

ROLE OF SEX AND OREXIN IN SLEEP DISRUPTION INDUCED WEIGHT GAIN

by

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ABSTRACT

Poor sleep time and quality associate with obesity in adults and women have a predisposition towards greater weight gain with sleep restriction. However, the mechanisms contributing to sex-specific sensitivity to weight gain following sleep loss remains unknown. Exposure to environmental noise is the only method of sleep disruption (SD) known to date that reduces sleep time and quality, stimulates weight gain and feeding and reduces distance traveled and energy expenditure (EE) (total and individual components) in male rats. The objective of this study was 1) validate the method of noise-induced sleep disruption in female rats, 2) determine mechanisms underlying the response to noise in male and female rats and 3) determine whether treatment with a sleep aid could block the effects of noise exposure independent of sex. Overall, findings from the study described in this dissertation demonstrate the following: 1) chronic exposure to noise increases weight gain and feeding and reduces total EE due to reductions in EE during spontaneous physical activity (SPA) and sleep without altering the length of the estrous cycle in female rats; 2) stress is not the primary mechanism underlying noise-induced weight gain in either male or female rats; and 3) Suvorexant, a Federal Drug Administration (FDA) approved dual orexin receptor antagonist for insomnia, significantly ameliorates noise-induced increases in time awake independent of sex but further exacerbates noise-induced increases in sleep fragmentation for males only. Collectively, these data have implications for future studies aimed to determine sex-specific sensitivity to weight gain following sleep loss. Furthermore, these data suggest that while Suvorexant may ameliorate reductions in sleep caused by noise equally in the sexes, the drug may have differential effects on the weight gain due to SD since Suvorexant further worsened sleep quality in males but not females.

Introduction

While the rates of obesity have nearly doubled in >70 countries since 1980 [1], the amount of adequate sleep (i.e. 7 hour of continuous sleep per night) achieved by adults has declined [2] and the incidence of physician-diagnosed sleep disorders has risen [3]. For example, 34.8% of respondents from the 2014 US Behavioral Risk Factor Surveillance System self-reported sleeping <7h per night in a 24-h period [4] and sleep-related visits to a physician increased by 29% between 1999 and 2010 [3]. The negative effect of inadequate sleep on overall health and well-being has been confirmed by a recent systematic review and meta-analysis which reported that inadequate sleep was associated with a greater risk for obesity, waist circumference, mortality and chronic diseases including diabetes mellitus, hypertension, cardiovascular and coronary heart disease [5]. While obesity and poor sleep are pervasive in society, population-based studies consistently report that prevalence of obesity is greater in women compared to men both globally [6] and in the US [7]. Moreover, recent studies report poorer sleep quality among women [8, 9] and a negative association between sleep duration and quality with waist circumference and body mass index (BMI) in women but not men [10] although one study found no sex differences [3]. Also, the biological transition to menopause in women is associated with increased prevalence of insomnia and weight gain [11]. Together, these data illustrate the influential role of sex on the relationship between disrupted sleep and risk of obesity. Thus, in this introduction, data are discussed addressing whether differences in sleep patterns and responses to sleep disruption influence sex disparities for weight gain.

Do sex differences modulate the relationship between sleep and obesity?

Clinical studies that reduce sleep: Clinical studies report that sleep restriction (SR) increases weight gain and calorie intake alone or in parallel with either increased or reduced EE in adults [12-20]. Yet, it is unclear whether men and women respond differently to SR since the duration of sleep restriction is dissimilar across studies, some studies do not report weight gain or do not include both men and women in their design. For example, two studies determined the effect of acute sleep loss on EE but not weight gain in either men or women. While one night of total sleep deprivation significantly reduced resting metabolic rate in men [20], two nights of SR (4h time in bed; TIB) significantly increased total EE in women compared to habitual sleep (8h TIB) [16]. Another study examined calorie intake, total EE with doubly-labeled water and resting metabolic rate but not weight gain in normal-weight men and women in a cross-over design after six nights of SR (4h TIB) and habitual sleep (8-h TIB) [13]. Despite that both sleep-restricted women and men non-significantly increased their calorie intake relative to the habitual sleep; only sleep-restricted women significantly increased intake of total and saturated fat. This result suggests that SR may differentially affect macronutrient selection but not total calorie intake between sexes.

A sex difference in weight gain after SR has been directly examined in studies, but with contradictory results. For example, in response to five consecutive nights of SR (5h TIB) compared with adequate sleep (9h TIB) [12], SR increased weight gain only in women despite that total EE and daily calorie intake (including foods high in carbohydrates and intake during late night hours) were increased in both men and women. However, others

report that men gain more weight compared to women in response to SR [12, 18] and the sex difference remained statistically significant after the data were analyzed by change in BMI or percent change in absolute weight gain from baseline (i.e. habitual or adequate sleep). This contrasts with a study reporting similar weight gain between sleep-restricted men and women despite significantly higher calorie intake in sleep-restricted men compared to sleep-restricted women [17]. In this study, when men and women were combined, sleep-restricted adults did not gain significantly more weight than controls after controlling for age, BMI, gender and race despite consuming significantly more calories [17].

Clinical studies that improve sleep: Given that reducing sleep time increases weight gain, extending time spent asleep might be expected to reduce weight gain. Yet, whether extending sleep reduces weight gain trajectories or if there is a sex difference remains unresolved. In a home-based intervention study, Tasali and colleagues examined the effects of sleep extension on sleep duration, appetite and desire for food with a cross-over design in ten overweight young adults with habitual sleep patterns of <6.5 hours per night [21]. Participants completed one week of baseline measurements, followed by a 2-week intervention period designed to achieve 8.5h of sleep per night. Sleep extension significantly increased sleep duration by 1.6h per night and physical activity by 7% as indicated by wrist actigraphy and reduced overall ratings for appetite, sweet and salty foods. A larger sleep extension study aimed to test whether extending sleep duration (i.e. up 7.5h per night) would improve weight in 125 obese adults [22] that completed three consecutive visits (e.g. screening, randomization and follow-up). Sleep, indicated by

sleep diaries and wrist actigraphy, significantly increased between the screening and randomization visits among adults who were later randomized to the intervention group (sleep extension) relative to those who were randomized to the comparison group despite that weight gain was similar between groups. Thus, it is currently unclear if sleep extension would indeed have had beneficial effects on weight gain as the placebo and Hawthorne effects were present in this study since differences in sleep were observed between screening and randomization [22].

Pre-clinical studies that reduce sleep: The discrepancy between population-based and clinical studies underscores the significance of developing animal models to investigate mechanisms contributing to sex differences in sleep loss-induced weight gain. Similar to humans, sleep differs between male and female rodents with females spending significantly more time awake and less time in non-rapid eye movement sleep (NREM) sleep [23, 24]. Compared to males, females also spend significantly less time in rapid eye movement sleep (REM) sleep across the light/dark periods [25, 23] despite that one study reported no sex differences in REM sleep across 24h [24]. Gonadectomy eliminates sleep disparities between sexes [24], highlighting the influence of sex hormones on differences in sleep between male and female rodents (reviewed by [26]). Early studies in female rodents reported differences in sleep and locomotor activity across the four phases of the estrous cycle (e.g. proestrus, estrus, metestrus/diestrus one and diestrus two); yet this outcome appears species-dependent. While one study reported that sleep and locomotor activity were similar across phases of the estrous cycle in three different mouse strains [27], another reported female Sprague-Dawley rats spend significantly more time awake,

less time in both NREM and REM sleep and have greater motor activity during the dark period in proestrus (e.g. high estradiol and progesterone levels) relative to other phases [28]. Our analysis of 24h weight gain and food intake corrected for uneaten food particles in female Sprague-Dawley rats across the estrous cycle indicates that weight gain and food intake is also significantly lower during proestrus relative to other phases [29] (Figure 1). These data demonstrate that sex hormones influence sleep, locomotor activity, calorie intake and weight gain within intact female rats. Thus, accounting for differences in these parameters would be imperative for studies examining whether sex moderates the effect of sleep on weight gain between males and intact females throughout the female estrous cycle.

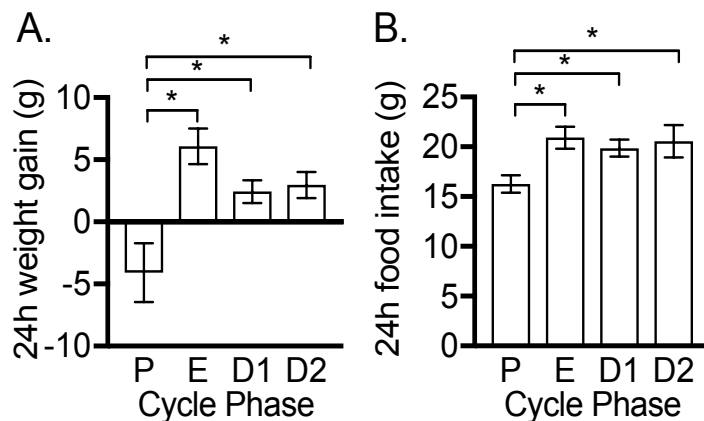


Figure 1. (A) Twenty-four hour bodyweight gain and (B) food intake (g) corrected for uneaten food in female Sprague-Dawley rats during the four phases of the estrous cycle including proestrus (P), estrus (E), diestrus 1 (D1) and diestrus 2 (D2). Data are expressed as mean \pm SEM; N = 4. Brackets indicate bars that are significantly different from each other (*P < 0.05). Note different scaling on y-axes.

Like humans, rodents show sex differences in response to SD; however, limited research has addressed the extent to which SD alters sleep parameters and outcomes related to weight gain between male and female rodents nor has it accounted for the effect of the estrous cycle. Early studies did not validate whether methods that had been shown to disrupt sleep in male rodents indeed disrupted sleep in females but instead focused on the recovery period after exposure to gentle handling [28] or SD during REM [30]. One study reported that the time required to recover from SD was similar between female rats that were subjected to sleep loss during proestrus and estrus [28]. This contrasts with data that showed the time required to return to baseline sleep after SD during REM differed between phases of the estrous cycle as well as between males and females [30]. Finally, sleep disruption (during non-REM and REM for over 3h) has yet to be validated within intact female rats across the entire estrous cycle, despite that one published conference abstract reported that the disk-over-water method completely abolished REM sleep during the 3h time period of SD in pregnant and non-pregnant female rats [31].

Sex differences in sleep and wake parameters after SD have also been examined in mice. Kohel and colleagues showed a similar change in NREM and REM sleep between males and females in response to 6h SD by the multiple platform method plus gentle handling as well as sleep during the recovery period [23]. These data contrast with results from Paul and colleagues who reported that 6h of SD by gentle handling reduced sleep more in male compared to female mice [24]. This same study showed sleep during the recovery period was greater in females compared to males when expressed as a percentage of baseline sleep, which suggests females were more sensitive to SD. Thus far, sleep

disruption by gentle handling or SD during REM reliably reduce sleep in male rodents [32, 33] but this has yet to be validated in female rodents [28, 30]. Despite this, these methods of SD cause weight loss in rodents [34-37, 32, 38], which contrasts with SD-induced weight gain in humans [18]. One study increased sleep fragmentation without reducing time spent asleep with a motorized mechanical sweeper for days 14-19 of gestation in pregnant dams and examined weight outcomes in male and female offspring [39]. They reported that sleep fragmentation only affected male offspring, which was indicated by an increase in their absolute bodyweight, food intake (g/day), visceral and subcutaneous fat compared to those who were not subjected to sleep fragmentation during gestation. While these data suggest that male offspring may be more sensitive to sleep fragmentation during gestation, it remains unknown whether a differential response to sleep fragmentation between male and female juvenile or adult rodents exists.

Future directions to address the contribution of sex to sleep and obesity: The discrepancies in SR-induced weight gain between men and women [18, 12], the inability of SR to increase weight gain among men in one study [12] and limitations in the sleep extension studies suggests that additional variables need to be considered when comparing sex differences within interventions that reduce or improve sleep. First, despite its inherent complexity, quantifying weight gain over longer periods of SR (i.e. weeks or 3 months) and reporting weight gain as percent gain of baseline bodyweight would be beneficial to gauge the clinical significance of sleep disruption-induced weight gain. The latter may be necessary to address whether sex modulates the effect of sleep restriction on weight gain since absolute bodyweight, energy intake, EE, and weight gain

trajectories differ between men and women. For example, a 3.5-kg and 4.5-kg weight gain represents a 5% weight gain for a women and man with an initial bodyweight of 70-kg and 90-kg, respectively, which is a clinically relevant change in weight in a 3-month period [40]. Thus, whether it is correct to conclude that weight gain was the same between the women and man since both had a 5% weight gain from their initial body weight is uncertain. Furthermore, using change in percent body fat or waist circumference in response to SR may better illustrate whether SR differentially alters risk for metabolic disease between men and women or augments weight redistribution. Second, as hormone changes alter energy balance, controlling for hormonal differences across the menstrual phase and completing measurements during the same phase of the menstrual cycle at baseline and during SR in premenopausal women could reduce variability in results for body weight, physical activity, sleep, calorie intake and EE. Third, it is also unclear whether SR has lasting effects on weight gain and metabolism. Hence, quantifying changes in these parameters after sleep restriction when individuals obtain adequate sleep would address maladaptive mechanisms that may promote weight gain after sleep restriction. Finally, analysis of sex differences in weight gain due to SR requires that studies be appropriately powered to substantiate the conclusion that lack of significant differences exists between men and women.

Since energy intake increases EE due to diet-induced thermogenesis and possibly weight gain if EE remains unchanged, standardizing methods for measuring energy intake and EE would aid in determining plausible mechanisms contributing to potential sex differences in weight gain due to SR. For example, providing free access to food during

studies allows one to test whether sleep restriction does indeed increase calorie intake from specific macronutrients or at different times of the day. As with weight gain, it would be ideal to compare the percent change in energy intake and EE relative to baseline calorie intake between SR men and women to account for inherent differences in calorie intake and EE between men and women during adequate sleep. Reporting not only total EE but its components (diet-induced thermogenesis, EE due to rest, sleep and spontaneous physical activity (SPA)) would allow one to design more effective behavioral interventions to prevent weight gain due to inadequate sleep. Finally, it is unknown whether SR augments volitional physical activity in free-living conditions based on studies conducted in whole room calorimeters since the latter limits volitional physical activity and is not recapitulated by involuntary sessions of physical activity during interventions. Collectively, implementing the aforementioned measures and types of analyses in future clinical studies may provide more conclusive evidence as to whether women or men are more sensitive to weight gain in response to sleep restriction while also providing more congruency between population-based and clinical studies.

Conclusion: The data presented in this introduction demonstrate that a relationship exists between insufficient sleep and weight gain but whether women or men are more sensitive to weight gain following sleep loss remains poorly understood. The inherent limitations of clinical studies underscore the significance of developing animal models in both sexes that mirror the human condition. We have previously shown that acute (12h) and chronic (8h/d for 9d) exposure to pre-recorded environmental noise reduces sleep time and quality [41-43], total EE and the EE due to spontaneous physical activity (SPA, for

example, low-intensity physical activity excluding exercise), and increases weight gain and feeding in male rats [41, 42]. This was a significant contribution to the scientific field as it is the only animal model of sleep disruption induced weight gain that translates to the human. However, the method of sleep disruption due to noise has yet to be validated in females, which thwarts efforts examining sex-specific sensitivity to weight gain following sleep loss and the mechanisms contributing to potential sex differences in the latter between men and women. Thus, the research presented in this dissertation was designed to address several of the conclusions discussed in this introduction, namely, the purpose was to: 1) determine the effect of environmental noise exposure on sleep, metabolism, and weight gain in female rats and decipher underlying influences by phases of the estrous cycle; 2) investigate the role of stress in noise-induced weight gain in both sexes since stress is another factor aside from changes in food intake and EE that can promote weight gain; 3) determine neural mechanisms underlying noise responses with a specific focus on orexin-A, which is a neuropeptide produced in discrete hypothalamic areas that regulates sleep-wake stages and metabolism by binding to orexin 1 and 2 receptors; and 4) decipher sex differences in response to noise exposure and whether improving sleep with Suvorexant, an Food Drug Administration (FDA) approved dual orexin receptor antagonist (DORA) for treatment of inadequate sleep in men and women can block the effects of noise in a manner that is sex-dependent.

CHAPTER ONE:

**Noise-induced sleep disruption increases weight gain and
reduces energy metabolism in female rats**

RATIONALE

Noise exposure reduces sleep in humans and lack of sleep increases risk for obesity. Identifying mechanisms underlying weight gain due to sleep disruption requires animal models that mirror the human condition in both men and women. Chronic exposure to environmental noise reduced sleep time and quality and promoted weight gain by increasing feeding and reducing energy expenditure (total and individual components) in male rats [42]. While this validation in male rats mirrors the weight gain observed among sleep restricted men, this method of sleep disruption (SD) had yet to be validated in female rodents. Without this validation study, we would not know if this same model could be used in male and female rats and thus, we would be unable to determine whether males or females are more sensitive to weight gain following sleep loss and mechanisms contributing to the latter. Thus, the first experiment (chapter one) sought to validate the method of SD due to noise in females by testing the hypothesis that chronic exposure to pre-recorded environmental noise would disturb sleep, increase weight gain and feeding and reduce total energy expenditure (EE) and its components without disrupting the length of the estrous cycle.

INTRODUCTION

The prevalence of inadequate sleep has increased [2, 44], and increases risk for weight gain [45, 46] Noise is an environmental factor that causes sleep disruption (SD) [47-53]. In a population sensitive to noise, women report lower sleep quality than men [54] and women with a short duration of sleep display a greater likelihood of weight gain after a 5-7 year follow-up [55]. Thus, women exhibiting SD have a predisposition for obesity and noise exposure can mediate this relationship.

Experimental sleep restriction (SR) promotes weight gain in women [12, 18, 56] though the underlying mechanisms are unknown. In women, SR increases calorie intake [12, 18, 17, 56] but effects on energy expenditure (EE) are contradictory since total EE increased [12, 16] or remained unchanged [15]. Moreover, although SR reduced resting metabolic rate [17] others report no change to this component of total EE[15], sleeping metabolic rate [16] or physical activity [56]. These contradictions are likely due to methodological differences across studies (e.g. methods used to measure EE, inclusion of women only [16, 15, 56] and some but not all studies accounted for the effect of the menstrual cycle on sleep [57] and EE [58]).

The discrepancies between these SR studies underscore the importance of developing an animal model for sleep restricted females. Yet, studies in gonodally intact female rodents report weight loss after SD, and thus, are not in agreement with the studies of SR in women [12, 18, 56]. Furthermore, despite reports in male rodents [32, 41, 42] and ovariectomized females [34, 24], the effect of SD methods on energy intake or EE have yet to be evaluated in gonodally intact females. A previous study showed that SD by the multiple platform method disrupted estrous cycle length [59] but whether SD methods disrupt sleep across all phases of the estrous cycle remains unknown. In female

rodents, sleep time and propensity increase during a recovery period after the gentle handling or multiple platform methods of SD [28, 30, 23] though in these studies sleep during the SD period was not reported [28, 30, 23] nor tested in all phases of the estrous cycle [28, 23]. An acute exposure to the sweeping bar method of SD reduced sleep during proestrus and diestrus only without altering estrous cycle length [60] but weight gain was not measured despite that this occurs in male rodents exposed to this method of SD [61]. The estrous cycle does add a further complication in any rodent study focused on weight gain in females due to SD but is particularly important given the high prevalence of SD and obesity in premenopausal women and data demonstrating that sleep [28, 25, 62], food intake [29] and EE [63-65] vary across the estrous cycle. Thus, these data demonstrate that previously tested methods of SD elicit weight loss in female rodents and do not mirror the human condition. The lack of a pre-clinical model for weight gain due to SD among women therefore hinders efforts to determine mechanistic underpinnings that alter sleep and energy balance to promote weight gain.

