Reduced Orexin System Function Contributes to Resilience to Repeated Social Stress

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ABSTRACT

Exposure to stress increases the risk of developing affective disorders such as depression and Post-Traumatic Stress Disorder (PTSD). However, these disorders only occur in a subset of individuals, those that are more vulnerable to the effects of stress, whereas others remain resilient. The coping style adopted to deal with the stressor, either passive or active coping, is related to vulnerability or resilience, respectively. Important neural substrates that mediate responses to a stressor are the orexins. These neuropeptides are altered in the cerebrospinal fluid of patients with stress-related illnesses such as depression and PTSD. The present experiments used a rodent social defeat model that generates actively coping rats and passively coping rats, which we have previously shown exhibit resilient and vulnerable profiles, respectively, to examine if orexins play a role in these stress-induced phenotypes. In situ radiolabeling and qPCR revealed that actively coping rats expressed significantly lower prepro-orexin mRNA compared with passively coping rats. This led to the hypothesis that lower levels of orexins contribute to resilience to repeated social stress. To test this hypothesis, rats first underwent five days of social defeat to establish active and passive coping phenotypes. Then, orexin neurons were inhibited prior to each social defeat for three additional days using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Inhibition of orexins increased social interaction behavior and decreased depressive-like behavior in the vulnerable population of rats. Indeed, this suggests that lowering orexins promoted resilience to social defeat, and may be an important target for treatment of stress-related disorders.
Significance Statement

Stress-related mental illnesses only occur in a subset of individuals, whereas others are resilient to the effects of stress. Our work used an animal model of social stress to identify a substrate of resilience, the neuropeptides orexins, which are known to be altered in patients with MDD and PTSD. We found that orexins are decreased in rats resilient to social stress. To test whether low orexins contribute to resilience, orexins were inhibited during three days of a social defeat stress paradigm, which increased subsequent social interaction behavior and decreased depressive-like behaviors in a previously vulnerable population of rats. This suggests that lowering orexins is important in promoting resilience to stress, and are an important target for treatments of stress-related illness.
Exposure to chronic stress is associated with the onset and increased incidence of stress-related mental illness such as depression, anxiety-related disorders, and PTSD (McEwen and Stellar, 1993; Yehuda et al., 1994; Ehlert et al., 2001). However, these disorders only occur in a subset of individuals that are more vulnerable to the effects of stress, whereas others remain resilient to the effects of stress. The neurobiological basis for these vulnerable and resilient phenotypes is not fully understood. Determining the neural substrates underlying vulnerability or resilience could lead to individualized treatment to either prevent vulnerability or promote resilience to stress.

Many stress-related disorders are associated with alterations in arousal. For example, PTSD is characterized by hypervigilance and hyperarousal to stimuli related to the traumatic event (Yehuda, 2000). An important neural substrate that mediates arousal, wakefulness, and vigilance are the neuropeptides orexins (de Lecea et al., 1998; Sakurai et al., 1998). Extending beyond their role in mediating general arousal and wakefulness, orexins are important in the response to stressful stimuli which requires the animal to shift from a basal to a reactive state (Berridge and España, 2005). More specifically, orexins are known to promote the stress response including activation of both the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis (Jászberényi et al., 2000; Kuru et al., 2000; Winsky-Sommerer et al., 2005; Spinazzi et al., 2006; Heydendael et al., 2011; Kuwaki, 2011; Johnson et al., 2012; Messina et al., 2014). Conversely, orexin neurons are activated by stressors such as forced swim and can also be activated by direct administration of the stress regulatory peptide corticotropin releasing hormone (Winsky-Sommerer et al., 2005; Chang et al., 2007; Furlong et al., 2009; Chen et al., 2013). Importantly, orexin levels are altered in the cerebrospinal fluid of patients with depression and PTSD. Together, both preclinical and clinical data suggest that orexins are involved in the processes by which stress leads to some psychiatric disorders (Strawn et al., 2010; Johnson et al., 2012). However, it is not known whether orexins contribute to individual differences that occur in...
response to stress, which are important in determining an individual's resilience or vulnerability to some psychiatric disorders.

One factor relating to susceptibility and resiliency is the coping style adopted to deal with the stressor (Veenema et al., 2003). Both active coping, characterized by the fight or flight response, and passive coping, characterized by heightened immobility, could be engaged during exposure to threatening stimuli (i.e. stressors) (Engel and Schmale, 1972; Koolhaas et al., 1999; Southwick et al., 2005; Wood and Bhatnagar, 2015). Clinical studies have indicated that humans demonstrating passive coping are more likely to develop depression than those who display active coping (Folkman and Lazarus, 1980; Billings and Moos, 1984). The present experiments used an animal model of social stress in which coping strategies vary and are associated with resilience or vulnerability to stress, as assessed by measures in the neuroendocrine system, behavior (Wood et al., 2010, 2015a; Chen et al., 2015b; Finnell et al., 2017b) and inflammatory processes (Pearson-Leary et al., 2017).

These experiments aimed to examine orexins as a potential substrate underlying differences in vulnerability and resilience in response to social defeat stress in rats. First, orexin expression was measured by in situ radiolabeling and by qPCR in passive coping (vulnerable) and active coping (resilient) rats, and revealed that orexin expression was lower in resilient rats. This led to the hypothesis that lower levels of orexin underlie resilience to repeated social stress. To test this hypothesis, after rats had established active or passive coping phenotypes over 5 days of social defeat, orexin neurons were inhibited prior to each social defeat for three additional days using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Dampening orexin action in passively coping rats prior to each defeat increased social interaction and decreased depressive-like behavior, promoting resilience. These studies establish that low orexin function contributes to the active/resilient behavioral phenotypes in response to repeated social defeat stress.
Materials and Methods

Animals

Adult, male Sprague Dawley rats (275–300 g at time of stress) were used as controls or intruders (Charles River, Wilmington, MA), and male Long-Evans retired breeders (650 – 850 g) served as residents (Charles River, Wilmington, MA). Rats were individually housed with a 12-h light, 12-h dark cycle (lights on at 0700 h) in a climate-controlled room with ad libitum food and water. Rats were given 5 days of acclimation prior to experimentation. Studies were approved by [Author University] Institutional Animal Care and Use Committee and conformed to the National Institutes of Health Guide for the Use of Laboratory Animals.

Social Defeat Paradigm

The social defeat paradigm used in this study was based on the resident-intruder model originally developed by (Miczek, 1979) (See Figure 1A).

Sprague-Dawley rats were randomly assigned to either a control or social defeat group. During social defeat, each rat was placed into the home cage of an unfamiliar Long Evans retired breeder (resident) for each of 5-8 consecutive days. Typically, the resident and intruder investigate each other for a short period of time (1-3 minutes), followed by attacks by the resident which result in a defeat of the intruder. A defeat was determined when the intruder assumed a supine posture and froze for at least 2-3 seconds. Upon assuming the defeat posture, the resident and intruder were separated by a wire mesh barrier until 30-minute had elapsed from time of initial placement into the cage of the resident. The barrier allowed for visual, auditory, and olfactory contact but prevented physical contact and further attacks upon the intruder. The latency to be defeated was then recorded. If no defeat occurred within 15 minutes, the rats were separated with a wire mesh barrier for the remaining 15 minutes. Control rats were placed into a clean novel cage behind a wire mesh barrier for 30 minutes. Once the 30-minute social stress was complete, each rat was placed back in their home cage. The average latency of each rat over the course of 7 days was entered into an R
script used to perform cluster analyses on average defeat latencies (code available at www.github.com/cookpa/socialdefeat). The bootstrap classification starts from the assumption that the average latencies are drawn from a bi-modal distribution. An initial classification of the average latencies is performed using "Partitioning Around Medoids" (PAM) implemented in R's cluster package (Reynolds, A; Richards, G; de la Iglesia, B; Rayward-Smith, 1992). PAM is a robust implementation of k-means clustering, which separates data into a pre-defined number of clusters (in this case, 2, one for passive coping, and one for active coping). The bootstrap algorithm resamples the data to assess the uncertainty in the classification. For each bootstrap iteration, we sample with replacement from the original latencies, and re-run the PAM clustering. After 10000 iterations, we define the probability of active coping classification for each of the average latencies as the fraction of the 10000 bootstrap iterations in which that latency is classified as active coping. Latencies that are consistently classified as active coping have probability 1.0, and those classified consistently as passive coping have probability 0.0. Rats with a value between 0.1 and 0.9 changed their classification in more than 10% of the bootstrap samples, and these animals were excluded from the experiment. 4 animals out of 42 were excluded based on this criterion.

