



## Orexin/hypocretin receptor modulation of anxiolytic and antidepressive responses during social stress and decision-making: potential for therapy

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### Abstract

Hypothalamic orexin/hypocretin (Orx) neurons in the lateral and dorsomedial perifornical region (LH-DMH/PeF) innervate broadly throughout the brain, and receive similar inputs. This wide distribution, as well as two Orx peptides (Orx<sub>A</sub> and Orx<sub>B</sub>) and two Orx receptors (Orx<sub>1</sub> and Orx<sub>2</sub>) allow for functionally related but distinctive behavioral outcomes, that include arousal, sleep-wake regulation, food seeking, metabolism, feeding, reward, addiction, and learning. These are all motivational functions, and tie the orexin systems to anxiety and depression as well. We present evidence, that for affective behavior, Orx<sub>1</sub> and Orx<sub>2</sub> receptors appear to have opposing functions. The majority of research on anxiety- and depression-related outcomes has focused on Orx<sub>1</sub> receptors, which appear to have primarily anxiogenic and pro-depressive actions. Although there is significant research suggesting contrary findings, the primary potential for pharmacotherapies linked to the Orx<sub>1</sub> receptor is via antagonists to block anxious and depressive behavior. Dual orexin receptor antagonists have been approved for treatment of sleep disorders, and are likely candidates for adaptation for affect disorder treatments. However, we present evidence here that demonstrates the Orx<sub>2</sub> receptors are anxiolytic and antidepressive. Using a new experimental pre-clinical model of anxious and depressive behavior stimulated by social stress and decision-making that produces two stable behavioral phenotypes, Escape/Resilient and Stay/Susceptible, we tested the effects of intracerebroventricular injections of Orx<sub>2</sub> agonist and antagonist drugs. Over ten behavioral measures, we have demonstrated that Orx<sub>2</sub> agonists promote resilience, as well as anxiolytic and antidepressive behavior. In contrast, Orx<sub>2</sub> antagonists or knockdown kindle anxious and pro-depressive behavior plus increase susceptibility. The results suggest that the Orx<sub>2</sub> receptor may be a useful target for pharmacotherapies to treat anxiety and depression.

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## Keywords

Anxiety; Depression; Fear conditioning; Orexin 2 receptor; Social defeat; Stress-Alternatives Model

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### 1. Orexin / hypocretins' regulatory role

The orexins (Orx; also called hypocretins) have an extremely broad distribution of projections and terminals from a tiny number of perikarya in the lateral-dorsomedial hypothalamus near the perifornical area (LH-DMH/PeF) (Baldo et al., 2003; Ch'ng and Lawrence, 2015; Chen et al., 1999; Cluderay et al., 2002; Marcus et al., 2001; Nambu et al., 1999; Peyron et al., 1998; Schmitt et al., 2012; Trivedi et al., 1998). The input to orexinergic perikarya is equally extensive (Yoshida et al., 2006), suggesting a broadly integrative behavioral regulatory role for the orexins (Bai et al., 2009; Bentzley and Aston-Jones, 2015; Blais et al., 2017; Borgland et al., 2009; Burgess, 2010; Choi et al., 2010; Sakurai, 2014; Scammell and Saper, 2005; Thompson and Borgland, 2011; Thorpe et al., 2005; Tsujino and Sakurai, 2013). As the list of orexin mediated behavioral outcomes includes arousal, emotional behavior, feeding and food seeking, locomotion, metabolism, reward and addictive behavior, sexual behavior, vigilance, and waking, it seems clear that orexins are involved in promoting motivational behavior associated with adaptive responses to stressful stimuli (Giardino and de Lecea, 2014; James et al., 2017b; Mahler et al., 2014; Sakurai, 2014). Importantly, brief and protracted exposure to stressors may differentially involve orexinergic systems, such that acute stress increases activation of Orx neurons (Furlong et al., 2009; Ida et al., 2000), and chronic social defeat stress reduces Orx levels in hypothalamus and elsewhere, which can be ameliorated by food restriction (Lutter et al., 2008; Nocjar et al., 2012). Motivational behavior is essential to affective condition, and the core suite of behaviors, such as arousal and reward, that orexin systems regulate. These are the fundamental stress-related elements that in dysfunctional states yield psychological disorders such as anxiety and depression.

### 2. Regulatory neurocircuitry for anxiety and depression

Recent studies of the efficacy of preclinical research using animal models to examine the predictive value of classical tests for clinical translation for patients with anxiety and depression, suggest that their effectiveness is low (Haller and Alicki, 2012; Haller et al., 2013). The misalignment between preclinical paradigms, animal models, and clinical translation suggests that the neurocircuitry involved in affective behavior must be carefully distinguished, and sorted to reflect specific neurochemical and molecular mechanisms. The mechanisms that generate anxiety and depression, comorbid as often as 63–81% (current vs lifetime) of the time (Lamers et al., 2011), are broadly associated with traumatically stressful events, and stress-related neurocircuitry, such that higher severity levels of depression and/or anxiety increase comorbidity (Schoevers et al., 2003). It is important to note, that while these affective states are frequently comorbid, they do not often occur simultaneously. That the neural mechanisms involved in fear and anxiety are similar in humans and other animals is substantiated by copious evidence (Davis et al., 2010; Tovote et al., 2015). As these

systems and circuits are highly conserved over evolutionary time, and because non-human vertebrates also show distinctively anxious and depressive behavioral inhibition, it seems clear that the use of non-human vertebrate systems is not the problem: We have not been careful in the tests of affective behavior that we use, and therefore the specific neurocircuitry that we evaluate may be inappropriate (Blanchard et al., 2013; Keifer and Summers, 2016; Øverli et al., 2007; Vindas et al., 2016). It is time to carefully analyze anxious and depressive behavior experienced by other vertebrate animals that may be used as models, and be sure the tests designed to measure the species specific anxiety and depression are also species-appropriate (Blanchard et al., 2013; Pearson et al., 2017; Robertson et al., 2015).

Neurocircuitry elements that modulate chronic social stresses characteristic of stimuli that induce depression and anxiety include circuits involved in fear, decision-making, learning and memory, stress, and reward (Kent and Rauch, 2003; Shin and Liberzon, 2010). While the neural circuitries for stress, anxiety, and depression undoubtedly have unique attributes, much of the pathways (and diagnostic criteria) overlap, suggesting to some that these conditions are intrinsically related (Kalueff and Nutt, 2007; Roffman et al., 2005; Stein, 2009). We therefore, have not attempted to create separate descriptions of the neural circuitries for anxiety and depression here. Output from fear-related brain regions, such as the central amygdala (CeA) contribute potently to anxious and depressive behavior (Tasan et al., 2010; Walker and Davis, 2002a; Walker and Davis, 2002b; Zhang et al., 2011). Similarly, the hippocampus also appears to be involved in depression and anxiety (Erickson et al., 2012; Hong et al., 2015; Hritcu and Gorgan, 2014; Kajiyama et al., 2010; Maghsoudi et al., 2014; Mlyniec et al., 2015; Ye et al., 2011), and especially related to neurogenesis in the dentate gyrus (Aboul-Fotouh et al., 2018; Brymer et al., 2018; Gao et al., 2018; Hill et al., 2015; Jesulola et al., 2018; Zhang et al., 2016).

The development of the Social-Defeat Model along with the notion of stress-coping strategies clarified the existence of behavioral phenotypes that are susceptible or resistant to affective disorders (Berton et al., 2007; Iniguez et al., 2014; Keifer and Summers, 2016; Koolhaas et al., 1999; Koolhaas et al., 2007; Korzan and Summers, 2007; Vidal et al., 2011). Discovery of Susceptible and Resilient phenotypes led to the inclusion of reward circuitry, the ventral tegmental area (VTA) and its dopaminergic projections to the nucleus accumbens (NAc) and striatum, as structures that are critically involved in anxiety and depression, through its effects on susceptibility to stress (Krishnan et al., 2007; Muir et al., 2018). The NAc is often considered part of the extended amygdala, along with bed nucleus of the stria terminalis (BNST), which plays an important role in anxiety (Avery et al., 2016; Davis and Shi, 1999; Walker et al., 2003), as well as the amygdala *per se* (Davis, 1992; Gray et al., 2015; Liebsch et al., 1995; Shekhar et al., 2005). Specialized fear- and extinction-related neurons in the basolateral amygdala (BLA) project specifically to prelimbic (PrL) and infralimbic (IL) cortices respectively (Senn et al., 2014). Local inhibitory circuits associated with the intercalated cells and basolateral amygdala appear to play an important role regulating fear learning and extinction (Amano et al., 2010; Likhtik et al., 2008; McDonald and Mascagni, 2001; Trouche et al., 2013). Orexin-related signaling or inhibition has been demonstrated to have effects in these brain regions, influencing depressive (Arendt et al., 2013; Ito et al., 2009; Lutter et al., 2008; Nocjar et al., 2012; Nollet et al., 2011; Nollet et al.,

2012; Ozsoy et al., 2017) and anxious behavior (Arendt et al., 2014; Azogu and Plamondon, 2017; Heydendael et al., 2014; Johnson et al., 2010; Johnson et al., 2012a; Johnson et al., 2012c; Khalil and Fendt, 2017; Lungwitz et al., 2012; Ozsoy et al., 2017). With interactive circuitries (Winsky-Sommerer et al., 2004; Winsky-Sommerer et al., 2005), Orx and corticotropin releasing factor (CRF or CRH) innervate stress-related brain regions (Achua et al., 2014; Ronan et al., 2016), which is important because CRF also directly modulates stress vulnerability along with anxious and depressive behaviors (Hauger et al., 2009; Holsboer and Ising, 2010; Litvin et al., 2007; Takahashi et al., 2001; Waters et al., 2015; Zorrilla et al., 2002).

