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2021

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Andrew T. Gargiulo

Bryn Mawr College, agargiulo1@brynmawr.edu

Varuna Jasodanand

Bryn Mawr College

Sandra Luz

Children's Hospital of Philadelphia, Philadelphia, PA

Lauren O'Mara

Children's Hospital of Philadelphia, Philadelphia, PA

Leszek Kubin

School of Veterinary Medicine, University of Pennsylvania

See next page for additional authors

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Citation

Andrew T. Gargiulo, Varuna Jasodanand, Sandra Luz, Lauren O'Mara, Leszek Kubin, Richard J. Ross, Seema Bhatnagar & Laura A. Grafe. 2021. "Sex differences in stress-induced sleep deficits." *Stress*.

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Authors

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Sex differences in stress-induced sleep deficits

Andrew T. Gargiulo¹, Varuna Jasodanand¹, Sandra Luz², Lauren O'Mara², Leszek Kubin³, Richard J. Ross^{4,5}, *Seema Bhatnagar^{2,4}, *Laura A. Grafe¹

¹Department of Psychology, Bryn Mawr College, Bryn Mawr, PA 19010,

²Department of Anesthesiology and Critical Care, Children's Hospital of Philadelphia,

³Department of Biomedical Sciences, School of Veterinary Medicine, University of Pennsylvania,⁴Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA and ⁵Behavioral Health Service, Crescenzo Veterans Affairs Medical Center Philadelphia, PA 19104, USA

*** Co-Senior Authors**

Correspondence:

Laura Grafe
Bettws-Y-Coed Building, Room 201
Department of Psychology
Bryn Mawr College
Bryn Mawr, PA 19010
Phone: 610-526-5020

Key words: stress, sex differences, slow-wave sleep, REM sleep, restraint, orexin

Figures: 6

Acknowledgments: National Institutes of Health (USA) grant R01 MH109975

ABSTRACT

Sleep disruptions are hallmarks in the pathophysiology of several stress-related disorders, including Major Depressive Disorder (MDD) and Post-Traumatic Stress Disorder (PTSD), both known to disproportionately affect female populations. Although previous studies have attempted to investigate disordered sleep in women, few studies have explored and compared how repeated stress affects sleep in both sexes in either human or animal models. We have previously shown that male rats exhibit behavioral and neuroendocrine habituation to 5 days of repeated restraint, whereas females do not; additional days of stress exposure are required to observe habituation in females. This study examined sex differences in sleep measures prior to, during and after repeated restraint stress in adult male and female rats. Our data reveal that repeated stress increased time spent awake and decreased slow-wave sleep (SWS) and REM sleep (REMS) in females, and these effects persisted over 2 days of recovery. In contrast, the effects of stress on males were transient. These insomnia-like symptoms were accompanied by a greater number of exaggerated motor responses to waking from REMS in females, a phenotype similar to trauma-related nightmares. In sum, these data demonstrate that repeated stress produces disruptions in sleep that persist days after stress is terminated in female rats. These disruptions in sleep produced by 5 days of repeated restraint may be due to their lack of habituation.

Introduction

Stress is associated with the onset of several psychiatric disorders including Major Depressive Disorder (MDD) and Posttraumatic Stress Disorder (PTSD) (Whitaker et al., 2014; Abdallah et al., 2019). Sleep disturbances are common in stress-related psychiatric disorders, often presenting as recurrent nightmares and hyperarousal in the form of insomnia (Ross et al., 1989). Incidence rates for stress-related psychiatric disorders are twice as high in women compared with men. Thus, biological sex appears to be an important factor in the etiology of these diseases (Bangasser and Valentino, 2014).

There is robust evidence suggesting that sex modifies the circuitry of stress response systems as females generally present increased endocrine and neurobehavioral responses to repeated stress (Grafe et al., 2017a; Bangasser and Wiersielis, 2018; Grafe and Bhatnagar, 2018). Further, there are differences in the prevalence of certain sleep disorders in males and females. For instance, rapid eye movement sleep (REMS) behavior disorder is higher in men, whereas insomnia disorder is more common in women (Mong and Cusmano, 2016).

Although previous studies have investigated disordered sleep in women, few studies have methodically compared how repeated stress affects sleep macroarchitecture and microarchitecture in both sexes (Mallampalli and Carter, 2014). Thus, the goal of this study was to better understand sex differences in sleep measures after repeated stress using a rat model. Specifically, we examined sleep architecture before, during, and after five consecutive days of restraint stress in male and female rats. We have previously shown that male rats exhibit behavioral and neuroendocrine habituation to 5 days of restraint, whereas females require additional days of restraint in order to habituate (Grafe et al., 2017a). In this study, we observed that repeated restraint stress caused increases in time spent awake and decreases in Slow-wave sleep (SWS) and REMS in female, but not male, rats. Moreover, female rats compared with males demonstrated a higher number of exaggerated motor responses to waking from REMS.

The neuropeptides orexins are important in promoting stress and wake (Winsky-Sommerer et al., 2003; Johnson et al., 2012; Xu et al., 2013), and have been shown to contribute to sex differences in the stress response (Grafe et al., 2017a). Importantly, female rats demonstrate heightened orexin activity and expression, which is associated with exacerbated behavioral and neuroendocrine responses to stress. Thus, we predicted that orexins would contribute to increased wake after stress in female rats. To test this hypothesis, orexin system activity was reduced in females prior to each restraint stress using an inhibitory DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) construct. However, inhibition of orexin neurons prior to each restraint did not alter the stress-induced sleep changes in female rats. In sum, our data suggest that repeated stress results in persistent sleep disturbances in female rats and that orexins released by daily stress are not a significant contributor to the sleep deficits observed in females.

Materials and Methods

Animals

Sprague-Dawley rats 65-75 days of age were obtained from Charles River Laboratories ($n = 10$ males, 26 females). More specifically, Cohort 1: Sex difference Study: 10 males, 10 females; Cohort 2 : DREADDs inhibition in female rats: 8 veh females, 8 CNO females. (Wilmington, MA, USA). They were singly housed under a 12-h light/dark cycle (lights on at 0700h and off at 1900h), had food and water available *ad libitum*, and were acclimated to the housing and lighting

conditions for at least five days prior to any experimental procedures. The Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia Research Institute approved all experimental procedures.

Instrumentation Surgery, Telemetric Recordings and Restraint Stress Procedures

Surgical procedures were performed under sterile conditions, and Isoflurane (2-3%) was used to induce and maintain anesthesia throughout surgery. A midline incision was performed into the abdominal cavity to suture a telemetric transmitter (Physiotel HD-S02; Data Science International, St. Paul, MN, USA) to the peritoneal wall. It was equipped with sensors that measured animals' core body temperature and motor activity, and had two biopotential amplifiers.

Two screws were implanted into the skull (1 mm to the right and to the left of lambda) and connected to an amplifier to measure cortical electroencephalogram (EEG) activity. Postural muscle electromyogram (EMG) activity was recorded via two insulated stainless steel wire electrodes attached bilaterally to dorsal neck muscles. After instrumentation, the scalp and the opening into the intraperitoneal cavity were sutured and the analgesic meloxicam was administered (2 mg/kg, *sc*). Animals were given one week to recover from surgery.