We have established a rodent model of SD-induced weight gain in male rats whereby exposure to pre-recorded environmental noise resulted in SD, hyperphagia, increased weight gain and reduced spontaneous physical activity (SPA), total EE and its components [42, 41, 43]. Here, by using this SD method in gonodally intact female rats we sought to validate this methodology in the female. Our hypothesis was that exposure to noise in these rats would result in SD, as indicated by an increase in wake, sleep fragmentation and sleep propensity and also stimulate weight gain due to hyperphagia and reductions in total EE and multiple components of total EE.

MATERIALS & METHODS

Animals: Three-month old female Sprague-Dawley rats (N = 26, Charles River Laboratories, Kingston, New York USA) were housed individually in plexiglass cages that had a perforated floor without bedding (21-22 °C, 12-h light/12-h dark periods, lights on 0600h). Rodent chow (Harlan Teklad 8604) and water were allowed *ad libitum*. Study procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Surgery: Rats were surgically implanted with a radiotelemetric transmitter connected to electroencephalogram (EEG, 40-EET, Data Sciences International (DSI), Saint Paul, Minnesota USA) and electromyogram (EMG) leads (stereotaxic coordinates, -3.1 mm posterior and ± 1.5 mm lateral to bregma [66, 67]. Experiments began 10d post-surgery.

Determining sleep-wake states: Wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep were manually scored from 15-sec. epochs of EEG and EMG recordings [66]. Time spent in sleep-wake states and indicators of sleep fragmentation including the number and duration of episodes, transitions between sleep-wake states, and NREM delta power, an indicator of sleep propensity [68]) were calculated as previously described [69, 66]. Time spent in sleep/wake stages will be referred to as WAKE_{TIME}, SLEEP_{TIME}, NREM_{TIME}, and REM_{TIME}.

Concurrent energy expenditure, physical activity, and sleep-wake states: A receiver was placed beneath the test cage to allow EEG and EMG signals to be recorded from the

implanted EEG and EMG electrodes [66]. EE was determined with a pull mode open circuit indirect calorimeter that measured O₂, CO₂, water vapor continuously each second from each chamber (Promethion-C, Sable Systems Inc. Las Vegas, Nevada USA) that was calibrated as previously described [70]. SPA was determined from infrared beam break sensors (Sable Systems Inc. Las Vegas, Nevada USA) as previously described [71, 70]. Thus, SPA and EE were measured concurrently each second from each chamber. Data were processed with Expedata software v1.9.13 (Sable Systems Inc. Las Vegas, Nevada USA) [70].

Determining components of total energy expenditure: Total EE was calculated with the Weir equation [72]. Individual components of total EE include: EE during SPA, Rest, NREM, and REM. These components were derived from time-stamped EEG/EMG recordings, SPA determined from infrared beam break sensor data, and total EE and calculated as previously described [42, 71]. For each component, total calories and the metabolic rate were calculated since a change in the EE of a specific component can be due to a change in the efficiency of energy utilization (indicated by metabolic rate) with or without a change in the amount of time spent in that component [42]. Total calories (kcal) for each component will be referred to as SPA_{EE}, Rest_{EE}, NREM_{EE} and REM_{EE} and metabolic rates (kcal/h) as SPA_{EE-MR}, Rest_{EE-MR}, NREM_{EE-MR}, REM_{EE-MR}.

Sleep disruption by environmental noise exposure: Rats were exposed to pre-recorded environmental noise (8h/d beginning at 0700; 1h after lights on) during the light phase by placing two speakers in front the rat's cages. This method of SD, has been validated to disrupt sleep in male rats [41, 42]. The 15 min. recording of noises (random street noises, vehicle horn, ambulance siren, hammering, sudden braking vehicle, bell, alarm, air plane

sound etc.) was repeated throughout the 8h period. To prevent habituation, the noise events, the duration of these events, the frequency range of sound (800 to 20000 Hz), the amplitude (65 to 100 dB, with average intensity of 85 db) and inter-noise interval were randomly distributed. The recording also contained periods of silence followed by a sharp attack rate (85 to 100 dB) with noises randomly distributed in the sound sequence. Exposure to sounds of this amplitude and frequency has been shown previously to produce no damage to the rodent cochlea [73]. Since the rodent audiogram differs from the human audiogram and rodents detect higher frequency sounds, the frequency range of the recording (800 to 20000 Hz) was matched to the rat audiogram (Audacity <https://www.audacityteam.org/>). The noises were selected from the Best Service Studio Box DVD3-Technical sample library (Best Service GmbH, Munchen, Germany).

Experimental Design: Female rats were randomized by bodyweight to sleep undisturbed (control) or be exposed to noise (8h/d, light period) for 17d (n = 10/group). Bodyweight and food intake, corrected for uneaten food, were measured manually every 48h. A separate group of females (N = 6) were implanted with EEG/EMG leads. Vaginal smears were collected for 12d following the post-surgical recovery period to validate normal estrous cycles [74]. Then, rats were acclimated to the indirect calorimetry chambers for 3d prior to a 9d baseline period (undisturbed sleep-wake), which was then followed by 9d of noise exposure (8h/d, light period) and a 9d recovery period (undisturbed sleep-wake) [41, 42]. SPA, EE, and EEG/EMG were measured continuously for 27d (baseline through recovery). Bodyweight and feeding, corrected for uneaten food were measured manually once daily and vaginal smears were performed daily (0600-0700h) to determine estrous

cycle phase (proestrus, estrus, diestrus 1 and 2) and estrous cycle duration [74] in this group of rats (Figure 1).

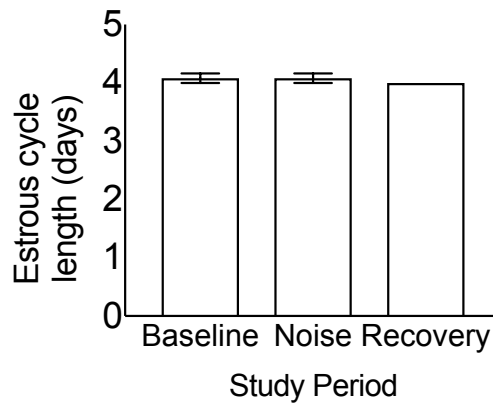


Figure 1. Female rats maintained 4-5d estrous cycles throughout the study. Vaginal smears were performed daily (0600-0700h) to determine estrous cycle phase (e.g. proestrus, estrus, diestrus 1 and -2) and the length of the estrous cycle during 9d of undisturbed sleep, 9d of noise exposure (8h/d for 9d during the light period), and 9d of recovery. Significant difference ($P < 0.05$) was determined by ANOVA. Data expressed as mean \pm SEM; $N = 6$.

Statistics: Data were analyzed with Prism 7.0d (GraphPad Software, San Diego, California USA) and are displayed as mean \pm SEM. Alpha was 0.05 for statistical tests and normality was determined with the Shapiro-Wilk test. Separate analyses were completed for each time period of the circadian cycle (lights-on (light period), lights-off (dark period) and over 24h). To determine the effect of noise exposure on weight gain and feeding, data were analyzed with two-way ANOVA (time and treatment) followed by paired t-tests with FDR correction for multiple comparisons [75]. The effect of estrous cycle phase during baseline on sleep-wake states, fragmentation, and EE was determined

by repeated measures ANOVA followed by paired t-tests with FDR correction for multiple comparisons [75]. To determine the effects of noise exposure and recovery on sleep-wake states and EE and to control for the effect of estrous cycle phase on these parameters, change from baseline (during noise exposure and recovery) was calculated by subtracting values within the same phase of the estrous cycle. Then, these values were summed to determine the cumulative change from baseline for each rat over the 9d period of noise exposure and recovery. To determine the duration of the change in sleep-wake states and EE during recovery, the cumulative change from baseline during recovery was summed in 3d bins. Endpoints were then analyzed with one sample t-tests for the null hypothesis of no change relative to baseline for the noise exposure and recovery periods. Sleep-wake data from one rat was excluded due to technical issues.

RESULTS

Noise exposure stimulates weight gain and hyperphagia in female rats: We first tested the hypothesis that exposure to pre-recorded noise would increase weight gain and feeding. Noise exposure significantly increased weight gain and food intake with respect to female rats that slept undisturbed (Figure 2A-2B, treatment: $F_{(1,18)} = 9.2$, $P < 0.007$ and $F_{(1,18)} = 11.0$, $P < 0.004$; time: $F_{(8,144)} = 36.3$, $P < 0.0001$ and $F_{(8,144)} = 3793$, $P < 0.0001$; time x treatment: $F_{(8,144)} = 2.7$, $P < 0.008$ and $F_{(8,144)} = 6.1$, $P < 0.0001$, respectively). Hence, exposure to noise resulted in increased weight gain and hyperphagia.

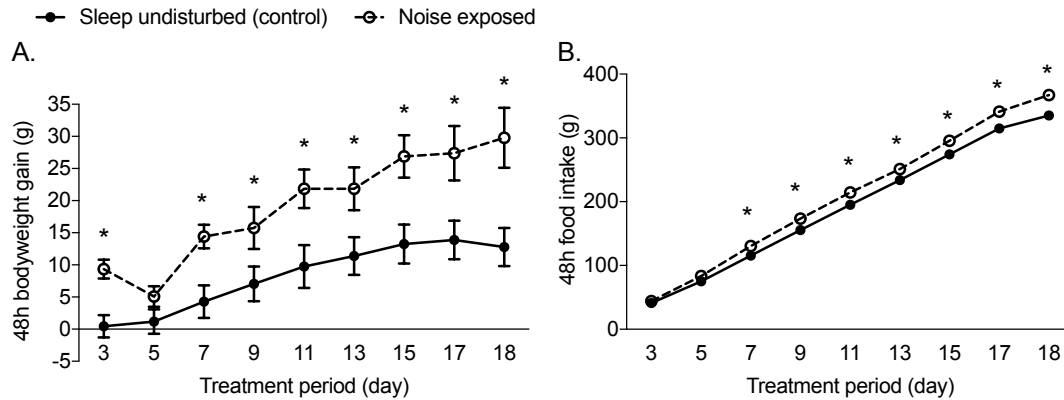


Figure 2. Noise exposure increases weight gain and food intake in female rats.

Female Sprague-Dawley rats (12 weeks old) were allowed to sleep undisturbed or exposed to pre-recorded noise (8h/d for 17d during the light period). (A) Weight gain and (B) food intake was determined every 48h. Significant differences ($P < 0.05$) were determined by two factor ANOVA followed by multiple comparison tests corrected for the false discovery rate. * $P < 0.05$ compared to undisturbed controls for a specific treatment day. Data represent mean \pm SEM. $n = 10/\text{group}$.

Sleep-wake states, SPA and EE differ across phases of the estrous cycle during undisturbed sleep-wake: To validate the effect of exposure to noise on sleep-wake states and metabolism, we first tested the hypothesis that sleep-wake, SPA, total EE and its components varied across phases of the estrous cycle under control conditions [28, 25, 62-65]. Consistent with prior reports, we found that the duration of sleep-wake states and several indicators of sleep fragmentation differed across phases of the estrous cycle during the light, dark and 24h periods with proestrus having the greatest effect (Figure 3A-3I).

Like sleep-wake states, SPA, total EE, and its components differed significantly across phases of the estrous cycle under control conditions. Over the dark and 24h periods, SPA and total EE were significantly higher during proestrus than during other phases with the exception of total EE over 24h (Figure 3J-3K, $P < 0.05$ pairwise comparisons between phases). Higher total EE during the dark period in proestrus was due to higher SPA_{EE} since REST_{EE} was similar and NREM_{EE} and REM_{EE} were actually significantly lower in proestrus during this time period (Figure 3L-3N, $P < 0.05$ pairwise comparisons between phases, data not shown for REST_{EE}). During the dark period, SPA_{MR} was also significantly higher in proestrus compared to diestrus-1 (Figure 3O, $P = 0.01$) while REST_{MR} was significantly higher in proestrus than in both diestrus-1 and -2 (Figure 3P, $P = 0.02$ and $P = 0.02$, respectively). In contrast, NREM_{MR} and REM_{MR} were significantly higher in proestrus than in other phases during the dark period (Figure 3R-S, $P < 0.05$ pairwise comparisons between phases). Taken together, these data demonstrate that parameters which affect weight gain and are known to be affected by SD due to noise exposure [42, 41] differ across phases of the estrous cycle.

Exposure to noise increases time spent awake and sleep fragmentation: We next tested the hypothesis that exposure to noise for 9d would disturb sleep as indicated by greater sleep-wake state fragmentation. During the light period, noise exposure significantly increased WAKE_{TIME} and decreased SLEEP_{TIME} due to significant reductions in NREM_{TIME} and REM_{TIME} (Figure 4A, $P < 0.05$). In contrast, during the dark period, WAKE_{TIME} was significantly reduced and SLEEP_{TIME} was significantly increased due to

prolonged REM_{TIME} (Figure 4B, $P < 0.05$). However, over 24h, WAKE_{TIME} was also significantly prolonged with respect to baseline (Figure 4C, $P = 0.01$). Noise exposure also increased sleep fragmentation during the light and 24h periods. This was due to significantly more episodes of NREM that were of shorter duration during the light period, more episodes of REM during both the light and 24h periods, and significantly more transitions between sleep-wake states during the light and 24h periods (Figure 4E-H, $P < 0.05$). Furthermore, we note that noise exposure significantly increased NREM delta power with respect to baseline during the 24h period. This was due to higher NREM delta power during the dark (Figure 4I, $P < 0.05$) but not the light period (Figure 4I, $P = 0.06$). These data demonstrate that exposure to noise resulted in SD by reducing sleep duration and increasing sleep fragmentation.

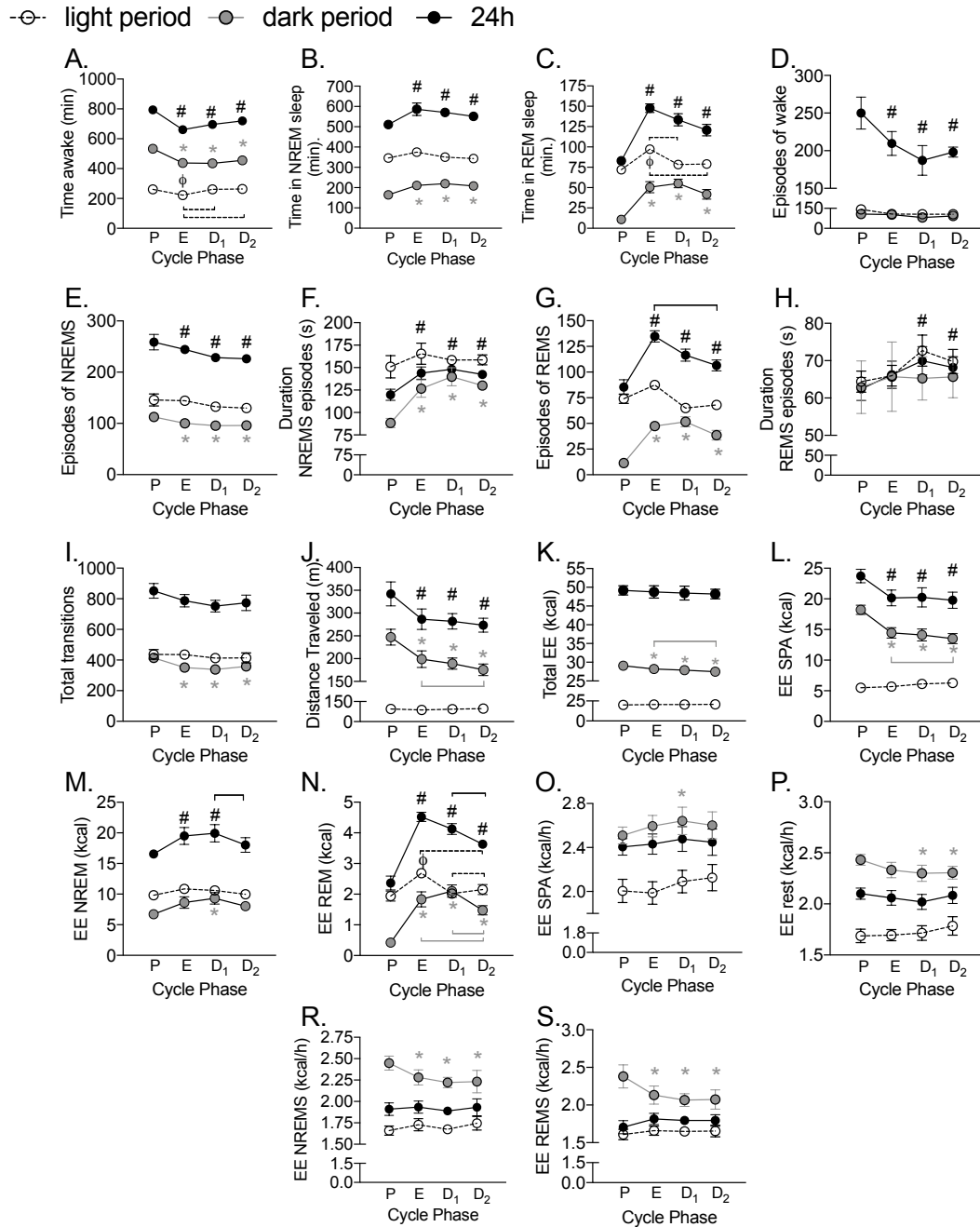


Figure 3. Estrous cycle phase affects sleep-wake states, SPA and EE. During 9d of undisturbed sleep, EEG and EMG signals were measured continuously from female Sprague-Dawley rats (12 weeks old) implanted with EEG/EMG leads. (A-C) Time spent in wake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep, measures of sleep fragmentation including the (D) number of wake episodes, (E-F)

number and duration of NREM episodes, (G-H) number and duration of REM episodes, and (I) total transitions between sleep-wake states, (J) SPA, (K) total EE, and (L-O) components of EE (kcal) including their (P-S) metabolic rates (kcal/h) across phases of the estrous cycle during undisturbed sleep. Significant differences between phases of the estrous cycle were determined with repeated measures ANOVA followed by paired t-tests with FDR correction for multiple comparisons. Data expressed as mean \pm SEM; N = 5-6. ϕ $P < 0.05$, * $P < 0.05$ and # $P < 0.05$ for the light period (dashed lines), dark period (gray), and 24h period (black), respectively as compared to proestrus (P). Brackets indicate significant differences between estrus (E), diestrus-1 (D₁) and diestrus-2 (D₂) within each time period.

