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The ability of stress to alter sleep in mice is sensitive to reproductive hormones

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Abstract

Though stress causes complex sleep disruptions that are different in females and males, little is known about how sex influences the ability of stress to alter sleep. To date there have been no comprehensive examinations of whether effects of stress on sleep are sensitive to determinants of sex, such as reproductive hormones. Since restraint stress produces a sexually dimorphic increase in rapid eye movement sleep (REMS) amount in mice that is greater in males than females, in the current study we sought to determine whether estrogens and androgens influence the ability of restraint stress to alter sleep states. We removed the gonads from adult female and male C57BL/6J mice and implanted the mice with recording electrodes to monitor sleep-wake states. Gonadectomized females and males exhibited similar amounts of REMS in response to restraint stress. Mice were then implanted with continuous release hormone pellets. Females received 17 β -estradiol and males received testosterone. Hormone replacement (HR) in females decreased the REMS response to restraint stress while HR in males increased the REMS response to restraint stress. The combined effects of HR in females and males restored the sex difference in the ability of restraint stress to alter REMS. These results demonstrate that sex differences in the effects of stress on REMS are dependent on reproductive hormones and support the view that endogenous or exogenous changes in the reproductive hormone environment influence sleep responses to stress.

Keywords

gender; sex; restraint; estrogen; androgen

Introduction

Adults exhibit gender differences in sleep amount, sleep-wake distribution and sleep pressure (for a review, see Paul, 2008). Furthermore, sleep ailments such as insomnia and excessive daytime sleepiness (EDS) exhibit gender disparities in risk, severity, and efficacy of available treatments (Armitage and Hoffmann, 2001; Armitage et al., 2001; Doi and Minowa, 2003; Kim and Young, 2005; Zhang and Wing, 2006). Since sleep is sensitive to stress and women

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and men react differently to stress, it is important to determine whether sleep responses to stress contribute to gender disparities in sleep quality and the risk for sleep disruptions.

Stress can influence sleep in at least two ways: 1) directly, by keeping the subject awake and generating a subsequent homeostatic sleep rebound, and 2) indirectly, by activating a physiological stress response such as the hypothalamic-pituitary-adrenal (HPA) axis, which in turn, acts on sleep regulatory mechanisms (for a review, see Basta et al., 2007). In fact, the effect of sleep deprivation on subsequent sleep-wake states appears to be partially determined by whether the depriving stimulus provokes a stress response. Environmental challenges that provoke acute HPA responses (most of which are accompanied by sleep loss) have a variety of different effects on subsequent sleep-wake states (for a review, see Pawlyk et al., 2008). Furthermore, the ability of acute stress to alter sleep is partially determined by the specific stress modality administered as well as other parameters such as the time of day that the subject is exposed to the modality, and the duration of exposure.

Studies in animal models have provided valuable information about how specific stressors alter subsequent sleep states. For instance, in rats a 2-hour span of restraint stress applied near the onset of the active period produces an increase of REMS amount during the active phase but comparably smaller alterations of non-rapid eye movement sleep (NREMS) amount (Rampin et al., 1991). Repeated bouts of restraint stress attenuate the REMS response. Longer durations of restraint stress (22 hours) produce decreases in REMS and NREMS during the subsequent recovery period (Papale et al., 2005). In mice, 1–2 hours of restraint stress during the rest phase produces increases in REMS and NREMS that are predominant during the subsequent active phase (Meerlo et al., 2001). Other stress modalities produce different effects on REMS and NREMS (for a full review, see Pawlyk et al., 2008). For instance, a study by Adrien et al., (1991) found that during a learned helplessness paradigm, 1 hour of inescapable footshocks in helpless rats did not substantially alter sleep-wake patterns. However, after shuttle-box sessions in which rats were given the opportunity to avoid footshocks, the helpless rats exhibited more REMS than controls and a reduced latency to REMS. Vazquez-Palacios and Velazquez-Moctezuma, (2000) report only transient changes in REMS and NREMS amount in rats after 5 min of unavoidable footshocks that are administered near the onset of the rest phase. However, Papale et al., (2005) reports decreases of REMS and NREMS on the last two days of a four-day paradigm of inescapable footshocks that are administered for 1 hour twice a day. In mice, when inescapable footshocks are administered near the onset of the rest phase, they produce a decrease of REMS during the rest and active phases (Sanford et al., 2003a; Sanford et al., 2003c). The magnitude of these changes are strain dependent. Anxiety also alters sleep-wake states as evidenced by a study in several strains of rats in which exposure to an open field near the onset of the rest phase caused an immediate decrease and subsequent increase of REMS and NREMS during the recovery period (Tang et al., 2005b). In mice, however, the effects of open field anxiety as well as restraint stress on sleep are strain dependent (Meerlo et al., 2001; Tang and Sanford, 2005a).