24-hour continuous EEG and EMG recordings were initiated by remote activation of a magnetic switch in the transmitter. In each animal, the first two days of the protocol were used to collect baseline activity. Then, each animal was exposed to 5 consecutive days of 30-min restraint, and recording was continued for 2 days after the last restraint day (**Fig. 1A**). Restraint stress was applied within 2 hours after lights-on using plexiglas restraint tubes, as described previously (Grafe et al., 2017a).

6 animals run in the study had unreadable telemeter recordings, and thus, were not included in analysis. Thus, final n are as follows: Cohort 1: Sex Difference Study: 8 males, 7 females; Cohort 2: DREADDs inhibition in female rats: 7 veh females, 8 CNO females.

Sleep Scoring and Analysis

Sleep-wake states were scored in 10-sec epochs using Neuroscore software (Data Science International, St. Paul, MN, USA). Wake and sleep percentage data were calculated using the automated Rodent Sleep Scoring Module 2 in Neuroscore. Using a probability matrix, Neuroscore determines the most likely sleep or wake states: wake (denoted as W on traces), slow wave sleep (denotes as S on traces) and REM sleep (denoted as P on traces for paradoxical sleep); see **Fig. 1B** for examples.

The EEG data were analyzed using a fast Fourier transform (FFT), which efficiently calculates either the discrete Fourier transform (DFT) of a signal or its inverse (IDFT), a representation of the signal in the frequency domain. In EEG, the two bands of interest examined are Delta power (frequencies between 0.05 and 4 Hz) and Theta power (frequencies between 4 and 8 Hz).

The EMG signal was analyzed by amplitude in relation to baseline. Motor activity data are derived using an algorithm that compares the signal strength generated by the telemeter implant with that received by the receiver antenna. While this signal strength naturally fluctuates, the algorithm generates activity counts only when it changes by a certain amount, indicating that the subject is active. For more information, see <https://support.datasci.com/hc/en-us/articles/115005030328-Understanding-the-Ponemah-Activity-Derived-Parameter>.

Depending on EMG value and presence of activity (indicated by movement of the transmitter), the epoch will likely be scored as wake. If EMG and activity recordings are low, then EEG measurements are examined using Delta Ratio (Delta power divided by total power from 0.5 to 25 Hz) and the Theta Ratio (Theta power divided by Delta power). The 10-sec epoch is likely scored as slow-wave sleep when there is high Delta power and REM sleep when there is high Theta power. This algorithm is very reliable as long as the signals are relatively clean. Additionally, two research assistants blind to group conditions visually cross-checked the accuracy of the recognition of sleep-wake stages.

Percentage of time in wake and sleep states (wake, slow-wave sleep, and REM sleep), occurrences of total awakenings, exaggerated motor activation at awakening, and REM sleep continuity were analyzed. These data were averaged across the 2 baseline days, then calculated for 5 days of restraint, and finally averaged over 2 recovery days. Percentage of time in sleep and wake states were split into light and dark periods. In the light period (**Figure 2 A-C**), data were analyzed from 10 am to 7 pm on the days of restraint (in order to capture the light period after the end of restraint stress until lights-off). The same time frame (10 am - 7 pm) was analyzed for the baseline and recovery time points. For the dark period (**Figure 2 D-F**), data were analyzed from 7 pm to 6 am (starting at lights-off) at the same time periods.

Exaggerated motor responses to waking were analyzed over a 6-hr period (10 am- 4 pm) and averaged on baseline days, day 1 and 5 of restraint, and recovery days. They were identified by visual examination of the nuchal EMG and locomotor activity over a 10-sec period following the termination of each sleep bout. Awakenings were categorized as exaggerated when the burst of nuchal EMG crossed a pre-set threshold (200 μ V) and there was concurrent locomotor activity within the first 10 sec after awakening. The EMG threshold used in this analysis was validated in a separate group of rats in which a systematic search was conducted for a threshold value that effectively discriminated between awakenings with and without immediate transition into a motor response as previously described (Grafe et al., 2020). **Figures 3A and B** show examples of a typical awakening and an awakening associated with exaggerated motor activation, respectively.

Fragmentation of REMS was assessed by visually scoring the relative times of occurrence of individual REM sleep episodes. According to the classification introduced by (Amici et al., 1994a), two types of REMS episodes were distinguished: those that were followed by another REMS episode after an interval longer than 3 min (“single” episodes, sin-REMS), and those followed by another REMS episode after an interval of 3 min or less (“sequential” episodes, seq-REMS). The total numbers of episodes of each type were counted over 6 hrs of recording (10 am – 4 pm) at baseline, day 1 and 5 of restraint, and over 2 recovery days. Seq-REMS episodes are, on average, of shorter duration than sin-REMS episodes and tend to occur in clusters (Amici et al., 1994a). Importantly, a shift toward more seq-REMS was previously shown to be indicative of increased fragmentation of REMS (Amici et al., 1994a; DaSilva et al., 2011).

DREADDs Inhibition of Orexin Neurons in female rats

To elicit inhibition of orexin neurons for up to 4 hrs (Alexander et al., 2009; Farrell and Roth, 2013), an inhibitory DREADDs construct with an orexin promoter expressed in a recombinant adenovirus (rAAV2/1-Ple112-hM4Di-mCitrine) was used as previously described (Grafe et al., 2017a, 2017b, 2018) in a separate cohort of female rats (Cohort 2; See **Figure 5A** for timeline of experimental paradigm). In short, an hM4D DREADDs virus (10^9 titer, 1 μ l

bilaterally) was stereotactically injected into the lateral hypothalamus (2.5 mm caudal to bregma, 1.8 mm from mid-line and 8 mm ventral) of female rats anesthetized using a cocktail of ketamine, xylazine, and acepromazine. The viral injection surgery occurred 3 weeks prior to telemetric instrumentation surgery.

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 as previously described (Grafe et al., 2017a). Previous studies have shown that CNO treatment in non DREADDs-expressing rats do not show off-target behavioral effects (Eacret et al., 2019). Sleep scoring and analysis were conducted as described in the previous section. After behavioral experiments concluded, expression of the DREADDs construct was verified by immunofluorescence as previously described (Grafe et al., 2017a, 2017b, 2018) (see **Figure 5A and B**).

Statistical Analysis

Data are presented as the mean \pm standard error of the mean (SE). Two-way repeated measures ANOVAs were run to examine sleep-wake percentages (Sex x Stress), exaggerated motor responses (Sex x Stress), REM fragmentation (Sex x Stress), and sleep-wake percentages after DREADDs manipulation (CNO x Stress). Graphs were generated using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Multiple comparisons analyses were run to further investigate the interactions between sex and stress. In all analyses, $\alpha = 0.05$ was regarded statistically significant.