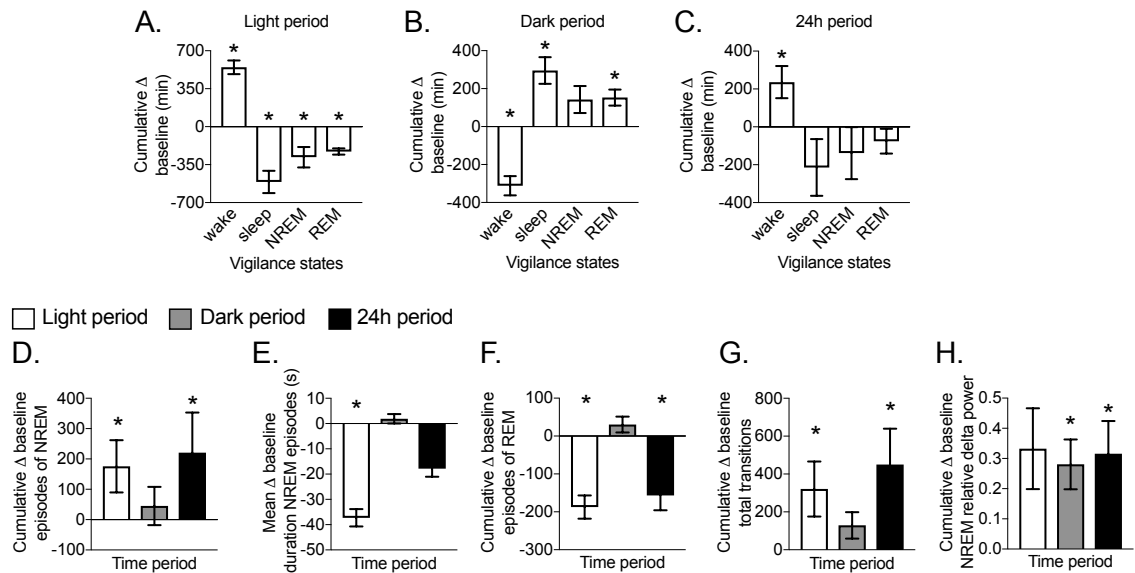


Figure 4. Noise exposure increases time awake and sleep fragmentation. Female Sprague-Dawley rats (12 weeks old) were implanted with EEG/EMG leads. EEG and EMG signals were measured continuously during exposure to noise (8h/d during the light period for 9d). (A-C) Time spent in wake, sleep, non-rapid eye movement (NREM) sleep

and rapid eye movement (REM) sleep and measures of sleep fragmentation including the (D-E) number and duration of NREM episodes, (F-G) number and duration of REM episodes, (H) total transitions between sleep-wake states, and (I) sleep propensity indicated by NREM delta power during the light, dark, and 24h periods. For each rat, change from baseline during noise exposure was calculated by comparing daily data in the same phase of the estrous cycle. Significant differences ($P < 0.05$) were determined with one sample t-tests. Data expressed as mean \pm SEM; $N = 5$. * $P < 0.05$.

Noise-induced SD reduces total EE: We then tested the hypothesis that noise-induced SD would reduce SPA and total EE and its components. In fact, noise-induced SD significantly decreased total EE with respect to baseline during the dark and 24h periods ($P = 0.009$ and $P = 0.03$, respectively, Figure 5A) despite the fact that NREM_{EE} and REM_{EE} were significantly lower with respect to baseline during the light period ($P = 0.001$ and $P = 0.003$, respectively, Figure 5B-C). The reduction in total EE during the dark and 24h periods was due to significant reductions in NREM_{EE} (24h: $P = 0.003$, Figure 5C), SPA_{EE} (dark: $P = 0.03$, Figure 5D) and SPA_{MR} (24h: -0.09 ± 0.03 , $P = 0.03$, data not shown). Despite reductions in SPA_{EE} and SPA_{MR}, overall SPA, as indicated by distance traveled, was not significantly different from baseline values during all time periods ($P > 0.05$ for all comparisons, data not shown). Taken together, these data show that noise-induced SD reduced total EE with respect to baseline during the dark and 24h periods; this was due to lower EE during SPA and NREM sleep.

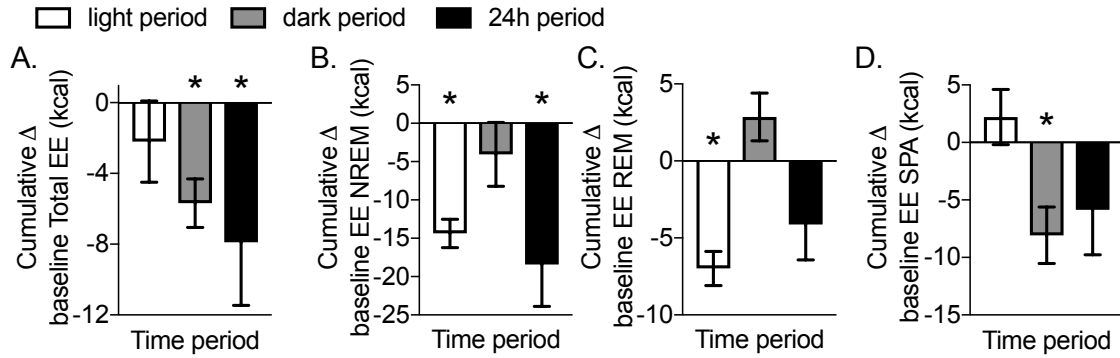


Figure 5. Noise-induced SD reduces total and individual components of EE. (A) total EE and components of EE including the EE during (B) non-rapid eye movement (NREM) sleep, and (C) rapid eye movement (REM) sleep, (D) SPA, and (E) rest were measured continuously during 9d of noise exposure in female Sprague-Dawley rats (12 weeks old), following a 3d acclimation period to the indirect calorimetry chambers. For each rat, change from baseline during noise exposure was calculated by comparing daily data in the same phase of the estrous cycle. Significant differences ($P < 0.05$) were determined with one sample t-tests. Data represent mean \pm SEM; $N = 5-6$. * $P < 0.05$.

Noise-induced SD causes rebound sleep: Given that noise exposure resulted in SD, we hypothesized that SD due to noise would elicit a compensatory increase in sleep during the recovery period. Recovery $SLEEP_{TIME}$ was indeed significantly greater with respect to baseline values during the dark and 24h periods ($P = 0.001$ and $P = 0.02$, respectively, Figure 6A-C). This was due to significantly prolonged $NREM_{TIME}$ in the dark period ($P = 0.04$) and significantly lower $WAKE_{TIME}$ during the dark and 24h periods ($P = 0.01$ and $P = 0.02$, respectively). Furthermore, noise-induced sleep fragmentation normalized during the recovery period. This was indicated by significantly fewer episodes of wake during

the light period and fewer NREM episodes during the light and 24h periods, in addition to significantly more episodes of REM that were of longer duration during all time periods. Furthermore, we noted significantly fewer transitions between sleep/wake stages during the light and 24h periods ($P > 0.05$ for all comparisons, Figure 6D-H). Recovery NREM delta power was also significantly higher than baseline delta power during the light and 24h periods ($P = 0.03$ and $P = 0.02$, respectively, Figure 6I). In summary, these results demonstrate that rebound sleep with reduced sleep fragmentation and increased NREM delta power occurred during the recovery period following noise-induced SD.

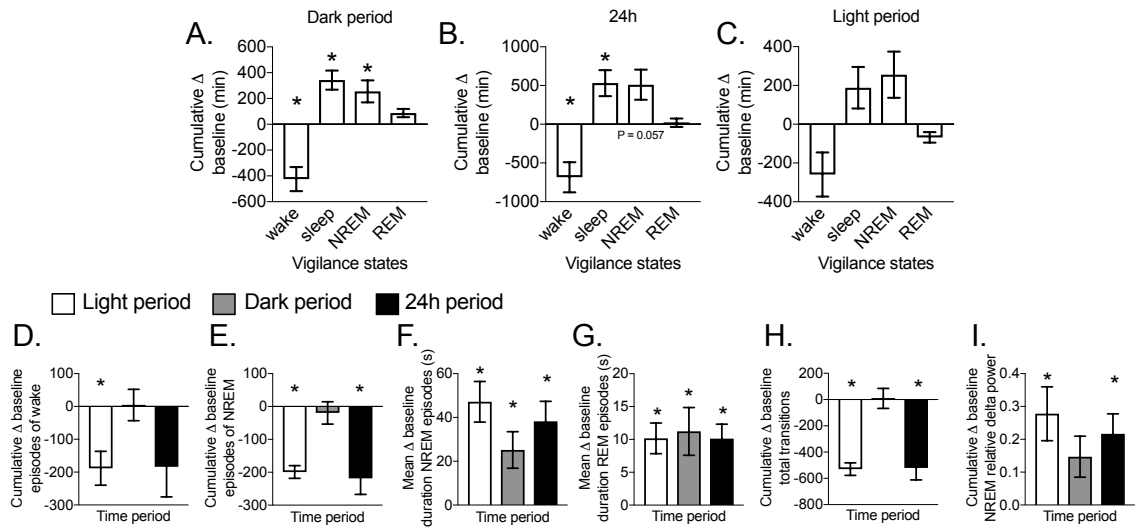


Figure 6. Female rats have rebound sleep during recovery after noise-induced SD.

Female Sprague-Dawley rats (12 weeks old) were implanted with EEG/EMG leads. EEG/EMG signals were measured continuously during 9d of recovery after exposure to noise (8h/d for 9d during the light period). (A-C) time spent in wake, sleep, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep and measures of sleep fragmentation including the (D) number of wake episodes, (E-F) number and duration of

NREM episodes, (G) duration of REM episodes, (H) total transitions between sleep-wake states, and (I) sleep propensity indicated by NREM delta power during the light, dark, and 24h periods. For each rat, change from baseline during recovery was calculated by comparing daily data in the same phase of the estrous cycle. Significant differences ($P < 0.05$) were determined with one sample t-tests. Data represent mean \pm SEM; $N = 5$. * $P < 0.05$.

Noise-induced SD causes rebound sleep for 9d during the dark period: We next considered the length of time that rebound sleep continued during the recovery period by binning the 9d sleep-wake data into 3d bins. For the first third of recovery, $WAKE_{TIME}$ was significantly lower and $SLEEP_{TIME}$ significantly higher due to increased $NREM_{TIME}$ during the light and 24h periods ($P < 0.05$ for all comparisons, Figure 7A-C, data not shown for $SLEEP_{TIME}$). In contrast, $WAKE_{TIME}$ was significantly lower and $SLEEP_{TIME}$ significantly higher due to increased $NREM_{TIME}$ and REM_{TIME} during the dark period throughout the 9d recovery period ($P < 0.05$ for $WAKE_{TIME}$ and $SLEEP_{TIME}$, data not shown for $SLEEP_{TIME}$, Figure 7A-C). Thus, 24h sleep-wake states normalized after 3d of recovery, but dark period rebound sleep remained prolonged throughout 9d.

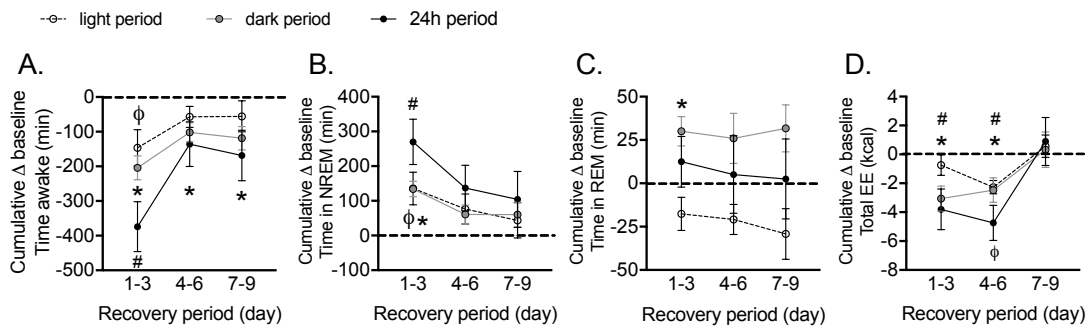


Figure 7. Female rats have prolonged rebound sleep and reduced total EE after noise-induced SD. Female Sprague-Dawley rats (12 week old) were implanted with EEG/EMG leads. During a 9d recovery period after exposure to noise (8h/d for 9d during the light period), EEG, EMG and total EE were measured continuously. Time course of time spent in (A) wake, (B) non-rapid eye movement (NREM) sleep, and (C) rapid eye movement (REM) sleep and (D) total EE in the light, dark, and 24h periods during recovery. For each rat, change from baseline during recovery was calculated by comparing daily data in the same phase of the estrous cycle. Significant differences ($P < 0.05$) were determined with one sample t-tests. Data represent mean \pm SEM; $N = 5-6$. ϕ $P < 0.05$, * $P < 0.05$ and # $P < 0.05$ for each tertile of the recovery period and for the light, dark and 24h periods, respectively.

Noise-induced SD reduced EE during rebound sleep: Based on the prolonged rebound sleep time during the recovery phase, we next investigated whether SPA, total EE and its components would be reduced during recovery. Dark and 24h period total EE was significantly lower for the first 6d of recovery ($P < 0.05$ for all comparisons, Figure 8D). This was due to significant differences in EE components since SPA_{EE} , SPA_{MR} , $REST_{EE}$, and $SLEEP_{MR}$ due to lower $NREM_{MR}$ were significantly lower than baseline during the dark and/or 24h periods ($P < 0.05$ for all comparisons, Figure 8A-E). In summary, reductions in total EE persisted for the first 6d of recovery and this was due to a lower EE during SPA and REST and reductions in the metabolic rates during sleep and NREM.

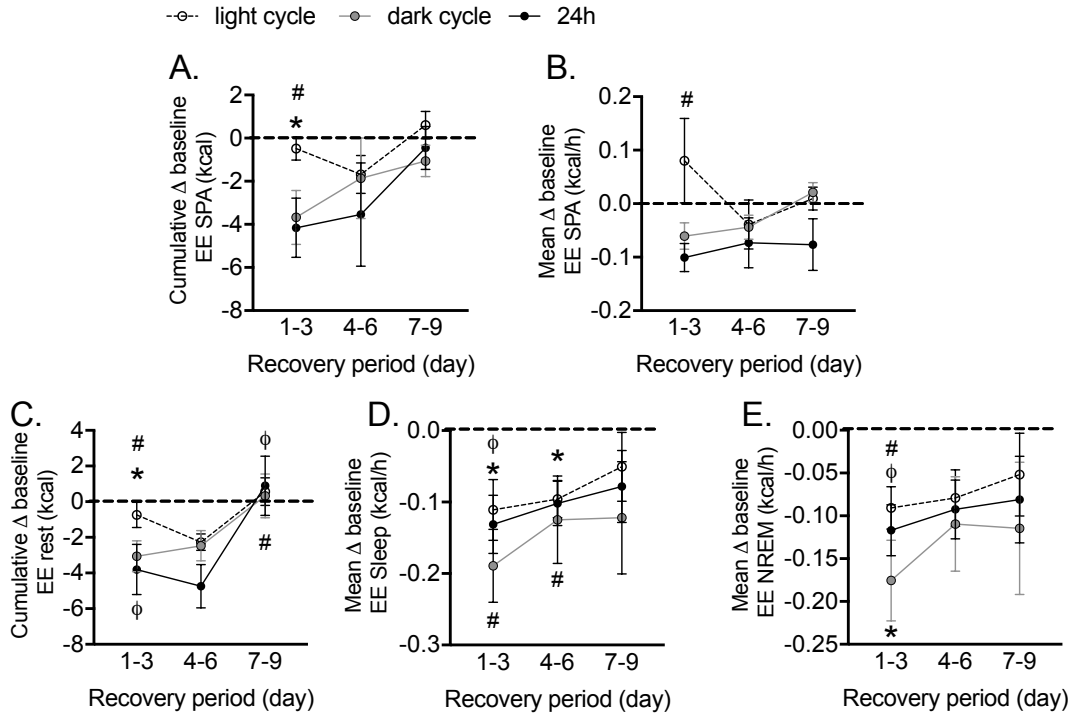


Figure 8. Components of total EE remained dampened during recovery after noise-induced SD. Time course of the components of EE including EE during (A) SPA, (B) metabolic rate for SPA, (C) EE during rest, (E) metabolic rate for EE sleep, and the (F) metabolic rate for non-rapid eye movement (NREM) sleep. Significant differences ($P < 0.05$) were determined with one sample t-tests for the null hypothesis of no change relative to baseline for each tertile of the recovery period. Data represent mean \pm SEM; $N = 5$. ϕ $P < 0.05$, * $P < 0.05$ and # $P < 0.05$ as compared to baseline for each sleep/wake stage and each tertile of recovery for the light, dark and 24h periods, respectively.

DISCUSSION

Environmental noise is a key factor that decreases sleep quality and duration [76, 52, 53] and increases obesity risk [55, 52], particularly among women [52, 54]. Yet, currently there is no pre-clinical model available to investigate the mechanisms underlying the effect of SD independent of [12, 18, 56, 55] or due to noise exposure [52] on weight gain in women. We previously reported that noise exposure in male rats caused SD and weight gain by increasing food intake and reducing EE (total and individual components) [42, 41]. By utilizing this same methodology of SD, we demonstrate here that environmental noise exposure in females increases time awake, sleep fragmentation, weight gain and feeding and reduces EE without disrupting estrous cycle length. Noise exposure in females decreased total EE due to lower SPA_{EE} and NREM_{EE}. These reductions in EE (total and individual components) persisted into the recovery period for 6d while rebound sleep indicated by higher SLEEP_{TIME} continued for 9d during the dark period of recovery. Finally, we show that females displayed 4-5d estrous cycles during control conditions, noise exposure, and the recovery period. These results are consistent with population and clinical studies reporting that SD increases the risk of obesity, promotes weight gain, and alters energy balance in women [55, 52, 12, 18, 56]. Thus, we propose that environmental noise is an appropriate SD method to investigate the mechanisms conferring the effects of SD on energy balance in females.

We are the first to report weight gain and hyperphagia in intact females by a method of SD. These results agree with our prior reports in males [41, 42] and the phenotype of sleep restricted women [12, 18, 17, 56] but contrasts the effect of other SD methods on weight gain in female rats [35, 37, 36] and one study demonstrating that food

intake was similar between ovariectomized SD females and undisturbed controls [34]. This discrepancy may be attributed to differences in technical aspects (e.g. SD duration, stress associated with SD, use of ovariectomized females, and not correcting for uneaten food particles); although, a direct comparison between different methods of SD including gentle handling, the multiple platform method, and environmental noise will be required to substantiate this hypothesis. In females, the increase in weight gain and feeding reached significance on day two and five, respectively compared to undisturbed controls. Interestingly, this contrasts the time course in male rats exposed to noise [41], suggesting a sex-specific effect of noise on these parameters. A quantitative comparisons between our previously published data in males [41] and the data reported here in females, shows that the direction of the effect is the same for males and females with an effect size (Cohen's *d*) of 0.9 and 0.6 for weight gain (e.g. expressed as weight gain/initial body weight to account for differences in weight between males and females) and 2.0 and 1.6 for food intake, respectively. However, future studies are warranted to decipher sex differences in response to noise since these studies were run in different geographical locations, at different times, and not concurrently. Nonetheless, our presentation of a SD method in females that parallels the phenotype of sleep-restricted women [12, 18] and elevated obesity risk in women exposed to environmental noise (15) underscores the significance of these data.

As noise increased weight gain, we next evaluated in a separate set of females whether noise caused SD and determined the effect of noise on EE and SPA since noise exposure dampens these parameters to promote weight gain in males [42]. Under control conditions, we demonstrate that females in proestrus spent more time moving and less

time in NREM and REM sleep during the dark and 24h periods which agrees with studies [28, 62] but contrasts others that report no difference in NREM sleep or physical activity across the estrous cycle [25], which may be related to differences in the timing of vaginal lavage since this influences estrous cycle staging [74]. Others have reported that 24h total EE is higher in proestrus [65] or similar across the phases [63], which contrasts our finding of higher total EE in proestrus during the dark period only. These discrepancies may be due to differences in measurement frequency, duration and timing [63, 65], or diets [63] and methods used to quantify EE [63]. Despite this, we are the first to report a detailed analysis of EE components across phases of the estrous cycle, which highlights new aspects of EE in females. For example, we show that higher total EE during the dark period during proestrus is driven by higher SPA_{EE}, paralleling increased SPA here and greater locomotor activity reported by others during proestrus [28, 62, 63, 77]. This may be related to higher estradiol levels during proestrus [78] since exogenous estradiol promotes arousal and PA in ovariectomized females [79]. Lastly, We show that calories and metabolic rate for specific components of EE can be disassociated. This follows from our finding that during control conditions, the metabolic rate does not always parallel the calories expended in a specific component of total EE. This was observed for REST_{EE}, as the total calories for this component was similar across phases of the estrous cycle while REST_{MR} during the dark period was higher in proestrus compared to all other phases. This result indicates that REST_{MR} is an inadequate estimate for REST_{EE} or its extrapolation to total EE over 24h [20, 17]. These data show that estrous cycle phase regulates energy metabolism with differential effects on metabolic rates and total calories for several components of EE.