Since stressors produce a variety of sleep responses, in the current study it was important to use a stress modality that had few additional parameters that could interact with sleep such as pain, social interaction, or noise. Restraint stress is considered a psychogenic stressor and satisfies these criteria. Though mice exhibit sex differences in the ability of restraint stress to alter sleep (Koehl et al., 2006) it is unknown whether the effects of stress on sleep are sensitive to reproductive hormones. When restraint stress is administered at any point during the light (rest) phase of a 24-hour light:dark cycle, the subsequent increase in REMS occurs mostly during the dark (active) phase (Koehl et al., 2002). Since mice exhibit a sex difference in which males exhibit more REMS in response to restraint stress than females, in the current study we examined whether gonadectomy (GDX) and reproductive hormone replacement (HR) in females and males alters the ability of restraint stress to increase REMS amount. The goal of

this study is to determine whether sex differences in the ability of restraint stress to alter sleep are dependent on estrogens and androgens.

Results

In this study, female and male mice that were either gonadally intact, GDX, or HR, underwent a 3-day protocol in a 14-hour light:10-hour dark cycle consisting of a control condition of 1-hour of gentle handling at ZT 7 (7 hours after light onset) on day 1 followed by a full day of recovery (day 2) and 1-hour of restraint stress (ZT 7) on day 3. Restraint was administered 7 hours into the light-phase because at this time sleep pressure has been sufficiently dissipated to minimize the homeostatic effects of sleep loss that occurs during restraint. This time point also allows 6 additional hours of light for homeostatic REMS rebound following restraint to insure that the REMS increase during the dark phase is not a homeostatic response to REMS loss during restraint. Therefore, the recovery period consisted of 16 hours, 6 hours of light and 10 hours of darkness. Gentle handling was administered to control against the effects of the sleep loss that occurred during the restraint stress procedure.

First we examined the effects of the following variables on the percentage of total recording time spent in REMS and NREMS during the recovery period: sex, phase (light or dark), treatment (restraint or control), and hormone status (intact, GDX, or HR). Since GD and HR were administered in the same subjects (see methods) they were analyzed as repeated measures. Post hoc analysis (t-test's) for hormone status was conducted for the following comparisons: intact/GDX, GDX/HR (paired), and intact/HR. MANOVA revealed effects of treatment (REMS: $F_{1,45} = 24.34$, $P = .001$; NREMS: $F_{1,45} = 5.11$, $P = .025$), phase (REMS: $F_{1,45} = 36.71$, $P = .001$; NREMS: $F_{1,45} = 149.86$, $P = .001$) and hormone status (REMS: $F_{2,45} = 5.54$, $P = .005$; NREMS: $F_{2,45} = 23.62$, $P = .001$), but no effect of sex (REMS: $F_{1,45} = 2.03$, $P = \text{n.s.}$; NREMS: $F_{1,45} = .586$, $P = \text{n.s.}$). Since phase exhibited such a robust influence on sleep we analyzed REMS and NREMS separately in the light and dark phases.