Experiment 1: Social Defeat and Prepro-Orexin Expression

In one cohort of either control rats or those exposed to 7 days social defeat, in situ hybridization was used to measure the level of prepro-orexin mRNA in the lateral hypothalamus. Briefly, 20 um sections of the lateral hypothalamus from brains of control, passively coping, and actively coping rats were collected on a cryostat (Rostral-caudal coordinates relative to bregma: -1.30 mm to -4.60 mm) and processed for in situ hybridization. Hybridization localization of mRNAs using 35S-labeled antisense mRNA probes was performed. In short, coronal brain slices encompassing the lateral hypothalamus were hybridized, in situ, with antisense to orexin (generously donated by Dr. Teresa Reyes, University of Cincinnati). Hybridizations for all slices were carried out in a
single lot followed by analysis of the signal on X-ray film. Routine controls
consisted of sense-strand probes labeled to similar specific activities as the
antisense probes. X-ray films were analyzed using ImageJ. Background
estimates were produced by optical density measurements over non-positively
hybridized regions.

Another cohort of either control rats or those exposed to 7 days of social
defeat was used to assess orexin mRNA by qPCR. Control, passively coping,
and actively coping rats were killed and fresh punches of lateral hypothalamus
were collected. RNA was extracted with Purelink mRNA kit according to the
manufacturer’s protocol (Thermo Fisher Scientific, Waltham, Massachusetts,
USA). RNA was reverse transcribed to cDNA using a high capacity cDNA
reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts,
USA). qPCR was performed using Taqman Gene Expression Assays (Thermo
Fisher Scientific, Waltham, Massachusetts, USA) with primers for prepro-orexin
(Hs01891339_s1) and Actb (Hs01060665_g1) and the Applied Biosystems 7500
Real Time PCR System.

Experiment 2: Inhibiting Orexins During Social Defeat using Designer Receptors
Exclusively Activated by Designer Drugs (DREADDs)

DREADDs are viruses that contain synthetic GPCRs and can be activated
by the otherwise pharmacologically inert ligand Clozapine-N-Oxide (CNO). We
obtained the CMV-hM4Di-mCitrine plasmid from Dr. Bryan Roth (University of
North Carolina, Chapel Hill, NC). Slice electrophysiology has demonstrated that
CNO application to hippocampal cells expressing this Gi coupled designer
receptor causes hyperpolarization and decreased firing rate (Armbruster et al.,
2007). Recent studies have found that CNO silencing of particular brain areas
can produce striking behavioral effects, such as a reduction in anxiety-like
behavior (McCall et al., 2015). We next obtained a 1295 bp promoter for human
preproorexin gene (Ple112) from Addgene plasmid No. 29004 (gift of Dr.
Elizabeth Simpson, Univ. of British Columbia). This promoter was subcloned
upstream of the hM4Di-mCitrine region to replace the construct's CMV promoter.
to drive transgene expression specifically in orexin neurons. The fragment Ple112-hm4Di-mCitrine was then subcloned between the Inverted Terminal Repeats (ITRs) of the AAV2 genome. In a separate study, we found AAV1 serotype displayed optimal tropism for Sprague Dawley rat hypothalamic neurons when we delivered in vivo and compared to AAV5,8,9 expression of GFP reporters driven by common constitutively active promoters Synapsin and CB7. Based on this finding, the [Author University] Vector Core produced a recombinant adenovirus rAAV2/1-Ple112-hM4Di-mCitrine (using AAV1 serotype capsid for optimal transduction in orexin neurons) for our use. Previous studies using this virus demonstrate that activation of this construct in vivo decreases cFos expression in orexin neurons, supporting the efficacy of this construct (Grafe et al., 2017a).

5 cohorts of male rats (20 rats per cohort) were anesthetized using a cocktail of ketamine, xylazine, and acepromazine. Using stereotaxic technique, virus containing the DREADDs construct (10^9 titer, 1ul bilaterally) was injected into the lateral hypothalamus (2.5 mm caudal to bregma, 1.8 mm from mid-line and 8 mm ventral). We verified the expression of the DREADDs constructs by immunofluorescence and determined that optimal expression occurs at 4 weeks post injection. Thus, virus was expressed for 4 weeks before social defeat procedures and subsequent behavior was assessed (See Experimental Paradigm in Figure 2A).

Male rats expressing DREADDs-containing virus were either assigned to a control condition or exposed to the social defeat paradigm. Defeated rats were exposed to 5 days of social defeat without orexin manipulation, allowing the emergence of passively and actively coping phenotypes based on average defeat latency over those 5 days. In the original study describing these naturally occurring differences in response to social defeat (Wood et al., 2010), the 5 day latencies are predictive of the 7 day defeat latencies. In addition, previous publications have demonstrated that 5 days of social defeat is sufficient to induce depressive-like behavior in passively coping rats (but not actively coping rats) (Wood et al., 2015b; Finnell et al., 2017a). Thus, 5 days of defeat is comparable
to the 7 days of defeat as performed in Experiment 1. Body weights were collected both prior to and following the 5 days of defeat. Then control, actively coping and passively coping rats were randomly assigned to a vehicle or CNO group. In these rats, on Days 6-8, either Vehicle (saline and 8% dimethyl sulfoxide) or CNO (2mg/kg; Sigma-Aldrich; St Louis, MO) was injected 60 min prior to each defeat (or at the same time of day in control rats). This dose is in accordance with doses used in previous DREADDs studies in rats (Farrell and Roth, 2013) and which we have previously used to inhibit orexin neurons using hM4Di DREADDs. This timing was chosen because CNO promotes behavioral effects in the rat within 30 minutes of administration and effects last up to 4 hours after administration (Alexander et al., 2009; Farrell and Roth, 2013; Hasegawa et al., 2014). Thus, the final groups were: Vehicle-treated control rats, CNO-treated control rats, Vehicle-treated passively coping rats, CNO-treated passively coping rats, Vehicle-treated actively coping rats, and CNO-treated actively coping rats.

On Day 9, two cohorts were sacrificed and in situ hybridization was used to measure the level of prepro-orexin mRNA in the lateral hypothalamus, as described in Experiment 1. Three other cohorts were exposed to a social interaction test in a 70cm x 70cm arena. In brief, rats were placed in the arena with another male Sprague Dawley rat of similar size and weight. Rats were allowed to interact in this arena for 15 minutes, and were videotaped and analyzed by Ethovision XT video tracking software (Noldus Information Technology, Leesburg, VA, USA). Latency to interact (time in seconds until experimental rat explores stimulus rat), total time interacting (number of seconds that the experimental rat explores stimulus rat), and distance moved were calculated. Total time interacting and latency to interact were verified by hand coding from an observer blind to experimental conditions.

On Days 11 and 12, rats were tested in the Porsolt Forced Swim Test (FST). Based on the work of Lucki (Lucki, 1997), FST was performed on two consecutive days: Rats were exposed to 15 minutes of forced swim (Day 1), followed 24 h later by a 5 min of forced swim (Day 2). The 5-min swim test was videotaped from directly above the clear glass cylinder [46 cm in height × 20 cm in diameter], filled to 35 cm with water at a temperature of 25 °C (±1 °C). Two
Trained observers categorized the rat’s videotaped behavior (Day 2) every 5 s for immobility, swimming, or climbing. Percent time swimming and climbing were also combined to analyze percent time active.

