2017

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Custom Citation

Grafe, Laura A., Amanda Cornfeld, Sandra Luz, Rita Valentino, Seema Bhatnagar. 2017. "Orexins Mediate Sex Differences in the Stress Response and in Cognitive Flexibility." Biological Psychiatry 81.8: 683–692.

Orexins mediate sex differences in the stress response and in cognitive flexibility

Short title: Orexins mediate sex differences in stress effects

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Key words: Orexins, Sex Differences, cognitive flexibility, Stress, DREADDs, Habituation

Abstract: 248 words

Article body: 4,050 words

Figures: 5

Tables: 0

Supplemental Information: 1 document

ABSTRACT

BACKGROUND: Women are twice as likely as men to suffer from stress-related psychiatric disorders. However, the biological basis of these sex differences is poorly understood. Orexins are altered in anxious and depressed patients. Using a rat model of repeated stress, we asked whether orexins contribute to sex differences in outcomes relevant to stress-related psychiatric diseases.

METHODS: Behavioral, neural, and endocrinal habituation to repeated restraint stress and subsequent cognitive flexibility was examined in adult male and female rats. In parallel, orexin expression and activation was determined in both sexes, and chromatin immunoprecipitation was used to determine transcription factors acting at the orexin promoter. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) were used to inhibit orexin activation throughout repeated restraint to determine if the stress related impairments in females could be reduced.

RESULTS: Female rats exhibited impaired habituation to repeated restraint with subsequent deficits in cognitive flexibility compared to male rats. Increased orexin expression and activation was observed in females compared to males. The higher expression of orexin mRNA in females was due to actions of glucocorticoid receptors on the orexin promoter, as determined by chromatin immunoprecipitation. Finally, inhibition of orexins using DREADDs in females throughout repeated restraint abolished their heightened HPA responsivity and reduced stress-induced cognitive impairments.

CONCLUSIONS: The results demonstrate that orexins mediate the impairments in adaptations to repeated stress and in subsequent cognitive flexibility exhibited by female rats and provide evidence for a broader role for orexins in mediating functions relevant to stress related psychiatric diseases.

Introduction

Stress-related psychiatric disorders are twice as common in women, however, the neurobiology underlying these sex differences is not fully understood (1–3). An important adaptation to repeated stress called habituation, is defined as decreased behavioral, hypothalamic pituitary adrenal (HPA) and autonomic responses to moderately intense stressors (4). Habituation to repeated stress, including the HPA response, is disrupted in stress-related illnesses such as post-traumatic stress disorder (PTSD) and panic disorder (5). The HPA axis integrates the response to a stressor at the paraventricular hypothalamus (PVN), which causes downstream release of ACTH from the anterior pituitary, and ultimately glucocorticoid release from the adrenal glands. Corticosterone, the primary glucocorticoid in rodents, binds to glucocorticoid and/or mineralocorticoid receptors (GR and MR, respectively) to exert its effects in the periphery and in the brain. Stressful life events can also impair cognitive function, including cognitive flexibility (6), which precipitates or exacerbates many psychiatric disorders (7). Patients with disorders such as panic disorder and PTSD exhibit altered concentrations of the hypothalamic peptides orexins in cerebrospinal fluid (5, 8, 9). Orexins regulate neuroendocrine and behavioral responses that are affected in stress-related illness including disruptions in the HPA axis, cognitive flexibility, arousal, food intake, and emotional memory (5, 10, 11). Sex differences in orexin precursor prepro-orexin mRNA have been reported (12), but neither the mechanisms underlying this sex difference nor the functional consequences of this disparity is understood.

To address these gaps in knowledge, we conducted a detailed examination of sex differences in the HPA response to repeated stress and in subsequent cognitive flexibility in an operant strategy shifting test (13). We then determined the role of orexins in mediating these effects of stress. The results indicate that elevated orexins in female rats are responsible for the heightened HPA responses to repeated stress and the stress-induced impairments in cognitive flexibility. Together, the results suggest a novel role for orexins in mediating sex differences in functions that are altered in stress-related psychiatric disorders.

Methods and Materials

Animals: Male and female Sprague-Dawley rats between 65-75 days of age were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were singly housed and had food and water available *ad libitum*, under 12-h light/dark cycle. Animals acclimated to the housing and lighting conditions for 5 days prior to any surgical or stress protocols. The Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia (CHOP) Research Institute approved all experimental procedures.

