Antagonism of intra-BLA Orx<sub>1</sub>R reduces conditioned fear responses in Stay animals while Orx<sub>2</sub>R inhibition diminishes fear freezing in Escape mice (N = 71). (B) Although both Escape and Stay phenotypes learn to associate a cue (tone, CS<sup>+</sup>) with social aggression (Phenotype Effect:  $F_{1,17} = 7.6$ ,  $p \leq 0.013$ ; CS Effect:  $F_{1,17} = 47.7$ ,  $p < 0.001$ ; Escape CS<sup>-</sup> vs CS<sup>+</sup>,  $t_6 = 3.9$ ,  $\#p \leq 0.008$ ; Stay CS<sup>-</sup> vs CS<sup>+</sup>,  $t_{11} = 5.7$ ,  $\#p < 0.001$ ), Stay mice exhibit heightened freezing behavior to both context (CS<sup>-</sup>;  $t_{17} = 2.8$ ,  $*p \leq 0.011$ ) and tone (CS<sup>+</sup>;  $t_{17} = 2.3$ ,  $*p \leq 0.033$ ). Baseline measurements of freezing are represented by a dotted line. Treatments of (D) Orx<sub>1</sub>R Ant. (CS Effect:  $F_{1,10} = 24.7$ ,  $p < 0.001$ ; Orx<sub>1</sub>R Ant. Escape CS<sup>-</sup> vs CS<sup>+</sup>,  $t_4 = 3.4$ ,  $\#p \leq 0.026$ ) or (E) Orx<sub>A</sub> (CS Effect:  $F_{1,9} = 26.8$ ,  $p < 0.001$ ; Orx<sub>A</sub> Escape CS<sup>-</sup> vs CS<sup>+</sup>,  $t_3 = 3.5$ ,  $\#p \leq 0.039$ ) do not alter conditioned fear behavior in Escape mice. However, (F) Escape animals treated with a drug cocktail (Orx<sub>A</sub> + MK-1064) designed to stimulate Orx<sub>1</sub>R (Orx<sub>1</sub>R Stim.; CS<sup>-</sup> vs CS<sup>+</sup>,  $t_5 = 1.8$ ,  $p \geq 0.140$ ) or (G) an Orx<sub>2</sub>R antagonist (CS Effect:  $F_{1,11} = 11.6$ ,  $\#p \leq 0.006$ ; Interaction Effect:  $F_{1,11} = 7.5$ ,  $p \leq 0.019$ ; CS<sup>-</sup> vs CS<sup>+</sup>,  $t_5 = 0.7$ ,  $p \geq 0.533$ ; Vehicle vs Orx<sub>2</sub>R Ant.,  $t_{11} = 2.7$ ,  $+p \leq 0.019$ ) do not display

conditioned fear responses. (H) In Stay animals, antagonism of Orx<sub>1</sub>R (Treatment Effect:  $F_{1,20} = 8.8$ ,  $p \leq 0.008$ ) reduces contextual (CS<sup>-</sup>; Vehicle Stay vs Orx<sub>1</sub>R Ant. Stay,  $t_{20} = 2.6$ ,  $^+p \leq 0.017$ ) and cued (CS<sup>+</sup>; Vehicle Stay vs Orx<sub>1</sub>R Ant. Stay,  $t_{20} = 2.7$ ,  $^+p < 0.001$ ) fear freezing but does not prevent the ability to associate the tone with social stress (CS Effect:  $F_{1,20} = 29.6$ ,  $p < 0.001$ ; Orx<sub>1</sub>R Ant. Stay CS<sup>-</sup> vs CS<sup>+</sup>,  $t_9 = 3.8$ ,  $^{\#}p < 0.001$ ). Stay mice treated with (I) Orx<sub>A</sub> (CS<sup>-</sup> vs CS<sup>+</sup>,  $t_4 = 4.2$ ,  $^{\#}p \leq 0.014$ ), (J) Orx<sub>1</sub>R Stim. (CS<sup>-</sup> vs CS<sup>+</sup>,  $t_8 = 4.0$ ,  $^{\#}p \leq 0.004$ ), or (K) Orx<sub>2</sub>R Ant. (CS Effect:  $F_{1,15} = 22.6$ ,  $^{\#}p < 0.001$ ) do not differ from vehicle controls in terms of fear freezing profile. (C) Mice exposed to social stress produce elevated levels of stress hormone (N = 39,  $F_{2,12} = 24.3$ ,  $p < 0.001$ ; Cage Control vs Vehicle Escape,  $t_5 = 3.1$ ,  $^{\wedge}p \leq 0.028$ ; Cage Control vs Vehicle Stay,  $t_9 = 9.9$ ,  $^{\wedge}p < 0.001$ ); however, Stay animals have the highest concentration (Vehicle Escape vs Stay,  $t_{10} = 2.6$ ,  $^-p \leq 0.025$ ). Inhibition of intra-BLA Orx<sub>1</sub>R reduces corticosterone levels in Stay mice (Vehicle Stay vs Orx<sub>1</sub>R Ant. Stay,  $t_{10} = 5.1$ ,  $^+p < 0.001$ ; Orx<sub>1</sub>R Ant. Stay vs Orx<sub>A</sub> Stay,  $t_6 = 3.3$ ,  $^!p \leq 0.002$ ).

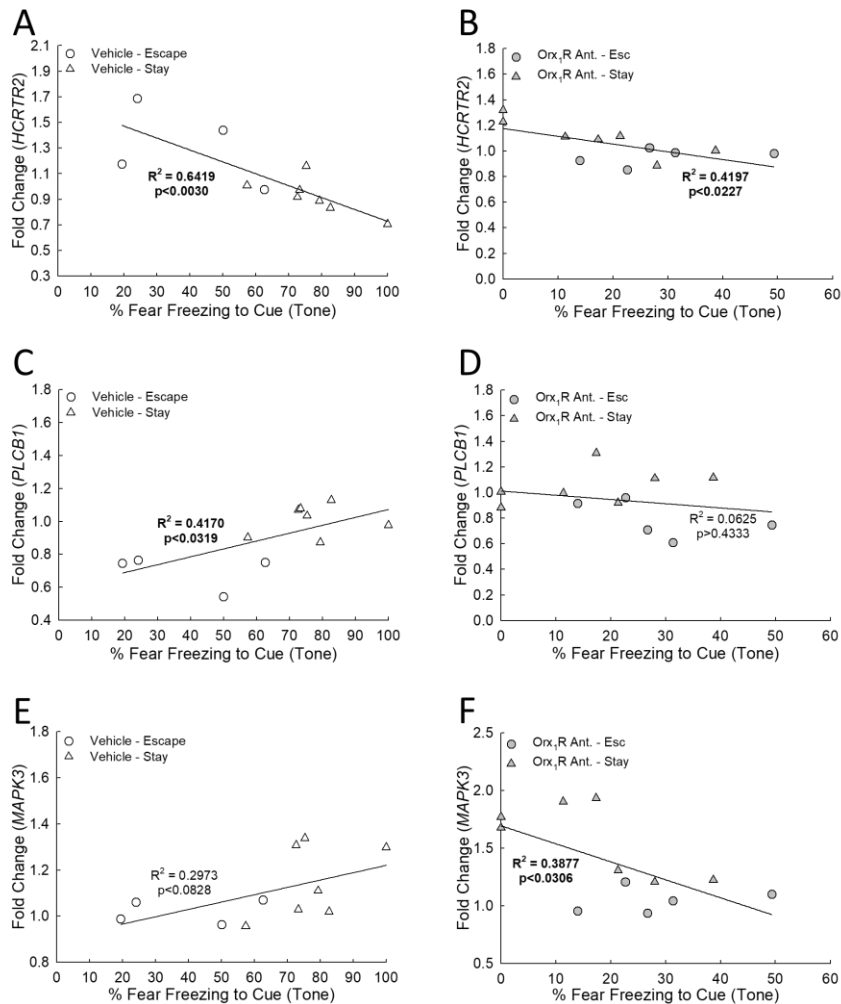




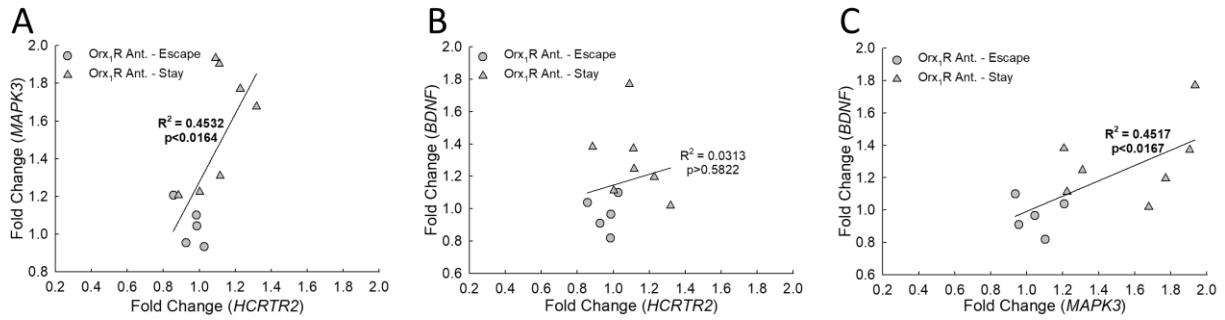
**Figure 6.** Transcriptional changes in the BLA after Orx<sub>1</sub>R or Orx<sub>2</sub>R antagonism shifts signaling profile.

(A) Antagonism of Orx<sub>1</sub>R in the BLA reduces *HCRTR1* expression (N = 45, Treatment Effect:  $F_{2,27} = 3.5$ ,  $p \leq 0.043$ ), but only significantly so in animals expressing the Escape phenotype (Cage Control vs Orx<sub>1</sub>R Ant. Escape,  $t_{11} = 2.2$ ,  $\hat{p} \leq 0.050$ ); whereas infusion of an Orx<sub>2</sub>R antagonist in the BLA reduces *HCRTR1* expression in Stay mice compared to vehicle animals of the same phenotype ( $t_{10} = 2.2$ ,  $^+p \leq 0.044$ ). (B) While Escape mice (Treatment Effect:  $F_{2,27} = 9.8$ ,  $p < 0.001$ ; Interaction Effect:  $F_{2,27} = 8.6$ ,  $p < 0.001$ ) treated with vehicle express higher *HCRTR2* levels compared to Stay mice ( $t_9 = 3.0$ ;  $^*p \leq 0.016$ ) and Orx<sub>1</sub>R- or Orx<sub>2</sub>R-antagonist-treated Escape animals (Vehicle vs Orx<sub>1</sub>R Ant.,  $t_7 = 2.6$ ,  $^+p \leq 0.035$ ; Vehicle vs Orx<sub>2</sub>R Ant.:  $t_7 = 4.5$ ,  $^+p < 0.001$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_8 = 3.5$ ,  $^1p < 0.001$ ), Orx<sub>1</sub>R antagonism results in elevated levels (Escape vs Stay,  $t_{10} = 2.2$ ,  $^*p \leq 0.05$ ; Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{12} = 2.4$ ,  $^+p \leq 0.034$ ) while Orx<sub>2</sub>R inhibition leads to a reduction (Vehicle vs Orx<sub>2</sub>R Ant.:  $t_{10} = 3.5$ ,  $^+p \leq 0.002$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_{10} = 4.7$ ,  $^1p < 0.001$ ) of *HCRTR2* in Stay mice. (C) A reduction of *PLCB1* (Phenotype Effect:  $F_{1,27} = 19.1$ ,  $p < 0.001$ ; Interaction Effect:  $F_{2,27} = 4.3$ ,  $p \leq 0.023$ ) that is found in Escape mice under control conditions (Cage control vs Vehicle Escape,  $t_{10} = 5.1$ ,  $\hat{p} < 0.001$ ; Escape vs Stay,  $t_9 = 5.0$ ,  $^*p < 0.001$ ) and Orx<sub>1</sub>R antagonism (Escape vs Stay,  $t_{10} = 3.1$ ,  $^*p \leq 0.012$ ; Cage Control vs Orx<sub>1</sub>R Ant.,  $t_{11} = 3.3$ ,  $\hat{p} \leq 0.007$ ) is eliminated with intra-BLA Orx<sub>2</sub>R antagonism (Vehicle vs Orx<sub>2</sub>R Ant.:  $t_7 = 2.8$ ,  $^+p \leq 0.017$ ). (D) While Stay mice treated with an Orx<sub>1</sub>R antagonist express higher levels of *MAPK3* (Phenotype Effect:  $F_{1,27} = 11.3$ ,  $p \leq 0.002$ ; Treatment Effect:  $F_{2,27} = 4.3$ ,  $p \leq 0.023$ ; Interaction Effect:  $F_{2,27} = 5.1$ ,  $p \leq 0.013$ ) in

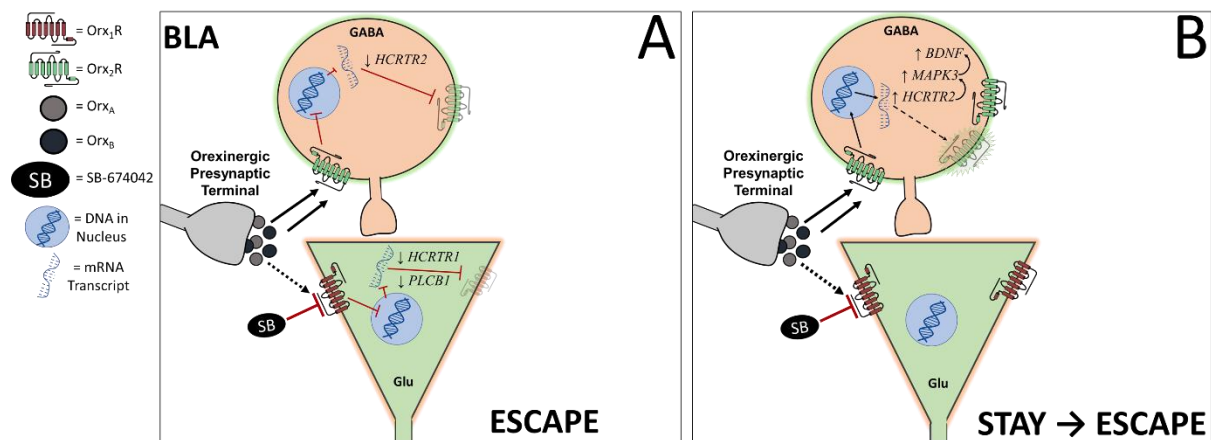
the BLA compared to Vehicle controls ( $t_{12} = 3.1$ ,  $^+p < 0.001$ ), administration of an Orx<sub>2</sub>R antagonist does not induce the same transcriptional response (Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_{10} = 2.7$ ,  $^!p \leq 0.022$ ). (E) Expression of *BDNF* in the BLA after treatment (Interaction Effect:  $F_{2,27} = 10.6$ ,  $p < 0.001$ ) with an Orx<sub>2</sub>R antagonist is enhanced in Escape mice (Orx<sub>2</sub>R Ant. Escape vs Stay:  $t_8 = 2.9$ ,  $^*p \leq 0.019$ ; Vehicle vs Orx<sub>2</sub>R Ant.:  $t_7 = 2.7$ ,  $^+p \leq 0.013$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_8 = 2.5$ ,  $^!p \leq 0.017$ ) and reduced in Stay animals (Vehicle vs Orx<sub>2</sub>R Ant.:  $t_{10} = 2.2$ ,  $^+p \leq 0.05$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_{10} = 3.9$ ,  $^!p < 0.001$ ); a phenotypically opposite effect is observed after Orx<sub>1</sub>R antagonism (Escape vs Stay,  $t_{10} = 2.8$ ,  $^*p \leq 0.018$ ; Orx<sub>1</sub>R Ant. Stay vs Vehicle Stay,  $t_{12} = 2.2$ ,  $^+p \leq 0.049$ ). Transcriptional changes after (F) intra-BLA Orx<sub>1</sub>R antagonism and (G) Orx<sub>2</sub>R inhibition are differentially regulated in a phenotype-dependent fashion.



**Figure 7.** Conditioned fear freezing response is related to gene expression changes resulting from intra-BLA Orx<sub>1</sub>R antagonism. In both (A) vehicle- (N = 11, F<sub>1,9</sub> = 16.1, R<sup>2</sup> = 0.6419, p ≤ 0.003) and (B) Orx<sub>1</sub>R antagonist-treated animals (N = 12, F<sub>1,10</sub> = 7.2, R<sup>2</sup> = 0.4197, p ≤ 0.023) a negative correlation exists between *HCRTR2* expression and cued fear freezing. (C) With vehicle treatment, relative *PLCB1* expression is positively associated with cued fear freezing (F<sub>1,9</sub> = 6.4, R<sup>2</sup> = 0.417, p ≤ 0.0319). (D) This relationship is not observed in mice that were administered an Orx<sub>1</sub>R antagonist (F<sub>1,10</sub> = 0.7, R<sup>2</sup> = 0.0625, p ≥ 0.4333). (E) While there is not a significant association between *MAPK3* expression and cued fear freezing after vehicle treatment (F<sub>1,9</sub> = 3.8, R<sup>2</sup> = 0.2973, p ≥ 0.0828), (F) a significant negative correlation is observed after Orx<sub>1</sub>R antagonism (F<sub>1,10</sub> = 6.3, R<sup>2</sup> = 0.3877, p ≤ 0.0306).



**Figure 8.** The BLA transcriptional changes that result from Orx<sub>1</sub>R antagonism form relationships that hint at molecular timelines and signaling dynamics. (A) While relative gene expression of *MAPK3* is positively correlated to the transcriptional changes of *HCRTR2* ( $N = 12$ ,  $F_{1,10} = 8.3$ ,  $R^2 = 0.4532$   $p \leq 0.0164$ ), (B) there is no association between *BDNF* and *HCRTR2* ( $F_{1,10} = 0.3$ ,  $R^2 = 0.0313$ ,  $p \geq 0.5822$ ). However, (C) a positive relationship emerges when comparing *BDNF* expression to that of *MAPK3* ( $F_{1,10} = 8.2$ ,  $R^2 = 0.4517$ ,  $p \leq 0.0167$ ).



**Figure 9.** Predicted circuit demonstrates the influence of intra-BLA  $\text{Orx}_1\text{R}$  antagonism, during endogenous stimulation through  $\text{Orx}_A$  and  $\text{Orx}_B$  release, on microcircuit dynamics in a phenotype-dependent fashion. (A) Escape mice treated with an  $\text{Orx}_1\text{R}$  Antagonist (SB-674042) undergo molecular shifts, including reduced *HCRTR1* and *PLCB1* transcription, leading to diminished orexin activity on glutamatergic neurons in the BLA. Escape mice also have decreased *HCRTR2* expression, potentially via (un-diagrammed) negative circuit feedback, even while  $\text{Orx}_2\text{R}$  are stimulated. (B) While  $\text{Orx}_B$  and  $\text{Orx}_A$  maintain stimulation of some GABAergic neurons through  $\text{Orx}_2\text{R}$ , antagonism of some pyramidal neurons via intra-BLA  $\text{Orx}_1\text{R}$  inhibition differentially modifies molecular mechanisms in Stay mice through enhancement of  $\text{Orx}_2\text{R}$  (*HCRTR2*),  $\text{ERK}_1$  (*MAPK3*), and *BDNF* transcription and increased orexin activity in  $\text{Orx}_2\text{R}$ -containing neurons (likely GABAergic cells).

## COMPLETE METHODS & MATERIALS

### Animals

Male C57BL/6NHsd mice (6-8 weeks old for pharmacology studies and 4-6 weeks old for shRNA studies) weighing ~22-26 g (Envigo, Indianapolis, IN; N = 108 [pharmacology experiment] & N = 32 [knockdown experiment]) were housed in groups (4-5 mice per cage) for a 5-day acclimatization period. For pharmacological studies (N = 92), bilateral stereotaxic surgeries were performed to implant guide cannula (26 ga cut to 4.0 mm) directed at the basolateral amygdala (intra-BLA). In orexin 1 receptor knockdown studies (N = 27), the insertion of an adeno-associated virus (AAV-U6-Orx<sub>1</sub>R-shRNA or AAV-scramble-shRNA) was performed stereotaxically by direct injection/perfusion. Following surgery, all animals were caged individually for the remainder of the experiments, including cage controls (N = 16 for pharmacology experiment; N = 5 for knockdown study). A separate cohort of retired male breeder Hsd:ICR (CD1) mice weighing ~50 g (Envigo) were housed in a similar fashion. These animals were used to provide aggression during social interaction in the Stress Alternatives Model (SAM), as they act aggressively towards C57BL/6N mice [59]. All mice were maintained on a 12:12 light-dark cycle (lights off at 6 pm) in rooms held at 22°C (35% Relative Humidity) and given *ad libitum* food and water. All behavioral experiments were performed during the animals' active phase (scotophase). Mice (C57BL/6N) were handled daily 48 hours after stereotaxic surgery for 7 days (pharmacological studies) or for the last 7 days of a 30-day viral incubation period (shRNA studies), followed by social engagement/aggression and behavioral testing for 5 days. Surgeries and behavioral experiments were all performed in a manner that minimized suffering and the number of animals used was 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.

### **Stereotaxic Surgery**

During stereotaxic surgery, mice were anesthetized using isoflurane (2% at 1.0 L/min flow rate) to allow for bilateral intra-BLA guide cannula (PlasticsOne, Roanoke, VA; 26 ga cut to 4.0 mm) implantation or AAV-U6-Orx<sub>1</sub>R-shRNA administration, then given a recovery period (7 days for pharmacological experiments; 30 days for AAV-U6-Orx<sub>1</sub>R-shRNA experiments which included time for virus incubation) before behavioral testing. Bilateral intra-BLA cannula placement and AAV-shRNA infusion were performed using the following stereotaxic coordinates: -1.35 mm AP,  $\pm$  3.30 ML, and -4.90 mm DV. During surgery, mice were kept on a warming pad to maintain core body temperature. After surgery, all animals were placed into home cages resting on a warming pad for post-surgery recovery and monitoring. Mice were injected subcutaneously with the analgesic ketorolac (5 mg/kg) immediately following surgery and 24 hours after receiving the first injection for a total of 48 hours post-surgery pain relief. Following behavioral experiments, brain tissue was dissected, fixed or frozen and later sectioned to distinguish correct injection placement directed at the BLA (Fig. S1A). Only animals in which the BLA was successfully targeted bilaterally (Fig. S1A; 71 mice out of 92 attempts for pharmacology experiment & 22 mice out of 27 attempts for shRNA study) were used for data analysis.

### **Drugs & Drug Infusions**

An Orx<sub>1</sub>R antagonist (SB-674042, IC<sub>50</sub> = 3.76 nM for Orx<sub>1</sub>R and 531 nM for Orx<sub>2</sub>R [60]; MedChemExpress, Monmouth Junction, NJ), Orexin A (Orx<sub>A</sub>; Tocris, Minneapolis, MN), Orx<sub>A</sub> plus an Orx<sub>2</sub>R antagonist for biased activation of Orx<sub>1</sub>R, an Orx<sub>2</sub>R antagonist (MK-1064, IC<sub>50</sub> =

18 nM for Orx<sub>2</sub>R and 292 nM for Orx<sub>1</sub>R (61; MedChemExpress) , or vehicle (artificial cerebrospinal fluid [aCSF] + DMSO) were bilaterally administered into the BLA (300 nL/side) on Day 3 (Fig. 1A), an hour before social interaction in the SAM. Dilutions for both treatments were prepared using a 3:1 ratio of aCSF to dimethylsulfoxide (DMSO). The dose of intra-BLA Orx<sub>1</sub>R antagonist (SB-674042; 0.3 nmol/0.3 μL) was adjusted from intracerebroventricular (icv) Orx receptor-targeting drugs with similar affinities, and preliminary results [28, 62]. The dose for the Orx<sub>2</sub>R antagonist (MK-1064; 0.1 nmol/0.3 μL) was 3x lower than previously used concentrations that produced anxiogenic effects when administered to the whole brain (icv) [28]. Similarly, the intra-BLA doses chosen for Orx<sub>A</sub> (0.1 nmol/0.3 μL) and the Orx<sub>1</sub>R activating cocktail (Orx<sub>A</sub>+MK-1064; 0.1 nmol/0.3 μL) were selected and adjusted based on icv administrations that produced anxious behaviors in mice [62]. After mixing, aCSF (8.59 g NaCl, 0.201 g KCl, 0.279 g, CaCl<sub>2</sub>, 0.16 g MgCl<sub>2</sub>, 0.124 g NaH<sub>2</sub>PO<sub>4</sub>, 0.199 g Na<sub>2</sub>HPO<sub>4</sub>/L H<sub>2</sub>O) was brought to a physiological pH (7.33) using NaOH, and then was filtered, degassed, and stored at 4°C. Drugs were administered by placing cannula (33 ga cut to 4.9 mm, extending 0.9 mm below each guide cannula) into the surgically implanted guide cannula, and injecting at a rate of ~0.5 μL/min using a 1.0 μL digital syringe (Model 7101 Zero Dead Volume, Knurled Hub 2.75", 22GA Needle; Hamilton Company, Reno, NV). After injections, the injector and syringe were left in place for 90 seconds. Home cage mobility was measured an hour later to assess locomotion changes that might be associated with drug interactions.

### **Short-hairpin Knockdown of Orx<sub>1</sub>R (AAV-U6-Orx<sub>1</sub>R-shRNA)**

The genetic reduction of Orx<sub>1</sub>R in adult mice was accomplished by intra-BLA injection of short-hairpin RNA packaged into an adeno-associated virus (serotype 2) vector. Preparations of Orx<sub>1</sub>R knockdown virus (AAV-U6-Orx<sub>1</sub>R-shRNA, sense target sequence:



CCAAAGGTCCCCACAGACATATTC) and scrambled control virus (AAV-scramble-shRNA, scrambled control sequence: CGGAATTTAGAAACCCGGCTCCAC) were performed at the Yale School of Medicine Virus Core (New Haven, CT). The oligonucleotides had SapI and XbaI overhangs to allow for ligation downstream of the mU6pro region of a modified pAAV-MCS vector, pAAV-shRNA. This vector was designed to co-express 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 polyadenylation sequence [25]. During stereotaxic surgery, mice were infused bilaterally using a 1.0  $\mu$ L syringe (Neuros Model 7001 KH Syringe, point style 3, 32GA; Hamilton Company, Reno, NV) with 400 nL of virus at a rate of  $\sim$ 0.25  $\mu$ L/min. After administration, the syringe was left in place for 5 min before being removed. Dental cement was used to seal the holes made in the skull before the incision was sutured together. Knockdown was validated using *in situ* hybridization (RNAscope) and was analyzed using ImageJ [63]. We observed a 59.4% knockdown ( $F_{2,13} = 35.4$ ,  $p < 0.001$ ) of Orx<sub>1</sub>R (Fig. S1D-G). Home cage mobility was performed after 30 days of recovery/viral incubation and animal weights were taken every week to assess virus-mediated changes in mobility and body weight, respectively (Fig. S5, S6D). Food weights were taken every day to assess changes in appetite or food-seeking behaviors that might have resulted from intra-BLA Orx<sub>1</sub>R knockdown (Fig. S6A-C).

### **Behavioral Design**

All behavioral measures were performed during the dark cycle when the animals are active, under red light ( $\sim$ 700 nm  $\lambda$ ). A GoPro (Hero 3 or Hero 7) video camera was used to record all behavioral interactions and testing for later analysis. Animal groups for pharmacological studies included home cage controls (N = 16), for comparisons of food intake, corticosterone levels, and

mRNA levels), vehicle-treated (N = 19), SB-674042-treated (N = 15), Or<sub>XA</sub>-treated (N = 9), Or<sub>XA</sub>+MK-1064-treated (N = 15), and MK-1064-treated animals (N = 13). Animal groups for shRNA knockdown experiments included cage controls (N = 5), AAV-U6-Or<sub>X1R</sub>-shRNA (N = 13), and AAV-scramble-shRNA (N = 9). All pharmacology treatment groups were subjected to 4 days of social aggression (Days 1-4; Fig. 1B) in the SAM with intra-BLA drug injections on Day 3. Virus-mediated knockdown experiments also included 4 days of aggressive social interaction (Days 1-4; Fig. 1B) in the SAM. All mice, excluding cage controls, were exposed to a fear conditioning (FC) paradigm after being inserted into the SAM (Fig. 1A) on the first 4 days of behavioral testing, using a tone (2500 Hz at 75 dB) as a conditioned stimulus (CS), but before exposure to an aggressive CD1 mouse, the unconditioned stimulus (US<sup>+</sup>), and at the end of test day (Day 5 for both pharmacology and AAV-shRNA experiments; Fig. 1B) to measure freezing behavior as a conditioned response (CR) in the absence of a CD1 mouse (US<sup>-</sup>). After undergoing the CR testing for FC, mice were anesthetized using isoflurane (5% at 1.0 min/L for 2 min) and rapidly decapitated. Whole brains and trunk blood plasma were collected and stored at -80°C until further analysis.

### **Stress Alternatives Model (SAM)**

The SAM apparatus (Fig. 1A) consists of a white rectangular box (91 cm x 22 cm x 30 cm) and two curved opaque dividers (r = 10.25 cm) that separate the box into three parts: an oval open field area (length = 71 cm, width = 22 cm, height = 30 cm) with two enclosed safety areas (10 cm x 22 cm x 30 cm) on both sides which are accessible via escape holes only the smaller C57BL/6N mice can fit through (Fig. 1A). Before social interaction in the SAM begins, an opaque cylinder (diameter = 15 cm, width = 20 cm) is positioned in the center of the open field, then the CD1 mouse is placed outside the cylinder in the open arena of the SAM. A C57BL/6N

mouse is put into the cylinder and subjected to a fear conditioning paradigm (see *Fear Conditioning (FC) Paradigm* section below).

After the cylinder is lifted, C57BL/6N mice are presented with the novel (novel on first day only) open field arena, containing two escape holes on both ends of the arena, and subjected to social aggression from a novel aggressive CD1 male, for 5 min. By the end of day 2 of the SAM, C57BL/6N mice will choose one of two phenotypes that they will express for the remaining days in the SAM (Days 3 & 4) [26-29]. Mice either utilize the escape holes which lead to the enclosed (safe) areas of the apparatus where they cannot be attacked by the CD1 mouse (Escape), or they remain in the open field arena and submit to social aggression (Stay). Previous studies from our laboratory show that Escape mice exhibit significantly lowered physiological and behavioral measurements of stress when compared to Stay mice [26-29], despite both groups of animals receiving high levels of social aggression from CD1 mice.

In instances where social aggression from a CD1 mouse was life threatening to a C57BL/6N mouse, a clear, perforated divider (15 cm wide and 20 cm high) was placed over the CD1 mouse to briefly interrupt the intense aggressive bouts, which include repeated bites to the head, neck, or underside of the test C57BL/6N mouse. After 5 min of SAM interaction each day, both mice were removed from the apparatus and placed back into their home cages. If a C57BL/6N mouse escaped, they were left in the enclosed area for the remainder of the 5 min, with a clear perforated sheet of plastic positioned in front of the escape hole in the open field to prevent the test mouse from going back into the SAM arena.

### **SAM Behavioral Analysis**

Behavioral analysis during the SAM was measured starting when the cylinder was lifted, until the 5 min of interaction ended, or when a test mouse utilized the escape routes. Mice self-

selected groups after the first two days of the SAM, being considered the Stay phenotype if the mouse did not escape on Days 1 or 2, or Escape if the mouse utilized the escape routes on Days 1 or 2. Behaviors recorded included time spent attentive to the escape holes and latency to escape.

Time spent attentive to the hole is defined as the amount of time that a mouse's head is within a 3 cm radius of either of the two escape holes and includes sniffing the holes or placing their head or body inside the escape route. This measurement is an indicator of stress-sensitive novelty exploration [28, 64, 65]. Even though the entire SAM apparatus is initially novel on Day 1, the escape routes are distinct in that they require a different physical path for movement, and the enclosed area on the other side of the escape routes remains unknown unless the mouse utilizes them. This measure provides a novel indicator of anxious behavior and decision-making that is unique to the SAM [27, 29], and tightly linked to Social Interaction/Preference (SIP) susceptible (for Stay) and resilient (for Escape) outcomes [28, 30]. Other studies from our lab show similar results for rainbow trout, suggesting an evolutionary conservation of the circuitry underlying these behaviors [35]. The Escape and Stay phenotypes are thought to be the result of decision-making as early responses are initially variable, then become stabilized with experience, and can be altered with learning or administration of anxiolytic or anxiogenic drugs [27-29, 33, 35, 66].

Latency to escape (or time spent with social aggressor) is determined by measuring the time it takes an animal to utilize an escape route once the cylinder has been lifted and social interaction begins. Previous experiments have shown that once an animal escapes, their latency to escape declines as escape becomes familiar, indicative of spatial and social learning [27, 29, 33].

Video recordings of the behavioral tests were analyzed using ANY-maze Video Tracking Software (Version 6.0, Stoelting Co., Wood Dale, IL). Between each animal trial, the arena was cleaned thoroughly using 70 % ethanol, disinfectant wipes, and dried using clean paper towels.

### **SAM Validation**

The SAM is a tool for assessing stress-related behavior that may be appropriately described as anxious and depressive behaviors, and as such has been progressively subjected to accepted validation testing, using the specific criteria for Construct, Predictive, and Face Validities [67]. Through incorporation of social defeat elements as well as active avoidance, the SAM construct combines elements of fear and anxiety [68-71], social stress and depression [72, 73], but also alleviation of these stress-related outcomes through Escape. This approach is both ecologically and ethologically relevant [26, 74, 75] and maintains similarities to relevant human disorders [76, 77], suggesting a degree of Construct Validity. Predictive validation of the SAM has been demonstrated through the induction of behavioral changes, including phenotype reversal, using known anxiolytic, antidepressive, or anxiogenic drugs (NPS, antalarmin, and yohimbine) [27, 29, 33]. The SAM has also been used in conjunction with, and produces comparable results to, the Social Interaction/Preference (SIP) Test [28, 66]. This additional test (SIP) has been validated as translationally and predictively reliable in demonstrating the effectiveness of pharmacotherapies used to treat anxiety (benzodiazepines) and depression (SSRIs) [7, 78-81]. Furthermore, elevated glucocorticoid levels establish an enhanced physiological stress response in animals encountering social aggression in the SAM, with the Stay phenotype expressing the greatest increase [26-29, 33]. With respect to Face Validity, SAM exposure results in behavioral outcomes, largely examples behavioral inhibition (FC freezing) and social avoidance (Escape, Escape Latency, and SIP test), that reflect those seen in human anxiety and depression. In 13

published papers on the Stress Alternatives Model, covering 43 experiments and approximately 1,032 animals sampled, the relationship between the number of Escape and Stay animals is very close to 50% for each phenotype. In most of these experiments, the SAM trials have been limited to 4 days, with fear conditioning tested on Day 5, and within these parameters, approximately 98% of phenotypes are stable after Day 2. In experiments to test the stability of these phenotypes, based on changes in the physical or social environment (prior exposure to the escape route, exercise, testing in the absence of a CD1 aggressor, or social stress prior to SAM) as well as the use of anxiolytic or anxiogenic drugs administered on Day 3, modification of phenotype expression can only be affected through manipulations that alter the stressful experience.

### **Fear Conditioning (FC) Paradigm**

Training and testing for FC took place in an opaque cylinder where C57BL/6N mice were placed prior to initiation of social aggression (unconditioned stimulus, US) in the open field of the SAM (4 days), and on test day (Day 5) in the absence of the US (US<sup>-</sup>). Freezing was measured in response to a conditioned stimulus (CS) tone, which indicated that an aggressive conspecific (US<sup>+</sup>) would appear shortly (after the divider was pulled). Before social interaction in the SAM on days 1-4, an opaque cylinder (diameter = 15 cm, width = 20 cm) was placed in center of the open field, and a novel CD1 mouse was placed in the arena outside of the cylinder. A C57BL/6N mouse was then introduced into the opaque cylinder to undergo a FC paradigm that consisted sequentially of a 30 sec acclimation period, the presentation of a 5 sec tone (2500 Hz at 75 dB, CS<sup>+</sup>), a 10 sec trace period, and the removal of the cylinder, exposing the test mouse to aggressive interactions from a CD1 mouse (US). On Day 5, the CS was presented without the US (US<sup>-</sup>) and freezing to the context and tone alone (CR) were measured.

## **FC Behavioral Analysis**

Freezing behavior analysis was performed using ANY-maze (Ver. 6.0) software. Measurements of freezing time were separated into time prior to the tone (30 sec acclimation period), which provides an indication of contextual conditioning (CS<sup>-</sup>), and after the tone (15 sec total: 5 sec tone and 10 sec trace), reflecting conditioning in response to the tone (CS<sup>+</sup>). Freezing behavior is often used to determine fear conditioning (28, 82), and was defined as bouts of immobility excluding normal breathing behavior, for one second or longer.

## **RNAscope**

Coronal slices of fresh frozen brains (N = 16; 16 µm) from AP -1.50 to -1.80 relative to bregma and incubated in cold (4°C) 10% formalin for 20 min before being washed (2x for 1 min) in 1x phosphate buffer solution (PBS). The sections then were dehydrated using sequential washes in ethanol (50%, 70%, and 100%; 5 min each). A final ethanol (100%) incubation period was performed overnight at -20°C. The next day, sections were processed. In short, proteins were digested using a protease treatment followed by rinses with distilled water. Sections were then bathed in RNAscope (Advanced Cell Diagnostics, Newark, CA) probes (*HCRTR1*, Cat. No. 466631; *HCRTR2*, Cat. No. 460881; *CALBI*, Cat No. 428431; *CAMKIIα*, Cat. No. 445231; *GADI*, Cat. No. 400951; *PVALB*, Cat. No. 421939) and allowed to incubate for 1 h in a hybridization oven (ACD HybEZ II oven, Cat. No. 321711) set to 40°C. Subsequent washes (RNAscope Wash Buffer Reagents [310091]: Wash Buffer 50x diluted to 1x) and incubation periods with amplification buffers (RNAscope Fluorescent Multiplex Detection Reagents [320851]: AMP1 [320852], AMP2 [320853], AMP3 [320854], AMP4 ALT A [320855], AMP4 ALT B [320856], AMP4 ALT C [320857]) linked fluorophores and enhanced signaling of targeted mRNA molecules. Tissue sections on microscope slides (Fisher Scientific, Pittsburgh,

PA; Superfrost Plus, Cat. No. 12-550-15) were stained with DAPI (20 sec) before adding a drop of mounting medium (Fisher Scientific; Prolong Gold Antifade Mountant, Cat. No. P10144) and a coverslip.

Images were visualized and captured using a fluorescence microscope (Nikon NIE with a Photometrics CoolSNAP MYO camera or Leica DM RA2 with Leica DFC3000 G camera). Regions of interest were identified from images and analyzed for fluorescence using ImageJ software. Colocalization of mRNA for cell markers and/or receptors were recognized as overlap of fluorescence signal or as distinct puncta overlaying DAPI signaling, suggesting mRNA expression in a single cell.

### **Stress Hormone (Corticosterone) Analysis**

After behavioral testing on day 5 (immediately following FC test), trunk blood was collected and centrifuged for 10 min in heparinized tubes to separate blood plasma. The plasma was then frozen immediately on dry ice and later transferred to a -80 °C freezer for storage until analysis could be completed. Concentrations of plasma corticosterone [B] were quantified in duplicate in a single run using corticosterone enzyme linked immunosorbent assay kit (Enzo Life Sciences, Farmingdale, NY).

### **qRT-PCR**

After behavioral testing on Day 5, mice brains were dissected and immediately frozen on dry ice. Brains were stored in a freezer set to -80°C until the tissue was processed. Fresh frozen brains were sectioned (Leica Biosystems, Buffalo Grove, IL; Leica CM1850 Clinical Cryostat, Cat. No. 047131148) to 200 µm, the BLA (AP -0.95 mm to -2.45 mm relative to Bregma) was microdissected on a cold plate using 25 GA punches (Stoelting Co., Wood Dale, IL; Brain Punch



Set, 0.25 to 1.25 mm; Cat. No. 57401) and immediately placed into 500  $\mu$ L of Trizol reagent (Thermo Fisher Scientific, Waltham, MA; Invitrogen TRIzol Reagent, Cat. No. 15-596-018).

Extraction of RNA took place using the Trizol method as previously described [83], but with some adjustments. In brief, BLA tissue was incubated in 500  $\mu$ L Trizol at room temperature for 5 min before the phase separation step which included the addition of 100  $\mu$ L of 1-bromo-3-chloropropane and centrifugation (4°C, 7,500xg, 15 min). The top aqueous layer was removed and used in the RNA precipitation step by mixing it with 250  $\mu$ L of isopropanol and 1  $\mu$ L glycol blue (Thermo Fisher Scientific, Waltham, MA; GlycoBlue Coprecipitant, Cat. No. AM9516) for pellet formation and identification. The RNA pellet was formed at the bottom of the tube by centrifugation (4°C, 12,000xg, 20 min). The fluid around the pellet was removed before it was washed with 75% ethanol and centrifuged at 16,000xg (4°C, 5 min). Most of the ethanol was removed while the remaining ethanol was evaporated by placing the sample in a hot plate oven set to 65°C for 20-40 min. The RNA pellet was then concentrated with 25  $\mu$ L RNase-free water and quantified using a nanodrop (Implen Inc., Westlake Village, CA; Nanophotometer N50 Spectrometer). Aliquots of RNA samples diluted to 20 ng/ $\mu$ L were created for PCR analysis before the samples were stored at -80°C.

Assays for the PCR analyses performed in this study were purchased from Thermo Fisher Scientific (Waltham, MA) and include *HCRTR1* (4351370, Mm01185776\_m1), *HCRTR2* (4351370, Mm01179312\_m1), *PLCB1* (4351370, Mm01329382\_m1), *MAPK1* (4448892, Mm00442479\_m1), *MAPK3* (4351370, Mm01278702\_gH), *BDNF* (4351370, Mm04230607\_s1), and *GAPDH* (4453320, Mm99999915\_g1) as the housekeeping gene. One-step RT-qPCR kits (Cat. No. 4392653) were used to build a master mix for each mRNA target and was combined with RNA samples in individual PCR tubes (MIDSCI, Valley Park, MO;

Pryme Ergonomic PCR Tubes; Cat. No. B77201). Tubes were loaded into Applied Biosystems QuantStudio 3 No. B77201 (Thermo Fisher Scientific, Waltham, MA; Cat. No. A28131) and, per vendor recommendations for Taqman Assays, were subject to 40 cycles at the following thermal cycling conditions: reverse transcription (48°C for 15 min), activation of DNA polymerase (95°C for 10 min), denaturation (95°C for 15 sec), and annealing/extension (60°C for 1 min).

Controls lacking either enzyme or template were used to identify possible contamination during PCR runs. Samples from 45 individuals (12 cage control mice, 11 vehicle-treated mice [4 Escape, 7 Stay], 12 Orx<sub>1</sub>R Antagonist-treated mice [5 Escape, 7 Stay], and 10 Orx<sub>2</sub>R Antagonist-treated mice [5 Escape, 5 Stay]) were used for PCR analysis. Each sample was run in duplicate, where the average Ct value was subtracted from the average housekeeping gene (*GAPDH*) Ct to give the  $\Delta$ Ct for analysis. Relative gene expression levels were determined using the  $2^{-\Delta\Delta Ct}$  method [84] and compared to the average  $\Delta$ Ct of the untreated controls (cage controls). Data are presented in graphical form as the average fold change.

### **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 (Orx receptor-targeting drug x behavioral phenotype x time in SAM interaction design), where phenotype was either Stay or Escape. In addition, two-way ANOVA (Orx receptor-targeting drug x Phenotype design) was utilized to determine the influence of manipulating activity of Orx receptors (Treatment Effects) relative to the expression of behavioral phenotypes (Stay vs Escape; Phenotype Effects) and Phenotype by Conditioning (Interaction Effects). Regression analyses were used for correlations of gene expression and behavioral (fear conditioning) responses, as well as for correlations between receptor or BDNF

gene expression and that of intracellular molecular signaling pathways. To compare changes occurring within a treatment group across SAM interaction days, a one-way repeated measures ANOVA (Orx receptor-targeting drug x day of SAM interaction design) was performed. Cage controls were necessary for interpretation of hormonal corticosterone levels and relative gene expression levels, because samples from SAM treatments were compared to baseline levels determined by the mean values 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 or Orx receptor-targeting drug) within a given phenotype (Escape or Stay) were analyzed by Student's t-tests. To determine differences in percentage of escape, chi-square and Fischer Exact statistical analyses were performed, where results from previous days were utilized as expected values.

Each animal was a singular sample source, from which multiple measures and analyses were taken. Five assumptions of parametric statistics were applied to the data, which were transformed when necessary, but also compared to non-parametric analyses, and graphed in their raw form. Analyses with both non-parametric and parametric statistics 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  $\alpha$  adjustment [85-90]. 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).

## COMPLETE RESULTS

### Orx<sub>1</sub>R are expressed in BLA glutamatergic neurons

Using RNAscope *in situ* hybridization, we identified neurons in the BLA which expressed Orx<sub>1</sub>R, CamKII $\alpha$ , and calbindin mRNA (Fig. 2). Consistent with previous reports in rodents [31, 32], we identified the majority (~80%) of BLA cells to express the glutamatergic marker, CamKII $\alpha$  (Fig. S2;  $F_{2,9} = 3,311.7, p < 0.001$ ; CamKII $\alpha^+$  vs Calb $^+$ :  $t_6 = 25.2, p < 0.001$ ; CamKII $\alpha^+$  vs Other:  $t_6 = 20.8, p < 0.001$ ). While Orx<sub>1</sub>R (*HCRTR1*) mRNA were observed in both glutamatergic (CamKII $\alpha$ -expressing [CamKII $\alpha^+$ ]) and calbindin-expressing (Calb $^+$ ) GABAergic neurons, the vast majority of Orx<sub>1</sub>R was expressed in CamKII $\alpha^+$  cells (>60%; Fig. 2;  $F_{2,9} = 386.8, p < 0.001$ ; CamKII $\alpha^+$  vs Calb $^+$ :  $t_6 = 12.0, p < 0.001$ ; CamKII $\alpha^+$  vs Other:  $t_6 = 4.3, p \leq 0.005$ ; Calb $^+$  vs Other:  $t_6 = 6.0, p < 0.001$ ). Further analyses revealed very few (<20%) of Orx<sub>1</sub>R $^+$  cells in the BLA to also express the gene for glutamate decarboxylase (*GADI*  $\rightarrow$  GAD67), a GABAergic neuron marker (Figs. 2G, H;  $t_8 = 29.5, p < 0.001$ ). Additionally, a small number (~10%) of Orx<sub>1</sub>R $^+$  cells also express parvalbumin mRNA (*PVALB*  $\rightarrow$  PV $^+$ ), a calcium-binding protein found in a proportion of BLA GABA neurons [91] (Figs. 2I, J;  $t_6 = 23.1, p < 0.001$ ). Importantly, our analyses suggest *HCRTR1* is expressed in 10-15% of BLA glutamatergic neurons, which is significantly more than expression in *GADI* $^+$  (GABAergic) neurons (Fig. 2K;  $t_7 = 3.2, p \leq 0.015$ ). Together, these results reveal Orx<sub>1</sub>R to be localized predominantly on glutamatergic neurons in the BLA (Fig. 2P).

### SAM social interactions produce Escape and Stay phenotypes

In the SAM, animals self-select one of two behavioral phenotypes, Escape or Stay (Fig. 1A, S1B & C), which are typically divided evenly within a population [26, 27, 29, 33, 34]. However, in some cohorts of animals this may be skewed [28, 30]. For the mice subjected to the

pharmacological experiments, we observed 39.4% Escape and 60.6% Stay phenotypes (Fig. S1B). The genetic knockdown experiments yielded Escape and Stay phenotype expression at the expected 50:50 ratio (Fig. S1C).

### **Intra-BLA Orx<sub>1</sub>R inhibition and knockdown increase attention toward the escape route**

Previous research from our laboratory demonstrated that the amount of time test subjects spend investigating the escape tunnel is an indicator of motivation to escape [28]. Similarly, time spent attentive to the escape hole was significantly different between phenotypes in vehicle-treated mice (Fig. 3A; Phenotype Effect:  $F_{1,51} = 16.4$ ,  $p < 0.001$ ; Time Effect:  $F_{3,51} = 1.3$ ,  $p \geq 0.299$ ; Interaction Effect:  $F_{3,51} = 1.5$ ,  $p \geq 0.235$ ; Escape vs Stay: Day 1,  $t_{17} = 2.6$ ,  $p \leq 0.018$ ; Day 2,  $t_{17} = 2.5$ ,  $p \leq 0.017$ ; Day 3,  $t_{17} = 1.6$ ,  $p \geq 0.125$ ; Day 4,  $t_{17} = 4.2$ ,  $p < 0.001$ ). Infusion of an Orx<sub>1</sub>R antagonist (SB-674042) stimulates attention to the escape hole on treatment day (Day 3) compared to vehicle-treated Escape mice (Figs. 3B, C; Treatment Effect:  $F_{1,30} = 7.7$ ,  $p \leq 0.019$ ; Time Effect:  $F_{3,30} = 2.3$ ,  $p \geq 0.098$ ; Interaction Effect:  $F_{3,30} = 0.9$ ,  $p \geq 0.470$ ; Day 3 Vehicle Escape vs Orx<sub>1</sub>R Antagonist Escape,  $t_{10} = 2.5$ ,  $p \leq 0.018$ ). In Stay mice, pharmacological inhibition of Orx<sub>1</sub>R (SB-674042) increases the time attentive to the escape route (Figs. 3B, D; Day 4 Vehicle x Orx<sub>1</sub>R Antagonist,  $t_{20} = 2.1$ ,  $p \leq 0.05$ ), however, the effect is smaller compared to that observed in Escape mice (Fig. 3C). Knockdown of Orx<sub>1</sub>R (AAV-Orx<sub>1</sub>R-shRNA) significantly increased attention toward the escape hole on the third day of the SAM relative to scramble controls (AAV-Scramble-shRNA) of the same phenotype (Fig. 3E; Day 3 Scramble vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{20} = 2.4$ ,  $p \leq 0.024$ ).