REMS

During the light-phase, a MANOVA revealed a hormone status x treatment interaction ($F_{2,45} = 6.6$, $P = .002$) on REMS, but no main effects of sex ($F_{1,45} = 1.98$, $P = \text{n.s.}$), treatment ($F_{1,45} = 0.26$, $P = \text{n.s.}$), or hormone status ($F_{2,45} = 2.5$, $P = \text{n.s.}$). In the control condition, GDX resulted in less REMS than both intact ($t_{29} = 3.8$, $P = .001$) and HR mice ($t_{28} = 4.8$, $P = .001$), but there were no differences between intact and HR mice ($t_{29} = 0.8$, $P = \text{n.s.}$). During the dark phase there was an effect of sex ($F_{1,45} = 13.12$, $P = .001$) and treatment ($F_{1,45} = 61.16$, $P = .001$), but no effect of hormone status ($F_{2,45} = 2.7$, $P = \text{n.s.}$). In all mice, males had more REMS than females, and restrained mice had more REMS than handled mice. Interactions included sex x treatment ($F_{1,45} = 7.9$, $P = .006$). During the dark phase restrained females and males and exhibited higher REMS than controls ($t_{44} = 4.63$, $P = .001$ and $t_{44} = 5.98$, $P = .001$ respectively).

NREMS

During the light-phase, a MANOVA revealed an effect of hormone status ($F_{2,45} = 15.41$, $P = .001$) but no effects of sex ($F_{1,45} = .54$, $P = \text{n.s.}$) or treatment ($F_{1,45} = 1.26$, $P = \text{n.s.}$), and no interactions on NREMS. GDX mice had less NREMS than intact ($t_{29} = 4.29$, $P = .001$) and HR mice ($t_{28} = 6.79$, $P = .001$). During the dark phase, there was an effect of treatment ($F_{1,45} = 18.75$, $P = .001$) and hormone status ($F_{2,45} = 9.4$, $P = .001$), but no effect of sex ($F_{1,45} = 0.12$, $P = \text{n.s.}$) and no interactions. Restrained mice had more NREMS than controls. GDX mice had less NREMS than intact ($t_{29} = 3.43$, $P = .001$) and HR mice ($t_{28} = 4.44$, $P = .001$). There were no differences between intact and HR mice ($t_{29} = .87$, $P = \text{n.s.}$).

GDx and HR alter the effects of restraint stress on sleep

Since we found effects of hormone status on REMS and NREMS and several sex interactions during REMS, we examined REMS and NREMS under each hormonal condition separately in females and males.

REMS

Females—In intact females, when the recovery period was averaged into 2-hour intervals a repeated-measures ANOVA detected no main effect of restraint stress ($F_{7,14} = 1.3$, $P = \text{n.s.}$) on REMS but did reveal a restraint $\times$ time interaction ($F_{7,98} = 4.9$, $P = .005$; Figure 1a). Restrained females exhibited less REMS than controls during the light-phase ($t_{14} = 2.7$, $P = .02$; Figure 1a) but more REMS than controls during the dark phase ($t_{14} = 5.2$, $P = .001$), verifying previous findings that the stress-induced increase of REMS occurs primarily during the dark phase (Koehl et al., 2002).

In GDx females, there was an effect of restraint stress on REMS amount ($F_{7,12} = 44.2$, $P = .001$) and a restraint $\times$ time interaction ($F_{7,84} = 9.9$, $P = .005$). This was the only female group in which restraint stress caused a net increase of total REMS amount (Figure 1b). REMS amount in restrained GDx females was not different from controls during the light-phase ($t_{12} = 1.1$, $P = \text{n.s.}$) but was greater than controls during the dark phase ($t_{12} = 8.1$, $P = .001$).

In HR females there was no main effect of restraint stress ($F_{7,14} = 1.1$, $P = \text{n.s.}$), but there was a restraint $\times$ time interaction ($F_{7,98} = 3.5$, $P = .003$; Figure 1c). REMS amount in restrained HR females was lower than in controls during the light-phase ($t_{14} = 5.2$, $P = .001$) and was not different from controls during the dark phase ($t_{14} = .79$, $P = \text{n.s.}$).