Experiment 1: Habituation to Repeated Restraint: Behavioral, Neural, and Endocrine Measures

Two cohorts of animals were used in these experiments (n = 48 animals, 8/treatment group: See **Figure 1A** for treatment groups and experimental paradigm). Animals were restrained in Plexiglas restrainers differently sized for male or female rats for 30min/day for 5 consecutive days. As orexins exhibit a circadian rhythm (14), animals were restrained within 2h after lights on so that levels of orexin remained consistent. Noldus software was used to quantify time spent struggling from videos of the first 10 min of restraint, as previously described (4, 15). Blood samples were taken on Day 1 and Day 5 of restraint for ACTH and corticosterone as previously described (16).

To assess activation in the parvocellular division of the PVN (pPVN) after restraint, rats were rapidly decapitated 30 minutes following restraint on day 5 and brains were flash frozen in 2-methyl butane. Sections through the pPVN (-1.72 mm to -1.92 mm from bregma) were fixed in 4% paraformaldehyde, immunostained for c-Fos (1:1250, sc-52; Santa Cruz Biotechnology, Santa Cruz, CA) , and analyzed in NIH Image J by two investigators blind to treatment conditions.

Experiment 2: Effects of Repeated Restraint on Cognitive Flexibility

Three cohorts of animals were used in this experiment (n= 48 animals, 12/ treatment group: Control Males, Control Females, 5 Day Restrained Males, or 5 Day Restrained Females). Male and female rats were either left in their home cages or restrained for five days. On the 6th day, an operant set shifting protocol was performed and analyzed as in Snyder et al ((13); See SI and **Figure 2A**).

Experiment 3: Orexin Expression and Activation in Male and Female Rats

Brains of control animals from *Experiment 1* were used to assess prepro-orexin mRNA (as described in (17, 18)) and brains from control, 1 day, or 5 day restrained rats were used to measure activation of orexin neurons. A separate cohort of rats was used for cerebrospinal fluid collection (12/group: Control Males and Control Females). Activation of orexinA-immunoreactive neurons was assessed by a dual stain for c-Fos (as described above) and orexinA (1:50, sc-8070; Santa Cruz Biotechnology, Santa Cruz CA), followed by biotinylated Horse Anti-Goat antibody (1:500, BA-9500; Vector Laboratories, Burlingame, CA) and a Nova Red reaction (SK-4800, Vector Laboratories, Burlingame, CA). Dual labeled cells (Fos nuclear black stain and orexinA cytoplasmic red stain) were counted by observers blind to experimental conditions.

Cerebrospinal fluid was collected from the cisterna magna of anesthetized rats and orexinA concentrations were assayed by radioimmunoassay (Phoenix Pharmaceuticals; Burlingame, CA). The minimum levels of detection for orexinA was 80 pg/ml. Intra-and interassay variability was 5-7% and 12-15%.

Experiment 4: Examining the Glucocorticoid Receptor (GR) regulation of prepro-orexin mRNA

Sections of the LH from control males and females in *Experiment 1* were used to examine GR expression in orexin neurons. Immunofluorescence was performed with primary antibodies for Orexin A and GR (1:50, SC-1004, Santa Cruz Biotechnology; Santa Cruz, CA), followed by

AlexaFluor488 Donkey anti-goat and AlexaFluor647 Donkey anti-rabbit secondary antibodies (1:200, A-11055 and A-31573; Life Technologies, Carlsbad, CA). A colocalization plugin was used in ImageJ to determine percent orexin neurons that express GR.

To examine potential transcription factors that act on the prepro-orexin promoter, the sequence for prepro-orexin was entered into the ALGGEN PROMO virtual laboratory (See SI), which identified the GR (α and β). Chromatin immunoprecipitation (ChIP) was performed for GR in the LH of control male and female rats (8/treatment group) using a standard kit (Catalog # 17-295, EMD Millipore). qPCR was performed at specific primer set locations along the prepro-orexin promoter, where GR was predicted to bind (See SI).