Results

Percentages of time in wake and different sleep stages during the light period were calculated before, during, and after repeated restraint stress in male and female rats (**Fig. 2 A-C**). A two-way ANOVA revealed a significant main effect of Stress and an interaction between Stress and Sex for percent time in wake (wake time/total recording period time; **Fig. 2A**; Stress, $F(3,22) = 10.3, p < 0.001$; Stress x Sex $F(3,30) = 3.7, p < 0.05$). Post hoc tests revealed that both day 1 and day 5 of restraint stress increased percent wake in both sexes, and this persisted for females (but not males) during the recovery period. We further examined locomotor activity on days 1-5 of restraint, and found that anticipatory activity increased 30 minutes prior to each restraint in both sexes compared to baseline and recovery measures. Though females had higher anticipatory activity to restraint than males overall, it was most apparent on Days 2 and 4 of restraint (data not shown). For SWS, a two-way ANOVA revealed a main effect of Stress (**Fig. 2B**; Stress, $F(3, 25) = 5.2, p < 0.05$). Specifically, post hoc tests indicated decreased SWS in both sexes on day 5 of restraint. There were no significant main effects of stress or sex on percent time in REMS during the light period. We next compared the latency to the first REMS episode in males and females after 30-minute restraint on Day 1 versus Day 5. However, there was no effect of restraint day or sex on latency to REMS (data not shown). In sum, repeated restraint stress increases wake and decreases SWS in both sexes, but these changes persist during the recovery period in females (but not males). That is, repeated restraint stress causes persistent disruptions in sleep in female rats during the light period (when sleep should be prominent) compared with male rats.

Percentages of time in wake and different sleep stages during the dark period were also calculated before, during, and after repeated restraint stress in males and females (**Fig. 2 D-F**).

Both percent wake and percent SWS showed main effects of Sex in two-way ANOVAs (Wake Sex effect: $F(1,11) = 5.8, p < 0.05$; SWS Sex effect: $F(1,11) = 5.7, p < 0.05$). Specifically, during the dark period, females demonstrated more wake and less SWS than males after restraint. There was an effect of stress on percent REM during the dark period ($F(3,28) = p < 0.05$). Post hoc tests revealed that Day 5 of restraint resulted in significantly less percent REM than Day 1 of restraint in both sexes.

In order to analyze sleep to wake transitions, we calculated the number of exaggerated motor responses to waking, as defined in the Methods. There were no significant main effects of Stress or Sex on the total number of exaggerated motor responses to waking (**Fig. 3C**). However, when awakenings were further categorized into those terminating SWS or REMS, a two-way ANOVA revealed a main effect of Stress and Sex on exaggerated motor responses to waking from REM sleep (**Fig. 3D**, Stress, $F(3,22) = 3.7, p < 0.05$; Sex, $F(1,10) = 5.6, p < 0.05$). Post hoc tests revealed that females had significantly more exaggerated responses to waking from REMS after restraint compared with males. However, Stress and Sex did not affect the number of exaggerated motor responses to waking from SWS (**Fig. 3E**).

As restraint stress induced sex-specific effects on arousal from REMS, we also investigated REMS continuity by examining REMS microarchitecture (**Figure 4**). The number of single REMS (sin-REMS) episodes did not significantly differ between males and females at any time point. Similarly, there were no significant main effects of stress or sex on sequential REMS (seq-REMS) episodes. Thus, 5 days of restraint stress does not significantly impair REM sleep continuity in male and female rats.

Previous studies have demonstrated that the neuropeptides orexins are important in modulating both stress and sleep (Winsky-Sommerer et al., 2003; Johnson et al., 2012). Based on our earlier study (Grafe et al., 2017a), we hypothesized that orexins contribute to the stress-induced sleep deficits observed in females. To test this hypothesis, orexin system activity was reduced in females using an inhibitory DREADDs construct (for experimental paradigm and representative images of viral expression, see **Figure 5**). Wake and sleep percentages were analyzed in the light and dark period after orexin neural inhibition by Clozapine-N-Oxide (CNO) administration prior to each restraint (**Fig. 6**). There were no significant effects of CNO on percentage of wake, SWS, or REMS in either the light or dark period. Additionally, orexin neural inhibition did not significantly affect sleep to wake transitions. Specifically, CNO administration prior to each restraint did not change the number of exaggerated motor responses to waking in females (data not shown). Thus, inhibiting orexin action during repeated restraint did not significantly change sleep in female rats either before or after stress.

Discussion

Repeated stress increases the risk of developing psychiatric illnesses including MDD and PTSD. Sleep disturbances are common phenotypes in these disorders, often presenting as hyperarousal in the form of insomnia or recurrent nightmares (Ross et al., 1989). Interestingly, these stress-related disorders disproportionately affect females. Although previous research has examined the effect of stress on sleep in women, there are limited studies comparing the effects of stress on sleep amounts and architecture in males and females in either humans or preclinical models (Mallampalli and Carter, 2014).

To fill this gap in knowledge, our study examined sex differences in the effects of repeated stress on sleep in adult male and female rats. Specifically, we measured sleep quantity and continuity before, during, and after repeated restraint stress. Our data revealed that repeated

restraint stress increased the percent of time spent awake, and decreased percentages of SWS and REMS to a greater degree in females compared to males, and these disruptions were still apparent during recovery days in females. Female rats also demonstrated more exaggerated motor responses to waking from REMS compared with male rats. However, restraint stress did not significantly impair REMS continuity in either sex (assessed by single and sequential REM episodes). All together, these data indicate that repeated restraint stress leads to persistent disruptions in sleep in female rats.

The neuropeptides orexins are important in regulating both stress and sleep (Winsky-Sommerer et al., 2003; Johnson et al., 2012; Xu et al., 2013), and have been shown to contribute to sex differences in the stress response (Grafe et al., 2017a). Therefore, we tested whether orexins might contribute to these stress-induced sleep deficits observed in females. However, inhibition of orexin neurons with DREADDs prior to each restraint did not cause significant changes in sleep in female rats. These data suggest that orexins released in temporal proximity to the restraint exposure are not important in producing the sleep deficits in females. However, these results do not rule out the possibility that actions of orexins over the remainder of the day are involved in producing the sleep deficits in females.

Stress effects on percent time spent in wake, SWS, and REMS

We found that repeated restraint stress increased the percentage of wake and decreased the percentage of SWS in both sexes during the light period, when sleep should be prominent. Sex differences became even more apparent in the dark period, where females spent more time awake and less time in SWS in the two days following repeated restraint stress. Thus, females were unable to compensate for decreased SWS at any point throughout the day. These data partially support previous clinical findings of lower SWS in women relative to men (Spoormaker and Montgomery, 2008; Mong and Cusmano, 2016). Moreover, in clinical populations, lower amounts of slow-wave sleep indicates lighter sleep, which increases the likelihood to be awakened (Fuller et al., 1994).