Males—In intact males, a repeated-measures ANOVA detected an effect of restraint stress ($F_{7,14} = 62.5$, $P = .003$), and a restraint $\times$ time interaction ($F_{7,98} = 1.9$, $P = .05$) on REMS amount (Figure 2a). REMS amount in restrained males was not different from controls during the light-phase ($t_{14} = .37$, $P = \text{n.s.}$) but was greater than controls during the dark phase ($t_{14} = 5.1$, $P = .001$). Consequently, in males the ability of restraint stress to increase REMS amount was predominant during the dark phase.

In GDx males, there was an effect of restraint stress ($F_{7,14} = 43.1$, $P = .001$) and a restraint $\times$ time interaction ($F_{7,98} = 5.6$, $P = .025$; Figure 2b). REMS amount in restrained GDx males was greater than in controls during the light ($t_{14} = 2.8$, $P = .01$) and dark ($t_{14} = 11.2$, $P = .001$) phases. In HR males, there was an effect of restraint stress ($F_{7,12} = 23.1$, $P = .003$) and a restraint $\times$ time interaction ($F_{7,84} = 1.5$, $P = .05$; Figure 2c). REMS amount in restrained HR males was not different from controls during the light-phase ($t_{12} = .18$, $P = \text{n.s.}$) but was greater than controls during the dark phase ($t_{12} = 3.8$, $P = .002$).

NREMS

Females—In intact females, a repeated-measures ANOVA did not detect an effect of restraint stress ($F_{7,14} = .028$, $P = \text{n.s.}$) or a restraint $\times$ time interaction ($F_{7,98} = 3.5$, $P = \text{n.s.}$) on NREMS amount (Figure 3a). In GDx females there was neither an effect of restraint stress ($F_{7,12} = 2.85$, $P = \text{n.s.}$) nor a restraint $\times$ time interaction ($F_{7,84} = 1.89$, $P = \text{n.s.}$; Figure 3b). In HR females, there was an effect of restraint stress ($F_{7,14} = 18.3$, $P = .001$) but there was no restraint $\times$ time interaction ($F_{7,98} = 1.5$, $P = \text{n.s.}$; Figure 3c). Restrained HR females exhibited more NREMS than controls during the light ($t_{14} = 2.9$, $P = .02$) and dark ($t_{14} = 2.8$, $P = .013$) phases.

Males—In intact males, a repeated-measures ANOVA did not detect an effect of restraint stress ($F_{7,14} = .703$, $P = \text{n.s.}$) or a restraint $\times$ time interaction ($F_{7,98} = 2.37$, $P = \text{n.s.}$) on NREMS

amount (Figure 3d). In GDX males there was neither an effect of restraint stress ($F_{7,14} = .001$, $P = \text{n.s.}$) nor a restraint $\times$ time interaction ($F_{7,98} = 2.031$, $P = \text{n.s.}$; Figure 3e). In HR males, there was no main effect of restraint stress ($F_{7,12} = 1.55$, $P = \text{n.s.}$) but there was a restraint $\times$ time interaction ($F_{7,84} = 24.27$, $P = .001$; Figure 3f). Restrained HR males exhibited less NREMS than controls during the light-phase ($t_{12} = -3.1$, $P = .010$) and more NREMS than controls during the dark phase ($t_{12} = 2.3$, $P = .034$).

When adjusted for sleep loss, sex differences in the REMS response to restraint stress are eliminated by GDX and restored by HR

Since the effects of restraint stress were predominant during the dark phase, we examined the specific effects of hormone status on REMS during the light and dark phases separately. We expressed the average difference in REMS recovery between the restraint stress and control conditions in each animal (restraint stress - control) as a percentage of the total recording time in each phase. This value is referred to as the REMS response to restraint stress.