Two cohorts of female rats were used to test whether GR regulates prepro-orexin expression in females (8/treatment group: Females injected with scrambled siRNA or Females injected with siRNA directed at the GR). Oligodeoxynucleotides (ODNs) (Eurofins MWG Operon; Huntsville, AL) directed at the GR were used to knock down its expression in the LH as previously described (19). In order to confirm knockdown of the GR by siRNA, slides were stained immunofluorescently for GR and orexinA. Additionally, LH punches from another cohort of control or siRNA treated female rats were collected 24 hours after the third day of ODN injections (6/group). QPCR on LH punches was performed using a Sybr Green Master Mix (Thermo Fisher Scientific; Pittsburgh, PA) and primers for GR (forward: AGG GGA GGG GGA GCG TAA TGG reverse: CCT CTG CTG CTT GGA ATC TGC) and GAPDH (forward: GAC ATG CCG CCT GGA GAA AC reverse: AGC CCA GGA TGC CCT TTA GT).

Experiment 5: Inhibiting Orexin via Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) throughout Repeated Restraint

Four cohorts of animals were used in these studies (n = 64 animals, 8/group: Females injected with Vehicle or Clozapine N-Oxide (CNO). Both groups had the DREADDs virus expressed in the LH (see SI for more construct information). Half of the animals were analyzed for pPVN and

orexin activation after the fifth restraint and half continued on to the strategy shifting test.

Female rats received injections of vehicle (saline and 8% DMSO) or CNO (Sigma-Aldrich; St Louis, MO 2 mg/kg ip) 60 minutes prior to the start of the 30-minute restraint for 5 consecutive days. This timing was chosen because CNO promotes behavioral effects in the rat within 30 minutes and effects last up to 4 hours (20, 21). Blood was collected on day 1 and day 5 of restraint and assayed for ACTH and corticosterone. Some females were sacrificed after 5 days of restraint to assess c-Fos in the pPVN and in orexin neurons as described above, while others continued to the strategy shifting test to be assessed for cognitive flexibility, as described above.

A new cohort of female rats (n 16, 8/group) underwent 5 consecutive days of 30-minute restraint with SB334867 (an orexin 1 receptor antagonist) administered 30 min prior to daily restraint. Tail blood for these rats were collected on day 1 and 5 of restraint.

Statistical Analysis: Two-way ANOVAs examined Sex and Stress variables for habituation and strategy shifting data. T-tests compared control males and females for orexin expression/activation and ChIP data. For DREADDs data: t-tests compared vehicle- and CNO-treated stressed females for pPVN activation, orexin activation, and basal plasma corticosterone; two-way ANOVAs were used to examine ACTH and corticosterone data; one-way AVOVA were used to analyze strategy shifting data. T-tests compared control and GR SiRNA treated rats for QPCR and prepro-orexin analysis. Statistical analysis was conducted with GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Females exhibit impaired habituation to repeated stress

Female rats spent significantly more time struggling in response to the 1st and 5th restraint compared with males (**Figure 1A**). Moreover, on day 5, time spent struggling in males was

reduced to 46% of its value on day 1, but the reduction in females was only to 69% of its value on day 1, indicating reduced behavioral habituation to repeated restraint in females.

cFos activation in the pPVN was significantly increased by acute restraint in males and reduced by day 5, comparable to that of control males (**Figure 1B**). In contrast, in females, pPVN activation was significantly increased acutely, and remained elevated on day 5 of restraint compared to control females, indicating continued activation of the pPVN with repeated restraint in female rats. Consistent with these results, integrated plasma ACTH levels were significantly decreased in males but not females by day 5 of restraint compared with day 1 (**Figure 1C**). The same sex difference was observed in integrated plasma corticosterone concentrations. Additionally, females displayed significantly higher plasma corticosterone concentrations at both day 1 and 5 compared with males. Interestingly, females but not males had significantly higher basal corticosterone concentrations on day 5 of restraint compared with their respective day 1 of restraint. These results indicate that behavioral, neural and neuroendocrine habituation to repeated stress is diminished in female rats compared to male rats and that female rats exhibited heightened baseline glucocorticoid release after repeated restraint.

Female rats exhibit impaired cognitive flexibility after repeated stress

Control and repeatedly restrained males and females trained in an operant strategy shifting test (see **Figure 2A**), followed by a test day, which consisted of three tasks: Side Discrimination, Side Reversal, and Light Discrimination**.** Stressed males required significantly fewer trials than control males to complete the side reversal task **(Figure 2B)**. In contrast, stressed females required significantly more trials than control females to complete the side reversal task. Thus, stress improved performance in males but impaired performance in females in the side reversal task. In the light discrimination task, stress increased the number of trials to criterion in females, thereby impairing performance in this task.