While repeated restraint stress did not significantly reduce percentage of time spent in REMS in the light period, there was a significant effect of stress on percent REMS in the dark period. Specifically, when comparing day 5 of restraint with day 1 of restraint, there was a decrease in percent time in REMS. Data concerning REMS percentages in stress-related disorders such as PTSD are equivocal, with some showing a higher percentage of REMS in PTSD, while others show no changes between clinical and non-clinical populations (Pace-Schott et al., 2015). The time after trauma where sleep is examined could be a critical factor in the effects observed (Mellman et al., 2014). At this point, the literature provides more evidence that stress may influence the continuity of REMS, rather than the quantity of this type of sleep (Pace-Schott et al., 2015).

Taken together, the data indicate that repeated restraint stress decreases percentages of SWS and REMS, with sex differences becoming more apparent by day 5 of restraint and persisting during the recovery period. This is congruent with a previous finding showing that women tend to take longer to recover from sleep deprivation (Corsi-Cabrera et al., 2003). Ultimately, our data demonstrate pervasive insomnia-like symptoms in rats following exposure to repeated stress, with significant increases in wake and decreases in REMS and SWS, especially in females. Increases in wake are clearly related to the maladaptive state of hyperarousal, a key marker of PTSD. Hyperarousal, by causing hypervigilance and restlessness, contributes to

perpetuating sleep impairments over time (Mellman et al., 2002).

Stress effects on sleep-wake transitions

Recurring nightmares, defined by the DSM-5 as long frightening dreams that causes one to awaken, are a hallmark of PTSD (Campbell and Germain, 2016). Treatment focused on alleviating nightmare frequency and intensity significantly improve patients' quality of life (Spoormaker and Montgomery, 2008). Moreover, previous studies have indicated a strong relationship between the experience of distressing dreams following a trauma and the development of PTSD (Zadra and Germain, 2009). Trauma-related nightmares are associated with exaggerated motor activation and autonomic arousal (Campbell and Germain, 2016). In our study and others, exaggerated motor responses to waking from sleep are used to represent stress-related nightmares (Yu et al., 2015; Grafe et al., 2020).

Our analyses of sleep to wake transitions during the light period showed a main effect of sex on the number of exaggerated motor responses to wake from REM sleep in particular. Specifically, female rats demonstrated significantly more exaggerated motor responses to wake from REMS than male rats during recovery from 5 days of repeated restraint stress. However, neither time nor sex had an effect on exaggerated motor responses from SWS. As nightmares are primarily a phenomenon of REMS (Germain, 2013), our study may model these sleep disturbances that appear soon after traumatic stress in humans. Importantly, previous studies have indicated that women report experiencing more nightmares than men (Habukawa et al., 2018). Because nightmares reinforce stressful memories, they may contribute to the increased rates of PTSD in women compared to men (Kobayashi and Mellman, 2012; Germain, 2013).

Stress effects on REMS continuity

As exaggerated motor responses to waking appeared to be more prevalent during REMS, we next examined REMS microarchitecture. Fragmented REMS episodes are predominant in PTSD patients and thus represent a neural marker of the deleterious consequences of psychological trauma on sleep quality (Yehuda et al., 2015; Habukawa et al., 2018). In our study, REMS continuity was measured by classifying REM episodes as single or sequential as described in previous studies (Amici et al., 1994b; Grafe et al., 2020). Ultimately, more frequent sequential REMS episodes indicate increased REMS fragmentation (DaSilva et al., 2011). Our data revealed that repeated restraint stress did not significantly alter the number of single or sequential REMS episodes in male or female rats. In short, 5 days of repeated stress did not produce REM fragmentation. Thus, although females spent more time awake and showed more frequent exaggerated motor activations to waking from REMS, they did not significantly differ from males in terms of REM fragmentation. However, it is possible that other measures of REMS quality not assessed, such as REM density, may show sex differences.

Role of orexins in stress-induced sleep impairments

While the neural substrates underlying stress-induced hyperarousal in females are unknown, the neuropeptides orexins are one likely candidate. First, orexins have a well-established role in promoting arousal and wakefulness (Winsky-Sommerer et al., 2003; Sakurai, 2007). Moreover, previous studies have indicated that orexins reciprocally interact with the HPA

system (Winsky-Sommerer et al., 2005) and are important mediators of sex differences in the response to repeated stress (Grafe et al., 2017a). Importantly, female rats demonstrate heightened orexin activity and expression, which is associated with exacerbated behavioral and neuroendocrine responses to stress. Thus, it seemed plausible that orexins might explain increased wake after stress in females.

However, when we inhibited orexin neural activity with DREADDs prior to each restraint stress, we did not observe significant changes in sleep. Previous studies have shown that CNO promotes behavioral effects in the rat within 30 minutes and effects last up to 4 hours (Alexander et al., 2009; Farrell and Roth, 2013). Thus, in our study in which DREADDs were activated 60 min prior to onset of the five daily restraint episodes, orexin release would have been inhibited for the period of time around the stress exposure. The lack of effect of orexin inhibition suggests that the orexins released proximal to restraint exposure are not important in the sleep deficits that occur throughout the day. One explanation for these lack of effects is that orexins acting at other times of the day are involved in producing the sleep deficits. Alternatively, other neural substrates known to promote arousal, including norepinephrine, histamine, and acetylcholine (Mignot et al., 2002; Cano et al., 2008; Zeitzer, 2013), may have compensated in response to reduced orexin action.

Study limitations and future directions

Our study reveals the presence of observed sex differences in insomnia-like and nightmare-like sleep parameters after repeated restraint stress. Indeed, female rats exhibited increased percentages of wakefulness as well decreased percentages of time spent in REM and SWS. Females further experienced a greater number of exaggerated motor responses to wake from REM. Though orexins are known to contribute to heightened stress in female rats, inhibition of orexins proximal to the time of stress did not ameliorate stress-induced sleep deficits. While we verified sufficient expression of our orexin DREADDs virus, we did not colabel with a neural marker, thus, it is possible that glial inhibition could contribute to the observed lack of effects. Moreover, we did not verify successful inhibition of orexin neurons. Thus, future studies should incorporate electrophysiological measures or an easily observable behavioral change upon CNO inhibition of orexin neurons to verify its efficacy.

As our rats are singly housed, it is important to consider the effects of single housing on each sex, and how this could have influenced our results. Previous research has indicated that corticosterone levels are elevated in adult females when singly-housed compared to when group-housed; in contrast, males may have lower corticosterone levels when singly-housed compared to when group-housed (Brown and Grunberg, 1995). Thus, isolation housing may influence susceptibility to restraint stress (Goel et al., 2014). However, housing status does not appear to exacerbate or attenuate sex differences in sleep physiology directly (Ehlers et al., 1993). Future research could investigate the potential interaction between housing status and sex differences in stress-related sleep deficits.

Future studies could also examine how other neural substrates of arousal (besides orexins) contribute to these sex-specific stress-induced sleep effects. Moreover, gonadal hormones should be considered as a factor affecting stress-induced sleep changes. Indeed, gonadal hormones are known to affect the stress response and have been reported to affect different measures of sleep (Corsi-Cabrera et al., 2000; Paul et al., 2009). Ultimately, it is crucial to explore how complex neurotransmitter and hormonal systems interact in the development of sex-specific symptoms in

stress-related illnesses.