Environmental noise caused SD during exposure and a rebound increase in sleep for the first 6d of the recovery period independent of the estrous cycle. Importantly, SD in response to noise is similar between female and male rats [42, 41]. Likewise, we show that sleep rebound following noise exposure is similar to that observed in female rodents exposed to methods that reduce sleep in males [28, 30, 23, 80] and women exposed to environmental noise [47]. We also report a lack of rebound in REM_{TIME}, which is consistent with the suppressive effect of exogenous estrogen on REM_{TIME} that was reported in sleep disrupted ovariectomized females [81].

Our finding that estrous cyclicity was unaltered during noise exposure and the recovery period demonstrates that noise did not disrupt the estrous cycle. These results are critical as prior reports show that other methods of SD [59], stress [82] or corticosterone [83] can lead to constant diestrus (e.g. anestrus) and that stress disrupts ovarian hormone secretion in rodents [84]. While our data do not rule out an effect of environmental noise on other measures of physiological or behavioral stress [85, 86], they suggest that stress is not the primary driver of its effects on sleep/wake behavior in females. Thus, our data show that the effects noise exposure on sleep are independent of disruption in estrous cyclicity.

A novel finding from this study is that, in females, environmental noise exposure persistently reduced total EE by decreasing both total calories and metabolic rates of EE components. This coincides with the effect of environmental noise in male rats [42] and humans [17, 20]. In female rats, noise exposure reduced total EE by reducing SPA_{EE} and SPA_{MR} as well as NREM_{EE} due to a decrease in NREM_{TIME}. Despite that SLEEP_{Time} increased during recovery, total EE remained dampened for the first 6d of recovery after

noise exposure ended, which was driven by reductions in SPA_{EE} due to lower SPA_{MR}, REST_{EE}, and SLEEP_{MR} due to lower NREM_{MR} only. These data show that environmental noise caused a sustained reduction in total EE (due to multiple components of EE) and SLEEP_{MR} and SPA_{MR} in female rats. Taken together, these data suggest that environmental noise exposure would promote weight gain in female rodents independent of an effect by the estrous cycle since cyclicity was maintained from baseline to recovery.

The mechanistic underpinnings of our current findings are unclear. The reduction in total EE and weight gain might be related to dampened orexin function in response to environmental noise exposure and SD. Orexins (i.e., hypocretins), are neuropeptides that bind to orexin 1 and 2 receptors [87], and regulate sleep/wake states. The absence of the orexin signal causes narcolepsy [88], a sleep disorder associated with elevated body mass index. Orexins also promote negative energy balance by stimulating arousal, PA, total EE and several of its components [89, 90, 71, 91]. Likewise, antagonism at both orexin receptors blocks orexin-A stimulated increases in these endpoints and reduces total EE and several of its components [71], highlighting the role of orexin in energy metabolism. We showed that environmental noise exposure blocks the orexin-A stimulated increases in arousal, SPA, EE and EE during SPA in male rats [43]. Thus, noise exposure and SD may reduce the efficacy of endogenous orexin-A to increase PA and EE, which would be expected to contribute to the weight gain here and in our prior reports [42, 41]. These studies must now be replicated in female rats to confirm that the response to orexin-A before and after environmental noise exposure coincides with our prior reports in males [43]. Finally, it is plausible that peripheral or central estrogen may have influenced our results since estradiol administration increases PA [79] and EE [92] in ovariectomized

females and reverses weight gain and hyperphagia caused by loss of estrogen due to ovariectomy [93]. This also remains to be tested since it is unknown if environmental noise augments estrogen.

Overall, the data presented here show that environmental noise disturbs sleep, energy metabolism and feeding, leading to increased bodyweight gain in female rats. Together with our prior studies in males, these data indicate that the response to environmental noise exposure in male and female rats models the weight gain and hyperphagia observed in sleep restricted men and women, which supports the internal and face validity of this model for studying the metabolic consequences of SD in humans. Thus, these data provide a foundation for future mechanistic studies aimed at elucidating metabolic consequences of SD by environmental noise.

CHAPTER TWO:

Role of stress in sleep disruption induced weight gain

RATIONALE

There are other factors that contribute to weight gain aside from sleep disruption (SD) such as stress. Other methods of SD that alter bodyweight also increase stress indicated by elevated corticosterone levels and prolonged the duration of the estrous cycle. The experiment described in chapter one demonstrated that chronic exposure to noise in female rats reduces sleep and promotes weight gain by altering feeding and energy expenditure in a manner that was similar to reports in male rats. Moreover, noise exposure in females failed to alter the normal cyclicity of the estrous cycle. This suggests that the effects of noise on weight gain may not be secondary to stress. Nonetheless, to determine the contribution of stress to weight gain in response to noise-induced sleep disruption, male and female rodents were exposed to noise during the dark period in this experiment (chapter two) in an effort to dissociate stress due to noise from noise-induced SD. Playing noise during the dark period causes such a dissociation since the dark period is the most active period for the rats. Thus, by exposing the rats to the noise during the dark period, they are more so exposed to the stress of the noise and not the sleep loss due to noise exposure. The overall hypothesis was that dark cycle noise exposure would have no effect on sleep duration or quality, weight gain or factors known to affect weight gain including food intake or physical activity independent of sex.

MATERIALS AND METHODS

Animals: Two sets of three-month old male and female Sprague-Dawley (N = 15) rats (Charles River Laboratories, Kingston, New York USA) were housed individually in solid-bottom cages in a temperature-controlled room (21-22 °C) with a 12-h light/12-h dark period (lights on at 0600h). Rodent chow (Harlan Teklad 8604) and water were allowed *ad libitum*. Study procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Surgery: Rats were anesthetized with ketamine (50-75mg/kg, i.p.) and xylazine (7 mg/kg, i.p) and surgically implanted with a radiotelemetric transmitter connected to electroencephalogram (EEG) and electromyogram (EMG) electrodes (F40-EET, Data Sciences International, Saint Paul, Minnesota USA) as described [66, 70]. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson [67]. Coordinates for the cannula and EEG electrodes, respectively, were as follows: 3.1 mm posterior and +/-1.5 mm lateral to bregma. Experimental trials began 10-d post-surgery.

Verification of estrous cycle phase: Vaginal smears were performed daily at 1700h-1800h, respectively to determine proestrus, estrus, diestrus 1 (e.g. metestrus) and diestrus 2 as described [94] to verify normal 4-5d estrous cyclicity [74, 95]. For all treatment days, female rats were tested during diestrus 2, when estrogen levels are low [78] to minimize the effect of estrous cycle phase on weight gain, food intake, sleep-wake and physical activity [28, 29, 95].

Concurrent EEG, EMG and spontaneous physical activity: A receiver was placed beneath the test cage to allow EEG and EMG signals to be recorded from the implanted

transmitter [66]. Spontaneous physical activity (SPA) was measured by infrared sensors placed around an acrylic cage ($425 \times 265 \times 305$ mm, TSE Systems, Chesterfield, MO) as described [70]. Briefly, ambulation was detected by two infrared arrays along the x- and y-axes, and vertical movement was detected by a third elevated x array. Movement was therefore simultaneously detected in all dimensions. Components of PA (distance traveled and vertical activity [e.g., rearing], and velocity) were determined from the infrared beam-break data.

Determining sleep-wake stages: Electroencephalogram and EMG data were visualized with Neuroscore software (version 2.0.1, Data Sciences International, Saint Paul, Minnesota USA) [70]. Consecutive 15-second epochs of EEG and EMG were manually scored to determine wake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep [66]. To be scored as a valid behavior state, the appropriate EEG and EMG activity patterns needed to persist for a minimum of 15 s. Time spent (min) in each state was calculated from the scored data. The total number and mean duration of sleep-wake episodes for each behavioral state and total number of transitions between different stages were determined by Neuroscore based on the manual scoring results.

Sleep disruption by environmental noise exposure: Rats were exposed to pre-recorded environmental noise (8h for 1d beginning at 1800; start of the dark period) during the light phase by placing two speakers in front the rat's cages. This method of SD, has been validated to disrupt sleep in male rats [41, 42]. The 15 min. recording of noises (random street noises, vehicle horn, ambulance siren, hammering, sudden braking vehicle, bell, alarm, air plane sound etc.) was repeated throughout the 8h period. To prevent

habituation, the noise events, the duration of these events, the frequency range of sound (800 to 20000 Hz), the amplitude (65 to 100 dB, with average intensity of 85 db) and inter-noise interval were randomly distributed. The recording also contained periods of silence followed by a sharp attack rate (85 to 100 dB) with noises randomly distributed in the sound sequence. Exposure to sounds of this amplitude and frequency has been shown previously to produce no damage to the rodent cochlea [73]. Since the rodent audiogram differs from the human audiogram and rodents detect higher frequency sounds, the frequency range of the recording (800 to 20000 Hz) was matched to the rat audiogram (Audacity <https://www.audacityteam.org/>). The noises were selected from the Best Service Studio Box DVD3-Technical sample library (Best Service GmbH, Munchen, Germany).

Experimental design: Male and female Sprague Dawley rats (n = 8 and 6, respectively) were allowed to sleep freely and exposed to pre-recorded environmental noise (8h beginning at 1800h; start of the dark period) in a repeated measures design with ≥ 48 h between treatments. EEG, EMG and spontaneous physical activity were measured for 8h. Twenty-four hour weight gain and food intake (i.e. corrected for uneaten food particles) was also measured. The following endpoints were analyzed: sleep duration and sleep fragmentation as indicated above, distance traveled, vertical counts, velocity, 24h weight gain and food intake.

Statistical Analysis: Data were analyzed with Prism 7.0d (GraphPad Software, Inc., San Diego, California USA). Data are expressed as mean \pm SEM. Alpha was 0.05 for all statistical tests. Analyses were completed for the 0-8h time period during the control

night and exposure to dark period environmental noise Data from males and females were analyzed separately by paired t-tests to determine the effect of dark period noise exposure on each endpoint. Then, change from baseline was calculated for each endpoint and unpaired t-tests were performed to determine differences between male and females on all endpoints. Sleep-wake data and physical activity was excluded for one rat due to technical issues.

RESULTS

Dark period noise exposure has a sex-dependent effect on sleep-wake duration and sleep fragmentation: To begin to address the contribution of stress to noise-induced weight gain [41, 42], male and females were exposed to an acute bout of environmental noise (8h) during the dark period when sleep is inherently low. Thus, we intended to expose rats to the same stress caused by the noise during the light period without disrupting sleep. We first determined whether exposing rats to noise during the dark period had any effect on sleep-wake duration. Contradictory to our expectation, dark period noise exposure augmented sleep-wake with a differential response between the sexes (Figure 1). Dark period noise exposure had no effect on time awake or asleep in males during all time periods ($P > 0.05$ compared to control, Fig. 1A-C) but dark period noise exposure in females significantly increased time awake and reduced both NREM and REM relative to control (no noise) ($P < 0.05$ compared to control, Figure 1A-C). Based on these results, the response to dark period noise for sleep-wake duration was significantly different between the sexes with one exception ($P < 0.05$ compared to noise-exposed males, Figure 1A-C). Rapid-eye movement (REM) was similar between noise-exposed males and females 0-8h ($P = 0.08$, Figure 1C). These data demonstrate that the effect of noise

exposure in the dark period on sleep-wake duration is sex-dependent since it altered sleep-wake duration in female but not male rats.

Next, we determined whether dark period noise augmented indicators of sleep fragmentation and determined whether this effect was sex dependent. Dark period noise exposure had no overall effect on sleep fragmentation in females (Figure 2). But females, dark period noise exposure increased sleep fragmentation in male rats indicated by increased episodes of sleep-wake stages that were of shorter duration and more transitions between these stages (Figure 2A-G). In males, dark period noise exposure significantly increased the number and reduced the duration of wake episodes (Figure 2A-B). In parallel, dark period noise exposure significantly increased the number and reduced the duration of NREM episodes in males compared to control (no noise) ($P < 0.05$ compared to control, Figure 2 C-D). Despite no effect of dark period noise on the number of REM episodes ($P > 0.05$ compared to control, Figure 2E) in males, the duration of these episodes was significantly shorter relative to control ($P < 0.05$ compared to control, Figure 2F). In males, dark period noise exposure also significantly increased the number of transitions between sleep-wake stages relative to control ($P < 0.05$ compared to control, Figure 2G). Between sexes, only episodes of REM were significantly greater in noise-exposed males relative to noise-exposed females ($P < 0.05$ compared to noise-exposed females, Figure 2E). These data demonstrate that unlike sleep-wake duration, dark period noise exposure has no effect on sleep quality in females but promotes sleep fragmentation in males by increasing episodes of wake and NREM, shortening their duration, and promoting more transitions between sleep-wake stages.

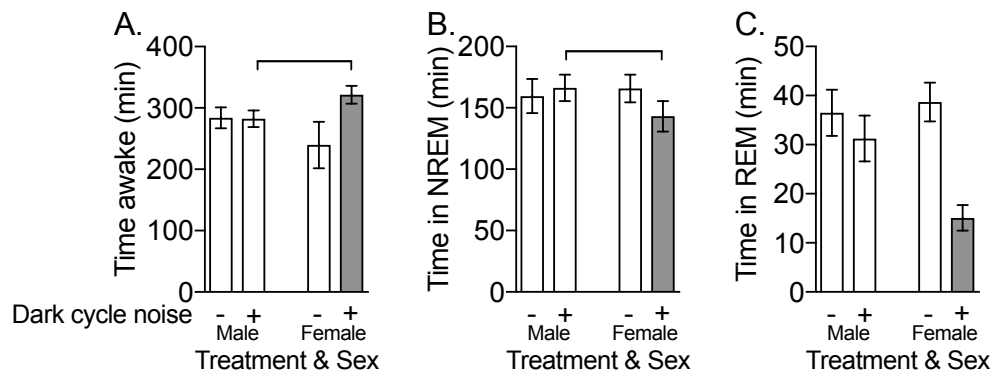


Figure 1. Environmental noise exposure during the dark period disturbs sleep in

female but not male rats. Male and female Sprague-Dawley rats (12 week old age) were implanted with EEG/EMG leads and EEG and EMG were measured continuously during exposure to environmental noise (8h/d during the dark period) for 1d. (A-C) Time spent in wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Significant differences ($P < 0.05$) within each sex were determined by paired t-tests. To determine differences between males and females, change from baseline was calculated for each rat and significant differences ($P < 0.05$) were determined by unpaired t-tests. Data are expressed as mean \pm SEM; $n = 8$ for males and $n = 6$ for females. The grey bar indicates a significant difference from control (no noise) and the brackets indicate significant differences between noise-exposed males and females. Note different scaling on y-axes.

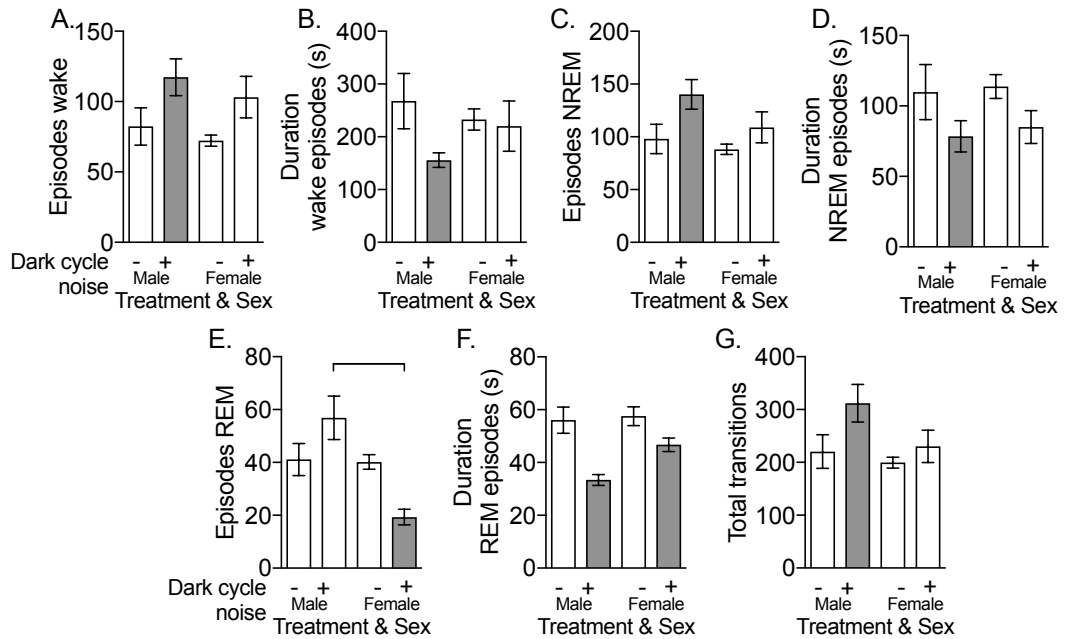


Figure 2. Environmental noise exposure during the dark period increases sleep fragmentation in male but not female rats. Male and female Sprague-Dawley rats (12 week old age) were implanted with EEG/EMG leads and EEG and EMG were measured continuously during exposure to environmental noise (8h/d during the dark period) for 1d. Indicators of sleep fragmentation including (A-B) number and duration of wake, (C-D) number and duration of non-rapid eye movement (NREM) sleep episodes, (E-F) number and duration of rapid eye movement (REM) sleep episodes, and (G) total transitions between sleep-wake stages. Significant differences ($P < 0.05$) within each sex were determined by paired t-tests. To determine differences between males and females, change from baseline was calculated for each rat and significant differences ($P < 0.05$) were determined by unpaired t-tests. Data are expressed as mean \pm SEM; $n = 8$ for males and $n = 6$ for females. The grey bar indicates a significant difference from control (no

noise) and the brackets indicate significant differences between noise-exposed males and females. Note different scaling on y-axes.

Dark period noise exposure has no effect on weight gain, food intake or SPA in female and male rats: In contrast to the stimulatory effect of chronic noise exposure during the light period on weight gain and food intake [41, 42], dark period noise exposure failed to increase weight gain or food intake relative to control in both sexes ($P > 0.05$ compared to control, Fig. 1A-C). Spontaneous physical activity indicated by distance traveled was also not significantly different between dark period noise-exposed males and females compared to their respective controls (Figure 3C). These data demonstrate that despite changes in sleep-wake duration or sleep fragmentation, dark period noise had no effect on weight gain or factors (e.g. SPA and food intake) known to promote weight gain due to chronic sleep loss [41, 42, 18, 17].