Further analyses demonstrate changes in the time animals investigate the escape routes after alternative treatments (Fig. S3; Escape: Treatment Effect,  $F_{4,23} = 7.9$ ,  $p < 0.001$ ; Day 3: Phenotype Effect,  $F_{1,61} = 36.1$ ,  $p < 0.001$ , Treatment Effect,  $F_{4,61} = 7.3$ ,  $p < 0.001$ , Interaction

Effect,  $F_{4,61} = 4.9$ ,  $p \leq 0.002$ ; Day 4: Phenotype Effect,  $F_{1,61} = 46.0$ ,  $p < 0.001$ , Treatment Effect,  $F_{4,61} = 5.2$ ,  $p < 0.001$ , Interaction Effect,  $F_{4,61} = 3.2$ ,  $p \leq 0.019$ ). For Escape mice, Orx<sub>A</sub> treatment reduced attention toward escape on Day 4 relative to Vehicle-treated animals of the same phenotype (Figs. S3A & B;  $t_9 = 2.6$ ,  $p \leq 0.013$ ). Additionally, on both Days 3 and 4, Escape mice treated with an Orx<sub>1</sub>R antagonist displayed higher attention toward escape compared to animals in the Orx<sub>A</sub> (Figs. S3A & B, Day 3:  $t_7 = 2.8$ ,  $p \leq 0.007$ ; Day 4:  $t_7 = 4.3$ ,  $p < 0.001$ ) and Orx<sub>1</sub>R stimulation (Figs. S3A & D; Day 3:  $t_9 = 2.7$ ,  $p \leq 0.023$ ; Day 4:  $t_9 = 3.1$ ,  $p \leq 0.003$ ) treatment groups. Infusion of an intra-BLA Orx<sub>1</sub>R antagonist also produced greater Day 4 attention toward escape behavior in Escape mice compared to those administered an Orx<sub>2</sub>R antagonist (Figs. S3A & F; Day 4:  $t_9 = 2.5$ ,  $p \leq 0.015$ ). In Stay mice, investigation of the Escape route was higher on Day 4 for animals treated with an Orx<sub>1</sub>R antagonist relative to those infused with Orx<sub>A</sub> (Figs. S3A & C;  $t_{13} = 2.3$ ,  $p \leq 0.036$ ) or an Orx<sub>2</sub>R antagonist (Figs. S3A & G;  $t_{15} = 2.3$ ,  $p \leq 0.034$ ). Attention toward escape on Day 3 was lower in Stay mice of the Orx<sub>1</sub>R stimulation (Orx<sub>A</sub> + MK-1064) treatment group compared to those treated with an Orx<sub>1</sub>R antagonist (Figs. S3A & E;  $p \leq 0.017$ ). Together these results characterize an important function of intra-BLA Orx<sub>1</sub>R in modulating adaptive motivation in a social stress environment, where inhibition or knockdown of these receptors promote increased motivation for Escape behavior and indirectly more avoidance behavior (Fig. 3F).

### **Inhibition and knockdown of BLA Orx<sub>1</sub>R promote escape**

In the pharmacological experiments, behavioral phenotypes for both Escape and Stay vehicle-treated animals were stable as demonstrated previously [27-29, 33]. However, intra-BLA injections of an Orx<sub>1</sub>R antagonist (SB-674042) resulted in a substantial number of Stay mice exhibiting Escape behavior on Days 3 and 4 of the SAM (Fig. 4A), with a 30% shift on the day

of treatment ( $\chi^2$ :  $F_1 = 2.0$ ,  $p \geq 0.078$ ) and a significant increase the day after (70% increase;  $\chi^2$ :  $F_1 = 9.3$ ,  $p < 0.001$ ). Interestingly, intra-BLA activation of both Orx receptors with Orx<sub>A</sub> or biased activation of Orx<sub>1</sub>R with a combination of Orx<sub>A</sub> and an Orx<sub>2</sub>R antagonist (MK-1064) blocked Escape behavior in a small proportion of mice on Days 3 and 4 (Fig. S4), and though not statistically significant does support the pro-stress role of Orx<sub>1</sub>R. Treatment with an Orx<sub>2</sub>R antagonist did not affect Escape behavior (Fig. S4).

In knockdown experiments, scramble control animals, like vehicle-treated mice, committed to Escape or Stay on the second day of SAM interaction and did not deviate from these self-selected behavioral phenotypes on the third and fourth days (for both:  $\chi^2$ :  $F_1 = 0.2$ ,  $p \geq 0.64$ ). While 54% of animals treated with AAV-Orx<sub>1</sub>R-shRNA chose to Stay in the SAM arena with the aggressive CD1 mouse on Day 2 of social stress, this number decreased incrementally on the last two days of SAM exposure (Fig. 4B;  $\chi^2$ :  $F_1 = 0.0$ ,  $p \geq 1.0$ ;  $\chi^2$ :  $F_1 = 0.2$ ,  $p \geq 0.69$ ). By the end of Day 4 of SAM interaction, 72.7% of AAV-Orx<sub>1</sub>R-shRNA-treated mice displayed the Escape phenotype compared to 54.5% of those that received the scramble control (Fig. 5B;  $\chi^2$ :  $F_1 = 0.0$ ,  $p \geq 0.87$ ).

For Escape and Stay phenotypes, the duration of social interaction with the aggressive CD1 mouse was significantly different (Phenotype Effect:  $F_{1,45} = 175.3$ ,  $p < 0.001$ ; Time Effect:  $F_{3,45} = 26.1$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,45} = 26.1$ ,  $p < 0.001$ ; Escape vs Stay: Day 1,  $t_{17} = 1.1$ ,  $p \geq 0.259$ ; Day 2,  $t_{17} = 5.8$ ,  $p < 0.001$ ; Day 3  $t_{17} = 10.6$ ,  $p < 0.001$ ; Day 4  $t_{17} = 11.9$ ,  $p < 0.001$ ) and changed for Escape mice over the four-day course of the experiments (Fig. 4C;  $F_{3,18} = 17.8$ ,  $p < 0.001$ ). As Escape mice learned to use one of two escape tunnels provided [26, 27, 29, 33, 35], they spent significantly less time in the SAM arena with an aggressive CD1 mouse on Days 2 through 4; i.e. escape latency was reduced (Fig. 4D; Day 1 vs Day 2,  $t_6 = 2.8$ ,  $p \leq 0.011$ ; Day 1

vs Day 3,  $t_6 = 5.7$ ,  $p < 0.001$ ; Day 1 vs Day 4,  $t_6 = 6.5$ ,  $p < 0.001$ ; Day 2 vs Day 3,  $t_6 = 2.9$ ,  $p \leq 0.009$ ; Day 2 vs Day 4,  $t_6 = 3.7$ ,  $p \leq 0.002$ ; Day 3 vs Day 4,  $t_6 = 0.8$ ,  $p \geq 0.437$ ); whereas vehicle-treated Stay mice remained submissively within the SAM arena for the entire 5 min period (Fig. 4D). Stay animals treated with the Orx<sub>1</sub>R antagonist (SB-674042), however, spent significantly less time with the aggressive CD1 mouse on Day 4 of the SAM paradigm compared to those administered vehicle (Fig. 4D; Treatment Effect:  $F_{1,54} = 2.8$ ,  $p \geq 0.111$ ; Time Effect:  $F_{3,54} = 2.9$ ,  $p \leq 0.043$ ; Interaction Effect:  $F_{3,54} = 2.9$ ,  $p \leq 0.043$ ; Day 4 Vehicle Stay vs Orx<sub>1</sub>R Antagonist Stay,  $t_{20} = 3.4$ ,  $p < 0.001$ ).

Treatment of the Orx<sub>1</sub>R antagonist (SB-674042) administered on the third day of the SAM had no effect on latency to escape in animals exhibiting the Escape phenotype compared to vehicle-treated mice (Fig. 4D; Treatment Effect:  $F_{1,30} = 0.2$ ,  $p \geq 0.675$ ; Time Effect:  $F_{3,30} = 26.9$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,30} = 0.3$ ,  $p \geq 0.856$ ). Knockdown of intra-BLA Orx<sub>1</sub>R (AAV-Orx<sub>1</sub>R-shRNA) had no effect on the latency to escape compared to the scramble control (AAV-Scramble-shRNA) group, although there was a similar decrease over interaction days (Fig. 4E; Treatment Effect:  $F_{1,60} = 0.0$ ,  $p \geq 0.960$ ; Time Effect:  $F_{3,60} = 5.5$ ,  $p \leq 0.002$ ; Interaction Effect:  $F_{3,60} = 0.0$ ,  $p \geq 0.995$ ). These results, in combination, suggest that intra-BLA Orx<sub>1</sub>R promote coping strategies associated with responses to increased stress, and acute inhibition of these receptors allows for greater expression of behavior that is derived from reduced output of pro-stress neurocircuitry (Escape).

### **BLA Orx<sub>1</sub>R regulate weight gain and food-seeking behavior, but do not increase locomotion**

As the orexin system is important for motivated behaviors, such as food-seeking, we assessed food intake throughout these experiments to determine if the combination of stress and Orx<sub>1</sub>R



treatments infused into the BLA would alter normal feeding behavior. In the pharmacological experiments, social stress caused fluctuations in feeding behavior throughout four days of SAM exposure (Fig. S6A). While vehicle-treated mice exhibited lower food consumption on Day 1 of the SAM compared to Day 2 (Treatment Effect:  $F_{1,99} = 1.9, p \geq 0.177$ ; Time Effect:  $F_{3,99} = 7.9, p \leq 0.001$ ; Interaction Effect:  $F_{3,99} = 1.6, p \geq 0.192$ ;  $t_{18} = 3.0, p \leq 0.008$ ) and Day 3 compared to Days 2 ( $t_{18} = 4.2, p \leq 0.001$ ) and 4 ( $t_{18} = 2.8, p \leq 0.012$ ), there were no significant differences between cage control animals (not exposed to social stress), which consumed relatively equal amounts of food throughout all four days of the SAM (Fig. S6A). Inhibition of Orx<sub>1</sub>R resulted in greater food consumption on treatment day (Day 3), and less intake on Day 4 compared to vehicle-treated mice (Fig. S6B; Treatment Effect:  $F_{1,96} = 0.001, p \geq 0.974$ ; Time Effect:  $F_{3,96} = 12.0, p \leq 0.001$ ; Interaction Effect:  $F_{3,96} = 6.0, p \leq 0.001$ ; Orx<sub>1</sub>R Antagonist Day 1 vs Day 2,  $t_{14} = 4.2, p \leq 0.001$ ; Orx<sub>1</sub>R Antagonist Day 2 vs Day 3,  $t_{14} = 3.3, p \leq 0.005$ ; Orx<sub>1</sub>R Antagonist Day 2 vs Day 4,  $t_{14} = 6.7, p \leq 0.001$ ; Day 3 Vehicle vs Orx<sub>1</sub>R Antagonist,  $t_{32} = 2.4, p \leq 0.021$ ; Day 4 Vehicle vs Orx<sub>1</sub>R Antagonist,  $t_{32} = 3.1, p \leq 0.004$ ). Further, home cage locomotion was unaltered by the dose of the Orx<sub>1</sub>R antagonist (SB-674042) or the other treatments (Orx<sub>A</sub>, Orx<sub>1</sub>R Stimulation [Orx<sub>A</sub> + MK-1064], & Orx<sub>2</sub>R antagonist) used in this set of experiments (Fig. S5A;  $t_{32} = 1.1, p \geq 0.299$ ), nor was it upset by knockdown of Orx<sub>1</sub>R (Fig. S5B;  $F_{2,24} = 1.4, p \geq 0.267$ ).

Since the administration of adeno-associated viruses aimed to knockdown Orx<sub>1</sub>R in the BLA was followed by 30 days to allow for viral incubation (Fig. 1B), animal weights were taken weekly to assess the influence of intra-BLA Orx<sub>1</sub>R on this metabolic process as it relates to food-seeking behavior. No differences in body weight gain were observed between cage control animals, which did not undergo surgery, and scramble controls; however, Orx<sub>1</sub>R knockdown (AAV-U6-Orx<sub>1</sub>R-shRNA) resulted in a reduction in weight gain that became more prominent as

the incubation period progressed with Days 1 and 5 revealing significant decreases compared to the cage control group (Fig. S6D; Treatment Effect:  $F_{2,120} = 1.9, p \geq 0.172$ ; Time Effect:  $F_{5,120} = 142.5, p \leq 0.001$ ; Interaction Effect:  $F_{10,120} = 2.2, p \leq 0.022$ ; Day 1 Cage Control vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{16} = 2.8, p \leq 0.014$ ; Day 5 Cage Control vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{16} = 2.7, p \leq 0.017$ ). Food consumption in scramble control animals, which experienced social stress, differed from cage controls only after four days of SAM social interaction (Fig. S6C; Treatment Effect:  $F_{2,120} = 4.7, p \leq 0.019$ ; Time Effect:  $F_{5,120} = 1.2, p \geq 0.302$ ; Interaction Effect:  $F_{10,120} = 2.6, p \leq 0.007$ ; Day 5 Cage Control vs AAV-Scramble-shRNA,  $t_{12} = 2.8, p \leq 0.016$ ). While modest reductions in food intake were observed in AAV-U6-Orx<sub>1</sub>R-shRNA animals compared to scramble controls, a significant difference was only detected after introduction of social stress (Day 1) of the experimental protocol (Fig. S6C; Day 1 AAV-Scramble-shRNA vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{20} = 2.8, p \leq 0.005$ ; Day 5 Cage Control vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{16} = 4.1, p \leq 0.001$ ). Together these results identify a role of intra-BLA Orx<sub>1</sub>R in maintaining normal feeding behavior and weight gain.

### **Activity of Orx<sub>1</sub>R in the BLA regulates cued fear responses**

On test day (Day 5 for both pharmacological and knockdown experiments; Fig. 1B), after four days of SAM social stress exposure (US<sup>+</sup>) paired with a conditioned stimulus (tone; Fig. 1A), fear conditioning was assessed in mice by measuring freezing behavior (CR) in the absence of a social aggressor (US<sup>-</sup>). While cued fear responses (enhanced freezing associated with the CS) were observed in both Escape and Stay phenotypes under control conditions (Figs. 5A, B; CS Effect:  $F_{1,17} = 47.7, p < 0.001$ ; Escape CS<sup>-</sup> vs CS<sup>+</sup>,  $t_6 = 3.9, p \leq 0.008$ ; Stay CS<sup>-</sup> vs CS<sup>+</sup>,  $t_{11} = 5.7, p < 0.001$ ), Stay mice displayed heightened freezing behavior to both context (CS<sup>-</sup>, opaque cylinder divider) and cue (CS<sup>+</sup>) compared to Escape animals (Fig. 5B; Phenotype Effect:  $F_{1,17} =$

7.6,  $p \leq 0.013$ ; Escape vs Stay: CS<sup>-</sup>,  $t_{17} = 2.8$ ,  $p \leq 0.011$ ; CS<sup>+</sup>,  $t_{17} = 2.3$ ,  $p \leq 0.033$ ). Inhibition of intra-BLA Orx<sub>1</sub>R (SB-674042) did not affect the fear freezing profile in Escape mice (Figs. 5A, D; Treatment Effect:  $F_{1,10} = 1.1$ ,  $p \geq 0.313$ ; CS Effect:  $F_{1,10} = 24.7$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,10} = 0.3$ ,  $p \geq 0.574$ ; Orx<sub>1</sub>R Antagonist Escape CS<sup>-</sup> vs CS<sup>+</sup>,  $t_4 = 3.4$ ,  $p \leq 0.026$ ); however, antagonist-treated Stay mice exhibited significantly reduced contextual (CS<sup>-</sup>) and cued (CS<sup>+</sup>) fear responses (Figs. 5A, H; Treatment Effect:  $F_{1,20} = 8.8$ ,  $p \leq 0.008$ ; CS Effect:  $F_{1,20} = 29.6$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,20} = 0.0$ ,  $p \geq 0.869$ ; CS<sup>-</sup> vs CS<sup>+</sup>: Vehicle Stay,  $t_{11} = 3.9$ ,  $p < 0.001$ ; Orx<sub>1</sub>R Antagonist Stay,  $t_9 = 3.8$ ,  $p < 0.001$ ; CS<sup>-</sup> Vehicle Stay vs Orx<sub>1</sub>R Antagonist Stay,  $t_{20} = 2.6$ ,  $p \leq 0.017$ ; CS<sup>+</sup> Vehicle Stay vs Orx<sub>1</sub>R Antagonist Stay,  $t_{20} = 2.7$ ,  $p < 0.001$ ). Like mice of the Escape phenotype, knockdown of BLA Orx<sub>1</sub>R (AAV-Orx<sub>1</sub>R-shRNA) did not affect conditioned freezing behavior (Fig. S7; Treatment Effect:  $F_{1,20} = 0.1$ ,  $p \geq 0.776$ ; CS Effect:  $F_{1,20} = 19.3$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,20} = 0.1$ ,  $p \geq 0.747$ ; CS<sup>-</sup> vs CS<sup>+</sup>: AAV-Scramble-shRNA,  $t_8 = 2.6$ ,  $p \leq 0.016$ ; AAV-Orx<sub>1</sub>R-shRNA,  $t_{12} = 3.7$ ,  $p < 0.001$ ). Importantly, activation of intra-BLA Orx<sub>1</sub>R and Orx<sub>2</sub>R with Orx<sub>A</sub> did not change the fear freezing profile in Escape (Treatment Effect:  $F_{1,9} = 0.9$ ,  $p \geq 0.364$ ; CS Effect:  $F_{1,9} = 26.8$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,9} = 0.2$ ,  $p \geq 0.655$ ) or Stay mice (Treatment Effect:  $F_{1,15} = 0.1$ ,  $p \geq 0.733$ ; CS Effect:  $F_{1,15} = 47.7$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,15} = 1.2$ ,  $p \geq 0.295$ ) compared to Vehicle control (Figs. 5A, E, & I). However, biased stimulation of Orx<sub>1</sub>R in the BLA with a combination of Orx<sub>A</sub> and an Orx<sub>2</sub>R antagonist (MK-1064) eliminated the conditioned response in Escape (Figs. 5A & F; Treatment Effect:  $F_{1,11} = 1.0$ ,  $p \geq 0.332$ ; CS Effect:  $F_{1,11} = 16.4$ ,  $p \leq 0.002$ ; Interaction Effect:  $F_{1,11} = 3.6$ ,  $p \geq 0.084$ ; Orx<sub>1</sub>R Stim. CS<sup>-</sup> vs CS<sup>+</sup>:  $t_8 = 2.1$ ,  $p \leq 0.073$ ), but not Stay mice (Figs. 5A & J; Treatment Effect:  $F_{1,19} = 4.1$ ,  $p \geq 0.060$ ; CS Effect:  $F_{1,19} = 45.1$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,19} = 0.0$ ;  $p \geq 0.955$ ; Orx<sub>1</sub>R Stim. CS<sup>-</sup> vs CS<sup>+</sup>:  $t_7 = 4.6$ ,  $p \leq 0.003$ ).

### **Corticosterone levels are reduced with intra-BLA Orx<sub>1</sub>R antagonism**

Immediately following assessment of conditioned fear (within 5 min of each trial), trunk blood plasma was collected and used to measure the concentration of rodent stress hormone, corticosterone, which was released in response to exposure of conditioned fear stimuli (Fig. 5C). As in previous studies [27, 28], corticosterone levels were elevated in both Escape and Stay animals relative to unstressed cage controls (Fig. 5C;  $F_{2,13} = 32.7$ ,  $p < 0.001$ ; Cage Control vs Vehicle Escape,  $t_{11} = 8.1$ ,  $p < 0.001$ ; Cage Control vs Vehicle Stay,  $t_{12} = 8.1$ ,  $p < 0.001$ ); however, Stay mice have higher levels of B compared to those of the Escape phenotype (Fig. 5C; Vehicle Escape vs Vehicle Stay,  $t_{10} = 2.8$ ,  $p \leq 0.015$ ). Inhibition of Orx<sub>1</sub>R (SB-674042) in the BLA, while having no effect on Escape stress hormone concentrations, decreased Stay B levels compared to vehicle-treated animals of the same phenotype (Fig. 5C;  $F_{1,16} = 17.5$ ,  $p < 0.001$ ; Phenotype Effect:  $F_{1,16} = 1.2$ ,  $p \geq 0.298$ ; Interaction Effect:  $F_{1,16} = 6.3$ ,  $p \leq 0.023$ ; Vehicle Escape vs Stay,  $t_{10} = 2.7$ ,  $p \leq 0.015$ ; Orx<sub>1</sub>R Antagonist Escape vs Stay,  $t_6 = 0.9$ ,  $p \geq 0.358$ ; Vehicle Escape vs Orx<sub>1</sub>R Antagonist Escape,  $t_6 = 1.1$ ,  $p \geq 0.283$ ; Vehicle Stay x Orx<sub>1</sub>R Antagonist Stay,  $t_{10} = 5.1$ ,  $p < 0.001$ ). Although antagonist-treated Escape animals had significantly higher B levels compared to non-stressed cage controls ( $F_{2,9} = 7.9$ ,  $p \leq 0.010$ ; Cage Control vs Orx<sub>1</sub>R Antagonist Escape  $t_6 = 6.1$ ,  $p < 0.001$ ), Orx<sub>1</sub>R antagonist-treated Stay mice B levels did not differ significantly from B levels in non-stressed mice (Fig. 5C; Cage Control x Orx<sub>1</sub>R Antagonist Stay,  $t_6 = 2.3$ ,  $p \geq 0.062$ ). No correlations, however, between corticosterone concentrations and fear freezing were observed (Vehicle:  $F_{1,10} = 2.4$ ,  $p \geq 0.1525$ ; Orx<sub>1</sub>R Ant.:  $F_{1,6} = 0.006$ ,  $p \geq 0.9402$ ). Activation of Orx<sub>1</sub>R and Orx<sub>2</sub>R with Orx<sub>A</sub> or selective stimulation of Orx<sub>1</sub>R (Orx<sub>A</sub> + MK-1064) did not impact corticosterone levels relative to vehicle controls (Treatment Effect,  $F_{3,28} = 1.1$ ,  $p \geq 0.351$ ; Phenotype Effect,  $F_{1,28} = 4.9$ ,  $p \leq 0.034$ ; Interaction

Effect,  $F_{3,28} = 0.8$ ,  $p \geq 0.489$ ); however, the phenotypic separation that is witnessed under control conditions was not observed after these treatments (Fig. 5C). Further, these stimulation treatments resulted in elevated corticosterone levels relative to cage control mice (Fig. 5C; Orx<sub>A</sub> Escape vs Cage Control,  $t_5 = 3.7$ ,  $p \leq 0.014$ ; Orx<sub>A</sub> Stay vs Cage Control,  $t_5 = 3.2$ ,  $p \leq 0.025$ ; Orx<sub>1</sub>R Stim. Escape vs Cage Control,  $t_5 = 5.0$ ,  $p \leq 0.004$ ; Orx<sub>1</sub>R Stim. Stay vs Cage Control,  $t_5 = 5.1$ ,  $p \leq 0.004$ ). These results highlight a role for BLA Orx<sub>1</sub>R in mediating the expression of behavioral and physiological fear responses.

### **Antagonism of intra-BLA Orx<sub>1</sub>R recruits alternative signaling pathways**

To determine how the shift in behavioral patterns observed with intra-BLA Orx<sub>1</sub>R inhibition coincides with adjustments to transcriptional levels for molecular mechanisms associated with Orx<sub>1</sub>R signaling, we performed RT-qPCR analyses of specific genes of interest within the BLA. As phenotype and treatment may influence receptor gene (*HCRTR1* & *HCRTR2*) expression, we analyzed levels in BLA tissue (Figs. 6A & B). While vehicle treatment did not result in changes in *HCRTR1* expression (Phenotype Effect:  $F_{1,19} = 0.3$ ,  $p \geq 0.585$ ; Treatment Effect:  $F_{1,19} = 5.5$ ,  $p \leq 0.03$ ; Interaction Effect:  $F_{1,19} = 0.2$ ,  $p \geq 0.690$ ), Orx<sub>1</sub>R antagonist-treated mice of the Escape phenotype exhibited significantly reduced *HCRTR1* gene expression compared to non-stressed cage controls (Fig. 6A;  $t_{11} = 2.2$ ,  $p \leq 0.050$ ). Escape animals in the vehicle control group exhibited elevated *HCRTR2* expression (Phenotype Effect:  $F_{1,19} = 2.8$ ,  $p \geq 0.111$ ; Treatment Effect:  $F_{1,19} = 1.6$ ,  $p \geq 0.221$ ; Interaction Effect:  $F_{1,19} = 14.1$ ,  $p < 0.001$ ) relative to control Stay mice ( $t_9 = 3.0$ ;  $p \leq 0.016$ ) and Escape animals treated with an Orx<sub>1</sub>R antagonist (Fig. 6B;  $t_7 = 2.6$ ,  $p \leq 0.035$ ). However, antagonist treatment resulted in elevated *HCRTR2* expression in Stay mice compared to those that escaped ( $t_{10} = 2.2$ ,  $p \leq 0.05$ ) and vehicle-treated animals of the same phenotype (Fig. 6B;  $t_{12} = 2.4$ ,  $p \leq 0.034$ ). Changes in both *HCRTR1* and *HCRTR2* gene

expression following Orx<sub>1</sub>R inhibition appear to occur in a phenotype-dependent way in the BLA.

Since PLC<sub>β1</sub> mRNA (*PLCBI*) is expressed in the amygdala [13], and its protein product likely plays a dominant role in Orx<sub>1</sub>R signaling [36] in the BLA, we quantified relative fold changes in cage control, vehicle-, and Orx<sub>1</sub>R antagonist-treated mice (Fig. 6C). In both vehicle and Orx<sub>1</sub>R antagonist groups, Escape mice expressed significantly lower amounts of PLC<sub>β1</sub> (*PLCBI*) compared to Stay animals of the same treatment (Fig. 6C; Phenotype Effect:  $F_{1,19} = 27.8$ ,  $p < 0.001$ ; Treatment Effect:  $F_{1,19} = 1.4$ ,  $p \geq 0.259$ ; Interaction Effect:  $F_{1,19} = 0.2$ ,  $p \geq 0.664$ ; Vehicle Escape vs Stay,  $t_9 = 5.0$ ,  $p < 0.001$ ; Orx<sub>1</sub>R Antagonist Escape vs Stay,  $t_{10} = 3.1$ ,  $p \leq 0.012$ ). Further, while Stay animals had similar expression levels of *PLCBI* compared to non-stressed controls ( $F_{2,19} = 0.3$ ,  $p \geq 0.723$ ), Escape mice in both vehicle- and Orx<sub>1</sub>R antagonist-treated groups had lower levels compared to these cage control animals (Fig. 6C;  $F_{2,14} = 11.2$ ,  $p < 0.001$ ; Cage Control vs Vehicle,  $t_{10} = 5.1$ ,  $p < 0.001$ ; Cage Control vs Orx<sub>1</sub>R Antagonist,  $t_{11} = 3.3$ ,  $p \leq 0.007$ ). These data seem to suggest that adaptive physiological shifts in intra-BLA *PLCBI* expression may play a role in, or result from, phenotype development, without identifying how Orx<sub>1</sub>R antagonism is involved.

As ERK signaling is one of the alternative molecular pathways that can be recruited during G<sub>q</sub> receptor activation, we evaluated intra-BLA transcriptional shifts in ERK genes (*MAPK1* & *MAPK3*) that result as an effect of Orx<sub>1</sub>R antagonism (Figs. 6D & S8). There were no significant differences in relative ERK<sub>2</sub> (*MAPK1*) mRNA in any group (Fig. S8; Phenotype Effect:  $F_{1,19} = 0.3$ ,  $p \geq 0.611$ ; Treatment Effect:  $F_{1,19} = 2.8$ ,  $p \geq 0.113$ ; Interaction Effect:  $F_{1,19} = 0.0$ ,  $p \geq 0.97$ ). However, in Stay mice treated with an Orx<sub>1</sub>R antagonist (SB-674042), we observed a robust increase in ERK<sub>1</sub> (*MAPK3*) expression that was significantly different

compared to Escape animals in the same treatment group (Phenotype Effect:  $F_{1,19} = 13.5$ ,  $p \leq 0.002$ ; Treatment Effect:  $F_{1,19} = 6.3$ ,  $p \leq 0.021$ ; Interaction Effect:  $F_{1,19} = 4.9$ ,  $p \leq 0.039$ ; Orx<sub>1</sub>R Antagonist Escape vs Stay,  $t_{10} = 3.5$ ,  $p \leq 0.006$ ), vehicle-treated animals of the Stay phenotype (Stay Vehicle vs Orx<sub>1</sub>R Antagonist,  $t_{12} = 3.1$ ,  $p < 0.001$ ), and non-stressed cage control mice (Fig. 6D;  $t_{13} = 4.1$ ,  $p < 0.001$ ). The results for molecular signaling pathways suggest potential links amongst Orx<sub>1</sub>R inhibition, phenotype plasticity, and the relationship between PLC $\beta_1$  (*PLCB1*) and ERK<sub>1</sub> (*MAPK3*) signaling recruitment in behaviorally distinctive groups, which also differ in stress sensitivity.

Since brain-derived neurotrophic factor (BDNF) is tied to neuroplasticity [37, 38] that may result in behavioral changes, like extinction of fear memories [39], we assessed its relative gene expression which we predicted would be increased with intra-BLA Orx<sub>1</sub>R antagonist treatment (Fig. 6E). As hypothesized, intra-BLA Orx<sub>1</sub>R inhibition resulted in elevated *BDNF* (Phenotype Effect:  $F_{1,19} = 8.4$ ,  $p \leq 0.009$ ; Treatment Effect:  $F_{1,19} = 3.5$ ,  $p \leq 0.077$ ; Interaction Effect:  $F_{1,19} = 1.9$ ,  $p \leq 0.181$ ) in Stay compared to Escape mice ( $t_{10} = 2.8$ ,  $p \leq 0.018$ ) and vehicle-treated Stay mice (Fig. 6E;  $t_{12} = 2.2$ ,  $p \leq 0.049$ ). As Stay mice treated with an Orx<sub>1</sub>R antagonist (SB-674042) experienced shifts from stress-vulnerable to resilient behavioral responses, the alterations in gene expression reported here may be implicit in this behavioral plasticity.

### **Molecular restructuring following intra-BLA Orx<sub>1</sub>R inhibition is related to fear responsiveness**

Expression levels of *HCRTR2* in vehicle- (Regression Analysis:  $F_{1,9} = 16.1$ ,  $p \leq 0.003$ ) and Orx<sub>1</sub>R antagonist-treated mice were negatively correlated to cued freezing (Figs. 7A & B; Regression Analysis:  $F_{1,10} = 7.2$ ,  $p \leq 0.023$ ). Relative expression levels of *PLCB1* were positively correlated to cued freezing behavior in vehicle-treated mice (Fig. 7C; Regression

Analysis:  $F_{1,9} = 6.4, p \leq 0.032$ ); however, this relationship is not observed following intra-BLA Orx<sub>1</sub>R inhibition (Fig. 7D; Regression Analysis:  $F_{1,10} = 0.7, p \geq 0.433$ ). Contextual freezing behavior was associated with *MAPK3* expression in only vehicle-treated mice (Figs. S9I & J; Vehicle,  $F_{1,9} = 5.5, p \leq 0.044$ ; Orx<sub>1</sub>R Antagonist,  $F_{1,10} = 2.6, p \geq 0.137$ ). Unlike contextual freezing behavior (Fig. S9J; Orx<sub>1</sub>R Antagonist,  $F_{1,10} = 2.6, p \geq 0.137$ ), intra-BLA antagonism of Orx<sub>1</sub>R cued freezing behavior is negatively correlated to ERK<sub>1</sub> (*MAPK3*) expression (Fig. 7F; Regression Analysis:  $F_{1,10} = 6.3, p \leq 0.031$ ). This relationship is not observed in vehicle-treated mice (Fig. 7E;  $F_{1,9} = 3.8, p \geq 0.083$ ). Finally, there are no associations of *HCRTR1* (Vehicle,  $F_{1,9} = 0.7, p \geq 0.416$ ; Orx<sub>1</sub>R Antagonist,  $F_{1,10} = 1.2, p \geq 0.302$ ) and *BDNF* (Vehicle,  $F_{1,9} = 0.2, p \geq 0.639$ ; Orx<sub>1</sub>R Antagonist,  $F_{1,10} = 0.9, p \geq 0.369$ ) expression with respect to fear freezing behavior (see also Figs. S9 & S10). Overall, gene expression was not largely correlated with cued fear freezing when phenotypes were assessed independently (Fig. S10); however, after intra-BLA Orx<sub>1</sub>R antagonism, *HCRTR2* expression was negatively correlated with cued fear freezing only in Stay animals (Fig. S10D;  $F_{1,5} = 13.7, R^2 = 0.7324, p \leq 0.014$ ). Mostly these results indicate that behavioral and transcriptional relationships exist within collective operational adaptations that link behavioral change to molecular modification. These relationships suggest a function-related connection between Orx<sub>1</sub>R antagonist-induced shifts in *HCRTR2* and *MAPK3* gene expression and fear-related behaviors.

### **Cells expressing Orx<sub>1</sub>R in the BLA do not co-express Orx<sub>2</sub>R**

Given that Orx<sub>1</sub>R antagonism within the BLA alters expression of Orx<sub>2</sub>R (*HCRTR2*) mRNA, a change which is related to cued fear behavioral responses in mice, we decided to investigate whether both orexin receptor subtypes are expressed within the same amygdalar cells using *in situ* hybridization (Figs. 2L-O). Most cells within the BLA express neither *HCRTR1* nor



*HCRTR2* (Fig. 2N,  $F_{2,9} = 42.1$ ,  $p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Other,  $t_6 = 7.5$ ,  $p < 0.001$ ; Orx<sub>2</sub>R<sup>+</sup> vs Other,  $t_6 = 8.4$ ,  $p < 0.001$ ). However, in BLA cells, *HCRTR1* and *HCRTR2* mRNA largely do not overlap (Fig. S11;  $t_{16} = 192.4$ ,  $p < 0.001$ ), and approximately 80% of Orx<sub>1</sub>R<sup>+</sup> cells do not co-express Orx<sub>2</sub>R (Figs. 2L, M, & O;  $t_6 = 10.1$ ,  $p < 0.001$ ).

### **Fear response after Orx<sub>2</sub>R inhibition is phenotypically different from Orx<sub>1</sub>R antagonism**

As blocking Orx<sub>1</sub>R in the BLA produced a dominant effect on conditioned fear freezing in Stay mice (Fig. 5H), an effect that is linked to an increase in *HCRTR2* gene expression (Fig. 6B), we predicted antagonism of Orx<sub>2</sub>R (MK-1064) might have a more prominent effect on fear behavior in Escape mice. Effects of intra-BLA Orx<sub>2</sub>R antagonism on fear responses were compared to vehicle- (Escape Mice: Treatment Effect:  $F_{1,11} = 2.1$ ,  $p \geq 0.178$ ; CS Effect:  $F_{1,17} = 12.4$ ,  $p \leq 0.005$ ; Interaction Effect:  $F_{1,17} = 8.0$ ,  $p \leq 0.017$ ; Stay Mice: Treatment Effect:  $F_{1,17} = 0.1$ ,  $p \geq 0.719$ ; CS Effect:  $F_{1,17} = 37.0$ ,  $p < 0.001$ ; Interaction Effect:  $F_{1,17} = 0.5$ ,  $p \geq 0.476$ ) animals (Figs. 5A, G, & K).

In Escape mice, acute inhibition of Orx<sub>2</sub>R in the BLA eliminated the cued-induced (CS<sup>+</sup>) freezing response observed after vehicle treatment (Figs. 5A & G;  $t_5 = 0.7$ ,  $p \geq 0.533$ ). Further, during the post-tone period (CS<sup>+</sup>), Orx<sub>2</sub>R antagonist-treated Escape mice exhibited reduced freezing compared to vehicle-treated animals of the same phenotype (Figs. 5A & G;  $t_{11} = 2.3$ ,  $p \leq 0.045$ ).

While the slope of the freezing profile was steeper in Stay mice treated with an Orx<sub>2</sub>R antagonist directed at the BLA compared to Stay animals in the vehicle control group, there were no statistical differences in the levels of contextual (CS<sup>-</sup>) and cued (CS<sup>+</sup>) freezing observed (Figs. 5A & K). These results suggest Orx receptor activity in the BLA influences social stress-

induced fear behavior in a phenotype dependent way.

### **Transcriptional changes after Orx<sub>2</sub>R antagonism contrast those observed after Orx<sub>1</sub>R inhibition**

Since blocking Orx<sub>1</sub>R produced changes in mRNA expression relevant to BLA cell signaling dynamics, we predicted Orx<sub>2</sub>R antagonism to induce opposing changes to these transcriptional relationships (Fig. 6). While Orx<sub>1</sub>R inhibition (Treatment Effect:  $F_{2,27} = 3.5$ ,  $p \leq 0.043$ ) resulted in a reduction in *HCRTR1* gene expression in Escape mice (Cage Control vs Orx<sub>1</sub>R Ant. Escape,  $t_{11} = 2.2$ ,  $p \leq 0.050$ ), Orx<sub>2</sub>R antagonism presented a similar decrease, but only in Stay animals (Fig. 6A; Vehicle vs Orx<sub>2</sub>R Ant.:  $t_{10} = 2.2$ ;  $p \leq 0.044$ ). Expression of *HCRTR2* in the BLA (Treatment Effect:  $F_{2,27} = 9.8$ ,  $p < 0.001$ ; Interaction Effect:  $F_{2,27} = 8.6$ ,  $p < 0.001$ ) was reduced in both Escape (Vehicle vs Orx<sub>2</sub>R Ant.:  $t_7 = 4.5$ ,  $p < 0.001$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_8 = 3.5$ ,  $p < 0.001$ ) and Stay phenotypes (Vehicle vs Orx<sub>2</sub>R Ant.:  $t_{10} = 3.5$ ,  $p \leq 0.002$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.:  $t_{10} = 4.7$ ,  $p < 0.001$ ) after blocking Orx<sub>2</sub>R, which contrasts with Orx<sub>1</sub>R antagonism which enhanced mRNA levels in Stay mice (Fig. 6B; Orx<sub>1</sub>R Ant. Escape vs Stay:  $t_{10} = 2.2$ ,  $p \leq 0.05$ ; Vehicle Stay vs Orx<sub>1</sub>R Ant. Stay:  $t_{12} = 2.4$ ,  $p \leq 0.034$ ). Further, intra-BLA Orx<sub>2</sub>R inhibition muted the reduction in PLC $\beta_1$  (*PLCBI*; Phenotype Effect:  $F_{1,27} = 19.1$ ,  $p < 0.001$ ; Interaction Effect:  $F_{2,27} = 4.3$ ,  $p \leq 0.023$ ) observed in Escape mice under Vehicle treatment ( $t_7 = 2.8$ ,  $p \leq 0.017$ ) conditions (Fig. 6C) while having no effect on *MAPK3* gene expression (Phenotype Effect:  $F_{1,27} = 11.3$ ,  $p \leq 0.002$ ; Treatment Effect:  $F_{2,27} = 4.3$ ,  $p \leq 0.023$ ; Interaction Effect:  $F_{2,27} = 5.1$ ,  $p \leq 0.013$ ), which was increased in Stay mice after Orx<sub>1</sub>R antagonist treatment (Fig. 6D; Vehicle vs Orx<sub>1</sub>R Ant.:  $t_{12} = 3.1$ ,  $p < 0.001$ ; Orx<sub>1</sub>R Ant. Vs Orx<sub>2</sub>R Ant.:  $t_{10} = 2.7$ ,  $p \leq 0.022$ ). Finally, Orx<sub>2</sub>R antagonist treatment enhanced *BDNF* expression (Interaction Effect:  $F_{2,27} = 10.6$ ,  $p < 0.001$ ) in Escape mice (Orx<sub>2</sub>R Ant. Escape vs Stay:  $t_8 = 2.9$ ,  $p \leq 0.019$ ; Vehicle Escape vs

Orx<sub>2</sub>R Ant. Escape:  $t_7 = 2.7, p \leq 0.013$ ; Orx<sub>1</sub>R Ant. Escape vs Orx<sub>2</sub>R Ant. Escape:  $t_8 = 2.5, p \leq 0.017$ ), while diminishing transcription in Stay animals (Vehicle vs Orx<sub>2</sub>R Ant.:  $t_{10} = 2.2, p \leq 0.05$ ), an effect that is phenotypically opposite to that observed after Orx<sub>1</sub>R inhibition (Fig. 6E; Orx<sub>1</sub>R Ant. Escape vs Stay:  $t_{10} = 2.8, p \leq 0.018$ ; Vehicle Stay vs Orx<sub>1</sub>R Ant. Stay:  $t_{12} = 2.2, p \leq 0.049$ ; Orx<sub>1</sub>R Ant. Stay vs Orx<sub>2</sub>R Ant. Stay:  $t_{10} = 3.9, p < 0.001$ ). Importantly, no relationships between gene expression and conditioned fear freezing were observed for any of the tested cell signaling markers after Orx<sub>2</sub>R antagonism except for *BDNF* (Figs. S12A-D), in which a significant negative correlation was revealed (Fig. S12E;  $F_{1,8} = 15.2, R^2 = 0.6548, p \leq 0.0046$ ).

### **Gene expression uncovers a potential molecular mechanism behind intra-BLA Orx<sub>1</sub>R antagonism**

To help generate a theoretical mechanism to explain the physiological basis surrounding the observed behavioral (and phenotypic) shifts resulting from intra-BLA inhibition of Orx<sub>1</sub>R, we explored transcriptional relationships in systems that exhibited similar regression patterns (Figs. 6, 7, & 8). With antagonism of Orx<sub>1</sub>R, there is a strongly positive relationship between *HCRTR2* and *MAPK3* expression (Fig. 8A; Regression Analysis:  $F_{1,10} = 8.3, p \leq 0.016$ ). Importantly, this association does not exist after vehicle treatment (Fig. S13A;  $F_{1,9} = 1.1, p \geq 0.322$ ). While there are no observed relationships between *BDNF* and *HCRTR2* expression levels in either treatment group (Fig. 8B & S13B; Vehicle,  $F_{1,9} = 0.1, p \geq 0.732$ ; Orx<sub>1</sub>R Ant.,  $F_{1,10} = 0.3, p \geq 0.582$ ), *BDNF* expression is positively correlated to ERK<sub>1</sub> (*MAPK3*) expression in animals treated with an Orx<sub>1</sub>R antagonist (Fig. 8C; Vehicle [Fig. S13C],  $F_{1,9} = 0.0, p \geq 0.951$ ; Orx<sub>1</sub>R Ant.,  $F_{1,10} = 8.2, p \leq 0.017$ ). Importantly, no relationships exist between *HCRTR1* expression and the other genes of interest (Fig. S13D-I). The connections reported here allowed us to predict a working model to help explain how Orx<sub>1</sub>R function within the BLA to establish

behavioral patterns consistent with stress-induced phenotype development (Fig. 9).

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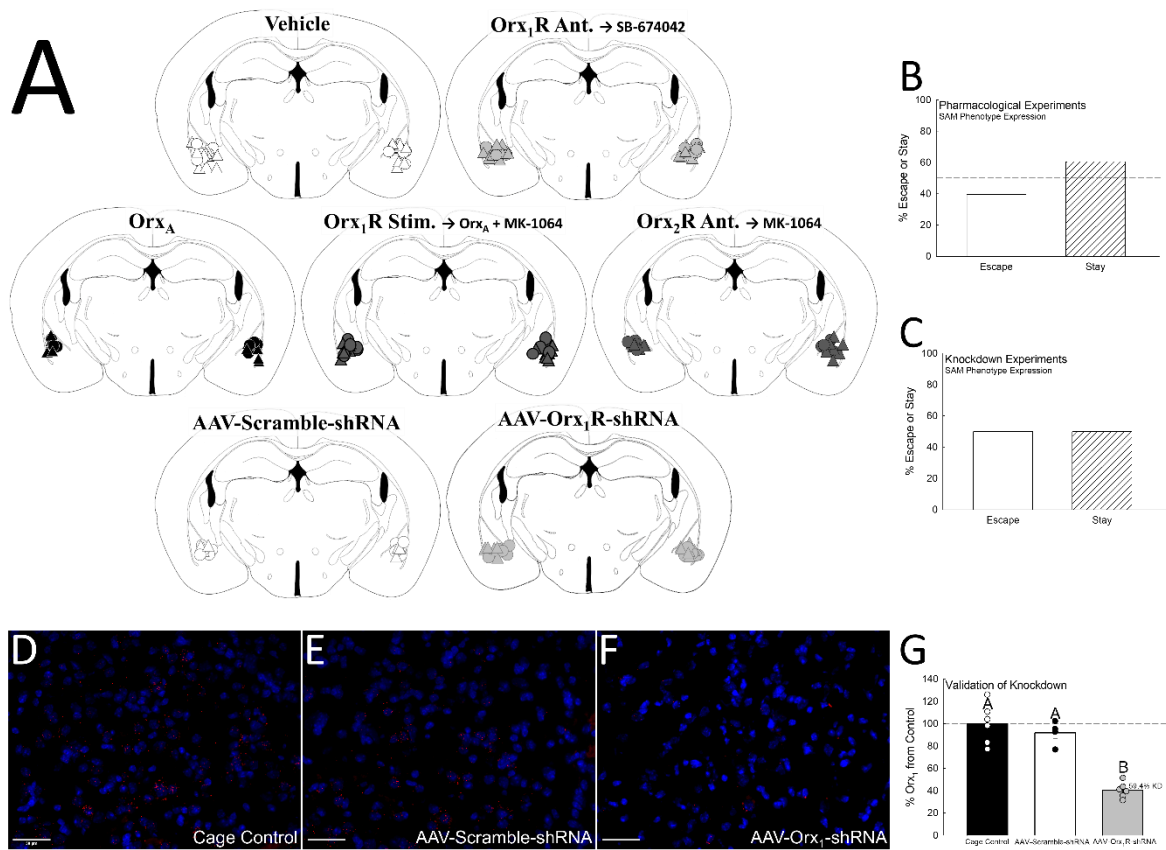
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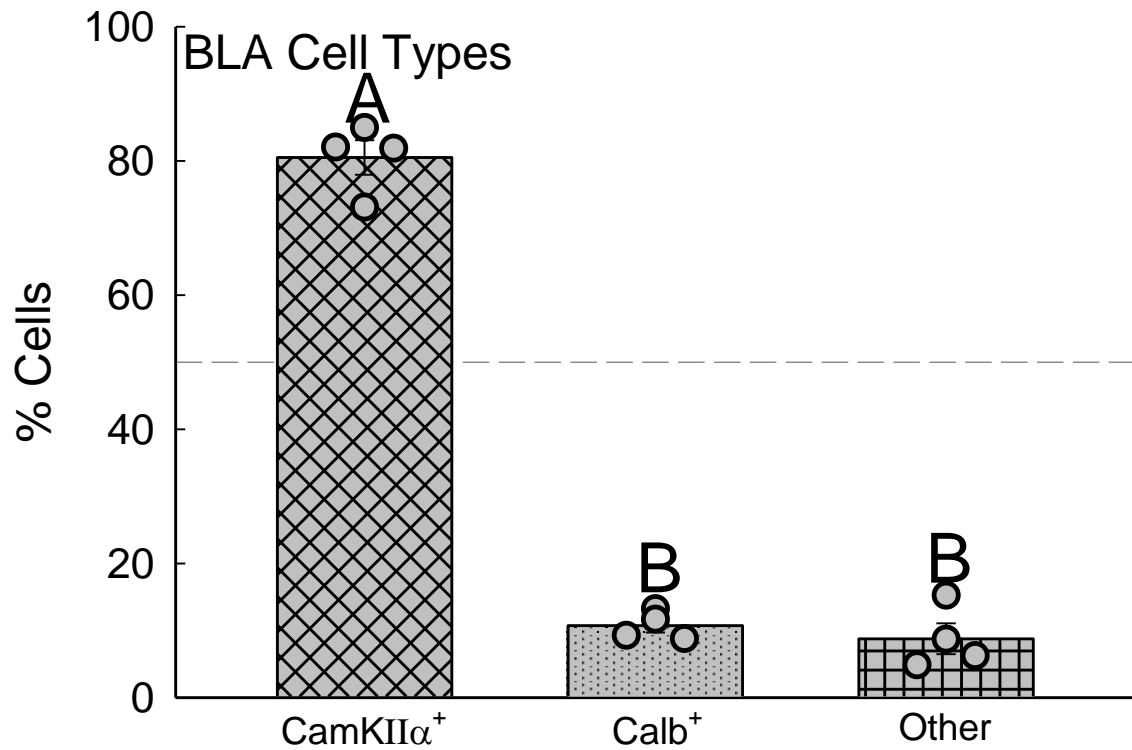
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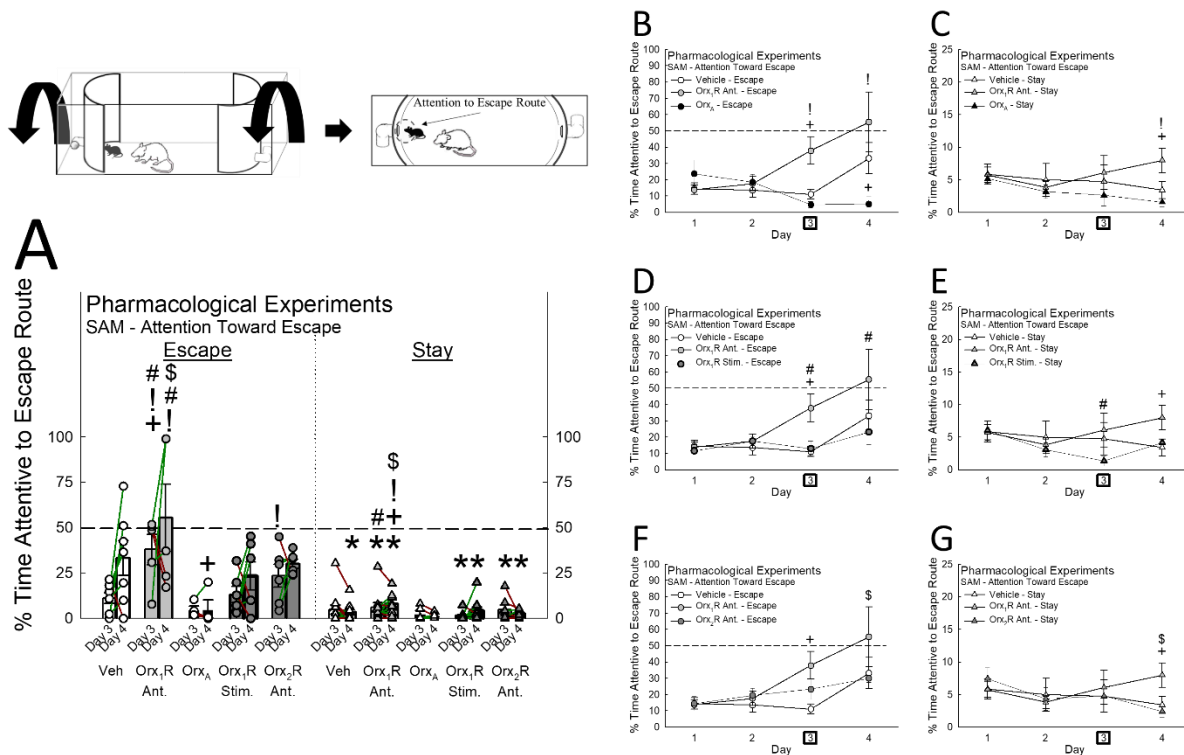
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**Figure S1.** (A) Injection sites for pharmacology (top & middle rows) and viral infusions (bottom row) successfully targeted BLA in test mice. In the SAM, (B) pharmacological experiment groups self-selected into a 40:60 split of Escape and Stay animals, respectively, by the end of Day 2 (N = 71); while (C) mice of the genetic knockdown experimental group more evenly (50:50) divided into Escape and Stay phenotypes by the end of Day 2 of the experimental design (N = 22). Expression levels of intra-BLA Orx<sub>1</sub>R (*HCRTR1*) mRNA in (D) Cage Controls, (E) Scramble Controls, and (F) Orx<sub>1</sub>R-shRNA animals revealed an (G) approximately 60% knockdown of Orx<sub>1</sub>R with viral treatment (N = 16,  $F_{2,13} = 35.4$ ,  $p < 0.001$ ; bars are statistically different from one another as illustrated with unique letters, e.g. A is significantly different from B;  $p < 0.001$ ).