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Figure Legends

Figure 1. Experimental paradigm and Representative Sleep Traces

A: Experimental Paradigm depicting the timeline for implantation of telemetry devices, continuous wireless EEG/EMG recordings, and 5 days of repeated restraint stress. The light period (analyzed from 10 am to 7 pm) is represented by open white boxes, while the dark period (analyzed from 7 pm to 6 am) is represented by black boxes. 30-minute restraint took place during the light period. **B:** Representative EEG, EMG, and activity traces of Wake (denoted as W), slow wave sleep (denoted as S) and REM sleep (denoted as P for paradoxical sleep).

Figure 2. Percent Wake, SWS, and REM in the light (A-C) and dark (D-F) periods before, during, and after repeated restraint

A: Percent time spent awake during the light period in male and female rats at baseline, restraint, and recovery. Repeated restraint stress increases wake in both sexes, but these changes persist during the recovery period in females (but not males). **B:** Percent time spent in slow wave sleep (SWS) during the light period in male and female rats at baseline, restraint, and recovery. 5 days of repeated restraint stress decreases SWS in both sexes. **C:** Percent time spent in REMS during the light period in male and female rats at baseline, restraint, and recovery. Repeated restraint stress does not significantly affect percent time in REMS. **D:** Percent time spent awake during the dark period in male and female rats at baseline, restraint, and recovery. Females demonstrated more wake than males after restraint. **E:** Percent time spent in slow wave sleep (SWS) during the light period in male and female rats at baseline, restraint, and recovery. Females exhibited less SWS than males on post-restraint stress days. **F:** Percent time spent in REMS during the light period in male and female rats at baseline, restraint, and recovery. 5 days of restraint stress decreases percent REM compared to 1 day of restraint stress in both sexes. $*p < 0.05$

Figure 3. Number of exaggerated motor responses during transitions to waking from SWS or REMS in the light period before, during, and after repeated restraint

A: Representative typical awakening: a trace of EEG, EMG, and locomotor activity data during arousal from a REMS episode (initiated at arrow). Note that EMG spikes do not go above the 200 μ V threshold in red within 10 sec of the sleep transition. **B:** Representative exaggerated motor response at awakening: a trace of EEG, EMG, and locomotor activity data during arousal from a REMS episode (initiated at arrow). Note the recurrent EMG spikes greater than 200 μ V (denoted with black arrows) that occur within 10 sec of the sleep transition with concurrent locomotor activity. **C:** Total counts of exaggerated motor responses to waking during 6 h of the light period at baseline, over 5 days of restraint, and during the recovery period. There were no significant main effects of Stress or Sex on the total number of exaggerated motor responses to waking. **D and E:** Exaggerated motor responses counted separately following arousals from REMS and SWS, respectively. Females had significantly more exaggerated responses to waking from REMS after restraint compared with males. However, stress and sex did not affect the number of exaggerated motor responses to waking from SWS. $*p < 0.05$

Figure 4. Continuity of REMS (single versus sequential episodes of REMS) in the light period before, during, and after repeated restraint

Repeated restraint stress did not significantly alter the number of single or sequential REMS episodes in male or female rats.

Figure 5. Experimental paradigm and representative viral expression (Experiment: inhibiting orexin neurons throughout repeated restraint in female rats via DREADDs)

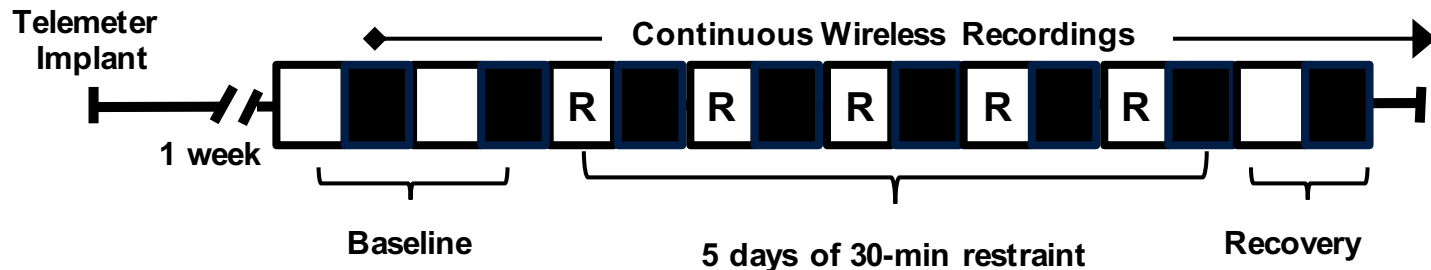
A: Experimental Paradigm depicting the timeline for DREADDs viral injection, implantation of telemetry devices, continuous wireless EEG/EMG recordings, and 5 days of repeated restraint stress in female rats. The light period (analyzed from 10 am to 7 pm) is represented by open white boxes, while the dark period (analyzed from 7 pm to 6 am) is represented by black boxes. 30-minute restraint took place during the light period. **B:** Representative images displaying viral expression of DREADDs in the LH of female rats at 4 weeks. **C:** A composite image displaying the spread of viral expression along the LH in female rats is depicted using rat brain atlas images (Paxinos and Watson, 1998). Each red dot represents a cell expressing the viral tag.

Figure 6. Inhibiting orexin neurons throughout repeated restraint does not change percent wake, SWS, and REMS in the light or dark period in female rats

A-C: Percent time spent in wake, slow wave sleep (SWS) and REM during the light period in female rats at baseline, restraint, and recovery. Clozapine-N-oxide (CNO)-treated females (orexins inhibited before each restraint) do not show significant changes in wake or sleep compared to vehicle-treated female rats. **D-F:** Percent time spent in wake, slow wave sleep (SWS) and REM during the dark period in female rats at baseline, restraint, and recovery. Clozapine-N-oxide (CNO)-treated females (orexins inhibited before each restraint) do not show significant changes in wake or sleep compared to vehicle-treated female rats.