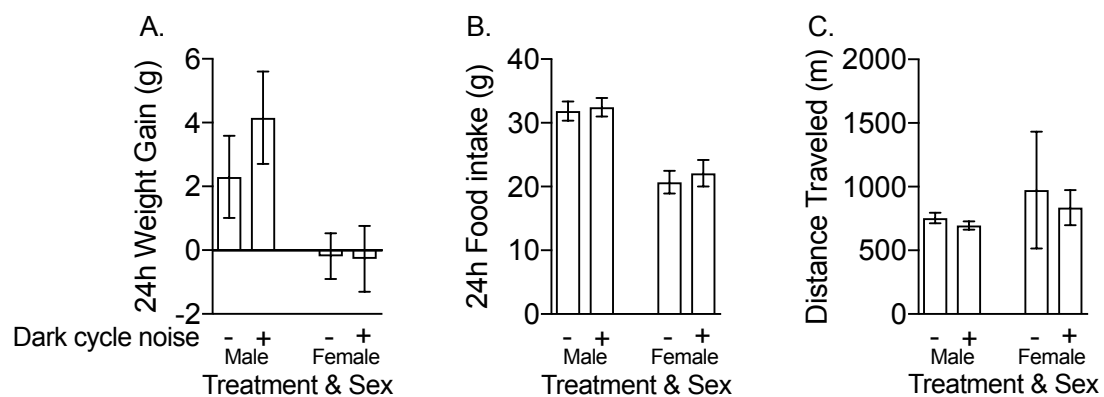


Figure 3. Dark period environmental noise exposure has no effect on weight gain, food intake or spontaneous physical activity (SPA) in male and female rats. Male and female Sprague-Dawley rats (12 week old age) were implanted with EEG/EMG leads and then exposed to environmental noise (8h/d during the dark period) for 1d. (A) 24h weight gain and (b) food intake was measured manually. (C) SPA was measured for the duration of exposure to environmental noise during the dark period (8h). Significant differences ($P < 0.05$) within each sex were determined by paired t-tests. To determine differences between males and females, change from baseline was calculated for each rat and significant differences ($P < 0.05$) were determined by unpaired t-tests. Data are expressed as mean \pm SEM; $n = 8$ for males and $n = 6$ for females. Note different scaling on y-axes.

DISCUSSION

Given the inherent stress of sleep disruption, we exposed both sexes to an acute exposure of noise during the dark period when sleep is inherently low to dissociate stress from noise-induced sleep disruption and thus determine its subsequent effect on weight gain [42, 41]. In contrast to noise exposure during the light period [42, 41], acute dark period noise exposure failed to stimulate weight gain or hyperphagia in males and females despite the fact that effects of dark period noise on sleep-wake and sleep fragmentation were dissimilar between the sexes. Collectively, our findings suggest that the weight gain observed during chronic noise-induced SD during the light period [42, 41] is not secondary to stress in either sex.

The relationship between sleep and stress has been debated since poor sleep is a stressor, indicated by elevated corticosterone levels. It's plausible that physiological

responses caused by sleep disturbance may be secondary to stress or that there may be additive effects between stress and sleep disturbance on weight gain or feeding. This is relevant for SD induced weight gain since stress does contribute to weight gain [96]. Disassociating stress from sleep disturbance by isolating the effect of sleep disturbance from stress may be experimentally unfeasible [97]. Nonetheless, we aimed to address the contribution of stress to noise-induced weight gain. To account for non-specific effects of stress, the noise-exposed group obtained an equal amount of noise exposure as obtained during the light period, but during the dark period when rats are normally awake. In contrast to light period noise exposure [41, 42, 95], which reduces both sleep time and quality in males and females, we show that 0-8h of noise exposure in the dark period reduced NREM and REM and increased time awake in female but not male rats (Figure 1). Furthermore, 0-8h of dark period noise exposure increased episodes of wake and NREM and shortened the duration of all sleep-wake episodes in male but not female rats (Figure 2). Due to the latter differences on sleep time and quality we also demonstrate that noise-exposed females spent more time awake, whereas sleep fragmentation was higher in noise-exposed males relative to the noise-exposed females (Figure 1 and 2). Collectively, these data demonstrate that dark period noise exposure disrupts sleep that occurs in the dark period in a sex-dependent manner.

Despite the fact that dark period noise exposure disturbed sleep time in females and sleep quality in males, weight gain, feeding and SPA were similar between noise-exposed males and females and their respective controls (no noise) (Figure 3). It is plausible that chronic exposure to dark period noise may augment weight gain and feeding since the acute exposure here did disrupt sleep time in females and sleep quality

in males. However, our preliminary study in male rats exposed to chronic noise during the dark period (8h/d for 9d) show similar weight gain [noise vs. control (mean \pm SEM): 14.5 ± 2.6 vs. 16.6 ± 3.8 , $P = 0.44$] and food intake [noise vs. control (mean \pm SEM): 262.6 ± 16.6 vs. 269.1 ± 12.5 , $P = 0.76$] over the 9d period compared to the control group that sleep ad libitum ($n = 7-8$ /group). Thus, the weight gain observed in our prior reports [95, 41, 42] following chronic noise-induced SD is likely mediated by mechanisms that are not solely dependent on stress since 9d of noise exposure during the dark period had no effect on weight gain or feeding in male rats. However, this would need to be confirmed by testing weight gain and feeding in response to chronic noise exposure during the dark period in female rats.

Noise exposure in male and female rats promotes weight gain by modulating food intake and energy expenditure, which maybe independent of stress based on the data presented here. Despite this, the neural mechanisms contributing to alterations in sleep, metabolism and weight gain following noise exposure in both sexes remains unknown.

Overall, the data presented here provide the first demonstration that the weight gain and hyperphagia observed during light period noise exposure may not be secondary to the effect of stress on these outcomes since dark period noise failed to alter weight gain and feeding independent of sex. Collectively, these data provide a foundation for future studies aimed at elucidating other mechanisms independent of stress contributing to noise-induced weight gain in both males and females and deciphering whether the latter mechanistic effects are sex-dependent.

CHAPTER THREE:

**Role of orexin-A in the ventrolateral preoptic area on components
of total energy expenditure**

RATIONALE

The precise neural mechanisms contributing to the noise-induced alterations in sleep-wake stages and energy expenditure (total and individual components) in are unknown. Thus, chapter three aimed at exploring neural mediators involved in the regulation of both sleep and energy metabolism with a specific focus on orexin-A, which is a neuropeptide that modulates arousal and metabolism. Central administration of orexin-A into the ventrolateral preoptic area (VLPO) (i.e. a brain site involved in sleep-wake regulation) increased time awake and total EE due to increased EE during PA [70]. This experiment (chapter three) sought to extend knowledge regarding the role of orexin-A and its receptors on energy metabolism in the VLPO by determining the effect of orexin receptor stimulation and antagonism on sleep-wake stages, total EE and other components of EE in addition to the EE during physical activity.

INTRODUCTION

Orexin-A (i.e. hypocretin-1) is an endogenous neuropeptide synthesized in lateral, dorsomedial and perifornical hypothalamic areas [98, 87] that modulates the sleep-wake cycle, energy balance, reward and autonomic function [99]. Physiological effects of orexin-A are mediated by two G protein-coupled receptors referred to as orexin 1 and 2 receptors (OXR1 and OXR2) [87]. Mutations in the canine OXR2 [100] and loss of orexin neurons in mice and humans [101, 88] are associated with narcolepsy [102, 103], which is characterized by disorganized sleep-wake transitions. Mice lacking orexin neurons exhibit narcolepsy, obesity, hypophagia and low physical activity [103], which illustrates the role of orexin in integrating sleep-wake stages and energy balance.

Central orexin-A administration reduces sleep and enhances wakefulness [89, 104], spontaneous physical activity (SPA, e.g. low intensity physical activity excluding exercise) [89, 105], energy expenditure (EE) [90], and food intake in a brain-site dependent manner [106]. Blocking both OXRs with dual orexin receptor antagonists (DORAs) promotes sleep and reduces wakefulness in several species [107, 108]. Dual orexin receptor antagonists also reduce basal [107, 108] and orexin-A-induced locomotor activity [109]. These data demonstrate that orexin-A impacts key behavioral processes, maintaining normal sleep-wake status and energy balance; however, the brain sites involved and the energy balance components affected are not fully defined.

The ventrolateral preoptic area (VLPO) is a brain site critical to sleep-wake regulation [110, 111]. The VLPO receives innervation from orexin neurons [112], contains both OXR subtypes [113, 66] and orexin-A infusion in the VLPO increases wakefulness and decreases sleep [114]. We recently confirmed the effects of orexin-A in

the VLPO on sleep-wake and also showed orexin-A increased SPA, total EE, and EE during SPA (i.e. non-exercise activity thermogenesis) [115], with no effect on feeding [70]. Together, these data imply that the VLPO may be an important node for integration of orexin-A signals that influence sleep-wake and metabolism.

The contribution of orexin-A in the VLPO to sleep-wake status, SPA, and total EE for regulating energy metabolism is evident. However, a better understanding of how orexin-A in the VLPO contributes to overall increases in total EE is warranted. Here we determined the effect of orexin-A in the VLPO on individual components of total EE; whether blocking both OXRs with a DORA reduced effects of orexin-A; and the effect of a DORA alone on sleep-wake, SPA, total EE, and components of total EE. We hypothesized that 1) orexin-A in the VLPO would increase components of total EE, 2) the DORA would reduce orexin-A-stimulated increases in wakefulness, SPA, total EE, components of EE and prevent the reduction in sleep and 3) a DORA alone would reduce basal total EE and its components.

MATERIALS AND METHODS

Animals: One set of three-month old male Sprague-Dawley (N = 7) rats (Charles River Laboratories, Kingston, New York USA) were housed individually in solid-bottom cages in a temperature-controlled room (21-22 °C) with a 12-h light/12-h dark period (lights on at 0600h). Rodent chow (Harlan Teklad 8604) and water were allowed *ad libitum* except during the 2.5h test period. Study procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Surgery: Rats were anesthetized and surgically implanted with a 26-gauge stainless steel

cannula (Plastics One, Roanoke, Virginia USA) targeted towards the VLPO and a radiotelemetric transmitter connected to electroencephalogram (EEG) and electromyogram (EMG) electrodes (F40-EET, Data Sciences International, Saint Paul, Minnesota USA) as described [66, 70]. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson [67]. Coordinates for the cannula and EEG electrodes, respectively, were as follows: -0.12 mm and 3.1 mm posterior to bregma, +/- 1.5 mm lateral to bregma and 0.8 mm below the skull surface. Experimental trials began ten days after surgery.

Drugs: Orexin-A (American Peptides, Sunnyvale, California USA) was dissolved in artificial cerebrospinal fluid (Sigma-Aldrich, Saint Louis, Missouri USA), which served as the vehicle-control for orexin-A. The DORA (TCS-1102; Tocris Bioscience, Saint Paul, Minnesota USA) was dissolved in DMSO/methanol HCl/sterile water, which served as the vehicle-control for the DORA. All drugs were stored frozen and then at 4 °C for < 48-h.

Injections: A volume of 0.5 µL was injected over 30-seconds with a 33-gauge injector (Plastics One, Roanoke, Virginia USA) that extended 1.0 mm beyond the tip of the guide cannula [70]. Injections were performed between 0800-1000h (>48-h between injections). Previous studies demonstrate that repeated injections do not cause tissue damage as measured by lack of gliosis around the injection site [116] or reduce the efficacy of orexin-A to stimulate SPA [117], suggesting maintenance of tissue integrity and behavioral responses to orexin-A with repeated injections.

Verification of cannula placement by histology: Brains were dissected out and stored in

10% formaldehyde. Cannulae were deemed incorrectly placed if >0.25 mm from the targeted site. This rationale is based on diffusion coefficients of the injection volume delivered [118]. All cannulae were correctly placed (Figure 1).

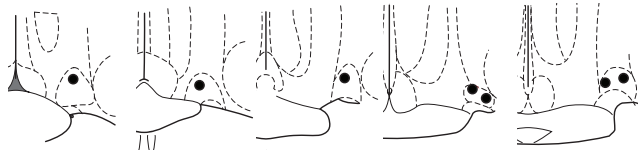


Figure 1. Histological verification map showing correct placement of injection sites into the ventrolateral preoptic area.

Concurrent EEG, EMG, spontaneous physical activity and indirect calorimetry measurements: A receiver was placed beneath the test cage to allow EEG and EMG signals to be recorded from the implanted EEG and EMG electrodes and transmitter [66]. Energy expenditure and SPA were determined with an indirect calorimeter and infrared beam break sensors (e.g. 1 cm spacing), respectively that measured O_2 , CO_2 , water vapor for the calculation of energy expenditure and distance traveled for the calculation of SPA continuously each second from each chamber simultaneously (Promethion-C, Sable Systems Inc. Las Vegas, Nevada USA) [70]. Gas analyzers were calibrated prior to each test with primary gas standards (100% Nitrogen and 1% CO_2) [119, 70]. The flow rate was maintained at 2500 mL/min. Rats were acclimated to the chambers (3-h/day for three consecutive days) with food and water *ad libitum* prior to the test injections. Water was available *ad libitum* during testing. Data were processed with Expedata software v1.7.30 (Sable Systems Inc. Las Vegas, Nevada USA) [70]. The respiratory quotient was defined as the mean respiratory exchange ratio during the measurement period.

Determining sleep-wake stages: Electroencephalogram and EMG data were visualized with Neuroscore software (version 2.0.1, Data Sciences International, Saint Paul, Minnesota USA) [70]. Consecutive 15-second epochs of EEG and EMG were manually scored to determine active wake (AW), quiet wake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep [66].

Determining components of total energy expenditure: Total EE was calculated with the Weir equation [72]. Individual components of total EE include: EE during SPA, wake (active wake + quiet wake), active wake, quiet wake, rest, sleep (NREM sleep + REM sleep), NREM sleep, and REM sleep. These individual components of EE will be referred to as EE during SPA, EE during wake, EE during active wake, EE during quiet wake, resting EE, EE during sleep, EE during NREM sleep, and EE during REM sleep. These data were derived from EEG and EMG recordings (scored as active wake, quiet wake, NREM sleep and REM sleep), distance traveled indicated by infrared beam break sensors, and total EE based on the time-stamp [70]. Individual components of total EE were calculated in accordance with previously described methods[42]. Briefly, EE during SPA was calculated as the sum of EE when rats were awake and moving based on the infrared beam break sensors and EEG/EMG recordings. Resting EE was calculated as the calories when the rat was awake but not moving or sleeping based on infrared beam break sensors, EEG, and EMG. Active wake and quiet wake EE was calculated as the calories when the animal was awake and either moving (i.e. active wake) or not moving (i.e. quiet wake) based on EMG radiotelemetric activity counts. Energy expenditure during NREM sleep and REM sleep was calculated as the calories when the rat was either in NREM sleep or REM sleep based on EEG and EMG. Energy expenditure during

total wake was calculated as the calories when the animal was in either active or quiet wake. Total sleep EE was calculated as the calories during both NREM sleep and REM sleep. We did not distinguish diet-induced thermogenesis from resting EE since diet-induced thermogenesis was likely minimal because food was unavailable during testing and tests were performed in the early light period. We have previously shown that this rat strain consumes < 10% of their total 24-h caloric intake within this time interval [120].

Experimental Design: The DORA (62.5 nmol/0.5 μ L) or vehicle-control was injected into the VLPO through the cannula 20 min prior to an injection of orexin-A (62.5 pmol/0.5 μ L) or vehicle-control. Treatments were given in a randomly assigned latin-square unblinded design. Measurements (EEG, EMG, SPA and EE) were taken for 2.5-h post-injection. Duration of measurements and doses for the DORA and orexin-A were based on previous reports [114, 70]. All experimental procedures were completed once.

Statistical Analysis: Data were analyzed with Prism 6.0f (GraphPad Software, Inc., San Diego, California USA). Data are expressed as mean \pm SEM. Alpha was 0.05 for all statistical tests. Data were analyzed with repeated measures ANOVA followed by Fischer's tests to determine differences between individual treatments. All assumptions for repeated measures ANOVA were met. Sample size was based on power calculations from previous report [70]. Since handling involved in the injection procedure augments wakefulness and SPA for up to 20 min post-injection independent of treatment, the first 20 min of data collection post-injection were excluded from data analysis [121]. Therefore, data were analyzed in the 20-80, 80-140, and 20-140 minute post-injection time periods, which will be referred to as the 1-h, 1-2 h, and 2-h post-injection. A

separate analysis was completed for each time period and endpoint (Table 1).

The following endpoints were analyzed: 1) sleep-wake status: percent time spent in wake, active wake, quiet wake, total sleep, NREM sleep, and REM sleep, number of episodes and mean duration of each sleep-wake state; total transitions between sleep-wake stages; latency to sleep onset; 2) physical activity and EE: respiratory quotient, total EE, SPA indicated by distance traveled based on infrared sensors and EE during SPA; 3) EE during sleep-wake stages: EE during wake, EE during active wake, resting EE, EE during sleep, EE during NREM sleep, and EE during REM sleep. There were no main effects on quiet wake for the aforementioned endpoints (data not shown). Thus, the effect of treatments on wake was due to active wake rather than quiet wake since wake was defined as the sum of active plus quiet wake (Table 1).

RESULTS

The DORA prevents the effects of orexin-A in the VLPO on sleep-wake stages: We hypothesized that the DORA in the VLPO would reduce orexin-A-stimulated increases in arousal and reductions in sleep. The DORA reduced the effect of orexin-A on sleep-wake stages and improved sleep quality (Figure 2, Table 1). Orexin-A significantly increased wake and active wake 1-h and 2-h post-injection compared to control (Figures 2A-2B, $P < 0.05$ for all comparisons). Orexin-A significantly reduced sleep (total, NREM sleep and REM sleep) 1-h post-injection relative to control (Figures 2C, 2D, $P < 0.05$ for all comparisons). The DORA reduced the orexin-A-stimulated increase in wake and active wake (Figures 2A, $P < 0.05$ for all comparisons). Likewise, the DORA reversed the orexin-A-stimulated reduction in sleep and NREM sleep but not REM sleep 1-h post-injection (Figures 2C-2D, $P < 0.05$ for all comparisons except REM sleep: $P = 0.06$).

Two hours post-injection, the DORA failed to significantly reduce orexin-A-stimulated increase in wake and active wake (Figure 2B, $P > 0.05$ for all comparisons, data not shown).

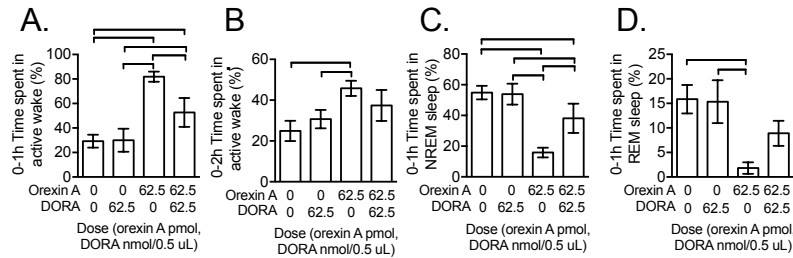


Figure 2. Orexin-A infusion in the ventrolateral preoptic area (VLPO) significantly increases time spent in (A, B) active wake and decreases time spent in (C) non-rapid eye movement (NREM) sleep and (D) rapid eye movement (REM) sleep compared to control. Pre-treatment with the dual orexin receptor antagonist (TCS-1102, DORA) in the VLPO reduces orexin-A-stimulated increases in (A) time spent in active wake and the orexin-A-stimulated reduction in (C) time spent in NREM sleep. Data are expressed as mean \pm SEM; $N = 7$. Brackets indicate bars that are significantly different from each other ($P < 0.05$). Note different scaling on y-axes.