During the light-phase, intact restrained females exhibited less REMS than controls resulting in a negative REMS response to restraint stress (Figure 4a). GDX females exhibited a positive REMS response which was greater than that of intact females ($t_{13} = 2.37$, $P = .04$) and HR decreased REMS amount in females ($t_{13} = 2.86$, $P = .003$) causing a negative REMS response which was similar to that of intact females. Intact restrained males exhibited less REMS than controls resulting in a negative REMS response (Figure 4a). GDX males exhibited a larger REMS response to restraint stress than intact males ($t_{14} = 2.18$, $P = .04$) and HR decreased REMS amount in males ($t_{13} = 1.6$, $P = \text{n.s.}$) causing a positive but negligible REMS response.

During the dark phase, the ability of restraint stress to increase REMS exhibited a sex difference in which intact females exhibited less REMS in response to restraint stress than intact males ($t_{14} = 2.5$, $P = .03$; Figure 4b). REMS amount in response to restraint stress in GDX females was not different from GDX males ($t_{13} = .671$, $P = \text{n.s.}$). Therefore, GDX in females and males eliminated the sex difference in the REMS response to restraint stress. In HR females, REMS amount in response to restraint stress was less than in HR males ($t_{13} = 3.3$, $P = .005$) resulting in a sex difference in HR groups that was similar to that of the intact groups. Therefore, HR in females and males restored the sex difference in the REMS response to restraint.

Intact females exhibited approximately 19.2 min less REMS in response to restraint stress than intact males during the dark phase while the difference in GDX groups was approximately 1.2 min (Figure 4b). HR in females decreased REMS amount in response to restraint stress (approximately 8.3 min) while HR in males increased REMS amount (approximately 18.5 min). The combined difference in REMS amount in HR groups was approximately 25.5 min.

GDX and HR alter the ability of stress to increase corticosterone (CORT)

To determine the effects of hormone status on stress reactivity we examined whether GDX and HR in females and males alters the ability of restraint stress to stimulate an HPA response. A subset of GDX mice that received hormone pellets ($n = 6$ in each group) were euthanized immediately following restraint stress and CORT levels were determined using RIA. It is important to note that these mice were not the same as those used for sleep recording, therefore the HR mice in this subset were naïve to restraint stress. One-way ANOVAs revealed an effect of hormone status on the ability of restraint stress to increase serum CORT levels in females ($F_{2,17} = 11.6$, $P = .001$), and males ($F_{2,17} = 9.7$, $P = .002$; Figure 5). In all groups, restraint stress increased CORT significantly above basal levels (which we measured at near 50 ng/ml in males and 80 ng/ml in females during the mid-day). After restraint stress, CORT levels in intact females were higher ($P < .05$, Tukey) than in all male groups (intact, GDX, and HR).

GDX in females caused a slight but non-significant reduction of CORT levels in response to stress. CORT levels in GDX females were higher than intact males ($P < .001$, Tukey) but not different from GDX or HR males. HR in females raised CORT levels above those above all groups ($P < .05$, Tukey) except for intact females.

Serum CORT levels in response to stress in intact males were lower than all groups ($P < .05$, Tukey). CORT levels in GDX males were higher than in intact males ($P = .008$) but lower than in intact ($P = .032$) and HR females ($P < .001$). Interestingly HR did not influence the CORT response to stress in GDX males.

Discussion

The major finding from these studies is that GDX in females and males eliminated the sex difference in the ability of restraint stress to increase dark-phase REMS, and HR restored it. These results demonstrate that gonadal hormones are necessary for the sex difference in the REMS response to restraint stress during the dark phase and confirm the hypothesis that sex differences in the ability of stress to acutely alter REMS are dependent on reproductive hormones. Interestingly, during the light phase there was an interaction between hormone status and treatment (restraint/handling) on REMS. This was because female controls from the intact and HR groups exhibited more light-phase REMS than those that were restrained, however this effect was not observed in GDX females. Conversely, male controls from the intact and HR group had similar amounts of REMS as restrained mice, but male GDX controls exhibited more light-phase REMS than those that were restrained.