After all behavioral experiments were complete, animals were killed, brains were collected, and 20 μm lateral hypothalamic slices were analyzed for both prepro-orexin mRNA expression (as previously described) and viral expression. Specifically, immunofluorescence for visualizing the virus tag was conducted as follows. Tissue was incubated with primary antibodies for both Orexin A (1:250, sc-8070; Santa Cruz Biotechnology, Santa Cruz CA) and GFP (1:500, ab290; AbCam, Cambridge, UK). As the mCitrine tag on the DREADDS virus originates from Aequorea victoria jellyfish, GFP antibodies are known to react with these proteins (Le et al., 2006). Sections were then incubated with AlexaFluor488 Donkey anti-goat and AlexaFluor647 Donkey anti-rabbit secondary antibodies (1:200, A-11055 and A-31573; Life Technologies, Carlsbad, CA). Images were acquired with a Leitz DMR microscope with a digital camera (Leica) (Figure 2B). The NIH Image J colocalization plugin was used to determine percent orexin cells transduced by the virus. Approximately 70% of the orexin cells are transfected at this time, consistent with previous studies. The number of DREADDs expressing cells and orexinA labeled cells were also methodically counted from anterior to posterior extent of the lateral hypothalamus (-2.12 mm to -3.60 mm) (Figure 2C).

**Statistical Analysis**

Data are presented as the mean ± the standard error of the mean. For orexin expression by *in situ* and qPCR, and body weight gain, a one-way ANOVA was performed, followed by Tukey’s post hoc t-test. For orexin expression (before and after behavior), social interaction and forced swim test data, a two-way ANOVA (Stress [Control, passive coping, or active coping] by Drug [Veh or CNO] treatments) was used, followed by tukey’s post hoc t-tests. All analyses used α=0.05 as the criterion level of significance. Statistical analysis was
conducted with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) in order to identify statistical differences.

Results

Prepro-orexin expression in rats vulnerable or resilient to defeat

After 7 days of social defeat (Figure 1A), rats were split into passive coping and active coping clusters based on average latency to defeat. Rats that displayed passive coping had an average defeat latency of 182 seconds while rats that displayed active coping had an average defeat latency of 419 seconds (Figure 1B; p < 0.001, t-test, n = 16/group). Prepro-orexin expression was then examined in two separate cohorts of control, passively coping, and actively coping rats; one cohort was used for in situ radiolabeling and the other was used for qPCR. Quantification of in situ radiolabeling in each treatment group revealed that actively coping rats had significantly less prepro-orexin mRNA in the lateral hypothalamus compared with passively coping rats. (Figure 1C, F(2,23) = 8.4, p = 0.002, one-way ANOVA followed by Tukey’s t-test; n=8/group, 4 slices per animal). Moreover, there was a trend for a negative correlation between average defeat latency and prepro-orexin mRNA as measured by in situ radiolabeling (Figure 1C, R² = 0.260, p = 0.062). QPCR analysis of prepro-orexin levels in another cohort of rats demonstrated a consistent result: Rats that displayed active coping expressed significantly less prepro-orexin mRNA than passively coping rats. (Figure 1D, F(2,22 = 4.4, p = 0.025, one-way ANOVA followed by Tukey’s t-test; n = 8/group). Average defeat latency was negatively correlated with orexin mRNA as quantified by qPCR (R² = 0.276, p = 0.044). Together, these results demonstrate that lower orexin expression is associated with an active coping strategy, and thus, based on previous findings (Wood et al., 2010), a resilient phenotype.

Inhibition of orexins during the last three days of social defeat using DREADDs

To determine the effects of orexin inhibition on behavioral outcomes produced by social defeat, rats were first injected with an inhibitory DREADDs...
viral construct which was allowed to express for 4 weeks (For experimental paradigm and confirmation of DREADDs expression, see Figure 2A-C). Next, rats underwent social defeat for 5 days and were split into passive and active coping groups based on average defeat latency (See Methods section for more detail on how this analysis was performed). As expected, body weight gain was significantly different between control and defeated groups (data not shown; F(2,38) = 6.766, p = 0.003, one-way ANOVA followed by Tukey's t-test; n = 16/group). Specifically, control rats that did not undergo social defeat stress showed significantly more weight gain than those that did. In addition, rats that displayed passive coping had less weight gain compared with actively coping rats.

Two cohorts of rats were sacrificed prior to social interaction behavior to examine the effect of social defeat and vehicle or CNO treatment on prepro-orexin mRNA expression (Figure 2D). Prepro-orexin mRNA levels appeared to differ between treatment groups (Defeat effect, F(2,27) = 3.2, p = 0.055; CNO effect, F(1,27) = 3.6, p = 0.067, 2-way ANOVA, followed by t-tests, n = 6/group). Particularly, vehicle-treated actively coping rats had lower levels of prepro-orexin mRNA than vehicle-treated passively coping rats. Thus, this phenotype of lower orexin expression in rats that demonstrate an active coping strategy is stable. Additionally, CNO treatment during the last 3 days of defeat reduced prepro-orexin mRNA levels in passive coping rats to that of control and active coping rats. To examine whether reducing orexins promotes behavioral correlates of resilience, 3 additional cohorts of rats were exposed to 5 days of social defeat followed by vehicle or CNO treatment prior to each defeat on days 6-8. These cohorts were then assayed for anxiety-like behavior in the social interaction test and depressive-like behavior in the forced swim test.

On day 9 of the experimental paradigm, rats were tested for social interaction with a stimulus rat (Figure 3). The amount of time spent interacting was significantly different between treatment groups (Figure 3A; Defeat effect, F(2,48) = 3.3, p = 0.043; Interaction effect, F(2,48) = 3.4, p = 0.045, 2-way ANOVA followed by Tukey’s t-test, n = 12/group). Importantly, in vehicle-injected
groups, actively coping rats spent significantly more time interacting than passively coping rats, replicating previous findings (Chen et al., 2015b; Pearson-Leary et al., 2017). CNO treatment (inhibition of orexin neurons during the last three days of social defeat) increased time spent interacting in passively coping rats but had no effect in actively coping rats or control rats. There were no significant differences in latency to interact between the treatment groups (Figure 3B). Additionally, there were no significant differences in the distance moved between the treatment group, indicating the manipulation of orexin action did not simply change general arousal or locomotor activity (Figure 3C). Thus, dampening of orexin action during the last three days of social defeat specifically increased social interaction in the vulnerable population of rats to the level of resilient rats.

On days 11 and 12 of the experimental paradigm, rats were tested in the Porsolt Forced Swim paradigm to assess depressive-like behavior (Figure 4). Analysis of percent time immobile revealed significant differences between treatment groups (Figure 4A; Interaction effect, $F(2,47) = 6.6, p = 0.003$, 2-way ANOVA followed by Tukey’s t-test, $n = 12$ /group). Specifically, vehicle treated passively coping rats spent significantly more time immobile than control rats, replicating previous findings (Wood et al., 2010). CNO treatment (inhibition of orexin neurons during the last three days of social defeat) decreased time spent immobile in passively coping rats but had no effect in actively coping rats. CNO treatment in control animals increased time spent immobile. Percent time spent active was next analyzed, revealing that vehicle-injected passively coping rats spent less time active than control rats, and inhibition of orexin neurons during social defeat reversed this effect (Figure 4B; Interaction effect, $F(2,46) = 6.7, p = 0.003$, 2-way ANOVA followed by Tukey’s t-test, $n = 12$ /group). Activity was then subdivided into swimming and climbing behaviors. Analysis of swimming behavior revealed that vehicle-injected passively coping rats spent significantly less time swimming than vehicle-injected control rats (Figure 4C; Interaction effect, $F(2,46) = 4.1, p = 0.022$, 2-way ANOVA followed by Tukey’s t-test, $n = 12$ /group). Moreover, CNO treatment decreased time spent swimming in control
rats but had no effect on passively or actively coping rats. Lastly, there were no significant differences between the treatment groups in climbing behavior (Figure 4D). Thus, it appears that the differences in activity between treatment groups can mostly be attributed to swimming behavior. However, CNO treatment in passively coping rats appeared to increase a combination of both swimming and climbing; these measures were only significantly increased when summed together as total activity.