Table 1. Repeated measures ANOVA for all endpoints

		Time period		
		<u>0-1h</u>	<u>1-2 h</u>	<u>0-2h</u>
Time spent in sleep-wake stages				
total wake	$F_{3,18} = 15.4, P < 0.0001$	$F_{3,18} = 1.2, P = 0.3330$	$F_{3,18} = 3.7, P = 0.0296$	
active wake	$F_{3,18} = 15.3, P < 0.0001$	$F_{3,18} = 1.2, P = 0.3318$	$F_{3,18} = 3.9, P = 0.0247$	
quiet wake	$F_{3,18} = 1.1, P = 0.3642$	$F_{3,18} = 0.6, P = 0.6165$	$F_{3,18} = 1.9, P = 0.1585$	
total sleep	$F_{3,18} = 15.0, P < 0.0001$	$F_{3,18} = 1.2, P = 0.3329$	$F_{3,18} = 2.8, P = 0.0696$	
NREM sleep	$F_{3,18} = 13.6, P < 0.0001$	$F_{3,18} = 1.5, P = 0.2416$	$F_{3,18} = 1.9, P = 0.1697$	
REM sleep	$F_{3,18} = 7.1, P = 0.0024$	$F_{3,18} = 0.5, P = 0.6805$	$F_{3,18} = 2.5, P = 0.0873$	
Sleep quality				
<u>Episodes</u>				
total wake	$F_{3,18} = 1.0, P = 0.4016$	$F_{3,18} = 0.6, P = 0.6192$	$F_{3,18} = 1.4, P = 0.2823$	
active wake	$F_{3,18} = 1.6, P = 0.2270$	$F_{3,18} = 0.8, P = 0.4972$	$F_{3,18} = 1.7, P = 0.2007$	
quiet wake	$F_{3,18} = 1.6, P = 0.2326$	$F_{3,18} = 0.4, P = 0.7800$	$F_{3,18} = 1.7, P = 0.1930$	
total sleep	$F_{3,18} = 4.4, P = 0.0176$	$F_{3,18} = 1.1, P = 0.3557$	$F_{3,18} = 2.7, P = 0.0784$	
NREM sleep	$F_{3,18} = 3.0, P = 0.0566$	$F_{3,18} = 1.0, P = 0.3924$	$F_{3,18} = 1.9, P = 1.609$	
REM sleep	$F_{3,18} = 4.4, P = 0.0169$	$F_{3,18} = 0.7, P = 0.5794$	$F_{3,18} = 2.4, P = 0.1037$	
<u>Mean duration of episodes of</u>				
total wake	$F_{3,18} = 0.2, P = 0.8921$	$F_{3,18} = 1.4, P = 0.2784$	$F_{3,18} = 0.1, P = 0.9815$	
active wake	$F_{3,18} = 0.2, P = 0.9103$	$F_{3,18} = 1.3, P = 0.3150$	$F_{3,18} = 0.0, P = 0.9848$	
quiet wake	$F_{3,18} = 1.5, P = 0.2520$	$F_{3,18} = 0.6, P = 0.6189$	$F_{3,18} = 1.3, P = 0.2943$	
total sleep	$F_{3,18} = 6.0, P = 0.0051$	$F_{3,18} = 0.6, P = 0.6241$	$F_{3,18} = 2.5, P = 0.0925$	
NREM sleep	$F_{3,18} = 4.1, P = 0.0213$	$F_{3,18} = 1.2, P = 0.3487$	$F_{3,18} = 1.4, P = 0.2750$	
REM sleep	$F_{3,18} = 4.9, P = 0.0117$	$F_{3,18} = 0.6, P = 0.5941$	$F_{3,18} = 1.9, P = 0.1636$	
<u>latency sleep onset</u>	$F_{3,18} = 11.3, P = 0.0002$	$F_{3,18} = 1.8, P = 0.1822$	$F_{3,18} = 2.0, P = 0.1424$	
<u>total transitions</u>	$F_{3,18} = 3.0, P = 0.0555$	$F_{3,18} = 1.2, P = 0.3258$	$F_{3,18} = 2.6, P = 0.0829$	
Distance traveled	$F_{3,18} = 17.6, P < 0.0001$	$F_{3,18} = 1.9, P = 0.1610$	$F_{3,18} = 6.6, P = 0.0034$	
RQ	$F_{3,18} = 1.3, P = 0.2995$	$F_{3,18} = 3.0, P = 0.0568$	$F_{3,18} = 2.2, P = 0.1204$	
Total EE	$F_{3,18} = 21.6, P < 0.0001$	$F_{3,18} = 2.8, P = 0.0668$	$F_{3,18} = 8.5, P = 0.0010$	
Components of total EE				
EE SPA	$F_{3,18} = 22.5, P < 0.0001$	$F_{3,18} = 1.6, P = 0.2231$	$F_{3,18} = 9.1, P = 0.0007$	
EE wake	$F_{3,18} = 13.5, P < 0.0001$	$F_{3,18} = 1.4, P = 0.2884$	$F_{3,18} = 11.8, P = 0.0002$	
EE active wake	$F_{3,18} = 15.7, P < 0.0001$	$F_{3,18} = 2.4, P = 0.1058$	$F_{3,18} = 25.0, P < 0.0001$	
EE rest	$F_{3,18} = 18.2, P < 0.0001$	$F_{3,18} = 3.3, P = 0.0474$	$F_{3,18} = 17.9, P < 0.0001$	
EE sleep	$F_{3,18} = 5.9, P = 0.0056$	$F_{3,18} = 2.9, P = 0.0627$	$F_{3,18} = 3.3, P = 0.0431$	
EE NREM sleep	$F_{3,18} = 6.9, P = 0.0028$	$F_{3,18} = 3.2, P = 0.0499$	$F_{3,18} = 3.3, P = 0.0453$	
EE REM sleep	$F_{3,18} = 3.6, P = 0.0858$	$F_{3,18} = 2.6, P = 0.0820$	$F_{3,18} = 3.3, P = 0.0452$	

EE: energy expenditure, NREM: non-rapid eye movement sleep, REM: rapid eye movement sleep, RQ: respiratory quotient, SPA: spontaneous physical activity.

The DORA reduced the effect of orexin-A on sleep quality (Figure 3, Table 1). Pre-treatment with the DORA reversed orexin-A-stimulated reductions in the number of total and REM sleep episodes ($P < 0.05$ for all comparisons, Figure 3A, 3B). The DORA failed to significantly reduce the mean duration of sleep episodes 1-h post-injection ($P > 0.05$, Figures 3C). Orexin-A significantly prolonged the latency to sleep onset while the DORA blocked the orexin-A-stimulated increase in the latency to sleep onset 1-h post-injection ($P < 0.05$ for all comparisons, Figure 3F).

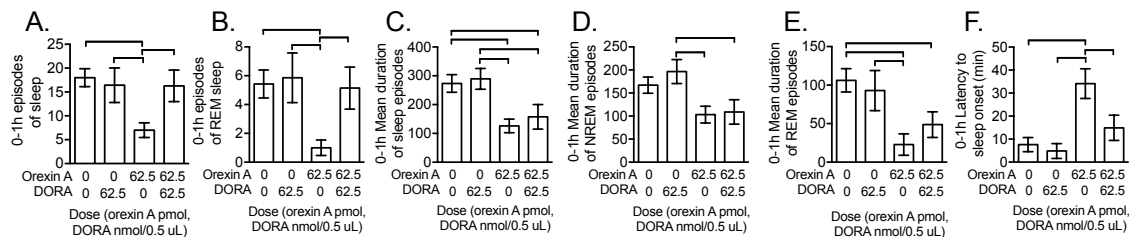


Figure 3. Orexin-A in the ventrolateral preoptic area (VLPO) significantly (F) prolongs the latency to sleep onset and reduces (A) episodes of sleep, (B) episodes of rapid eye movement (REM) sleep, (C) mean duration of sleep episodes, and (E) mean duration of REM sleep episodes but not (D) mean duration of non-rapid eye movement (NREM) sleep episodes ($P = 0.058$) compared to control. Pre-treatment with the dual orexin receptor antagonist (TCS-1102, DORA) in the VLPO significantly reversed the orexin-A-stimulated increase in the (A) episodes of sleep and (B) episodes of REM sleep and the latency to sleep onset. Data are expressed as mean \pm SEM; $N = 7$. Brackets indicate bars that are significantly different from each other ($P < 0.05$). Note different scaling on y-axes.

The DORA prevents the effects of orexin-A in the VLPO on SPA and total EE: We

hypothesized that blocking OXRs would reduce basal and orexin-A-stimulated increase in SPA and total EE. The DORA reduced the effects of orexin-A on SPA and total EE (Figure 4, Table 1). Orexin-A significantly increased SPA and total EE 1 and 2-h post-injection (Figures 4A-4D, $P < 0.05$ for all comparisons). The DORA significantly blocked orexin-A-stimulated increases in total EE (Figure 4C-4D) and reduced SPA stimulated by orexin-A 1 but not 2-h post-injection (Figure 4A-4B). The DORA alone significantly reduced basal total EE 2-h post-injection relative to control (Figure 4D).

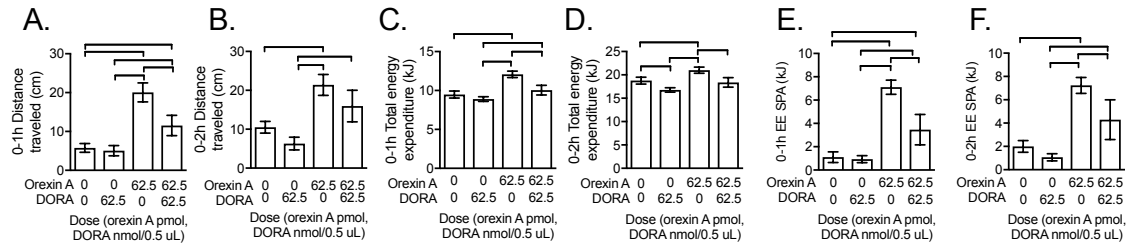


Figure 4. Pre-treatment with the dual orexin receptor antagonist (TCS-1102, DORA) in the ventrolateral preoptic area (VLPO) significantly reduced orexin-A stimulated increases in (A, B) distance traveled and (C, D) total energy expenditure (EE) and (E, F) EE during spontaneous physical activity (SPA) (D) The DORA alone significantly reduced (D) total EE compared to control 2-h post-injection. Data are expressed as mean \pm SEM; $N = 7$. Brackets indicate bars that are significantly different from each other ($P < 0.05$). Note different scaling on y-axes.

The DORA prevents the effects of orexin-A on components of total EE:

We hypothesized that the DORA would reduce orexin-A-stimulated effects on EE during SPA, wake, rest and sleep as well as reduce basal levels of total EE and its components.

Orexin-A significantly increased EE during SPA, active wake, rest, and during NREM sleep compared to control 1-h post-injection (Figure 4E, Table 2, $P < 0.05$ for all comparisons). Two hours post-injection, orexin-A significantly increased EE during SPA, active wake, and rest (Figure 4F, Table 2, $P < 0.05$ for all comparisons).

The DORA significantly reduced orexin-A stimulated increases in EE during SPA, active wake, rest, and NREM sleep 1 and 2-h post-injection (Figure 4E, 4F, Table 1 and 2, $P < 0.05$ for all comparisons). The DORA alone significantly reduced EE during active wake, REM sleep, rest, and NREM sleep (Table 2, $P < 0.05$ for all comparisons).

Table 2. Components of total energy expenditure (kilojoules/hr) for each treatment during the 0-1, 1-2 and 0-2h post-injection time period.

Components of total EE		Treatment			
		control/control	DORA/control	control/orexin A	DORA/orexin A
EE-active wake					
	0-1h:	10.04 ± 0.67	9.24 ± 0.53	12.47 ± 0.40* [§]	10.82 ± 0.52 ^{§φ}
	1-2h:	9.54 ± 0.50	7.85 ± 0.19	8.85 ± 0.42	8.87 ± 0.66
	0-2h:	9.87 ± 0.44	8.66 ± 0.33*	12.17 ± 0.40* [§]	10.23 ± 0.51 ^{§φ}
EE-Rest					
	0-1h:	9.72 ± 0.62	8.98 ± 0.45	12.23 ± 0.36* [§]	10.20 ± 0.61 ^{§φ}
	1-2h:	9.44 ± 0.49	7.82 ± 0.20*	8.84 ± 0.42	8.50 ± 0.42
	0-2h:	9.41 ± 0.43	8.41 ± 0.26*	11.38 ± 0.27* [§]	9.54 ± 0.50 ^{§φ}
EE-NREM sleep					
	0-1h:	9.34 ± 0.32	8.89 ± 0.25	11.25 ± 0.59* [§]	9.62 ± 0.56 ^φ
	1-2h:	9.23 ± 0.52	7.91 ± 0.23*	8.98 ± 0.33 [§]	8.13 ± 0.33*
	0-2h:	9.28 ± 0.35	8.39 ± 0.20*	9.36 ± 0.32 [§]	8.53 ± 0.30 ^φ
EE-REM sleep					
	0-1h:	8.95 ± 0.32	8.12 ± 0.15	9.69 ± 0.47	8.38 ± 0.24
	1-2h:	9.01 ± 0.53	7.83 ± 0.22	8.81 ± 0.34	8.06 ± 0.27
	0-2h:	8.99 ± 0.36	7.97 ± 0.19*	8.84 ± 0.34 [§]	8.22 ± 0.25

EE: energy expenditure, NREM: non-rapid eye movement sleep, REM: rapid eye movement sleep. DORA: dual orexin receptor antagonist. Data expressed as mean ± SEM; N = 7. * $P < 0.05$ as compared to vehicle/vehicle, $^{\S}P < 0.05$ as compared to antagonist/vehicle, $^{\phi}P < 0.05$ as compared to vehicle/orexin-A.

DISCUSSION

Previously we showed that orexin-A in the VLPO decreased sleep and enhanced wakefulness, SPA, total EE, and EE during SPA, with no effect on feeding [70]. Our data here verify and extend those results [114, 70]. The current study demonstrates that orexin-A in the VLPO affects individual components of total EE, while blockade of both OXRs reduces stimulation of EE (total and individual components) by orexin-A. In addition, total EE and its components were lower after OXR blockade. The stimulatory effect of orexin-A on total EE is due to enhancement of several individual components of total EE (i.e. resting EE and EE during SPA, wake, and NREM sleep, Figure 4 and Table 2), and antagonizing both OXRs prevents these effects. Finally, we demonstrate that DORA blockade of endogenous OXR stimulation in the VLPO reduces total EE by decreasing resting EE and EE during wake, active wake, NREM sleep, and REM sleep. These data are novel and show that the stimulatory effect of orexin-A in the VLPO on total EE involves an increase in several components of EE. In addition, blocking OXRs lowers total EE through reductions in non-SPA related EE independent of the time spent asleep. Given the role of orexins in obesity resistance [122], narcolepsy [102], and insomnia [123], these data have implications for developing effective treatments to combat obesity and sleep disorders.

Orexin-A in the VLPO significantly increased time in active wake and decreased sleep (Figure 2). These data parallel results from other studies of orexin-A administration in the VLPO [114, 70] and other brain nuclei central to sleep-wake regulation [89, 124]. Prolongation of wakefulness by orexin-A was due to a reduction in the duration and number of sleep episodes [114] (Figure 3). We have previously shown [70] a positive

effect of orexin-A in the VLPO on SPA, total EE, and EE during SPA. The results here (Figures 4) are concordant with those data [70] and agree with others [90, 89, 125, 105, 126].

Based on our work [70] and others, which shows that orexin-A increases resting metabolism [90], body temperature [127] brown adipose tissue thermogenesis [128], and autonomic function [129]; we hypothesized that orexin-A in the VLPO would increase other components of total EE. Our data show that the stimulatory effect of orexin-A in the VLPO on total EE is due to other EE components in addition to the EE during SPA, which is a novel finding. Moreover, the metabolic and sleep-wake effects of orexin-A can be dissociated since orexin-A decreased time spent in NREM sleep and REM sleep but only increased EE during NREM sleep. Finally, the latter suggests that orexin-A in this brain site may have an independent effect on EE during NREM sleep versus REM sleep. Together, our data suggest that orexin-A contributes to negative energy balance by increasing multiple components of total EE in addition to SPA [91].

Next, we tested whether blocking both OXRs reduced the aforementioned effects of orexin-A. The DORA reduced orexin-A-stimulated increases in active wake, SPA, total EE, resting EE, and EE during SPA, active wake and NREM sleep. Moreover, the DORA reduced orexin-A-induced reductions in NREM sleep and sleep quality. The reduction of orexin-A-induced active wake by the DORA agrees with our prior work with this specific antagonist [70] and others who have reported that another DORA, Almorexant, reversed SPA stimulated by orexin-A [109]. Antagonism of OXR1 also reverses orexin-A-stimulated SPA and total EE [125]. Together, these data highlight the importance of OXR stimulation for increasing arousal and total EE. Future studies are

needed to determine whether either receptor has a more prominent role in sleep-wake regulation or EE, or whether each receptor contributes equally to these processes.

Based on the efficacy of DORAs to increase sleep [107, 130] and suppress SPA [108], we hypothesized that preventing endogenous OXR stimulation with the DORA alone would reduce total EE mainly through effects on SPA. As expected, blocking endogenous OXRs reduced total EE and several EE components. Most interestingly, the DORA alone failed to significantly alter sleep time or reduce SPA and its resultant EE, a result that contrasts with previously published data [107-109]. This discrepancy may be related to study design since the type and dose of the DORA used, animal species tested, route of administration and the measurement duration differed between our study and these previous reports[107-109]. We also show that the DORA alone did not reduce basal SPA or its corresponding EE, which agrees with studies that showed DORAs (Merck DORA-12 and Almorexant) had no effect on rotarod activity [131], but contrasts with another that reported that a DORA (Merck DORA-1) reduced basal dark period locomotion [108]. The fact that the DORA failed to reduce basal SPA here may be due to a “floor-effect” for physical activity, as the injections were performed in the early light period when SPA is inherently low. It is plausible that the DORA injections in the early dark period may reduce SPA, as shown previously [108], and thus its resultant EE. Interestingly, basal total EE was significantly lower in rats treated with the DORA alone 2-h post-injection (Figure 4D), which suggests that factors aside from SPA contribute to the DORA-induced decrease in basal total EE.

Blocking endogenous OXR stimulation in the VLPO with the DORA alone significantly reduced EE during active wake, rest, NREM sleep and REM sleep. This

demonstrates that blocking endogenous OXR stimulation may reduce baseline total EE independent of SPA, since the DORA alone had no effect on SPA or its corresponding EE. More than 50% of total daily EE is due to resting EE [132]. Thus, the DORA-induced reductions in resting EE and NREM sleep 2-h post-injection are significant factors contributing to the overall reduction in total EE. Moreover, that the DORA alone had no effect on time in active wake, REM sleep or NREM sleep, yet reduced their associated EE suggests that rats simply expended less energy in response to this specific DORA regardless of whether they were awake or asleep. The latter is significant since individuals with insomnia would be prescribed a DORA prior to bedtime to promote sleep. Hence, blocking endogenous OXR stimulation with the DORA alone may reduce EE during sleep in these individuals. The fact that basal total EE and several components (EE during active wake, NREM sleep, REM sleep and rest) were significantly lower after administration of the DORA alone underscores the importance of quantifying components of total EE, and the potential effect of OXR antagonism on energy balance. We tested a low dose of the DORA; thus, it is plausible that higher doses would further reduce basal total EE. This latter and our data therefore have implications for insomnia therapies as it is unclear whether long-term antagonism of OXRs would promote positive energy balance through reductions in total EE and favor weight-gain. The effects of chronic OXR antagonism on food intake, total EE, and weight gain remain to be tested.

The mechanism(s) underlying the physiological effects of orexin-A in the VLPO remain unclear. Orexin-A increases firing rates of arousal-promoting neurons, including the locus coeruleus noradrenergic cells [89], the tuberomammillary nucleus histaminergic cells [133], cholinergic neurons in the pedunculopontine and laterodorsal tegmental

nuclei [134], and the dorsal raphe serotonergic neurons [135]. Orexin-A also stimulates release of noradrenaline, histamine, acetylcholine, and serotonin [136, 124, 137, 138]. The arousal centers promote wakefulness to some extent by inhibiting GABA and galanin neurons in the VLPO [139-142]. Based on these data and OXRs in the VLPO, a hypothetical mechanism by which orexin-A in the VLPO may promote wakefulness is by enhancing the activity of arousal-promoting nuclei through binding to OXRs on the VLPO neurons directly, or to the terminals from arousal-promoting neurons. Orexin-A given in some brain areas (e.g. after ventricular injection) has also been shown to influence autonomic outflow [143, 129], through increases in heart rate, mean arterial blood pressure and temperature in conscious [129, 127] and anesthetized [144, 127] rodents, and could be one mechanism underlying increases in resting EE by orexin-A. Thus, blocking both OXRs with a DORA alone would be expected to reduce resting EE. Yet, the DORA (Almorexant) had no effect on body temperature [107] heart rate or mean arterial pressure [145] in normal rats independent of time of administration.