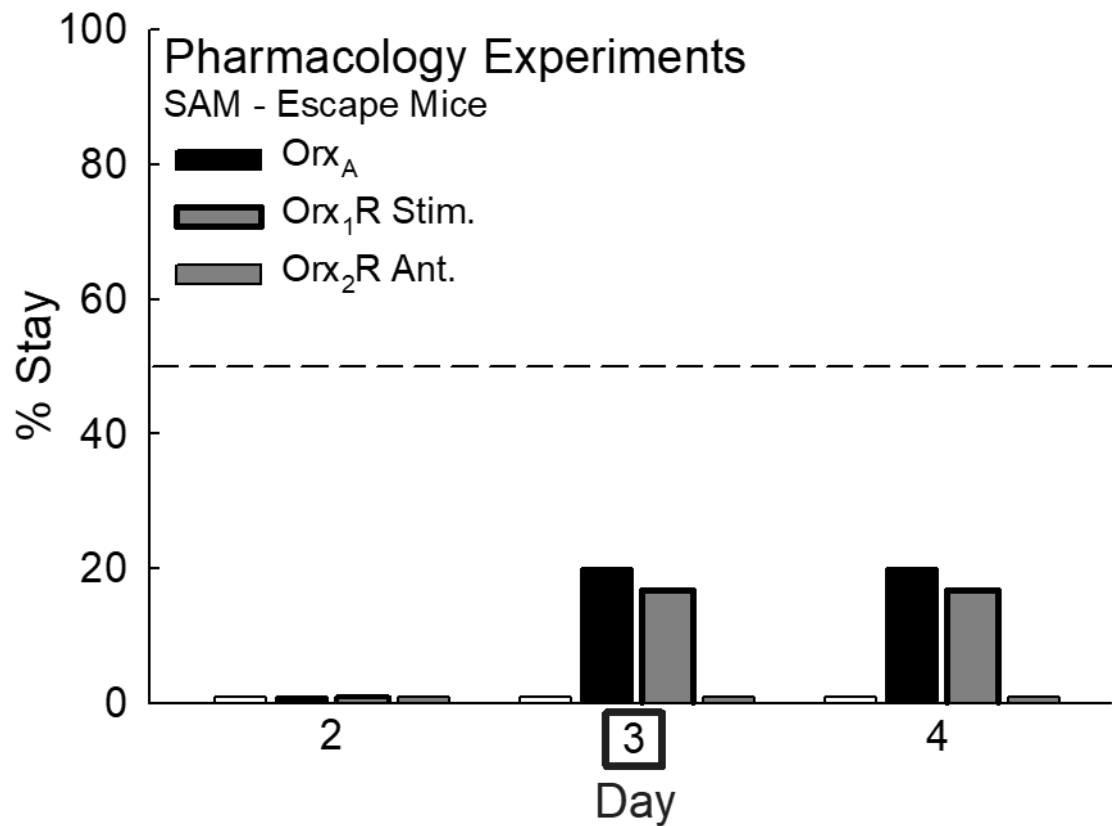


**Figure S2.** In the BLA, the majority of cells express the glutamatergic cell marker, CamKII $\alpha$ , and very few cells express mRNA for the calcium-binding protein, calbindin ( $N = 4$ ,  $F_{2,9} = 3,311.7$ ,  $p < 0.001$ ; CamKII $\alpha^+$  vs Calb $^+$ ,  $t_6 = 25.2$ ,  $p < 0.001$ ; CamKII $\alpha^+$  vs Other,  $t_6 = 20.8$ ,  $p < 0.001$ ; bars are statistically different from one another as illustrated with unique letters, e.g. A is significantly different from B;  $p < 0.001$ ).



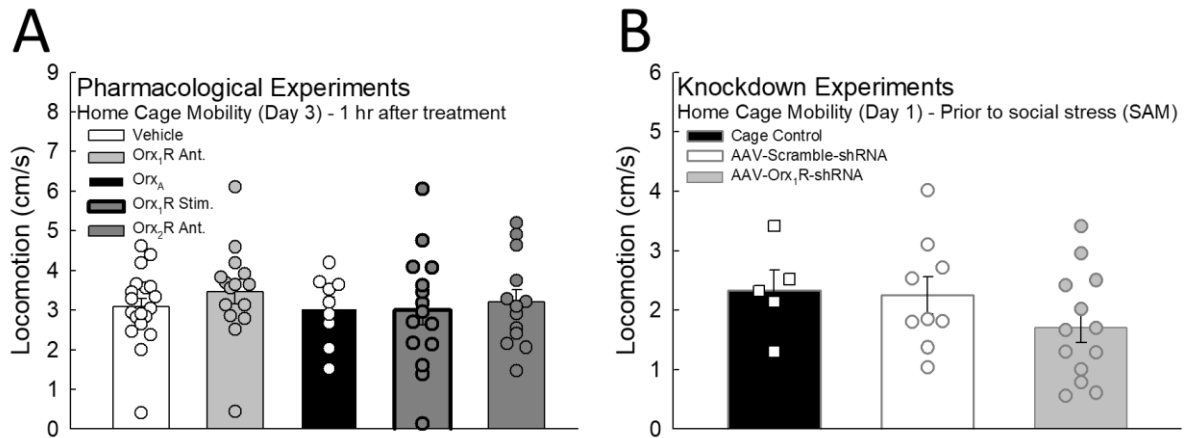
**Figure S3.** The amount of time mice spend attentive to the escape route is influenced by Orx receptor activity in the BLA. (A) While Stay mice, in general, spend less time attentive to SAM escape routes, investigation of the Escape routes on Days 3 & 4 is heightened in both Escape and Stay mice after Orx<sub>1</sub>R antagonism and reduced with Orx<sub>A</sub> treatment (N = 71; Day 3: Phenotype Effect,  $F_{1,61} = 36.0$ ,  $p < 0.001$ , Treatment Effect,  $F_{4,61} = 7.3$ ,  $p < 0.001$ , Interaction Effect,  $F_{4,61} = 4.8$ ,  $p \leq 0.002$ ; Day 4: Phenotype Effect,  $F_{1,61} = 46.0$ ,  $p < 0.001$ , Treatment Effect,  $F_{4,61} = 5.2$ ,  $p < 0.001$ , Interaction Effect,  $F_{4,61} = 3.2$ ,  $p \leq 0.019$ ). Individual analyses based on *a priori* hypotheses reveal significant reductions in attention toward escape in (B) Escape (Treatment Effect,  $F_{2,39} = 5.8$ ,  $p \leq 0.016$ ; Interaction Effect,  $F_{6,39} = 3.0$ ,  $p \leq 0.016$ ; Day 3: Orx<sub>1</sub>R Ant. vs Orx<sub>A</sub>,  $t_7 = 2.8$ ,  $^1p \leq 0.007$ ; Day 4: Vehicle vs Orx<sub>A</sub>,  $t_9 = 2.6$ ,  $^+p \leq 0.013$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>A</sub>,  $t_7 = 4.3$ ,  $^1p < 0.001$ ) and (C) Stay mice after intra-BLA Orx<sub>A</sub> administration (Day 4: Orx<sub>1</sub>R Ant. vs Orx<sub>A</sub>,  $t_{13} = 2.3$ ,  $^1p \leq 0.036$ ). (D) Escape mice in the Orx<sub>1</sub>R Stim. group exhibited lower attention toward escape compared to animals treated with an Orx<sub>1</sub>R Ant. (Treatment Effect,  $F_{2,45} = 3.8$ ,  $p \leq 0.045$ ; Day 3: Orx<sub>1</sub>R Ant. vs Orx<sub>1</sub>R Stim.,  $t_9 = 2.7$ ,  $p \leq 0.023$ ; Day 4: Orx<sub>1</sub>R Ant. vs Orx<sub>1</sub>R Stim.,  $t_9 = 3.1$ ,  $^#p \leq 0.003$ ). (E) Stay mice administered the Orx<sub>1</sub>R Stim. treatment presented lower attention toward escape

on Day 3 compared to animals in the Orx<sub>1</sub>R Ant. group (<sup>#</sup> $p \leq 0.017$ ). Mice treated with an Orx<sub>2</sub>R antagonist displayed lower attention toward escape on Day 4 compared to Orx<sub>1</sub>R Ant. treatment in both (F) Escape (Treatment Effect,  $F_{2,45} = 3.8$ ,  $p \leq 0.045$ ; Orx<sub>1</sub>R Ant. vs Orx<sub>2</sub>R Ant.,  $t_9 = 2.5$ ,  $^{\$}p \leq 0.015$ ) and (G) Stay ( $t_{15} = 2.3$ ,  $^{\$}p \leq 0.034$ ) phenotypes. Symbols represent significant differences compared to Escape mice in the same treatment group (\*), Vehicle (+), Orx<sub>A</sub> (!), Orx<sub>1</sub>R Stim. (#), and Orx<sub>2</sub>R Ant. (\$).

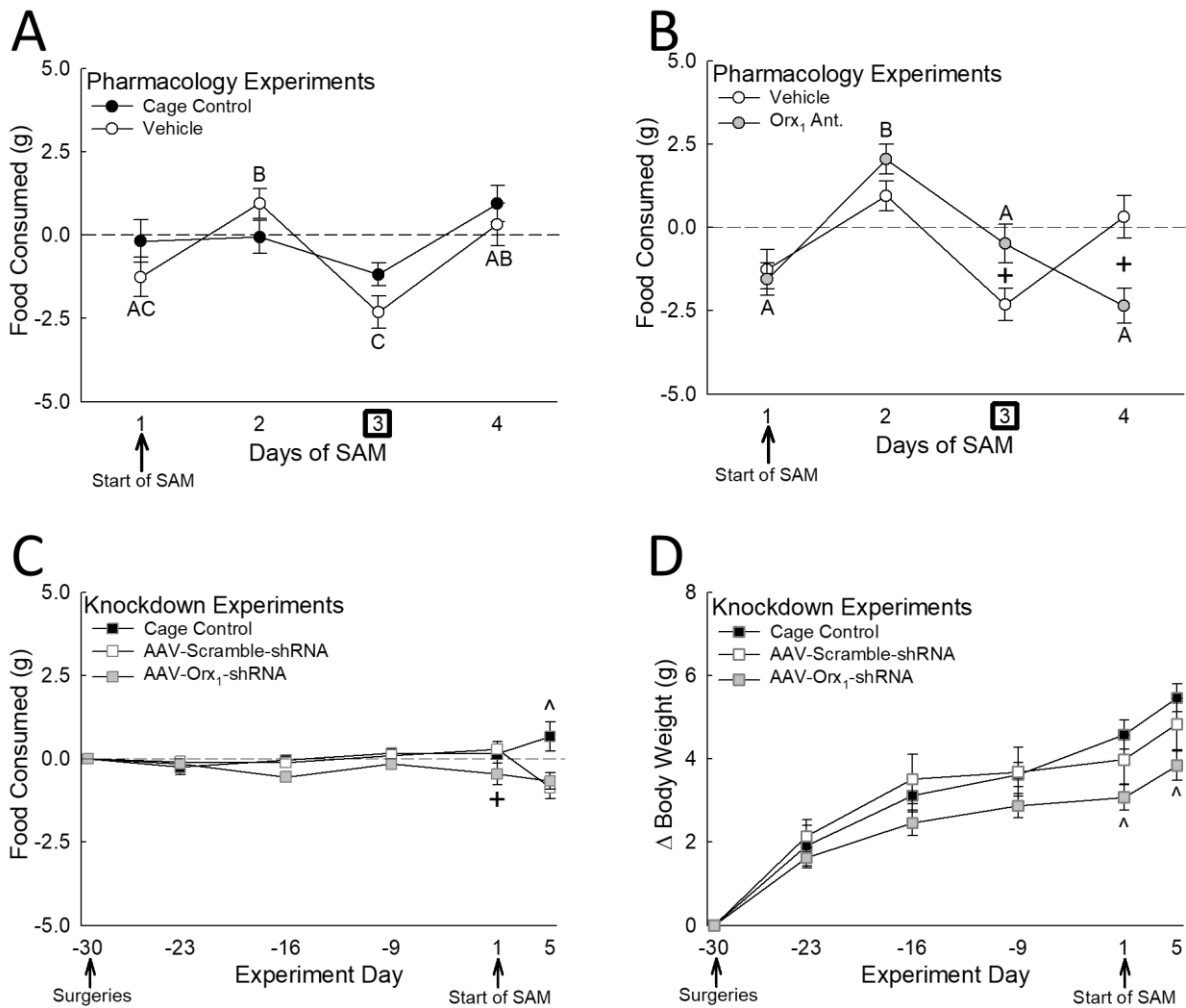


**Figure S4.** Activation of intra-BLA Orx<sub>1</sub>R and Orx<sub>2</sub>R with Orx<sub>A</sub> or biased stimulation of Orx<sub>1</sub>R (Orx<sub>1</sub>R Stim. = Orx<sub>A</sub> + Orx<sub>2</sub>R Ant.) promotes Stay behavior in a small, though not significant, percentage of Escape mice (N = 23).



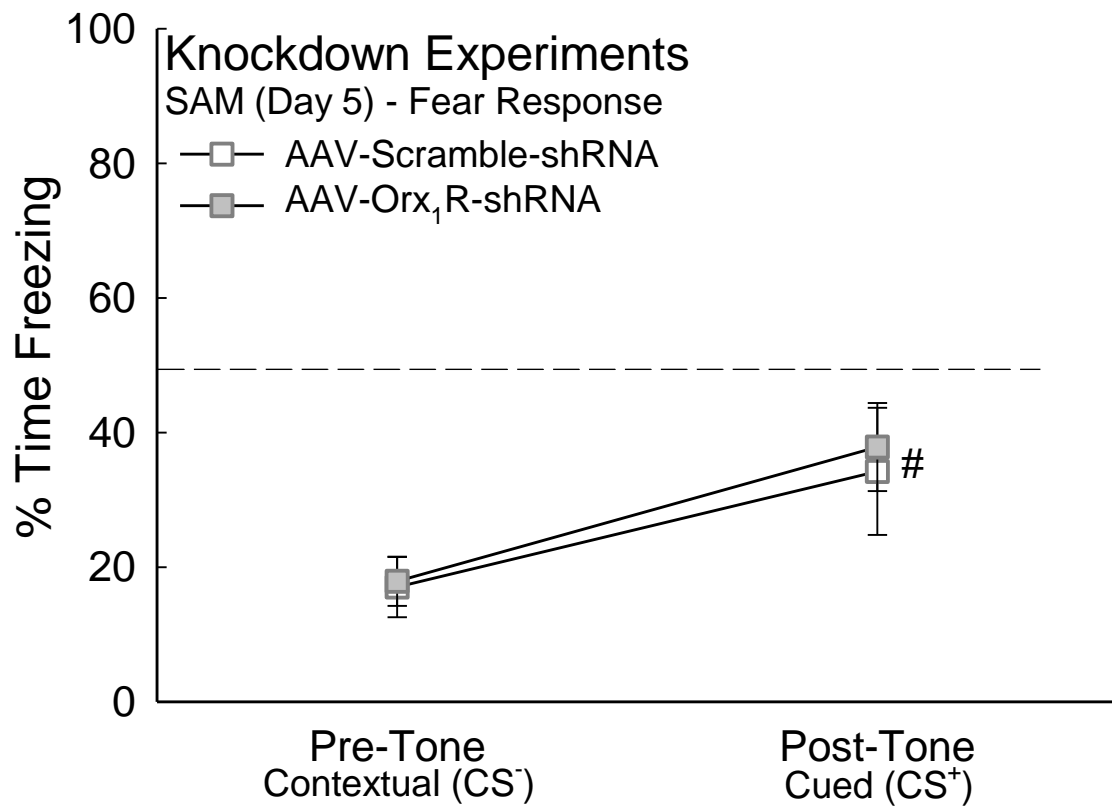


**Figure S5.** Intra-BLA Orx<sub>1</sub>R activity does not influence home cage mobility. (A) In the BLA, infusion of an Orx<sub>1</sub>R antagonist, Orx<sub>1A</sub>, biased activation of Orx<sub>1</sub>R (Orx<sub>1</sub>R Stim.), or an Orx<sub>2</sub>R antagonist does not affect locomotion in the animal's home cage environment ( $N = 71$ ,  $F_{4,66} = 0.813$ ,  $p \geq 0.813$ ). (B) Similarly, genetic knockdown of intra-BLA Orx<sub>1</sub>R has no influence over home cage mobility ( $N = 27$ ,  $F_{2,24} = 1.4$ ,  $p \geq 0.267$ ).

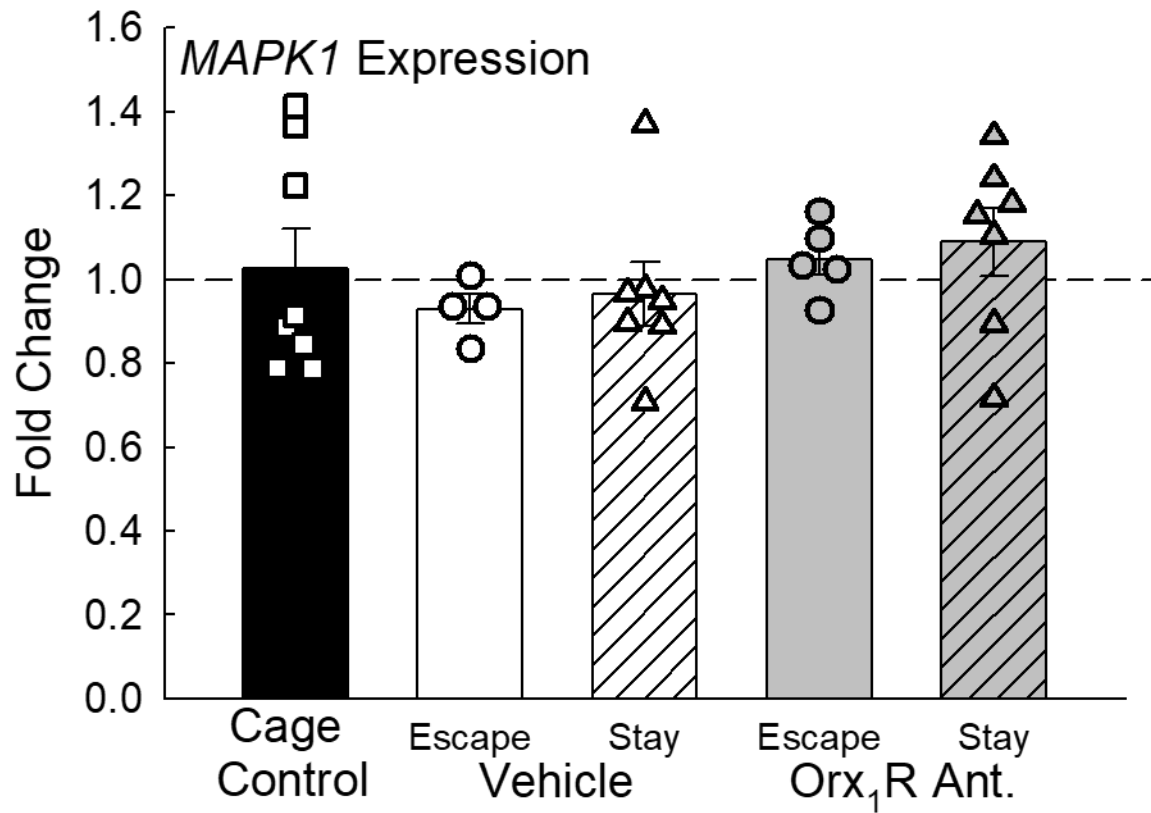


**Figure S6.** Intra-BLA Orx<sub>1</sub>R activity plays a role in controlling weight gain. (A) While social stress impacts an animal's eating patterns (N = 35, Time Effect:  $F_{3,99} = 7.9$ ,  $p \leq 0.001$ ; Vehicle Day 1 vs Day 2,  $t_{18} = 3.0$ ,  $p \leq 0.008$ ; Vehicle Day 2 vs Day 3,  $t_{18} = 4.2$ ,  $p \leq 0.001$ ; Vehicle Day 3 vs Day 4,  $t_{18} = 2.8$ ,  $p \leq 0.012$ ) (B) acute inhibition of intra-BLA Orx<sub>1</sub>R modifies this cycle on the day of treatment (Day 3) and the day after (N = 34, Day 4; Time Effect:  $F_{3,96} = 12.0$ ,  $p \leq 0.001$ ; Interaction Effect:  $F_{3,96} = 6.0$ ,  $p \leq 0.001$ ; Orx<sub>1</sub>R Ant. Day 1 vs Day 2,  $t_{14} = 4.2$ ,  $p \leq 0.001$ ; Orx<sub>1</sub>R Ant. Day 2 vs Day 3,  $t_{14} = 3.3$ ,  $p \leq 0.005$ ; Orx<sub>1</sub>R Ant. Day 2 vs Day 4,  $t_{14} = 6.7$ ,  $p \leq 0.001$ ; Day 3 Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{32} = 2.4$ ,  $^+p \leq 0.021$ ; Day 4 Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{32} = 3.1$ ,  $^+p \leq 0.004$ ; unique letters indicate significant differences, e.g. A is different from B). (C) Knockdown of Orx<sub>1</sub>R in the BLA does not influence food consumption behavior (N = 27, Treatment Effect:  $F_{2,120} = 4.7$ ,  $p \leq 0.019$ ; Interaction Effect:  $F_{10,120} = 2.6$ ,  $p \leq 0.007$ ) until social

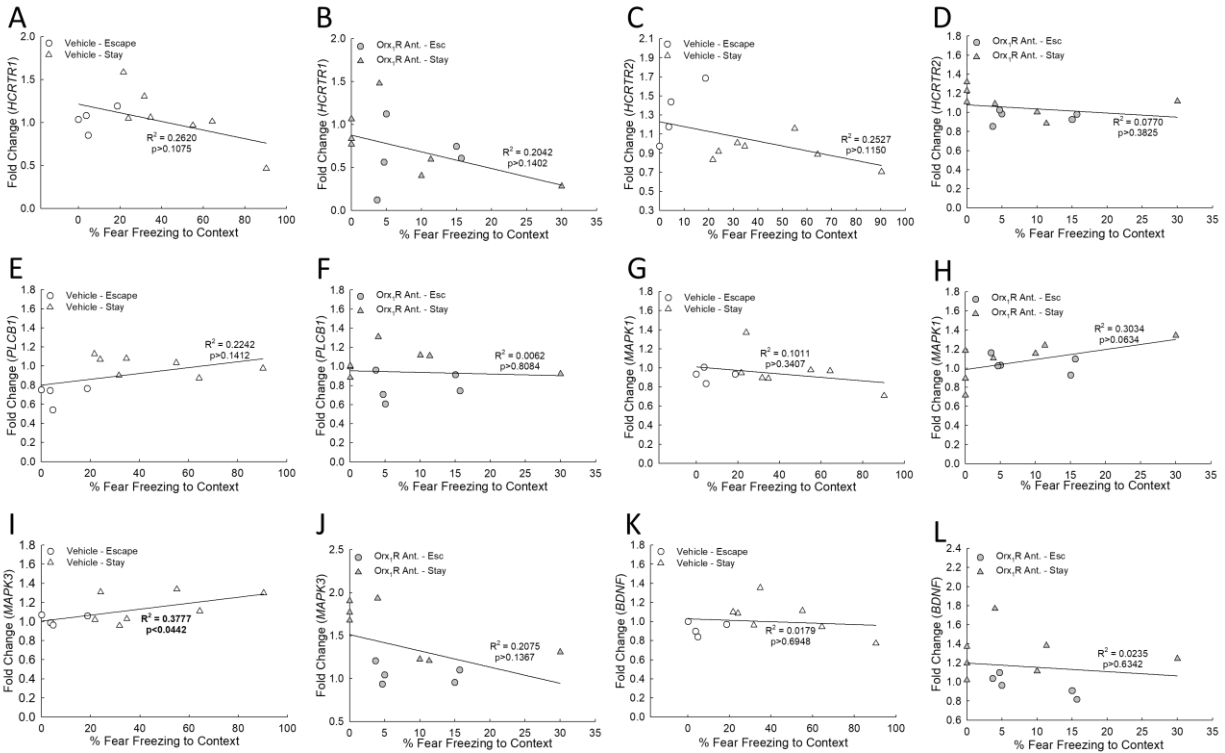
stress is introduced (Day 1; AAV-Scramble-shRNA vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{20} = 2.8$ ,  $^+p \leq 0.005$ ), at which point knockdown temporarily reduces food intake, an effect that is matched by scramble controls only after 4 days of social stress (Day 5) relative to non-stressed cage control animals ( $\hat{p} < 0.05$ ; Day 5 Cage Control vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{16} = 4.1$ ,  $\hat{p} \leq 0.001$ ; Cage Control vs AAV-Scramble-shRNA,  $t_{12} = 2.8$ ,  $\hat{p} \leq 0.016$ ). (D) While body weight gain is reduced in knockdown mice, this effect becomes significant (relative to cage controls) only after they are subjected to social stress (N = 27, Days 1-5; Time Effect:  $F_{5,120} = 142.5$ ,  $p \leq 0.001$ ; Interaction Effect:  $F_{10,120} = 2.2$ ,  $p \leq 0.022$ ; Day 1 Cage Control vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{16} = 2.8$ ,  $\hat{p} \leq 0.014$ ; Day 5 Cage Control vs AAV-Orx<sub>1</sub>R-shRNA,  $t_{16} = 2.7$ ,  $\hat{p} \leq 0.017$ ).



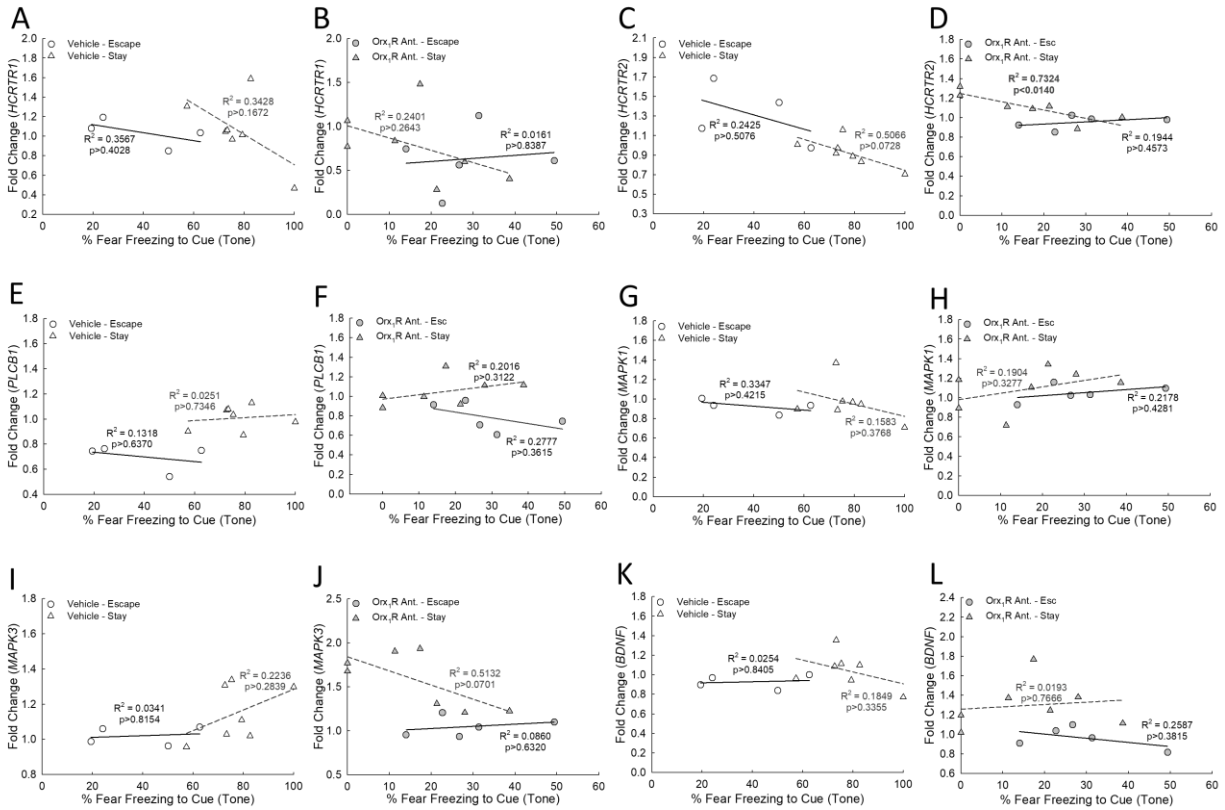
**Figure S7.** Knockdown of intra-BLA Orx<sub>1</sub>R prior to social stress-induced fear conditioning, does not affect an animal's fear learning response (N = 22, CS Effect:  $F_{1,20} = 19.3$ , # $p < 0.001$ ).



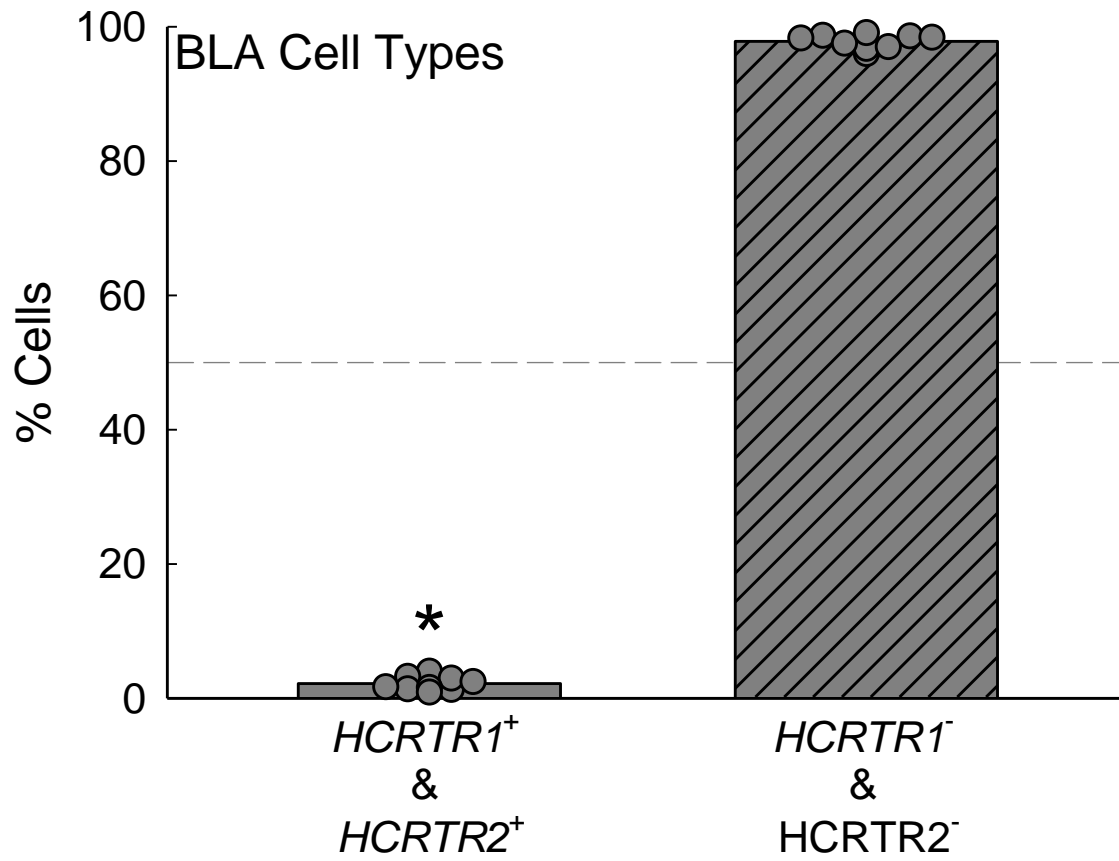
**Figure S8.** Treatment with intra-BLA Orx<sub>1</sub>R antagonist did not change expression levels of *MAPK1* (N = 31, Phenotype Effect,  $F_{1,19} = 0.3$ ,  $p \geq 0.611$ ; Treatment Effect,  $F_{1,19} = 2.8$ ,  $p \geq 0.113$ ; Interaction Effect,  $F_{1,19} = 0.001$ ,  $p \geq 0.970$ ).



**Figure S9.** Molecular changes in BLA signaling dynamics as a result of intra-BLA Orx<sub>1</sub>R antagonism are not related to contextual fear response. Transcription levels of *HCRTR1*, *HCRTR2*, *PLCB1*, and *MAPK1* are not related to contextual fear freezing in (A, C, E, & G) Vehicle- (N = 11) or (B, D, F, & H) Orx<sub>1</sub>R Antagonist-treated animals (N = 12). While a significant positive relationship was observed between contextual fear freezing and ERK<sub>1</sub> (*MAPK3*) mRNA in (I) Vehicle-treated mice, (J) this positive relationship is absent (becomes more closely resembling a negative relationship) in animals infused with the Orx<sub>1</sub>R Antagonist. Contextual fear freezing is not associated with *BDNF* transcription levels in (K) Vehicle- or (L) Orx<sub>1</sub>R Antagonist-treated mice.

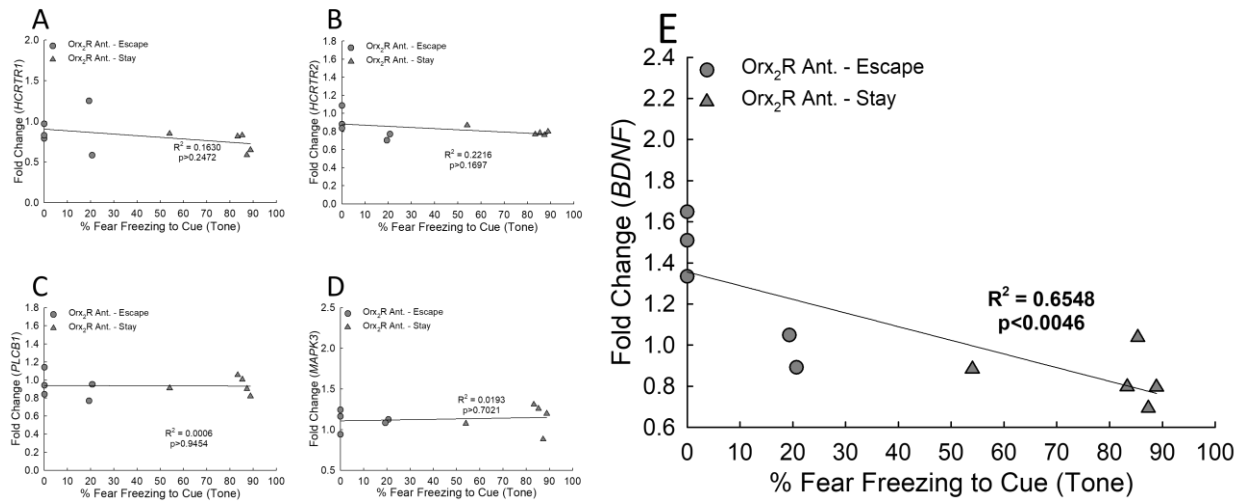


**Figure S10.** Phenotype-dependent cued fear learning is not correlated to changes in transcription levels. There are no phenotype-specific relationships observed between relative transcription levels of *HCRTR1*, *HCRTR2*, *PLCB1*, *MAPK1*, *MAPK3*, and *BDNF* and cued fear freezing response in (A, C, E, G, I, & K) Vehicle-treated mice (N = 11). While this trend holds true mostly for animals treated with an (B, F, H, J, L) Orx<sub>1</sub>R antagonist as well (N = 12), (D) there is exists a significant negative relationship between *HCRTR2* expression and cued fear freezing in only Stay mice administered the Orx<sub>1</sub>R antagonist ( $F_{1,5} = 13.7$ ,  $R^2 = 0.7324$ ,  $p \leq 0.014$ ).

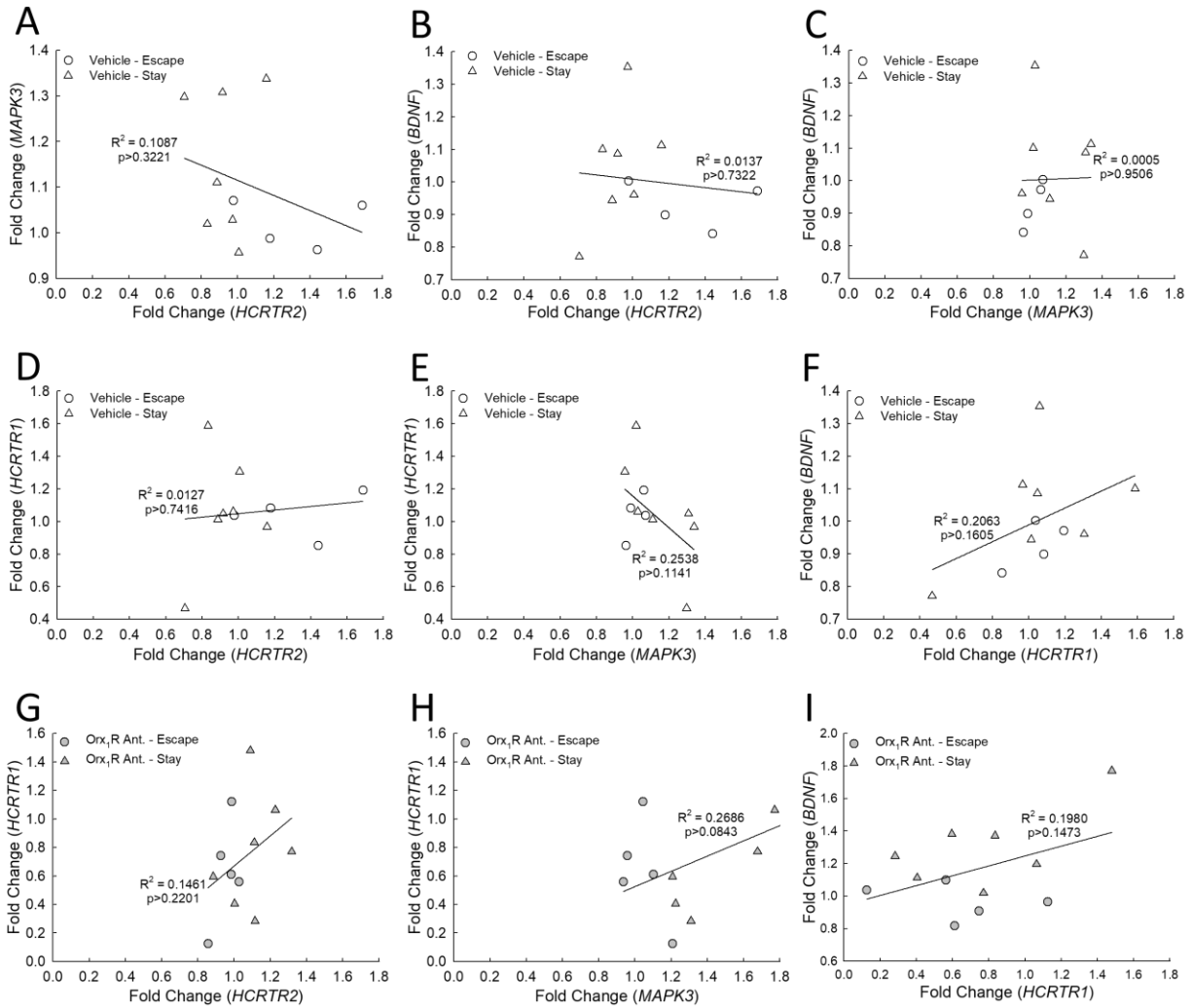


**Figure S11.** The proportion of BLA cells that express both *HCRTR1* and *HCRTR2* is small (~2%) compared to those that do not express the mRNA for both Orx receptors (N = 9,  $t_{16} = 192.4$ ,  $p < 0.001$ ).





**Figure S12.** Inhibition of Orx<sub>2</sub>R in the BLA is not reveal correlations between cued fear freezing and transcription of (A) *HCRTR1*, (B) *HCRTR2*, (C) *PLCB1*, or (D) *MAPK3* (N = 10). (E) However, after intra-BLA Orx<sub>2</sub>R antagonism, a significant negative relationship between BDNF and fear freezing was observed (N = 10,  $F_{1,8} = 15.2$ ,  $R^2 = 0.6548$ ,  $p \leq 0.0046$ ).



**Figure S13.** Statistical correlations of molecular changes in the BLA associated with Orx<sub>1</sub>R antagonism (Fig. 11) are not present in Vehicle-treated animals. (A) With vehicle administration, intra-BLA changes in *MAPK3* and *HCRTR2* mRNA levels are not correlated (N = 11). Similarly, in vehicle-treated mice (N = 11), relationships in BLA transcription levels do not exist for (B) *BDNF* and *HCRTR2* or (C) *BDNF* and *MAPK3*. Further, BLA analyses of mice administered vehicle (N = 11) did not reveal relationships between *HCRTR1* and (D) *HCRTR2*, (E) *MAPK3*, or (F) *BDNF*. After intra-BLA Orx<sub>1</sub>R antagonism (N = 12), *HCRTR1* gene expression levels in the BLA were not correlated with (G) *HCRTR2*, (H) *MAPK3*, or (I) *BDNF*.

### **Chapter 3: Orexin receptor modulation in basolateral amygdala reveals generalization of social stress learning**

#### **ABSTRACT**

Fear-associated memories and behavior are often expressed in contexts/environments distinctively different from those in which they are created. This generalization process contributes to psychological disorders, particularly PTSD. Stress-related neurocircuits in the basolateral amygdala (BLA) receive inputs from hypothalamic orexin (Orx) neurons. These neurons mediate activity by targeting orexin 1 (Orx<sub>1</sub>R) and orexin 2 (Orx<sub>2</sub>R) receptors which govern opposing behavioral functions. In the BLA, inhibition of Orx<sub>1</sub>R or activation of Orx<sub>2</sub>R ameliorate stress responsiveness and behavior. We discovered that most Orx<sub>1</sub>R<sup>+</sup> cells also express CamKII $\alpha$ , while a majority of Orx<sub>2</sub>R<sup>+</sup> cells are colocalized with GAD67. Further, *HCRTR1* expression was positively correlated, and *HCRTR2* expression was negatively correlated with freezing in a phenotype-dependent fashion (Escape vs Stay) in the Stress Alternatives Model (SAM). The SAM consists of 4-days of social interaction trials between test mice and novel larger aggressors. Exits positioned at opposite ends of the SAM oval arena provide opportunities to actively avoid aggression. By Day 2, mice commit to a behavioral phenotype: Escape or Stay. Pharmacologically manipulating Orx receptor activity in the BLA, before Day 3 of the SAM, was followed with standard tests of anxiety: Open Field (OF) and Elevated Plus Maze (EPM). Freezing and locomotion during SAM interaction were generalized to the non-social OF environment. This transference of behaviors was blocked by intra-BLA Orx<sub>1</sub>R antagonism, but not Orx<sub>2</sub>R antagonism in Stay mice. Moreover, the pattern of social avoidance in the SAM for Escape and Stay mice were recapitulated in the OF test, with behavioral transference being mediated by Orx<sub>1</sub>R and Orx<sub>2</sub>R activity.

## INTRODUCTION

Fear learning plays an important role in many psychological disorders, including anxiety, depression, and PTSD. In these disorders, particularly PTSD and related animal models, fear-associated memories and behavior are often expressed in contexts and/or environments that are distinctively different from those in which they are generated, a learning process known as generalization or transference [1-6]. Preclinical models for these disorders often use environmental or social stressors [7], and frequently demonstrate specific fear learning mechanisms, such as Pavlovian conditioning [8, 9]. However, several classical tests for anxiety and/or depression (such as Elevated Plus Maze [EPM] or Open Field [OF] Test) in rodents have failed to faithfully translate to successful clinical trials [10, 11]. We suggest that more efficacious models should carefully include ecologically and ethologically designed applications [7, 12] that specifically consider learning in the production of behavioral outcomes and decision-making [13].

During socially stressful interactions, such as aggression, behavioral responses are influenced by previous experience, environmental options, and the intensity of the social contact [8, 9, 14, 15]. These behavioral dynamics are controlled by neural and endocrine reactivity to stress. We designed the Stress Alternatives Model (SAM) to allow for a window onto the development of anxious and depressive behavior, and the mechanisms of decision-making that produce resilient and susceptible phenotypes. In an oval arena with apical escape routes, novel larger aggressive individuals interact with a smaller adult test subjects. Test animals self-select one of two phenotypes: Escape or Stay, which exhibit stress resilient and susceptible responses to social interaction/preference tests (SIP) and in plasma glucocorticoid concentrations [9, 16, 17]. Anxiolytic drugs (corticotropin releasing factor type 1 receptor [CRF<sub>1</sub>] antagonist

antalarmin, orexin 1 receptor [Orx<sub>1</sub>R] antagonist SB-674042, and orexin 2 receptor [Orx<sub>2</sub>R] agonist [Ala<sup>11</sup>, D-Leu<sup>15</sup>]-Orx<sub>B</sub>) prompt resilient Escape behavior in susceptible Stay animals [9, 16, 17]. Alternatively, anxiogenic drugs (Yohimbine, an  $\alpha_2$  adrenoreceptor antagonist, and Orx<sub>2</sub>R antagonist MK-1064) promote Stay behavior in Escape phenotype mice. Behaviors reflecting motivation to escape the SAM are also modified by stress-related neuromodulatory events [16, 17]. A fear conditioning protocol (tone = conditioned stimulus [CS]) precedes the aggressive interaction (unconditioned stimulus [US]) in the SAM, in which the cued conditioned response (CR = freezing) in Stay mice is reduced by intra-basolateral amygdala (intra-BLA) injection of an Orx<sub>1</sub>R antagonist [17].

Orexins (hypocretins) are comprised of two peptides, Orx<sub>A</sub> (HCrt<sub>1</sub>) and Orx<sub>B</sub> (HCrt<sub>2</sub>), cleaved from the same pro-peptide produced in equal proportions in the lateral, dorsomedial hypothalamus perifornical area (LH-DMH/PeF) [18, 19]. While Orx<sub>A</sub> has equally high binding affinity for Orx<sub>1</sub>R and Orx<sub>2</sub>R, Orx<sub>B</sub> has a modestly higher affinity for Orx<sub>2</sub>R over Orx<sub>1</sub>R [20, 21]. In the BLA, and in other regions such as the paraventricular thalamus, the two receptor types are functionally opposed [22-25]. We suspect therefore, that specific receptor binding is defined by cellular localization [17], such that functions of the G<sub>q</sub>-linked Orx receptors are married to the output of the neuronal types in which each primarily exists. During experiments examining the anti-stress properties of Orx<sub>1</sub>R antagonism in the BLA, it became clear that the classical fear conditioning we expected for stress-susceptible animals alone [9, 26], was more complex than originally hypothesized [17]. Instead of classical fear conditioning being limited to susceptible Stay mice, both Stay and Escape mice exhibit cued CRs. Moreover, Stay mice also exhibit enhanced contextual conditioning (freezing prior to tone in opaque divider) during the same SAM protocol [17]. Additionally, intra-BLA injection of an Orx<sub>1</sub>R antagonist and icv

delivery of an Orx<sub>2</sub>R agonist reduce cued fear conditioning [16, 17]. Although Escape individuals experience significantly reduced neural and endocrine reactivity to stress, as well as diminished stress-related behavior compared to Stay animals [9, 16, 26] they undergo classical and contextual conditioning. While the phenotypes are fundamentally different in the magnitude of responses (such as freezing), the fear learning processes can be induced in resilient (Escape) animals [17].

In addition to the Orx<sub>1</sub>R antagonist inhibiting secretion of the stress hormone corticosterone in both vulnerable (Stay) and resilient (Escape) mice, there were also specific alterations of signaling-related gene expression [17]. Specifically, inhibition of Orx<sub>1</sub>R in BLA promoted increased Orx<sub>2</sub>R (*HCRTR2*) gene expression in non-glutamatergic, presumably GABAergic, neurons [17]. In BLA, following Orx<sub>1</sub>R inhibition (likely in pyramidal neurons) there was also a significant increase in ERK<sub>1</sub> (*MAPK3*) and Brain-Derived Neurotrophic Factor (*BDNF*) transcripts [17]. We hypothesized that these changes occurred primarily in GABAergic cells containing Orx<sub>2</sub>R. This potential cross-neuron stimulation of Orx<sub>2</sub>R, ERK<sub>1</sub>, and BDNF mRNA production was only evident in vulnerable Stay mice following intra-BLA administration of an Orx<sub>1</sub>R antagonist. The data suggest the Orx system not only modifies behavioral activity through actions in the BLA, it also acts to shift the signaling systems that underlie those behaviors [17]. We surmised that if behavior and signaling systems were both altered following intra-BLA Orx<sub>1</sub>R antagonism, then the learning and memory systems that allow and support those behaviors might also be changed.

As inhibition of Orx<sub>1</sub>R in the BLA corresponded with transcriptional changes in Orx receptors and conditioned fear responses [17], we first predicted that mRNA levels of Orx receptors in the BLA would be related to phenotype-dependent socially induced freezing

behavior in the SAM arena. Additionally, we noticed adaptive adjustment of specific self-positioning strategies during avoidance of the aggressor appear during SAM trials. That is to say, resilient Escape mice seemed to make more use of SAM arena edge space on the way to using the escape route at the apical edge, even though the CD1 aggressors primarily patrol there. Not surprisingly, susceptible Stay mice make greater use of center areas. Those observations lead us to examine if the development of unique Escape and Stay coping strategies were being translated into equivalent behavior in subsequent trials using alternative testing models post-treatment. Since the open field (OF) and elevated plus (EPM) tests have uniquely defined movement strategies that have been associated with anxious responses [11,27,28], our surprising results suggested that the typical OF and EPM outcomes can be overridden. Based on these findings, we hypothesized that social stress-induced fear freezing behavior will be generalized (learned transfer of the adaptive response from one context to another) to a novel OF Test environment, and that Orx<sub>1</sub>R inhibition or Orx<sub>2</sub>R stimulation would mute this learning response. As intra-BLA Orx receptors are important for generalization of fearful responses, we also hypothesized that specific learning of environmental self-positioning strategies during social aggressor avoidance in the SAM may be transferred/generalized to the non-social OF Test with pharmacological manipulation of Orx receptor activity. Finally, we proposed that measures of anxious behavior in the classical EPM after social stress and behavioral testing may be unreliable and inconsistent with results from the SAM and OF Test learning trials.

## **MATERIALS & METHODS**

### **Social Stress and Decision-Making Paradigm**

In the SAM paradigm (Fig. 1A), social conflict between a larger novel CD1 mouse and smaller C57BL/6NHsd male mouse takes place for five minutes over four days, during which test animals

may shorten interaction with the aggressor by escaping out of size-limited tunnels at the ends of an oval open field arena. A tone given during isolation in the SAM apparatus prior to arena exposure conditions test subjects to the upcoming social interaction. As distinct and stable phenotypes (Escape & Stay) are established on Day 2, drug manipulation on Day 3 allows for behavioral comparisons between phenotypes and drug controls (vehicle) during the SAM (Days 3 & 4) and in tests of anxiety that follow SAM exposure (Open Field and Elevated Plus Maze). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the USD Institutional Animal Care and Use Committee.