A control experiment was performed to determine whether CNO alone had any impact on behavior in non DREADDs-expressing rats. Specifically, a separate naïve cohort of rats was injected with either vehicle or CNO for three consecutive days, followed by testing in the social interaction test and forced swim test. This was the same treatment regimen as in the original experiment above. The data indicate that total social interaction time did not differ between vehicle- and CNO-treated groups (395.6±10.5 vs 404.8±16.5 seconds, p = 0.600, t-test, n = 6/group). Latency to interact was also not different between treatment groups (13.0±2.9 vs 19.4±6.0 seconds, p = 0.534, t-test, n = 6/group). Moreover, distance traveled in the social interaction arena did not differ between treatment groups (21,458.2±3872.1 vs 28,746.6±9193.6 cm, p = 0.403, t-test, n = 6/group). Lastly, neither percent immobility nor total activity differed between vehicle- and CNO-treated groups in the forced swim test (immobility: 20.8±2.5 vs 28.9±4.2, p = 0.19, t-test, n = 6/group; activity: 79.2±4.2 vs 71.1±2.5, p = 0.19, t-test, n = 6/group). In summary, CNO treatment alone in non-DREADDs expressing rats did not cause significant changes in the social interaction or forced swim test when compared with vehicle-treated animals. These results indicate that the effects of DREADDs inhibition in the experiment above were not due to the effects of CNO alone.

Prepro-orexin mRNA levels differed between treatment groups after the forced swim test on day 12 (Figure 2E, CNO effect, F(1,38)= 8.0, p = 0.007, 2-way ANOVA followed by Tukey’s t-test, n = 12/group). Namely, vehicle-treated actively coping rats had lower prepro-orexin expression compared with vehicle-treated passively coping rats. Once again, this phenotype of lower orexin
expression in actively coping rats is stable. CNO treatment reduced prepro-
orexin expression in both control and passively coping rats. Overall, these results
indicate that reducing orexin action in the last three days of social defeat
increased social interaction time and reversed the depressive-like behavior
observed in the vulnerable population of rats.

### Statistical Table

| Data Structure | Type of Test | Confidence Interval (95%) |
|----------------|-------------|---------------------------|
| a Normal Distribution | T test | Passive vs. Active Latency: 189.8 to 284.3 |
| b Normal Distribution | One-Way ANOVA, Tukey’s T-test | Control vs. Active Coping Orexin mRNA: 1.3 to 2093 |
| c Normal Distribution | Correlation | Passive vs. Active Coping Orexin mRNA: 749.8 to 3180 |
| d Normal Distribution | One-Way ANOVA, Tukey’s T-test | Latency vs Orexin mRNA: -0.8 to 0.0 |
| e Normal Distribution | Correlation | Control vs. Active Coping Orexin mRNA: 0.2 to 1.2 |
| f Normal Distribution | One-Way ANOVA, Tukey’s T-test | Passive vs. Active Coping Orexin mRNA: 0.1 to 1.2 |
| g Normal Distribution | Two-Way ANOVA, Tukey’s T-test | Latency vs Orexin mRNA: -0.8 to 0.0 |
| h Normal Distribution | Two-Way ANOVA, Tukey’s T-test | Control vs. Passive Coping Body Weight: 5.6 to 19.5 |
| i Normal Distribution | Two-Way ANOVA, Tukey’s T-test | Control vs. Active Coping Body Weight: 0.2 to 13.8 |
| j Normal Distribution | Two-Way ANOVA, Tukey’s T-test | Vehicle-treated Control vs Vehicle-treated Passive Coping Orexin mRNA: -1157 to -73.81 |
| k Normal Distribution | Two-Way ANOVA, Tukey’s T-test | Vehicle-treated Passive Coping vs Vehicle-treated Active Coping Orexin mRNA: 80.6 to 1002 |
| l Normal Distribution | Two-Way ANOVA, Tukey’s T-test | Vehicle-treated Passive Coping vs CNO-treated Passive Coping Orexin mRNA: -26.99 to 1057 |
| m Normal Distribution | T test | Passive vs. CNO-treated Orexin mRNA: -125.7 to 102.8 |
| n Normal Distribution | T test | Passive vs. CNO-treated Orexin mRNA: -26.99 to 1057 |
| o Normal Distribution | T test | Vehicle vs CNO-treated Orexin mRNA: -125.7 to 102.8 |
| p Normal Distribution | T Test | Vehicle vs CNO-treated Orexin mRNA: -26.99 to 1057 |
Discussion

These experiments used a social defeat paradigm that generates two different populations of rats that demonstrate either passive or active coping strategies, based on their average latency to be defeated. Previous studies have indicated that rats displaying a passive coping strategy demonstrate subsequent anxiety- and depressive-like behaviors (Wood et al., 2010; Chen et al., 2015b; Pearson-Leary et al., 2017). This is consistent with human studies, which have demonstrated that passive coping is more often associated with the development of Major Depressive Disorder. Our results suggest a substrate of resilience, namely, the neuropeptides orexins, which are known to mediate the stress response and are altered in patients with MDD and PTSD (Kuru et al., 2000; Yehuda, 2000; Winsky-Sommerer et al., 2004; Spinazzi et al., 2006; Furlong et al., 2009; Strawn et al., 2010; Chen et al., 2015a). In short, we first discovered that lower orexin expression was associated with active coping strategies. We next inhibited orexin action during the last three days of social defeat, and this produced an increase in social interaction and a decrease in depressive-like behaviors in passively coping rats. Thus, we established that low orexin function contributes to the active/resilient behavioral phenotypes in response to repeated social defeat stress.

Both \textit{in situ} radiolabeling and qPCR approaches revealed that rats displaying active coping (resilient rats) expressed significantly lower levels of prepro-orexin mRNA compared with rats that displaying passive coping (vulnerable rats) as well as control rats. This lead to the hypothesis that it is the lower levels of orexins that underlie resilience to repeated social stress. To test this, rats first underwent five days of social defeat stress to establish active and passive coping phenotypes. Next, to determine whether reducing orexins promotes resilience, we inhibited orexin neurons prior to each social defeat for three additional days using DREADDs. Importantly, we found that CNO treatment...
during the last 3 days of defeat reduced prepro-orexin mRNA levels in passive coping rats to that of active coping rats. Additionally, inhibition of orexin neurons prior to each defeat resulted in increased social interaction behavior and decreased immobility during forced swim test in passively coping (vulnerable rats) to the level of actively coping (resilient rats). As expected, inhibition of orexin neurons prior to each defeat had no effect on subsequent behavior in resilient rats, as our experiments indicate they already express very low levels of orexins. Thus, inhibiting orexin action during the last three days of social defeat increased social interaction and decreased depressive-like behaviors specifically in the vulnerable population of rats, thereby promoting resilience. This indicates that dampened orexin function under conditions of stress contributes to resilience to social defeat.