It is plausible that diffusion of orexin-A into the supraoptic nucleus (SON) contributed to our results since this brain site contains OXR1 protein [146], orexin fibers [143], and expresses c-fos after ventricular orexin-A infusion [143]. However, no studies have discriminated between orexin-A action in the SON versus VLPO on sleep-wake stages or other endpoints reported here. Moreover, while SON might be involved in circadian processes [147], its precise role in sleep-wake remains undefined. Future studies should distinguish the metabolic and behavioral effects of orexin-A injections in the SON and VLPO.

In conclusion, we show that blocking OXRs with a DORA reduces the effects of orexin-A in the VLPO on total EE and several of its components. We also show that blocking endogenous OXR stimulation by the DORA alone reduces basal total EE primarily by reducing EE during rest and sleep. This is the first demonstration that stimulation and antagonism of OXRs has disparate effects on the components of total EE. Our results suggest that OXR stimulation may contribute to negative energy balance through increases in EE during SPA, rest and NREM sleep if orexin-A was given in the active period, while OXR may contribute to positive energy balance by decreasing non-SPA related EE. These results imply that current therapies for obesity and insomnia may have unintended and likely unwanted effects on body weight.

CHAPTER FOUR:

Sex-dependent effects of Suvorexant on sleep disruption due to environmental noise exposure

RATIONALE

Data from chapter three demonstrated that administration of orexin-A in the VLPO of male rats increased total EE by increasing several of its components and that pre-treatment with a dual orexin receptor antagonist (DORA) could block these effects by orexin-A. These data extended knowledge regarding the role of orexin-A and its receptors on energy metabolism, which resulted in a publication from our lab demonstrating that the efficacy of orexin-A to stimulate total EE and the EE during PA in the VLPO of male rats was reduced following both acute and chronic exposure noise-induced sleep disruption [43]. While the latter study and the experiment conducted as part of the current study (chapter three) must now be replicated in female rats, the data provides evidence that alterations in orexin signaling may underlie the responses to noise previously reported in males [41, 42] and observed during the experiment in females described in chapter one .

Results from chapter three also demonstrated that administration of a DORA alone reduced non-stimulated EE (total and its components). This finding is novel and provided the rationale for the experiment described in this chapter as it suggests that current orexin-based therapies to treat disordered sleep in men and women may have unintended effects on EE. Thus, the overall goal of the lab is to determine next whether Suvorexant (i.e. an FDA approved DORA) can ameliorate weight gain due to noise-induced sleep disruption, hyperphagia and the reductions in EE in both male and female rats. Thus, this experiment (chapter four) was designed to begin to address this question by performing a dose-response study to determine the lowest effective dose of

Suvorexant (i.e. FDA approved DORA for insomnia) that could block noise-induced sleep disruption independent of sex.

INTRODUCTION

Insomnia is a prevalent sleep disorder [148], characterized by difficulty initiating or maintaining sleep [149], that increases risk of obesity [150], cardiovascular disease [151] and psychiatric disorders [152]. Women are 50% more likely to report insomnia [153], have increased sensitivity to stimuli that disrupt sleep [52] and are more sensitive to weight gain following sleep disruption relative to men [55]. Pharmaceuticals are prescribed to treat insomnia but the recommended doses differ by sex and their effect on weight gain has been understudied. For example, the dose for Zolpidem, a hypnotic benzodiazepine receptor agonist, and Suvorexant, a dual orexin receptor antagonist (DORA), is half for women due to slower clearance relative to men despite administration of the same dose [154, 155]. These guidelines imply a sex-specific sensitivity to therapies that improve sleep. However, they do not address whether women may require a higher dose if women are indeed more sensitive to sleep disruption or if a different dose is required to mitigate the weight gain due to insomnia.

Orexins (orexin-A and orexin-B, also known as hypocretins) are neuropeptides synthesized in discrete hypothalamic areas [98, 87] that regulate sleep-wake, metabolism and reward [99] by acting at orexin 1 and 2 receptors (OXR) [87]. Lack of orexin neurons leads to the sleep disorder narcolepsy in humans [101, 88]. Moreover, central administration of orexin-A promotes wakefulness and reduces both non-rapid eye movement (NREM) and rapid eye movement sleep (REM) [104, 89]. Based on the crucial role of orexins in arousal and stabilization of sleep/wake states, antagonism of OXRs have been targeted for insomnia treatment.

Suvorexant (i.e. MK-4305) is currently the first, FDA approved DORA for insomnia. Like other DORAs that promote sleep [107, 108], Suvorexant reduces wakefulness and promotes sleep in males [156, 157] but has yet to be tested in non-pregnant females [155]. In healthy individuals and those with insomnia, Suvorexant improves subjective total sleep time and time to sleep onset while also reducing wake after sleep onset, latency to persistent sleep and REM sleep relative to placebo [130, 158, 159]. Despite the fact that women taking Suvorexant at lower doses than men self-reported more relative adverse events, sex differences in sleep/wake have not been analyzed while controlling for physiological differences in sleep between men and women due to the female menstrual cycle [160]. Thus, it remains unknown whether weight gain in response to Suvorexant differs between men and women with insomnia.

Exposure to pre-recorded environmental noise during the light period causes sleep disruption indicated by increased wakefulness and sleep fragmentation as well as stimulates weight gain through hyperphagia and reductions in energy expenditure in male and female rats [42, 41, 43, 95]. Since Suvorexant increases sleep, it's plausible that Suvorexant may also ameliorate weight gain caused by sleep disruption in a sex-dependent manner. To begin to address this, we performed a dose-response study to first determine whether Suvorexant could prevent acute noise-induced sleep disruption in a sex-dependent manner. We hypothesized that independent of sex, 1) Suvorexant alone would promote sleep; and 2) ameliorate noise-induced sleep disruption

MATERIALS AND METHODS

Animals: Two sets of three-month old male and female Sprague-Dawley (N = 15) rats

(Charles River Laboratories, Kingston, New York USA) were housed individually in solid-bottom cages in a temperature-controlled room (21-22 °C) with a 12-h light/12-h dark period (lights on at 0600h). Rodent chow (Harlan Teklad 8604) and water were allowed *ad libitum*. Study procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Surgery: Rats were anesthetized with ketamine (50-75mg/kg, i.p.) and xylazine (7 mg/kg, i.p) and surgically implanted with a radiotelemetric transmitter connected to electroencephalogram (EEG) and electromyogram (EMG) electrodes (F40-EET, Data Sciences International, Saint Paul, Minnesota USA) as described [66, 70]. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson [67]. Coordinates for the cannula and EEG electrodes, respectively, were as follows: 3.1 mm posterior and +/-1.5 mm lateral to bregma. Experimental trials began ten days after surgery.

Drugs: Suvorexant (4-80 mg/kg; AdooQ Bioscience, Irvine, CA) was dissolved in 15% DMSO, 30% ETOH and 55% Polyethylene glycol, which served as the vehicle-control for Suvorexant and noise exposure. Lyophilized Suvorexant was stored frozen and then the dilutions were stored at 4 °C for the duration of the study.

Verification of estrous cycle phase: Vaginal smears were performed daily at 0800-0900h and to determine proestrus, estrus, diestrus 1 (e.g. metestrus) and diestrus 2 as previously described [94] to verify normal 4-5d estrous cyclicity [74, 95]. For all studies, female rats were tested during diestrus 2, when estrogen levels are low [78] to minimize the effect of estrous cycle phase on weight gain, food intake, sleep/wake and physical activity [28, 29,

95].

EEG/EMG recordings and determination of sleep-wake behavioral states: A receiver was placed beneath the test cage to allow EEG and EMG signals to be recorded from the implanted transmitter [66]. Then, electroencephalogram and EMG data were visualized with Neuroscore software (version 2.0.1, Data Sciences International, Saint Paul, Minnesota USA) [70]. Consecutive 10-second epochs of EEG and EMG were manually scored to determine wake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep [66].

Sleep disruption by environmental noise exposure: Rats were exposed to pre-recorded environmental noise (8h/d beginning at 0900; 3h after lights on) during the light phase by placing two speakers in front the rat's cages. This method of SD, has been validated to disrupt sleep in male rats [41, 42]. The 15 min. recording of noises (random street noises, vehicle horn, ambulance siren, hammering, sudden braking vehicle, bell, alarm, air plane sound etc.) was repeated throughout the 8h period. To prevent habituation, the noise events, the duration of these events, the frequency range of sound (800 to 20000 Hz), the amplitude (65 to 100 dB, with average intensity of 85 db) and inter-noise interval were randomly distributed. The recording also contained periods of silence followed by a sharp attack rate (85 to 100 dB) with noises randomly distributed in the sound sequence. Exposure to sounds of this amplitude and frequency has been shown previously to produce no damage to the rodent cochlea [73]. Since the rodent audiogram differs from the human audiogram and rodents detect higher frequency sounds, the frequency range of the recording (800 to 20000 Hz) was matched to the rat audiogram (Audacity <https://www.audacityteam.org/>). The noises were selected from the Best Service Studio

Box DVD3-Technical sample library (Best Service GmbH, Munchen, Germany).

Experimental design:

Sex-specific effect of Suvorexant on noise-induced sleep disruption: Suvorexant (4, 40, 80 mg/kg, i.p.) and vehicle-control was administered in the presence and absence of noise exposure (8h beginning at 0900h, [41]) to males (n = 7) and females (n = 6) in a repeated measures design. All female rats were in diestrus 2. Doses were based on prior reports [156]. EEG and EMG were recorded for 8h post-injection. The following endpoints were analyzed: percent time spent awake, NREM, and REM sleep, sleep fragmentation indicated by the number and mean duration of episodes for each sleep/wake state, total transitions between these states, as well as the latency to both NREM and REM sleep. Injections were performed between 0830h and 0900h with ≥ 48 h between injections.

Statistical Analysis: Data were analyzed with Prism 7.0d (GraphPad Software, Inc., San Diego, California USA). Data are expressed as mean \pm SEM. Alpha was 0.05 for all statistical tests. Analyses for each endpoint were conducted for the 0-8h post-injection time period (Table 1). For study one, change from baseline (vehicle-control, no noise) was calculated for sleep-wake endpoints to account for sex-dependent differences in sleep-wake at baseline [24, 25]. A one sample t-test for the null hypothesis of no change relative to baseline (i.e. vehicle control without noise exposure) was performed to determine the effect of Suvorexant (80mg/kg) alone and noise exposure on all endpoints. Then data were analyzed by two-way repeated measures ANOVA (main effect of sex, main effect of treatment, sex x treatment interaction) followed by multiple comparisons with the FDR correction [75] to determine whether Suvorexant affected noise-induced

sleep-wake in a sex-dependent manner. Since handling involved in the injection procedure augments wakefulness for up to 30 min post-injection independent of treatment, the first 30 min of data collection were excluded from data analysis. Sleep-wake data was excluded for one rat due to technical issues.

RESULTS

Suvorexant reduces the effects of noise on sleep-wake states independent of sex: We first tested whether Suvorexant alone promoted sleep [156, 157, 161] and whether pre-recorded environmental noise exposure reduced sleep duration. In contrast to prior work [156, 157, 161], there was no effect of Suvorexant alone on sleep/wake duration; however, as previously published [42, 41, 95], noise exposure increased time awake and reduced sleep in both sexes (Figure 1). Relative to baseline (i.e. vehicle no noise), noise exposure significantly increased time awake and reduced both NREM and REM in males and females ($P < 0.05$ compared to baseline, Figure 1A-C, Table 1). Combined, these data demonstrate that exposure to environmental noise increases time awake 0-8h independent of sex.

Next, we tested whether Suvorexant when administered with noise exposure, would affect noise-induced changes in sleep/wake duration in a sex-dependent manner. In both sexes, Suvorexant (4-80mg/kg) prevented noise-induced increases in time awake and the reduction in sleep (Figure 1, Table 1). Suvorexant (4-80mg/kg) significantly reduced noise-induced time awake in males ($P < 0.05$ for all comparisons, Figure 1A, Table 1). Likewise in females, Suvorexant (4-80mg/kg) reduced noise-induced increases in time awake but the effect of SUV was dose-dependent ($P < 0.05$ for all comparisons,

Figure 1A, Table 1). There was no difference in the effect of Suvorexant on time awake ($P > 0.05$ for all comparisons, Figure 1A and Table 1). Thus, these data demonstrate that Suvorexant reduces noise-induced increases in wake duration independent of sex.

The reduction in noise-induced increases in time awake by Suvorexant was paralleled by increases in NREM and REM in both sexes (Figure 1, Table 1). Suvorexant (4-80mg/kg) significantly dampened the noise-induced reduction in NREM in males ($P < 0.05$ for all comparisons, Figure 1B, Table 1). In females, Suvorexant (40mg/kg and 80mg/kg) significantly dampened the noise-induced suppression of NREM ($P < 0.05$ for all comparisons, Figure 1B, Table 1). In males, Suvorexant (40mg/kg and 80mg/kg) significantly and dose-dependently dampened the noise-induced suppression of REM ($P < 0.05$ for all comparisons, Figure 1C, Table 1). In contrast, Suvorexant (4-80mg/kg) in females, significantly and dose-dependently dampened the noise-induced suppression of REM ($P < 0.05$ for all comparisons, Figure 1C, Table 1). There were no sex differences for time awake, NREM or REM ($P > 0.05$ for all comparisons, Figure 1A-C, Table 1). Collectively, these data demonstrate that Suvorexant prevents the noise-induced suppression of sleep in males and females, independent of differences between the sexes.

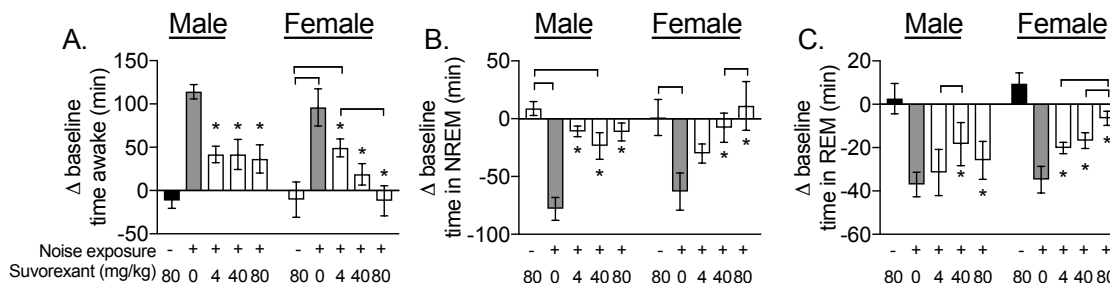


Figure 1. Suvorexant prevents noise-induced increases in wake independent of sex.

Male and female Sprague-Dawley rats (12 week old) were implanted with EEG/EMG leads and EEG and EMG were measured continuously during exposure to environmental noise (8h/d during the light period) for 1d. (A-C) Time spent in wake, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. For each rat change from baseline was calculated. The effect of noise exposure alone on sleep-wake stages within each sex was determined by one-sample t-tests for the null hypothesis of no change relative to baseline. Significant differences ($P < 0.05$) between all other treatments within each sex and between the sexes were determined by two factor repeated measures ANOVA followed by multiple comparisons tests corrected for the false discovery rate. Data are expressed as mean \pm SEM; $n = 7$ for males and $n = 6$ for females. Within each sex, the black bar indicates significant differences from all other treatments, the grey bar indicates a significant difference from baseline, and the brackets indicate significant differences from all other treatments. * $P < 0.05$ as compared to noise. Note different scaling on y-axes.

Table 1. Two factor repeated measures ANOVA to determine the effect of treatment and sex on sleep/wake stages and sleep quality.

	<u>Treatment</u>	<u>Sex</u>	<u>Interaction</u>
<u>Time spent</u>			
Wake Δ from baseline	$F_{(4,44)} = 25.4, P < 0.0001$	$F_{(1,11)} = 1.3, P = 0.27$	$F_{(4,44)} = 1.6, P = 0.18$
NREM Δ from baseline	$F_{(4,44)} = 15.6, P < 0.0001$	$F_{(1,11)} = 0.3, P = 0.60$	$F_{(4,44)} = 1.4, P = 0.26$
REM Δ from baseline	$F_{(4,44)} = 25.8, P < 0.0001$	$F_{(1,11)} = 0.9, P = 0.35$	$F_{(4,44)} = 1.4, P = 0.24$
<u>Episodes of</u>			
Wake Δ from baseline	$F_{(4,44)} = 18.3, P < 0.0001$	$F_{(1,11)} = 0.0, P = 0.95$	$F_{(4,44)} = 1.3, P = 0.22$
NREM Δ from baseline	$F_{(4,44)} = 15.6, P < 0.0001$	$F_{(1,11)} = 1.3, P = 0.27$	$F_{(4,44)} = 1.9, P = 0.13$
REM Δ from baseline	$F_{(4,44)} = 15.6, P < 0.0001$	$F_{(1,11)} = 2.5, P = 0.14$	$F_{(4,44)} = 2.0, P = 0.11$
<u>Duration of (s)</u>			
Wake Δ from baseline	$F_{(4,44)} = 11.7, P < 0.0001$	$F_{(1,11)} = 2.0, P = 0.20$	$F_{(4,44)} = 3.8, P = 0.001$
NREM Δ from baseline	$F_{(4,44)} = 18.2, P < 0.0001$	$F_{(1,11)} = 0.5, P = 0.49$	$F_{(4,44)} = 1.8, P = 0.13$
REM Δ from baseline	$F_{(4,44)} = 13.2, P < 0.0001$	$F_{(1,11)} = 2.5, P = 0.14$	$F_{(4,44)} = 2.0, P = 0.11$
<u>Transitions</u>			
Total Δ from baseline	$F_{(4,44)} = 11.1, P < 0.0001$	$F_{(1,11)} = 0.3, P = 0.60$	$F_{(4,44)} = 0.6, P = 0.67$
<u>Latency to (s)</u>			
NREM Δ from baseline	$F_{(4,44)} = 7.2, P = 0.0001$	$F_{(1,11)} = 0.2, P = 0.68$	$F_{(4,44)} = 1.2, P = 0.33$
REM Δ from baseline	$F_{(4,44)} = 5.6, P = 0.001$	$F_{(1,11)} = 0.4, P = 0.55$	$F_{(4,44)} = 1.5, P = 0.22$

Non-rapid eye movement sleep (NREMS). Rapid eye movement sleep (REMS).

Suvorexant increases noise-induced sleep fragmentation in males only: We next verified that Suvorexant alone improved sleep quality and that environmental noise exposure increased sleep fragmentation in both sexes [42, 41, 95] (Figure 2 and 3, Table 1). There was no effect of Suvorexant alone on indicators of sleep quality relative to control (Figure 2A-F and 3A-C). In contrast, consistent with prior work [42, 41, 95], noise exposure increased sleep fragmentation in both sexes. This was due to an increase in wake episodes for both sexes, an increase in NREM episodes for males, prolonged duration of wake episodes in males, reduced duration of NREM episodes in both sexes, reduced duration of REM episodes in males, and an increase in total transitions between sleep/wake states in males only ($P < 0.05$ for all comparisons, Figure 2A-F, Figure 3A, Table 1).