### 3. Orx-CRF reciprocal interactions

Recent results suggest direct and indirect reciprocal interaction between brain CRF and Orx systems. Immunolabeling for CRF is abundant in the Orx field of the LH-DMH/PeF, along with Orx immunoreactivity (Achua et al., 2014; Ronan et al., 2016). In addition, Orx perikarya in this field also interact with each other. However, the CRF-Orx inter-reactivity is much more complex, partially due to the distributed nature of CRF synthesis, which contributes to the input to the LH-DMH/PeF (Sakurai et al., 2005; Yoshida et al., 2006) primarily via the ventral BNST (vBNST) and the paraventricular nucleus (PVN) (Achua et al., 2014; Ronan et al., 2016). The molecular mechanisms for these interactions are also significantly more complex than previously thought (Winsky-Sommerer et al., 2004; Winsky-Sommerer et al., 2005). Specifically, receptors for CRF are found on Orx<sub>A</sub>-immunopositive neurons in the LH-DMH/PeF and CRF positive neurons in the PVN and CeA express Orx receptors (Achua et al., 2014; Ronan et al., 2016). Direct CRF innervation of Orx-expressing neurons yields depolarization via CRF<sub>1</sub> receptors, blocked by CRF<sub>1</sub> antagonists or knockout (Winsky-Sommerer et al., 2004; Winsky-Sommerer et al., 2005). In addition, Orx neurons connect reciprocally to the CRF system, such that CRF<sub>1</sub> receptors mediate stress-induced activation of orexinergic outputs that return activation in a positive feedback manner. New evidence suggests that both CRF<sub>1</sub> and CRF<sub>2</sub> receptors are involved in regulation of orexinergic neurons; both receptor types are found there (Winsky-Sommerer et al., 2004). While CRF<sub>1</sub> receptors have been clearly demonstrated to influence activity of Orx neurons in the LH-DMH/PeF, our immunohistochemical results suggest this CRF<sub>1</sub> effect may not be acting directly on Orx neurons. The CRF receptor type found on Orx cells appears to be distinctively CRF<sub>2</sub> (Slater et al., 2016). The LH-DMH/PeF CRF<sub>1</sub> receptor expression, which influences Orx cell firing rate, is localized to as yet unknown neuronal types (Winsky-Sommerer et al., 2004). Nevertheless, results clearly showing CRF<sub>2</sub> expression on Orx cells does not dismiss the reality that CRF can affect orexin neurons through a CRF<sub>1</sub>-mediated mechanism. This was demonstrated by the ability of astressin, a CRF receptor antagonist, to block CRF-induced depolarization of orexin neurons ex-Wra in hypothalamic tissue slices (Winsky-Sommerer et al., 2004). Recent evidence suggests that more complex circuits are involved, with a wide limbic input of CRF to LH-DMH/PeF, additional regulatory influences by CRF<sub>2</sub> and Orx<sub>2</sub> receptors plus CRF binding protein (CRF<sub>BP</sub>), and even indirect CRF influences on the orexin field via the lateral septum (LS) (Achua et al., 2014; Ronan et al., 2016; Slater et al., 2016). The strongest CRF projections directly to the orexin field in the LH-DMH/PeF come from the vBNST, with few

connections made directly from dorsal BNST (dBNST), CeA, or even nearby PVN (Achua et al., 2014; Ronan et al., 2016). Broad icv infusion of Orx<sub>A</sub> suggests that CRF systems are an important target, as 96% of CRFergic cells in the paraventricular nucleus of the hypothalamus (PVN; a major CRFergic area), and 45% in the central nucleus of the amygdala (CeA; also CRFergic), expressed fos activation (Sakamoto et al., 2004). Orexin receptors have been found in the PVN and application of Orx<sub>A</sub> in PVN has excitatory effects on CRF systems and the hypothalamo-pituitary-adrenal (HPA) axis (Hagan et al., 1999; Marcus et al., 2001). Microinjection of Orx<sub>A</sub> into the PVN results in large increases in plasma corticosterone levels (Samson et al., 2002). Importantly, BNST inputs, including CRF- and cholecystinin (CCK)-expressing projections, modulate orexinergic tone in the lateral hypothalamus and condition emotional output (Giardino et al., 2018). Furthermore, physiological control of adrenocorticotrophic hormone (ACTH) during acute stress is mediated by Orx<sub>2</sub> receptors (Grafe et al., 2017). It seems likely that any regulatory role for Orx in stress-related initiation or inhibition of anxiety or depression is manifest in conjunction with CRF systems, and that interplay between these two systems must always be considered regarding basic mechanisms or therapeutic interventions.

#### 4. Orx<sub>1</sub> actions in anxious behavior and fear conditioning

While the actions of Orx<sub>1</sub> receptors influence arousal and motivation (Mahler et al., 2014; Sakurai, 2014), they may also coincidentally, or separately, act in emotion-related regions of the brain (sometimes via systemic delivery) to promote anxious behavior or anxiety (Alo et al., 2016; Azogu and Plamondon, 2017; Heydendael et al., 2014; Johnson et al., 2010; Johnson et al., 2012a; Johnson et al., 2012c; Khalil and Fendt, 2017; Lungwitz et al., 2012; Ozsoy et al., 2017). Interestingly, narcoleptic patients, while rarely fitting a diagnosis of major depression, exhibit heightened levels of anxiety, particularly related to social phobias (Fortuyn et al., 2010). Effects of Orx<sub>A</sub> treatments, appear to be primarily related to Orx<sub>1</sub> receptor activity. Whole brain (icv) infusion of Orx<sub>A</sub> in mice increased anxiogenic behaviors in the Light-Dark Test (LDT) and Elevated Plus Maze (EPM) (Suzuki et al., 2005); while effective pharmacological reversal of the anxious behaviors stimulated by icv injection of Orx<sub>A</sub> or Orx<sub>B</sub> were highly dependent on the drugs' specificity for monoaminergic receptors (Matsuzaki et al., 2002). Stress induced through corticosterone administration in mice promoted anxious responses in EPM, commensurate with increased Orx<sub>A</sub> (Jalewa et al., 2014). Activation of Orx cells using chemogenetic tools (DREADDs) during social defeat promotes a passive coping phenotype, expressing anxiogenic behaviors and reduced memory (Eacret et al., 2018). The functional efficacy of Orx<sub>1</sub> antagonists as a pharmacotherapeutic pathway to limiting Orx<sub>A</sub> activity, and thereby reducing anxiety has merit, however, it also carries the inevitable side effect of modifying sleep-wake cycles, such that careful dosing would be necessary for their effective use.

Despite the uncertainty of its precise physiological role in modulating affect, the orexinergic system has a clear connection to depressive behaviors. Orexinergic cell function is reliably dysregulated by depression (Bowrey et al., 2017). In clinically depressed humans, the concentration of serum Orx was positively associated with incidences of physical or emotional neglect (Ozsoy et al., 2017). Similarly, chemogenetic inhibition (DREADDs) of Orx cells during social defeat increased social interaction, and decreased depressive

behavior in vulnerable individuals (Grafe et al., 2018). In contrast, CSF concentrations of Orx<sub>A</sub> are significantly lower in patients with major depression (Brundin et al., 2007a; Brundin et al., 2007b) and exhibit blunted diurnal variation in this peptide (Salomon et al., 2003). Additionally, severity of the symptoms of depression, negatively correlate with CSF and plasma Orx<sub>A</sub> (Brundin et al., 2007b; Rotter et al., 2011). Stress induced through corticosterone administration in mice promoted depressive reactions in Tail Suspension Test (TST) trials, but further was associated with increased Orx<sub>A</sub>-containing cells in the hypothalamus (Jalewa et al., 2014). 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 (Allard et al., 2004). Similarly, social defeat-induced depressive behavior in rats was accompanied by a reduction of Orx in the VTA, medial prefrontal cortex (mPFC), and hypothalamus (Nocjar et al., 2012). In the ventral pallidum, with direct projections of Orx neurons, Orx activates GABAergic neurons and prevents depressive behaviors, promoting resilience (Ji et al., 2018).

Furthermore, Orx-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 (Khalil and Fendt, 2017). In contrast, optogenetic excitation of Orx cells in rats increased anxiogenic aversion for a social target, increased exploratory behaviors, and resulted in the internalization of Orx<sub>1</sub> receptors in the paraventricular thalamus (PVT) and locus ceruleus (LC) (Heydendael et al., 2014). These examples describe an influential role of Orx in affective symptomology. In addition to its broad implications to central dysfunction, Orx administered into distinct areas of the stress circuit, namely the BNST (Lungwitz et al., 2012), the CeA (Avolio et al., 2011), and the BLA (Kim et al., 2015), produced anxious and depressive behavior.

In addition, chronic social defeat epigenetically reduced prepro-Orx mRNA, but calorie restriction thereafter enhances activation of Orx cells, which results in an antidepressive response (Lutter et al., 2008). Similarly, early life stress dampened restraint-stimulated Orx cell activity, and produced a depressive behavioral phenotype, all of which were reversed by exercise in adolescent male rats (James et al., 2014). These reports together suggest that reduced Orx is critically associated with depression and preclinical depressive behavior (Bowrey et al., 2017; James et al., 2017a; Yeoh et al., 2014). What is more, in genetic models of depression, reduced Orx levels have also been measured (Allard et al., 2004; Taheri et al., 2001). Rodents bred with the Orx<sub>1</sub> gene knocked out, exhibited increased anxious responses (Abbas et al., 2015), reduced depressive behaviors (Abbas et al., 2015; Scott et al., 2011), and impaired fear conditioning in reaction to cued and contextual stimuli (Flores et al., 2014; Soya et al., 2013). Collectively, these results suggest the potential importance of therapeutic antidepressive intervention by targeting Orx<sub>1</sub> receptors.