Stressed males made fewer errors than control males in the side reversal task, consistent with their fewer trials to criterion (**Figure 2C).** Moreover, stressed females made significantly more errors than control females in both the side reversal and light discrimination tasks, consistent with their impaired performance in trials to criterion for both tasks. Total errors were further categorized into perseverative or regressive errors (**Figure 2D**, for more detail, see SI and (13)). In the side reversal task, stressed males made less perseverative errors compared with control males. In contrast, stressed females made more perseverative errors than control females in both the side reversal and light discrimination tasks. There were no differences between the treatment groups in the number of regressive errors in either task. Stressed males took less time to complete the side reversal task than control males, while stressed females took significantly more time to complete the side reversal task (**Figure 2E)**. In addition, stressed females made a higher percentage of omissions compared with control females, contributing to this longer time to complete the task. In summary, stressed females made more omissions and perseverative errors in the side reversal task, contributing to their impaired performance in this task.

Female rats exhibit elevated baseline orexin expression and activation compared to male rats

Orexin system function was assessed at several levels. First, significantly higher prepro-orexin mRNA was observed in control females compared with control males **(Figure 3A**) as assessed by *in situ* hybridization. Next, control females exhibited significantly more activation of orexin neurons compared with control males as assessed by dual labeling for orexinA and cFos (**Figure 3B**). While day 1 of restraint induced significant activation of orexin neurons, orexin activation returned to its respective baseline by day 5 of restraint in both sexes. However, orexin neural activation in females was still significantly higher than males after repeated restraint. Finally, concentrations of orexinA were significantly higher in the cerebrospinal fluid of females

compared to males (**Figure 3C**). In summary, females displayed higher levels of baseline orexin expression and activation compared with control males.

Glucocorticoid receptors promote elevations of prepro-orexin mRNA in females

We next explored the mechanism by which orexins are increased in females compared with males. We examined candidate transcription factors that may bind to the orexin promoter and upregulate prepro-orexin mRNA in females, one of which was the GR, which is known to be expressed in orexin neurons ((22) and see **Figure 4A)**. Chromatin immunoprecipitation revealed that GR bound to several sites on the orexin promoter (**Figure 4B**) with higher enrichment of GR in control females compared with males in the HCRT1, HCRT5, and HCRT6 primer sets. We also showed amplification of the period 1 promoter, a positive control of which GR is known to bind, based on previous literature (23) and no amplification at the GAPDH promoter, a negative control. While these results indicated that the GR is enriched at the orexin promoter in females, they did not determine whether this enrichment leads to the upregulation of prepro-orexin in females. To test this, GR siRNA was administered into the LH in female rats. There was stable knockdown of GR in orexin neurons (**Figure 4C)** confirming the efficacy of the GR siRNA. Critically, this GR knockdown reduced prepro-orexin mRNA in females compared to scrambled control (**Figure 4D),** demonstrating that GR directly acts at the orexin promoter to upregulate orexin expression in female rats.

Orexins contribute to HPA activity and cognitive impairment after repeated restraint in females

The previous results lead to the hypothesis that higher orexin system activity in females produces their reduced habituation and impaired cognitive flexibility. To test this hypothesis, orexin system activity was reduced in females using two different approaches. First, an inhibitory DREADDs construct targeted to orexins (referred to as *hM4D-orexins*) was developed to inhibit orexin cells in females throughout repeated restraint (see **Figure 5A**). Inhibition of *hM4D-orexins*

transfected neurons by Clozapine N-Oxide (CNO) administration prior to each restraint significantly reduced neural activity in both orexin neurons and in pPVN cells in stressed females by day 5 of restraint (**Figure 5B and C**), decreased plasma ACTH concentrations by day 5 of restraint compared with day 1 (**Figure 5D**) and eliminated the heightened basal plasma corticosterone concentrations in stressed females by day 5 of restraint (**Figure 5E**). This augmented basal corticosterone on day 5 was also abolished through use of a second approach to reduce orexin activity, *ip* administration of the orexin 1 receptor antagonist SB334867 prior to each daily restraint. To summarize, inhibiting orexin action during repeated restraint was sufficient to induce habituation to repeated restraint in females and to lower their heightened baseline glucocorticoid release. Moreover, inhibiting orexin neurons throughout restraint reduced the trials to criterion, time to criterion, and number of omissions in the side reversal task in stressed females (**Figure 5F and 5G**). Thus, inhibition of orexins throughout repeated restraint ultimately improved cognitive flexibility in females.