Prepro-orexin expression was lower in resilient rats compared with vulnerable rats by both in situ radiolabeling and qPCR methods. This phenotype was stable in multiple cohorts of rats, immediately after repeated social defeat, as well as after several additional behavioral tests. We cannot determine whether prepro-orexin expression was lower in resilient rats before social stress or as a consequence of social stress, thus, it is possible it is a pre-existing difference. A previous study demonstrated that control female rats had higher orexin expression than male rats, and as a result, females had persistent HPA activation in response to repeated restraint stress, and were not able to habituate as fully as males (Grafe et al., 2017a). Just as females had higher levels of orexins prior to the stressor, and thus were inherently different than males, perhaps actively coping (resilient) rats are inherently different than passively coping (vulnerable) rats before social defeat occurs. In this respect, pre-existing differences in orexin expression impact future responses to stress. On the other hand, perhaps orexin function is also decreased with repeated exposure to social defeat in rats that become resilient to defeat; this is known to occur after repeated restraint stress (Grafe et al., 2017b). Currently, we are only able to measure cerebrospinal fluid levels of orexins terminally, and plasma levels of orexins are not a reliable indication of central orexin activity, thus, we cannot
directly determine whether this difference in orexin expression pre-exists exposure to stress.

Based on these data, we hypothesized that lower levels of orexin contribute to resilience to repeated social stress. After rats established active and passive coping phenotypes, orexins were inhibited by DREADDs in subsequent days of social defeat. Behavior was examined in both the social interaction and forced swim tests to determine how orexin action during defeat affects subsequent anxiety-like and depressive-like behaviors. Our results first demonstrated that vehicle-treated actively coping rats spent more time socially interacting than passively coping rats, replicating a previous finding (Wood et al., 2010). Orexin inhibition during the last three days of defeat increased the amount of time passively coping rats spent interacting with a stimulus rat, with total interaction time at a comparable level to that of actively coping rats. This result is consistent with earlier studies in which central injections of orexins produced anxiety-like behaviors in the light-dark test and elevated plus maze (Suzuki et al., 2005; Li et al., 2010; Avolio et al., 2011). As expected, inhibition of orexins in resilient rats during social defeat did not further increase their social interaction time likely because resilient rats already express low levels of orexins. In sum, orexins promote anxiety behaviors, and dampening orexin action throughout repeated stress allows rats that are initially vulnerable to exhibit the resilient phenotype.

The increase in social interaction observed in CNO-treated passively coping rats was independent of the total amount of movement in the social interaction chamber. As orexins have been shown to modulate spontaneous physical activity (Kotz et al., 2002), it is important to note that the increase in social interaction was not accompanied by an increase in activity, thus, it is a socially specific behavioral result and not an effect on global arousal. Moreover, the orexin manipulation only took place during the last three days of social defeat, and not during this social interaction test, thus, short term action of CNO treatment did not confound our results. However, our data indicate that CNO treatment on days 6-8 of social defeat can cause long lasting changes in prepro-
orexin mRNA expression, which then leads to changes in social interaction behavior.

In the FST, vehicle-treated passively coping rats spent significantly more time immobile than control rats, replicating a previous finding that passive coping during social defeat leads to depressive-like behavior (Wood et al., 2010). DREADDs-mediated inhibition of orexin neurons prior to three social defeat exposures reduced percent time spent immobile in the vulnerable rats. Hence, increased orexin action may contribute to depressive-like behavior. However, we found that repeated CNO treatment increased immobility in control, non-stressed animals. Thus, inhibiting orexins in non-stress conditions increases depressive-like behavior. Our data show that prepro-orexin expression does not differ between vehicle- and CNO-treated control (non-stressed) animals, so this cannot explain the differences in percent immobility in the FST. It is possible that other measures of orexin function, such as neuronal activation, may differ between vehicle- and CNO-treated control animals, explaining these differences in behavior. Ultimately, the effect of inhibiting orexins on immobility is dependent on whether the animal is stressed; different brain circuits involving orexins may be activated in these different conditions, explaining the opposing behaviors.

The link between the orexinergic system and depression remains equivocal, as clinical models report conflicting results. Specifically, different studies indicate that either hypoactivity (Brundin et al., 2007; Ito et al., 2008) or hyperactivity (Salomon et al., 2003; von der Goltz et al., 2011) of the orexinergic system is associated with Major Depressive Disorder (Brundin et al., 2007; Lutter et al., 2008). Some inconsistencies may result from limitation of methods, as orexin levels in plasma are close to the resolving limit of radioimmunoassay (Chen et al., 2015a). Moreover, whether measures of orexin A in plasma or cerebrospinal fluid are physiologically meaningful and can act as proxy for orexin system activity remains to be established. However, a recent preclinical study provided a causal link between orexins and depressive-like behavior: pharmacological blockade of the orexin system during unpredictable chronic mild stress reduced subsequent immobility in the tail suspension test. (Nollet et al.,
This result is consistent with our findings in that inhibition of orexins during stress decreases subsequent depressive-like behavior. As expected, inhibition of orexins in actively coping rats during the last three days of social defeat did not further decrease immobility, as our results indicate that actively coping (resilient) rats already express very low levels of orexins. Together, these data suggest that low levels of orexins may be a biomarker to predict resilience to stress and thus, a lower likelihood of developing depression.

Rats that displayed passive coping spent less time active (mostly due to a decrease in swimming) in the FST than control rats. However, blocking orexin action prior to three social defeat exposures appeared to increase both swimming and climbing in passively coping rats. Independently, these two measures were not significantly increased with CNO treatment. Specifically, CNO treatment increased the sum of these two behaviors together, measured as total activity. Swimming and climbing are known to be mediated by serotonin and norepinephrine, respectively, as antidepressants targeting these neurotransmitters can selectively increase these behaviors (Bogdanova et al., 2013). Indeed, it is known that orexins have direct connections with both serotonergic and noradrenergic neurons to regulate sleep/wakefulness, thus, it makes sense that manipulating orexins may affect swimming and climbing behavior (Tabuchi et al., 2013; Zitnik, 2016). However, the effect of manipulating orexins on these behaviors appears to be dependent on whether the animal is stressed: inhibiting orexins in a control animal decreases activity (and increases immobility), while inhibiting orexins in a socially defeated rat increases activity (and decreases immobility). Thus, repeated stress must change the way these neurotransmitters interact with orexins.

Recent findings have indicated that a high dose (10 mg/kg) of CNO may allow for non-specific effects of the metabolite clozapine on behavior (Gomez et al., 2017). Particularly, converted clozapine could have effects on the DREADDs or, if the levels are high enough, on endogenous clozapine binding sites as well. Moreover, another study found that 5mg/kg doses of CNO have behavioral effects in Long-Evans rats not expressing DREADDs (MacLaren et al., 2016).
We tested whether the lower dose of CNO (2mg/kg) used in the present studies had non-specific behavioral effects. We found that CNO treatment in non-DREADDs-expressing rats did not cause significant effects in the social interaction test or Porsolt forced swim test. Thus, we can conclude that the effects of DREADDs we observed in our studies were not due to actions of CNO or its metabolites but to DREADDs-induced inhibition of orexins.

The brain regions in which orexins act during stress to regulate subsequent anxiety-like and depressive-like behaviors are not fully elucidated. However, there are many key brain areas that likely play a role. For example, orexins have dense projections to brain areas relevant to anxiety-and depressive-like behaviors such as the paraventricular nucleus of the thalamus (PVT), locus coeruleus, prefrontal cortex, dorsal raphe, hippocampus, and amygdala (Peyron et al., 1998). Previous studies have demonstrated that orexins act in the PVT to induce anxiety-like behavior (Li et al., 2010; Heyndael et al., 2011, 2013). Another study found that orexin 1 receptors in the amygdala regulate stress-induced depressive-like behavior (Arendt et al., 2013). Other experiments indicate that orexin interaction with the dorsal raphe may be important for regulation of stress-induced depressive-like behavior (Brown et al., 2001; Muraki et al., 2004). Future studies should further examine the role of specific brain regions where orexins may be acting to promote resilience and identify genes mediating these orexin effects.

Because orexins are known to underlie arousal and appetite, it is possible that inhibition of these neuropeptides with DREADDs affected these physiological parameters, and thus, may have influenced our results (Sakurai, 2014). For example, if inhibiting orexin action allowed animals to sleep more, perhaps this could have subsequently decreased anxiety-like or depressive-like behavior. While our results show that there were no changes in general arousal between treatment groups in terms of total movement during behavioral tests, measuring sleep parameters after orexin manipulation may provide more insight. Additionally, though we did not measure food intake throughout the study, there were no differences in body weight gain between vehicle and CNO treated rats.
indicating that changes in appetite and food intake didn’t have a significant effect on the present results.