### **Experiment Overview** (*see also* COMPLETE MATERIALS & METHODS)

For these experiments, Orx receptor-targeting drugs (Orx<sub>1</sub>R antagonist: SB-674042, Orx<sub>2</sub>R antagonist: MK-1064, Orx<sub>A</sub>, concoction of Orx<sub>A</sub> & MK-1064 [for biased stimulation of Orx<sub>1</sub>R], and Orx<sub>2</sub>R agonist: YNT-185) were directed at the BLA 1h prior to SAM interaction on Day 3 (Fig. 1B). After SAM interaction on Day 4, mice were exposed to the OF Test (Day 4) and EPM (Day 5). Behavioral measurements were taken the active phase (dark cycle), and included freezing (conflict-associated [SAM] or generalized [OF Test]), locomotion (SAM, OF Test, and home cage), time spent in center area (SAM and OF Test), and standard EPM measurements (time in open/closed arms and intersection zone). Brains were collected and used for visual representations of mRNA (using RNAscope) or relative changes in gene expression (rt-qPCR) of Orx<sub>1</sub>R (*HCRTR1*) and Orx<sub>2</sub>R (*HCRTR2*) receptors, Ca<sup>++</sup>/Calmodulin Kinase type 2 alpha (*CAMKIIα*; glutamatergic cell marker), Glutamate Decarboxylase (*GAD1*; GABAergic cell marker), and parvalbumin (*PVALB*; calcium-binding protein found in a subset of GABA neurons). All statistical analyses were built from *a priori* hypotheses and performed using two-



way repeated measures ANOVA for SAM freezing behavior across days (Phenotype x Day), two-way ANOVA for *in situ* cellular localization studies and SAM, OF, or EPM behaviors (Phenotype x Treatment), Regression analyses where correlations between SAM and OF behaviors were made, and t-test, followed (where appropriate) by *post hoc* analyses.

## **RESULTS** (*see also* COMPLETE RESULTS)

### **Social stress-induced freezing linked to specific Orx receptors in specific neurons**

Freezing in response to social conflict is common and most pronounced on Day 4 of the SAM paradigm (Fig. 2A; Phenotype Effect:  $F_{1,168} = 4.8, p \leq 0.033$ ; Time Effect:  $F_{3,168} = 4.2, p \leq 0.007$ ; Interaction Effect:  $F_{3,168} = 4.1, p \leq 0.008$ ; Day 4 Escape vs Stay:  $t_{56} = 3.8, p < 0.001$ ).

This freezing behavior exhibits phenotype specific positive (Escape mice) and negative (Stay) regression relationships with *HCRTR1* (Escape; Fig. 2B;  $F_{1,8} = 7.8, R^2 = 0.4946, p \leq 0.0233$ ) and *HCRTR2* (Stay; Fig. 2C;  $F_{1,12} = 9.7, R^2 = 0.4481, p \leq 0.0088$ ). The Orx<sub>1</sub>R and Orx<sub>2</sub>R are found in a minority of BLA neurons (Fig. 2D;  $F_{3,44} = 134.0, p < 0.001$ ), suggesting that the strong functional relationship to freezing is determined by specific neurocircuits, presumably including CamKII-positive glutamatergic pyramidal cells for Orx<sub>1</sub>R effects on freezing in Escape mice (Figs. 2E-G; Interaction Effect:  $F_{2,30} = 37.4, p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> CamKII $\alpha$ <sup>+</sup> vs CamKII $\alpha$ <sup>-</sup>:  $t_{10} = 6.4, p < 0.001$ ; Orx<sub>2</sub>R<sup>+</sup> CamKII $\alpha$ <sup>+</sup> vs CamKII $\alpha$ <sup>-</sup>:  $t_{10} = 5.8, p < 0.001$ ; CamKII $\alpha$ <sup>+</sup> Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>2</sub>R<sup>+</sup>:  $t_{10} = 5.9, p < 0.001$ ; CamKII $\alpha$ <sup>+</sup> Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_{10} = 2.8, p \leq 0.009$ ), and likely GAD67-positive GABAergic neurons for Orx<sub>2</sub>R relationship with freezing in Stay mice (Figs. 2H-J; GAD67 Expression Effect:  $F_{1,24} = 322.9, p < 0.001$ ; Interaction Effect:  $F_{2,24} = 73.3, p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> GAD67<sup>+</sup> vs GAD67<sup>-</sup>:  $t_8 = 18.0, p < 0.001$ ; GAD67<sup>+</sup> Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 8.4, p < 0.001$ ; GAD67<sup>+</sup> Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 2.9, p \leq 0.008$ ; Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>

GAD67<sup>+</sup> vs GAD67<sup>-</sup>:  $t_8 = 12.3, p < 0.001$ ; GAD67<sup>-</sup> Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 2.9, p \leq 0.008$ ; GAD67<sup>-</sup> Orx<sub>2</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 5.5, p < 0.001$ ).

### **Orx receptor-dependent generalization of freezing and locomotion are phenotype specific**

Importantly, freezing in response to social conflict in the SAM is generalizable to the OF test for vehicle treated Stay mice (Fig. 3; Antagonist groups: Phenotype Effect,  $F_{1,43} = 16.0, p < 0.001$ ; Stimulation groups: Drug Effect,  $F_{3,46} = 17.1, p < 0.001$ ; Phenotype Effect,  $F_{1,46} = 9.1, p \leq 0.004$ ). These Stay mice exhibit significantly more conflict freezing than Escape mice (Fig. 3A;  $t_{19} = 2.67, p \leq 0.015$ ). The distinctive phenotype difference in behavior is eliminated by Orx<sub>1</sub>R antagonist treatment, but not by Orx<sub>2</sub>R antagonist (Fig. 3A;  $t_{15} = 2.8, p \leq 0.014$ ). Further, phenotype differences are abolished with Orx<sub>A</sub> and Orx<sub>1</sub>R stimulation as a result of Escape mice displaying more freezing, and after Orx<sub>2</sub>R agonist as both phenotypes experience a reduction in freezing (Fig. 3B; Escape Vehicle vs Orx<sub>A</sub>:  $t_{10} = 3.4, p \leq 0.007$ ; Stay Vehicle vs Orx<sub>A</sub>:  $t_{16} = 3.3, p \leq 0.005$ ; Stay Vehicle vs Orx<sub>1</sub>R Stim.:  $t_{21} = 3.0, p \leq 0.008$ ; Escape Vehicle vs Orx<sub>2</sub>R Stim.:  $t_9 = 2.3, p \leq 0.047$ ; Escape Orx<sub>A</sub> vs Orx<sub>2</sub>R Stim.:  $t_5 = 3.2, p \leq 0.023$ ; Stay Vehicle vs Orx<sub>2</sub>R Stim.:  $t_{17} = 3.3, p \leq 0.004$ ; Stay Orx<sub>A</sub> vs Orx<sub>2</sub>R Stim.:  $t_9 = 8.7, p < 0.001$ ; Stay Orx<sub>1</sub>R Stim. vs Orx<sub>2</sub>R Stim.:  $t_{13} = 8.5, p < 0.001$ ).

Generalization of locomotion is also transferable from SAM to OF for Escape mice (Fig. 4; Antagonist groups: Phenotype Effect,  $F_{1,43} = 9.0, p \leq 0.005$ ; Stimulation groups: Phenotype Effect,  $F_{1,46} = 8.2, p \leq 0.006$ ), with significant positive regressions between OF and SAM locomotion for Orx<sub>1</sub>R antagonist, but also for Orx<sub>A</sub> and the Orx<sub>2</sub>R agonist (Figs. 4E-H; Orx<sub>1</sub>R Ant.:  $F_{2,3} = 15.9, p \leq 0.028$ ; Orx<sub>A</sub>:  $F_{2,2} = 49.4, p \leq 0.02$ ; Orx<sub>2</sub>R Stim.:  $F_{2,1} = 351.5, p \leq 0.034$ ). Escape mice (vehicle treated) exhibit significantly more locomotion in the SAM and OF (Figs. 4A, C), but not in the home cage (Fig. S9; SAM:  $t_{19} = 2.7, p \leq 0.014$ ; OF:  $t_{19} = 2.5, p \leq 0.023$ ).

This phenotypic distinction is observed after Orx<sub>2</sub>R antagonism, but not Orx<sub>1</sub>R antagonism (Figs. 4A, C; SAM:  $t_{11} = 2.3, p \leq 0.039$ ) or Orx<sub>1</sub>R stimulation (Figs. 4B, D; SAM:  $t_{13} = 2.5, p \leq 0.026$ ; OF:  $t_{13} = 2.5, p \leq 0.028$ ). However, the correlation of locomotive behavior in SAM and OF is still present despite the reversal of the phenotypic divide (Orx<sub>1</sub>R Ant.:  $F_{2,3} = 15.9, p \leq 0.028$ ).

### **Generalization of phenotypic behavior patterns are modulated by Orx receptors**

As freezing and locomotion in response to social conflict are generalizable from SAM apparatus to OF, we sought to understand whether the basic patterns of phenotypic response (Stay and Escape), would be reflected in standard tests of anxious responsiveness, such as OF or EPM. There was a generalization effect of phenotypic behavior in OF (Fig. 5; Antagonist groups, SAM: Phenotype Effect,  $F_{1,43} = 7.4, p \leq 0.010$ ; OF: Phenotype Effect,  $F_{1,43} = 15.7$ ; Stimulation groups, SAM: Drug Effect,  $F_{3,45} = 5.5, p \leq 0.003$ ; OF: Phenotype Effect,  $F_{1,46} = 4.9, p \leq 0.032$ ). However, for treated mice this transference was strictly dependent on movement patterns learned during 4 days in the SAM. Escape mice, which used the edges of the SAM to locate apical escape routes located on the edge, also favored edges in the OF (Figs. 5F top; ). In contrast, Stay mice frequented the center of the SAM apparatus to avoid patrolling CD1 aggressors, and maintained that pattern in OF when treated with Orx<sub>1</sub>R or Orx<sub>2</sub>R antagonists or an Orx<sub>2</sub>R agonist (Figs. 5E, F bottom, G, H; Orx<sub>1</sub>R Ant.:  $F_{1,8} = 16.8, p \leq 0.003$ ; Orx<sub>2</sub>R Ant.:  $F_{1,5} = 13.5, p \leq 0.014$ ; Orx<sub>2</sub>R Stim.:  $F_{1,4} = 40.2, p \leq 0.003$ ).

The EPM results following 4 days of SAM interaction did not produce a phenotypic distinction between Escape and Stay mice for open arm, closed arm, or interaction zone times (Figs. S11; Open Arms: Phenotype Effect,  $F_{1,46} = 7.5, p \leq 0.009$ ; Closed Arms: Phenotype Effect,  $F_{1,46} = 8.5, p \leq 0.005$ ). Surprisingly, anxious, stress-vulnerable Stay mice [9, 24],

exhibited significantly more time in open arms following Orx<sub>A</sub> treatment ( $t_7 = 2.2, p \leq 0.037$ ), and Orx<sub>1</sub>R stimulation (Figs. S11B, D;  $t_{13} = 2.2, p \leq 0.044$ ); opposite of the expected finding relative to anxiety.

## **DISCUSSION**

The process of phenotype development in the SAM requires numerous learning phases. Decision-making for stress-vulnerable individuals (Stay) in the SAM paradigm shifts to resilient (Escape) responses after anxiolytic drugs or behavioral modifications (such as exercise) are administered [9, 13]. This is also true after intra-BLA Orx<sub>1</sub>R inhibition or icv Orx<sub>2</sub>R stimulation [16, 17]. Conversely, decisions in the SAM switch from stress resilient responses to stress susceptible responses in Escape phenotype animals following anxiogenic treatments that include Orx<sub>2</sub>R antagonism [9, 17]. Furthermore, social aggression-based contextual and cued fear conditioning are reduced by intra-BLA Orx<sub>1</sub>R antagonism and by icv Orx<sub>2</sub>R stimulation [16, 17], which suggested to us that amygdalar Orx receptors modify associative learning related to fear behavior. Activation of the Orx system amends performance in novel object recognition, while reducing social interaction following defeat [29], further suggesting that Orx and stress-related behavior together modify the conditions for learning.

In the SAM, following the CS cue for fear conditioning, socially induced freezing in response to aggressive contact also takes time to learn, and appears most consistently after 4 days of training (Fig. 2A). Importantly, SAM-induced behavioral changes, like freezing, after acute pharmacological intervention (Day 3) are long-lasting, showing behavioral exhibiting changes on the day of treatment but also later (Days 4 & 5) [9, 16, 17]. Social aggression-induced, phenotype-dependent freezing behavior on Day 4 is bidirectionally correlated,

positively for Escape with *HCRTR1* and negatively for Stay with *HCRTR2* gene expression in the BLA (Figs. 2B & C). The locations of these receptors are distinctively organized primarily with *HCRTR1* expressed (> 60%) in glutamatergic pyramidal (Figs. 2D-F, S5D, F) and *HCRTR2* in GABAergic neurons (Figs. 2G-I, S5E, F), suggesting separation of cellular function in the stress circuits of the BLA that are dependent on learning. Simply put, the data suggest that, in Escape mice, as Orx<sub>1</sub>R mRNA in BLA (mostly pyramidal neurons) increases, freezing also increases. Additionally, the data suggest that as Orx<sub>2</sub>R mRNA increases in Stay BLA (mostly GABA neurons), freezing also decreases. Pyramidal Orx<sub>1</sub>R-containing neurons in the BLA are located in a larger pro-stress circuitry, and activate anxiogenic and pro-depressive behaviors and conditioned fear learning [17, 30]. This circuitry is also innervated by noradrenergic neurons of the locus coeruleus (LC), which are modulated by Orx<sub>1</sub>R and mediate cue-dependent fear memories [31-33]. In the BLA, Orx<sub>2</sub>R-containing GABA neurons inhibit the pro-stress circuitry, but also result in anxiolytic and anti-depressive behaviors and reduce conditioned-fear learning [16, 24]. The data suggest that learned responses are not only tuned to the Orx receptor type and particular neurocircuitry element in which they exist, but also reflected by the gene expression changes that occur over the 4 days of training. Surprisingly, the coping strategies learned in the SAM is transferred to other behavioral tests for anxiety, such as the open field (OF; Figs. 3, 4, 5), but not the elevated plus maze (EPM; Fig. S11).

We have previously noted that Escape and Stay behavior both require learning social behavioral patterns and associative cues, to efficiently minimize vulnerability from attack while using the escape hole or remaining in the SAM arena [8, 14]. Escaping mice utilize one of the two tunnels for egress with progressively reduced latency, while Stay mice display socially induced conflict freezing with progressively increased duration (Fig. 2A). This suggests that

both Stay and Escape animals utilize coping strategies that include learning how to minimize vulnerability from aggression more efficiently with each trial. This means it is necessary to monitor the patrolling patterns of the dominant aggressive male, to avoid those spaces while freezing (for Stay animals), or to develop ballistic or secretive escape movements to safely accomplish Escape. As such, our model demonstrates more than one learned and adaptive reaction is possible in response to an unconditioned fearful stimulus in a fear conditioning paradigm. Social defeat is replete with contextually rich stimuli, including the elements of social rank dynamics, which in natural settings allows for more than one appropriate behavioral response. Others have demonstrated that Orx activation during social defeat reduces both social interaction and recognition learning in defeated mice [29], modifying appropriate behavioral responses. Thus, the generalization or transference of responses (freezing, locomotion, center preference in Stay vs. edge preference in Escape mice) from the SAM to the OF arena, suggests behavioral plasticity in coping strategies linked to specific stress phenotypes.

Regardless of the eventual phenotype, Escape and Stay mice must learn the patrolling routines of the novel, dominant, larger, aggressive (CD1) mice. These mice patrol the edges of the SAM, because it is ecologically safer, and can easily block escape this way, since the tunnels are located on the apical edge. Stress-vulnerable (Stay) mice learn to frequent the center of the SAM arena, to avoid the aggressor. After 4 days of SAM training, these anxious, stress-vulnerable Stay mice also frequent the center of the OF arena, just the opposite of what would be expected, since susceptible animals demonstrate reduced social interaction [29] in the SIP test [16, 34]. Conversely, Escape mice seek egress by following the edge of the arena to the tunnel, and thus in the OF, these stress-resilient (Escape) mice also keep close to the edges. Finally, the duration of socially induced freezing in the OF is correlated with prior freezing in the SAM,

reflecting the learning that occurred there (Fig. 3E).

The behavioral transference or generalization learning observed here likely results from distinctive Orx<sub>1</sub>R or Orx<sub>2</sub>R signaling within decision-making and anxiety/fear neurocircuitries that are inextricably tied to learning systems [17, 31-33]. When decision-making coincides with stress, recruitment of neural networks that define executive function, including the dorsolateral prefrontal, anterior cingulate, and orbitofrontal cortices, utilize connections with the emotion processing system of the amygdala [35, 36], which can be modified by other brain regions, such as the LC [31-33]. Likewise, learning events prompted by fear are mediated through potentially distinctive circuits involving hippocampus, lateral hypothalamus, LC, and amygdala [31-33]. In this way, the gating of stress-induced learning behavior, like those associated with transference and generalization, requires amygdalar engagement.

Importantly, we demonstrate transference or generalization is strongly modified by Orx receptor actions in the BLA. Antagonism of Orx receptors in the BLA impacts spatial memory, specifically during the consolidation/re-consolidation phase [37]. However, we report an observed caveat that Orx influence over BLA-gated learning events may depend on the anxious state of the individual. For example, in Stay mice, intra-BLA Orx<sub>1</sub>R antagonism promotes learning to increase time in the center of the SAM arena (Figs. 5A, C, E, F). These Orx<sub>1</sub>R reside predominantly in glutamatergic pyramidal cells of the BLA (Figs. 2A-F, S5F). Interestingly, while Orx<sub>2</sub>R inhibition promotes activity in the center of the OF, and is suggestive of Stay learning of center avoidance in the SAM (Figs. 5A, C, G), typically BLA Orx<sub>1</sub>R and Orx<sub>2</sub>R inhibition have opposite effects on anxious behaviors in the SAM [17].

Stress-induced generalization learning requires integration of anxiety elements of neurocircuitry [38, 39]. Stress-susceptible (Stay) mice exhibit enhanced socially induced

freezing behavior in the SAM (Fig. 3A), which is carried over (generalized or possibly overgeneralized) to the non-social OF Test arena (Fig. 3E). Uniquely, our behavioral design incorporates conditioning over four days to a naturalistic fear in the form of social aggression (US<sup>+</sup>). Our model captures fear generalization when mice are introduced to a new testing context (i.e. OF Test), where the absence of the unconditioned stimulus (social aggressor) should be more immediately distinguishable than the exclusion of a shock, as in less ethologically relevant stress paradigms. While it is true that timing and layout of experimental design may modify the intensity of the generalized behavior [40], the transferred fear response observed in our study is tied to additional learned coping strategies (i.e. time in center and locomotion) when mice are moved from the SAM to the OF Test. Thus, we posit that stress-induced generalization learning requires integration of both learning and anxiety elements of neurocircuitry [38, 39].

Like learning how to move (Fig. 4) and identifying safe areas in the social context of the SAM (Fig. 5), generalization of freezing behavior is influenced by Orx receptor activity in the BLA (Fig. 3). Activity from distinct neuronal populations within the lateral amygdala (LA) support the expression of generalized fear [41], and likely contribute to the observed transference of freezing behavior reported here. While a relationship of the Orx system and contextual fear response has been identified through indirect noradrenergic connections to the LA from the LC [33], we provide evidence for a more direct influence of Orx in the amygdala on fear generalization. In support of the relationship revealed between Orx<sub>2</sub>R expression in the BLA and socially induced freezing in the SAM (Fig. 2C), Stay mice display enhanced freezing behavior in both the SAM and OF Test environments after Orx<sub>2</sub>R antagonism (Figs. 3A, C), and reduced freezing with Orx<sub>2</sub>R stimulation (Figs. 3B, D). Curiously, Orx<sub>A</sub> treatment, which



activates both Orx<sub>1</sub>R and Orx<sub>2</sub>R, elevated SAM freezing behavior in both Escape and Stay mice (Fig. 3B). As Orx<sub>1</sub>R are expressed at higher levels in the BLA compared to Orx<sub>2</sub>R (Fig. 2D), it is reasonable to suggest that Orx<sub>A</sub> treatment would disproportionately activate Orx<sub>1</sub>R over Orx<sub>2</sub>R. Further, while Orx<sub>1</sub>R antagonism had no effect on socially induced freezing in the SAM (Fig. 3A), it reduced freezing in the OF Test in Escape animals (Fig. 3C). Biased activation of Orx<sub>1</sub>R enhanced freezing in both SAM and OF Test contexts in Stay animals (Figs. 3B, D). While generalization of freezing behavior was apparent in vehicle-treated Stay mice (Fig. 3E), manipulation of intra-BLA Orx receptor activity disrupted this behavior (Fig. S7). What is clear, however, is that Orx receptors in the BLA mitigate freezing behavior as learned in a social environment is carried over to a non-social context (Fig. 3), and these receptors appear to do so in a phenotype-dependent way (Figs. 2B, C).

The EPM results testing for anxiety relationships in vehicle-treated animals do not show socially induced phenotypic separation (Fig. S11). This was surprising at first, because both SAM and SIP results suggest a strong correlation between Escape and resilience, as well as Stay animals having high stress vulnerability. In the EPM, both Escape and Stay mice spend most of their time in the closed arms, with significant excursions into the open arms, which were not affected by either Orx<sub>1</sub>R or Orx<sub>2</sub>R antagonists (Figs. S11A, C), an observation consistent with previous studies [42, 43]. Similarly, animals tested on the EPM before SAM trials, where Escape and Stay phenotypes develop, also do not exhibit differences in open or closed arm times [13]. It may simply be that social and environmental stressors provide radically dissimilar results. However, with application of Orx<sub>A</sub>, Orx<sub>1</sub>R stimulation, or Orx<sub>2</sub>R stimulation, phenotypic differences are again revealed (Figs. S11B, D). In the OF, stimulation of Orx<sub>1</sub>R (Orx<sub>A</sub> and Orx<sub>1</sub>R stimulation) prompted Stay animals to spend more time in the open (and less

in the closed) arms (Figs. S11B, D). Again, the results seem to have been modified by previous experience in the SAM, which calls into question the value of both the OF and EPM tests. If the results of the tests can be dramatically skewed, or reversed, by previous experience in the SAM, they may also be slanted by additional, perhaps not obvious, environmental or social stresses in other experimental paradigms, or by other life experiences before experimentation. The clinical translatability of these tests has previously been called into question [10-12], and our results add reason to question their validity. We urge caution for all those planning to use EPM or OF in future experiments.

In conclusion, the Orx system interacts with BLA neurons to regulate fear learning and generalization during social stress. Additionally, neurons that synthesize Orx<sub>1</sub>R and Orx<sub>2</sub>R in the BLA are mostly distinct. While Orx<sub>1</sub>R are located primarily in glutamatergic neurons, a smaller majority of Orx<sub>2</sub>R are found in GABAergic interneurons. Although learning strategies are influenced by anxious state and behavioral phenotype, our results suggest that within the BLA, Orx receptors modulate learning outcomes and generalization, while concomitantly modifying stress-related behavior. The intra-BLA Orx receptors bidirectionally balance these learning states with Orx<sub>1</sub>R inhibition and, alternatively, Orx<sub>2</sub>R stimulation contributing to behavioral transference and a reduction in fear-induced generalization. While orexin's effect over learning extends beyond the BLA, including targets like the LC and hippocampus, we demonstrate an important role for intra-BLA Orx receptors to influence learning in a receptor- and anxious state-dependent manner.

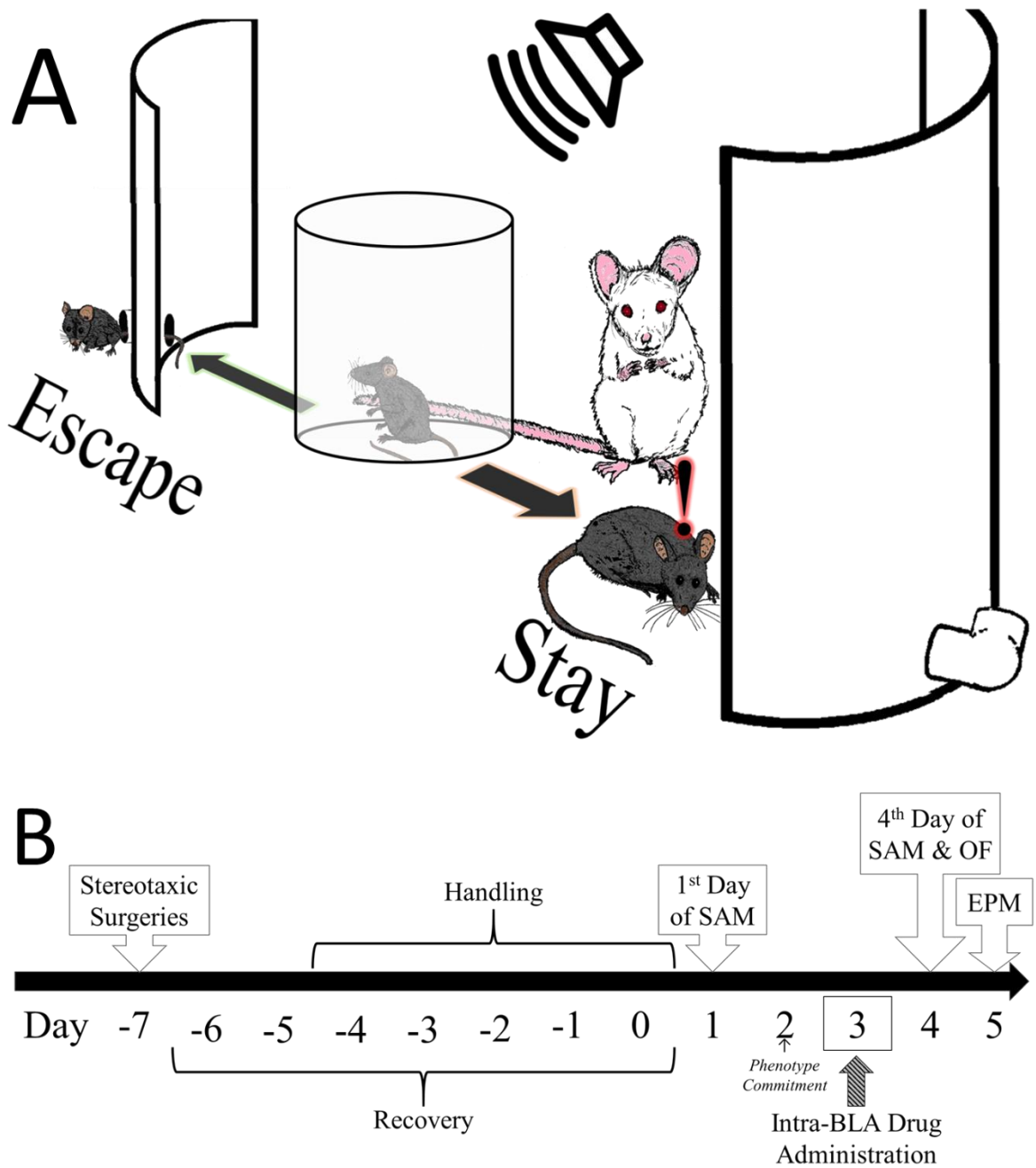
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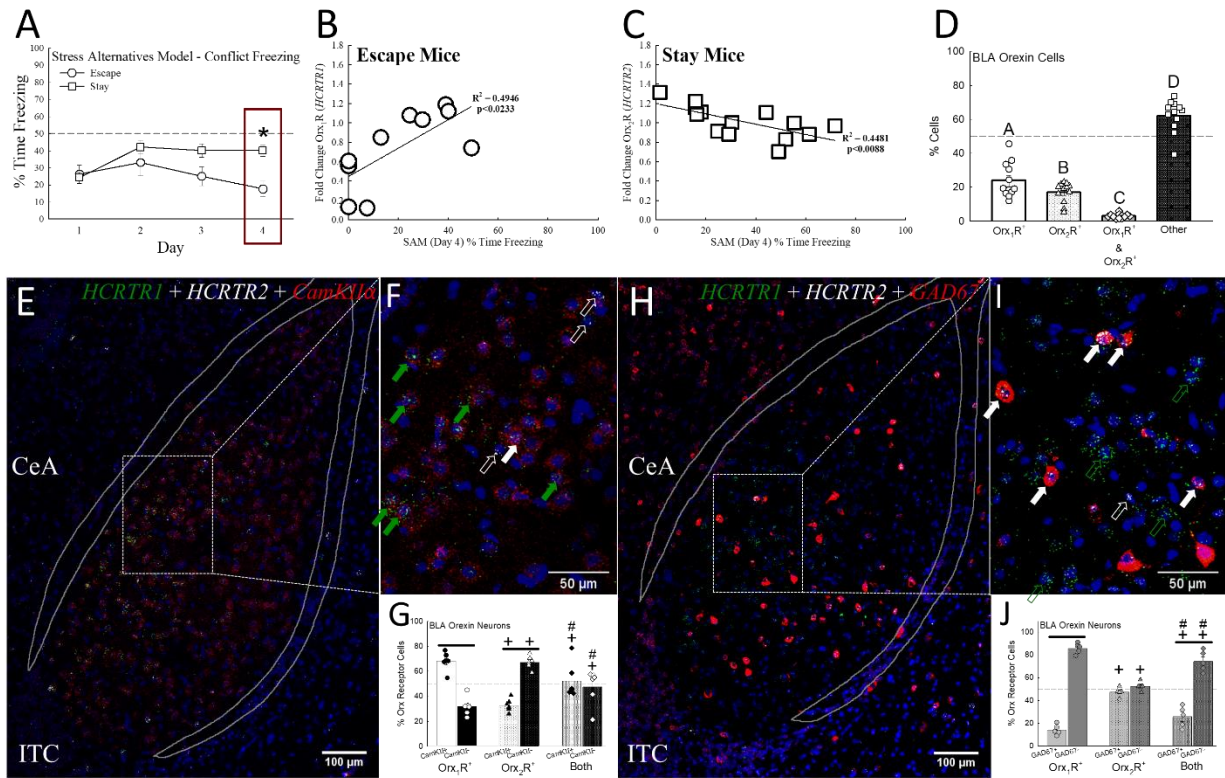
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**Figure 1.** The Stress Alternatives Model (SAM) results in phenotype establishment after two days of social stress. (A) The SAM is a 4-day paradigm in which test mice are conditioned to a tone (*middle*) before an opaque cylinder is lifted and animals must decide whether to Escape (*left*) or submit (Stay, *right*) to a large social aggressor. By the end of Day 2, test mice commit to the Escape or Stay behavioral phenotype. (B) Experimental design for behavioral trials include stereotaxic surgeries for cannula

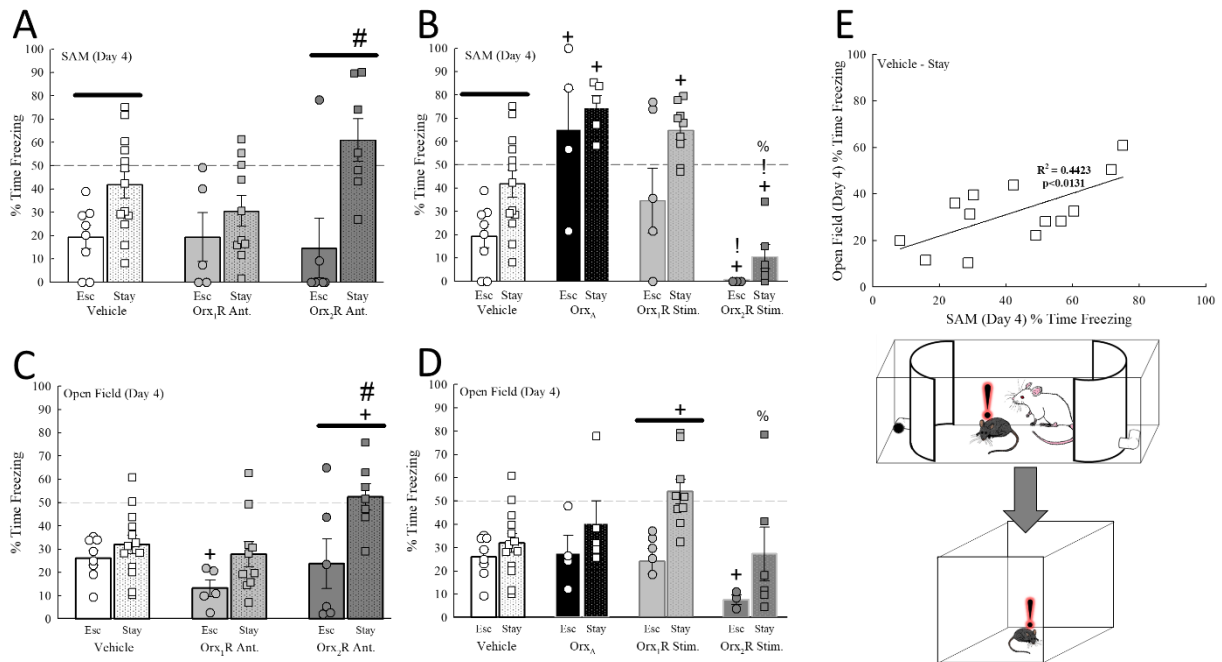
implantation followed by a recovery and handling period before the beginning of the SAM. On Day 3, 1 hr before SAM exposure, mice were administered Orx receptor targeting drugs into the BLA. Following SAM social interaction on Day 4, mice were exposed to the Open Field (OF) Test and on Day 5 they were introduced to the Elevated Plus Maze (EPM).



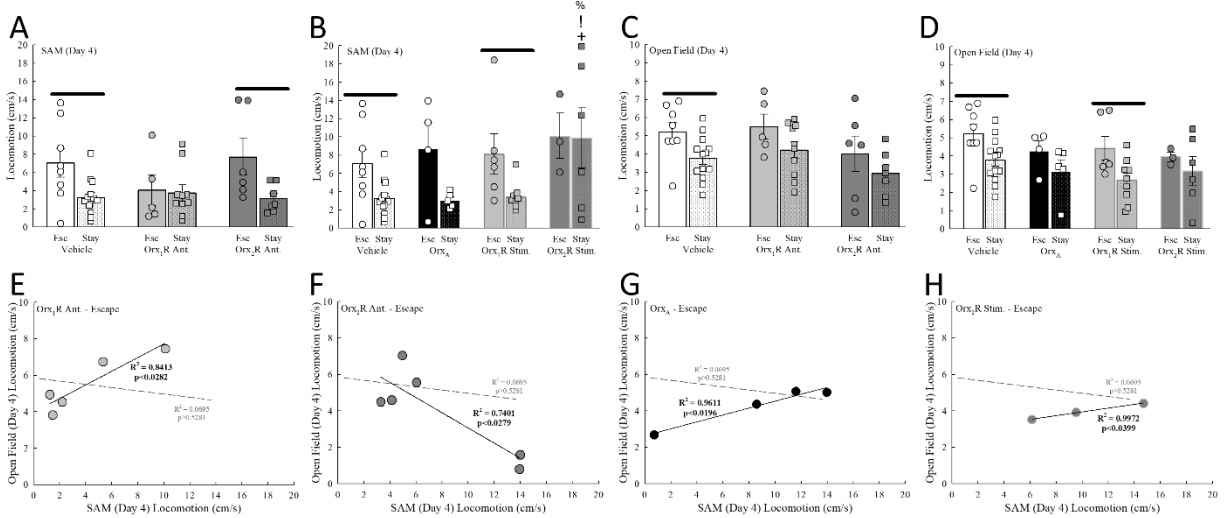


**Figure 2.** Stress-induced and phenotype-dependent freezing behavior is bidirectionally correlated with  $Orx_1R$  (*HCRTR1*), expressed predominantly in glutamatergic neurons, and  $Orx_2R$  (*HCRTR2*), in GABAergic neurons, gene expression in the BLA. (A) Phenotype (Escape & Stay) distinctions in SAM-derived conflict freezing are significant and most pronounced on Day 4 (Phenotype Effect:  $F_{1,168} = 4.8$ ,  $p \leq 0.033$ ; Time Effect:  $F_{3,168} = 4.2$ ,  $p \leq 0.007$ ; Interaction Effect:  $F_{3,168} = 4.1$ ,  $p \leq 0.008$ ; Day 4 Escape vs Stay:  $t_{56} = 3.8$ ,  $*p < 0.001$ ). (B) In Escape mice, intra-BLA  $Orx_1R$  (*HCRTR1*) transcription is positively associated with socially induced freezing (social stress-related freezing) in the SAM ( $F_{1,8} = 7.8$ ,  $R^2 = 0.4946$ ,  $p \leq 0.0233$ ). (C) Conversely, socially induced freezing behavior in the SAM is negatively related to  $Orx_2R$  (*HCRTR2*) mRNA levels in the BLA of Stay mice ( $F_{1,12} = 9.7$ ,  $R^2 = 0.4481$ ,  $p \leq 0.0088$ ). While  $Orx$  receptors are associated to phenotype and freezing behavior, (D) only a small percentage of the total number of BLA cells contain  $Orx_1R$ ,  $Orx_2R$ , or both receptor subtypes ( $F_{3,44} = 134.0$ ,  $p < 0.001$ ; significance is by unique symbol, e.g. A is significantly different from B, C, & D). (E & F) In the BLA,  $Orx_1R$  (green), but not  $Orx_2R$  (white), are highly co-expressed with the glutamatergic cell marker

CamKII $\alpha$  (red; some of the observed colocalizations are indicated with solid green arrows = Orx<sub>1</sub>R<sup>+</sup> + CamKII $\alpha$ <sup>+</sup>, solid white arrow = Orx<sub>2</sub>R<sup>+</sup> + CamKII $\alpha$ <sup>+</sup>, and unfilled white arrows = Orx<sub>2</sub>R<sup>+</sup> + CamKII $\alpha$ <sup>-</sup>). (G) The number of BLA Orx<sub>1</sub>R<sup>+</sup> cells expressing CamKII $\alpha$  is over 60% while Orx<sub>2</sub>R<sup>+</sup> cells co-express the glutamatergic marker ~30% of the time, and about 50% of the small proportion of BLA cells that express both Orx<sub>1</sub>R and Orx<sub>2</sub>R also express CamKII $\alpha$ . (H & I) Expression of Orx<sub>2</sub>R (white) overlaps with GAD67 (red) more than Orx<sub>2</sub>R (green) in the BLA (a few observed colocalizations are identified with unfilled green arrows = Orx<sub>1</sub>R<sup>+</sup> + GAD67<sup>-</sup>, solid white arrow = Orx<sub>2</sub>R<sup>+</sup> + GAD67<sup>+</sup>, and unfilled white arrows = Orx<sub>2</sub>R<sup>+</sup> + GAD67<sup>-</sup>). (J) Analyses reveal Orx<sub>2</sub>R are expressed in GABA neurons in a greater proportion than Orx<sub>1</sub>R or cells that co-express Orx<sub>1</sub>R & Orx<sub>2</sub>R.  $-p \leq 0.05$  for comparisons to CamKII $\alpha$ <sup>-</sup>/GAD67<sup>-</sup> cells in the same receptor (Orx<sub>1</sub>R<sup>+</sup> or Orx<sub>2</sub>R<sup>+</sup>) group;  $+p \leq 0.05$  for comparisons to Orx<sub>1</sub>R<sup>+</sup> of the same CamKII $\alpha$ <sup>+</sup>/GAD67<sup>+</sup> profile;  $\#p \leq 0.05$  for comparisons to Orx<sub>2</sub>R<sup>+</sup> of the same CamKII $\alpha$ <sup>+</sup>/GAD67<sup>+</sup> profile. CeA = central amygdala; ITC = intercalated cells of the amygdala

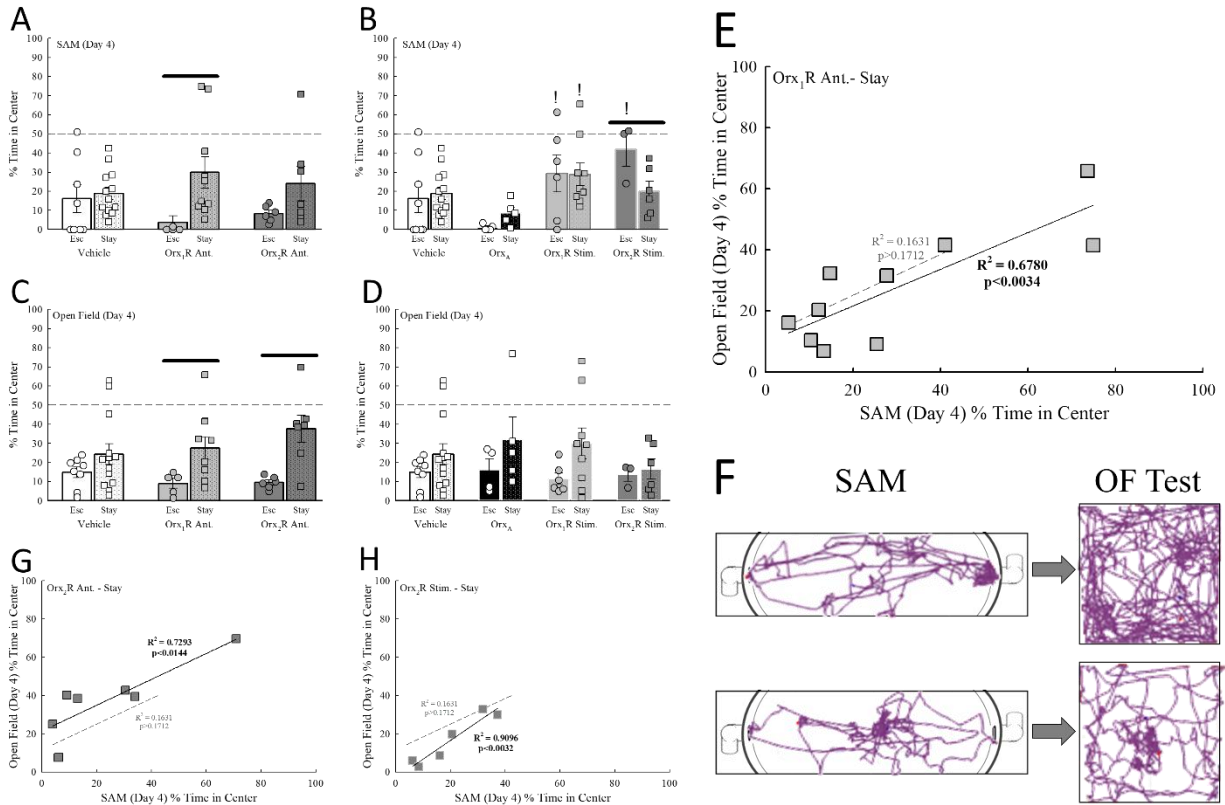


**Figure 3.** Socially induced freezing behavior in the SAM is transferred to the non-social OF Test in Stay mice. (A) Stay mice treated with intra-BLA infusion of an Orx<sub>2</sub>R antagonist, but not an Orx<sub>1</sub>R antagonist, exhibit enhanced freezing in the SAM. (B) Mice in Orx<sub>A</sub> and Orx<sub>1</sub>R stimulation groups exhibit enhanced freezing, while animals treated with an Orx<sub>2</sub>R agonist demonstrate significantly reduced freezing in the SAM. (C) Antagonism of Orx<sub>1</sub>R receptors in the BLA reduced generalized OF Test freezing in Escape mice only, while Orx<sub>2</sub>R antagonist treatment increased OF freezing in Stay animals. (D) Freezing in the OF Test was increased in Orx<sub>1</sub>R stimulation group mice, while intra-BLA agonism of Orx<sub>2</sub>R reduced freezing in both phenotypes. (E) In vehicle-treated control Stay mice, conflict freezing in the SAM is positively correlated to OF Test freezing ( $F_{1,11} = 8.7$ ,  $R^2 = 0.4423$ ,  $p \leq 0.0131$ ).  $-p \leq 0.05$  for comparisons between phenotypes in the same treatment group;  $+p \leq 0.05$  for comparisons to Vehicle-treated mice of the same phenotype;  $\#p \leq 0.05$  for comparisons to Orx<sub>1</sub>R Ant. group of the same phenotype;  $!p \leq 0.05$  for comparisons to Orx<sub>A</sub> treatment animals of the same phenotype;  $\%p \leq 0.05$  for comparisons to mice in the Orx<sub>1</sub>R stimulation group of the same phenotype.



**Figure 4.** Social stress-induced locomotion in SAM is generalized/transferred to a non-social OF Test environment after intra-BLA manipulation of Orx receptor activity in Escape animals. (A) Escape mice express higher locomotor activity compared to Stay animals during social stress in the SAM, but this phenotype difference is not observed after intra-BLA Orx<sub>1</sub>R antagonism. (B) Infusion of an Orx<sub>2</sub>R agonist into the BLA enhances locomotion in the SAM in Stay mice. (C) While Escape animals in the vehicle control group display higher locomotion compared to Stay mice in the OF Test, this divergent phenotype response is not observed after intra-BLA Orx<sub>1</sub>R or Orx<sub>2</sub>R antagonism. (D) Similarly, Orx<sub>A</sub> and Orx<sub>2</sub>R stimulation eliminates the difference in locomotion between Escape and Stay mice in the OF Test. (E) Unlike vehicle controls (gray dotted line represents linear regression line,  $F_{1,12} =$ ,  $R^2 = 0.0695$ ,  $p \geq 0.5281$ ), a significant positive relationship between SAM and OF Test locomotion is observed in Escape mice treated with an Orx<sub>1</sub>R antagonist ( $F_{2,3} = 15.9$ ,  $R^2 = 0.8413$ ,  $p \leq 0.0282$ ). (F) A significant negative correlation is revealed between SAM and OF Test locomotion in Escape mice treated with an Orx<sub>2</sub>R antagonist ( $F_{1,4} = 11.4$ ,  $R^2 = 0.7401$ ,  $p \leq 0.0279$ ). Like Orx<sub>1</sub>R antagonism, significant positive associations between locomotor activity in the SAM and OF Test for Escape mice treated with (G) Orx<sub>A</sub> ( $F_{2,2} = 49.4$ ,  $R^2 = 0.9611$ ,  $p \leq 0.02$ ) or (H) an Orx<sub>2</sub>R agonist ( $F_{2,1} = 351.5$ ,  $R^2 = 0.9972$ ,  $p \leq 0.034$ ).  $-p \leq 0.05$  for comparisons between phenotypes in the same treatment group;  $+p \leq 0.05$  for comparisons to Vehicle-treated mice of the same phenotype;  $!p \leq 0.05$  for comparisons to Orx<sub>A</sub> treatment animals of the

same phenotype;  ${}^{\%}p \leq 0.05$  for comparisons to mice in the Orx<sub>1</sub>R stimulation group of the same phenotype.



**Figure 5.** Treatments targeting Orx receptors in the BLA promote transfer learning from the SAM to the OF Test in Stay mice. (A) In the SAM, the amount of time spent in the center of the arena is not different between Escape and Stay animals in the vehicle control group, but phenotype divergence occurs after intra-BLA Orx<sub>1</sub>R antagonism with Stay mice spending more time in the center. (B) Escape mice treated with an Orx<sub>2</sub>R agonist display increased time in the center of the SAM arena. (C) While Escape and Stay vehicle-treated mice did not show differences in the amount of time spent in the center of the OF Test, both intra-BLA Orx<sub>1</sub>R antagonism and Orx<sub>2</sub>R antagonism prompted phenotype separation with Stay animals spending more time in the center of the OF. (D) No differences in time spent in the center of the OF Test were observed in Orx receptor stimulation groups. (E) Regression analysis revealed a significant and positive relationship ( $F_{1,8} = 16.8$ ,  $R^2 = 0.6780$ ,  $p \leq 0.0034$ ) between time spent in the center of the SAM and time spent in the center of the OF Test after intra-BLA Orx<sub>1</sub>R antagonism in Stay animals, but not in vehicle-treated Stay mice (dotted gray line represents regression line,  $F_{1,12} = 2.1$ ,  $R^2 = 0.1631$ ,  $p \geq 0.1712$ ). (F) Examples of tracking software maps for Orx<sub>1</sub>R antagonist-treated Stay mice

that prefer the edges of the SAM and OF Test (top) and those that bias the center regions (bottom). (*G*) Significant and positive correlations exist for time spent in the center of the SAM and time spent in the center of the OF Test for Stay mice treated with an Orx<sub>2</sub>R antagonist ( $F_{1,5} = 13.5$ ,  $R^2 = 0.7293$ ,  $p \leq 0.0144$ ) or (*H*) an Orx<sub>2</sub>R agonist ( $F_{1,4} = 40.2$ ,  $R^2 = 0.9096$ ,  $p \leq 0.0032$ ).  $\neg p \leq 0.05$  for comparisons between phenotypes in the same treatment group;  $!p \leq 0.05$  for comparisons to Vehicle-treated mice of the same phenotype.

## COMPLETE MATERIALS & METHODS

### Animals

Adult male C57BL/6NHsd mice (6-8 weeks old) weighing ~22-28 g were obtained from Envigo (Indianapolis, IN; N=194) and acclimated for a 5-day period in groups five, after which animals were singly housed in rooms held at 22°C and 35% relative humidity for the remainder of the experiments. Food and water were provided *ad libitum*. For studies involving pharmacological manipulations (N = 109), bilateral stereotaxic surgeries were performed where guide cannula (26 ga cut to 4.0 mm) were directed at the basolateral amygdala, or intra-BLA. A separate set of retired male breeder Hsd:ICR mice (CD1, N = 30) weighing ~50 g (Envigo) were individually housed, and used to initiate aggression in the Stress Alternatives Model (SAM; Fig. 1A).

Mice were subjected to a 12:12 light-dark cycle (with lights turning off at 6 p.m.), and behavioral experiments were performed during the animals' active phase (scotophase). Two days (48 h) after surgeries, test subjects (C57BL/6NHsd mice) were handled daily for 7 days before SAM exposure and behavioral testing the five proceeding days (Fig. 1B). All procedures (surgery and behavioral testing) were performed in a manner that minimized suffering. The number of animals used was 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.

### Stereotaxic Surgeries

Mice were anesthetized using isoflurane (2% at 1.0 L/min flow rate) before bilateral intra-BLA guide cannula (PlasticsOne, Roanoke, VA; 26 ga cut to 4.0 mm) implantation. Following surgery, mice were provided a recovery period (7 days) before behavioral testing. Cannula placement was performed using the following stereotaxic coordinates: -1.35 mm AP,  $\pm$  3.30 ML,



and -4.90 mm DV. During surgery and for ~45 minutes post-surgery, mice were kept on a warming pad to maintain core body temperature. Immediately following surgery and 24 hours after surgical procedures, mice were provided pain relief in the form of subcutaneous injections of the analgesic ketorolac (5 mg/kg).

### **Drugs & Drug Administration**

As several drugs were used to activate or inhibit intra-BLA Orx receptors, we broke the assessments into two broad categories: Orx receptor antagonist groups and Orx receptor stimulation groups. The Orx receptor antagonist groups consisted of mice treated with the Orx<sub>1</sub>R antagonist SB-674042 (N = 20; IC<sub>50</sub> = 3.76 nM for Orx<sub>1</sub>R; MedChemExpress, Monmouth Junction, NJ) and the Orx<sub>2</sub>R antagonist MK-1064 (N = 17; IC<sub>50</sub> = 0.5 nM for Orx<sub>2</sub>R; MedChemExpress). For Orx receptor stimulation groups, mice were administered Orx<sub>A</sub> (N = 13; EC<sub>50</sub> = 20 nM for Orx<sub>1</sub>R & Orx<sub>2</sub>R; ToCris, Minneapolis, MN), a concoction of Orx<sub>A</sub> & MK-1064 (N = 19; for biased Orx<sub>1</sub>R activation), or the Orx<sub>2</sub>R agonist YNT-185 (N = 12; EC<sub>50</sub> = 28 nM for Orx<sub>2</sub>R; Wako Chemicals, Richmond, VA). Drug effects were compared to vehicle-treated (N = 28; artificial cerebrospinal fluid; aCSF + 25% DMSO) control animals that underwent cannula implantation surgeries and were exposed to the same testing conditions and procedures as drug-treated mice. On Day 3 of the behavioral design (Fig. 1B), mice were infused bilaterally in the BLA (300 nL/side) with their designated treatment an hour before social interaction in the SAM.