Discussion

Inability to adapt to repeated stress, including an impaired habituation to familiar non-life-threatening stressors and disruptions in cognitive functions are common in illnesses such as depression and PTSD (5, 7). For the first time, a detailed examination of sex differences in the ability to habituate to stress and in subsequent cognitive flexibility revealed that female rats exhibited reduced habituation and enhanced baseline glucocorticoid concentrations after 5 days of restraint, a moderately intense and primarily cognitive stressor (24). Furthermore, female rats exhibited disruptions in cognitive flexibility after stress whereas cognitive flexibility improved in male rats after stress. To determine the mechanisms that contribute to these sex differences, we examined the neuropeptides orexins, since orexins have been shown to contribute to both the HPA response and cognitive function (10, 11, 25, 26). We discovered that nonstressed female rats had higher prepro-orexin expression, activation of orexin neurons, and higher orexin

levels in the cerebrospinal fluid compared with male rats. We then determined that GR acts on the prepro-orexin promoter to increase prepro-orexin mRNA. Using both inhibitory DREADDs specifically targeted to orexin neurons and pharmacological approaches to inhibit orexin receptors, we determined that the enhanced orexins produce both the impaired habituation to restraint and subsequent cognitive deficits in females. These results suggest that orexins are important mediators of sex differences in the response to repeated stress and subsequent cognitive function, providing important insights in both the etiology and treatment of psychiatric diseases.

Sex differences in habituation to stress

Though it is well established that female rats have a higher HPA response to acute stressors than male rats (27), fewer studies have examined sex differences in adaptations to repeated stress such as habituation. Through behavioral, neuronal, and endocrine measures, the results suggest that females do not habituate as fully as males to 5 consecutive days of 30-minute restraint. This does not preclude the possibility that females may habituate similarly to males with further exposure to the same stressor. While other studies from our lab have found that females still do not display habituation at 8 consecutive days of 30-min restraint (28), a recent study found that 10 days of 30-min restraint produced habituation in both sexes (29). However, by examining this point in time when sex differences in the stress response do occur, we are able to study the neurobiology underlying the increased sensitivity to stress displayed by females, shedding light on gender biased stress-related illness.

Sex differences in cognitive function after stress

In humans, stressful life events impair cognitive function, precipitating or exacerbating many psychiatric disorders (30). Aspects of executive functioning in humans can be assessed using the Wisconsin Card Sorting Task, a demonstration of cognitive flexibility (31). Stress impairs

cognitive flexibility using analogous tests in rodents including the attentional set shifting task and the operant strategy shifting task used here (13). However, there has been limited research directly examining sex differences in these paradigms (13, 32). Our results are the first to demonstrate that repeated stress leads to sex-specific deficits in cognitive flexibility in rats. Females performed worse on both the side reversal and light discrimination tasks after stress, as indicated by an increased number of trials to criterion, perseverative errors, and omissions. In contrast, repeated restraint improved male performance in the side reversal task. Perseveration is observed in stress-related psychiatric disorders such as PTSD, and impairs the ability of one to learn a new set of rules, ultimately impairing working memory (7). Omissions in attention tasks have also been noted in patients with PTSD, indicating slower cortical processing (7). As cognitive inflexibility is a prominent phenotype in stress-related psychiatric disorders, the results suggest that females may be more vulnerable to this type of cognitive impairment after repeated stress compared with males.

Sex differences in orexin expression are produced by actions of GRs

As orexins are known to contribute to both the stress response and cognitive function, it was possible that these neuropeptides could explain the sex differences we observed. Sex differences in prepro-orexin mRNA have been noted in Wistar Rats (12). We replicated and extended these initial findings, demonstrating that female Sprague Dawley rats not only exhibit higher baseline levels of prepro-orexin mRNA, but additionally display higher activation of orexin neurons and increased orexinA concentrations in the cerebrospinal fluid compared with male rats. While other studies indicate that female rats display higher prepro-orexin mRNA during proestrus (33, 34), a study that ovariectomized females showed no reduction in prepro-orexin mRNA, further suggesting that this sex difference is not mediated by female gonadal hormones (35). However, both the paucity and equivocal nature of this literature leaves the contribution of female gonadal hormones in regulating prepro-orexin expression unclear.