In addition to altering the total amounts of REMS in response to restraint stress, GDX and HR also appear to have altered REMS distribution during the dark phase, particularly in the males. Reductions of REMS in castrated males occurred almost exclusively near the beginning and end of the dark phase and were accompanied by a notable increase in REMS during the light-phase, thereby causing little change in total REMS during recovery. This observation suggests that instead of merely decreasing REMS, the castration may have reduced the latency of the REMS rebound. Though this result indicates little about the potential mechanisms responsible for the effects of gonadal hormones on REMS, it does suggest that the ability of GDX to alter REMS in males occurs through a different mechanism than that of females, whose REMS distribution appears to be only mildly affected by GDX.

The delay in the REMS rebound until the dark phase suggests that this effect may be under regulation of the circadian timing system. However, though castration appears to have altered the temporal distribution of the REMS response in restrained males in the current study, it has no effect on basal REMS amount or distribution in untreated males (Paul et al., 2006), suggesting that the circadian regulation of sleep was not substantially altered. In fact, gonadal hormones modulated the REMS response to restraint stress to a larger degree than they do for basal REMS or the REMS response to extended wakefulness (Paul et al., 2006; Paul et al., 2009). This finding suggests that their effects on REMS may be achieved by acting on stress response mechanisms, which are notoriously sensitive to reproductive hormones (Solomon and Herman, 2009), and not homeostatic sleep regulatory mechanisms which appear to be insensitive to reproductive hormones (Paul et al., 2006; Paul et al., 2009).

In the current study, GDX and HR altered CORT levels in restrained females however HR in restrained males did not reduce CORT levels. Increases in CORT levels are most often associated with increases in wakefulness and decreases of NREMS. For instance, in rats systemic injection of a CRH-1 receptor antagonist increases humoral CORT levels and reduces total sleep amount (Lancel et al., 2002) and central administration of the CRH antagonist astressin reduces serum CORT levels and increases sleep amount (Chang and Opp, 2000).

Furthermore, CORT replacement in adrenalectomized adult rats decreases NREMS (Bradbury et al., 1998). In intact mice immediately following restraint stress, NREMS and REMS were depressed and wake amount was elevated, while gentle handling which does not substantially increase serum CORT levels (Meerlo et al., 2001), was followed by an immediate sleep rebound. This finding suggests that CORT may have contributed to increases in wakefulness observed in the initial hours following restraint.

There is an abundance of evidence that CORT or other HPA metabolites have little to do with the ability of stress to increase REMS amount in mice. For instance, mice that lack the $\beta 2$ subunit of the nicotinic acetylcholine receptor exhibit sustained increases in CORT levels following 2 hours of restraint, but also exhibit a suppression of REMS amount (Lena et al., 2004). In addition, stressors that have been shown to increase serum CORT levels in mice such as shock stress (Sanford et al., 2003c) and fearful cues (Sanford et al., 2003a) have not been shown to increase REMS amount in rats (Vazquez-Palacios and Velazquez-Moctezuma, 2000). These reports reduce the likelihood that the ability of stress to increase REMS is mediated by HPA hormones. In fact, Marinesco et al., (1999) provides evidence from studies in male rats that CORT is responsible for decreases in REMS commonly observed after prolonged periods of restraint stress. Furthermore, this study reveals that in rats, plasma CORT concentrations return to basal levels 4 hours after restraint stress (1 hour), which is well before the onset of the REMS rebound in mice. The ability of restraint stress to increase REMS amount is more likely to be dependent on the pituitary hormone prolactin since the ability of ether stress to increase REMS is eliminated in prolactin deficient mice (Obal et al., 2005).