All drug treatments were diluted using a 3:1 ratio of aCSF to dimethylsulfoxide (DMSO); and all treatments, excluding SB-674042 and YNT-185, were brought to a 0.1 nmol/0.3 µL concentration. The dose for the Orx<sub>2</sub>R antagonist, MK-1064, was 3x lower than previously used concentrations that produced anxiogenic effects when administered to the whole brain (intracerebroventricularly; icv) [1]. Similarly, the intra-BLA dose for Orx<sub>A</sub> was selected and

adjusted based on icv administrations that produced anxious behaviors in mice [2]. As the Orx<sub>1</sub>R antagonist SB-674042 and the Orx<sub>2</sub>R agonist YNT-185 have lower binding affinities compared to the Orx<sub>2</sub>R antagonist (MK-1064), we chose a slightly higher doses (0.3 nmol/0.3  $\mu$ L for SB-674042 and 10 nmol/0.3  $\mu$ L for YNT-185) in order to compensate for these differences.

Artificial cerebrospinal fluid (aCSF; 8.59 g NaCl, 0.201 g KCl, 0.279 g, CaCl<sub>2</sub>, 0.16 MgCl<sub>2</sub>, 0.124 g NaH<sub>2</sub>PO<sub>4</sub>, 0.199 g Na<sub>2</sub>HPO<sub>4</sub>/L H<sub>2</sub>O) was mixed and brought to a physiological pH (~7.33) using NaOH before being filtered, degassed, and stored at 4°C. Drugs were infused using injector cannula (33 ga cut to 4.9 mm, extending 0.9 mm below each guide cannula) placed into implanted guide cannulae, and injecting with a 1.0  $\mu$ L digital syringe (Model 7101 Zero Dead Volume, Knurled Hub 2.75", 22GA Needle; Hamilton Company, Reno, NV) at a rate of 0.5  $\mu$ L/min. After drug administration, the injector and syringe were left in place for 90 sec. Home cage mobility was measured briefly (~3 min) after SAM interaction on Day 3 in order to note changes in locomotion that resulted, not from social stress, but instead from drug interactions.

### **Behavioral Design**

Behavioral procedures were performed during the dark cycle when the animals are active, under red light (~700 nm  $\lambda$ ). Video cameras (GoPro Hero 3 & Hero 7) were used to record behavioral measures for later analyses. To assess conflict freezing during the SAM (Figs. 2A, B), mice from three separate cohorts (N = 73) were run through four days of the SAM. For pharmacological experiments (Figs. 3-5), animal groups included mice treated with vehicle (N=28, used for drug treatment comparisons), SB-674042 (Orx<sub>1</sub>R Ant.; N=20), MK-1064 (Orx<sub>2</sub>R Ant.; N=17), Orx<sub>A</sub> (N=13), Orx<sub>A</sub> + MK-1064 (Orx<sub>1</sub>R Stim.; N=19), and YNT-185 (Orx<sub>2</sub>R Stim.; N = 12).

Each treatment group was subjected to 4 days of social stress in the SAM with intra-BLA drug administration occurring on Day 3 an hour before SAM exposure (Fig. 1A, B). Day 4 of the experimental design includes SAM social interaction followed immediately by Open Field (OF) testing (Fig. 1B). On the final day (Day 5), mice were tested in the Elevated Plus Maze (EPM; Fig. 1B). At the end of testing on Day 5, mice were briefly anesthetized using isoflurane (5% at 1.0 min/L for ~2 min) and rapidly decapitated. Whole brains were collected, stored at -80°C, and sectioned to discern accurate placement of cannula and injections into the BLA. Only animals in which the BLA was successfully targeted bilaterally (82 mice out of 109 total; Vehicle = 21 [8 Escape & 13 Stay]; Orx<sub>1</sub>R Ant. = 15 [5 Escape & 10 Stay]; Orx<sub>2</sub>R Ant. = 13 [6 Escape & 7 Stay]; Orx<sub>A</sub> = 9 [4 Escape & 5 Stay]; Orx<sub>1</sub>R Stim. = 15 [6 Escape & 9 Stay]; Orx<sub>2</sub>R Stim. = 9 [3 Escape & 6 Stay]) were used for behavioral analyses (Fig. S1)

### **Stress Alternatives Model (SAM)**

The Stress Alternatives Model (SAM) (Fig. 1A) includes the use of a white box (91 cm x 22 cm x 30 cm) with two concave dividers (r = 10.25 cm). In this way, the SAM apparatus consists of three parts, which include an oval open field area (length = 71 cm, width = 22 cm, height = 30 cm) and two enclosed (safe) areas (10 cm x 22 cm x 30 cm) which are accessible only to smaller C57BL/6N mice that choose to leave the open field area via provided escape tunnels (Fig. 1A). At the beginning of the SAM paradigm, an opaque cylinder (diameter = 15 cm, width = 20 cm) is placed in the center of the open field and an aggressive CD1 is positioned outside the cylinder in the open arena of the SAM. A test mouse (C57BL/6N) is placed into the cylinder and subjected to a fear conditioning paradigm, consisting sequentially of a 30 sec acclimation period, a 5 second tone (2500 Hz at 75 dB; conditioned stimulus), a 10 second post-tone trace period,

and finally removal of the cylinder resulting in aggressive social interaction with the CD1 (unconditioned stimulus) for a 5 min period.

During the first day of SAM exposure, the open field arena and the two escape routes on both ends are novel, but over the course of four days, mice are allowed to choose to utilize the escape route or remain in the SAM arena with the CD1 aggressor. Importantly, each test mouse encounters a different and unfamiliar CD1 aggressor throughout the 4-day SAM paradigm. By the end of Day 2 of the SAM, test mice will commit to one of two behavioral phenotypes that will be consistent for the remaining days (Days 3 & 4) of the SAM protocol: mice that choose to utilize the escape tunnels (escaping social aggression) are categorized as expressing the Escape phenotype, and those that remain in the SAM arena and submit to social aggression express the Stay phenotype (Fig. 1A). Earlier studies from our lab revealed that Escape mice exhibit significantly reduced physiological and behavioral measurements of stress when compared to Stay mice [1, 3-5]; however, both phenotypes receive equally high levels of social aggression from CD1 mice.

At the end of 5-minute interactions in the SAM, both mice (test mice and CD1 mice) were removed from the apparatus and placed into their home cages. When a test mouse escaped, they were left in the enclosed area for the remainder of the 5 minutes, with a clear perforated sheet of plastic being placed in front of the escape route. SAM interactions were recorded (GoPro Hero 3 & Hero 7) and analyzed using ANY-maze Video Tracking Software (Version 6.0, Stoelting Co., Wood Dale, IL). Between SAM trials, the apparatus was cleaned using 70 % ethanol, disinfectant wipes, and dried using clean paper towels.

Animals were separated into phenotype groups after the first two days of the SAM: Escape if they utilized the escape route on Days 1 and/or 2 and Stay if the mouse did not utilize the escape

tunnel by the end of Day 2. For pharmacological studies, animals within a given phenotype group were randomly assigned to receive treatments (vehicle, Orx<sub>1</sub>R Ant., Orx<sub>2</sub>R Ant., Orx<sub>A</sub>, Orx<sub>1</sub>R Stim., or Orx<sub>2</sub>R Stim.) on Day 3. Behavioral measures on Day 4 (24 hours after drug administration) were analyzed, including conflict freezing, time in the center of the SAM arena, and social stress-induced locomotion.

Conflict freezing describes motionless behavior in the presence of the social aggressor, and is defined as bouts of immobility, excluding normal breathing behavior, for one second or longer during social interaction in the SAM [1] or OF Test. Conflict freezing includes freezing in anticipation or in response to aggression, as well as contextual freezing in response to being in the SAM open field where social aggression previously took place or the OF Test during transference.

Time spent in the center of the SAM was defined as the amount of total time each mouse spent inside the center of the SAM (48 cm x 9 cm). As this measure is often used as an indicator of stress in the OF Test [6], we sought to compare time spent in the center of the SAM open field to traditional OF testing. We further explored how this behavior may change with phenotype expression: Stay and Escape.

Locomotor activity was measured in the SAM to compare social stress-induced locomotion to that occurring subsequently in the OF Test, in which no social aggressor was present. Home cage locomotion was also measured on Day 3 to assess whether behavioral measurements of locomotor activity in the SAM and OF Test were not simply drug-induced responses.

Locomotion in the SAM, OF Test, and Home cage Mobility was calculated taking the total distance the mice traveled and normalizing it to the amount of time spent in the SAM, OF, or Home cage environment (cm/s).

## **Validation of the SAM**

As the SAM is a resource for describing anxious and depressive behaviors, it has been subjected to validation testing, using criteria for Construct, Predictive, and Face Validities [7].

Incorporating aspects of social defeat and measures of active avoidance, the SAM construct explores behavioral fear and anxiety [8-11], depression and stress [12, 13], but further the mitigation of these stress-induced products via Escape, while being both ethologically and ecologically applicable [3, 14, 15] and preserving comparisons to pertinent human disorders [16, 17], suggesting a degree of Construct Validity. In the SAM, predictive validation has been confirmed through the induction of behavioral alterations, including the reversal of phenotypes, using antidepressive, anxiolytic, or anxiogenic drugs (antalarmin, NPS, and yohimbine) [4, 5, 18]. Further, the SAM has been used in combination with, and produces analogous results to, the Social Interaction/Preference (SIP) Test [1, 19], which has been validated as translationally and predictively reliable in representing the efficacy of pharmacotherapies used to treat anxiety (benzodiazepines) and depression (SSRIs) [20-24]. Additionally, raised glucocorticoid concentrations exhibit an enhanced physiological stress response in animals facing social aggression in the SAM, where Stay mice present the greatest increase [1, 3-5, 18, 25]. As far as Face Validity, SAM exposure results in behavioral effects, largely examples of behavioral inhibition (FC freezing, Conflict freezing), startle (Conflict Startle), and social avoidance (Escape, Escape Latency, and SIP test), that imitate those seen in human depression and anxiety.

## **Open Field (OF) Test**

On Day 4, after being subjected to 5 min of social aggression in the SAM, the OF Test [6] was conducted in an opaque, white square box (40 cm<sup>3</sup>), under red lighting (Fig. 1B). Test mice (C57BL/6N) were placed in the center of the arena at the beginning of testing and allowed to

explore the open field area for 5 min while being video recorded (GoPro Hero 3 & Hero 7). Videos were used to perform behavioral analyses (ANY-maze 6.0). Analyzed behaviors included freezing time (as defined and measured in the SAM), time spent in the center (20 cm<sup>2</sup>) of the apparatus, and locomotion (as defined and measured in the SAM and Home cage mobility assessment). Between each trial, the arena was cleaned using 70% ethanol, disinfectant wipes, and dried thoroughly with clean paper towels.

The OF Test has long been used as a model for anxiety in animal studies, specifically to determine how treatments alter anxious behavior [6]. Behavioral analysis in the OF began as soon as the animal was placed in the center of the arena, and continued for 5 min. Time spent in the center of the OF arena (20 cm<sup>2</sup>) and time spent around the edges of the arena were recorded, as more time spent in the center of the OF arena is typically interpreted as an animal experiencing decreased anxious behavior, and more time around the border as an indication of increased anxious behavior. For these experiments, it is important to note that we compared the OF behavior of this classic OF Test with SAM OF behavior.

### **Elevated Plus Maze (EPM)**

On Day 5, test mice (C57BL/6N) were exposed to the EPM [26], one day after SAM social interactions (Fig. 1B). The EPM apparatus includes an elevated (74 cm tall) plus-shaped platform with two open arms (50.5 cm x 10.5 cm x 1.5 cm edge height), two closed arms (50.5 cm x 10.5 cm x 40 cm wall height), and an intersection zone (10.5 cm<sup>2</sup>). Under red light, all animals were placed in the center of the EPM, with their heads facing a corner between an open and closed arm to avoid bias toward a specific arm. Trials were video recorded (GoPro Hero 3 & Hero 7) and behavioral measurements were scored by individuals blind to the treatment

groups. Between all trials, the arena was cleaned sequentially using 70% ethanol, disinfectant wipes, and thoroughly dried with paper towels.

Once mice were placed on the EPM apparatus, behavior was analyzed for 5 min. Time spent in the open and closed arms of the EPM were measured, as they are typically thought to reflect the anxious state of an animal. Increased time spent on the open arms is associated with less anxiety, while increased time spent on the closed arms reflects more generalized anxious behavior [26, 27]. Total time spent in intersection zone was also recorded. The results for a single animal in the Orx<sub>1</sub>R Ant. Stay group is not included in the reported results as there was a camera malfunction during behavioral testing and no video was available for analysis.

### **RNA Extraction**

Fresh frozen brains of mice exposed to the 4-day SAM paradigm and cage control animals not exposed to social stress (N = 30 Total; N = 6 Cage Controls, N = 10 Escape mice, & N = 14 Stay mice) were cut (Leica Biosystems, Buffalo Grove, IL; Leica CM1850 Clinical Cryostat, Cat. No. 047131148) into 200  $\mu$ m sections. Microdissection of the BLA (AP -1.00 mm to -2.45 mm relative to Bregma) was performed on a cold plate using 25 GA punches (Stoelting Co., Wood Dale, IL; Brain Punch Set, 0.25 to 1.25 mm; Cat. No. 57401) and submerged into 500  $\mu$ L of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA; Invitrogen TRIzol Reagent, Cat. No. 15-596-018).

The TRIzol method as described previously [28] was used with some modifications. In short, incubation of BLA tissue in 500  $\mu$ L Trizol at room temperature took place for 5 min followed by phase separation through the addition of 100  $\mu$ L of 1-bromo-3-chloropropane and centrifugation (4°C, 7,500xg, 15 min). The topmost aqueous layer was collected and RNA precipitation through the addition of 250  $\mu$ L of isopropanol and 1  $\mu$ L glycol blue (Thermo Fisher



Scientific, Waltham, MA; GlycoBlue Coprecipitant, Cat. No. AM9516) followed by centrifugation (4°C, 12,000xg, 20 min) formed a visible RNA pellet. The excess alcohol around the pellet was removed using a pipette, and the remaining RNA was washed with 75% ethanol before being centrifuged (4°C, 16,000xg, 5 min). The majority of the ethanol was collected and discarded and the samples were positioned in a hot plate oven set to 60°C for 20-40 min to evaporate the remaining ethanol. A concentrated RNA sample was created by adding 25 µL RNase-free water before quantification using a nanodrop (Implen Inc., Westlake Village, CA; Nanophotometer N50 Spectrometer). Sample aliquots were diluted to 20 ng/µL and stored at -80°C for later PCR analyses.

### **Quantitative Reverse Transcription PCR (qRT-PCR)**

Purchased assays (Thermo Fisher Scientific, Waltham, MA) for the PCR analyses included *HCRTR1* (4351370, Mm01185776\_m1), *HCRTR2* (4351370, Mm01179312\_m1), and *GAPDH* (4453320, Mm99999915\_g1) as the housekeeping gene. A master mix for each PCR target was created using a one-step qRT-PCR kit (Cat. No. 4392653) before being mixed with RNA samples from BLA tissue in individual PCR tubes (MIDSCI, Valley Park, MO; Pryme Ergonomic PCR Tubes; Cat. No. B77201). The PCR tubes were then loaded into Applied Biosystems QuantStudio 3 No. B77201 thermal cycler (Thermo Fisher Scientific, Waltham, MA; Cat. No. A28131) and, as per Taqman Assay vendor recommendations, were run through 40 cycles at the following conditions: reverse transcription (48°C, 15 min), DNA polymerase activation (95°C, 10 min), denaturation (95°C, 15 sec), and annealing & extension (60°C, 1 min).

No enzyme and no template control PCR sample tubes were created to rule out the possibility of contamination during PCR runs. Individual samples from non-stressed cage control mice (N = 6), Escape mice (N = 10), and Stay mice (N = 14) were used for PCR analysis. Duplicates for

each sample were run and the average Ct value was subtracted from the average housekeeping gene (GAPDH) Ct to give the  $\Delta$ Ct for analysis. Determination of relative gene expression levels was made using the  $2^{-\Delta\Delta Ct}$  method [29], which was then compared to the average  $\Delta$ Ct of the non-stressed cage controls. Regression curves were made for these data where average fold change is correlated to SAM freezing behavior.

### ***In situ* Hybridization (RNAscope)**

Fresh frozen brains (N = 12) of C57BL/6NHsd mice (9-10 weeks old) not exposed to social stress or behavioral testing were sectioned into 20  $\mu$ m coronal sections and positioned on slides (Fisher Scientific, Pittsburgh, PA; Superfrost Plus, Cat. No. 12-550-15). Tissue that incorporated the BLA from AP -1.50 to -1.80 relative to bregma was incubated in cold (4°C) 10% formalin for 20 min and then washed (2x for 1 min) in 1x phosphate buffer solution (PBS). Dehydration of tissue was performed by sequentially washing the sections in ethanol (50%, 70%, and 100%; 5 min each) followed by a final ethanol (100%) wash overnight in a -20°C freezer.

Proteins were digested in the tissue sections the next day with a protease treatment before being rinsed in distilled H<sub>2</sub>O. Bathing of tissue in RNAscope (Advanced Cell Diagnostics, Newark, CA) probes (*HCRTR1*, Cat. No. 466631; *HCRTR2*, Cat. No. 581631; *GAD1*, Cat. No. 400951; *CAMKII $\alpha$* , Cat. No. 445231; *PVALB*, Cat. No. 421931) took place at 40°C for 2 h in a specially designed hybridization oven (ACD HybEZ II oven, Cat. No. 321711). Next, sequential washes (RNAscope Wash Buffer Reagents [310091]: Wash Buffer 50x diluted to 1x) and bathing with amplification buffers (RNAscope Fluorescent Multiplex Detection Reagents [320851]: AMP1 [320852], AMP2 [320853], AMP3 [320854], AMP4 ALT A [320855], AMP4 ALT B [320856]) was performed to bind fluorophores and enhance the signaling of target mRNA. Lastly, the sections were stained with DAPI (20 sec) and a mounting medium (Fisher

Scientific; Prolong Gold Antifade Mountant, Cat. No. P10144) before being coverslipped and stored at 4°C in the dark until imaging.

Section visualization and image acquisition were performed using a confocal microscope (Nikon NIE) and camera (Photometrics CoolSNAP MYO camera). Areas of interest were selected from images and analyzed and counted for fluorescence using ImageJ software. The colocalization of fluorescence-tagged mRNA were identified as overlap of signal or as puncta of different fluorescence clustering on the same DAPI signaling, which would suggest that the mRNA expression is in a single cell.

### **Statistical Analyses**

Statistical analyses and experimental designs were based on *a priori* hypotheses. Two-way ANOVA (Orx receptor targeting drug x Phenotype design) was used to examine the contribution of drug effects relative to behavioral phenotype expression (Stay x Escape). Regression analyses were used to investigate correlations of gene expression (*HCRTR1* & *HCRTR2*) and SAM conflict freezing responses. Further, regression analyses were used to identify associations between SAM-dependent behavioral responses (Day 4) and OF Test behaviors (Day 4). Evaluations of locomotor activity in the home cage after drug treatment were assessed by one-way ANOVA. Comparisons between two treatments (Vehicle, Orx<sub>1</sub>R Ant., Orx<sub>2</sub>R Ant., Orx<sub>A</sub>, Orx<sub>1</sub>R Stim, or Orx<sub>2</sub>R Stim.) within a given phenotype (Escape or Stay) were investigated by Student's t-tests.

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 also compared to non-parametric analyses, and graphed in their raw form. Analyses with both non-parametric and parametric statistics 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  $\alpha$  adjustment [30-35]. 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).

## COMPLETE RESULTS

### **Socially induced conflict freezing response is positively correlated with BLA**

#### **transcriptional changes in Orx<sub>1</sub>R and negatively associated with changes in Orx<sub>2</sub>R**

We previously demonstrated that Orx<sub>1</sub>R and Orx<sub>2</sub>R activity in the BLA bidirectionally effects stress responsivity [25, 36]. To understand how these receptors in the basolateral amygdala (BLA) might influence stress-sensitive phenotype development and learning, we first assessed the relationship between Orx receptor transcription (*HCRTR1* & *HCRTR2*) levels and conflict freezing (Figs. 2A-C). In the Stress Alternatives Model (SAM; Fig. 1A), social stress-induced conflict freezing is an indicator of fear-based behavioral inhibition and is most pronounced on Day 4 of the SAM paradigm [36], where Stay mice express significantly elevated freezing behavior (individual results combined from 3 cohorts: Phenotype Effect:  $F_{1,168} = 4.8, p \leq 0.033$ ; Time Effect:  $F_{3,168} = 4.2, p \leq 0.007$ ; Interaction Effect:  $F_{3,168} = 4.1, p \leq 0.008$ ; Day 4 Escape vs Stay:  $t_{56} = 3.825, p < 0.001$ ) compared to Escape animals (Fig. 2A). Relative transcription levels of Orx<sub>1</sub>R (*HCRTR1*) in Escape, but not Stay, mice were positively associated with Day 4 freezing behavior in the SAM (Figs. 2B, S2A; Escape:  $F_{1,8} = 7.8, p \leq 0.0233$ ; Stay:  $F_{1,12} = 0.3, p \geq 0.6095$ ). Alternatively, Orx<sub>2</sub>R (*HCRTR2*) mRNA levels in the BLA were negatively correlated with SAM freezing behavior in only Stay mice (Figs. 2C, S2B; Stay:  $F_{1,12} = 9.7416, p \leq 0.0088$ ; Escape:  $F_{1,8} = 1.3, p \geq 0.2840$ ). These results indicate that Orx receptor activity in the

BLA may be important for stress-related fear- and learning-based behaviors, but these associations are closely linked to an animal's stress responsive state (Escape or Stay).

**In the BLA, Orx<sub>1</sub>R is primarily expressed on glutamatergic neurons and Orx<sub>2</sub>R on both glutamatergic and GABAergic cells**

In the BLA, approximately 25% of cells express Orx<sub>1</sub>R, while less than 20% contain Orx<sub>2</sub>R and even fewer (< 3%) co-express both Orx<sub>1</sub>R and Orx<sub>2</sub>R (Fig. 2D;  $F_{3,44} = 134.0, p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>2</sub>R<sup>+</sup>,  $t_{22} = 2.1, p \leq 0.050$ ; Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>,  $t_{22} = 7.0, p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Other,  $t_{22} = 9.6, p < 0.001$ ; Orx<sub>2</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>,  $t_{22} = 7.9, p < 0.001$ ; Orx<sub>2</sub>R<sup>+</sup> vs Other,  $t_{22} = 14.1, p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup> vs Other,  $t_{22} = 21.8, p < 0.001$ ). To consider how Orx receptors in BLA microcircuits influence behavioral states, we identified the relative relationships of Orx<sub>1</sub>R (*HCRTR1*) and Orx<sub>2</sub>R (*HCRTR2*) expression in CamKII $\alpha$ <sup>+</sup> (glutamatergic) and GAD67<sup>+</sup> (GABAergic) neurons. In the brain sections we sampled, ~70% of cells were CamKII $\alpha$ <sup>+</sup> and ~20% expressed the GABA marker GAD67 (Fig. S5D;  $t_9 = 16.8, p < 0.001$ ). We have previously shown that Orx<sub>1</sub>R in the BLA are predominantly located in glutamatergic neurons that do not express Orx<sub>2</sub>R [25].

To assess the cellular location of Orx<sub>2</sub>R and differences of Orx receptors in excitatory or inhibitory neurons, we used RNAscope *in situ* hybridization techniques, and evaluated proportional differences in Orx receptor-containing cells with respect to CamKII $\alpha$  expression (Figs. 2D-I, S3, S5). While >60% of Orx<sub>1</sub>R<sup>+</sup> cells co-expressed CamKII $\alpha$  (Receptor Effect:  $F_{2,30} = 0.0, p \geq 0.9$ ; CamKII $\alpha$  Expression Effect:  $F_{1,30} = 0.7, p \geq 0.398$ ; Interaction Effect:  $F_{2,30} = 37.4, p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> CamKII $\alpha$ <sup>+</sup> vs CamKII $\alpha$ <sup>-</sup>:  $t_{10} = 6.4, p < 0.001$ ), less than 40% of Orx<sub>2</sub>R<sup>+</sup> cells were colocalized with the glutamatergic marker (Orx<sub>2</sub>R<sup>+</sup> CamKII $\alpha$ <sup>+</sup> vs CamKII $\alpha$ <sup>-</sup>:  $t_{10} = 5.8, p < 0.001$ ), and ~51% of cells that colocalize both Orx receptors are glutamatergic (Fig. 2F).

Further, the amount of  $\text{CamKII}\alpha^+$  neurons that express  $\text{Orx}_2\text{R}$  were significantly lower than those that produce  $\text{Orx}_1\text{R}$  ( $t_{10} = 5.9, p < 0.001$ ) or both receptor subtypes (Fig. 2G;  $t_{10} = 3.1, p \leq 0.004$ ). Alternatively, a greater proportion of  $\text{Orx}_2\text{R}^+$  cells do not express  $\text{CamKII}\alpha$ , while less than 40% of  $\text{Orx}_1\text{R}^+$  cells and under 50% of cells expressing both receptors do not house the glutamate marker (Fig. 2G;  $\text{CamKII}\alpha^- \text{Orx}_1\text{R}^+$  vs  $\text{Orx}_2\text{R}^+$ :  $t_{10} = 6.3, p < 0.001$ ;  $\text{CamKII}\alpha^- \text{Orx}_2\text{R}^+$  vs  $\text{Orx}_1\text{R}^+ \& \text{Orx}_2\text{R}^+$ :  $t_{10} = 3.5, p \leq 0.002$ ;  $\text{CamKII}\alpha^- \text{Orx}_1\text{R}^+$  vs  $\text{Orx}_1\text{R}^+ \& \text{Orx}_2\text{R}^+$ :  $t_{10} = 2.8, p \leq 0.009$ ).

We next compared the proportion of Orx receptors with respect to GABA cells in the BLA, which express the glutamate decarboxylase gene (*GAD1*) for GAD67 (Figs. 2H-J, S4, S5; Receptor Effect:  $F_{2,24} = 0.0, p = 1.0$ ; GAD67 Expression Effect:  $F_{1,24} = 322.9, p < 0.001$ ; Interaction Effect:  $F_{2,24} = 73.3, p < 0.001$ ). Less than 20% of  $\text{Orx}_1\text{R}^+$  cells are GABAergic, which is significantly less than the number of  $\text{Orx}_1\text{R}^+$  cells that do not contain GAD67 ( $t_8 = 18.0, p < 0.001$ ) and less than  $\text{Orx}_2\text{R}^+$  cells that also express the GABA marker ( $t_8 = 8.4, p < 0.001$ ) or both receptor subtypes (Fig. J2;  $t_8 = 2.9, p \leq 0.008$ ). The number of  $\text{Orx}_2\text{R}^+$  BLA cells that express GAD67 is not significantly lower than those that do not express this GABA marker ( $t_8 = 1.3, p \geq 0.206$ ), as nearly 50% of  $\text{Orx}_2\text{R}^+$  cells produce GABA (Fig. 2J). Of the small number of BLA neurons that co-express both Orx receptors, most (~70%) are not GABAergic (Fig. 2J;  $\text{Orx}_1\text{R}^+ \& \text{Orx}_2\text{R}^+ \text{GAD67}^+$  vs  $\text{GAD67}^-$ :  $t_8 = 12.3, p < 0.001$ ;  $\text{GAD67}^- \text{Orx}_1\text{R}^+$  vs  $\text{Orx}_1\text{R}^+ \& \text{Orx}_2\text{R}^+$ :  $t_8 = 2.9, p \leq 0.008$ ;  $\text{GAD67}^- \text{Orx}_2\text{R}^+$  vs  $\text{Orx}_1\text{R}^+ \& \text{Orx}_2\text{R}^+$ :  $t_8 = 5.5, p < 0.001$ ).

We had previously predicted that the calcium-binding protein, parvalbumin (PV), which acts as a marker for a specific subpopulation of GABAergic neurons, might be important for Orx receptor activity in the BLA [1, 36], so we also looked for distributions of Orx receptors within

this specific type of neuron (Fig. S6; Receptor Effect:  $F_{2,18} = 0.0$ ,  $p = 1.0$ ; PV Expression Effect:  $F_{1,18} = 705.0$ ,  $p < 0.001$ ; Interaction Effect:  $F_{2,18} = 1.2$ ,  $p \geq 0.329$ ). All Orx receptor-expressing cells co-expressed PV at very low levels (Fig. S6F; Orx<sub>1</sub>R<sup>+</sup> PV<sup>+</sup> vs PV<sup>-</sup>:  $t_6 = 22.4$ ,  $p < 0.001$ ; Orx<sub>2</sub>R<sup>+</sup> PV<sup>+</sup> vs PV<sup>-</sup>:  $t_6 = 16.1$ ,  $p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup> PV<sup>+</sup> vs PV<sup>-</sup>:  $t_6 = 11.5$ ,  $p < 0.001$ ). Interestingly, there appears to be a topographical organization of Orx<sub>1</sub>R in the BLA, with the greatest expression being in the medial portion and very little in the lateral-most section of this amygdalar region (Fig. S5A). The distribution of Orx<sub>2</sub>R in the BLA seems to be less organized (Fig. S5B).

In order to fully consider the influence of Orx receptor activity on BLA neurons, we further assessed the proportion of CamKII $\alpha$ <sup>+</sup> or GAD67<sup>+</sup> BLA cells that expressed the genes for the Orx receptors (Figs. S5D-F). In the brain sections we sampled, over 15% of glutamatergic neurons also expressed Orx<sub>1</sub>R, while less than 10% expressed Orx<sub>2</sub>R, and under 5% co-expressed Orx<sub>1</sub>R and Orx<sub>2</sub>R (Fig. S5D;  $F_{2,15} = 84.6$ ,  $p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>2</sub>R<sup>+</sup>:  $t_{10} = 8.2$ ,  $p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_{10} = 12.8$ ,  $p < 0.001$ ; Orx<sub>2</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_{10} = 5.8$ ,  $p < 0.001$ ). Alternatively, very few GABAergic neurons express both Orx receptors and less than 10% express Orx<sub>1</sub>R; however, ~25% of GAD67<sup>+</sup> neurons co-express Orx<sub>2</sub>R (Fig. S5E;  $F_{2,12} = 84.0$ ,  $p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 9.7$ ,  $p < 0.001$ ; Orx<sub>1</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 2.6$ ,  $p \leq 0.023$ ; Orx<sub>2</sub>R<sup>+</sup> vs Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>:  $t_8 = 12.3$ ,  $p < 0.001$ ). These data suggest while the number of cells expressing Orx receptors in the BLA is relatively small (Fig. 2D), ~16.25% of BLA cells house Orx<sub>1</sub>R and are glutamatergic and ~7.5% of BLA cells are GABAergic and express Orx<sub>2</sub>R (Fig. S5F). Collectively, these results propose Orx<sub>1</sub>R activity influences BLA signaling primarily through glutamatergic pyramidal neurons while Orx<sub>2</sub>R receptors activate, through a small majority, inhibitory interneurons in BLA microcircuits.

### **Stress-induced freezing is ameliorated with acute intra-BLA Orx<sub>2</sub>R stimulation**

In the SAM, longer periods of freezing are observed in Stay mice compared to Escape mice, and this freezing behavior intensifies over the 4-day SAM paradigm [1], where we see the greatest difference on Day 4 (Fig. 2A). We have previously demonstrated that single treatments before Day 3 of SAM interaction, including drugs that target the Orx system, produce longer lasting behavioral changes observed on Days 4 and 5 [1, 5, 25]. Here, we compared freezing behavior on the last day of SAM exposure (Day 4) to freezing in the novel OF Test environment (also Day 4) where there is no social aggressor, to see if stress exposure leads to fear-related generalization in a non-threatening environment. As reported previously [1] Stay mice experienced periods of freezing in the SAM that were significantly increased relative to Escape mice under the control conditions of vehicle treatment (Figs. 3A, B, white bars;  $t_{19} = 2.67, p \leq 0.015$ ).

Since Orx<sub>1</sub>R and Orx<sub>2</sub>R gene expression in the BLA are related to socially induced conflict freezing behavior in a phenotype-dependent fashion (Figs. 2B, C), we assessed the influence of pharmacologically inhibiting intra-BLA Orx receptors on freezing during conflict behavior in the SAM (Fig. 3A; Drug Effect,  $F_{2,43} = 1.1, p \geq 0.339$ ; Phenotype Effect,  $F_{1,43} = 16.0, p < 0.001$ ; Interaction Effect,  $F_{2,43} = 2.2, p \geq 0.126$ ). Treatment with an Orx<sub>1</sub>R antagonist (SB-674042) eliminated differences in conflict freezing between Escape and Stay mice ( $t_{13} = 0.9, p \geq 0.365$ ), but did not result in significant differences compared to vehicle-treated controls (Fig. 3A, light gray bars; Escape: Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{11} = 0.001, p \geq 0.999$ ; Stay: Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{21} = 1.3, p \geq 0.212$ ). Antagonizing Orx<sub>2</sub>R (MK-1064) resulted in an increase in freezing behavior in Stay mice compared to vehicle-treated controls, though only significant at the  $p \leq 0.080$  level (Fig. 3A,  $t_{18} = 1.9$ ). Compared to Orx<sub>1</sub>R antagonist-treated mice, however, Stay



animals in the Orx<sub>2</sub>R antagonist group exhibited elevated freezing behavior (Fig. 3A;  $t_{15} = 2.8, p \leq 0.014$ ).

To further assess the role of Orx receptors in the BLA on stress-induced conflict freezing behavior, we pharmacologically stimulated intra-BLA Orx receptors (Fig. 3B; Drug Effect,  $F_{3,46} = 17.1, p < 0.001$ ; Phenotype Effect,  $F_{1,46} = 9.1, p \leq 0.004$ ; Interaction Effect,  $F_{3,47} = 0.7, p \geq 0.561$ ). Treatment with Orx<sub>A</sub>, which stimulates both Orx<sub>1</sub>R and Orx<sub>2</sub>R, significantly increased freezing behavior in both Escape ( $t_{10} = 3.4, p \leq 0.007$ ) and Stay ( $t_{16} = 3.3, p \leq 0.005$ ) relative to vehicle-treated control animals of the corresponding phenotype (Fig. 3B, black bars). While biased stimulation of Orx<sub>1</sub>R (accomplished through a drug mixture of Orx<sub>A</sub> and the Orx<sub>2</sub>R antagonist MK-1064) resulted in enhanced freezing in both Escape and Stay mice, only freezing in Stay animals reached statistical significance compared to vehicle-treated controls (Fig. 3B, light gray bars with dark gray outline;  $t_{21} = 3.0, p \leq 0.008$ ). Alternatively, agonizing Orx<sub>2</sub>R (YNT-185) reduced stress-induced conflict freezing in Escape mice compared to those treated with vehicle ( $t_9 = 2.3, p \leq 0.047$ ) and Orx<sub>A</sub> ( $t_5 = 3.2, p \leq 0.023$ ). In Stay animals, intra-BLA Orx<sub>2</sub>R stimulation reduced freezing behavior (Fig. 3B, dark gray bars with light gray outline) statistically below mice treated with vehicle ( $t_{17} = 3.3, p \leq 0.004$ ), Orx<sub>A</sub> ( $t_9 = 8.7, p < 0.001$ ), and Orx<sub>1</sub>R stimulation ( $t_{13} = 8.5, p < 0.001$ ). Together, these results suggest learned, phenotype-dependent, freezing behavior is mediated and generalized through Orx receptor activity in the BLA.

### **Stress-induced freezing in the SAM is transferred to the OF Test**

Following four days of social aggression exposure, mice were subjected to the Open Field (OF) Test (Fig. 1B) to determine how SAM-established behavioral phenotypes and Orx treatments would impact behavior in a novel, anxiety-producing environment. As the OF Test was

performed immediately following SAM social interaction on Day 4 of the behavioral proceedings (Fig. 1B), we first assessed freezing behavior in the OF Test that may be a result of fear-induced generalization (Figs. 3C, D). While there were no differences in freezing between phenotypes in vehicle- and Orx<sub>1</sub>R antagonist-treated groups (Drug Effect,  $F_{2,43} = 4.1, p \leq 0.024$ ; Phenotype Effect,  $F_{1,43} = 12.1, p < 0.001$ ; Interaction Effect,  $F_{2,43} = 2.1, p \geq 0.139$ ; Vehicle: Escape vs Stay,  $t_{20} = 1.0, p \geq 0.320$ ; Orx<sub>1</sub>R Ant.: Escape vs Stay,  $t_{13} = 1.8, p \geq 0.093$ ), Escape mice that underwent acute Orx<sub>1</sub>R antagonism exhibited reduced freezing in the OF Test compared to those mice administered vehicle treatment (Fig. 3C;  $t_{11} = 2.637, p \leq 0.023$ ). As predicted, intra-BLA Orx<sub>2</sub>R inhibition elevated OF Test freezing behavior relative to Escape mice that underwent the same treatment (Fig. 3C;  $t_{11} = 2.5, p \leq 0.029$ ). Further, these Stay mice that were administered an Orx<sub>2</sub>R antagonist experienced increased freezing in the OF Test compared to vehicle- ( $t_{19} = 3.0, p \leq 0.008$ ) and Orx<sub>1</sub>R Ant.-treated mice (Fig. 3C;  $t_{15} = 3.1, p \leq 0.008$ ).

In intra-BLA Orx receptor stimulation studies, Orx<sub>A</sub> treatment did not result in a change in freezing behavior in the OF Test that was different from vehicle-treated controls (Fig. 3D; Drug Effect,  $F_{3,46} = 3.2, p \leq 0.033$ ; Phenotype Effect,  $F_{1,47} = 12.0, p < 0.001$ ; Interaction Effect,  $F_{3,47} = 1.5, p \geq 0.217$ ; Escape: Vehicle vs Orx<sub>A</sub>,  $t_{10} = 0.2, p \geq 0.814$ ; Stay: Vehicle vs Orx<sub>A</sub>,  $t_{17} = 1.0, p \geq 0.336$ ). However, Orx<sub>1</sub>R stimulation produced a robust freezing response in Stay mice that was significantly different from Escape animals that experienced the same treatment ( $t_{13} = 3.8, p \leq 0.002$ ) and vehicle-treated Stay mice (Fig. 3D;  $t_{21} = 3.4, p \leq 0.003$ ). Acute activation of intra-BLA Orx<sub>2</sub>R resulted in reduced OF Test freezing in Escape mice compared to vehicle controls (Fig. 3D;  $t_9 = 3.4, p \leq 0.008$ ). While Stay animals infused with an Orx<sub>2</sub>R agonist were not different from Escape mice administered the same treatment ( $t_7 = 1.2, p \geq 0.284$ ) or vehicle

controls ( $t_{18} = 0.5$ ,  $p \geq 0.635$ ), they did experience reduced OF Test freezing compared to animals that received the Orx<sub>1</sub>R stimulation treatment (Fig. 3D;  $t_{13} = 2.4$ ,  $p \leq 0.034$ ).

To determine if freezing behavior in the OF Test was a result of generalization after social stress exposure, we performed correlational analyses comparing freezing behavior in the SAM to that of the OF Test (Figs. 3E, S7). There were no relationships observed in Escape mice following any Orx receptor treatment (Figs. S7A-F). Further, a significant and positive association was observed in vehicle-treated Stay animals (Fig. 3E;  $F_{1,11} = 8.7$ ,  $p \leq 0.0131$ ), but this relationship is lost in Orx<sub>1</sub>R antagonist treated mice (Fig. S7G). The lack of correlation following Orx<sub>1</sub>R antagonist suggests that Orx<sub>1</sub>R activity in vehicle-treated mice allowed for generalization of fear learning to be induced in these mice. Importantly, Stay animals in the Orx<sub>2</sub>R antagonist or Orx<sub>1</sub>R stimulation groups displayed robust freezing behavior in both the SAM and OF Test (Figs. 3A-D), and while regressions revealed no relationships in these mice (Figs. S7H-J); a potential ceiling effect may have impaired the search for meaningful analyses.

### **Stress-induced locomotion in the SAM explains OF test locomotion after acute alteration of intra-BLA Orx receptor activity in Escape mice**

As the Orx system plays a role in arousal [39, 40] and may initiate locomotor functions [41], we investigated locomotion in the SAM (Figs. 4A, B) and OF Test (Figs. 4C, D) as behaviors in both SAM and OF Tests (socially induced freezing and time in center) may be influenced by changes in locomotion. Importantly, the pharmacological manipulations of BLA Orx receptors had no effect on home cage mobility (Fig. S9;  $F_{5,76} = 0.7$ ,  $p \geq 0.658$ ; Escape:  $F_{5,26} = 1.3$ ,  $p \geq 0.302$ ; Stay:  $F_{5,44} = 1.0$ ,  $p \geq 0.433$ ); however, several significant differences were observed during social stress in the SAM (Figs. 4A, B; Antagonist groups: Drug Effect,  $F_{2,43} = 0.9$ ,  $p \geq 0.423$ ; Phenotype Effect,  $F_{1,43} = 9.0$ ,  $p \leq 0.005$ ; Interaction Effect,  $F_{2,43} = 1.7$ ,  $p \geq 0.191$ ;

Stimulation groups: Drug Effect,  $F_{3,46} = 2.7, p \geq 0.059$ ; Phenotype Effect,  $F_{1,46} = 8.2, p \leq 0.006$ ; Interaction Effect,  $F_{3,46} = 0.7, p \geq 0.564$ ) and afterwards in the OF Test (Figs. 4C, D; Antagonist groups: Drug Effect,  $F_{3,43} = 3.0, p \geq 0.061$ ; Phenotype Effect,  $F_{1,43} = 7.8, p \leq 0.008$ ; Interaction Effect,  $F_{3,43} = 0.1, p \geq 0.941$ ; Stimulation groups: Drug Effect,  $F_{3,46} = 1.7, p \geq 0.175$ ; Phenotype Effect,  $F_{1,46} = 9.7, p \leq 0.003$ ; Interaction Effect,  $F_{3,46} = 0.3, p \geq 0.858$ ).

In vehicle control animals, Escape mice displayed heightened locomotion compared to Stay animals in both the SAM (Figs. 4A, B, white bars;  $t_{19} = 2.7, p \leq 0.014$ ) and OF Test (Figs. 4C, D, white bars;  $t_{19} = 2.5, p \leq 0.023$ ). While not different from vehicle-treated mice, intra-BLA Orx<sub>1</sub>R antagonism removes phenotypic separation of Escape and Stay locomotor activity in the SAM (Fig. 4A, light gray bars;  $t_{13} = 0.2, p \geq 0.864$ ) and OF Test (Fig. 4C, light gray bars;  $t_{13} = 1.6, p \geq 0.141$ ). Acute inhibition of Orx<sub>2</sub>R in the BLA resulted in a maintained phenotype separation in SAM locomotion, with Escape mice expressing higher locomotor activity than Stay animals (Fig. 4A, dark gray bars;  $t_{11} = 2.3, p \leq 0.039$ ); however, this relationship was not observed in the OF Test (Fig. 4C, dark gray bars;  $t_{11} = 1.1, p \leq 0.314$ ).

Similar to vehicle-treated mice, animals in the Orx<sub>1</sub>R stimulation group exhibited phenotype differences in SAM ( $t_{13} = 2.5, p \leq 0.026$ ) and OF Test locomotion (Fig. 4B, light gray bars with dark gray outline;  $t_{13} = 2.5, p \leq 0.028$ ). This divergent phenotype response was not observed with Orx<sub>A</sub> or Orx<sub>2</sub>R stimulation treatments. However, agonism of BLA Orx<sub>2</sub>R resulted in Stay mice expressing elevated locomotion in the SAM that was significantly greater than Stay animals in vehicle ( $t_{18} = 2.9, p \leq 0.010$ ), Orx<sub>A</sub> ( $t_9 = 2.7, p \leq 0.009$ ), and Orx<sub>1</sub>R stimulation ( $t_{13} = 3.0, p \leq 0.005$ ) treatment groups (Fig. 4B). This heightened locomotor response in Orx<sub>2</sub>R agonist-treated Stay mice was not observed in the OF Test (Fig. 4D).

Regression analyses comparing locomotion in the SAM to locomotor activity in the OF Test revealed no associations in Stay mice (Figs. S8C-H). Further, no significant correlations were observed for Escape animals in vehicle ( $F_{1,6} = 0.4, p \geq 0.528$ ) and Orx<sub>1</sub>R stimulation ( $F_{1,4} = 5.0, p \geq 0.090$ ) treatment groups (Figs. S8A, B). Strong positive correlations between SAM and OF Test locomotion in Escape mice were revealed with intra-BLA treatments of an Orx<sub>1</sub>R antagonist (Fig. 4E;  $F_{2,3} = 15.9, p \leq 0.028$ ), Orx<sub>A</sub> (Fig. 4G;  $F_{2,2} = 49.4, p \leq 0.02$ ), and an Orx<sub>2</sub>R agonist (Fig. 4H;  $F_{2,1} = 351.5, p \leq 0.034$ ). In contrast, a significant negative relationship was observed between SAM and OF Test locomotor activity in Escape animals treated with an Orx<sub>2</sub>R antagonist (Fig. 4F;  $F_{1,4} = 11.4, p \leq 0.028$ ). Together, these results suggest that Orx receptors in the BLA influence generalization learning of stress-related locomotor activity, transferred from SAM to OF.

### **Transient manipulation of Orx receptor activity in the BLA promotes learning transference in Stay mice**

As both the SAM and OF Test arenas comprise open field environments, on Day 4 we compared behavior in the SAM arena to the behaviors exhibited in the OF Test, directly after the SAM social interaction (Fig. 5). Mice of Escape and Stay behavioral phenotypes administered vehicle treatments did not differ in the amount of time spent in the center of the SAM arena (Figs. 5A, B, white bars;  $t_{19} = 0.5, p \geq 0.699$ ) or the OF Test (Figs. 5C, D, white bars;  $t_{19} = 1.3, p \geq 0.213$ ). In the Orx receptor inhibition groups, there were no differences observed between animals administered Orx receptor targeting drugs and those of the vehicle control group in the SAM (Fig. 5A; Drug Effect,  $F_{2,43} = 0.02, p \geq 0.978$ ; Phenotype Effect,  $F_{1,43} = 7.4, p \leq 0.010$ ; Interaction Effect,  $F_{2,43} = 1.7, p \geq 0.197$ ; Escape: Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{11} = 1.3, p \geq 0.226$ ; Stay: Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{21} = 1.4, p \geq 0.183$ ; Escape: Vehicle vs Orx<sub>2</sub>R Ant.,  $t_{12} = 0.9, p \geq$

0.380; Stay: Vehicle vs Orx<sub>2</sub>R Ant.,  $t_{18} = 0.6, p \geq 0.530$ ) or OF Test behaviors (Fig. 5C; Drug Effect,  $F_{2,43} = 0.4, p \geq 0.679$ ; Phenotype Effect,  $F_{1,43} = 15.7, p < 0.001$ ; Interaction Effect,  $F_{2,43} = 1.4, p \geq 0.268$ ; Escape: Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{11} = 1.4, p \geq 0.186$ ; Stay: Vehicle vs Orx<sub>1</sub>R Ant.,  $t_{21} = 0.4, p \geq 0.699$ ; Escape: Vehicle vs Orx<sub>2</sub>R Ant.,  $t_{12} = 1.5, p \geq 0.151$ ; Stay: Vehicle vs Orx<sub>2</sub>R Ant.,  $t_{18} = 1.5, p \geq 0.163$ ). However, there was significant phenotype separation in mice that received the Orx<sub>1</sub>R antagonist as Stay animals spent more time in the center of the SAM (Fig. 5A, light gray bars;  $t_{13} = 2.2, p \leq 0.047$ ) and OF Test (Fig. 5C, light gray bars;  $t_{13} = 2.2, p \leq 0.05$ ) compared to Escape mice administered the same treatment. While Stay mice given the Orx<sub>2</sub>R antagonist did not statistically spend more time than Escape animals in the center of the SAM (Fig. 5A, dark gray bars;  $t_{11} = 1.6, p \geq 0.144$ ), phenotypic separation was clearly evident for time in the center when mice were exposed to the OF Test after Orx<sub>2</sub>R antagonist treatment (Fig. 5C, dark gray bars;  $t_{11} = 3.6, p \leq 0.004$ ).

In the Orx receptor stimulation groups, SAM (Fig. 5B; Drug Effect,  $F_{3,45} = 5.5, p \leq 0.003$ ; Phenotype Effect,  $F_{1,45} = 0.4, p \geq 0.516$ ; Interaction Effect,  $F_{3,45} = 1.4, p \geq 0.246$ ) and OF Test (Fig. 5D; Drug Effect,  $F_{3,46} = 0.4, p \geq 0.779$ ; Phenotype Effect,  $F_{1,46} = 4.9, p \leq 0.032$ ; Interaction Effect,  $F_{3,46} = 0.4, p \geq 0.750$ ), targeting the BLA, Orx<sub>A</sub> infusion resulted in a reduction, though not significant, in the amount of time spent in the center of the SAM in both Escape ( $t_{10} = 1.4, p \geq 0.186$ ) and Stay mice ( $t_{16} = 1.8, p \geq 0.089$ ) compared to vehicle controls (Fig. 5B, black bars). Animals in the Orx<sub>1</sub>R stimulation group spent more time in the center of the SAM than those mice treated with Orx<sub>A</sub> (Escape:  $t_8 = 2.3, p \leq 0.05$ ; Stay:  $t_{12} = 2.4, p \leq 0.033$ ), but did not differ from animals in the vehicle control group (Fig. 5B; Escape:  $t_{12} = 1.1, p \geq 0.286$ ; Stay:  $t_{20} = 1.6, p \geq 0.132$ ). Interestingly, a phenotype divergence was observed in mice treated with the Orx<sub>2</sub>R agonist, where Escape animals spent more time in the center of the SAM compared to Stay mice

(Fig. 5B, dark gray bars with light gray outline;  $t_7 = 2.3, p \leq 0.05$ ). These Escape mice also spent more time in the center of the SAM compared to Orx<sub>A</sub>-treated animals of the Escape phenotype (Fig. 5B;  $t_5 = 5.4, p \leq 0.003$ ). There were no significant differences in time spent in the center of the OF Test between any of the Orx receptor stimulation groups (Fig. 5D; Drug Effect,  $F_{3,46} = 0.4, p \geq 0.779$ ; Phenotype Effect,  $F_{1,46} = 4.9, p \leq 0.032$ ; Interaction Effect,  $F_{3,46} = 0.4, p \geq 0.750$ ).

We next performed regression analyses to assess whether time spent in the center of the SAM arena, where test mice primarily interact with a social aggressor, was being transferred to the non-social and novel OF Test environment, and if Orx receptor activity might play a role in transference learning (Figs. 5E-H). Significant positive regressions were revealed in Stay animals between center time and treatment with an Orx<sub>1</sub>R antagonist (Fig. 5E, F;  $F_{1,8} = 16.8, p \leq 0.003$ ), and an Orx<sub>2</sub>R agonist (Fig. 5H;  $F_{1,4} = 40.2, p \leq 0.003$ ). Curiously, there was also a significant positive relationship observed after an Orx<sub>2</sub>R antagonist was administered (Fig. 5G;  $F_{1,5} = 13.5, p \leq 0.014$ ). No significant correlations were observed in Escape mice in any treatment group (Figs. S10A-F), nor Stay animals in the vehicle (Fig. S10G;  $F_{1,11} = 2.1, p \geq 0.171$ ), Orx<sub>A</sub> (Fig. S10H;  $F_{1,3} = 3.5, p \geq 0.157$ ), and Orx<sub>1</sub>R stimulation groups (Fig. S10I;  $F_{1,7} = 1.3, p \geq 0.296$ ). These results seem to suggest Orx receptors play a role in regulating phenotypically dependent behavioral transference in Stay animals during periods of stress, perhaps by acting through alternate (such as locus coeruleus) pathways related to stress and learning neurocircuitries [37, 38].