Figure 1: Experimental Paradigm and Representative Sleep Traces

A



B

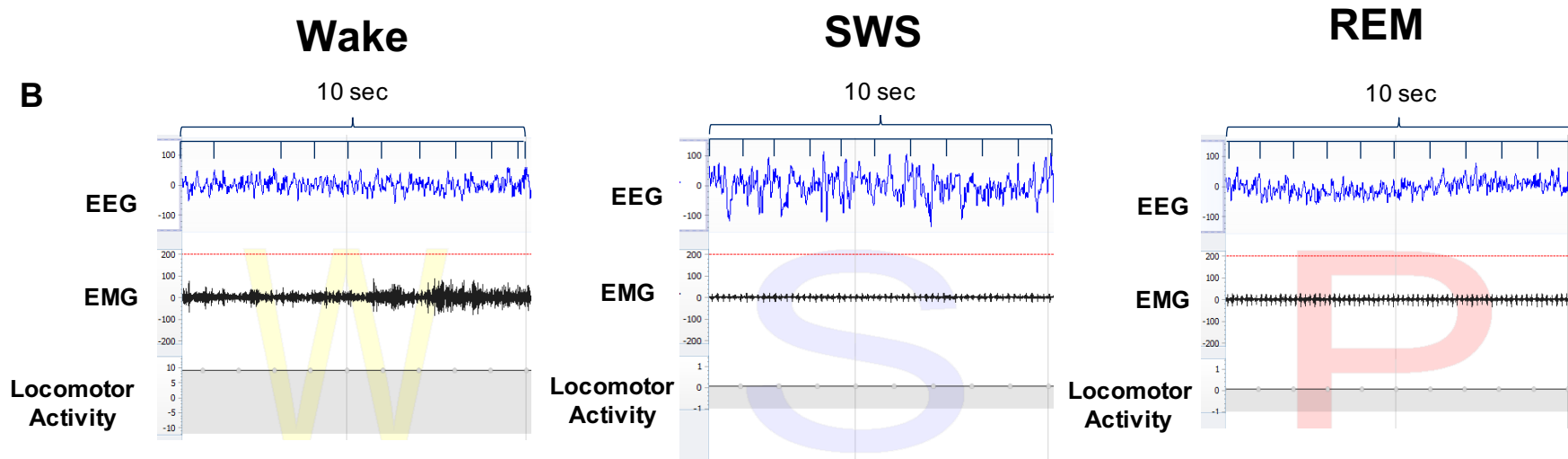


Figure 2. Percent Wake, SWS, and REM in the light (A-C) and dark (D-F) periods before, during, and after repeated restraint

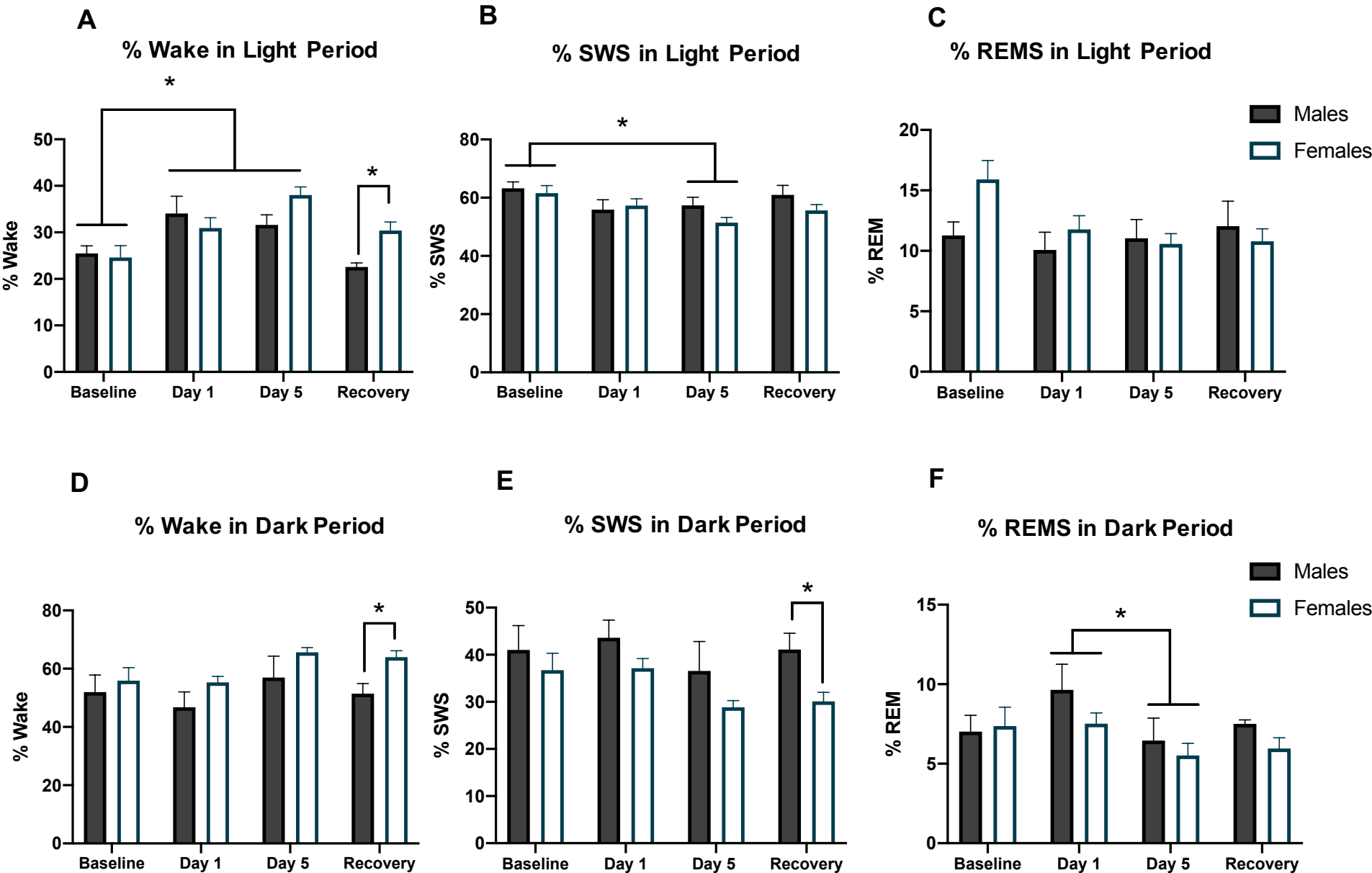


Figure 3. Number of exaggerated motor responses during transitions to waking from SWS or REMS in the light period before, during, and after repeated restraint