The results from this study demonstrate that orexin expression is lower in rats resilient to social defeat stress. To provide a causal link between decreased orexins and resilience, we inhibited orexins during the last three days of social defeat stress, and reversed the negative behavioral effects of social defeat in previously vulnerable rats. These findings highlight orexins as previously uncharacterized substrates of resilience.
References

Alexander GM, Rogan SC, Abbas AI, Armbruster BN, Pei Y, Allen JA, Nonneman RJ, Hartmann J, Moy SS, Nicolelis MA, McNamara JO, Roth BL (2009) Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. Neuron 63:27–39 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2751885&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Arendt DH, Ronan PJ, Oliver KD, Callahan LB, Summers TR, Summers CH (2013) Depressive behavior and activation of the orexin/hypocretin system. Behav Neurosci 127:86–94 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23398442 [Accessed January 20, 2014].

Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A 104:5163–5168 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1829280&tool=pmcentrez&rendertype=abstract [Accessed November 12, 2015].

Avolio E, Alò R, Carelli A, Canonaco M (2011) Amygdalar orexinergic–GABAergic interactions regulate anxiety behaviors of the Syrian golden hamster. Behav Brain Res 218:288–295 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21074570 [Accessed March 8, 2017].

Berridge CW, España RA (2005) Hypocretins: waking, arousal, or action? Neuron 46:696–698 Available at: http://www.sciencedirect.com/science/article/pii/S089662730500437X [Accessed August 30, 2014].

Billings AG, Moos RH (1984) Coping, stress, and social resources among adults with unipolar depression. J Pers Soc Psychol 46:877–891 Available at: http://www.ncbi.nlm.nih.gov/pubmed/6737198 [Accessed August 15, 2016].
Bogdanova O V., Kanekar S, D’Anci KE, Renshaw PF (2013) Factors influencing behavior in the forced swim test. Physiol Behav 118:227–239 Available at: http://proxy.library.upenn.edu:2067/science/article/pii/S0031938413001534 [Accessed May 4, 2017].

Brown RE, Sergeeva O, Eriksson KS, Haas HL (2001) Orexin A excites serotonergic neurons in the dorsal raphe nucleus of the rat. Neuropharmacology 40:457–459 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11166339 [Accessed March 14, 2017].

Brundin L, Björkqvist M, Petersén A, Träskman-Bendz L (2007) Reduced orexin levels in the cerebrospinal fluid of suicidal patients with major depressive disorder. Eur Neuropsychopharmacol 17:573–579 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17346943 [Accessed January 12, 2015].

Chang H, Saito T, Ohiwa N, Tateoka M, Deocaris CC, Fujikawa T, Soya H (2007) Inhibitory effects of an orexin-2 receptor antagonist on orexin A- and stress-induced ACTH responses in conscious rats. Neurosci Res 57:462–466 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17188385 [Accessed April 20, 2015].

Chen Q, de Lecea L, Hu Z, Gao D (2015a) The hypocretin/orexin system: an increasingly important role in neuropsychiatry. Med Res Rev 35:152–197 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25044006 [Accessed March 14, 2016].

Chen RJ, Kelly G, Sengupta A, Heydendael W, Nicholas B, Beltrami S, Luz S, Peixoto L, Abel T, Bhatnagar S (2015b) MicroRNAs as biomarkers of resilience or vulnerability to stress. Neuroscience 305:36–48 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26208845 [Accessed March 8, 2017].

Chen X, Wang H, Lin Z, Li S, Li Y, Bergen HT, Vrontakis ME, Kirouac GJ (2013) Orexins (hypocretins) contribute to fear and avoidance in rats exposed to a single episode of footshocks. Brain Struct Funct Available at: http://www.ncbi.nlm.nih.gov/pubmed/23955372 [Accessed January 28, 2014].
de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 95:322–327 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=18213&tool=pmcentrez&rendertype=abstract [Accessed January 23, 2015].

Ehlert U, Gaab J, Heinrichs M (2001) Psychoneuroendocrinological contributions to the etiology of depression, posttraumatic stress disorder, and stress-related bodily disorders: the role of the hypothalamus-pituitary-adrenal axis. Biol Psychol 57:141–152.

Engel GL, Schmale AH (1972) Conservation-withdrawal: a primary regulatory process for organismic homeostasis. Ciba Found Symp 8:57–75 Available at: http://www.ncbi.nlm.nih.gov/pubmed/4144967 [Accessed May 2, 2017].

Farrell MS, Roth BL (2013) Pharmacosynthetics: Reimagining the pharmacogenetic approach. Brain Res 1511:6–20 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3562395&tool=pmcentrez&rendertype=abstract [Accessed March 18, 2015].

Finnell JE, Lombard CM, Melson MN, Singh NP, Nagarkatti M, Nagarkatti P, Fadel JR, Wood CS, Wood SK (2017a) The protective effects of resveratrol on social stress-induced cytokine release and depressive-like behavior. Brain Behav Immun 59:147–157 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27592314 [Accessed December 4, 2017].

Finnell JE, Lombard CM, Padi AR, Moffitt CM, Wilson LB, Wood CS, Wood SK (2017b) Physical versus psychological social stress in male rats reveals distinct cardiovascular, inflammatory and behavioral consequences. Kavushansky A, ed. PLoS One 12:e0172868 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28241050 [Accessed July 31, 2017].

Folkman S, Lazarus RS (1980) An analysis of coping in a middle-aged community sample. J Health Soc Behav 21:219–239 Available at:
http://www.ncbi.nlm.nih.gov/pubmed/7410799 [Accessed August 15, 2016].

Furlong TM, Vianna DML, Liu L, Carrive P (2009) Hypocretin/orexin contributes to the expression of some but not all forms of stress and arousal. Eur J Neurosci 30:1603–1614 Available at:

http://www.ncbi.nlm.nih.gov/pubmed/19811530 [Accessed May 16, 2014].

Gomez JL, Bonaventura J, Lesniak W, Mathews WB, Sysa-Shah P, Rodriguez LA, Ellis RJ, Richie CT, Harvey BK, Dannals RF, Pomper MG, Bonci A, Michaelides M (2017) Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. Science (80- ) 357:503–507 Available at:

http://www.ncbi.nlm.nih.gov/pubmed/28774929 [Accessed November 21, 2017].

Grafe LA, Cornfeld A, Luz S, Valentino R, Bhatnagar S (2017a) Orexins Mediate Sex Differences in the Stress Response and in Cognitive Flexibility. Biol Psychiatry 81:683–692.

Grafe LA, Eacret D, Luz S, Gotter AL, Renger JJ, Winrow CJ, Bhatnagar S (2017b) Orexin 2 receptor regulation of the hypothalamic–pituitary–adrenal (HPA) response to acute and repeated stress. Neuroscience 348:313–323 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28257896 [Accessed March 8, 2017].

Hasegawa E, Yanagisawa M, Sakurai T, Mieda M (2014) Orexin neurons suppress narcolepsy via 2 distinct efferent pathways. J Clin Invest 124:604–616 Available at:

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3904620&tool=pmcentrez&rendertype=abstract [Accessed March 31, 2014].

Heyndendael W, Sengupta A, Beck S, Bhatnagar S (2013) Optogenetic examination identifies a context-specific role for orexins/hypocretins in anxiety-related behavior. Physiol Behav Available at:

http://www.ncbi.nlm.nih.gov/pubmed/24140988 [Accessed January 28, 2014].

Heyndendael W, Sharma K, Iyer V, Luz S, Piel D, Beck S, Bhatnagar S (2011)

Orexins/hypocretins act in the posterior paraventricular thalamic nucleus
during repeated stress to regulate facilitation to novel stress. Endocrinology 152:4738–4752 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3230061&tool=pmcentrez&rendertype=abstract [Accessed January 25, 2014].