Hyperactivity, a behavior characteristic of anxiety-related disorders, is heightened in prenatal pups exposed to alcohol, but can be alleviated using daily administration (ip) of SB-334867, a potent selective antagonist of Orx<sub>1</sub> receptors (Stettner et al., 2011). Curiously, systemic (ip) delivery of SB-334867 does not appear to impact general anxiety in the EPM (Rodgers et al., 2013; Scott et al., 2011; Staples and Cornish, 2014); however, this drug can reverse decreased time in the open arms associated with acute nicotine-induced anxious

behavior (Plaza-Zabala et al., 2010). Additionally, panic induced through exposure to hypercapnic air results in increased mean arterial pressure (MAP) and enhanced anxious behavior in the open field (OF), but these measures can be attenuated through injections (ip) of SB-334867 (Johnson et al., 2012c). Furthermore, panic-prone rats administered SB-334867 (ip) exhibited decreased freezing behavior (Johnson et al., 2010) and increased social interaction time (Johnson et al., 2015). Rats systemically-treated (ip) with SB-334867 more readily approached odors associated with naïve and familiar predators (Staples and Cornish, 2014). Administration (ip) of SB-334867 in mice heightened cellular activity in BLA extinction neurons (Flores et al., 2017), suggesting a direct pharmacological action on stress/fear neural circuits. Intracranial infusion of SB-334867 into the posterior PVT decreased time in closed arms (EPM) and reversed Orx<sub>A</sub>-promoted internalization of Orx<sub>1</sub> receptors in the PVT (Heydendael et al., 2011). In a similar way, intra-BLA injections of SB-334867 facilitated fear extinction in mice (Flores et al., 2014). Collectively, these examples demonstrate therapeutic viability of SB-334867 in the treatment of anxiety disorders. In addition to SB-334867, other Orx antagonists have been used to control anxious behavioral output. An fMRI study in rats demonstrated GSK-1059865, a selective Orx<sub>1</sub> antagonist, can attenuate abnormal activity in fronto-hippocampal and extended amygdalar regions after yohimbine-induced stress (Gozzi et al., 2013).

Injections of SB-334867 (ip) mimic the Orx<sub>1</sub> null mice showing reduced depressive reactions in tests of behavioral despair (FST and TST) (Scott et al., 2011) and fear conditioning paradigms (Flores et al., 2014). In contrast, SB-334867 (ip) inhibited the reported antidepressive actions of icv Orx<sub>A</sub> in the Forced Swim Test (FST) in ddY mice, and prevented Orx-induced proliferation in the dentate gyrus (Ito et al., 2008).

## 5. Dual orexin receptor antagonists (DORAs)

Developed for use in treatment of insomnia, DORAs are antagonists of both Orx<sub>1</sub> and Orx<sub>2</sub> receptors (Preskorn, 2018), and while effectively yielding hypnotic results (Howland, 2014; Owen, 2016), have been suggested to be potentially useful in treatment of addiction as well (Khoo and Brown, 2014). These drugs, though not selective for the Orx<sub>1</sub> receptor alone, have displayed promise as therapeutic tools for relieving signs of affect. Orally administered DORA-12 enhanced social interaction time in rats subjected to high levels of CO<sub>2</sub> to promote a panic state (Johnson et al., 2015). Almorexant, a competitive Orx receptor antagonist designed to treat insomnia, lowered blood pressure (BP) in hypertensive rats, while having no impact on the resting BP of wildtype animals (Li et al., 2013). Further, almorexant, in a dose-dependent fashion, reduced fear potentiated startle in conditioned rats, while having no myorelaxant effects (Steiner et al., 2012). Using an unpredictable chronic mild stress (UCMS) model for depression, mice administered daily almorexant experienced decreased anxious and depressive behaviors that were comparable to fluoxetine-treated animals (Nollet et al., 2012). The effectiveness of dual orexin receptor antagonists suggests that the orexin receptors function in similar fashion; however, before this type of drug becomes the standard therapy, it seems judicious to clarify the functions of each receptor separately.

## 6. A case for opposite actions of Orx<sub>1</sub> and Orx<sub>2</sub> receptors

A study of depressive behavior using the forced swim test (FST) suggested a divergent pattern between hippocampal and amygdalar Orx<sub>A</sub> activity, and Orx<sub>1</sub> gene expression associated with immobility (Arendt et al., 2013). The initial experimental results suggested to us that Orx<sub>1</sub> activity may be depressive and/or anxiogenic, because the FST results showed a positive linear correlation between the duration of immobility and amygdalar Orx<sub>1</sub> gene expression. In the following experiment, we saw that ten days of social defeat produced a similar increase of Orx<sub>1</sub> mRNA in BLA of stress susceptible, but not resilient mice (Arendt et al., 2014). Interestingly, the results for Orx<sub>2</sub> mRNA were opposite; mice susceptible to anxious and depressive behavior in the SIP test, and to increased anxious behavior in OF, exhibited decreased Orx<sub>2</sub> gene expression, not measured in resilient mice. Regression analysis showed the same opposing trend, a strong linear, negative relationship between social preference (in the Social Interaction/Preference test, SIP) and Orx<sub>1</sub> mRNA levels, similar to what we found for immobility (though in the opposite direction because the valence for SIP score and immobility is reversed), and strong positive linear relationship between Orx<sub>2</sub> gene expression and social preference. Together the results suggest that Orx<sub>1</sub> activity is anxiogenic, as has been demonstrated by other labs (Johnson et al., 2012b), but that Orx<sub>2</sub> receptor activity is anxiolytic (Arendt et al., 2013; Arendt et al., 2014; Staton et al., 2018). Knockdown of BLA Orx<sub>1</sub> or Orx<sub>2</sub> activity, using short-hairpin RNA (shRNA), also stimulated opposing results (Fig. 1)(Arendt et al., 2014).

## 7. Knockout and knockdown of Orx receptors

Mice lacking prepro-orexin gene expression, Orx knockout (Orx KO), have been demonstrated to exhibit reduced feeding, as might be expected, but also obesity (Hara et al., 2001), which suggest that the metabolic effects of Orx are even more important than those associated with food seeking and feeding. These feeding-related orexinergic effects appear to be differentiated by sex, such that increased body weight in females is seen in Orx KO mice, but increased insulin resistance occurs (Tsuneki et al., 2008) in both sexes (Ramanathan and Siegel, 2014). In Orx KO mice abnormal sleep-wake patterns (Blumberg et al., 2007; Diniz Behn et al., 2010; Espana et al., 2007; Nakamura et al., 2007), elevated body temperature during sleep (Mochizuki et al., 2006), and narcolepsy are produced (Chemelli et al., 1999; Hara et al., 2001), and exacerbated when the Orx<sub>2</sub> receptor is also deleted in the ventrolateral periaqueductal gray using a neurotoxin (Kaur et al., 2009). Combining feeding and narcoleptic effects, Orx KO mice further exhibit feeding elicited cataplexy (Clark et al., 2009). In addition, mice with Orx KO experience reduced cardiovascular and behavioral responses to social stress in the resident intruder test (Kayaba et al., 2003). This latter response is suggestive of a role for the orexin system in depressive behavior and social anxiety. Our model of social defeat, and species-specific anxious and depressive behavior (Carpenter and Summers, 2009; Smith et al., 2014; Summers et al., 2017), also suggests that Orx receptor activation promotes several kinds of learning (see below), and Orx KO impairs spatial working memory (Dang et al., 2018).

In Orx<sub>2</sub> receptor KO mice, basal concentration of hypothalamic norepinephrine (NE), acetylcholine (ACh), and histamine (H) are significantly higher, but stimulated release of



these transmitters was reduced (Ortega et al., 2012). By contrast, in prefrontal cortex (PFC), stimulated release of serotonin (5-HT), NE, and dopamine (DA) is significantly increased. A potential inhibitory Orx<sub>2</sub> function in hypothalamus on basal concentrations of transmitters may be mirrored by Orx<sub>2</sub> receptor down-regulation of evoked release in PFC. Therefore, genetic deletion of Orx<sub>2</sub> receptors modulates neurotransmitter systems (Ortega et al., 2012) that are involved in depressive and anxious behavior in regions that are also involved. This idea is supported by the fact that Orx KO mice display anhedonia in the sucrose preference test (Matsuo et al., 2011). Additionally, Orx<sub>2</sub> KO mice also display increased behavioral despair in FST and TST (Scott et al., 2011).

Following experiments in our lab that examined Orx<sub>1</sub> and Orx<sub>2</sub> gene expression in BLA and prelimbic prefrontal cortex, which suggested opposing functions for these two receptors in response to ten days of social defeat, we employed viral vectors designed to knock down the expression of the orexin receptors in unstressed mice, via bilateral injections targeting the BLA (Arendt et al., 2014). Separate plasmids with short hairpin RNA structures were designed to target specific regions of the Orx<sub>1</sub> (CCAAAGGTCCCCACAGACATATTC) and Orx<sub>2</sub> (AGAAACCCTTCAGTGGGACTTAAC) mRNAs (Scrambled control: CGGAATTTAGAAACCCGGCTCCAC). The unstressed Orx<sub>2</sub> BLA-KD mice exhibited anxiogenic responses in the SIP (Orx<sub>2</sub>:  $t_{11} = 2.4$ ,  $p = 0.037$ ) and open field (OF; Orx<sub>1</sub>: $t_{12} = 1.0$ ,  $p = 0.33$ ; Orx<sub>2</sub>:  $t_{11} = 2.7$ ,  $p = 0.021$ ) tests (Fig. 1; from Arendt et al., 2014), without any change (Orx<sub>2</sub>:  $t_{12} = 1.5$ ,  $p = 0.16$ ) in locomotion (Arendt et al., 2014). These results suggest an anxiolytic role for Orx<sub>2</sub> receptors. After Orx<sub>1</sub> KD in the BLA, mice had a significant reduction in locomotion (Orx<sub>1</sub>: $t_{12} = 2.5$ ,  $p = 0.033$ ), and an insignificant (Orx<sub>1</sub>: $t_{10} = 0.9$ ,  $p = 0.39$ ) rise in SIP score. The results suggested differential, if not strictly opposing, functional manifestations of unstressed Orx<sub>1</sub> and Orx<sub>2</sub> KD mice. The broader implication of the research is that Orx<sub>2</sub> receptors may not play an anxiogenic role, as other research has suggested, but rather reduce anxious and depressive behavior, even in unstressed mice. The results also suggested the need for examining the effects of Orx<sub>2</sub> receptors in mice in response to severe stressors.