### **EPM results are muted by prior experience**

On Day 5 of the experimental design, mice were exposed to the Elevated Plus Maze (EPM) to assess behavioral measures of generalized anxiety as a comparator to SAM and OF Test results observed the day prior (Day 4; Fig. 1B). In the Orx receptor antagonist groups, there were no

phenotype differences nor differences with respect to vehicle-treated control mice for time in the open (Fig. S11A, B; Drug Effect,  $F_{2,42} = 1.8$ ,  $p \geq 0.178$ ; Phenotype Effect,  $F_{1,42} = 0.00006$ ,  $p \geq 0.994$ ; Interaction Effect,  $F_{2,42} = 0.07$ ,  $p \geq 0.933$ ) or closed arms (Fig. S11C, D; Drug Effect,  $F_{2,42} = 5.8$ ,  $p \leq 0.006$ ; Phenotype,  $F_{1,43} = 0.2$ ,  $p \geq 0.671$ ; Interaction Effect,  $F_{2,42} = 0.1$ ,  $p \geq 0.871$ ) of the EPM. Contrary to our predictions and behavioral results in the SAM (Fig. 3A), Stay mice administered intra-BLA infusion of an Orx<sub>2</sub>R antagonist exhibited more time in the open arms ( $t_{14} = 2.1$ ,  $p \leq 0.05$ ) and less time in the closed arms ( $t_{14} = 3.1$ ,  $p \leq 0.008$ ) compared to Stay animals treated with an Orx<sub>1</sub>R antagonist (Figs. S11A, C). Further, in assessing time spent in the intersection zone of the EPM (Drug Effect,  $F_{2,42} = 2.0$ ,  $p \geq 0.149$ ; Phenotype Effect,  $F_{1,42} = 5.1$ ,  $p \leq 0.029$ ; Interaction Effect,  $F_{2,42} = 1.2$ ,  $p \geq 0.313$ ), Stay animals in the Orx<sub>2</sub>R antagonist group spent more time in this area between the open and closed arms when compared to Escape mice in the same treatment group ( $t_{11} = 2.5$ ,  $p \leq 0.018$ ), as well as vehicle- ( $t_{18} = 2.3$ ,  $p \leq 0.028$ ) and Orx<sub>1</sub>R antagonist-treated Stay mice (Fig. S11E;  $t_{14} = 2.5$ ,  $p \leq 0.016$ ).

In the Orx receptor stimulation groups, there were no effects observed for the amount of time mice spent in the intersection zone of the EPM (Fig. S11F; Drug Effect,  $F_{3,46} = 0.7$ ,  $p \geq 0.533$ ; Phenotype Effect,  $F_{1,46} = 2.3$ ,  $p \geq 0.138$ ; Interaction Effect,  $F_{3,46} = 0.9$ ,  $p \geq 0.461$ ); however, overall effects were observed for time in the open (Fig. S11B; Drug Effect,  $F_{3,46} = 2.0$ ,  $p \geq 0.126$ ; Phenotype Effect,  $F_{1,46} = 7.5$ ,  $p \leq 0.009$ ; Interaction Effect,  $F_{3,46} = 1.3$ ,  $p \geq 0.296$ ) and closed arms (Fig. S11D; Drug Effect,  $F_{3,46} = 1.9$ ,  $p \geq 0.135$ ; Phenotype Effect,  $F_{1,46} = 8.5$ ,  $p \leq 0.005$ ; Interaction Effect,  $F_{3,46} = 1.4$ ,  $p \geq 0.318$ ). Stay mice treated with Orx<sub>A</sub> spent more time in the open arms of the EPM than those of the Escape phenotype (Fig. S11B, black bars;  $t_7 = 2.2$ ,  $p \leq 0.037$ ), but this divergence of phenotypes was not observed for time in the closed arms (Fig. S11D, black bars;  $t_7 = 1.8$ ,  $p \geq 0.078$ ). While Stay mice in the Orx<sub>1</sub>R stimulation group spent



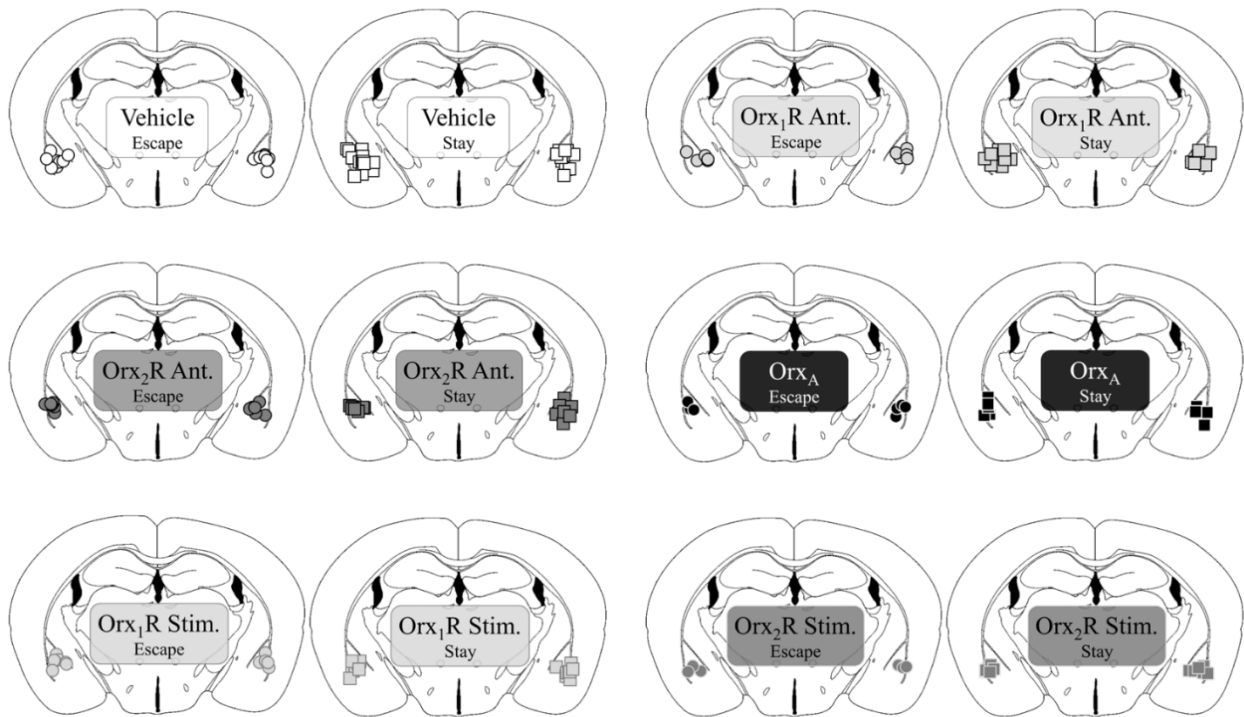
more time in the open arms compared to their Escape counterparts ( $t_{13} = 2.2, p \leq 0.044$ ), the Escape animals in this treatment group spent less time in the open arms compared to vehicle controls of the same phenotype (Fig. S11B, light gray bars with dark gray outline;  $t_{12} = 2.6, p \leq 0.024$ ). The opposite result was observed for time in the closed arms of the EPM (Fig. S11D), where Orx<sub>1</sub>R stimulation resulted in Escape mice spending more time in the closed arms compared to Stay animals in the same treatment group ( $t_{13} = 2.7, p \leq 0.020$ ) and vehicle-treated Escape mice ( $t_{12} = 2.7, p \leq 0.021$ ). Again, counter to our predictions, Escape mice infused with an intra-BLA Orx<sub>2</sub>R agonist spent significantly less time in the open arms (Fig. S11B) and more time in the closed arms (Fig. S11D) compared to Escape animals in the vehicle control (Open Arms:  $t_9 = 3.5, p \leq 0.006$ ; Closed Arms:  $t_9 = 4.1, p \leq 0.003$ ) and Orx<sub>A</sub> treatment groups (Open Arms:  $t_5 = 3.6, p \leq 0.015$ ; Closed Arms:  $t_5 = 2.9, p \leq 0.034$ ). Together, these results suggest that anxiety-related learning in the SAM (or perhaps any environment) functionally modifies behavioral responses to novel anxious conditions, such that standard ethological manifestations no longer apply.

## Supplemental References

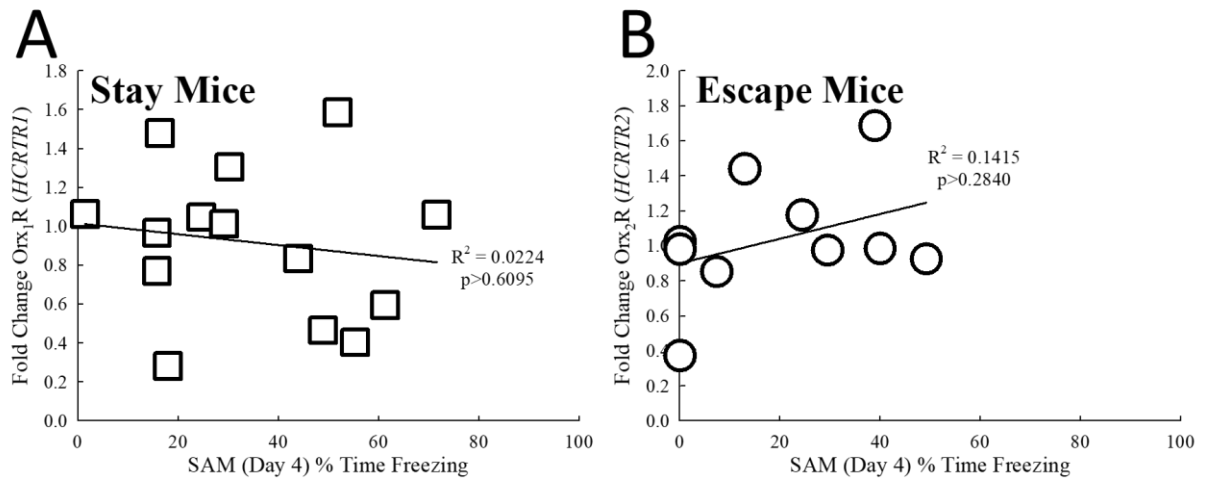
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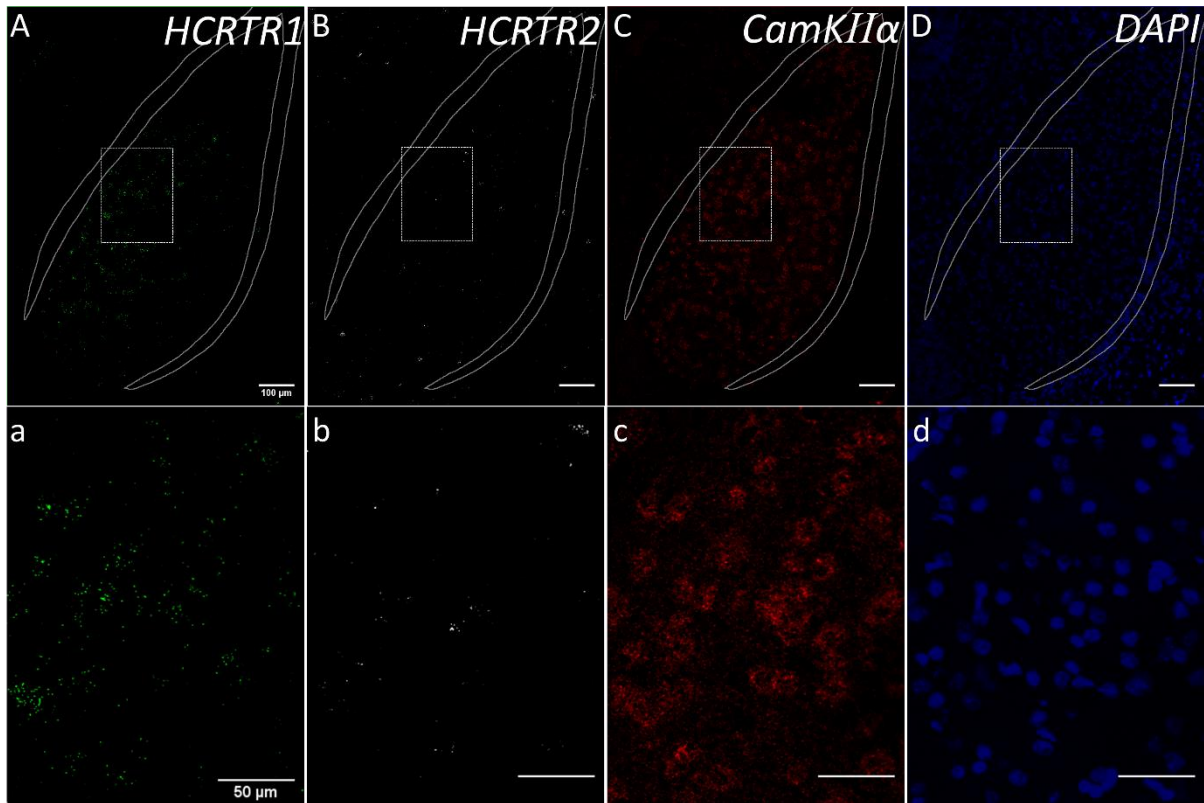
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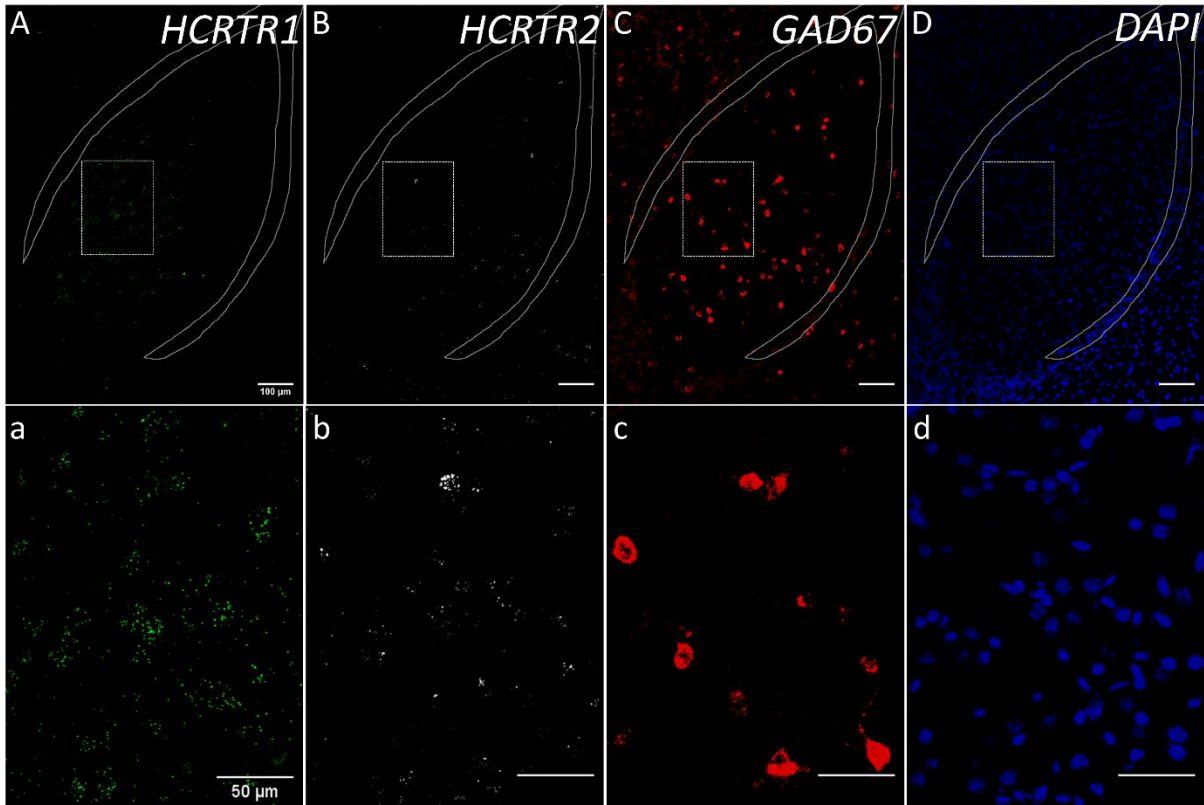
**Figure S1.** Schematic shows successfully targeted intra-BLA injections of Escape and Stay mice treated with Vehicle, Orx<sub>1</sub>R Antagonist (SB-674042), Orx<sub>2</sub>R Antagonist (MK-1064), Orx<sub>A</sub>, Orx<sub>1</sub>R Stimulation (Orx<sub>A</sub> + MK-1064 combination), and Orx<sub>2</sub>R Stimulation (YNT-185) treatments. Escape mice are identified as circles and Stay mice are symbolized with squares.



**Figure S2.** (A) While relative expression levels of *HCRTR1* are positively correlated with SAM-induced conflict freezing in Escape mice (see Fig. 2B of main manuscript), correlation analyses in Stay mice does not reveal this relationship ( $F_{1,12} = 0.3$ ,  $R^2 = 0.0224$ ,  $p \geq 0.6095$ ). (B) Similarly, Escape mice conflict freezing in the SAM is not associated with *HCRTR2* transcription levels ( $F_{1,8} = 1.3$ ,  $R^2 = 0.1415$ ,  $p \geq 0.2840$ ); however, there is a negative relationship between freezing *HCRTR2* expression in Stay animals (see Fig. 2C of main manuscript).

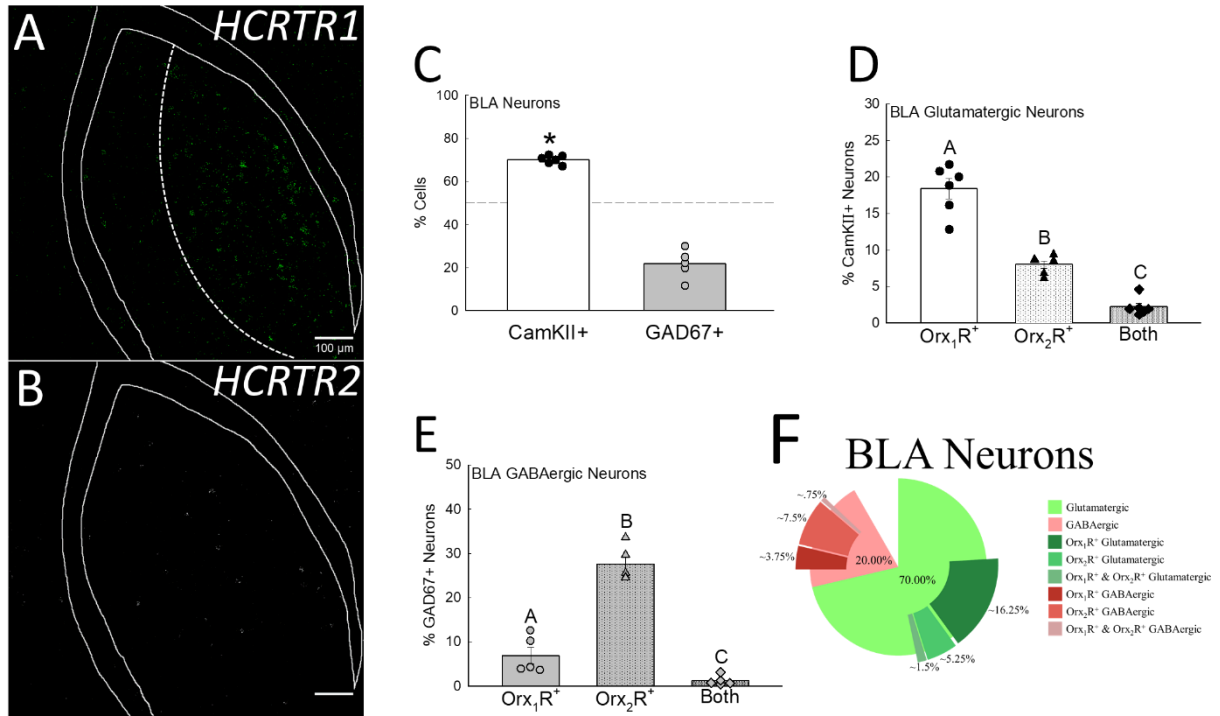


**Figure S3.** Expression of (A, *a*) *HCRTR1* (green), (B, *b*) *HCRTR2* (white), (C, *c*) *CamKIIα* (red), and (D, *d*) DAPI (blue) in BLA cells. Images in *a*, *b*, *c*, & *d* are enlarged figures from square outlines in A, B, C, & D. Note- images align with Figs. 2E & F of primary manuscript.

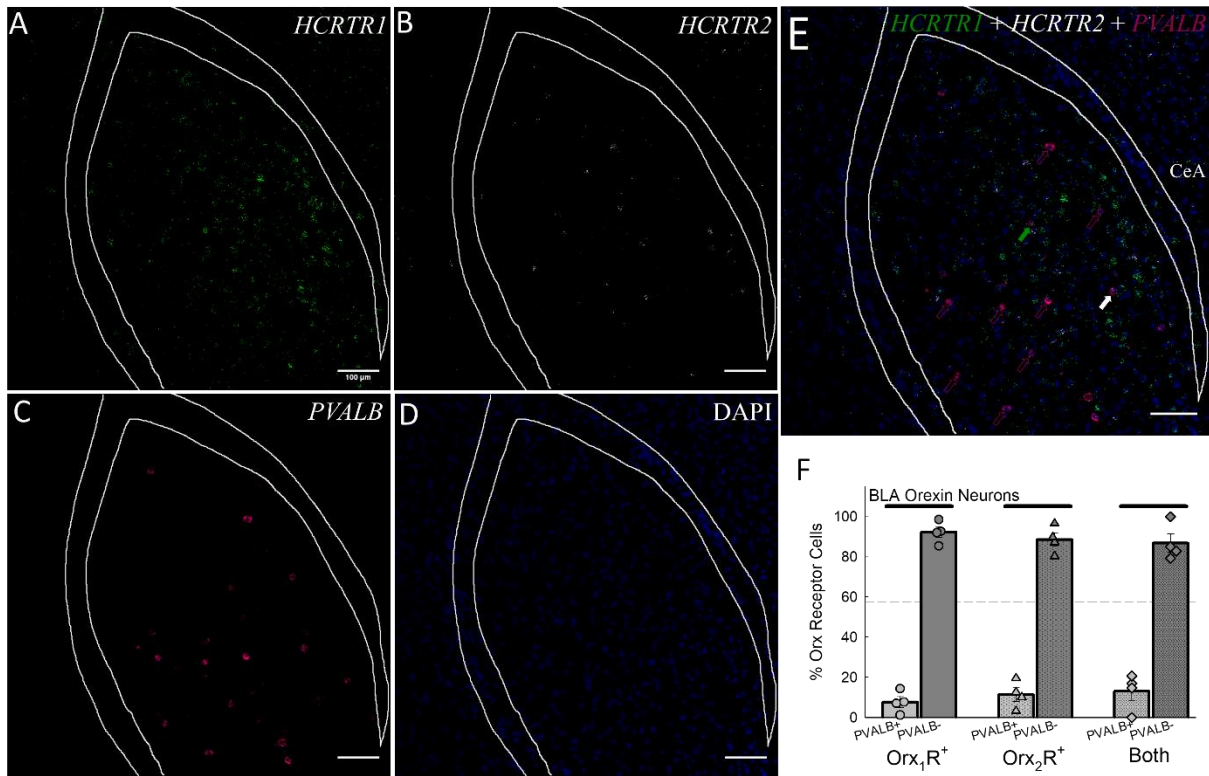


**Figure S4.** Expression of (A,*a*) *HCRTR1* (green). (B, *b*) *HCRTR2* (white), (C, *c*) *GAD67* (red), and (D, *d*) DAPI (blue) in BLA cells. Images in *a*, *b*, *c*, & *d* are enlarged figures from square outlines in A, B, C, & D. Note- images align with Figs. 2H & I of primary manuscript.

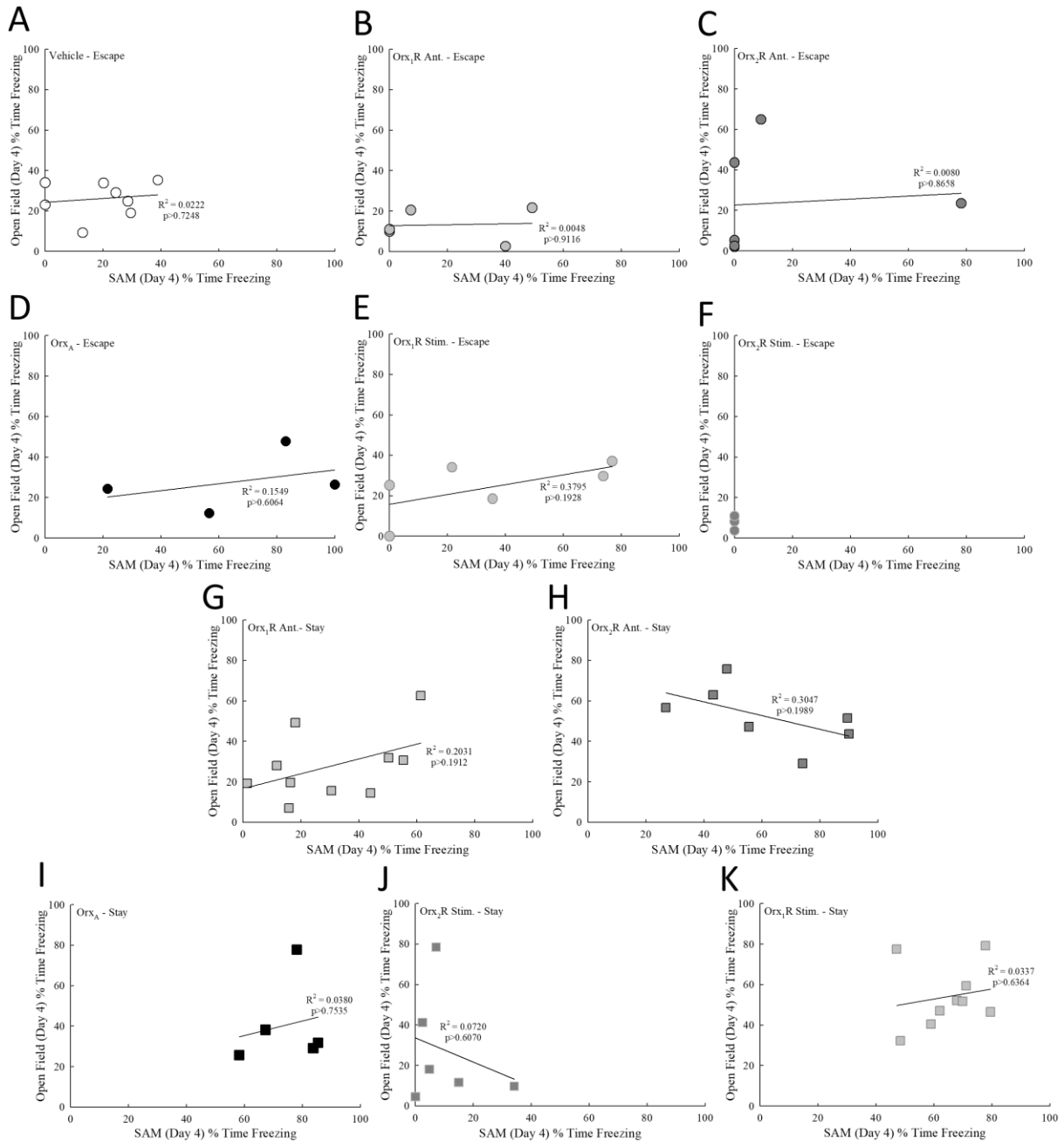




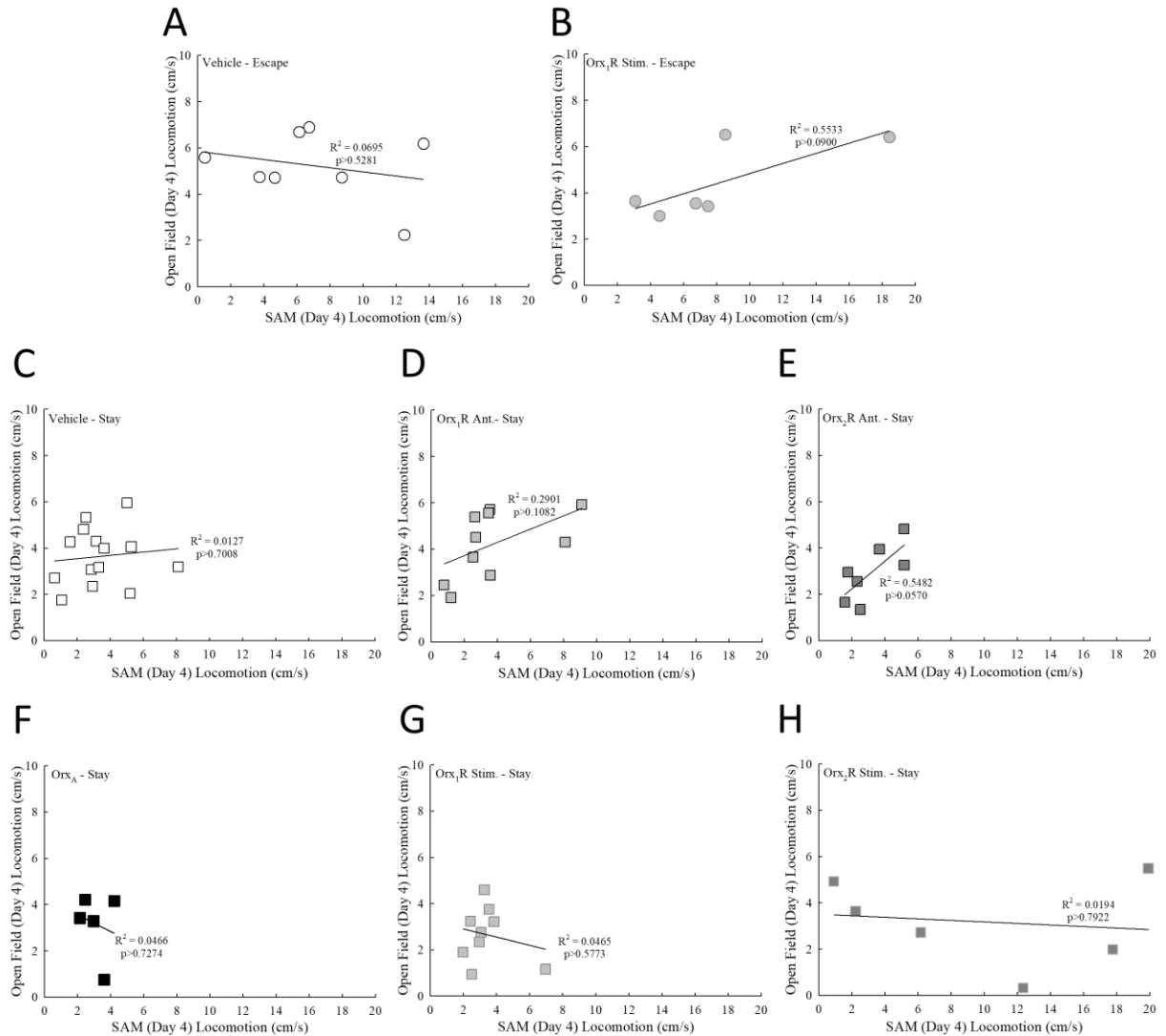
**Figure S5.** (A) While Orx<sub>1</sub>R mRNA (*HCRT1*) expression has a topographical organization in the BLA (most abundant in medial portion - separated with white dotted line), (B) *HCRT2* does not exhibit an obvious pattern of expression. (C) Approximately 70% of neurons in the BLA expressed the glutamatergic marker CamKII $\alpha$ , while only around 20% expressed GAD67 ( $t_9 = 16.8, p < 0.001$ ). (D) Of CamKII $\alpha$ -expressing cells, ~18% express Orx<sub>1</sub>R, less than 10% express Orx<sub>2</sub>R, and even less express both Orx receptors ( $F_{2,15} = 84.6, p < 0.001$ ). (E) Over 25% of GABA neurons in the BLA expressing GAD67 also express Orx<sub>2</sub>R, while less than 10% express Orx<sub>1</sub>R or both Orx receptors ( $F_{2,12} = 84.0, p < 0.001$ ). (F) A pie chart illustrates estimated proportions of BLA neurons and Orx receptors from reported results.



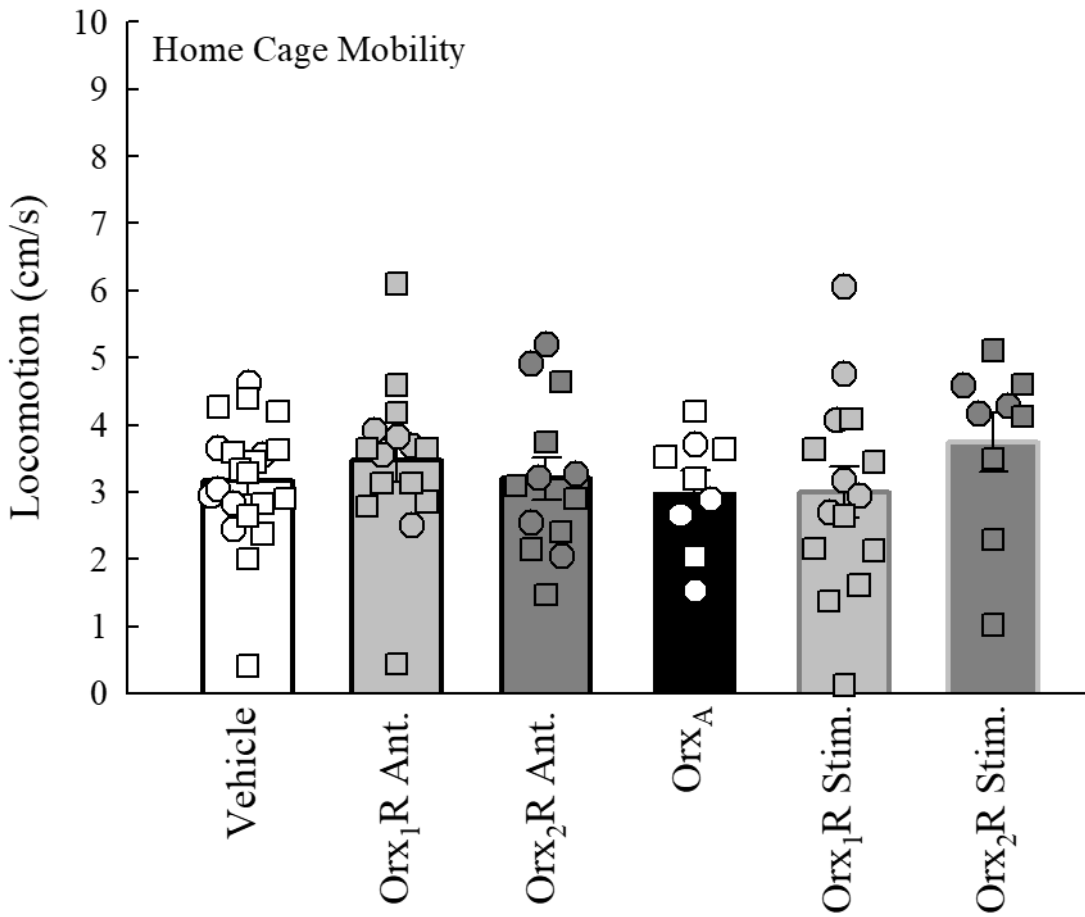
**Figure S6.** Orexin receptors in the BLA are expressed on *PVALB*<sup>+</sup> GABA neurons at very low levels. (A) Expression of *HCRTR1* (green), (B) *HCRTR2* (white), (C) *PVALB* (magenta), and DAPI (blue) when (E) merged (some observed colocalizations are identified with filled green arrow = Orx<sub>1</sub>R<sup>+</sup> + *PVALB*<sup>+</sup>, solid white arrow = Orx<sub>2</sub>R<sup>+</sup> + *PVALB*<sup>+</sup>, and unfilled magenta arrows = Orx<sub>1</sub>R<sup>-</sup> + Orx<sub>2</sub>R<sup>-</sup> + *PVALB*<sup>+</sup>) reveals (F) low overlap of Orx receptor mRNA with *PVALB*<sup>+</sup> GABA neurons (compared to *PVALB*<sup>-</sup> cells in the same receptor [Orx<sub>1</sub>R<sup>+</sup>, Orx<sub>2</sub>R<sup>+</sup>, or Orx<sub>1</sub>R<sup>+</sup> & Orx<sub>2</sub>R<sup>+</sup>] group,  $p \leq 0.05$ ). CeA = central amygdala



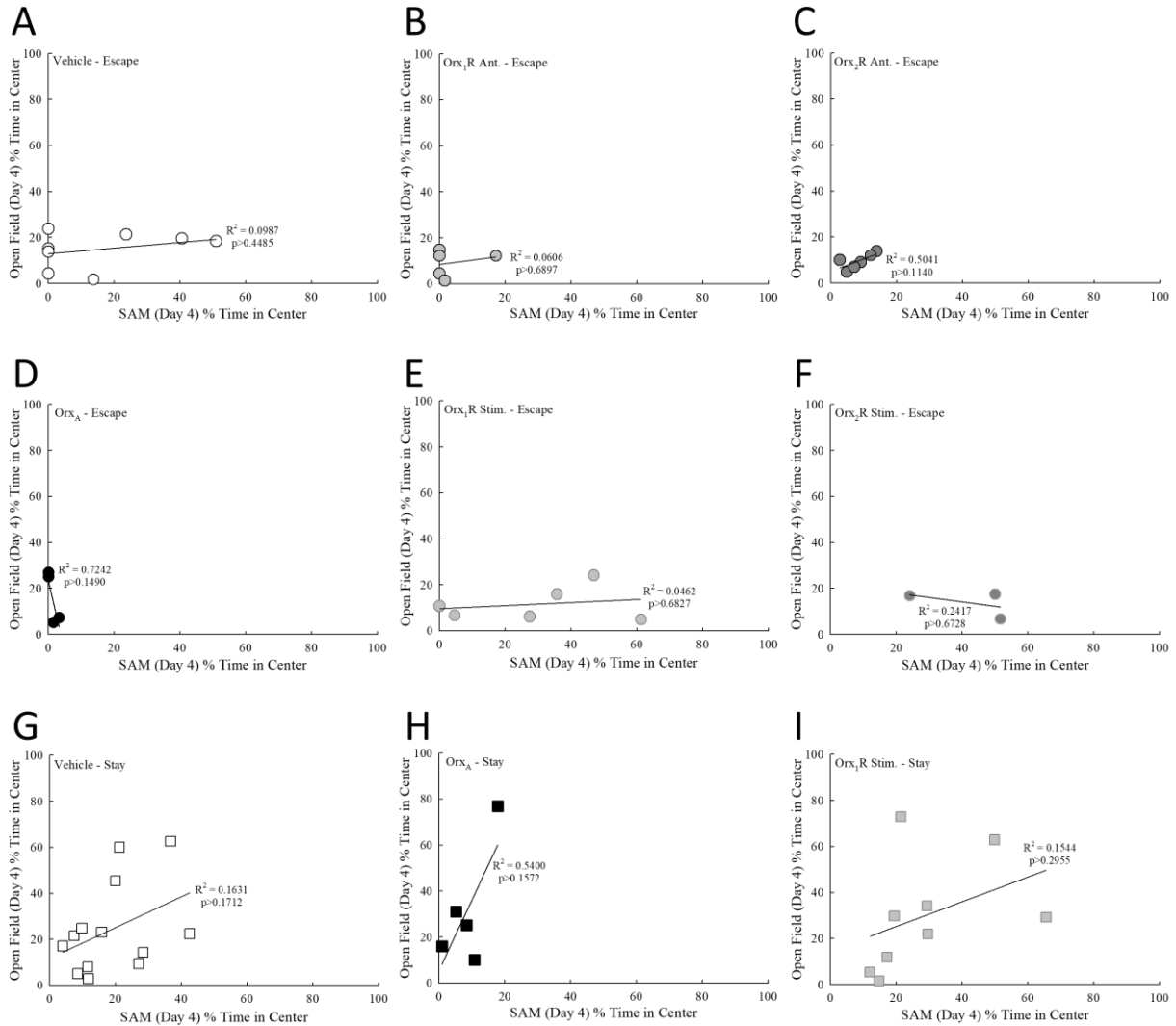
**Figure S7.** (A-F) No significant correlations exist between freezing behavior in the SAM and freezing response in the OF Test for Escape mice in any treatment group. Further, no freezing relationships were observed for Stay animals in the (G) Orx<sub>1</sub>R Antagonist ( $F_{1,8} = 2.0, p \geq 0.1912$ ), (H) Orx<sub>2</sub>R Antagonist ( $F_{1,5} = 2.2, p \geq 0.1989$ ), (I) Orx<sub>A</sub> ( $F_{1,3} = 0.1, p \geq 0.7535$ ), (J) Orx<sub>1</sub>R Stimulation ( $F_{1,7} = 0.2, p \geq 0.6364$ ), and (K) Orx<sub>2</sub>R Stimulation ( $F_{1,4} = 0.3, p \geq 0.607$ ) groups.



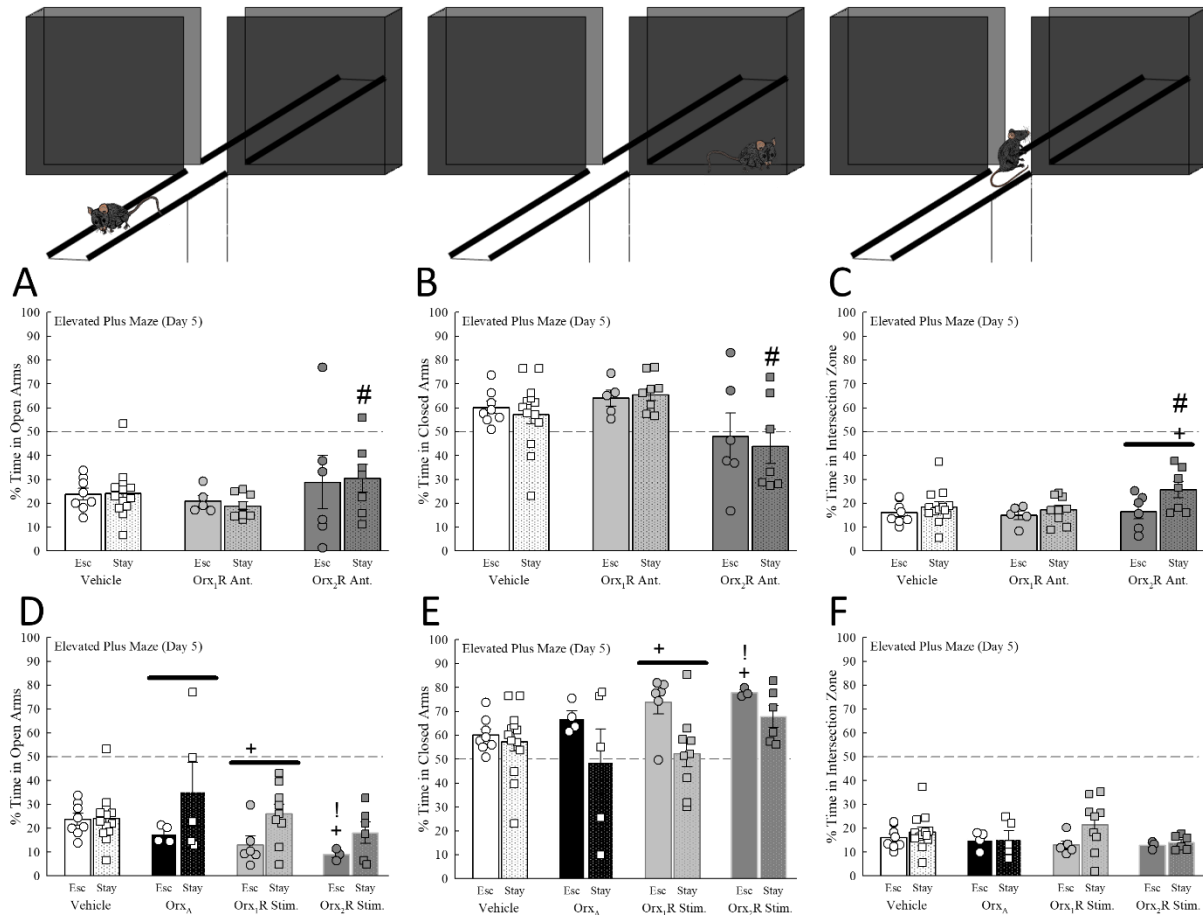
**Figure S8.** (A) For Escape mice under control conditions (vehicle administration) there is no significant correlation between locomotion in the SAM and locomotion in the OF Test ( $F_{1,6} = 0.4$ ,  $p \geq 0.5281$ ). (B) Similarly, there is no significant relationship between SAM and OF Test locomotion for Escape animals in the Orx<sub>1</sub>R Stimulation group ( $F_{1,4} = 5.0$ ,  $p \geq 0.09$ ). (C-H) No significant correlations exist between locomotor activity in the SAM and locomotion in the OF Test for Stay mice in any treatment group; although, regression analyses for those (E) animals treated with an Orx<sub>2</sub>R antagonist exposed a positive relationship between SAM and OF Test locomotion that is significant at the  $p < 0.06$  level ( $F_{1,5} = 6.1$ ,  $p \leq 0.057$ ).



**Figure S9.** While locomotion in the SAM and OF Test are altered by Orx receptor-targeting drugs, home cage mobility is not affected ( $F_{5,76} = 0.7, p \geq 0.658$ ; Escape:  $F_{5,26} = 1.3, p \geq 0.302$ ; Stay:  $F_{5,44} = 1.0, p \geq 0.433$ ). Circles represent Escape animals in the designated treatment group and squares signify Stay mice.



**Figure S10.** (A-F) In animals exhibiting the Escape phenotype, there were no significant correlations between time in the center of the SAM arena and time in the center of the OF Test. Additionally, there were no relationships observed in Stay mice for those in the (G) Vehicle ( $F_{1,11} = 2.1$ ,  $p \geq 0.1712$ ), (H) Orx<sub>A</sub> ( $F_{1,3} = 3.5$ ,  $p \geq 0.1572$ ), or (I) Orx<sub>1</sub>R Stimulation ( $F_{1,7} = 1.3$ ,  $p \geq 0.2955$ ) treatment groups.



**Figure S11.** Phenotype separation is observed after Orx receptor manipulation in post-SAM EPM, but results are not consistent with anxious state. (A) Stay mice treated with an intra-BLA Orx<sub>2</sub>R antagonist spent more time in the open arms of the EPM than Stay animals treated with an Orx<sub>1</sub>R antagonist. (B) Escape mice in Orx<sub>1</sub>R and Orx<sub>2</sub>R stimulation groups spent significantly less time in the open arms of the EPM compared to vehicle-treated control animals. (C) Administration of an Orx<sub>2</sub>R antagonist into the BLA or Stay mice resulted in less time in the closed arms of the EPM compared to animals of the same phenotype that were treated with an Orx<sub>1</sub>R antagonist. (D) Stimulation of Orx<sub>1</sub>R or Orx<sub>2</sub>R in the BLA promoted Escape animals to spend more time in the closed arms of the EPM compared to vehicle controls. (E) Antagonizing intra-BLA Orx<sub>2</sub>R led to more time spent in the intersection zone of the EPM. (F) No differences in time in the intersection zone of the EPM were observed for the Orx receptor stimulation groups. # $p \leq 0.05$  for comparisons to mice in the Orx<sub>1</sub>R antagonist group of the same

phenotype; \*  $p \leq 0.05$  for comparisons to Escape phenotype in the same treatment group; + $p \leq 0.05$  for comparisons to Vehicle-treated mice of the same phenotype; † $p \leq 0.05$  for comparisons to Orx<sub>A</sub> treatment animals of the same phenotype.



## Chapter 4: Female social defeat avoidance is modified by orexin 2 receptor activity

### ABSTRACT

Stress responsive states require signaling balance in brain regions associated with the promotion of pro- and anti-stress behavioral output. Biases in these circuits shift behavioral patterns and define phenotypes that exhibit stress resilience or vulnerability. The Stress Alternatives Model (SAM) is a behavioral paradigm that separates mice into social stress-induced behavioral phenotypes: active avoidance (Escape) and accepting confrontation (Stay). Manipulation of intra-BLA orexin 2 receptor (Orx<sub>2</sub>R) activity shifted phenotype-specific behaviors. As the orexin system mediates stress responses in a sex-dependent fashion, we developed a model for investigating social stress in female mice using shock-induced aggression (SIA). Unlike males exposed to the SAM paradigm, all females display Escape behavior, which can be altered by changing the paradigm to enhance the stress state. Further, female mice possess more BLA cells that express Orx<sub>2</sub>R mRNA (*HCRTR2*) compared to males. Antagonizing Orx<sub>2</sub>R subcutaneously at a low dose (30 nmol) in female animals resulted in phenotype divergence with a proportion of mice displaying slower escape (Escape<sup>S</sup>), a result replicated in all females administered yohimbine ( $\alpha_2$  receptor antagonist). Like yohimbine-treated mice, females of the Escape<sup>S</sup> phenotype also showed reduced social preference and enhanced cued fear freezing. Additionally, Escape<sup>S</sup> animals had more *HCRTR2*-positive cells in the BLA. These results suggest that Orx<sub>2</sub>R mediate stress responsivity, likely by balancing pro- and anti-stress circuitry.

## INTRODUCTION

Females are reported as having twice the rates of affective disorder diagnoses compared to males [1-3]. As stress prompts affective dysfunction and initiates disorder development, possibly in a sex-dependent manner [4], understanding behavioral and neurophysiological consequences of stress in female populations is paramount. Models incorporating social defeat, while potent tools for delineating differential outcomes of stress-induced behavior and neurophysiology [5], are limited in their ability to provoke equivalent stress states in female populations. In this way, much of our current understanding of stress neurocircuitry is biased toward the physiology and behavior of males.

An impediment to understanding the heightened propensity of females to exhibit stress-related psychiatric conditions such as anxiety and depression is an animal model with highly translatable results. As the major impetus for developing such disorders is social stress, three recently developed models of social defeat for females have been put forth [6-8]. While these models proved to be important advances, they had limited effectiveness in producing social defeat in females, because the rate of aggression from males was low. We report that our model of social stress, the Stress Alternatives Model (SAM), can be utilized to produce aggressive interaction in females. This paradigm pits aggressive CD1 males with smaller female C57Bl/6 test mice, and occurs in an oval arena provided with escape routes that allow for escape. Four daily trials with a novel aggressor produces two behavioral phenotypes in males: Escape and Stay [9, 10]. Pharmacological treatments are given on Day 3, with anxiogenic drugs (such as the  $\alpha_2$  antagonist yohimbine) reversing Escape (become Stay), and anxiolytic drugs (such as the CRF<sub>1</sub> antagonist antalarmin) allow Stay animals to Escape [11].