Next, we tested whether Suvorexant when administered in combination with noise exposure would block the increase in sleep fragmentation due to noise in a sex-dependent manner. Suvorexant promoted the noise-induced increase in sleep fragmentation in males but had no overall effect in females despite that the highest dose of Suvorexant significantly dampened the suppression of the duration of NREM episodes by noise (Figure 2, Table 1). In males, Suvorexant (4-80mg/kg for wake and 40mg/kg and 80mg/kg for NREM), significantly increased the noise-induced increase in episodes of wake and NREM but not REM ($P < 0.05$ for all comparisons, Figure 2A-C, Table 1). In males, Suvorexant (4-80mg/kg) also significantly reduced the noise-induced increase in the duration of wake episodes ($P < 0.05$ for all comparisons, Figure 2D and Table 1). In contrast, Suvorexant failed to augment the reduction in the duration of NREM and REM

episodes by noise in males ($P > 0.05$ for all comparisons, Figure 2E-F and Table 1). Lastly, in males, Suvorexant (40mg/kg) significantly heightened the noise-induced increase in total transitions between sleep/wake states despite reducing the latency to NREM but not REM ($P < 0.05$ for all comparisons, Figure 3A-C and Table 1). Collectively, these data demonstrate that Suvorexant exacerbates noise-induced sleep fragmentation in male but not female rats.

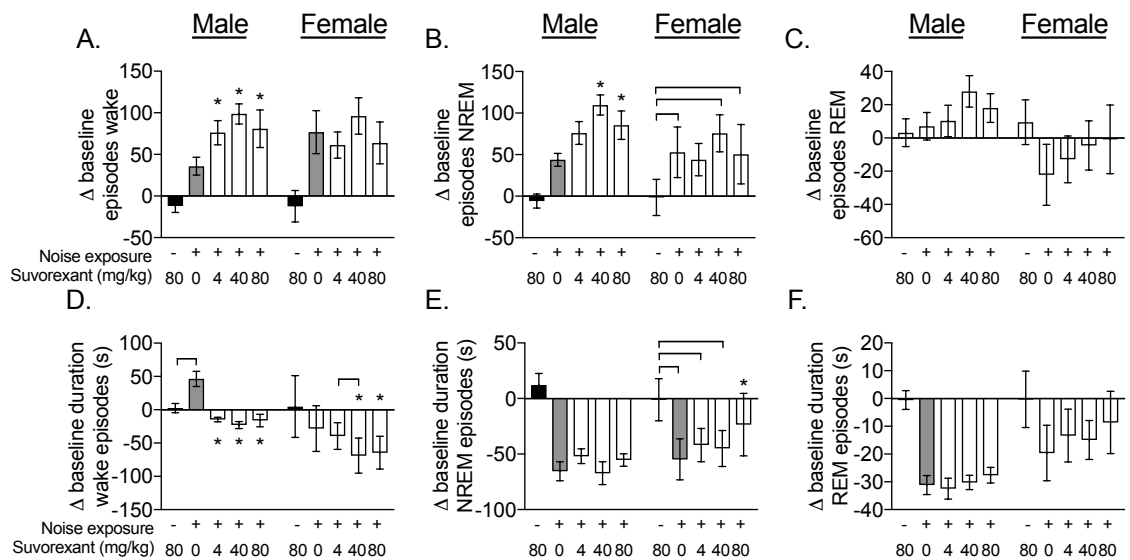


Figure 2. Suvorexant exacerbates the noise-induced increase in sleep fragmentation in male but not female rats. Male and female Sprague-Dawley rats (12 week old) were implanted with EEG/EMG leads. Both sexes were treated with increasing doses of Suvorexant in the presence or absence of exposure to environmental noise (8h/d for 1d during the light period) and EEG and EMG were measured continuously. (A-C) Number of wake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep episodes, and (D-F) duration of wake, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep episodes. For each rat change from baseline was calculated.

The effect of noise exposure alone on sleep-wake stages within each sex was determined by one-sample t-tests for the null hypothesis of no change relative to baseline. Significant differences ($P < 0.05$) between all other treatments within each sex and between the sexes were determined by two factor repeated measures ANOVA followed by multiple comparisons tests corrected for the false discovery rate. Data are expressed as mean \pm SEM; $n = 7$ for males and $n = 6$ for females. Within each sex, the black bar indicates significant differences from all other treatments, the grey bar indicates a significant difference from baseline, and the brackets indicate significant differences from all other treatments. * $P < 0.05$ as compared to noise. Note different scaling on y-axes.

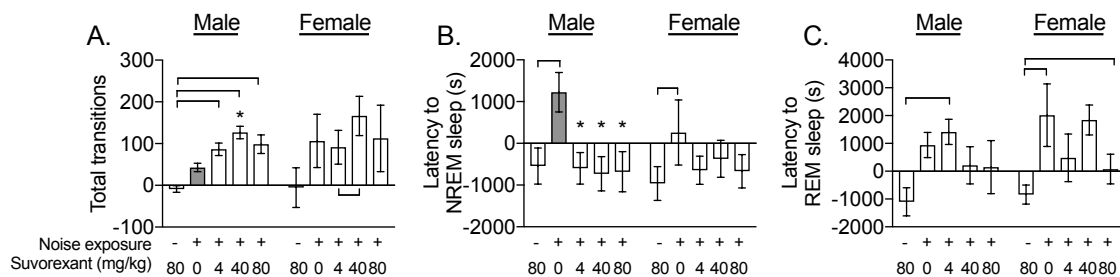


Figure 3. Suvorexant increases total transitions between sleep-wake stages in male but not female rats. Male and female Sprague-Dawley rats (12 week old) were implanted with EEG/EMG leads. Both sexes were treated with increasing doses of Suvorexant in the presence or absence of exposure to environmental noise (8h/d for 1d during the light period) and EEG and EMG were measured continuously. (A) Number of total transitions between sleep-wake stages, and (B-C) latency to non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. For each rat change from baseline was calculated. The effect of noise exposure alone on sleep-wake stages

within each sex was determined by one-sample t-tests for the null hypothesis of no change relative to baseline. Significant differences ($P < 0.05$) between all other treatments within each sex and between the sexes were determined by two factor repeated measures ANOVA followed by multiple comparisons tests corrected for the false discovery rate. Data are expressed as mean \pm SEM; $n = 7$ for males and $n = 6$ for females. Within each sex, the grey bar indicates a significant difference from baseline, and the brackets indicate significant differences from all other treatments. * $P < 0.05$ as compared to noise. Note different scaling on y-axes.

DISCUSSION

Sex-dependent effects of therapeutic interventions to treat disordered sleep and mitigation of sleep-loss induced weight gain remains understudied. To begin to address this, we determined whether Suvorexant in the context of a disordered sleep paradigm could promote sleep in a sex-dependent manner. Utilizing our validated model of noise-induced sleep disruption in male and female rodents [42, 41], we demonstrate that Suvorexant ameliorates noise-induced increases in time awake and the suppression of sleep independent of sex. However, Suvorexant exacerbated the noise-induced increase in sleep fragmentation in males only. Collectively, our findings indicate that Suvorexant displays sex-dependent effects on noise-induced sleep fragmentation but not sleep time. These findings have implications for future studies aimed to determine whether the sleep promoting effects of Suvorexant coincides with a reduction in the weight gain that is associated with sleep restriction in men and women [18].

Consistent with chronic noise-induced sleep disruption in male and female rats [41, 43, 95] and 12h of noise-induced sleep disruption in male rats [43, 42], we show here that an acute 8h exposure to noise during the light period significantly suppressed both NREM and REM and increased time awake as well as sleep fragmentation in both sexes (Figure 1). The increased sleep fragmentation was due to increased episodes of wake and shorter duration of NREM episodes in males and females and more transitions between sleep/wake states in males only (Figure 2 and 3). The latter is consistent with our prior reports [43, 42] with the exception that chronic noise-induced sleep disruption significantly increased the number of transitions between sleep/wake states in females [95] whereas the acute exposure here had no effect, which is a discrepancy that is probably related to the duration of noise exposure. We also demonstrate that the effect of 8h noise exposure during the light period is similar between male and female rats, which suggests that sensitivity to noise exposure is similar between the sexes. This finding contrasts population based studies demonstrating increased sensitivity to poor sleep due environmental noise among women [52], which may be attributed to differences in study design since the species tested (humans vs. rodents), use of subjective versus objective measures of sleep, and duration of noise exposure between our study and theirs. Therefore, future studies should aim to determine whether sensitivity to chronic noise-induced sleep disruption differs between male and female rodents.

Based on the efficacy of Suvorexant to promote sleep [156, 157, 130, 158-161], we hypothesized that Suvorexant in the absence of noise exposure would promote sleep. However, we show Suvorexant alone relative to control (e.g. no noise) failed to alter sleep/wake or sleep quality in either sex (Figure 1, 2 and 3). This contradiction with prior

work is likely due to a “ceiling effect” since Suvorexant was administered in the early light period when sleep is inherently high since most studies administer Suvorexant in the dark period [157, 161, 156]. Furthermore, this discrepancy may be due to differences in species [157, 161], dose of Suvorexant [157, 161, 156], timing of administration [161, 156] and duration of measurements [157, 161, 156]. Importantly, the lack of an effect of Suvorexant in gonadally intact female animals may be related to testing during diestrus 2 and thus, the response to Suvorexant may vary across phases of the estrous cycle since sleep/wake states [95], sleep quality [95], and expression of orexin receptors are estrous cycle dependent [162]. Future studies are warranted to ascertain this hypothesis.

The effect of Suvorexant has only been reported in male animals [156, 157, 161] or pregnant females [155] in the absence of a stimulus known to cause sleep disruption in both sexes [41, 43, 95]. We are the first to show that Suvorexant significantly dampened noise-induced increases in time awake in both males and females due to an increase in both NREM and REM (Figure 1), which parallels clinical studies demonstrating that Suvorexant increases total sleep time in adults with insomnia [130, 158, 159] independent of sex [24]. In contrast, we demonstrate that Suvorexant in males but not females exacerbated noise-induced increases in sleep fragmentation (e.g. increases episodes of wake and NREM, shortened the duration of wake episodes and promoted more transitions between sleep/wake states) relative to noise alone (Figure 2 and 3). This finding aligns with another report, which demonstrated that Suvorexant increased total awakenings in male mice following administration in the light period [157] but contrasts with clinical studies showing that Suvorexant improves indicators of sleep quality in healthy men and women and those with insomnia [21-23]. The reason for the discrepancy

between our study and the effect of Suvorexant on sleep fragmentation in humans is at present unclear. Taken together, these data demonstrate dissimilar effects of Suvorexant on sleep/wake and sleep quality between males and females in the context of a stimulus that disrupts sleep and suggests that Suvorexant may be more efficacious at reversing noise-induced weight gain in females since sleep fragmentation was increased in males following treatment with Suvorexant.

The mechanistic underpinnings contributing to the sex-dependent effects shown here are unclear. The exacerbation of sleep fragmentation by Suvorexant in males but not females may be related to sex differences in orexin signaling since Suvorexant promotes sleep by antagonizing both orexin receptors. Studies report that levels of orexin-A and mRNA for both orexin receptors differ between the sexes [163, 164]. Specifically, while females display higher hypothalamic prepro-orexin and orexin receptor 1 mRNA [165, 164], gene expression for both orexin receptors in the pituitary and adrenal gland is higher in males compared to females [164], these results have yet to be verified at the protein levels. Moreover, while these data suggest sex-dependent effects of orexins, it remains unknown whether sleep/wake in response to Suvorexant administered in the hypothalamus, pituitary and adrenal glands or other central regions known to regulate sleep/wake differs between males and females. It is also plausible that peripheral or central alterations in sex hormones may have contributed to our results since sex hormones including estradiol and progesterone promote wakefulness in females [166] and testosterone and progesterone increase NREM and REM, respectively in males [167, 168]. In females, the multiple platform method of sleep deprivation has also been shown to reduce estradiol and increase testosterone and progesterone levels relative to control

[35]. This hormonal response may be related to the abnormal change in estrus cycle with the multiple platform method of sleep disruption [59]. While our prior work shows noise-induced sleep disruption doesn't alter the estrus cycle [95], it remains unknown if noise-induced sleep disruption alters sex hormones in females. Together, these data demonstrate an opposing role of sex hormones on sleep/wake between males and females and illustrates that the levels of these hormones are affected by sleep loss. However, the contribution of sex hormones to our results here can only be determined by testing how environmental noise independent of or coincided with administration of Suvorexant augments sex hormones differentially in males and females.

Overall, the data presented here provide the first demonstration that Suvorexant prevents noise-induced reductions in sleep duration independent of sex in parallel to a sex-dependent effect of Suvorexant on sleep quality since Suvorexant exacerbated noise-induced sleep fragmentation in male but not female rats. Collectively, these data provide a foundation for future studies aimed at elucidating sex-dependent effects of Suvorexant on prevention of noise-induced weight gain due to feeding and energy metabolism.

CHAPTER FIVE:
Conclusions & Future perspectives

Noise-induced sleep disruption increases weight gain and reduces energy metabolism in female rats: Studies report sex disparities for sleep disturbance and obesity prevalence.

Identifying mechanisms contributing to these differences requires animal models that mirror the human condition. Our lab previously validated the only animal model of sleep disruption induced weight gain that mirrors weight gain due to insufficient sleep for men. The experiment described in Chapter one of this dissertation sought to extend these findings to females while also deciphering the role of the estrous cycle on weight gain due to sleep disruption. As previously published in males, the data presented in chapter one demonstrated that chronic exposure to environmental noise (8h/d for 9d) significantly reduced sleep and increased weight gain by modulating food intake and reducing total EE due to lower EE during PA and sleep. The reduction in total EE also remained dampened during recovery despite a compensatory increase in rebound sleep. Lastly, despite noise-induced sleep disruption, the data in chapter one also demonstrated the estrous cyclicity of female rats remained normal during noise exposure and a recovery period from sleep disruption. Thus, the effects of noise on weight gain, sleep and metabolism did not affect estrous cycle phase. Other methods of sleep disruption have been reported to extend the length of the female estrous cycle [59]. Thus our finding that cyclicity remained normal throughout noise exposure and recovery is important as it suggests that stress is not the driving factor of noise-induced alterations in sleep, metabolism and weight gain in female rats.

Our observation of weight gain, increased feeding and lower EE in females following noise exposure may be related to alterations in sex hormones since estradiol administration increases PA [79] and EE [92] in ovariectomized females and reverses

weight gain and hyperphagia caused by loss of estrogen due to ovariectomy [93]. Future studies in females should determine the peripheral and central effects of estradiol administration on sleep and metabolism both before and after exposure to environmental noise. Collectively, The validation of noise-induced sleep disruption in female rats is the first demonstration in the literature of a sleep disruption method that mirrors the weight gain observed in sleep restricted women [18, 12]. Coincident with our validation in males, these data now have implications for future studies aimed at determining mechanisms contributing to sex disparities for disordered sleep and prevalence of obesity between men and women.

Role of stress in sleep disruption-induced weight gain: Sleep disruption is inherently stressful and stress associates often with weight gain. Noise-induced sleep disruption failed to alter the rhythmicity of the estrous cycle in females (chapter one). While these data suggests that stress may not underlie the weight gain observed during noise-induced sleep disruption during the light cycle, we sought to dissociate stress due to noise from noise-induced sleep loss in the experiment described in chapter two by exposing rats to an acute bout of noise (8h for 1d) during the dark period, when sleep is inherently low. In contrast to our hypothesis, we demonstrated that dark period noise exposure significantly reduced sleep time in females and increased sleep fragmentation in males relative to control (no noise). However, despite an effect of dark period noise on sleep time for females and sleep fragmentation for males, weight gain, feeding, and physical activity was similar between noise-exposed male and females and their respective controls (no noise). Our preliminary studies in males also demonstrate that chronic exposure (8h/d for

9d) to noise during the dark period has no effect on weight gain or feeding. While this study must now be replicated in females and corticosterone levels measured in both sexes during noise exposure in the light and dark periods, these data suggest that the weight gain observed during noise-induced sleep disruption during the light period is not secondary to the stress of noise.

Role of orexin-A in the ventrolateral preoptic area on components of total energy expenditure: The neural mechanisms contributing to noise-induced weight gain and reductions in EE (total and individual components) remain poorly understood. The results from chapter three extended knowledge regarding the role of orexin-A and its receptors to energy metabolism by demonstrating that administration of orexin-A in the VLPO increased total EE due to an increase in several components (i.e. EE during rest, NREM) in addition to the EE during PA. The data from chapter three also demonstrated that pre-treatment with a DORA could block the effects of orexin-A on total EE and its components. While these findings highlight new aspects of orexin-A to energy metabolism they are also important to our model of noise-induced sleep disruption as it suggests that the reduction in total EE and its components by noise may be related to dampened orexin function in response to environmental noise exposure. In fact, our lab demonstrated that acute and chronic exposure to environmental noise blocks orexin-A stimulated increases in arousal, PA, EE and EE during PA in male rats [43]. These data suggest that low orexin function may contribute to the weight gain observed following noise-induced sleep disruption in male rats [41, 42]. Despite this finding, few studies have characterized the central regulation of orexin-A on sleep, total EE, or its components in females. Performing studies to decipher the latter is imperative given that

disordered sleep and obesity is highly prevalent in women and orexins have a prominent role in the regulation of both sleep/wake and energy metabolism. Furthermore, the study from chapter three of this dissertation along with our prior study in males [43] must be replicated in female rats to determine the response to orexin-A both before and after exposure to environmental noise [43]. Finally, it should be determined whether the orexin-A responses to sleep, total EE and its components both before and after environmental noise exposure differs between the sexes and whether the effect of orexin-A on these endpoints between male and female rats is brain site dependent. Acquiring this knowledge will allow for the development of more personalized therapies to treat disordered sleep and obesity in men and women.

Sex-dependent effects of Suvorexant on sleep disruption due to environmental noise

exposure: Suvorexant is an FDA approved DORA for insomnia treatment in men and women that has been shown to promote sleep in humans and animals [156, 157, 161].

The efficacy of Suvorexant to promote sleep suggests that treatment with this therapy may also be efficacious at preventing the weight gain due to noise-induced sleep disruption in both male and female rats. To begin to address this question, a dose-response study was performed (cf. chapter four) to determine the lowest effective dose of Suvorexant that could block noise-induced sleep disruption independent of sex.

Suvorexant prevented the noise-induced reduction in sleep duration independent of sex.

In contrast, there was a sex-dependent effect of Suvorexant on sleep fragmentation as all doses further exacerbated the noise-induced increase in sleep fragmentation in male but not female rats. This is the first report to show that Suvorexant affects sleep duration and

sleep quality after noise disturbance in intact female rats, which underscores the significance of this data. Furthermore, these data provide the first demonstration that the effect of Suvorexant on noise-induced sleep disruption is dependent on sex. That Suvorexant worsened sleep fragmentation in noise-exposed males suggests the capacity of this medication to prevent the increase in weight gain due to sleep disruption may be more efficacious in female compared to male rats. Since an acute exposure to noise was performed in this experiment (chapter four), it is also plausible that the ability and dose of Suvorexant required to block noise-induced sleep disruption and responses between the sexes may differ depending on the length of environmental noise exposure. Future studies should aim to determine the following 1) can Suvorexant block weight gain due to sleep disruption independent of sex?; 2) how does Suvorexant alter factors that affect weight gain including food intake, total EE and its components during noise-induced sleep disruption?; and 3) are different doses of Suvorexant required to block noise-induced wake and fragmentation versus weight gain and is the dose of Suvorexant dependent on the length of sleep disruption?. Lastly, future studies should aim to determine sex differences in orexin signaling, as this may be one plausible mechanism contributing to the sex-dependent effect of Suvorexant on the exacerbation of noise-induced sleep fragmentation in male but not female rats.

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