## 8. The Stress-Alternatives Model

The Stress-Alternatives Model (SAM) is designed to examine the development of stress-related behaviors in response to aggressive social interaction (Fig. 2). We submit that these behaviors (See 9. Validation of the SAM) may reflect species-specific anxious and depressive behavior (Keifer and Summers, 2016). The SAM conceptual model and arena are designed to be ethologically relevant for the particular test species (Blanchard et al., 2013; Keifer and Summers, 2016; Pearson et al., 2017), while considering the necessity of social stress as a prerequisite for translational results (Haller and Alicki, 2012; Haller et al., 2013). The plan for the SAM apparatus devolved from two other highly successful models, the visible burrow system (Arakawa et al., 2007; Blanchard et al., 1995; Blanchard and Blanchard, 1989; Pobbe et al., 2010) and the social defeat model (Berton et al., 2007; Golden et al., 2011; Krishnan et al., 2007), such that the task involved was ethologically relevant, socially stressful, provided active behavior to measure, and included decision-making and learning (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014; Summers et al., 2017). We consider Escape and Stay behavioral outcomes to be the

result of decision-making, because early responses are variable, and become stable with experience, and are modifiable by learning as well as anxiogenic or anxiolytic drugs (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014; Smith et al., 2016; Summers et al., 2017; Yaeger et al., 2018). The SAM apparatus has been adapted to accommodate studies on rodents (mice, rats, and slightly modified for hamsters) or fish (Rainbow trout) with results that indicate that stress-related behavior, and the neurocircuitry that mediates them, is highly conserved evolutionarily (Arendt et al., 2012; Carpenter and Summers, 2009; Keifer and Summers, 2016; Robertson et al., 2015; Smith et al., 2016; Summers et al., 2017). We propose that anxious and depressive behavior, considered in a species appropriate manner, may also be evolutionarily conserved, as it appears to also be primarily derived from stress-associated circuitry. The basic construct is a neutral oval open field arena in which a small test adult experiences social aggression from a novel and much larger conspecific each day for several days, but with escape routes available (Fig. 2). Surprisingly, in rodents and fish, on average over numerous experiments, only about half of the animals use the escape hole (Escape), while the other half remain submissively, and continue to be aggressively abused by the larger individual (Stay). While there is variability in the proportion of Escape and Stay animals with each cohort tested, and stressful animal care conditions can skew the ratio, Escape animals are reliably resilient to stress as measured by the social interaction or preference test (SIP: susceptibility/resilience scores are measured by time near container with a social target (at one edge of a 40<sup>3</sup> cm box) / time near a novel container lacking a social target × 100; such that a score < 100 indicates susceptibility, and a score > 100 indicates resilience) used in the social defeat model (Arendt et al., 2014; Golden et al., 2011; Krishnan et al., 2007; Staton et al., 2018; Yaeger et al., 2018). Similarly, Stay animals are reliably susceptible to stress and anti-social behavior measured by SIP; by which we mean that untreated Stay animals have SIP scores below 100, which indicates anxiety due to the lack of approach to potentially fearful animals. We suggest that these results may also indicate mouse-specific depressive tendencies, because this normally socially curious and gregarious test animal shuns social interaction after SAM-induced Stay phenotype development. This is an example of what we consider may be species-specific anxious and depressive responses. What is more, these Stay animals exhibit experience modified anxious behavior in the OF and EPM tests, as well as increased fear conditioned and conflict-stimulated freezing, elevated conflict-evoked startle, increased corticosterone concentrations, increased indecisiveness associated with anxiety (or CRF)-induced headshaking, and decreased novelty exploration (Carpenter and Summers, 2009; Robertson et al., 2015; Robertson et al., 2017; Smith et al., 2014; Smith et al., 2016; Staton et al., 2018; Summers et al., 2017; Yaeger et al., 2018); suggesting multiple stress-related, and potentially anxious and depressive, behavioral expressions, to validate equating the stay phenotype with susceptibility to these species-specific outcomes, which appear to be affective responses. These socially defeated animals (Stay) represent the end of an intensity gradient of anxious behavior produced by the SAM paradigm (Robertson et al., 2015; Smith et al., 2016). The gradient of anxiety that spans the contextual settings of escaping the open field, escaping from aggression, and submitting to aggression corresponds with increasing levels of plasma corticosterone and increasing levels of neuropeptide S (NPS) and brain-derived neurotrophic factor (BDNF) in the central amygdala. As behavioral responses escalate in concordance with physiological reactions associated with stress, anxiety and learning, and with each

ameliorated differentially by endogenous neuropeptides, physical apparatus, and pharmaceutical interventions, it allows for parsing anxious SAM behavior into contextual niches along a species-specific gradient (Robertson et al., 2015; Smith et al., 2016). The addition of a conditioned stimulus (CS = tone) during the initial separation period (small test animal in an opaque cylindrical divider) prior to aggression (= unconditioned stimulus; US), with CS + US paired for 4 days of SAM interaction, results in a conditioned response (CR, freezing in mice, increased cortisol in trout) in the Stay phenotype only (Carpenter and Summers, 2009; Smith et al., 2014; Staton et al., 2018).

While the Escape/Resilient and Stay/Susceptible phenotypes are relatively stable, they are not innate or immutable. Each phenotype, as well as the anxious or resilient behaviors expressed by Escape or Stay individuals, can be modified by environmental or neurochemical factors that are either anxiogenic (yohimbine - an  $\alpha_2$ -adrenoreceptor antagonist, social aggression) or anxiolytic (wheel running exercise, prior exposure to the escape hole, prior-experience escaping in the SAM, NPS, antalarmin (a CRF<sub>1</sub> receptor antagonist) (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014; Smith et al., 2016; Summers et al., 2017; Yaeger et al., 2018).

The act of escape requires learning where the escape route is, at the distal ends of the oval OF for mice and in the upper corner of the 3-D OF aquarium for trout, which happens during the first moments of the initial social interaction in the SAM. The more complex aspect of learning has to do with the large aggressor, that must be avoided to affect the escape (Carpenter and Summers, 2009; Robertson et al., 2015; Summers et al., 2017). The SAM arena provided a unique test apparatus with multiple layers of behavioral evidence for our experiments on Orx<sub>2</sub> receptor modulation of anxious and depressive behavior during socially stressful decision-making.

## 9. Validation of the SAM

The SAM is a recently devised preclinical behavioral model, designed to specifically address ecologically relevant, species-specific, anxious and depressive behavior (Blanchard et al., 2013) in mice, but using modified versions for rats and fish as well (Robertson et al., 2015). This model has only existed a few years, with its first published paper in 2009 (Carpenter and Summers, 2009), and the first data from a mouse version published in 2014 (Smith et al., 2014), and thus while we take great comfort in the results that suggest strong evolutionary conservation of stress-derived behavioral inhibition (Smith et al., 2016; Summers et al., 2017), which is similar to anxious and depressive behavior (Keifer and Summers, 2016), we understand that removing some caution regarding the results from this model awaits further validation. For this purpose, we consult an accepted set of criteria for assessing the validity of animal models, which has been used and refined for 5 decades (McKinney and Bunney, 1969; Willner, 1984; Willner, 1991; Willner and Mitchell, 2002). They are typically grouped into three categories: 1) Construct Validity, relating to the model's theoretical rationale 2) Predictive Validity, relating to the success of predictions made from the model, such as similarity in treatment effectiveness; and 3) Face Validity, relating to similarities between the symptomatology and behaviors resulting from the animal model (Willner, 1984). Validations for models of depression and anxiety are difficult due to

largely or exclusively behavioral symptom profiles, as well as etiology and biological mechanisms involved that are imperfectly understood. As a result, none of the extant animal models are completely validated; nor are the predisposing factors, antecedent conditions, circuits, mechanisms of action, or even descriptive characteristics of common symptomology completely known for the disorders themselves.

**Construct Validity** - is usually treated as the most important of the three criteria (Sarter and Bruno, 2002) and may involve all elements that characterize the disorder, such as inclusion in the Diagnostic and Statistical Manual (DSM) or the International Classification of Diseases (ICD). It typically includes substantial attention to predisposing factors and antecedent conditions (and sometimes behavior), as components of the pattern of similarity of a model to factors contributing to the concept/definition of the target condition. These include the use of threatening or painful events to elicit behaviors or behavioral changes that are presumed to be related to anxiety, and chronic stressors used to induce changes that are treated as measures of depression. Models based on chronic social defeat, take advantage of the relationship between fear and anxiety (Allcoat et al., 2015; Barros and Tomaz, 2002; Haegler et al., 2010; Lang et al., 2000), but also social stress and depression (Grandjean et al., 2016; Heim and Binder, 2012), maintaining ecologically, ethologically, and species-specific relevance for the model (Blanchard et al., 2013; Keifer and Summers, 2016; Robertson et al., 2015) and relevance to the human disorder (Hendrie et al., 2013; Hunt et al., 2012). The development of the SAM, has been aimed at the observation that social stress produces the most significant elevation in stress hormones (Koolhaas et al., 1997) plus a high degree of metabolic cost in addition to the dimension of unpredictability (Koolhaas et al., 2011; Summers et al., 2005), and the fact that the incidence rates of adult affective disorders steeply rises during adolescence in parallel with a structural and functional reorganization of the neural circuitry underlying stress reactivity (Andersen, 2003; Bernstein et al., 1996; Coppens et al., 2011; Hankin et al., 1998; Romeo and McEwen, 2006). Since stress circuits and neuromodulatory factors are predisposing factors for human depression (Binder and Nemeroff, 2010; Swaab et al., 2005; Waters et al., 2015) and anxiety (Binder and Nemeroff, 2010; Swaab et al., 2005) as well as mouse depressive (Grandjean et al., 2016; Waters et al., 2015) and anxious behavior (Meng et al., 2015), the SAM fits the basic criteria for a preclinical model with substantial construct validity (Robertson et al., 2015; Smith et al., 2016; Summers et al., 2017).