We next examined possible mechanisms responsible for higher orexin expression and activation in females. We hypothesized that transcription factors more prevalent or active in females may bind to the orexin promoter and cause upregulation of orexin expression. A transcription factor binding prediction program revealed that the consensus sequence for the GR may bind to the orexin promoter. Using chromatin immunoprecipitation, we found GR to be more highly enriched at the orexin promoter in females compared with males. GR expression was inhibited in the LH of female rats using siRNA and this inhibition did indeed produce a decrease in prepro-orexin expression suggesting that GR is responsible for the upregulation of prepro-orexin in females. These data suggest that the GRs directly on the orexin promoter increase prepro-orexin expression in females, providing a novel regulatory mechanism for the control of orexin system activity.

Our studies focused on baseline changes in orexin regulation of habituation and cognitive function and did not characterize relationship between orexins and GRs in stressed animals. It is possible that this relationship is different than in control animals. The activation of the GR changes with stress which could obscure our understanding of the relationship between glucocorticoids and orexins under basal conditions, as investigated here. Future studies are necessary to delineate the relationship between orexins and glucocorticoids throughout repeated stress.

Inhibiting orexins rescues habituation to stress and cognitive flexibility in females

We next examined the role of orexins in regulating the sex differences that we observed in habituation to stress and subsequent cognitive function. We found that inhibiting orexin neurons prior to each restraint using *hM4D-orexins* reduced pPVN activation, integrated ACTH levels and basal corticosterone concentrations at day 5 of restraint. This reduction of basal corticosterone levels by day 5 of restraint was also induced by pharmacological means through administration of the orexin 1 receptor antagonist SB334867, indicating that the orexin 1 receptor

is responsible for higher basal corticosterone in females after repeated restraint. Thus, these data suggest that the relationship between glucocorticoids and orexins is bidirectional: not only can GR increase central orexin expression, but decreasing orexin action lowers basal glucocorticoid levels. Further, these results suggest a novel mechanism for regulation of basal glucocorticoid release. This is important because basal glucocorticoids are higher in patients with depression (36). Thus, if orexins contribute to an increase in basal glucocorticoids, inhibiting these neuropeptides may aid in treatment of certain aspects of this neuropsychiatric disorder. By extension, understanding how orexins regulate basal glucocorticoids may also inform aspects of disorders where basal glucocorticoids are low, such as in PTSD (37).

We then examined whether inhibiting orexins throughout repeated restraint impacted subsequent cognitive flexibility. We found that inhibiting orexin neurons prior to each restraint with *hM4D-orexins* reduced subsequent cognitive impairments in strategy shifting in stressed females. Specifically, inhibiting orexin neurons markedly reduced the number of trials to criterion, the number of omissions, and the time it took to complete the side reversal task. Overall, these results indicate that orexins actions during repeated stress are important for the subsequent impairments in strategy shifting in female rats.

Conclusions and Future Directions

The present studies demonstrated that elevated expression and activation of orexin neurons in female rats, due in part to GR actions at the prepro-orexin promoter, underlies their inability to habituate to repeated stress. Furthermore, orexins are involved in behavioral consequences of stress, contributing significantly to stress-induced impairment in cognitive flexibility in females. Together, these results demonstrate that orexins are novel regulators of sex differences in neural, behavioral and neuroendocrine adaptations to repeated stress and in the cognitive consequences of repeated stress exposure. The results suggest that orexins could be

important in the etiology of those stress-related psychiatric disorders that present differently in men and women.

Acknowledgements: This work was supported by The Defense Advanced Research Projects Agency (DARPA) and the Army Research Office award number W911NF1010093 and by NIMH grant number R01MH109975. LG was supported by award number T32NS007413 from the National Institute of Neurological Disorders and Stroke (NINDS) and award number F32MH109269-01 from the National Institute of Mental Health. The content is the sole responsibility of the authors and does not necessarily represent the official views of the NINDS or NIMH of the National Institutes of Health. We gratefully acknowledge the dedicated assistance of Jane Dobkin and Herminio Guajardo. We also greatly appreciate the expertise of Dr. Ted Abel for his assistance with Chromatin Immunoprecipitation.

Financial Disclosures: All authors declare no biomedical financial interests or potential conflicts of interest.

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Figure Legends

Figure 1. Habituation is diminished in female compared to male rats.