Hypothalamus-derived orexins mediate stress responsive states in a sex-dependent fashion

[12, 13]. The end result of post-translational processing in orexinergic neurons is the production of two similar, yet distinct, neuromodulators: Orexin A (Orx<sub>A</sub>) and Orexin B (Orx<sub>B</sub>). Targeted cells express type 1 and 2 orexin receptor subtypes (Orx<sub>1</sub>R and Orx<sub>2</sub>R) which, upon activation by Orx<sub>A</sub> (EC<sub>50</sub> = 30 nM for Orx<sub>1</sub>R and 38 nM for Orx<sub>2</sub>R) or Orx<sub>B</sub> (EC<sub>50</sub> = 2,500 nM for Orx<sub>1</sub>R and 36 nM for Orx<sub>2</sub>R), initiate G<sub>q</sub> signaling pathways [14]. Orexinergic innervation is widespread and influences reward and arousal, but also motivation and stress [15-17]. Blocking Orx<sub>1</sub>R in the fear learning region of the brain, the basolateral amygdala (BLA), reduces anxious behavior (changes Stay to Escape), fear conditioning, and increases resilient behavior and motivation to Escape [18].

We adapted the SAM paradigm to include classical conditioning (a brief shock during anogenital sniffing) for the CD1 aggressor mice to effectively induce male aggression towards females. With CD1 male mice behaving aggressively toward females, we were able to evaluate the behavioral profile of socially stressed females, the phenotypes that were produced, and how the relationship of exhibited behaviors to balanced pro- and anti-stress neurocircuitries of the BLA. Females and males have been demonstrated to react differently to stressors, which involves distinct neurochemistry, including that of orexin [12, 13]. Those differences are reflected in unique female orexin signaling and behavior.

## **METHODS & MATERIALS**

### **Animals**

Adult (6–12 weeks) female (N = 117) and male (N = 144) C57BL/6N mice (Envigo, Indianapolis) were briefly group housed (4–5 per cage for 5 days) before being individually housed on a 12:12 light-dark cycle (lights off at 6 P.M.) at 22°C, with *ad libitum* food and water.

Male Hsd:ICR (CD1) retired breeder mice (N = 50) were singly housed and used as aggressor mice in behavioral paradigms. Test mice (C57BL/6N) were exposed to daily handling for 7 days prior to behavioral trials. Procedures were performed in ways that minimized suffering and the use of animals, and were in accordance with the NIH's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the IACUC of USD.

### **Surgeries and intra-BLA injections**

Stereotaxic surgeries were performed on a cohort of male C57BL/N mice (N = 50) where guide cannulae were positioned bilaterally above the basolateral amygdala (relative to bregma: AP, -1.5 mm; ML, +/-3.3 mm; DL, 4.5 mm). Mice were allowed to recover for 7 days and were provided pain relief (ketorolac, sc, 5 mg/kg) for 48 h following surgeries. On the day of drug delivery (Day 3), injector cannula (4.8 mm) were inserted into the guide cannula and drugs were infused at a rate of 1  $\mu$ l/min for 20 s to deliver 300 nL, after which the injector cannula was left in position for 90 s.

### **Drugs**

For intra-BLA manipulation of Orx<sub>2</sub>R receptor, an Orx<sub>2</sub>R antagonist (MK-1064, IC<sub>50</sub> = 18 nM for Orx<sub>2</sub>R; MedChemExpress, Monmouth Junction, NJ), Orx<sub>2</sub>R agonist (YNT-185 for fear response test, EC<sub>50</sub> = 28 nM for Orx<sub>2</sub>R and [Ala<sup>11</sup>,D-Leu<sup>15</sup>]-Orx<sub>B</sub> for Social Interaction/Preference (SIP) test, EC<sub>50</sub> = 0.13 nM for Orx<sub>2</sub>R; Tocris, Minneapolis, MN), and Vehicle, mixed in a 25% DMSO solution (75% artificial cerebrospinal fluid [aCSF; 8.59 g NaCl, 0.201 g KCl, 0.279 g, CaCl<sub>2</sub>, 0.16 g MgCl<sub>2</sub>, 0.124 g NaH<sub>2</sub>PO<sub>4</sub>, 0.199 g Na<sub>2</sub>HPO<sub>4</sub>/L H<sub>2</sub>O brought to a pH of 7.3]), were administered on Day 3 of the experimental plan (Fig. 2a). For subcutaneous (sc) pharmacological experiments, an Orx<sub>2</sub>R antagonist (MK-1064), an  $\alpha_2$  receptor antagonist (yohimbine), and vehicle (1:3, Saline to DMSO ratio) were similarly delivered on Day 3 (Fig. S2). Doses for drugs infused into the

BLA (0.1 nmol/300 nL/side for MK-1064 and [Ala<sup>11</sup>,D-Leu<sup>15</sup>]-OrxB; 10 nmol/300 nL/side for YNT-185) were selected and adjusted based on previous experiments from our laboratory [18, 19]. As we were unsure which dose of MK-1064 to use systemically on female mice, for sc studies we performed a dose response, where our high dose (1  $\mu$ mol) was chosen to be lower than that necessary to induce sleep in mice [20]. For our yohimbine control group, a dose of 5 mg/kg, which blocked escape in male mice [11], was used for pharmacological studies in females.

### **Shock-induced aggression (SIA)**

Classical Pavlovian conditioning was used to train male CD1 mice to behave aggressively toward female C57BL/6N mice. Male CD1 mice, investigating (specifically anogenital sniffing) female mice introduced into the males' home cage, were delivered a brief mild shock (1 mA, <1 s) to the rump region, resulting in intense aggression directed toward the female mice. Once aggression was displayed by the CD1, mice were separated. Typical interactions lasted under 5 min, as aggression was often immediately obtained through the pairing of the female with a mild shock. This procedure was repeated for 4 days prior to the use of the male CD1 mice in behavioral paradigms. In the actual experiments, CD1 mice trained to associate a female mouse (scent) with a mild shock, seldom required shock to display aggression. In cases where the CD1 mouse did not behave aggressively toward the female during experimentation, a mild shock was applied to produce aggressive behavior.

### **Behavioral paradigms**

The primary behavioral paradigm used in these studies utilized the SAM. The SAM involves a 4-day (5 min/day) experimental plan that starts with test animals (C57BL/6N mice) being introduced to an opaque cylinder in the center of an oval-shaped, open field arena (Fig. 1). An aggressor (male CD1 mouse) roams the arena outside of the cylinder. After a 30 s resting

period, a 5 s tone (conditioned stimulus, CS) and 10 s trace proceed the lifting of the cylinder divider allowing the aggressive CD1 mouse to attack (unconditioned stimulus, US) the test animal. Importantly, no test mouse encounters the same CD1 mouse twice throughout the 4-Day paradigm. In the SAM arena, two escape routes existing on opposite apical ends of the open field space allow the smaller test mouse to escape aggressive encounters. In male mice, the end of the second day marks a commitment to one of two stable behavioral phenotypes: Escape and Stay. This commitment allows for pharmacological intervention on Day 3, which may reverse the chosen phenotype. Anxiogenic drugs delay escape time and promote Stay behavior, whereas, anxiolytic drugs initiate escape behaviors in Stay mice. A purpose of the following studies was to investigate how female behavior in the SAM differs from that in males.

To explore female behavior with our SIA method, we exposed female C57BL/6N mice to several unique paradigms (Fig. S1). A group of female mice (N = 22) were presented with the standard 4-Day SAM paradigm. A separate cohort (N = 10) were introduced into a SAM-like setting, but without escape routes for four days (Inescapable Social Stress group). In preliminary trials, all females introduced into the 4-Day SAM escaped. We next tested an experimental plan in which a cohort of females (N = 16) was presented with a shorter SAM protocol (2-Day SAM group) to determine if Escape and Stay phenotypes could be differentiated in females. Finally, to see how elevated stress levels might affect SAM behavior, we tested a group of females (N = 20) that were presented with brief bouts of aggression (less than 1 min) in a male CD1 home cage for four days preceding the standard 4-Day SAM paradigm (Prior Stress + 4-Day SAM). A separate experimental design, involving both female (N = 41) and male (N = 17) mice, utilized the standard 4-Day SAM paradigm with sc drug administration on Day 3.

In all experiments, the behavioral paradigms were followed with the Social Interaction/Preference (SIP) and Fear Response tests as previously described [10, 18, 19, 21]. In brief, the SIP test involves introduction of a test mouse to a square (40 cm<sup>2</sup>) open field environment. An empty perforated jar (Novel target) is positioned alongside on wall and the test mouse is allowed to explore this area, including the Novel target, for 1.5 min. The mouse is briefly removed and the empty jar is replaced by an identical jar that contains an aggressive CD1 mouse (Social target) not previously used in preceding behavioral trials. Again, the test mouse is allowed to explore the environment and social target for 1.5 min. More time spent within 3 cm of the Social target compared to the Novel target is considered social preference behavior, and increased time spent in the corners when the Social target is social avoidance behavior. Fear Response is tested by placing test mice within the same cylinder divider used during daily fear conditioning (context, CS<sup>-</sup>) and measuring freezing for 30 s. Afterwards, freezing is measured during a 5 s tone and 10 s trace period (cue, CS<sup>+</sup>). Importantly, during the fear response test, no CD1 aggressor is present. At the end of the Fear Response test, mice were briefly anesthetized (5% isoflurane, 2 min) and rapidly decapitated. Trunk blood was spun down (5 min) in heparinized tubes and plasma was collected. Brains were extracted and flash frozen in cold isopentane on dry ice.

All behavioral paradigm was performed during the animals' awake period (Dark hours) under red light. Each behavioral trial was recorded using GoPro (Hero 7) cameras. Videos were analyzed using ANY-maze (version 6.0) software.

### **Estrous cycle**

Vaginal lavages were performed daily based on a previously described protocol [22] with modifications. In brief, 50 µL of distilled water (dH<sub>2</sub>O) was gently flushed 3-6 times into the

vaginal cavity and the contents were placed onto microscope slides. Samples were dried under a heat lamp and stained for 1 min with Cresyl violet (0.1 g/100 mL dH<sub>2</sub>O), 1 min dH<sub>2</sub>O twice, and air dried. The samples were viewed under a microscope and the stages of estrous were identified by the abundance of three distinct cell types (Fig. S3): proestrus samples included mostly round nucleated epithelial cells, estrus samples were characterized by dense clusters of cornified squamous epithelial cells, metestrus samples contained predominantly leukocytes with few cornified epithelial cells, and diestrus samples involved a mix of all cell types and was distinguished from metestrus by the presence of nucleated epithelial cells. As our preliminary results do not definitively expose whether the stage of the estrous cycle might impact stress-related behaviors, for drug treatment studies (Fig. S2) we only started female animals through behavioral the paradigm when they were in proestrus.

#### ***In situ* hybridization - RNAscope**

Sections of fresh frozen brains (coronal; 20 µm; relative to bregma AP -1.40 to -2.0) were placed in cold (4°C) 10% formalin for 20 min and subsequently washed (2x for 1 min) in 1x phosphate buffer solution (PBS), before dehydration with ethanol (50% x 1, 70% x 1, and 100% x 3; 5 min each with the final ethanol being kept at -20°C overnight). The following day, proteins were digested using a protease treatment and rinsed with dH<sub>2</sub>O. Brain sections were incubated for two hours in RNAscope (Advanced Cell Diagnostics, Newark, CA) probes (*HCRTR2*, Cat. No. 460881) in a hybridization oven (ACD HyBEZ II oven) set to 40°C. Fluorophores were linked to probes and signaling was enhanced through application of a series of amplification buffers (RNAscope Fluorescent Multiplex Detection Reagents). Finally, tissue was briefly stained with DAPI (20 sec) and coverslipped. Image acquisition was performed fluorescence microscope (Nikon A1; 10x/0.30 Plan Fluor and 20x/0.75 Plan Apo VC Nikon objectives) and NIS Elements



software. The BLA was identified from images and analyzed using QuPath 3.0 and ImageJ programs.

## **Statistics**

Experimental design and analyses were based on *a priori* hypotheses. For comparisons that involved SAM (and other behavioral paradigm) trials across days, SIP test results for Novel and Social target, and Fear Response test (CS<sup>-</sup> & CS<sup>+</sup>) analyses, we utilized two-way repeated measures ANOVA. For changes occurring across treatment/experimental groups we applied a one-way ANOVA. Non-stressed cage controls were added for comparisons of corticosterone levels, *in situ* hybridization results, and home cage mobility measurements, in which one-way ANOVA was used. Assessments between two treatments/experimental conditions were performed by Student's two-tailed t-tests. To determine differences in percentage of escape or estrous cycle stage, chi-square and Fischer Exact statistical analyses were utilized. Each mouse provided a single unit for analyses involving *a priori* hypotheses. The five assumptions of parametric statistics were applied to the data, transformed when necessary, compared to non-parametric analyses, and graphed in raw form. Analyses for parametric and non-parametric statistics were used along with an examination for multiple comparisons applying the Holm-Sidak method. If the statistical analyses match, as they do for the data herein, we report the parametric results without  $\alpha$  adjustment [23-28]. 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).

## RESULTS

### Pharmacological manipulation of intra-BLA Orx<sub>2</sub>R activity modifies stress-related behavior

The Stress Alternatives Model (SAM) is a 4-day social stress paradigm in which male mice diverge into distinguishable behavioral phenotypes, by actively avoiding (Escape) or accepting (Stay) confrontation (Fig. 1). As the BLA appears to be important for SAM-promoted phenotype development [18], we targeted this area (intra-BLA) with Orx<sub>2</sub>R-targeting drugs to see if we could influence phenotype-specific behaviors in Social Interaction/Preference (SIP) and Fear Response tests (Figs. 2A, B, C). In both Escape and Stay male mice, Orx<sub>2</sub>R antagonism reduced social preference (Figs. 2D, E; Treatment Effect:  $F_{2,30} = 8.1, p \leq 0.002$ ; Target Effect:  $F_{1,30} = 22.7, p < 0.001$ ; Social, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{19} = 2.4, p \leq 0.02$ ; Vehicle, Novel vs Social,  $t_{10} = 2.9, p \leq 0.02$ ; Treatment Effect:  $F_{2,14} = 10.4, p \leq 0.002$ ; Target Effect:  $F_{1,14} = 20.0, p < 0.001$ ; Interaction Effect:  $F_{2,14} = 15.2, p < 0.001$ ; Social, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{10} = 2.5, p \leq 0.02$ ). Agonism of Orx<sub>2</sub>R increased preference for both the novel and social target in Escape and Stay mice (Figs. 2D, E; Escape: Novel, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_{21} = 2.1, p \leq 0.05$ ; Novel, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{20} = 4.1, p < 0.001$ ; Social, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{20} = 2.6, p \leq 0.016$ ; Orx<sub>2</sub>R Agonist, Novel vs Social,  $t_{11} = 3.8, p \leq 0.003$ ; Stay: Novel, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 3.2, p \leq 0.009$ ; Social, Vehicle vs Orx<sub>2</sub>R agonist,  $t_7 = 2.9, p \leq 0.01$ ; Social, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 6.1, p < 0.001$ ; Orx<sub>2</sub>R Agonist, Novel vs Social,  $t_4 = 14.3, p < 0.001$ ). While Orx<sub>2</sub>R antagonism increased social avoidance in Escape mice (Fig. 2F; Treatment Effect:  $F_{2,30} = 4.9, p \leq 0.01$ ; Social, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{19} = 2.5, p \leq 0.02$ ; Social, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{20} = 2.7, p \leq 0.01$ ), there was no effect in Stay animals (Fig. 2G). Curiously,

both Orx<sub>2</sub>R antagonism and agonism reduced cued fear freezing and eliminated the learning response (more freezing post-tone than pre-tone) in Escape mice (Fig. 2H; Treatment Effect:  $F_{2,13} = 5.9, p \leq 0.02$ ; CS Effect:  $F_{1,13} = 9.6, p \leq 0.008$ ; Interaction Effect:  $F_{2,13} = 4.6, p \leq 0.03$ ; Post-Tone, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{11} = 2.5, p < 0.001$ ; Post-Tone, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_8 = 3.7, p < 0.001$ ; Vehicle, Pre- vs Post-Tone,  $t_7 = 4.8, p < 0.001$ ). However, intra-BLA agonism of Orx<sub>2</sub>R diminished both contextual and cued fear freezing in Stay animals (Fig. 2I; Treatment Effect:  $F_{2,22} = 7.2, p \leq 0.004$ ; CS Effect:  $F_{1,22} = 37.2, p < 0.001$ ; Pre-Tone, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_{16} = 2.3, p \leq 0.03$ ; Pre-Tone, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 2.9, p \leq 0.02$ ; Post-Tone, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_{16} = 3.9, p < 0.001$ ; Post-Tone, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 3.6, p < 0.001$ ; Vehicle, Pre- vs Post-Tone,  $t_{13} = 4.8, p < 0.001$ ; Orx<sub>2</sub>R Antagonist, Pre- vs Post-Tone,  $t_8 = 4.7, p < 0.001$ ; Orx<sub>2</sub>R Agonist, Pre- vs Post-Tone,  $t_5 = 2.9, p \leq 0.04$ ).

### **Orx<sub>2</sub>R expression is higher in female vs male BLA**

In the BLA, Orx<sub>2</sub>R are expressed at low levels in male mice [18]. Male and female mice differ in the amount of BLA cells that express Orx<sub>2</sub>R mRNA (*HCRTR2*) with females having higher levels (>20%) compared to male (~10%) mice (Fig. 3;  $t_{11} = 3.5, p \leq 0.005$ ).

### **Shock-induced aggression (SIA) initiates attacks toward female mice**

In order to investigate the effects of social stress on female populations, we developed a method for producing male CD1 retired breeder mice that act aggressively toward female mice (Fig. 4A). As males investigated (including anogenital sniffing) female mice, a mild shock (1 mA, <1 s) was applied to the rump region, which resulted in intense attacks of female conspecifics. The number of shocks necessary to promote aggression varies (average = 2.85). However, all males introduced to our protocol successfully exhibited aggression toward female mice (Fig. 4B).

Surprisingly, all females subjected to the SAM choose to avoid social aggression by the end of Day 3, which is different from males, in which ~45% escape (Fig. 4C; Day 1:  $X^2 = 18.6, p < 0.001$ ; Day 2:  $X^2 = 16.3, p < 0.001$ ; Day 3 & 4:  $X^2 = 19.5, p < 0.001$ ). However, manipulating the stress state of females by exposing them to brief bouts of social aggression for 4-Days prior to SAM exposure, the percentage of Escape mice can be reduced (Fig. 4D; Day 1:  $X^2 = 4.9, p \leq 0.03$ ; Day 2:  $X^2 = 3.2, p \leq 0.04$ ). The escape latency of males of the Escape phenotype mimics that of females, except for Day 1 where male mice utilize the escape routes at a slower rate (Fig. 4E; Day Effect:  $F_{3,141} = 51.8, p < 0.001$ ; Day 1:  $t_{47} = 2.4, p \leq 0.02$ ). Interestingly, females of the Prior Stress + 4-Day SAM experimental conditions, display enhanced latency to escape times on Days 1-3; however, the learning profile, as indicated by the curve of the plot, remains similar (Fig. 4F; Paradigm Effect:  $F_{1,120} = 12.9, p < 0.001$ ; Day Effect:  $F_{3,120} = 51.0, p < 0.001$ ; Day 1:  $t_{40} = 4.3, p < 0.001$ ; Day 2:  $t_{40} = 2.8, p < 0.001$ ; Day 2:  $t_{40} = 2.6, p \leq 0.01$ ). Importantly, while males of the Stay phenotype encounter greater levels of aggression in the SAM compared to both females and Escape males (Phenotype Effect:  $F_{2,192} = 42.9, p < 0.001$ ; Day Effect:  $F_{3,192} = 5.1, p \leq 0.002$ ), there is no difference in aggression received when comparing female and males that avoid social aggression (Fig. 4G). Further, no differences in the amount of aggression received exist between females exposed only to the SAM or those that encountered prior stress before the SAM; however, females presented with an inescapable social stress environment using SIA experience the greatest amount of aggression (Fig. 4H; Paradigm Effect:  $F_{2,147} = 32.4, p < 0.001$ ), which resembles that of Stay male mice.

### **Social preference and avoidance in female mice is affected by stress state**

As male mice of the Escape phenotype tend to show more social preference and less avoidance compared to Stay animals in the SIP test [19] (Figs. 2D-G), we wanted to examine how females

subjected to various SIA paradigms (Fig. S1) would behave in this context (Fig. 5). Female mice exposed to the standard 4-Day SAM paradigm (Fig. S1) exhibited social preference (Fig. 5A; Paradigm Effect:  $F_{3,64} = 41.3, p < 0.001$ ; Target Effect:  $F_{1,64} = 37.6, p < 0.001$ ; Interaction Effect:  $F_{3,64} = 5.8, p < 0.001$ ; 4-Day SAM, Novel vs Social,  $t_{21} = 7.1, p < 0.001$ ) and reduced social avoidance (Fig. 5B; Paradigm Effect:  $F_{3,64} = 9.4, p < 0.001$ ; Target Effect:  $F_{1,64} = 12.2, p < 0.001$ ; Interaction Effect:  $F_{3,64} = 3.3, p \leq 0.03$ ; 4-Day SAM, Novel vs Social,  $t_{21} = 3.0, p \leq 0.007$ ). Animals subjected to inescapable social stress, a 2-Day SAM paradigm, or prior stress before 4-Day SAM exposure displayed reduced social preference (Fig. 5A; Novel, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 4.0, p < 0.001$ ; Novel, 4-Day SAM vs 2-Day SAM,  $t_{36} = 6.6, p < 0.001$ ; Novel, 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 7.1, p < 0.001$ ; Social, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 5.7, p < 0.001$ ; Social, 4-Day SAM vs 2-Day SAM,  $t_{36} = 9.8, p < 0.001$ ; Social, 4-Day SAM vs Social Stress + 4-Day SAM,  $t_{40} = 10.3, p < 0.001$ ; Social, Inescapable Social Stress vs 2-Day SAM,  $t_{26} = 2.6, p \leq 0.01$ ; Social, Inescapable Social Stress vs Prior Stress + 4-Day SAM,  $t_{28} = 2.6, p \leq 0.01$ ; Inescapable Social Stress, Novel vs Social,  $t_9 = 2.6, p \leq 0.01$ ) and enhanced social avoidance (Fig. 5B; Novel, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 3.7, p < 0.001$ ; Novel, 4-Day SAM vs 2-Day SAM,  $t_{36} = 2.7, p \leq 0.01$ ; Novel, 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 3.5, p < 0.001$ ; Social, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 4.8, p < 0.001$ ; Social, 4-Day SAM vs 2-Day SAM,  $t_{36} = 3.4, p < 0.001$ ; Social, 4-Day SAM vs Social Stress + 4-Day SAM,  $t_{40} = 5.3, p < 0.001$ ; Inescapable Social Stress, Novel vs Social,  $t_9 = 2.0, p \leq 0.05$ ; 2-Day SAM, Novel vs Social,  $t_{15} = 2.1, p \leq 0.04$ ; Prior Stress + 4-Day SAM, Novel vs Social,  $t_{19} = 3.4, p < 0.001$ ).

As hormonal changes associated with the estrous cycle may further modify stress-related behaviors [32], we investigated whether behaviors exhibited in the SIP test were related to the

stage of the estrous cycle (Figs. 5C-D, S2). Mice in proestrus exhibited the highest amount of social preference (Fig. 5C; Stage Effect:  $F_{3,49} = 6.7, p < 0.001$ ; Target Effect:  $F_{1,49} = 34.8, p < 0.001$ ; Interaction Effect:  $F_{3,49} = 7.6, p < 0.001$ ; Novel, Proestrus vs Diestrus,  $t_{28} = 3.1, p \leq 0.005$ ; Social, Proestrus vs Estrus,  $t_{21} = 3.5, p < 0.001$ ; Social, Proestrus vs Metestrus,  $t_{18} = 4.5, p < 0.001$ ; Social, Proestrus vs Diestrus,  $t_{28} = 5.7, p < 0.001$ ; Proestrus, Novel vs Social,  $t_9 = 5.9, p < 0.001$ ; Estrus, Novel vs Social,  $t_{12} = 3.5, p < 0.001$ ), while animals in diestrus showed the greatest amount of social avoidance (Fig. 5D; Interaction Effect:  $F_{3,49} = 3.1, p \leq 0.04$ ; Social, Proestrus vs Diestrus,  $t_{28} = 3.1, p \leq 0.002$ ; Diestrus, Novel vs Social,  $t_{19} = 3.0, p \leq 0.004$ ).

### **Fear response in female mice is impacted by stress responsive state**

While male mice of the Stay phenotype display enhanced contextual and cued fear freezing behavior relative to both male Escape animals and female mice (all of which escape in the SAM paradigm), male Escape animals exhibit enhanced cued fear freezing compared to females (Fig. 6A; Phenotype Effect:  $F_{2,82} = 19.9, p < 0.001$ ; CS Effect:  $F_{1,82} = 72.5, p < 0.001$ ; Pre-Tone, Male Stay vs Male Escape,  $t_{61} = 3.5, p < 0.001$ ; Pre-Tone, Male Stay vs Female,  $t_{56} = 4.8, p < 0.001$ ; Post-Tone, Male Stay vs Male Escape,  $t_{61} = 2.5, p \leq 0.02$ ; Post-Tone, Male Stay vs Female,  $t_{56} = 6.1, p < 0.001$ ; Post-Tone, Male Escape vs Female,  $t_{57} = 3.5, p < 0.001$ ). Importantly, both phenotypes from male mice as well as female mice show cued fear learning, characterized by elevated freezing post-tone compared to pre-tone (Fig. 6A; Male Stay,  $t_{35} = 5.8, p < 0.001$ ; Male Escape,  $t_{26} = 6.5, p < 0.001$ ; Female,  $t_{21} = 2.9, p \leq 0.005$ ). Female mice presented with our various stress paradigms (Fig. S1) all display cued fear learning (Fig. 6B; Paradigm Effect:  $F_{3,64} = 8.1, p < 0.001$ ; CS Effect:  $F_{1,64} = 87.6, p < 0.001$ ; Interaction Effect:  $F_{3,64} = 5.1, p \leq 0.003$ ; 4-Day SAM, Pre-Tone vs Post-Tone,  $t_{21} = 2.6, p \leq 0.01$ ; Inescapable Social Stress, Pre-Tone vs Post-Tone,  $t_9 = 6.2, p < 0.001$ ; 2-Day SAM, Pre-Tone vs Post-Tone,  $t_{15} = 5.2, p < 0.001$ ; Prior

Stress + 4-Day SAM, Pre-Tone vs Post-Tone,  $t_{19} = 4.1, p < 0.001$ ). However, mice introduced to the experiments involving inescapable social stress, 2-Day SAM, and prior stress before 4-Day SAM demonstrate increased cued fear relative to standard 4-Day SAM animals (Fig. 6B; 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 5.5, p < 0.001$ ; 4-Day SAM vs 2-Day SAM,  $t_{36} = 3.9, p < 0.001$ ; 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 3.9, p < 0.001$ ). Further, those mice in the prior stress + 4-Day SAM experimental group display enhanced contextual fear freezing (Fig. 6B; 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 3.4, p \leq 0.002$ ). Interestingly, mice in proestrus do not display fear learning and exhibit reduced cued freezing relative to animals in metestrus and diestrus (Fig. 6C; Stage Effect:  $F_{3,49} = 4.3, p \leq 0.009$ ; CS Effect:  $F_{1,49} = 30.2, p < 0.001$ ; Post-Tone, Proestrus vs Metestrus,  $t_{18} = 2.7, p \leq 0.008$ ; Post-Tone, Proestrus vs Diestrus,  $t_{28} = 3.7, p < 0.001$ ; Estrus, Pre-Tone vs Post-Tone,  $t_{12} = 2.9, p \leq 0.005$ ; Metestrus, Pre-Tone vs Post-Tone,  $t_9 = 2.6, p \leq 0.01$ ; Diestrus, Pre-Tone vs Post-Tone,  $t_{19} = 5.2, p < 0.001$ ).

### **Systemic administration of an Orx<sub>2</sub>R antagonist reveals stress-related phenotypes in female mice**

As it is unclear whether the hormonal fluctuations associated with stages of the estrous cycle are responsible for shifts in stress-related behaviors, we controlled for potential influence by selecting only female animals in proestrus for systemic (subcutaneous) pharmacological experiments (Figs. 7-9, S3). Pharmacological treatments, while not statistically significant, seemed to shift the stage of the estrous cycle (Fig. S3). Treatment with yohimbine, an  $\alpha_2$  adrenergic receptor antagonist known for anxiogenic effects, increased escape latency in females on the day of drug administration (Fig. 7B; Day Effect:  $F_{3,108} = 17.2, p < 0.001$ ; Day 3, Vehicle vs Yohimbine,  $t_{11} = 4.9, p < 0.001$ ; Day 3, MK-1064 – 1  $\mu\text{mol}$  vs Yohimbine,  $t_{12} = 5.8, p < 0.001$ ), an effect that is similarly seen in male mice [11]. While minor deviations in latency to

escape were observed with low doses of an Orx<sub>2</sub>R antagonist (300 nmol & 30 nmol), there was no significant differences compared to vehicle-treated animals (Fig. 7B). However, in the 30 nmol-treated group, we observed disparities in how female mice responded to drug treatment, where we defined a distinct separation phenotypes: Slow Escape (Escape<sup>S</sup>) and Fast Escape (Escape<sup>F</sup>). The females in the Escape<sup>S</sup> classification exhibited delayed escape behavior similar to that observed in our yohimbine control mice (Fig. 7C; Treatment Effect:  $F_{3,66} = 3.9, p \leq 0.02$ ; Day Effect:  $F_{3,66} = 11.0, p < 0.001$ ; Day 3, Vehicle vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_9 = 2.6, p \leq 0.03$ ; Day 3, MK-1064 – 30 nmol – Escape<sup>F</sup> vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_{11} = 3.0, p \leq 0.01$ ). Escape males administered the same dose (30 nmol) of the Orx<sub>2</sub>R antagonist did not display the phenotype emergence observed in female mice (Fig. 7D), however, males already display distinct phenotypes (Fig. 4C). Further, on the day of drug delivery (Day 3), home cage locomotion was impaired in yohimbine-treated mice, but not animals given MK-1064 at varying doses (Fig. S4A;  $F_{5,38} = 3.0, p \leq 0.021$ ; Cage Control vs Yohimbine,  $t_8 = 6.3, p < 0.001$ ; Vehicle vs Yohimbine,  $t_{11} = 4.6, p < 0.001$ ; MK-1064: 1  $\mu$ mol,  $t_{12} = 3.7, p \leq 0.003$ ; 300 nmol,  $t_{13} = 3.3, p \leq 0.006$ ; 30 nmol,  $t_{18} = 3.1, p \leq 0.006$ ). Importantly, home cage locomotion was restored to normal levels in the yohimbine treatment group 24 hours after treatment (Fig. S4B)

### **Systemic delivery of Orx<sub>2</sub>R antagonist reduces social preference**

While female mice administered vehicle treatment, as well as low doses of an Orx<sub>2</sub>R antagonist (300 nmol & 30 nmol), displayed social preference in the SIP test, yohimbine-treated animals exhibited a decrease in social preference (Fig. 8A; Target Effect:  $F_{1,36} = 27.8, p < 0.001$ ; Social, Vehicle vs Yohimbine,  $t_{11} = 3.1, p \leq 0.003$ ; Vehicle, Novel vs Social,  $t_5 = 3.8, p < 0.001$ ; MK-1064 – 300 nmol, Novel vs Social,  $t_7 = 2.1, p \leq 0.045$ ; MK-1064 – 30 nmol, Novel vs Social,  $t_{12} = 3.0, p \leq 0.005$ ). However, mice of the Escape<sup>S</sup> phenotype displayed a reduction in social



preference similar to that observed after yohimbine treatment (Fig. 8B; Target Effect:  $F_{1,16} = 24.1$ ,  $p < 0.001$ ; Social, Vehicle vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_9 = 2.7$ ,  $p \leq 0.01$ ; MK-1064 – 30 nmol – Escape<sup>F</sup>, Novel vs Social,  $t_7 = 2.8$ ,  $p \leq 0.01$ ). Only vehicle-treated mice, similar to untreated females (Fig. 5B), saw a reduction in social avoidance (Figs. 8C, D;  $t_5 = 2.5$ ,  $p \leq 0.05$ ).

### **Fear response is enhanced in females with Orx2R antagonism**

All treatment groups exhibited fear learning behavior (Fig. 9A; CS Effect:  $F_{1,36} = 42.0$ ,  $p < 0.001$ ; Vehicle, Pre-Tone vs Post-Tone,  $t_5 = 2.5$ ,  $p \leq 0.05$ ; MK-1064 – 1  $\mu$ mol, Pre-Tone vs Post-Tone,  $t_6 = 2.4$ ,  $p \leq 0.02$ ; MK-1064 – 300 nmol, Pre-Tone vs Post-Tone,  $t_7 = 2.5$ ,  $p \leq 0.02$ ; MK-1064 – 30 nmol, Pre-Tone vs Post-Tone,  $t_{12} = 4.7$ ,  $p < 0.001$ ; Yohimbine, Pre-Tone vs Post-Tone,  $t_6 = 4.2$ ,  $p < 0.001$ ); however, low dose (30 nmol) Orx2R antagonism promoted increased contextual freezing behavior while yohimbine-treated female mice demonstrated enhanced freezing to the cue (Fig. 9A; Pre-Tone, MK-1064 – 1  $\mu$ mol vs MK-1064 – 30 nmol,  $t_{18} = 2.4$ ,  $p \leq 0.03$ ; Post-Tone, Vehicle vs Yohimbine,  $t_{11} = 2.5$ ,  $p \leq 0.03$ ). After low dose Orx2R antagonist treatment, mice exhibited the Escape<sup>S</sup> phenotype experienced elevated cued freezing compared to vehicle-treated control animals, an effect mimicking yohimbine treatment (Fig. 9B; CS Effect:  $F_{3,22} = 44.7$ ,  $p < 0.001$ ; Post-Tone, Vehicle vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_9 = 2.8$ ,  $p \leq 0.009$ ; MK-1064 – 30 nmol – Escape<sup>S</sup>, Pre-Tone vs Post-Tone,  $t_4 = 4.0$ ,  $p < 0.001$ ; MK-1064 – 30 nmol – Escape<sup>F</sup>, Pre-Tone vs Post-Tone,  $t_7 = 3.5$ ,  $p \leq 0.002$ ).

### **HCRTR2 expression is distinct in pharmacologically induced phenotypes**

The number of cells in the BLA expressing Orx2R mRNA is higher in Escape<sup>S</sup> mice compared to Escape<sup>F</sup> animals (Fig. 10;  $t_8 = 2.3$ ,  $p \leq 0.048$ ). This suggests the presence of a physiological difference in these phenotypes, which may define the stress responsive state.

## DISCUSSION

Pro- and anti-stress neurocircuitries are divided functionally into prelimbic (PrL) prefrontal cortical connections to anterior BLA and infralimbic (IL) connections to posterior regions (pBLA), which correspond to non-overlapping cells with genetic markers *Rspo2* (coding for the protein R-spondin 2) and *Ppp1r1b* (coding for the protein DARPP-32) (29, 31). In the male BLA, Orx<sub>1</sub>R are found in a minority of cells, which are primarily glutamatergic (CamKII-expressing) pyramidal cells [18]. Balancing pro- and anti-stress systems appears to involve BLA microcircuits, but the relationship of Orx<sub>2</sub>R in these circuits is largely unexplored, with related studies providing only a glimpse into this complicated system. For example, oral administration of an Orx<sub>2</sub>R antagonist in a clinical setting improves sleep, but in the process also produces antidepressive effects [33]. In contrast, systemic brain delivery (intracerebroventricular injection) of Orx<sub>2</sub>R agonists produces anxiolytic and antidepressive effects in an animal model of social defeat and avoidance [19]. Additionally, Orx<sub>2</sub>R null mice display enhanced behavioral despair [34] and reduced contextual freezing [35], as Orx<sub>2</sub>R knockout decreases stress responsivity [36]. While inhibition of Orx<sub>2</sub>R in the BLA diminished cued freezing in male Escape mice (Fig. 2H), we have previously revealed a possible anxiogenic outcome of blocking receptor function [21]. Inhibiting Orx<sub>1</sub>R reduces fear/panic-induced freezing [37-40], however, Orx<sub>2</sub>R antagonism directed at the BLA appears to eliminate fear learning in Escape mice (Fig. 2H) and agonism reduces fear freezing in Stay mice (Fig. 2I), suggesting a phenotype-dependent effect. This response may further be specific to the BLA, as Orx<sub>2</sub>R activity in the nucleus accumbens shell, PrL, and paraventricular thalamus may enhance anxious behavior [41-44]. These collective qualities of Orx<sub>2</sub>R action suggest this receptor subtype, unlike Orx<sub>1</sub>R activity which is consistently linked to pro-stress outcomes [37, 38, 45],

differentially regulates behavioral responses in a way that reflects the initial, possibly innate, stress state [46].

In the BLA, Orx<sub>2</sub>R expressing cells are more abundant in females compared to males (Fig. 3), and female mice exposed to the SAM arena always escape and do so faster than males on Day 1 (Figs. 4E). Further, females show greater social preference and diminished fear responses compared to males (Figs. 5A, 6A). These sexually dimorphic responses may be altered by manipulating the female stress state (Figs. 5A-B, 6B), suggesting, like males [10, 11, 18, 19], SAM-induced escape behavior in females is linked to anti-stress outcomes.

We suspected these behavioral differences in females and males might be associated with Orx<sub>2</sub>R function, and attempted to manipulate escape-related behaviors by pharmacologically inhibiting Orx<sub>2</sub>R activity. In our dose response, the highest concentration (1  $\mu$ mol) of MK-1064, selected to be slightly under the amount necessary to promote sleep [20], produced minimal changes to female behavior; however, the lowest dose (30 nmol) modified behaviors more noticeably (Figs. 8, 9). Closer examination revealed a divergence in behavioral outcomes after treatment of low dose MK-1064 (Fig. 7C): animals exhibiting delayed escape (Escape<sup>S</sup>) and mice showing normal escape (Escape<sup>F</sup>). Female mice defined as Escape<sup>S</sup> animals further displayed pro-stress responses, which mimicked those of the anxiogenic  $\alpha_2$  receptor antagonist yohimbine, in SIP and Fear Response tests (Figs. 8B, 9B). We postulate the prominent effect of the lowest dose of MK-1064 to be a result of enhanced biased signaling of Orx<sub>2</sub>R, over Orx<sub>1</sub>R, as a result of limiting the drug load. Interestingly, this low dose of Orx<sub>2</sub>R had no effect on male Escape mice, further highlighting sex differences in stress behavioral responses.

The orexin system is plastic, and the number of hypothalamic cells that produce orexins fluctuate diurnally [47] and during dependency [48, 49]. We demonstrate a mechanism for

potential plasticity of Orx<sub>2</sub>R-expressing cells in the BLA that may define an animals' stress responsive state as Escape<sup>S</sup> mice have more of these cells (Fig. 10). Importantly, it is unclear from the results reported here if the higher expression of *HCRTR2*-positive cells in Escape<sup>S</sup> mice precedes drug administration. If this were true, however, it would establish a neurophysiological difference that may explain phenotype emergence after drug administration. In males, antagonism of intra-BLA Orx<sub>2</sub>R in Escape animals lowers cued freezing behavior (Fig. 2H); however, male Escape mice have higher *HCRTR2* expression in the BLA compared to Stay animals [18]. In females, high Orx<sub>2</sub>R-expressing cells appears to be the stable state (Fig. 3); however, phenotype emergence promotes neurophysiological adaptations and plasticity within the BLA-contained orexin system. If this is true, higher *HCRTR2*-expressing cells in the BLA acts as a homeostatic mechanism to help establish balance to counteract a bias in pro-stress signaling.

## **Conclusions**

Shifts in microcircuits regulate stress responsivity and higher reactive states (Stay). In males, these stress responsive states are modified by Orx<sub>2</sub>R activity in the BLA, where sex-defined differences in intra-BLA *HCRTR2* may help explain sexual dimorphism in stress-induced behaviors. By manipulating Orx<sub>2</sub>R activity in females, behavioral phenotypes emerge. These phenotypic differences are further defined by the number of *HCRTR2*-expressing cells in the BLA. Together, these results suggest the balance of Orx<sub>2</sub>R activity in the BLA is important for mediating stress responsivity in both males and females.

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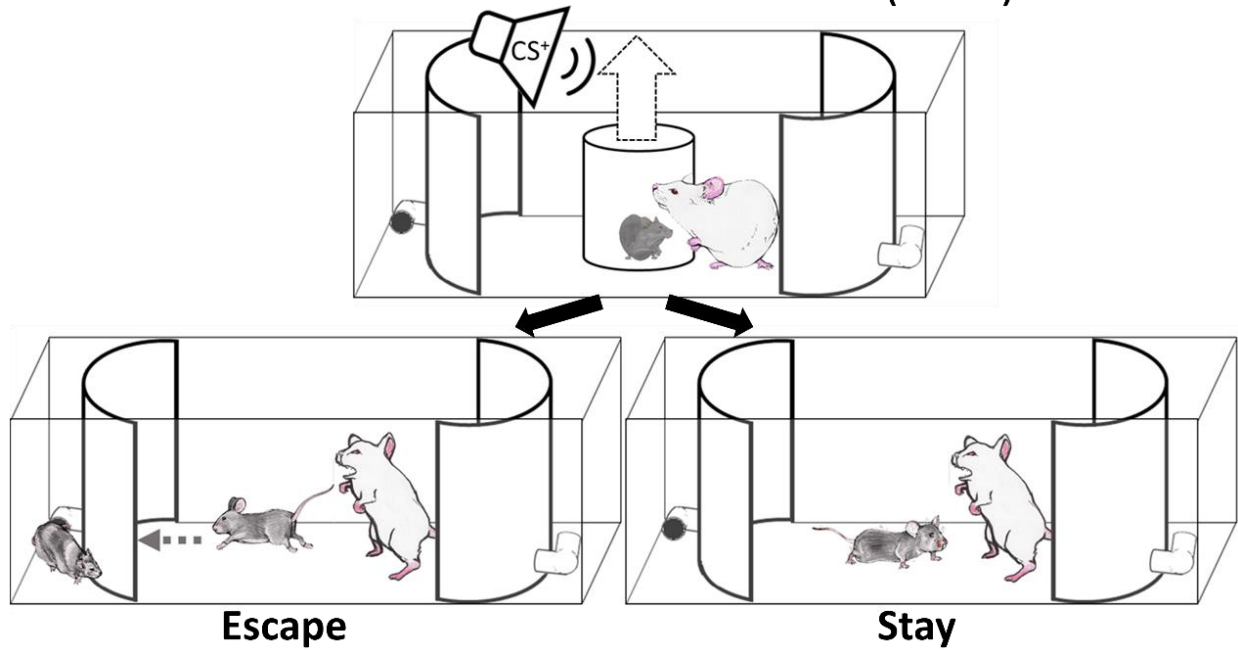
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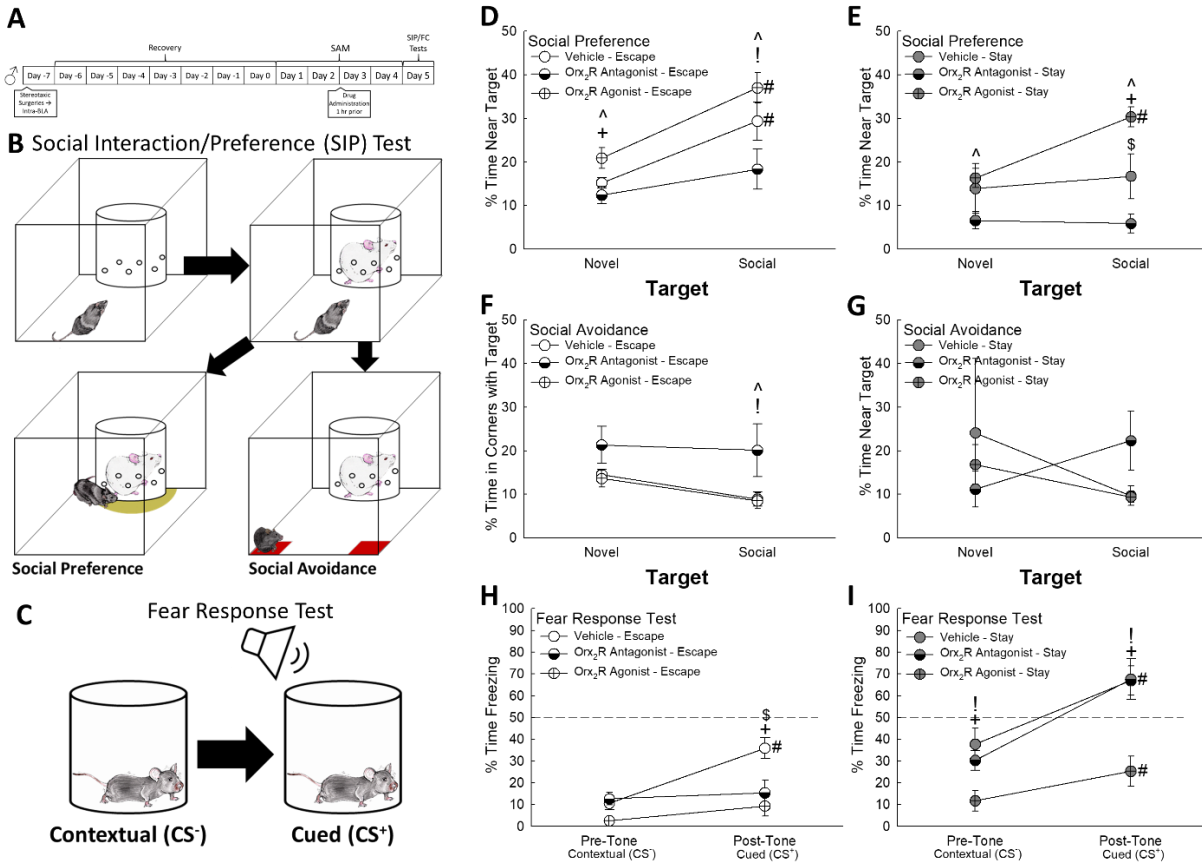
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## Stress Alternatives Model (SAM)

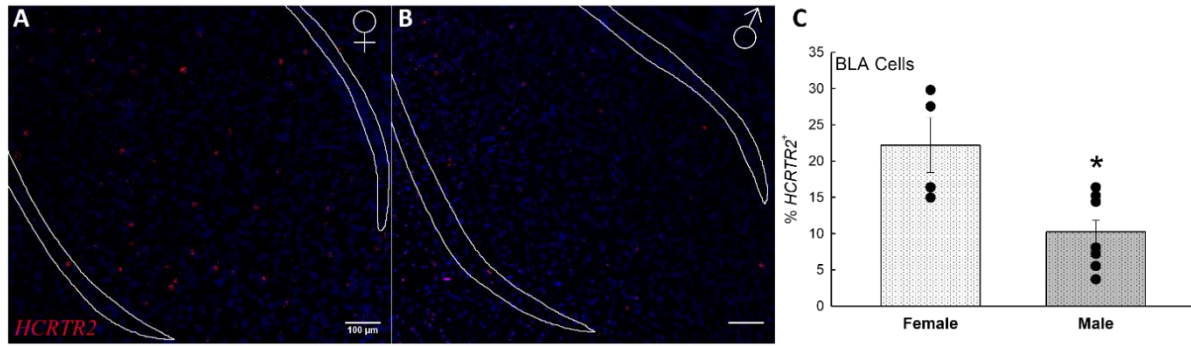


**Figure 1.** The Stress Alternatives Model (SAM) is a 4-Day social stress paradigm in which mice are conditioned (Tone, CS<sup>+</sup>) to social aggression (US) and commit to a behavioral phenotype by the end of Day 2: Escape (active avoidance of social aggression) and Stay (accepting confrontation from aggressor).