**Predictive Validity** - The use of drugs, through both induction and reduction, as part of the definition of some pathological behavioral states remains a common approach, a predictive validity. For models of anxiety and depression, predictive validity involves the ability of established anxiolytics and antidepressants (respectively) to normalize behaviors that are induced or changed by some type of condition or manipulation (such as social defeat or social stress) that produces potentially anxiety- or depression-relevant patterns. (Iny et al., 1994) The problem of course, for anxiety and depression, is that “established” anxiolytics (like benzodiazepines) or antidepressants (such as serotonin or norepinephrine reuptake inhibitors), while being the primary drugs used for treatment, have not established a broad enough level of patient satisfaction (Young et al., 2001), and often include side-effects like addiction and withdrawal. Simplistic interpretations of the predictive validity concept tend to

deflect attention from complex biochemical and behavioral etiologies and outcomes. As only one in three anxiety or depression patients receive effective treatment (Young et al., 2001), classical and established anxiolytic or antidepressant drugs have not been our priority for SAM validation, and we admit that this is a significant shortcoming. We have however, examined other drugs with known anxiety or depression related effects, including anxiolytics such as NPS, the anxiolytic and antidepressant antalarmin, as well as the anxiogenic drug yohimbine. For example, icv injection of the CRF<sub>1</sub> receptor antagonist antalarmin on the 3<sup>rd</sup> day of a 4 day SAM protocol in mice, or on the 4<sup>th</sup> day of the 7 day protocol in trout, allows for Escape behavior in 40% of mice and 60% of trout that were previously Stay individuals. Anxiolytic factors also decrease the latency to escape, which is dramatically reduced by 2 days of familiarity with the task. Since the icv CRF<sub>1</sub> antagonist treatment was after this 2 day period, escape latency reveals that Stay animals had also learned the behavioral strategies necessary to escape prior to treatment with the anxiolytic drug, but did not make use of these strategies until anxiolysis made it possible, at which time these CRF<sub>1</sub> antagonist-treated animals escape with the same latency as those that had been escaping all along. These known anxiolytic, antidepressant, or anxiogenic drugs (NPS, antalarmin, yohimbine), provide some predictive validation for the relationship between the uniquely SAM behavioral measures and anxiety or depression (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2014; Smith et al., 2016; Summers et al., 2017). An additional test, Social Interaction testing (including the SIP), that we often use along with the SAM has been validated against drugs commonly used therapeutically for anxiety (benzodiazepines) and depression (SSRIs), (File, 1980; Johnson et al., 2010; Lightowler et al., 1994; Molosh et al., 2018; Sanders and Shekhar, 1995). In addition, site-dependent positive and negative *in vivo* markers of depression such as BDNF, also are suggestive of a predictive validity for social defeat models, such as the SAM (Berton et al., 2007; Smith et al., 2014). What is more, similar to anxiety- and depression-related physiological dysfunction in humans, the SAM results depict elevated glucocorticoid concentrations in response to putatively anxious and depressive events and behaviors (Carpenter and Summers, 2009; Robertson et al., 2015; Smith et al., 2016; Staton et al., 2018).

**Face Validity** - Behavioral similarities, described as providing ‘face validity’, have typically been the least appreciated criteria for animal models. While normal behavioral actions of rodents or other nonhuman animals are substantially different from human actions having exactly the same function, motivational or incentive states may differ. We argue that adaptive functioning, based on the general evolutionary history of the animal, responsive to subject, stimulus, and situational characteristics, and characterized in terms of its immediate and longer-term outcomes, can constitute a highly efficient basis on which to evaluate parallels between behaviors in an animal model, and those of people (Blanchard et al., 2013; Keifer and Summers, 2016; Robertson et al., 2015). We argue that depressive behavioral inhibition, anhedonia, and despair, or enhanced anxious startle and an impending sense of danger that are commonly seen in human depression and/or anxiety patients, are also evident for nonhuman preclinical models. In our model, depressive behavioral inhibition is seen in absence of escape, delayed escape, enhanced fear conditioning, elevated conflict-induced freezing, and avoidance of the escape route. The lack of escape behavior or interest in the escape route are also indications of despair and anhedonia, because reducing stress levels

promotes both behaviors. Anxious startle is evident by increased startle behavior during social interaction. An impending sense of danger, a classic symptom of anxiety, is evident in SAM fear conditioning. (Smith et al., 2016; Staton et al., 2018).

## 10. Evidence for potential pharmacotherapies targeting the Orx<sub>2</sub> receptor

Our work, making use of the FST and social defeat model, clearly suggested that Orx<sub>2</sub> receptors had a different behavioral profile than Orx<sub>1</sub> receptors (Arendt et al., 2013; Arendt et al., 2014). Those results led us to hypothesize that Orx<sub>2</sub> receptors were functionally anxiolytic, a deduction that was corroborated by experiments in which shRNA was used to knockdown the Orx<sub>1</sub> and Orx<sub>2</sub> receptors in the BLA (Fig. 1). While we did not see any anxiety- or depression-related behavioral effects from the shRNA Orx<sub>1</sub> KD, aside from decreased locomotion, the Orx<sub>2</sub> KD mice exhibited anxiogenic and pro-depressive effects in OF and SIP tests (Fig. 1). Neither KD had any effect on the elevated plus maze, however, since these were unstressed animals, we were not sure what the effect of Orx receptors would be after social defeat, although the gene expression data for defeated mice suggested the effect would be opposite for Orx<sub>1</sub> and Orx<sub>2</sub> (Arendt et al., 2014). Our next step was to apply Orx receptor treatments to stressed animals using the SAM apparatus.

We began by working with the Orx<sub>2</sub> receptor drugs for two reasons. The first was that our work indicated the potential for anxiolytic and antidepressive effects from stimulation of the Orx<sub>2</sub> receptor, and second, more was already known about Orx<sub>1</sub>. Additionally, it occurred to us that actual treatment for anxiety and depression via Orx<sub>2</sub> pharmacotherapy would be necessarily systemic, and so our initial approach considered a need for understanding systemic effects, but at the same time we didn't want to have central Orx<sub>2</sub> actions be complicated by peripheral effects on metabolism or other physiological functions (Tsuneki et al., 2008; Zhang et al., 2005). We settled on an intracerebroventricular (icv) approach, targeting all or most of the brain, and used an Orx<sub>2</sub> agonist ([Ala<sup>11</sup>-D-Leu<sup>15</sup>]-Orx<sub>B</sub>) and an antagonist (MK-1064), hypothesizing complementary but opposing results. In this set of SAM experiments, the results clearly suggest reduced anxious and depressive behavior associated with increased Orx<sub>2</sub> activity.

The SAM provides a variety of potentially anxiety- and depression-related measures, the first being whether mice that have demonstrated a reliable pattern of Escape or Stay behavior on days one and two, can be induced to switch the behavior from that of their established behavioral phenotype after Orx<sub>2</sub> drug icv injection. First, icv injection of vehicle has no effect on behavioral phenotype. However, similar to what was seen in earlier experiments with the anxiogenic  $\alpha_2$ -adrenoreceptor antagonist yohimbine (Smith et al., 2016), in Escape mice (escaping 100% of the time on days 1 and 2) the Orx<sub>2</sub> antagonist MK-1064 injected on day 3 of SAM interactions blocked 50% of those mice from escaping (Staton et al., 2018). It is important to remember that the Escape phenotype is stress resilient in the SIP test (Fig. 3E), and Stay animals are stress susceptible, so this result is the equivalent of taking resilient individuals, and making half of them susceptible to stress, and putatively anxious and/or depressive behavior. The other results from this set of experiments confirm this notion. When we started with Stay phenotype mice, those that are susceptible after the first 2 days of SAM interactions, and give an Orx<sub>2</sub> agonist, there was a very small reversal to Escape in

11% of Stay mice, and this happened on day 4, one day after the day of injection (Staton et al., 2018). Clearly more work needs to be done to determine if there is an effect, but it is suggestive of a potential anxiolytic response.

We measured freezing in two ways in these experiments. The first is a part of a fear conditioning paradigm in which the conditioned stimulus (CS) is a tone played during the brief period of isolation for the test animal (in an opaque cylinder in the SAM apparatus) prior to aggressive interaction with a novel CD1 mouse. Social interaction with a CD1 (which was placed outside the cylinder) constitutes the unconditioned stimulus (US) that follows. The CS and US are paired for each of the 4 days of SAM social interaction. Freezing is measured during the trace period after the tone and prior to the divider being removed. On the 5<sup>th</sup> day, no larger CD1 mouse is present outside the cylinder, and the CS is presented alone, to test for a conditioned response (CR). We also measured conflict freezing during the SAM social interaction itself, something the small C57 black mouse did in response to, the presence of a larger CD1 mouse, or aggression from that animal. For both cued fear conditioning (Fig. 3A;  $t_{13} = 2.7, p = 0.018$ ) and conflict freezing (Fig. 3B;  $t_{16} = 2.4, p = 0.03$ ), vehicle-treated mice of the Stay phenotype did significantly more freezing (normalized for each mouse by the time spent in the SAM arena) than Escape mice did. Only Stay mice exhibit fear conditioning (CR = fear freezing ratio > 100%) in this measure on test day (Fig. 3A). The Orx<sub>2</sub> agonist blocked the fear conditioned response in Stay phenotype mice on test day (Fig. 3A;  $F_{2,31} = 4.86, p = 0.015$ ), and significantly reduced conflict freezing ( $F_{2,25} = 4.8, p = 0.017$ ) during the period of actual social aggression on day 3 (Fig. 3B), when the drug was injected icv (Staton et al., 2018). That means that the Orx<sub>2</sub> agonist had an immediate effect on conflict freezing, but also a prolonged effect, blocking the CR two days later. While the Orx<sub>2</sub> antagonist did not affect the conditioned response, it did stimulate a continued increase in conflict freezing on injection day (day 3; Fig. 1B), which was not seen in vehicle-treated mice.

Fear enhanced startle is a common method for examining anxious behavior (Davis, 1992), and our model produces significant stress during the social interaction. Startle responses (whole-body flinching) measure an animal's reaction to sudden environmental stimuli, often acoustic (Heesink et al., 2017; Sallinen et al., 1998). We surmised that as the intensity and amplitude of the acoustic-startle response are affected by stress-related events (Belzung et al., 2001; Risbrough et al., 2004), the frequency of socially-induced startle might also be enhanced by increasing stress during aggressive interactions. With this in mind, we pirated the concept of startle for use in the SAM, because we noticed that active approach of an aggressor mouse also elicits a similar startle response. Therefore, we made use of a stimulus that exists as a part of aggressive social interaction, the threat of attack, and measured the number of flinches or recoils that were induced by the approach of the larger CD1 mouse during each entire social interaction. We examined the number startle responses, rather than the amplitude of startle, stimulated by the approach of the aggressor mouse. The frequency of startle responses grew in vehicle-treated Stay phenotype mice, over each of the 4 days of the SAM social interactions (Staton et al., 2018), and was significantly greater ( $t_{14} = 2.7, p = 0.019$ ) in vehicle-treated Stay compared to Escape phenotype mice (Fig. 3C). In these Stay mice, the icv-injected Orx<sub>2</sub> agonist on day 3 significantly ( $F_{2,21} = 3.89, p = 0.036$ ) reduced the frequency of startle responses.