Panel A. Top: Repeated restraint paradigm. **Bottom:** Time spent struggling while in the restrainer is quantified. Males reduce cumulative duration of movement to 46% of day 1 by day 5, whereas females only reduce to 69% of day 1 by day 5. Females display significantly higher struggle behavior on both day 1 and 5 compared with males. (Sex effect, $F(1,24) = 36.7$, $p<0.0001$; Stress effect, $F(1,24) = 17.5$, $p<0.001$; two-way ANOVA followed by Tukey's t-test; n = 8/group) **Panel B.** pPVN activation in control, 1 day restrained, and 5 days repeatedly restrained male and female rats. Representative images of cFos staining, with dotted lines outlining the pPVN. While day 1 of restraint induces significant cFos staining in both males and females, males have significantly lower cFos activation by day 5, whereas pPVN neurons remain activated in females on day 5. (Stress effect, $F(2,28) = 7.1$, $p<0.01$; Interaction trend, $F(2,28) = 3.1$, $p = 0.06$; two-way ANOVA followed by Tukey's t-test; n = 8/group) **Panel C.** Integrated plasma ACTH and corticosterone on day 1 and 5 of restraint in male and female rats. Both plasma ACTH and corticosterone decrease by day 5 compared with day 1 of restraint in males, but not in females. Regardless of stress, plasma corticosterone levels are significantly higher in females compared with males. (ACTH: Stress effect, $F(1,17) = 5.9$, $p<0.05$; Cort: Sex effect, $F(1,18) = 20.0$, $p<0.001$; two-way ANOVA followed by Tukey's t-test). Basal corticosterone (0 minute time point) is significantly higher in females on day 1 of restraint compared with males on day 1. Moreover, females display higher basal corticosterone on day 5 of restraint compared with day 1, whereas males do not (Sex effect, $F(1,39) = 25.6$, $p<0.001$; Stress effect, $F(1,39) = 9.2$, $p<0.01$; Interaction effect, $F(1,39) = 11.5$, $p<0.01$; two-way ANOVA followed by Tukey's t-test; n = 8/group). *P<0.05, **P<0.01, ***P<0.001

Figure 2. Repeated stress impairs cognitive flexibility and cortical activation in females.

Panel A. Schematic illustrating the operant set shifting paradigm training days. § Adapted from *Behavioural Brain Research*, Volume 190 (Issue 1). Floresco SB, Block AE, and Tse M. "Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure", p85-96, 2008, with permission from Elsevier. **Panel B.** Trials to criterion for each task on test day. In the side reversal task, stress improved performance in males but impaired performance in females (Stress effect, $F(1,23) = 6.0$, $p<0.05$; Interaction effect, $F(1,23) = 31.7$, $p<0.0001$; two-way ANOVA followed by Tukey's t-test). In the light discrimination task, stress impaired performance in females while it did not affect males (Stress trend, ($F(1,25) = 2.8$, $p = 0.1$); two-way ANOVA followed by Tukey's t-test; n = 12/group). **Panel C.** Number of errors for each task on test day. Stress decreases errors made in males but increases errors made in females in both the side reversal and light discrimination tasks (Side Reversal: Interaction effect, $F(1,28) = 6.5$, $p<0.05$; Light Discrimination: Interaction trend, $F(1,28) = 3.4$, $p = 0.08$; two-way ANOVA followed by Tukey's t-test). **Panel D.** Perseverative and regressive error characterization. Stress decreases perseverative errors made in males but increases perseverative errors made in females in both the side reversal and light discrimination tasks (Side Reversal: Interaction effect, $F(1,26) = 6.7$, $p<0.05$; Light Discrimination: Stress effect, ($F(1,26) = 5.3$, $p<0.05$; two-way ANOVA followed by Tukey's t-test). **Panel E.** Time to criterion and % trials omitted in the side reversal task. Stress decreases the time it takes males but increases the time required by females to complete the task. This may be explained by increased number of omissions exhibited by females after stress (Time: Interaction effect, $F(1,28) = 7.8$, $p<0.01$; two-way ANOVA followed by Tukey's t-test). #P≤0.10, *P<0.05, ****P<0.0001

Figure 3. Females have higher orexin expression and activation than males.