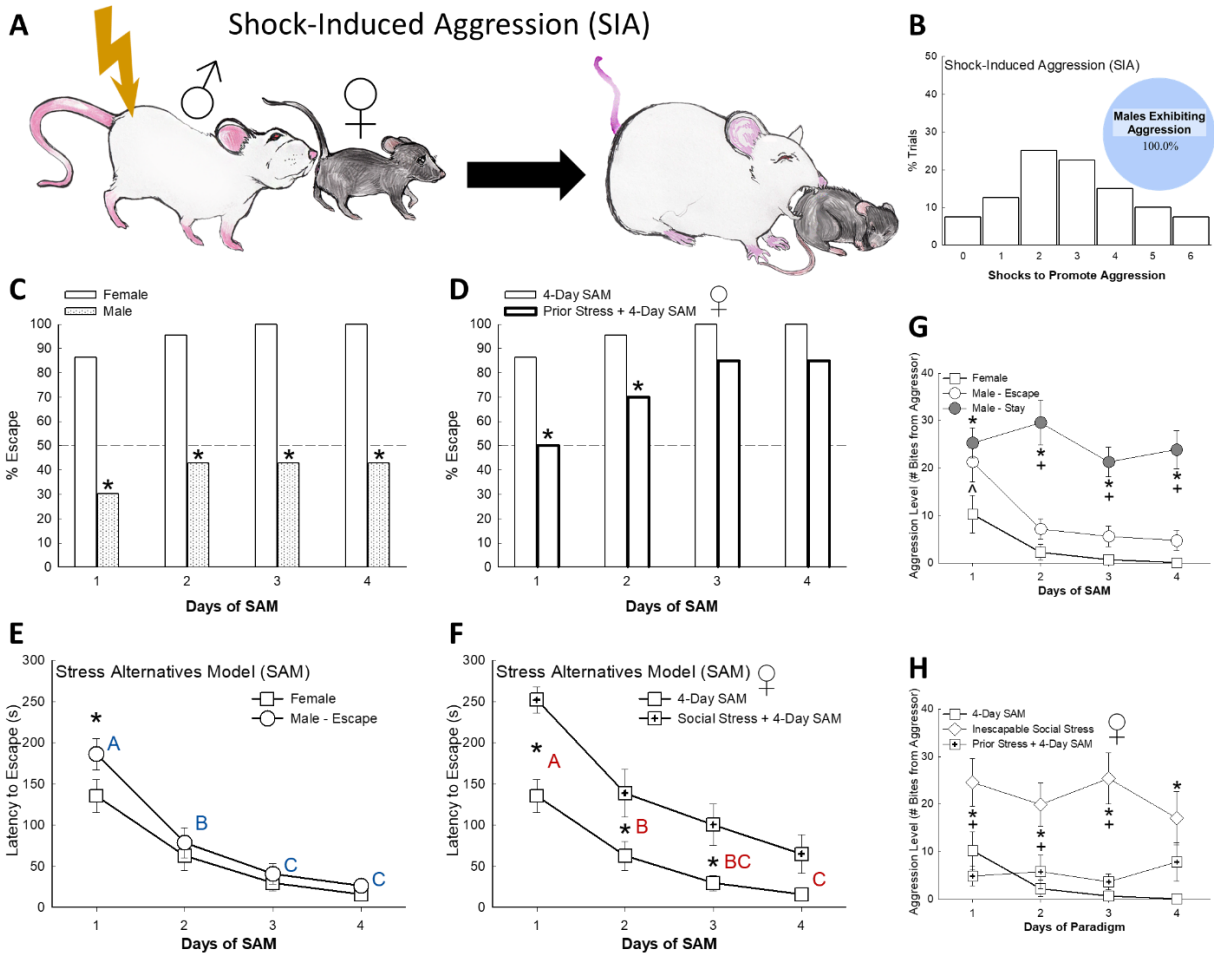


**Figure 2.** Administration of intra-BLA Orx<sub>2</sub>R-targeting drugs alters stress-related behavior in SIP and Fear Response tests. (A) Experimental design for experiment pharmacologically targeting male intra-BLA Orx<sub>2</sub>R. (B) Social Interaction/Preference (SIP) test measures social preference (bottom left) and avoidance (bottom right). (C) Fear Response test indicates if fear learning takes place and whether fear responses are observed in response to the context (CS<sup>-</sup>) or cue (CS<sup>+</sup>). (D) Escape mice treated with an Orx<sub>2</sub>R antagonist exhibit reduced social preference (n = 33, Treatment Effect:  $F_{2,30} = 8.1, p \leq 0.002$ ; Target Effect:  $F_{1,30} = 22.7, p < 0.001$ ; Novel, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_{21} = 2.1, +p \leq 0.05$ ; Novel, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{20} = 2.6, ^*p \leq 0.016$ ; Social, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{19} = 2.4, !p \leq 0.02$ ; Social, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{20} = 4.1, ^*p < 0.001$ ; Vehicle, Novel vs Social,  $t_{10} = 2.9, #p \leq 0.02$ ; Orx<sub>2</sub>R Agonist, Novel vs Social,  $t_{11} = 3.8, #p \leq 0.003$ ). (E) In Stay mice, social preference is enhanced after Orx<sub>2</sub>R agonist treatment and reduced after administration of an Orx<sub>2</sub>R antagonist (n = 17, Treatment Effect:  $F_{2,14} = 10.4, p \leq 0.002$ ; Target Effect:  $F_{1,14} = 20.0, p < 0.001$ ; Interaction Effect:  $F_{2,14} = 15.2, p < 0.001$ ; Novel, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 3.2, ^*p \leq$

0.009; Social, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{10} = 2.5$ ,  $\$p \leq 0.02$ ; Social, Vehicle vs Orx<sub>2</sub>R agonist,  $t_7 = 2.9$ ,  $+p \leq 0.01$ ; Social, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 6.1$ ,  $\wedge p < 0.001$ ; Orx<sub>2</sub>R Agonist, Novel vs Social,  $t_4 = 14.3$ ,  $\#p < 0.001$ ). (F) Social avoidance is enhanced in Orx<sub>2</sub>R antagonist-treated Escape mice ( $n = 33$ , Treatment Effect:  $F_{2,30} = 4.9$ ,  $p \leq 0.01$ ; Social, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{19} = 2.5$ ,  $!p \leq 0.02$ ; Social, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{20} = 2.7$ ,  $\wedge p \leq 0.01$ ). (G) There is no effect of treatment on social avoidance behavior in Stay mice. (H) Cued fear freezing is reduced in Escape mice following either Orx<sub>2</sub>R antagonism or agonism ( $n = 16$ , Treatment Effect:  $F_{2,13} = 5.9$ ,  $p \leq 0.02$ ; CS Effect:  $F_{1,13} = 9.6$ ,  $p \leq 0.008$ ; Interaction Effect:  $F_{2,13} = 4.6$ ,  $p \leq 0.03$ ; Post-Tone, Vehicle vs Orx<sub>2</sub>R Antagonist,  $t_{11} = 2.5$ ,  $\$p < 0.001$ ; Post-Tone, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_8 = 3.7$ ,  $+p < 0.001$ ; Vehicle, Pre- vs Post-Tone,  $t_7 = 4.8$ ,  $\#p < 0.001$ ). (I) Agonism of intra-BLA Orx<sub>2</sub>R reduces both contextual and cued fear freezing in Stay animals ( $n = 29$ , Treatment Effect:  $F_{2,22} = 7.2$ ,  $p \leq 0.004$ ; CS Effect:  $F_{1,22} = 37.2$ ,  $p < 0.001$ ; Pre-Tone, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_{16} = 2.3$ ,  $+p \leq 0.03$ ; Pre-Tone, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 2.9$ ,  $+p \leq 0.02$ ; Post-Tone, Vehicle vs Orx<sub>2</sub>R Agonist,  $t_{16} = 3.9$ ,  $+p < 0.001$ ; Post-Tone, Orx<sub>2</sub>R Antagonist vs Orx<sub>2</sub>R Agonist,  $t_{11} = 3.6$ ,  $!p < 0.001$ ; Vehicle, Pre- vs Post-Tone,  $t_{13} = 4.8$ ,  $\#p < 0.001$ ; Orx<sub>2</sub>R Antagonist, Pre- vs Post-Tone,  $t_8 = 4.7$ ,  $\#p < 0.001$ ; Orx<sub>2</sub>R Agonist, Pre- vs Post-Tone,  $t_5 = 2.9$ ,  $\#p \leq 0.04$ ).



**Figure 3.** Expression of Orx<sub>2</sub>R mRNA in the BLA is higher in female compared to male mice. (A) Representative image of female BLA with Orx<sub>2</sub>R mRNA (*HCRTR2*) expression (red = *HCRTR2*, blue = DAPI). (B) Expression of *HCRTR2* in male BLA. (C) Female mice have more BLA cells that express Orx<sub>2</sub>R mRNA compared to males (n = 13, t<sub>11</sub> = 3.5, \*p ≤ 0.005).

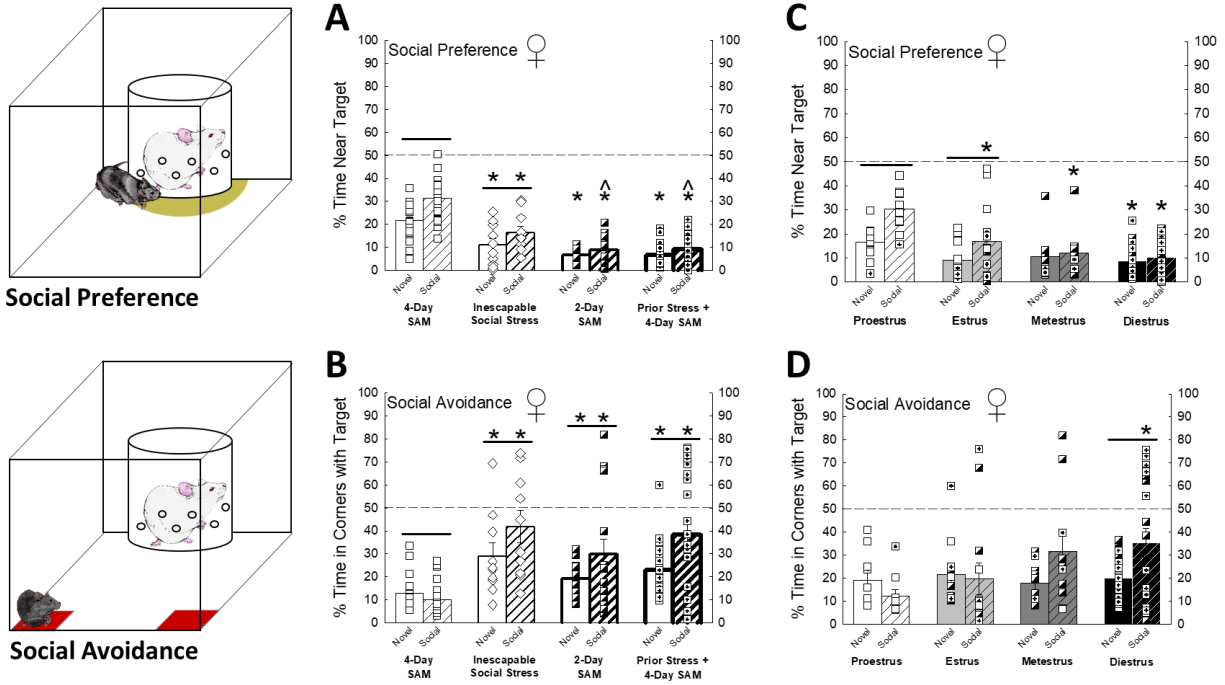


**Figure 4.** Female social stress and behavioral phenotype development is distinct from that in male mice. (A) Shock-induced aggression (SIA) involves applying a mild shock (1 mA, <1 s) to male (CD1 retired breeder) mice as they perform anogenital sniffing of female conspecifics. (B) The number of shocks needed to promote aggression varies, but all preliminary trials ( $n = 40$ ) resulted in aggressive interactions. (C) Female mice experiencing social aggression in the SAM all escaped by Day 3; whereas, ~45% of male mice committed to the Escape phenotype by the end of Day 2 ( $n = 85$ , Day 1:  $X^2 = 18.6$ ,  $*p < 0.001$ ; Day 2:  $X^2 = 16.3$ ,  $*p < 0.001$ ; Day 3 & 4:  $X^2 = 19.5$ ,  $*p < 0.001$ ). (D) Female mice that experienced 4-Days of social stress prior to SAM introduction exhibited more Stay behavior ( $n = 42$ , Day 1:  $X^2 = 4.9$ ,  $*p \leq 0.03$ ; Day 2:  $X^2 = 3.2$ ,  $*p \leq 0.04$ ). (E) Male mice that chose the Escape phenotype experienced a slower latency to escape on Day 1 compared to female animals ( $n = 49$ , Day Effect:  $F_{3,141} = 51.8$ ,  $p < 0.001$ ; Day 1:  $t_{47} = 2.4$ ,  $*p \leq 0.02$ ; unique letters indicate differences from other Days, e.g. A is

different from B,  $p \leq 0.02$ ). (F) Latency to escape in females that experienced social stress prior to SAM exposure displayed slower escape times on Days 1-3 ( $n = 42$ , Paradigm Effect:  $F_{1,120} = 12.9$ ,  $p < 0.001$ ; Day Effect:  $F_{3,120} = 51.0$ ,  $p < 0.001$ ; Day 1:  $t_{40} = 4.3$ ,  $*p < 0.001$ ; Day 2:  $t_{40} = 2.8$ ,  $*p < 0.001$ ; Day 3:  $t_{40} = 2.6$ ,  $*p \leq 0.01$ ; unique letters indicate differences from other Days, e.g. A is different from B,  $p < 0.001$ ).

(G) Male Stay mice experience more aggression than Escape mice, which receive similar levels of aggression as Female mice in the SAM arena ( $n = 85$ , Phenotype Effect:  $F_{2,192} = 42.9$ ,  $p < 0.001$ ; Day Effect:  $F_{3,192} = 5.1$ ,  $p \leq 0.002$ ).

(H) Females in an inescapable social stress paradigm receive more aggression than females exposed to the SAM where they can avoid aggressive encounters ( $n = 52$ , Paradigm Effect:  $F_{2,147} = 32.4$ ,  $p < 0.001$ ).



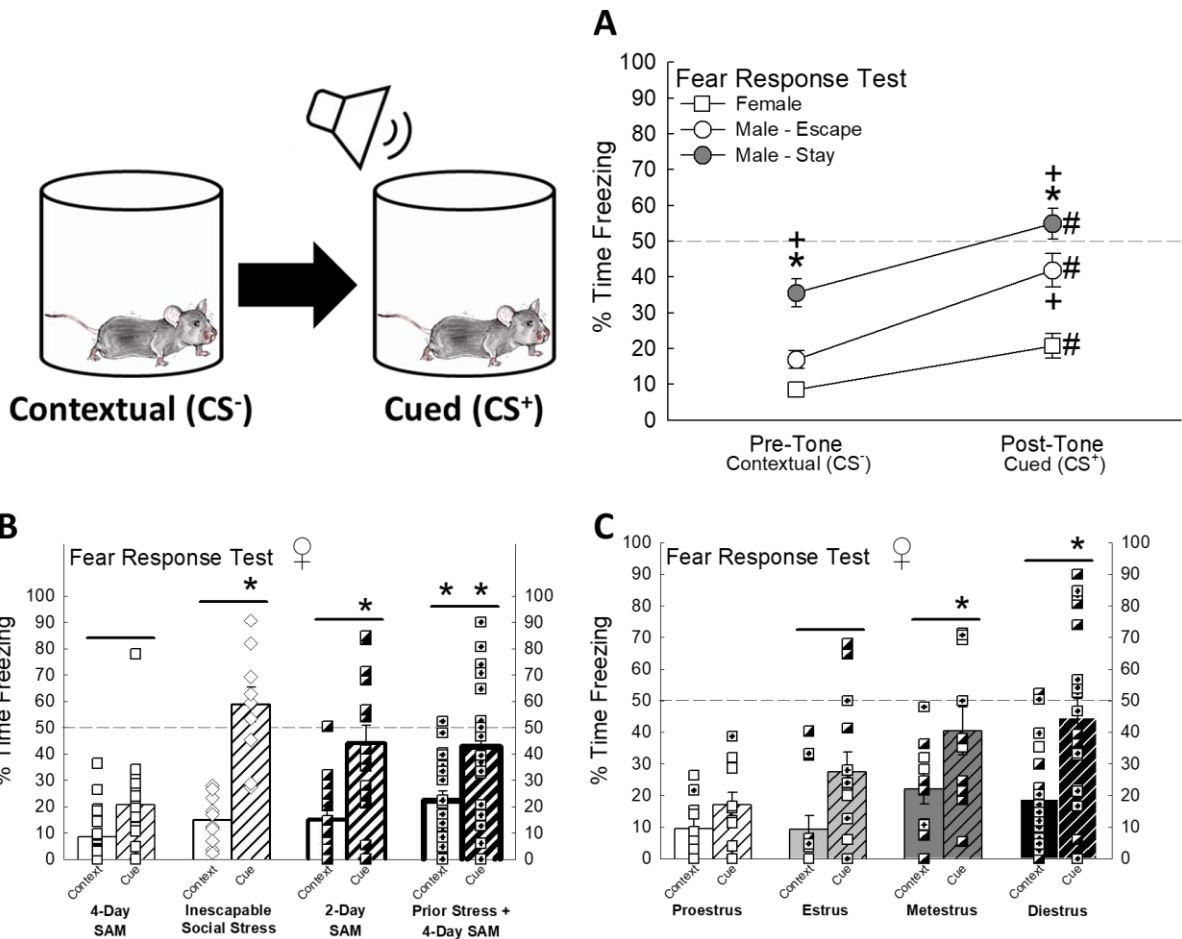
**Figure 5.** Female stress state impacts results in SIP test. (A) Enhanced stress in females reduces preference for both novel and social targets ( $n = 68$ , Paradigm Effect:  $F_{3,64} = 41.3$ ,  $p < 0.001$ ; Target Effect:  $F_{1,64} = 37.6$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,64} = 5.8$ ,  $p < 0.001$ ; Novel, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 4.0$ ,  $*p < 0.001$ ; Novel, 4-Day SAM vs 2-Day SAM,  $t_{36} = 6.6$ ,  $*p < 0.001$ ; Novel, 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 7.1$ ,  $*p < 0.001$ ; Social, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 5.7$ ,  $*p < 0.001$ ; Social, 4-Day SAM vs 2-Day SAM,  $t_{36} = 9.8$ ,  $*p < 0.001$ ; Social, 4-Day SAM vs Social Stress + 4-Day SAM,  $t_{40} = 10.3$ ,  $*p < 0.001$ ; Social, Inescapable Social Stress vs 2-Day SAM,  $t_{26} = 2.6$ ,  $^{\wedge}p \leq 0.01$ ; Social, Inescapable Social Stress vs Prior Stress + 4-Day SAM,  $t_{28} = 2.6$ ,  $^{\wedge}p \leq 0.01$ ; 4-Day SAM, Novel vs Social,  $t_{21} = 7.1$ ,  $-p < 0.001$ ; Inescapable Social Stress, Novel vs Social,  $t_9 = 2.6$ ,  $-p \leq 0.01$ ). (B) In females, social avoidance is increased in paradigms involving inescapable social stress, 2-Day SAM, and prior stress before 4-Day SAM ( $n = 68$ , Paradigm Effect:  $F_{3,64} = 9.4$ ,  $p < 0.001$ ; Target Effect:  $F_{1,64} = 12.2$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,64} = 3.3$ ,  $p \leq 0.03$ ; Novel, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 3.7$ ,  $*p < 0.001$ ; Novel, 4-Day SAM vs 2-Day SAM,  $t_{36} = 2.7$ ,  $*p \leq 0.01$ ; Novel, 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 3.5$ ,  $*p < 0.001$ ; Social, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 4.8$ ,  $*p < 0.001$ ; Social, 4-Day SAM vs 2-Day SAM,  $t_{36} = 3.4$ ,  $*p$

< 0.001; Social, 4-Day SAM vs Social Stress + 4-Day SAM,  $t_{40} = 5.3$ ,  $*p < 0.001$ ; 4-Day SAM, Novel vs Social,  $t_{21} = 3.0$ ,  $-p \leq 0.007$ ; Inescapable Social Stress, Novel vs Social,  $t_9 = 2.0$ ,  $-p \leq 0.05$ ; 2-Day SAM, Novel vs Social,  $t_{15} = 2.1$ ,  $-p \leq 0.04$ ; Prior Stress + 4-Day SAM, Novel vs Social,  $t_{19} = 3.4$ ,  $-p < 0.001$ ).

(C) Mice in proestrus exhibit enhanced social preference compared to other stages of the estrous cycle ( $n = 53$ , Stage Effect:  $F_{3,49} = 6.7$ ,  $p < 0.001$ ; Target Effect:  $F_{1,49} = 34.8$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,49} = 7.6$ ,  $p < 0.001$ ; Novel, Proestrus vs Diestrus,  $t_{28} = 3.1$ ,  $*p \leq 0.005$ ; Social, Proestrus vs Estrus,  $t_{21} = 3.5$ ,  $*p < 0.001$ ; Social, Proestrus vs Metestrus,  $t_{18} = 4.5$ ,  $*p < 0.001$ ; Social, Proestrus vs Diestrus,  $t_{28} = 5.7$ ,  $*p < 0.001$ ; Proestrus, Novel vs Social,  $t_9 = 5.9$ ,  $-p < 0.001$ ; Estrus, Novel vs Social,  $t_{12} = 3.5$ ,  $-p < 0.001$ ).

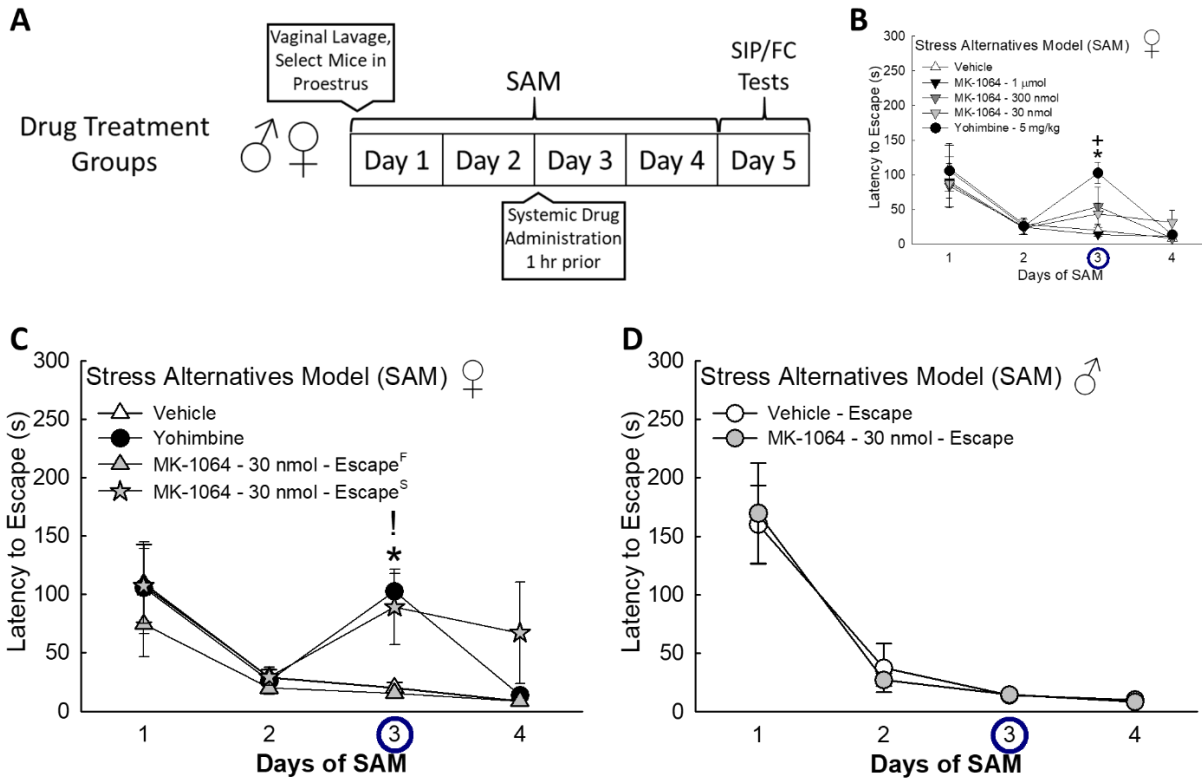
(D) Animals in diestrus display higher social avoidance behavior ( $n = 53$ , Interaction Effect:  $F_{3,49} = 3.1$ ,  $p \leq 0.04$ ; Social, Proestrus vs Diestrus,  $t_{28} = 3.1$ ,  $*p \leq 0.002$ ; Diestrus, Novel vs Social,  $t_{19} = 3.0$ ,  $-p \leq 0.004$ ).



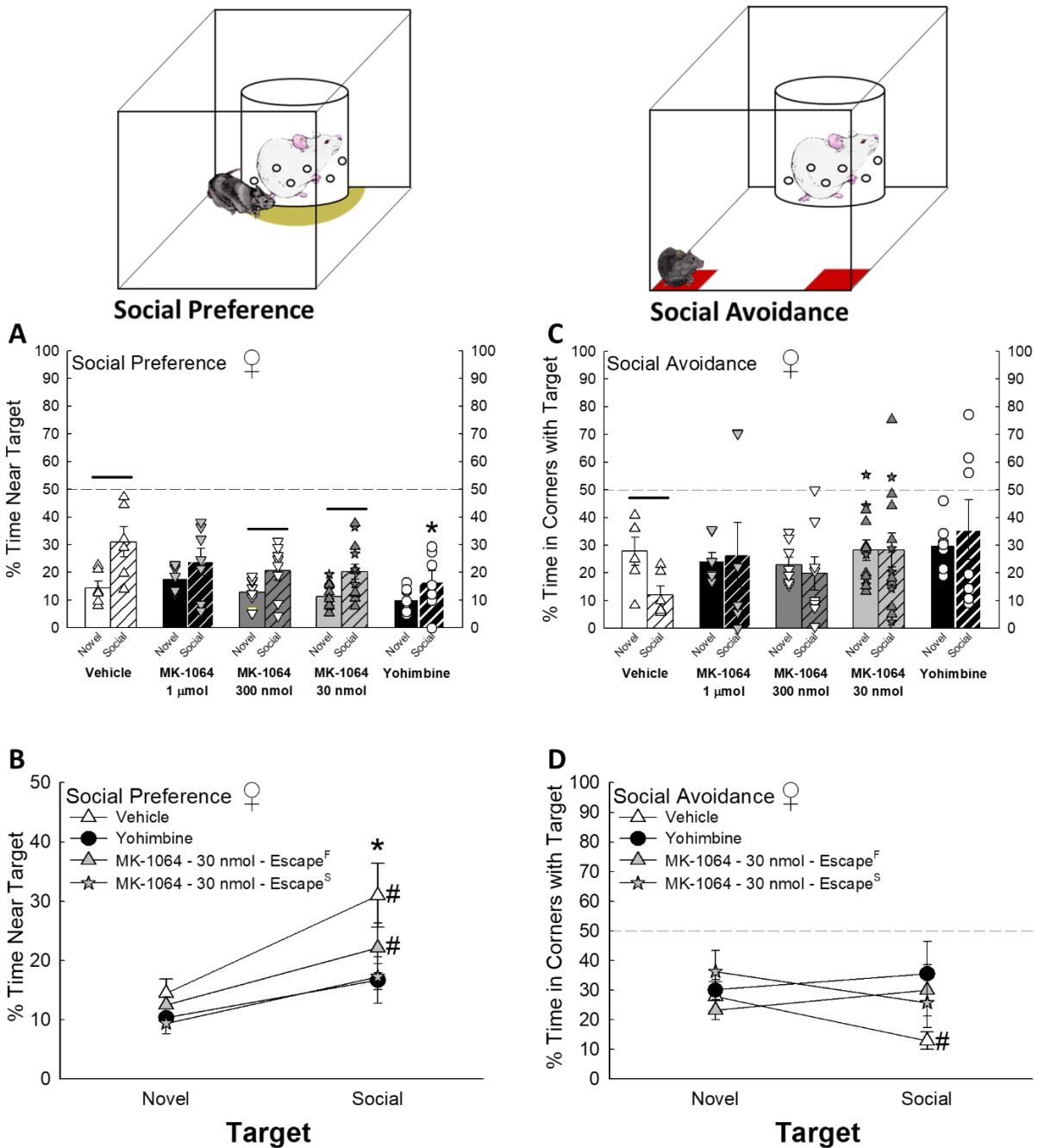


**Figure 6.** Fear response in female mice is dependent on stress state. (A) Male Stay mice exhibit the greatest amount of contextual and cued freezing, but male Escape animals freeze more to the cue than females ( $n = 85$ , Phenotype Effect:  $F_{2,82} = 19.9$ ,  $p < 0.001$ ; CS Effect:  $F_{1,82} = 72.5$ ,  $p < 0.001$ ; Pre-Tone, Male Stay vs Male Escape,  $t_{61} = 3.5$ ,  $*p < 0.001$ ; Pre-Tone, Male Stay vs Female,  $t_{56} = 4.8$ ,  $+p < 0.001$ ; Post-Tone, Male Stay vs Male Escape,  $t_{61} = 2.5$ ,  $*p \leq 0.02$ ; Post-Tone, Male Stay vs Female,  $t_{56} = 6.1$ ,  $+p < 0.001$ ; Post-Tone, Male Escape vs Female,  $t_{57} = 3.5$ ,  $+p < 0.001$ ; Male Stay, Pre-Tone vs Post-Tone,  $t_{35} = 5.8$ ,  $\#p < 0.001$ ; Male Escape, Pre-Tone vs Post-Tone,  $t_{26} = 6.5$ ,  $\#p < 0.001$ ; Female, Pre-Tone vs Post-Tone,  $t_{21} = 2.9$ ,  $\#p \leq 0.005$ ). (B) In female mice, enhancing the stress state promotes increased freezing in the fear response test ( $n = 68$ , Paradigm Effect:  $F_{3,64} = 8.1$ ,  $p < 0.001$ ; CS Effect:  $F_{1,64} = 87.6$ ,  $p < 0.001$ ; Interaction Effect:  $F_{3,64} = 5.1$ ,  $p \leq 0.003$ ; Pre-Tone, 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 3.4$ ,  $*p \leq 0.002$ ; Post-Tone, 4-Day SAM vs Inescapable Social Stress,  $t_{30} = 5.5$ ,  $*p < 0.001$ ; Post-Tone,

4-Day SAM vs 2-Day SAM,  $t_{36} = 3.9$ ,  $*p < 0.001$ ; Post-Tone, 4-Day SAM vs Prior Stress + 4-Day SAM,  $t_{40} = 3.9$ ,  $*p < 0.001$ ; 4-Day SAM, Pre-Tone vs Post-Tone,  $t_{21} = 2.6$ ,  $-p \leq 0.01$ ; Inescapable Social Stress, Pre-Tone vs Post-Tone,  $t_9 = 6.2$ ,  $-p < 0.001$ ; 2-Day SAM, Pre-Tone vs Post-Tone,  $t_{15} = 5.2$ ,  $-p < 0.001$ ; Prior Stress + 4-Day SAM, Pre-Tone vs Post-Tone,  $t_{19} = 4.1$ ,  $-p < 0.001$ ). (C) Female mice in metestrus and diestrus display elevated cued fear freezing behavior ( $n = 53$ , Stage Effect:  $F_{3,49} = 4.3$ ,  $p \leq 0.009$ ; CS Effect:  $F_{1,49} = 30.2$ ,  $p < 0.001$ ; Post-Tone, Proestrus vs Metestrus,  $t_{18} = 2.7$ ,  $*p \leq 0.008$ ; Post-Tone, Proestrus vs Diestrus,  $t_{28} = 3.7$ ,  $*p < 0.001$ ; Estrus, Pre-Tone vs Post-Tone,  $t_{12} = 2.9$ ,  $-p \leq 0.005$ ; Metestrus, Pre-Tone vs Post-Tone,  $t_9 = 2.6$ ,  $-p \leq 0.01$ ; Diestrus, Pre-Tone vs Post-Tone,  $t_{19} = 5.2$ ,  $-p < 0.001$ ).

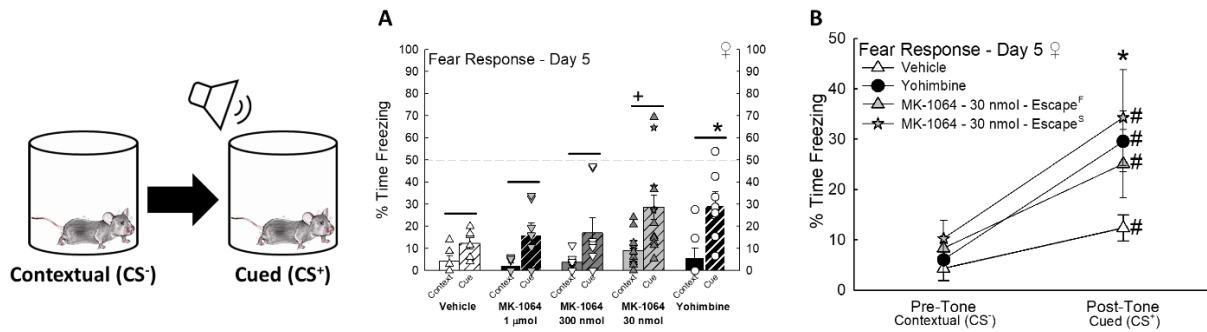


**Figure 7.** Systemic antagonism of Orx<sub>2</sub>R reveals female phenotype formation. (A) Experimental design for pharmacological experiments. (B) Yohimbine, an  $\alpha_2$  receptor antagonist, significantly increases latency to escape, while low doses of an Orx<sub>2</sub>R antagonist (MK-1064) moderately alter latency to escape ( $n = 41$ , Day Effect:  $F_{3,108} = 17.2$ ,  $p < 0.001$ ; Day 3, Vehicle vs Yohimbine,  $t_{11} = 4.9$ ,  $*p < 0.001$ ; Day 3, MK-1064 – 1  $\mu\text{mol}$  vs Yohimbine,  $t_{12} = 5.8$ ,  $+p < 0.001$ ). (C) Phenotype separation in female mice after low dose (30 nmol) treatment of an Orx<sub>2</sub>R antagonist (MK-1064) reveals fast (Escape<sup>F</sup>) and slow (Escape<sup>S</sup>) escapers, where Escape<sup>S</sup> animals express an enhanced latency to escape comparable to that observed after yohimbine treatment ( $n = 26$ , Treatment Effect:  $F_{3,66} = 3.9$ ,  $p \leq 0.02$ ; Day Effect:  $F_{3,66} = 11.0$ ,  $p < 0.001$ ; Day 3, Vehicle vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_9 = 2.6$ ,  $*p \leq 0.03$ ; Day 3, MK-1064 – 30 nmol – Escape<sup>F</sup> vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_{11} = 3.0$ ,  $!p \leq 0.01$ ). (D) Male Escape mice administered MK-1064 at 30 nmol did not display increased latency to escape.

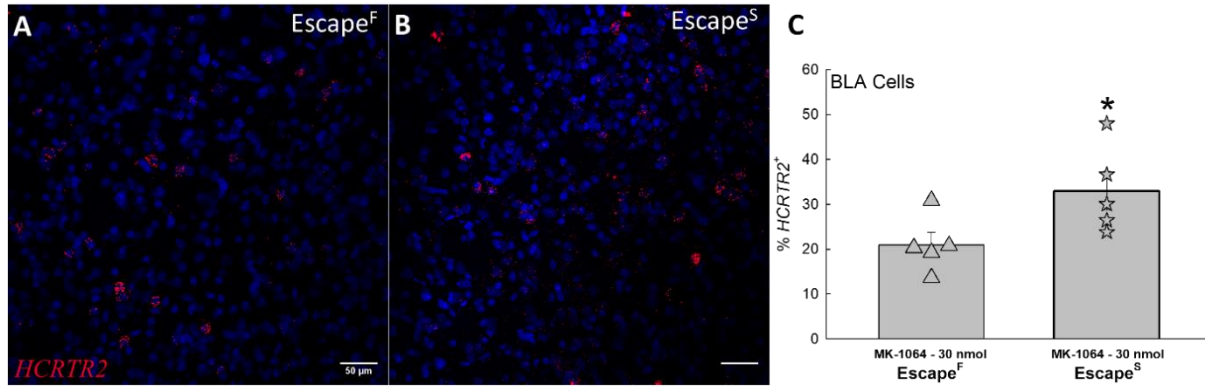


**Figure 8.** Pharmacologically revealed phenotypes in females show different responses in SIP test. (A) Social preference is reduced with Yohimbine treatment ( $n = 41$ , Target Effect:  $F_{1,36} = 27.8$ ,  $p < 0.001$ ; Social, Vehicle vs Yohimbine,  $t_{11} = 3.1$ ,  $*p \leq 0.003$ ; Vehicle, Novel vs Social,  $t_5 = 3.8$ ,  $-p < 0.001$ ; MK-1064 – 300 nmol, Novel vs Social,  $t_7 = 2.1$ ,  $-p \leq 0.045$ ; MK-1064 – 30 nmol, Novel vs Social,  $t_{12} = 3.0$ ,  $-p \leq 0.005$ ). (B) Female mice treated with MK-1064 at the 30 nmol dose that exhibited slow escape behavior (Escape<sup>S</sup>) displayed reduced social preference comparable to that observed in yohimbine-treated

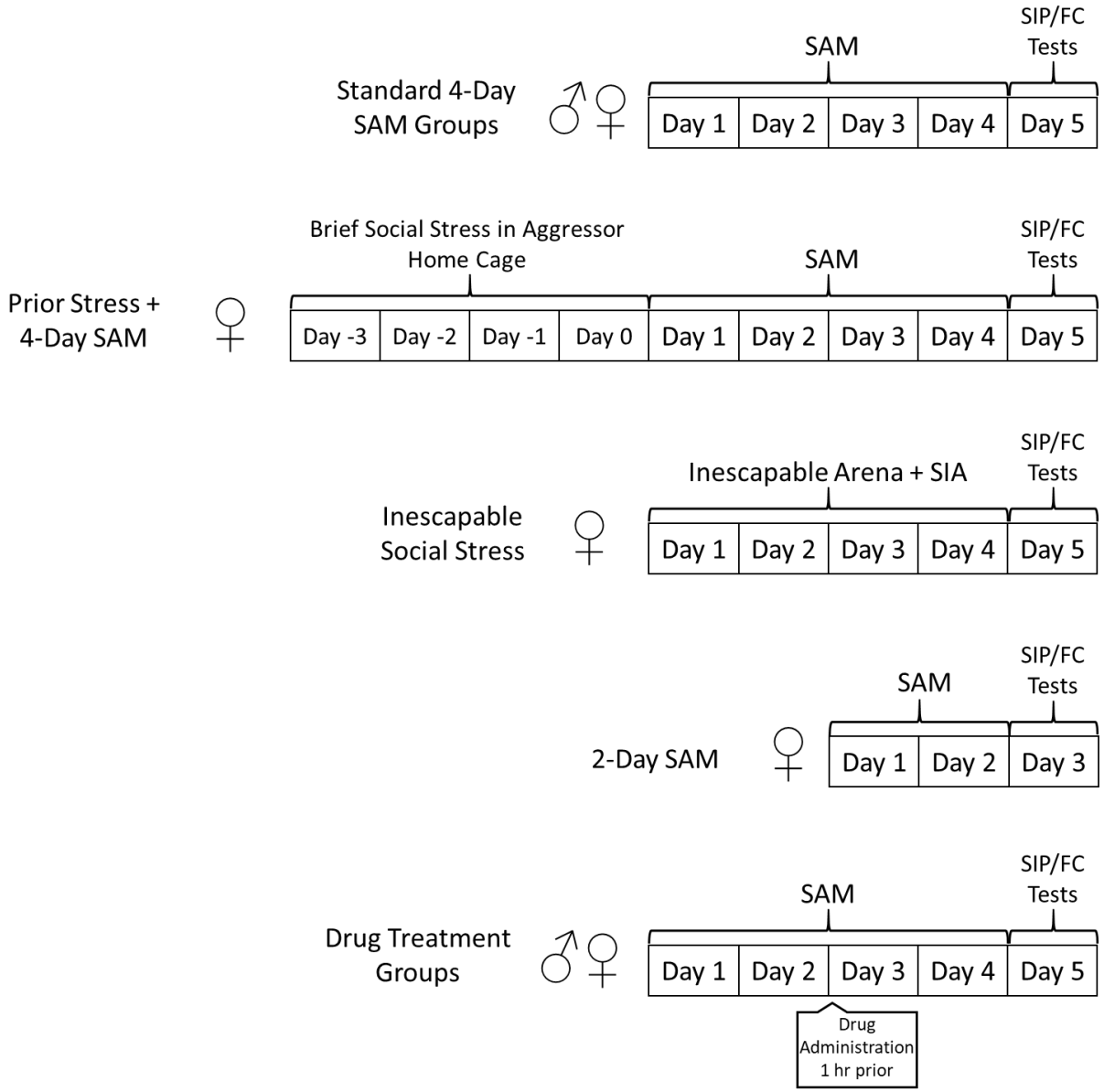
mice ( $n = 26$ , Target Effect:  $F_{1,16} = 24.1$ ,  $p < 0.001$ ; Social, Vehicle vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_9 = 2.7$ ,  $*p \leq 0.01$ ; MK-1064 – 30 nmol – Escape<sup>F</sup>, Novel vs Social,  $t_7 = 2.8$ ,  $\#p \leq 0.01$ ). (C-D) Only vehicle-treated mice exhibited reduced social avoidance behavior ( $t_5 = 2.5$ ,  $-/\#p \leq 0.05$ ).



**Figure 9.** Systemic antagonism of Orx<sub>2</sub>R affects fear response in female mice in a phenotype-dependent fashion. (A) Yohimbine increases cued fear freezing ( $n = 41$ , CS Effect:  $F_{1,36} = 42.0$ ,  $p < 0.001$ ; Pre-Tone, MK-1064 – 1  $\mu\text{mol}$  vs MK-1064 – 30 nmol,  $t_{18} = 2.4$ ,  $+p \leq 0.03$ ; Post-Tone, Vehicle vs Yohimbine,  $t_{11} = 2.5$ ,  $*p \leq 0.03$ ; Vehicle, Pre-Tone vs Post-Tone,  $t_5 = 2.5$ ,  $-p \leq 0.05$ ; MK-1064 – 1  $\mu\text{mol}$ , Pre-Tone vs Post-Tone,  $t_6 = 2.4$ ,  $-p \leq 0.02$ ; MK-1064 – 300 nmol, Pre-Tone vs Post-Tone,  $t_7 = 2.5$ ,  $-p \leq 0.02$ ; MK-1064 – 30 nmol, Pre-Tone vs Post-Tone,  $t_{12} = 4.7$ ,  $-p < 0.001$ ; Yohimbine, Pre-Tone vs Post-Tone,  $t_6 = 4.2$ ,  $-p < 0.001$ ). (B) Female Escape<sup>S</sup> phenotype displays elevated cued freezing behavior similar to yohimbine-treated female mice ( $n = 26$ , CS Effect:  $F_{3,22} = 44.7$ ,  $p < 0.001$ ; Post-Tone, Vehicle vs MK-1064 – 30 nmol – Escape<sup>S</sup>,  $t_9 = 2.8$ ,  $*p \leq 0.009$ ; MK-1064 – 30 nmol – Escape<sup>S</sup>, Pre-Tone vs Post-Tone,  $t_4 = 4.0$ ,  $\#p < 0.001$ ; MK-1064 – 30 nmol – Escape<sup>F</sup>, Pre-Tone vs Post-Tone,  $t_7 = 3.5$ ,  $\#p \leq 0.002$ ).

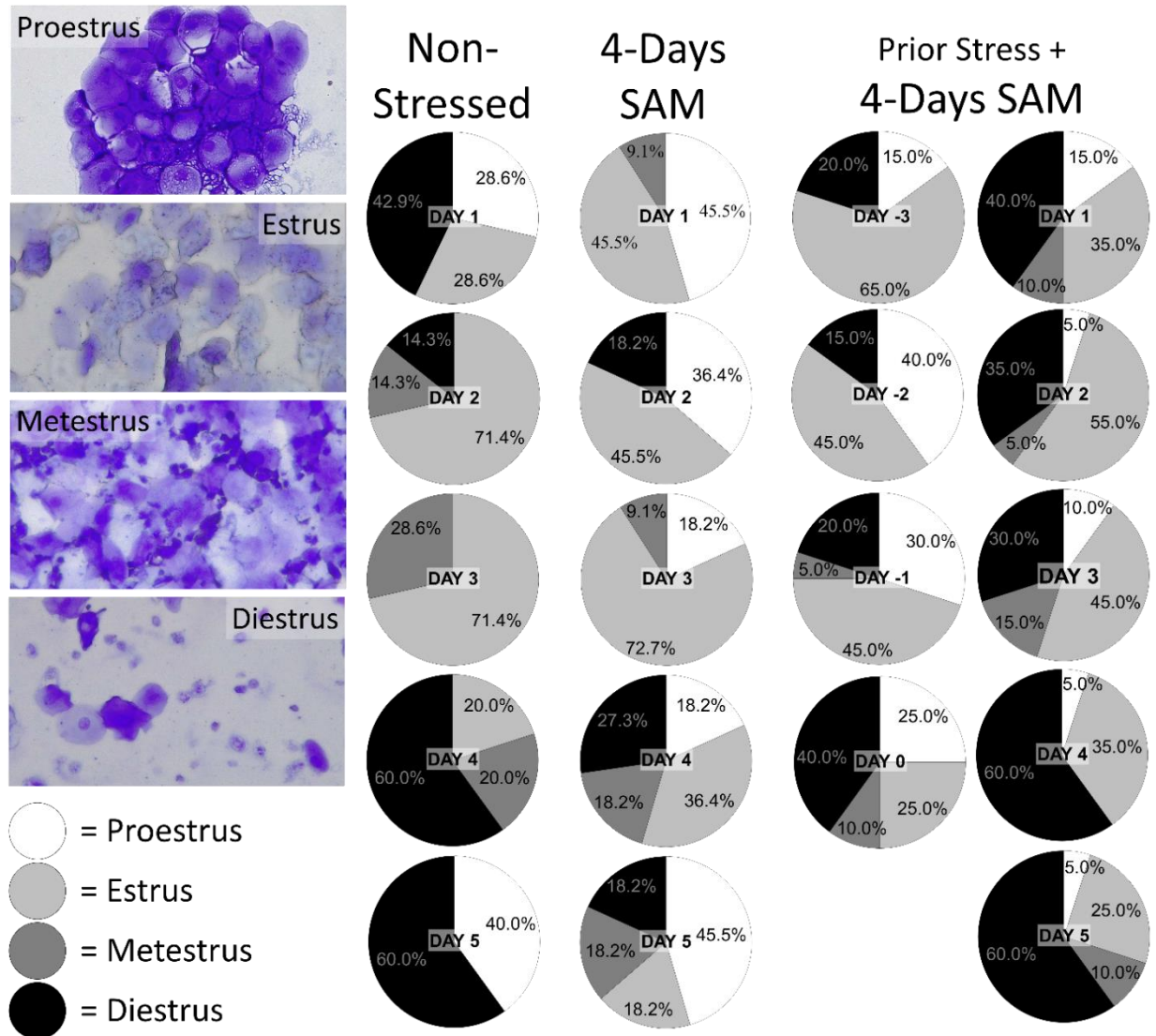


**Figure 10.** Cells in BLA expressing Orx<sub>2</sub>R are unique in antagonist-induced phenotypes. (A) Image representing *HCRTR2* expression in BLA cells of mice defined as Escape<sup>F</sup> (red = *HCRTR2*, blue = DAPI) and (B) Escape<sup>S</sup>. (C) Mice exhibiting the Escape<sup>S</sup> phenotype have more Orx<sub>2</sub>R mRNA expression compared to Escape<sup>F</sup> animals (n = 10, t<sub>8</sub> = 2.3, \*p ≤ 0.048).

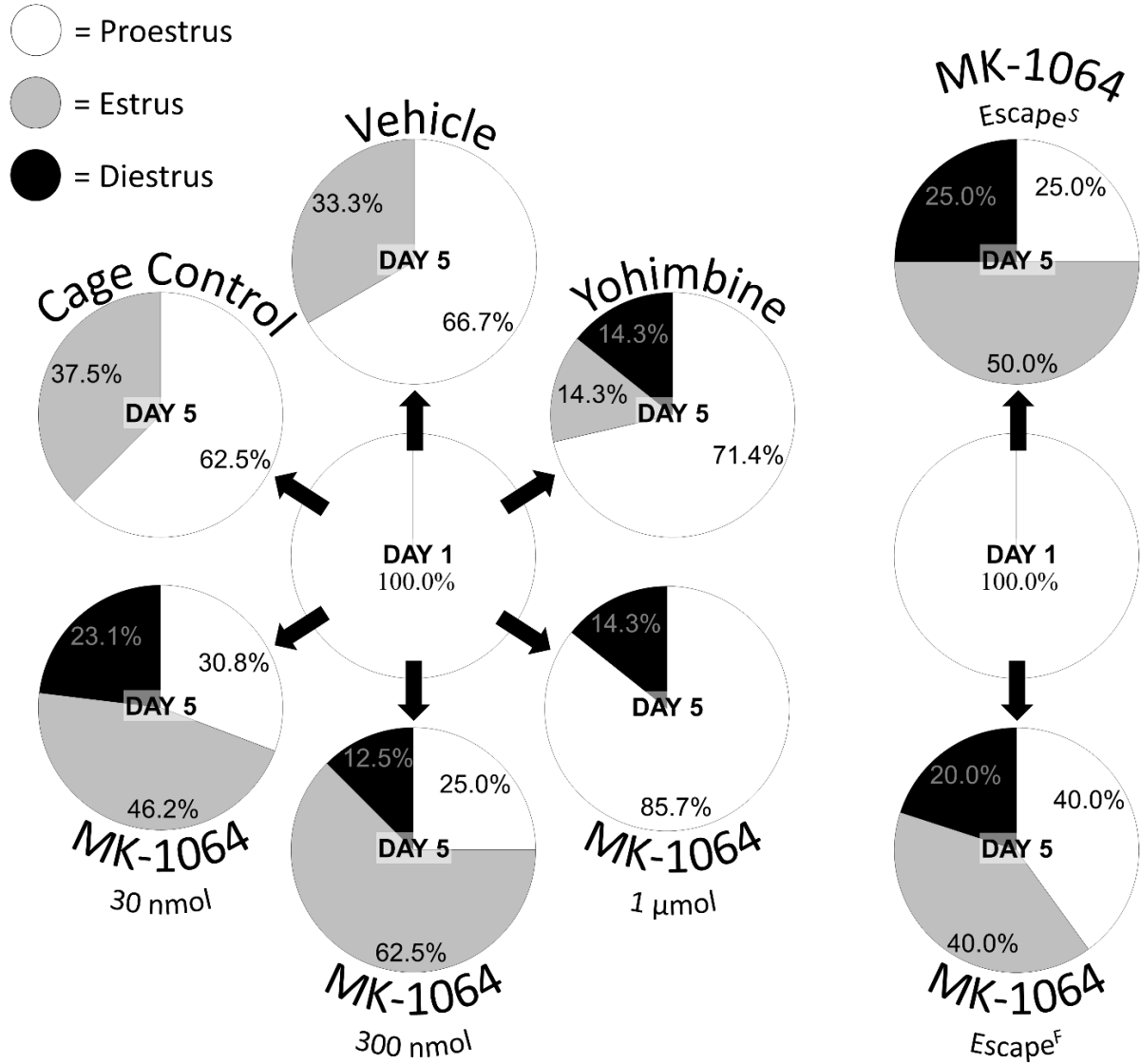


**Figure S1.** Social stress paradigm outlines used for exploring female social stress.

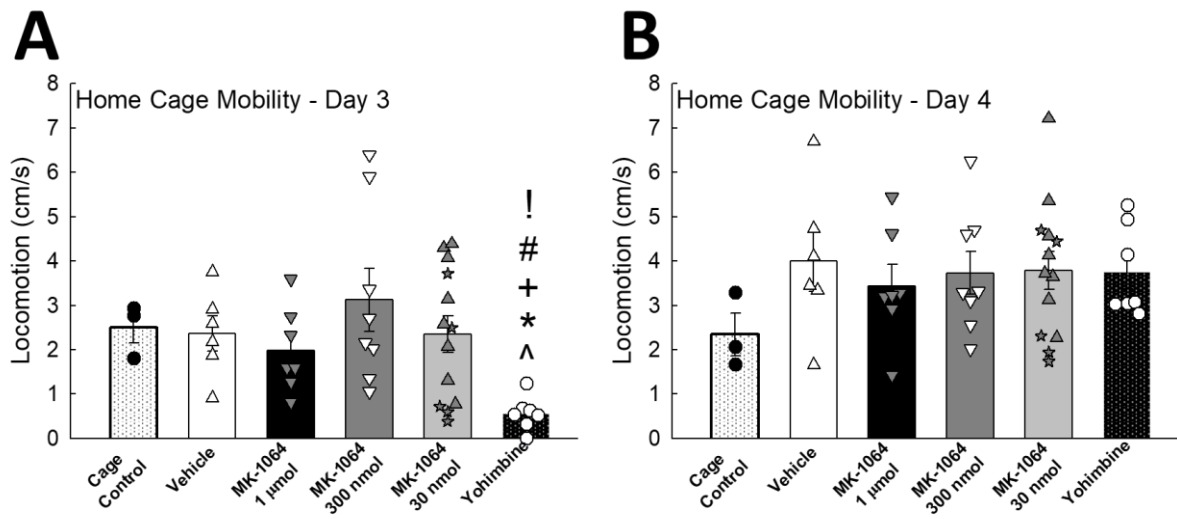




**Figure S2.** Lavage samples were collected daily to determine the stage of the estrous cycle for Non-Stressed Cage Control mice and those of experimental groups: 4-Days SAM & Prior Stress + 4-Days SAM.



**Figure S3.** While all animals started experimental trials in the proestrus stage of the estrous cycle, variation was observed in the number of animals in a particular stage of the cycle after drug treatment (Yohimbine or MK-1064).



**Figure S4.** (A) Yohimbine reduces home cage locomotion on Day 3, 1 hr after drug administration ( $F_{5,38} = 3.0$ ,  $p \leq 0.021$ ). Differences are observed relative to cage control ( $t_8 = 6.3$ ,  $^{\wedge}p < 0.001$ ), vehicle- ( $t_{11} = 4.6$ ,  $^*p < 0.001$ ), and MK-1064-treated mice (1  $\mu\text{mol}$ :  $t_{12} = 3.7$ ,  $^+p \leq 0.003$ ; 300 nmol:  $t_{13} = 3.3$ ,  $^{\#}p \leq 0.006$ ; 30 nmol:  $t_{18} = 3.1$ ,  $^!p \leq 0.006$ ). (B) There were, however, no effects on home cage mobility on Day 4 (24 hr after) drug treatment ( $F_{5,38} = 0.7$ ,  $p \geq 0.647$ ).