As the SAM apparatus has escape routes at each end of the oval interaction arena, we developed a novel measure of exploration, which appears to be suggestive of intent to use the escape route. By examining the time that each mouse spent specifically attentive to the hole (inspecting, sniffing, partially entering, or in very close proximity [3 cm] to the escape tunnel) relative to the total time they were in the arena, it is possible to compare this set of behaviors with Escape or Stay phenotype and with other time sensitive behaviors that signal intention to escape (Staton et al., 2018). Not surprisingly, mice of the Escape phenotype (vehicle-treated in these experiments) are the most attentive ( $t_{16} = 4.67, p < 0.001$ ) to the hole (Fig. 3D). What is somewhat surprising is that, although all mice rapidly notice and inspect, Stay mice spend so little time investigating the hole. The Orx<sub>2</sub> antagonist actually reduces the amount of time, although not significantly as the amount of time spent is already below 1% of the total time in the SAM arena. Additionally, Escape mice spend proportionally more time investigating the hole as the time of egress approaches. In an experiment in which mice were pre-exposed to the escape tunnel, while 80% of mice escaped when SAM social interaction actually convened, 20% of the mice were irrevocably of the Stay phenotype. Although both groups had experienced the safety of the tunnel and the small chamber that it leads to, escaping mice spent more time attentive to the hole (Yaeger et al., 2018). However, the Orx<sub>2</sub> agonist significantly ( $F_{2,24} = 12.37, p < 0.001$ ) increases the percentage of time examining the escape route on the day of icv injection (3) and on the next day as well (Fig. 3D). The day after icv Orx<sub>2</sub> agonist injection (4) is significantly higher than on the day of injection, at which point the Stay animals, which are mostly still *not* escaping, are nearly as attentive to the hole as mice of the Escape Phenotype. What is more, the day 4 expression of greatest attentiveness to the hole coincides with the small 11% reversal of Stay to Escape phenotype described earlier. This conjunction of day 4 events suggests the possibility of neural plasticity relative to the prolonged effect. We see a similar prolonged effect in fear conditioning (measured on test day = 5) and the SIP test, also measured on the day after SAM interactions conclude.

Escape and Stay mice very clearly behaviorally self-select their phenotype, which corresponds later to Social Interaction/Preference (SIP; Fig. 3E). This apparent self-determination is evident, and Escape or Stay are not simply coping responses, because earlier studies clearly indicate that the magnitude or timing of aggression does not promote escape, even though early Escape-Stay responses are variable, and become stable with experience. Additionally, a stable phenotype is modifiable by anxiogenic and anxiolytic drugs, but also by learning (Carpenter and Summers, 2009; Prince et al., 2015; Robertson et al., 2015; Smith et al., 2014; Smith et al., 2016; Summers et al., 2017; Yaeger et al., 2018). Escape vehicle-treated mice spend significantly more time in close proximity to a container enclosing an unfamiliar aggressor mouse (CD1), than to that same novel container that is empty (Staton et al., 2018). This type of behavior (susceptibility/resilience scores = time near container with a social target / time near a novel container lacking a social target  $\times 100$ ; such that a score  $< 100$  indicates susceptibility, and a score  $> 100$  indicates resilience) has been interpreted to be resilient to stress, anxious and depressive behavior, because the mice exhibiting social preference also score low on independent measures of stress, and species specific anxiety and depression (Berton et al., 2007; Golden et al., 2011; Krishnan et al., 2007). Stay mice (vehicle-treated) avoid the social target in the container, and spend more



time in proximity to the container without a social target. These results suggest that Escape and Stay ( $t_{18} = 3.82, p < 0.001$ ), which are evoked as a reliable phenotypic response early (by day 2) in the SAM behavioral regimen, are essentially equivalent markers of Resilience and Susceptibility (Staton et al., 2018; Yaeger et al., 2018). Remarkably, the Orx<sub>2</sub> agonist delivered icv on day 3 reverses Susceptibility in Stay mice making them Resilient ( $F_{2,30} = 4.6, p = 0.018$ ) and icv-administration of the Orx<sub>2</sub> antagonist negates Resilience in Escape mice to render them Susceptible ( $t_{11} = 2.6, p = 0.025$ ; Fig. 3E). Importantly, none of the potentially anxiety- or depression-related behaviors induced by Orx<sub>2</sub> receptor action or inhibition was attributable to changes in activity, as home cage locomotion was unchanged by the icv injection of the drugs ( $F_{2,35} = 0.39, p = 0.68$ ; Fig. 3F)

## 11. Clinical implications of DORA versus Orx<sub>1</sub> versus Orx<sub>2</sub> treatments

While Orx receptor antagonist drugs have been approved for treatment of insomnia (Citrome, 2014; Osborne, 2013), virtually no large-scale clinical examinations of their effectiveness in affective disorder treatment has been undertaken. The DORA hypnotic drug suvorexant (Belsomra) is clinically approved as a treatment to promote sleep by reducing arousal and wakefulness (Howland, 2014). While this drug effectively reduces sleep onset times and increases sleep duration, without rebound or withdrawal (Owen, 2016), it also lowered stress hormone levels cortisol and norepinephrine, and reduced the severity of anxiety and depression in psychiatric patients with insomnia (Nakamura and Nagamine, 2017). These results are consistent with the Orx<sub>1</sub> antagonist activities of the drug, but contrasting with those expected for its Orx<sub>2</sub> receptor antagonist actions. Alarming, these results, contrast sharply with a case study in which a renal dialysis patient with insomnia plus major depressive and generalized anxiety disorders, the latter two treated somewhat effectively with an antidepressant, found that the severity of depression worsened with suvorexant treatment (Petrous and Furmaga, 2017). Interestingly, in a preclinical trial of the DORA almorexant, a competitive Orx<sub>1</sub> + Orx<sub>2</sub> antagonist that had been in phase II clinical trials, anxious and depressive behavior were reduced to the same extent as with the antidepressant fluoxetine (Nollet et al., 2012). As chronic insomnia is highly comorbid with affective disorders, and is associated with up to a 4-fold increased risk of developing major depression (Baglioni and Riemann, 2012; Blake et al., 2018; Staner, 2010), it seems appropriate to test interactions between drugs, such as DORAs, taken for insomnia and antidepressants (Cruz et al., 2014). This study examined the drug interactions between desipramine and almorexant, in part because the enzyme primarily responsible for metabolism of the antidepressant is inhibited by almorexant. Not surprisingly, the DORA almorexant increased exposure to desipramine by nearly 4-fold, whereas the antidepressant had no relevant pharmacokinetic effects on almorexant. There was however, a slight increase in calmness in patients using almorexant. Ironically, this DORA with potential for reducing affective symptoms was removed from clinical trials on the basis of its safety profile. Another Orx receptor antagonist in phase II trials is Filorexant (MK-6096); these trials in patients with major depression were terminated early, without showing a significant difference in depression rating, but with one of the two most common adverse events being suicidal ideation, common in this type of patient (Connor et al., 2017).

The clinical implications for DORAs, as used clinically for treatment of insomnia, are not clear with respect to anxiety or depression. Interestingly, a preclinical optogenetic study found that although sleep disturbance impairs memory, dual and single Orx receptor antagonists used to promote sleep also improve cognitive processes (Li et al., 2018). However, comparisons may be difficult, since the dosages used, at least in relation to our own experiments, are on the order of ten-fold or more higher in hypnotic treatments or arousal studies (Staton et al., 2018)(Table 1). 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. In contrast, anxious and panic behavior can be inhibited by doses of Orx<sub>1</sub> antagonist (or DORA) similar to that used for sleep induction (Johnson et al., 2010; Johnson et al., 2012b; Johnson et al., 2012c; Johnson et al., 2015)(Table 1), suggesting that some Orx drugs may have a wide range of efficacy. The low doses necessary to produce anxiolytic or antidepressive actions from Orx<sub>2</sub> agonist treatment, may prove to be a therapeutic advantage (Table 1). Thus, inhibition of Orx<sub>1</sub> receptors, and stimulation of Orx<sub>2</sub> receptors, both appear to have the potential for anxiolytic and antidepressive actions. While DORA treatment confounds the opposing actions of Orx<sub>1</sub> and Orx<sub>2</sub> receptors, it seem likely that it's anti-panic effects derive mostly from antagonizing Orx<sub>1</sub> actions (Johnson et al., 2015), given the evidence that blocking Orx<sub>2</sub> receptors is anxiogenic and pro-depressive (Staton et al., 2018).