Ito N, Yabe T, Gamo Y, Nagai T, Oikawa T, Yamada H, Hanawa T (2008) I.c.v. administration of orexin-A induces an antidepressive-like effect through hippocampal cell proliferation. Neuroscience 157:720–732 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18952152 [Accessed January 26, 2015].

Jászberényi M, Bujdosó E, Pataki I, Telegdy G (2000) Effects of orexins on the hypothalamic-pituitary-adrenal system. J Neuroendocrinol 12:1174–1178 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11106974 [Accessed August 11, 2014].

Johnson PL, Molosh A, Fitz SD, Truitt WA, Shekhar A (2012) Orexin, stress, and anxiety/panic states. Prog Brain Res 198:133–161 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3665356&tool=pmcentrez&rendertype=abstract [Accessed January 27, 2014].

Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MA, Blokhuis HJ (1999) Coping styles in animals: current status in behavior and stress-physiology. Neurosci Biobehav Rev 23:925–935 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10580307 [Accessed July 20, 2017].

Kotz CM, Teske JA, Levine JA, Wang C (2002) Feeding and activity induced by orexin A in the lateral hypothalamus in rats. Regul Pept 104:27–32 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11830273 [Accessed March 8, 2017].

Kuru M, Ueta Y, Serino R, Nakazato M, Yamamoto Y, Shibuya I, Yamashita H (2000) Centrally administered orexin/hypocretin activates HPA axis in rats. Neuroreport 11:1977–1980 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10884055 [Accessed January 6, 2016].

Kuwaki T (2011) Orexin links emotional stress to autonomic functions. Auton
Le T, Liang Z, Patel H, Yu MH, Sivasubramaniam G, Slovitt M, Tanentzapf G, Mohanty N, Paul SM, Wu VM, Beitel GJ (2006) A new family of Drosophila balancer chromosomes with a w- dfd-GMR yellow fluorescent protein marker. Genetics 174:2255–2257 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1698648&tool=pubmed&rendertype=abstract [Accessed November 12, 2015].

Li Y, Li S, Wei C, Wang H, Sui N, Kirouac GJ (2010) Orexins in the paraventricular nucleus of the thalamus mediate anxiety-like responses in rats. Psychopharmacology (Berl) 212:251–265 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20645079 [Accessed March 18, 2014].

Lucki I (1997) The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. Behav Pharmacol 8:523–532 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9832966 [Accessed March 7, 2017].

Lutter M, Krishnan V, Russo SJ, Jung S, McClung CA, Nestler EJ (2008) Orexin signaling mediates the antidepressant-like effect of calorie restriction. J Neurosci 28:3071–3075 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2713756&tool=pubmed&rendertype=abstract [Accessed August 8, 2014].

MacLaren DAA, Browne RW, Shaw JK, Krishnan Radhakrishnan S, Khare P, España RA, Clark SD (2016) Clozapine N-Oxide Administration Produces Behavioral Effects in Long-Evans Rats: Implications for Designing DREADD Experiments. eNeuro 3 Available at: http://www.ncbi.nlm.nih.gov/pubmed/27822508 [Accessed January 5, 2017].

McCall JG, Al-Hasani R, Siuda ER, Hong DY, Norris AJ, Ford CP, Bruchas MR (2015) CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. Neuron 87:605–620 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26212712 [Accessed July 23, 2015].
McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153:2093–2101 Available at: http://www.ncbi.nlm.nih.gov/pubmed/8379800 [Accessed August 11, 2016].

Messina G, Dalia C, Tafuri D, Monda V, Palmieri F, Dato A, Russo A, De Blasio S, Messina A, De Luca V, Chieffi S, Monda M (2014) Orexin-A controls sympathetic activity and eating behavior. Front Psychol 5:997 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25250003 [Accessed March 3, 2017].

Miczek KA (1979) A new test for aggression in rats without aversive stimulation: differential effects of d-amphetamine and cocaine. Psychopharmacology (Berl) 60:253–259 Available at: http://www.ncbi.nlm.nih.gov/pubmed/108702 [Accessed August 15, 2016].

Muraki Y, Yamanaka A, Tsujino N, Kilduff TS, Goto K, Sakurai T (2004)
Serotonergic Regulation of the Orexin/Hypocretin Neurons through the 5-HT1A Receptor. J Neurosci 24:7159–7166 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15306649 [Accessed March 14, 2017].

Nollet M, Gaillard P, Tanti A, Girault V, Belzung C, Leman S (2012) Neurogenesis-independent antidepressant-like effects on behavior and stress axis response of a dual orexin receptor antagonist in a rodent model of depression. Neuropsychopharmacology 37:2210–2221 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3422486&tool=pmcentrez&rendertype=abstract [Accessed February 4, 2015].

Pearson-Leary J, Eacret D, Chen R, Takano H, Nicholas B, Bhatnagar S (2017)
Inflammation and vascular remodeling in the ventral hippocampus contributes to vulnerability to stress. Transl Psychiatry 7:e1160 Available at: http://www.ncbi.nlm.nih.gov/pubmed/28654094 [Accessed July 20, 2017].

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9822755 [Accessed August 11, 2014].

Reynolds, A; Richards, G; de la Iglesia, B; Rayward-Smith V (1992) Clustering rules: A comparison of partitioning and hierarchical clustering algorithms. J
Sakurai T et al. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573–585 Available at: http://www.ncbi.nlm.nih.gov/pubmed/9491897 [Accessed February 15, 2015].

Sakurai T (2014) The role of orexin in motivated behaviours. Nat Rev Neurosci 15:719–731 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25301357 [Accessed October 10, 2014].

Salomon RM, Ripley B, Kennedy JS, Johnson B, Schmidt D, Zeitzer JM, Nishino S, Mignot E (2003) Diurnal variation of cerebrospinal fluid hypocretin-1 (Orexin-A) levels in control and depressed subjects. Biol Psychiatry 54:96–104 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12873798 [Accessed March 13, 2017].

Southwick SM, Vythilingam M, Charney DS (2005) The Psychobiology of Depression and Resilience to Stress: Implications for Prevention and Treatment. Annu Rev Clin Psychol 1:255–291 Available at: http://www.ncbi.nlm.nih.gov/pubmed/17716089 [Accessed July 20, 2017].

Spinazzi R, Andreis PG, Rossi GP, Nussdorfer GG (2006) Orexins in the regulation of the hypothalamic-pituitary-adrenal axis. Pharmacol Rev 58:46–57 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16507882 [Accessed August 5, 2014].

Strawn JR, Pyne-Geithman GJ, Ekhaor NN, Horn PS, Uhde TW, Shutter L a, Baker DG, Geraciocit TD (2010) Low cerebrospinal fluid and plasma orexin-A (hypocretin-1) concentrations in combat-related posttraumatic stress disorder. Psychoneuroendocrinology 35:1001–1007 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20116928 [Accessed July 15, 2014].

Suzuki M, Beuckmann CT, Shikata K, Ogura H, Sawai T (2005) Orexin-A (hypocretin-1) is possibly involved in generation of anxiety-like behavior. Brain Res 1044:116–121 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15862796 [Accessed January 26,
Tabuchi S, Tsunematsu T, Kilduff TS, Sugio S, Xu M, Tanaka KF, Takahashi S, Tominaga M, Yamanaka A (2013) Influence of Inhibitory Serotonergic Inputs to Orexin/Hypocretin Neurons on the Diurnal Rhythm of Sleep and Wakefulness. Sleep 36:1391–1404 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23997373 [Accessed May 4, 2017].

Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM (2003) Genetic selection for coping style predicts stressor susceptibility. J Neuroendocrinol 15:256–267 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12588514 [Accessed July 20, 2017].

von der Goltz C, Koopmann A, Dinter C, Richter A, Grosshans M, Fink T, Wiedemann K, Kiefer F (2011) Involvement of orexin in the regulation of stress, depression and reward in alcohol dependence. Horm Behav 60:644–650 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0018506X11002091 [Accessed March 13, 2017].

Winsky-Sommerer R, Boutrel B, de Lecea L (2005) Stress and arousal: the corticotrophin-releasing factor/hypocretin circuitry. Mol Neurobiol 32:285–294 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16385142 [Accessed February 15, 2015].

Winsky-Sommerer R, Yamanaka A, Diano S, Borok E, Roberts AJ, Sakurai T, Kilduff TS, Horvath TL, de Lecea L (2004) Interaction between the corticotropin-releasing factor system and hypocretins (orexins): a novel circuit mediating stress response. J Neurosci 24:11439–11448 Available at: http://www.ncbi.nlm.nih.gov/pubmed/15601950 [Accessed August 11, 2014].

Wood SK, Bhatnagar S (2015) Resilience to the effects of social stress: evidence from clinical and preclinical studies on the role of coping strategies. Neurobiol Stress 1:164–173 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25580450 [Accessed September 13, 2016].

Wood SK, Walker HE, Valentino RJ, Bhatnagar S (2010) Individual differences in
reactivity to social stress predict susceptibility and resilience to a depressive phenotype: role of corticotropin-releasing factor. Endocrinology 151:1795–1805 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2850230&tool=pmcentrez&rendertype=abstract [Accessed January 27, 2014].

Wood SK, Wood CS, Lombard CM, Lee CS, Zhang X-Y, Finnell JE, Valentino RJ (2015a) Inflammatory Factors Mediate Vulnerability to a Social Stress-Induced Depressive-like Phenotype in Passive Coping Rats. Biol Psychiatry 78:38–48 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25676490 [Accessed July 31, 2017].

Wood SK, Wood CS, Lombard CM, Lee CS, Zhang X-Y, Finnell JE, Valentino RJ (2015b) Inflammatory Factors Mediate Vulnerability to a Social Stress-Induced Depressive-like Phenotype in Passive Coping Rats. Biol Psychiatry 78:38–48 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25676490 [Accessed December 4, 2017].

Yehuda R (2000) Biology of posttraumatic stress disorder. J Clin Psychiatry 61 Suppl 7:14–21 Available at: http://www.ncbi.nlm.nih.gov/pubmed/10795605 [Accessed August 11, 2016].

Yehuda R, Teicher MH, Levengood RA, Trestman RL, Siever LJ (1994) Circadian regulation of basal cortisol levels in posttraumatic stress disorder. Ann N Y Acad Sci 746:378–380 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7825891 [Accessed July 21, 2016].

Zitnik GA (2016) Control of arousal through neuropeptide afferents of the locus coeruleus. Brain Res 1641:338–350 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26688115 [Accessed March 30, 2017].
Figure Legends

Figure 1. Social defeat paradigm and prepro-orexin expression in control and defeated rats.

Panel A. Social defeat paradigm. Panel B. Average defeat latency over 7 days of social defeat. Passively coping rats have an average defeat latency under 300 seconds while actively coping rats have an average defeat latency over 300 seconds. Panel C. Prepro-orexin expression in control, passive coping, and active coping rats. Top: Representative images of in situ radiolabeling for prepro-orexin in control, passive coping, and active coping rats. Bottom: Quantification of in situ radiolabeling in each treatment group reveals that actively coping rats have significantly less prepro-orexin mRNA in the lateral hypothalamus compared with control or passively coping rats. There is a negative correlation between average defeat latency and in situ radiolabeled orexin mRNA. Panel D. Prepro-orexin mRNA expression in control, passive coping, and active coping rats as measured by qPCR. Actively coping rats express significantly less prepro-orexin mRNA than control and passively coping rats. There is a negative correlation between average defeat latency and qPCR quantified orexin mRNA. *P<0.05, **P<0.01, ***P<0.001

Figure 2. Expression of DREADD-containing virus and inhibiting orexins during social defeat.

Panel A. A timeline of the experimental paradigm. 4 weeks after DREADDs injection, rats are exposed to 8 days of social defeat (the latter 3 days Veh or CNO is injected prior to defeat), followed by social interaction and forced swim test. Panel B. Representative images displaying viral expression of DREADDs in the lateral hypothalamus (LH) at 4 weeks. Panel C. A composite image displaying the spread of viral expression along the LH is depicted using rat brain atlas images (Paxinos and Watson, 1998). Each red dot represents a cell.
expressing the viral tag. **Panel D.** Prepro-orexin expression in vehicle- and CNO-treated control, passive coping, and active coping rats on Day 9 (before further behavioral testing). **Top:** Representative images of in situ radiolabeling for vehicle- and CNO-treated prepro-orexin in control, passive coping, and active coping rats. **Bottom:** Quantification of in situ radiolabeling in each treatment group reveals that actively coping rats have significantly less prepro-orexin mRNA in the lateral hypothalamus compared with passively coping rats. CNO treatment reduces prepro-orexin expression in passively coping rats to levels similar to that of actively coping rats. **Panel E.** Prepro-orexin expression in vehicle- and CNO-treated control, passive coping, and active coping rats on Day 12 (after social interaction and forced swim test behaviors). **Top:** Representative images of in situ radiolabeling for vehicle- and CNO-treated prepro-orexin in control, passive coping, and active coping rats. **Bottom:** Quantification of in situ radiolabeling in each treatment group reveals that actively coping rats have less prepro-orexin mRNA in the lateral hypothalamus compared with passively coping rats. CNO treatment reduces prepro-orexin expression in both control and passively coping rats to levels similar to that of actively coping rats.

*P<0.05, #P < 0.10

**Figure 3. Social Interaction behavior after the social defeat paradigm**

**Panel A.** Time spent interacting with the stimulus rat. Actively coping rats spend significantly more time interacting than passively coping rats. CNO treatment (inhibition of orexin neurons) increases time spent interacting in passively coping rats. **Panel B.** Latency to interact with the stimulus rat. There were no significant differences in latency to interact between the treatment groups. **Panel C.** Distance moved in the social interaction arena. There were no significant differences in the distance moved between the treatment groups.

*P<0.05

**Figure 4. Forced Swim Test behavior after the social defeat paradigm**
Panel A. Percent of time spent immobile in the forced swim test. Passively coping rats spend significantly more time immobile than control rats. CNO treatment (inhibition of orexin neurons) decreases time spent immobile in passively coping rats. CNO treatment (inhibition of orexin neurons) increases time spent immobile in control rats. Panel B. Percent of time spent active in the forced swim test. Vehicle treated passively coping rats spend significantly less time active than control rats. While CNO treatment decreases time spent active in control rats, it increases time spent active in passively coping rats. Panel C. Percent of time spent swimming in the forced swim test. Passively coping rats spend significantly less time swimming than control rats. CNO treatment decreases time spent swimming in control rats. Panel D. Percent of time spent climbing in the forced swim test. There were no significant differences in time spent climbing between the treatment groups.

*P<0.05, **P<0.01
Figure 1

A) Rats randomly assigned to control or defeat group
   Intruder placed into resident cage
   Intruder separated from resident by wire partition after either 1 supine defeat or 15 minutes of resisting attacks
   Intruder returns to home cage
   Brains collected and prepared for in situ hybridization or qPCR
   30 min/day for 7 days

B) Average Defeat Latency (%)

C) Control  Passive Coping  Active Coping

D) Oxenon mRNA
   Fold change (X:Y) compared with control

   Average Defeat Latency (s)
Figure 3

A

Time spent interacting (s)

Control Passive Coping Active Coping

300 350 400 450 500

B

Latency to interact (s)

Control Passive Coping Active Coping

0 10 20 30

C

Distance Moved (cm)

Control Passive Coping Active Coping

0 10000 20000 30000 40000

Vehicle CNO
Figure 4