Panel A. Representative photomicrographs of *in situ* radiolabeling for prepro-orexin mRNA in the LH in control male and female rats. Dark black grains indicate prepro-orexin expression. Control females express significantly more prepro-orexin mRNA than control males ($p<0.05$; t-test; n = 8/group). **Panel B.** Orexin neural activation in control, 1 day restrained, and 5 days repeatedly restrained male and female rats. Representative 10x images of an orexin/cFos dual stain in the LH in control male and female rats. *f* denotes the fornix. The box with dashed lines in the representative control female image is displayed at 40x. Orexin neurons can be visualized by red cytoplasmic stains (NovaRed reaction, Δ symbol) and cFos can be visualized with black nuclear stains (Nickel DAB reaction). The arrow denotes a dual cFos and orexin labeled cell. Control females have significantly more activated orexin neurons than males. While day 1 of restraint induces significant activation of orexin neurons, orexin activation returns to its respective baseline by day 5 of restraint in both sexes. However, orexin neural activation in females is still significantly higher than males after repeated restraint. (Stress effect, $F(2,97) = 36.5$, $p<0.0001$; Sex effect, $F(1,97) = 21.0$, $p<0.0001$; Interaction effect, $F(2,97) =8.7$, $p<0.001$; two-way ANOVA followed by Tukey's t-test; n = 8/group). **Panel C.** Cerebrospinal fluid was collected from the cisterna magna and assayed for Orexin A. Females have significantly higher levels of Orexin A in the cerebrospinal fluid than males ($p<0.05$; t-test; n = 12/group). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Figure 4. GR is highly enriched at the orexin promoter in females compared to males.

Panel A. A representative image of dual staining for both orexin (green, Δ symbol) and GR (red, arrow symbol) reveals that most orexin neurons express GR (colabeling in yellow). Specifically, colocalization analysis revealed that roughly 80% of orexin neurons express GR. **Panel B.** ChIP and qPCR quantified GR bound to the orexin promoter in control male and female rats. At HCRT primer set 1, 5, and 6, control females were more highly enriched with the GR compared with control males (Primer set 1: $p = 0.10$; Primer Set 5: $p = 0.10$; Primer Set 6: $p<0.05$; t-test; n =

8/group). **Panel C.** Representative images of dual stain for orexin neurons and GR in control females (left) and females injected with siRNA directed against the GR (right). GR siRNA reduced visible GR staining in the LH. Additionally, GR siRNA significantly reduced GR expression in the LH as measured by qPCR ($p<0.001$; t-test; n = 6/group). **Panel D.** *In situ* radiolabeling of prepro-orexin mRNA in control females and females injected with siRNA directed against the GR. Representative images of prepro-orexin expression (top) and quantification of the dark black grains (bottom). siRNA directed against the GR injected into the LH decreases prepro-orexin mRNA expression in females compared with controls ($p<0.05$; t-test; n = 8/group). #P≤0.10, *P<0.05, ***P<0.001

Figure 5. Inhibiting orexin neurons throughout repeated restraint promotes habituation and reduces baseline HPA activity and subsequent cognitive impairment in female rats. **Panel A.** Representative images displaying viral expression of DREADDs in the LH at 4 weeks. 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. A timeline of the experimental paradigm is pictured below. **Panel B.** Representative images of dual cfos/orexin staining in the LH in repeatedly restrained female rats. CNO treated females (orexins inhibited prior to each restraint) exhibited significantly less activation in orexin neurons compared with vehicle treated females ($p<0.05$, t-test, n = 8/group). **Panel C.** Representative images of cFos staining in the pPVN in repeatedly restrained female rats. CNO treated females (orexins inhibited prior to each restraint) exhibited significantly less activation in the pPVN compared with vehicle treated females. ($p<0.05$; t-test; n = 8/group). **Panel D.** Integrated plasma ACTH and corticosterone responses to Day 1 and Day 5 of restraint. CNO treated females exhibited significantly lower plasma ACTH by day 5 of restraint (Stress effect, $F(1,13) = 5.3$, $p<0.05$; two-way ANOVA followed by Tukey's t-test). **Panel E.** Basal plasma corticosterone levels on day 5 in repeatedly restrained females. CNO treated females exhibited

significantly lower basal plasma corticosterone levels on day 5 of restraint ($p<0.01$; t-test). Treatment with orexin receptor antagonist SB334867 also significantly reduced basal corticosterone levels by day 5 of restraint ($p<0.05$; t-test; n = 8/group). **Panel F.** Trials to criterion in repeatedly restrained females. CNO treated females exhibited a reduced number of trials to criterion in the side reversal task. ($p<0.05$; t-test; n = 8/group). **Panel G.** Time to criterion and percent trials omitted in the side reversal task in repeatedly restrained females. CNO treated females exhibited both reduced time and reduced percent trials omitted in the side reversal task (Time: $p=0.10$, Omissions: $p<0.01$; t-test). #P≤0.10, *P<0.05, **P<0.01