## 12. Synopsis

Our results, as well as that of others, indicate that the orexinergic system contributes significantly to mediation of stress, as well as anxious and depressive behavior. These lines of evidence are reason enough to posit that pharmacotherapies involving the orexin/hypocretin neuromodulators and hormones may be beneficial in patients with anxiety and depression. While the orexinergic system has two active neuropeptides, Orx<sub>A</sub> and Orx<sub>B</sub>, it appears to be the receptors, Orx<sub>1</sub> and Orx<sub>2</sub>, which primarily give rise to opposing functions relative to stress and affective behaviors. In keeping with the theme of this special issue, antagonists do have potential for limiting Orx<sub>1</sub> receptor generated anxiety and panic (Johnson et al., 2010; Johnson et al., 2012b). Interestingly, in unstressed mice, Orx<sub>1</sub> receptor KD had no effect on anxious behaviors, where Orx<sub>2</sub> KD produced anxiogenic response in SIP and OF tests (Arendt et al., 2014). In socially stressed animals in the SAM apparatus, Orx<sub>2</sub> receptors stimulated by icv injection of the agonist [Ala<sup>11</sup>, d-Leu<sup>15</sup>]-Orx<sub>B</sub> produced anxiolytic responses to fear conditioning, conflict stimulated freezing, startle, attentiveness to adaptive behavioral opportunities (attention to the escape hole), SIP Resilience/Susceptibility test, and potentially reversed the socially defeated Stay phenotype to Escape in a small percentage of animals (Staton et al., 2018). The Orx<sub>2</sub> antagonist MK-1064 created anxiogenic responses by reversing Escape to Stay behavior, increasing conflict freezing, increasing Susceptibility responses in the SIP test, and increased plasma corticosterone in anxious stay mice. Taken together, these ten behavioral and physiological results, based on Orx<sub>2</sub> receptor stimulation or inhibition, indicate a possible specific role for the Orx<sub>2</sub> receptor in regulation of anxiety and depression. As only Orx<sub>2</sub> receptor action among orexinergic systems, appears to regulate anxious or depressive behaviors in both unstressed and socially stressed animals, it may be that this receptor subtype plays an important role in the etiology

of anxiety and depression (Arendt et al., 2014; Staton et al., 2018). These factors suggest that Orx<sub>2</sub> receptor physiology may be an important target for anxiety and depression-related pharmacotherapeutic investigations.

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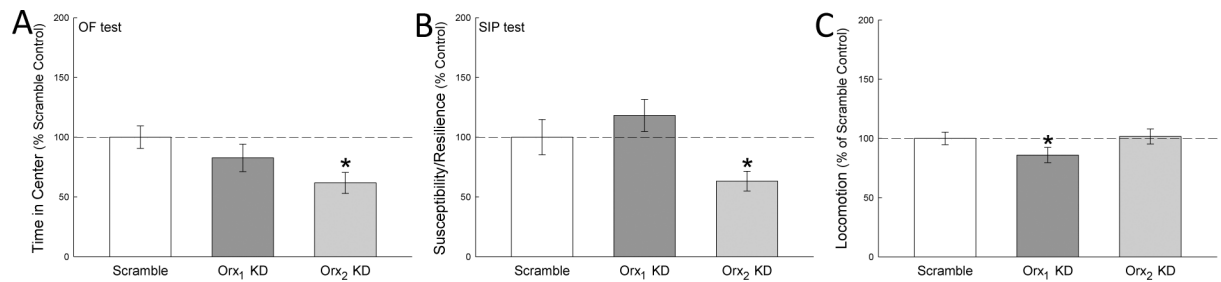
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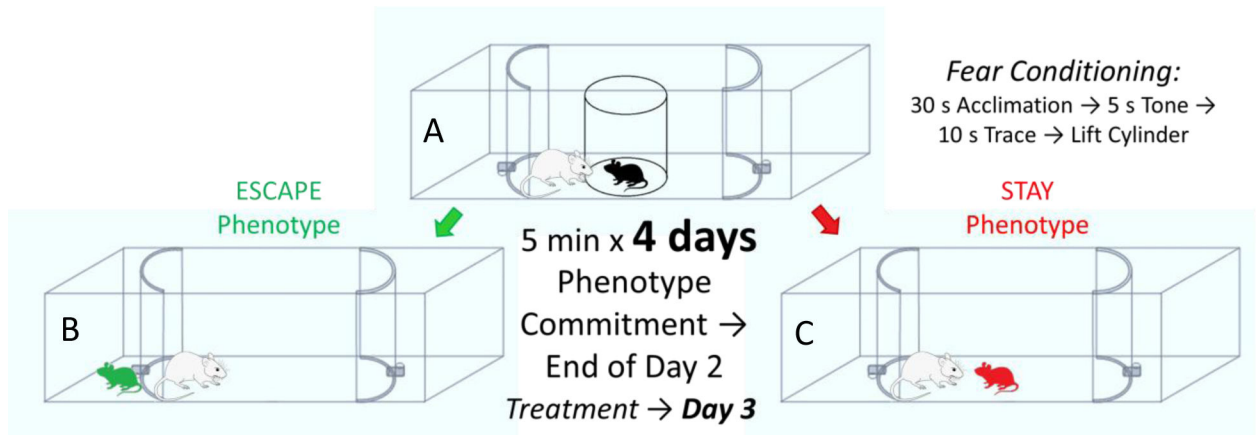
- Orexin/hypocretins innervate limbic regions associated with anxiety and depression
- Orexins are reciprocally innervated with CRF
- Orexin 1 receptors promote anxious and pro-depressive behavior
- Orexin 2 receptor knockdown promotes anxious and depressive behaviors
- Orexin 2 agonists are anxiolytic and anti-depressive



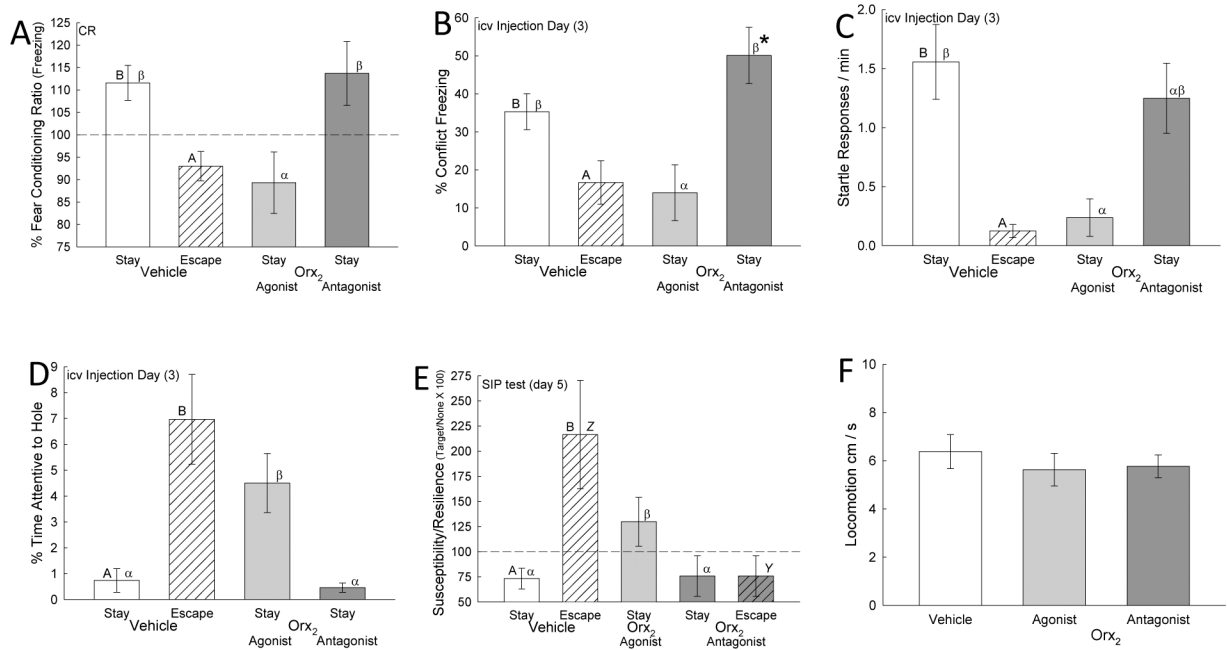
**Fig. 1—.**

Intracranial injection of shRNA in the BLA produced knockdown (KD) of the Orx<sub>1</sub> or Orx<sub>2</sub> receptors, with scramble shRNA as control [from (Arendt et al., 2014)]. Intra-BLA KD of Orx<sub>1</sub> receptors (dark gray bars) had no effect on measures of anxious behavior such as **A**) time spent in the center of the open field (OF; Orx<sub>1</sub>:t<sub>10</sub> = 1.0, *p* = 0.33), **B**) Susceptibility / Resilience scores (time near container with a social target / time near a novel container lacking a social target × 100; such that a score < 100 indicates susceptibility, and a score > 100 indicates resilience) in the Social Interaction/Preference test (SIP Orx<sub>1</sub>:t<sub>10</sub> = 0.9, *p* = 0.39), or EPM (data not shown), but did **C**) reduce locomotion (Orx<sub>1</sub>:t<sub>12</sub> = 2.5, *p* = 0.033) compared to scramble controls (white bars). Conversely, Orx<sub>2</sub> receptor KD in the BLA (light gray bars) was anxiogenic, reducing **A**) OF center time (Orx<sub>2</sub>: t<sub>11</sub> = 2.7, *p* = 0.021), **B**) Susceptibility / Resilience scores in SIP test (Orx<sub>2</sub>: t<sub>11</sub> = 2.4, *p* = 0.037), but **C**) did not reduce locomotion (Orx<sub>2</sub>: t<sub>12</sub> = 1.5, *p* = 0.16) compared to scramble shRNA controls.



**Fig. 2—.**

The Stress-Alternatives Model (SAM) social interaction regimen, begins with **A**) placement of a young adult male C57BL/6 N mouse in the center of the oval arena, but hidden by an opaque cylinder, while a novel and much larger CD1 mouse is also placed in the arena, but outside of the cylinder. After a 30s acclimation period (during which freezing is measured for comparison), a 5s tone is played as a conditioned stimulus (CS), which is followed by a 10s trace period (freezing is also measured during the tone and trace). The opaque cylinder is then removed and social interaction, including vigorous aggression, ensues for 5 min, repeated with a new CD1 aggressor each day for 4 days. During the first 2 days, mice choose a relatively stable behavioral phenotype, either **B**) Escape, which consists of making use of one of the available tunnels at the ends of the oval interaction arena and shortening the duration of their aggressive interaction, or **C**) Stay, in which mice remain in the SAM arena for the full 5 minutes and continues to receive attacks from the CD1. Importantly, both phenotypes receive significant aggression, and the phenotypes, while relatively stable can be reversed by anxiolytic or anxiogenic drugs. Drug treatments occur on day 3, after stable phenotypes have been formed.