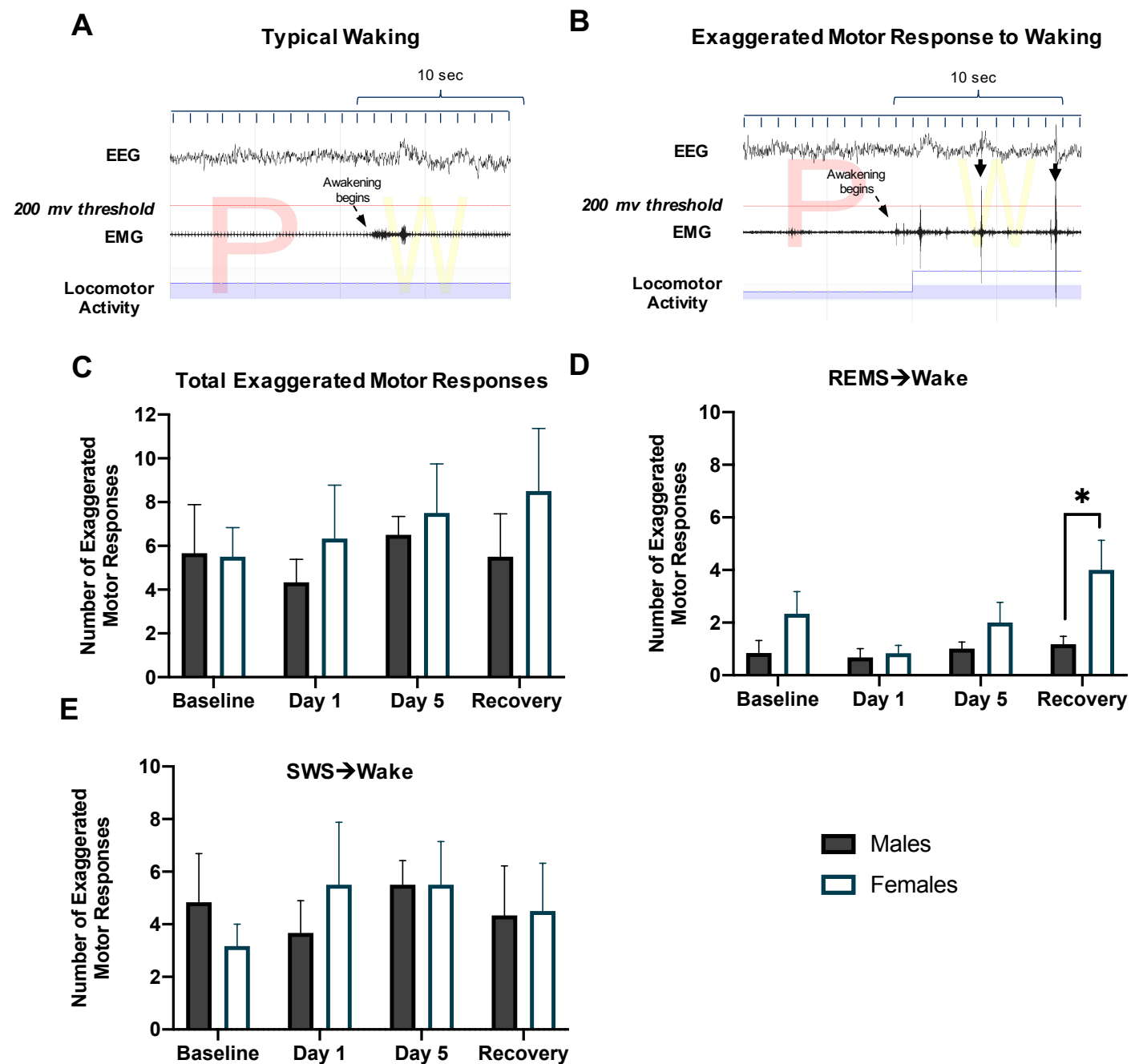
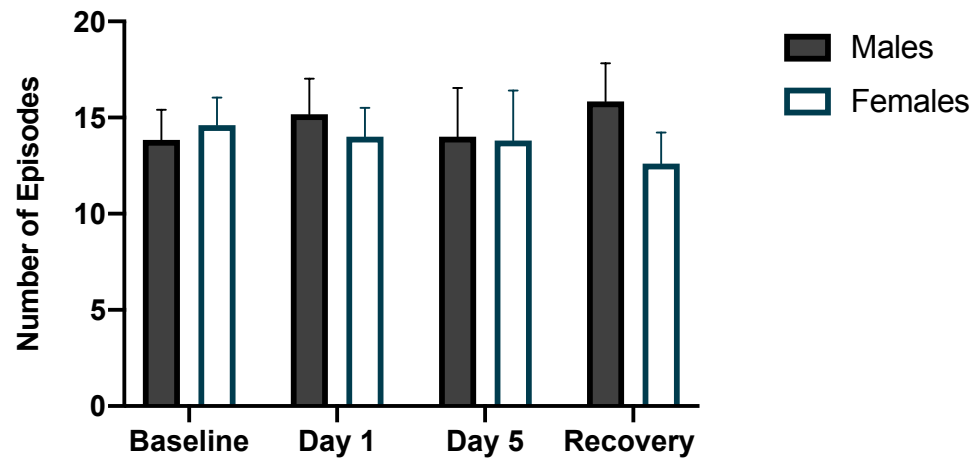


Figure 4. Continuity of REMS (single versus sequential episodes of REMS) in the light period before, during, and after repeated restraint

A

Single REMS Episodes



B

Sequential REMS Episodes

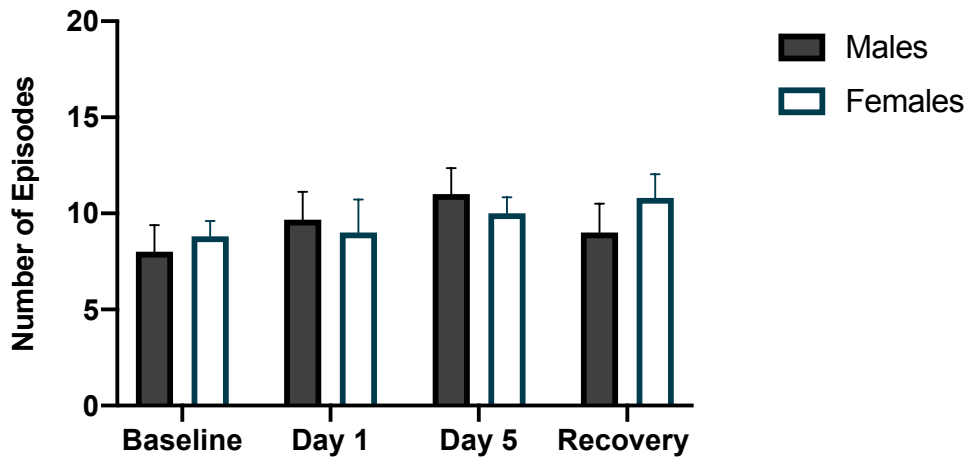


Figure 5: Experimental Paradigm and Representative Viral Expression (Experiment: Inhibiting orexin neurons throughout repeated restraint in female rats via DREADDs)