**Fig. 3—**

Results from icv Orx<sub>2</sub> agonist and antagonist experiments (Staton et al., 2018) that activation of Orx<sub>2</sub> receptors is anxiolytic and anti-depressive. Behavioral phenotypes derived from social interaction in the Stress-Alternatives Model (SAM) differed, Stay (clear bar) vs Escape (hatched bar; statistical significance between behavioral phenotypes is represented by differing Roman letters atop the bar, such that A is different from B), with more anxious behavior evident in Stay mice by increased **A**) Mean ( $\pm$  SEM) freezing ( $t_{13} = 2.7$ ,  $P = 0.018$ ) in response to fear conditioning ( $[\text{Freezing after CS}/\text{Freezing before}] \times 100$ ); **B**) Freezing during SAM social aggression (% time freezing;  $t_{16} = 2.4$ ,  $P = 0.03$ ); and **C**) Startle responses (/min;  $t_{14} = 2.7$ ,  $P = 0.019$ ); but enhanced anxiolytic responses in Escape phenotype mice by increased **D**) Mean ( $\pm$  SEM) % Time attentive to the hole ( $t_{16} = 4.67$ ,  $P = 0.001$ ), and **E**) Resilience score in the Social Interaction Preference test (SIP;  $t_{18} = 3.82$ ,  $P < 0.001$ ), but **F**) did not affect locomotion. Activation of the Orx<sub>2</sub> receptor by Ala<sup>15</sup>, D-Leu<sup>11</sup>-Orx<sub>B</sub> (light gray bars) reduces **A**) Freezing during fear conditioning in Stay mice ( $F_{2,31} = 4.86$ ,  $P = 0.015$ ; statistical significance between Stay groups is represented by differing Greek letters atop the bar, such that α is different from β), **B**) Conflict induced freezing ( $F_{2,25} = 4.8$ ,  $P < 0.017$ ), and **C**) Startle responses ( $F_{2,21} = 3.89$ ,  $P = 0.036$ ), but increases **D**) % Time attentive to the hole ( $F_{2,24} = 12.37$ ,  $P < 0.001$ ), which may promote an 11% increase in escape behavior, and **E**) SIP resilience score “(time near container with a social target / time near a novel container lacking a social target  $\times$  100; such that a score  $< 100$  indicates susceptibility, and a score  $> 100$  indicates resilience) in Stay mice ( $F_{2,30} = 4.6$ ,  $P = 0.018$ ; comparing Escape mice:  $t_{11} = 2.6$ ,  $P = 0.025$ ). Inhibition of the Orx<sub>2</sub> receptor by MK-1064 (dark gray bars) in Stay mice increases **B**) Conflict freezing on injection day compared to day 2, and plasma corticosterone (data not shown). In Escape phenotype mice the Orx<sub>2</sub> antagonist (dark gray hatched bar) increases **E**) Susceptibility score in SIP test (statistical significance between Escape groups is represented by differing *Italic* letters atop the bar,

such that Y is different from Z), and reduces the number of escaping mice by half (data not shown), but **F**) did not affect locomotion ( $F_{2,35} = 0.39$ ,  $P = 0.68$ ).

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**Table 1.**

Orexin drug treatments for anxious and depressive behaviors

Drug Description ( <i>animal</i> )	Delivery Method	Dose (nmol)	Description of effect on anxious or depressive behavior	Citation
<b>Orx<sub>A</sub></b>				
Orx <sub>A</sub> ( <i>mice</i> )	icv	1 nmol	↑ Anxiogenic behaviors in LDT & EPM	Suzuki et al., 2005
Orx <sub>A</sub> ( <i>rats</i> )	icv	10 nmol	↑ Locomotion, face-washing, grooming, wet-dog shaking	Matsuzaki et al., 2002
Orx <sub>A</sub> ( <i>rats</i> )	iBNST	3 nmol	↑ Anxious behavior in SIP & EPM	Lungwitz et al., 2012
Orx <sub>A</sub> ( <i>mice</i> )	iBLA	0.0001488 nmol (0.53 ng/0.5 µl)	↑ Immobility in FST ↓ Social interaction in U-field test	Kim et al., 2015
Orx <sub>A</sub> ( <i>hamster</i> )	iCeA	0.02 nmol (20 nM)	↑ Anxious and depressive behavior (LDT & EPM but not mentioned in review)	Avolio et al., 2011
Orx <sub>A</sub> ( <i>rats</i> )	i-pPVT	28.08 nmol (0.1mg/250 nl)	↑ Anxious behavior in EPM	Heydendael et al., 2011
Orx <sub>A</sub> ( <i>rats</i> )	iVP	500 nmol (1 µM)	↓ Anhedonia (Sucrose Preference) and behavioral despair (FST) after LV-shOrx1R knockdown	Ji et al., 2018
<b>Orx<sub>B</sub></b>				
Orx <sub>B</sub> ( <i>rats</i> )	icv	10 nmol	↑ Locomotion, face-washing, grooming, wet-dog shaking	Matsuzaki et al., 2002
Orx <sub>B</sub> ( <i>hamster</i> )	iCeA	0.06 nmol (60 nM)	↑ Anxious and depressive behavior (LDT & EPM but not mentioned in review)	Avolio et al., 2011
<b>Orx<sub>1</sub> antagonist</b>				
SB-334867 ( <i>mice</i> )	ip	782.9 nmol (10 mg/kg)	↓ Behavioral despair in FST & TST	Scott et al., 2011
SB-334867 ( <i>mice</i> )	ip	234.9 nmol, 391.45 nmol, 782.9 nmol (3,5& 10 mg/kg)	↓ Reaction to cued & contextual stimuli (freezing time to fear tests)	Flores et al., 2014
SB-334867 ( <i>mice</i> )	ip	234.9–2,349 nmol <sup>1</sup> (3–30 mg/kg) 782.9 nmol <sup>2</sup> (10 mg/kg)	No general anxiety impact in EPM	Rodgers et al, 2013 <sup>1</sup> Scott et al., 2011 <sup>2</sup> Staples & Cornish 2014 <sup>2</sup>
SB-334867 ( <i>mice</i> )	ip	391.45 nmol & 782.9 nmol (5 & 10 mg/kg)	↓ Anxiety induced by acute nicotine administration in EPM	Plaza-Zabala et al. 2010
SB-334867 ( <i>rats</i> )	ip	1,879 nmol (20 mg/kg)	↓ Hyperactivity in prenatal pups exposed to alcohol	Stettner et al., 2011
SB-334867 ( <i>mice</i> )	ip	2,350 nmol (30 mg/kg)	↓ Anxiety in OF induced by hypercapnic air	Johnson et al., 2012c
SB-334867 ( <i>rats</i> )	ip	30,530 nmol (30 mg/kg)	↓ Freezing in Sprague-Dawley rats	Johnson et al., 2010
SB-334867 ( <i>rats</i> )	ip	30,530 nmol (30 mg/kg)	↓ Anxiety in SIP induced by hypercapnic air	Johnson et al., 2015
SB-334867 ( <i>rats</i> )	ip	7,830 nmol (10 mg/kg)	↓ Latency to approach novel & familiar predator odors	Staples & Cornish, 2014
SB-334867 ( <i>ddY mice</i> )	ip	3,290 nmol (30 mg/kg)	↓ Antidepressive action of icv Orx <sub>A</sub> in FST	Ito et al. 2008
SB-334867 ( <i>rats</i> )	i-pPVT	313.2 nmol (0.1 mg/250 nl)	↓ Anxious behavior in EPM	Heydenael et al. 2011

Drug Description ( <i>animal</i> )	Delivery Method	Dose (nmol)	Description of effect on anxious or depressive behavior	Citation
SB-334867 ( <i>mice</i> )	iBLA	10 nmol	↑ Contextually induced fear extinction	Flores et al. 2014
GSK-1059865 ( <i>rats</i> )	ip	20,630 nmol (30 mg/kg)	↓ Attenuate yohimbine induced stress fMRI	Gozzi et al. 2013
<b>Orx<sub>2</sub> agonist</b>				
Ala <sup>11</sup> -D-Leu <sup>15</sup> -Orx <sub>B</sub> ( <i>mice</i> )	icv	0.3 nmol	↓ Anxious behaviors in stress-susceptible animals in the SAM	Staton et al. 2018
<b>Orx<sub>2</sub> antagonist</b>				
TCSOX <sub>2</sub> 29 ( <i>mice</i> )	ip	576.1 nmol & 864.1 nmol (10 & 15 mg/kg)	↓ Freezing during contextual fear No effect w/ cued fear consolidation	Flores et al. 2014
MK-1064 ( <i>mice</i> )	icv	0.3 nmol	↑ Anxious behaviors in stress resilient animals in the SAM	Staton et al. 2018
<b>DORA</b>				
DORA-12 ( <i>rats</i> )	orally	21,600 nmol (30 mg/kg)	↓ Anxiety in SIP induced by hypercapnic air	Johnson et al. 2015
Almorexant ( <i>rats</i> )	orally	14,600–146,000 nmol Dose dependent (30–300 mg/kg)	↓ Fear potentiated startle in conditioned rats	Steiner et al. 2012
Almorexant ( <i>mice</i> )	orally	4,880 nmol per day (100 mg per day)	↓ Anxious & depressive behaviors in UCMS model	Nollet et al. 2012

Drugs modifying orexin system and receptor activity used for preclinical and clinical treatments of anxious and depressive behaviors. Dosages presented as as converted to nmol based on either reported animal weights, or average weight for the development stage utilized in the study (and as published). A brief description of the drug's effects on anxious and depressive behavior is included. Abbreviations: DORA = Dual Orexin Receptor Antagonist, EPM = Elevated Plus Maze, fMRI = functional Magnetic Resonance Imaging, FST = Forced Swim Test, iBLA = intra-Basolateral Amygdala, iBNST = intra-Bed Nucleus of the Stria Terminalis, iCeA = intra-Central Amygdala, icv = intracerebroventricular, ip = intraperitoneal, i-pPVT = intra-posterior Paraventricular Thalamus, iVP = intra-Ventral Pallidum, LDT = Light-Dark Test, OFT = Open Field Test, SAM = Stress-Alternatives Model, SIP = Social Interaction/Preference test, TST = Tail Suspension Test, UCMS = Unpredictable Chronic Mild Stress