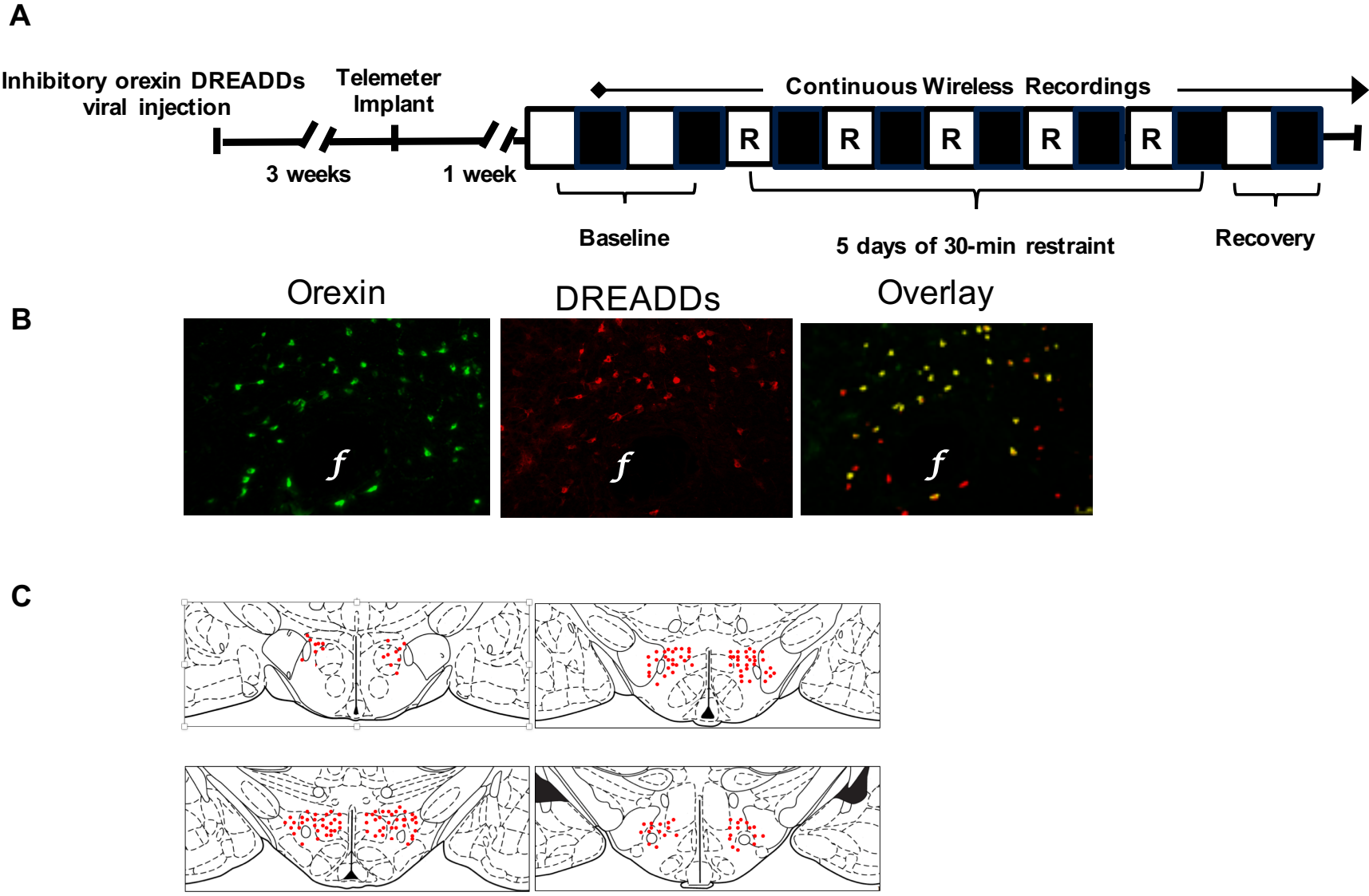
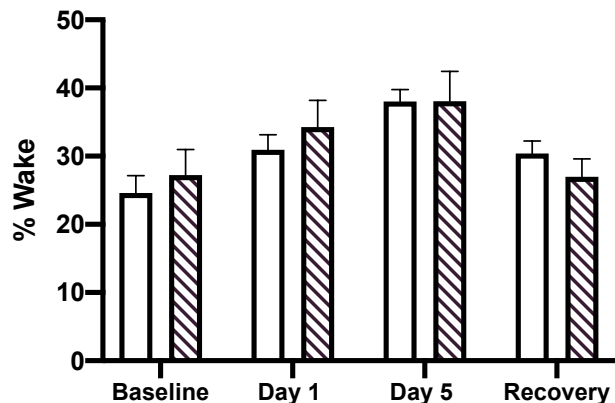


Figure 6: Inhibiting orexin neurons throughout repeated restraint does not change percent wake, SWS, and REMS in the light or dark period in female rats

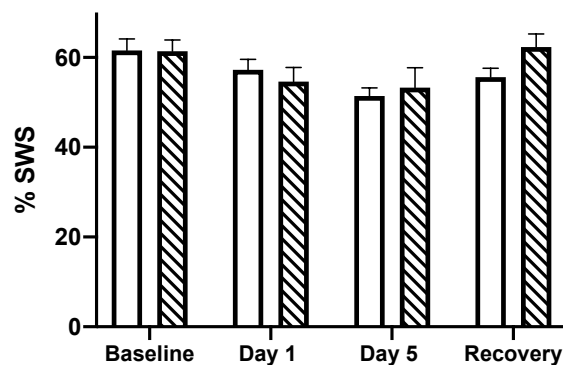
A

% Wake in Light Period



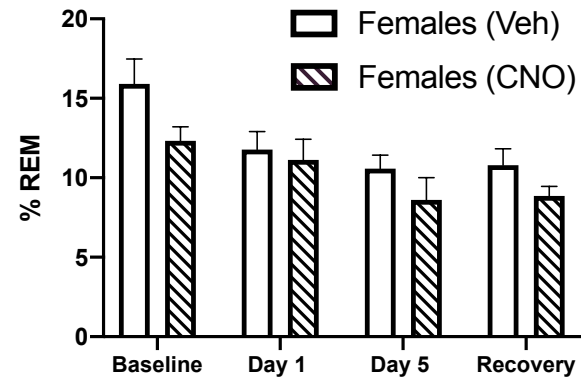
B

% SWS in Light Period



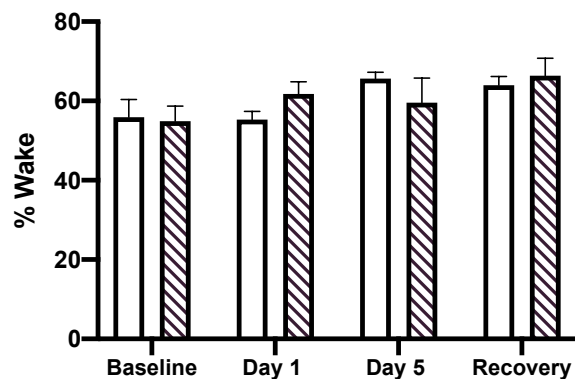
C

% REMS in Light Period



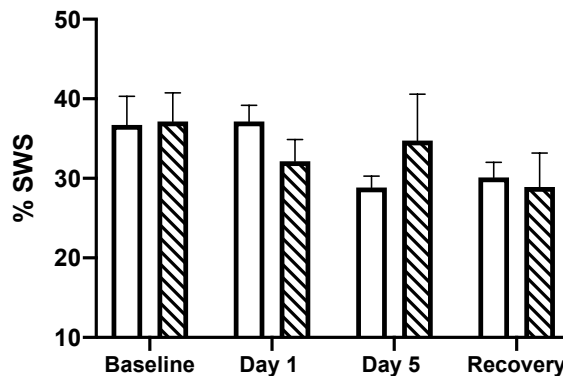
D

% Wake in Dark Period



E

% SWS in Dark Period



F

% REMS in Dark Period