Several limbs of the REMS regulatory pathway are sensitive to stress and must therefore be considered as potential targets of gonadal hormones and their influence on the REMS response to stress. For instance, suppression of noradrenergic neurotransmission in the locus coeruleus (LC) reduces the ability of restraint stress to increase REMS in male rats (Gonzalez et al., 1995). A recent study suggests that neurons of the LC, a primary REMS regulator, are a potential substrate of gonadal hormones (Pendergast et al., 2008). Serotonergic systems have also been implicated in the ability of stress to increase REMS. In male rats the serotonin inhibitor para-chlorophenylalanine prevents the ability of restraint stress to alter REMS (Sinha, 2006). Furthermore, mice bearing a selective knockout of the 5-HT_{1A} serotonin receptor do not exhibit an increase of REMS following restraint stress (Boutrel et al., 2002). Examples of cholinergic influences include that restraint stress-induced increases of REMS are prevented in male mutant mice that lack the $\beta 2$ subunit of the nicotinic acetylcholine receptor (Lena et al., 2004). In rats, sex differences in the ability of stress to augment acetylcholine release in the dorsal hippocampus, a regulator of REMS (Jouvet, 1998), are completely dependent on gonadal hormones (Mitsushima et al., 2009) suggesting that sex may interact with cholinergic regulation of REMS. Alternatively, inhibition of the histamine H1 receptor in male rats also prevents the ability of restraint stress to increase REMS (Rojas-Zamorano et al., 2009). Furthermore, the ability of stress to impair histamine release from hypothalamic neurons in rats is greater in males than in females. This effect appears to be due to desensitization of H3 receptors (Ferretti et al., 1998) which exhibit a gonadal hormone-dependent sex difference characterized by greater cortical density in adult females than males (Ghi et al., 1991).

Though there were no effects of sex on REMS and NREMS recovery in the control condition, there were effects of hormone status. One hour of sleep deprivation is very mild and does not produce a significant increase of REMS or NREMS over basal levels (unpublished results). However, in the hours immediately following sleep deprivation, intact and HR mice of both sexes exhibited more total sleep than restrained mice. The most likely explanation is that following restraint stress the mice remained aroused until CORT levels decreased sufficiently enough to allow them to return to sleep. However, since 1 hour of gentle handling does not noticeably increase CORT levels (Meerlo et al., 2001), control mice returned to sleep

immediately following sleep deprivation and therefore obtained more sleep in the hours immediately following sleep loss than the restrained mice. Curiously, control GDX mice of either sex exhibited sleep amounts that were similar to restrained mice in the hours immediately following sleep deprivation. Though we do not currently have an explanation for this paradoxical occurrence, it appears to result from slight reductions in sleep in control mice, and slight increases in restrained mice. A similar effect was observed in GDX females and males after 6 hours of sleep deprivation (Paul et al., 2009). In that study, in the hours immediately following sleep deprivation HR mice exhibited slightly more sleep than GDX mice.

It is important to note that in the current study, stage of estrous in female mice was not taken into consideration. Estrous stage does not have substantial effects on REMS or NREMS (Koehl et al., 2003). However this does not eliminate the possibility that the ability of stress to alter sleep may be sensitive to the estrous cycle, as are the effects of stress on other outcome measures. For instance, the ability of shock stress to impair associative learning in female rats is sensitive to the estrous cycle and is most evident during proestrus, when estradiol levels are relatively elevated (Dalla et al., 2009). Estrous stage also interacts with the ability of restraint stress to increase c-fos expression in cortical and hippocampal regions of female rats (Figueiredo et al., 2002). Conversely, there are no effects of estrous stage on c-fos levels in the medial amygdala or ventrolateral septum of restrained females. Neither are there influences of estrous stage on the ability of restraint stress to facilitate hippocampal-dependent spatial recognition in female rats (Conrad et al., 2004).

Since several genetic and hormonal sleep regulatory pathways are conserved between mice and humans (for a review see Mackiewicz et al., 2008), the finding that sex differences in sleep responses to stress are biologically driven in mice suggests that similar response mechanisms may exist in humans. Interestingly, as first suggested by (Armitage and Hoffmann, 2001), when it comes to altering sleep wake states, the interaction of hormonal state with stress responses appears to override the effects of hormonal state alone. The present results support the hypothesis that the ability of hormonal fluctuations (common during puberty, pregnancy, and menopause) to disturb sleep is sensitive to stress.

Sex influences sleep and potentially causes sex differences in the risks for several sleep disorders. More importantly however, it is the potential interaction of sleep mechanisms with stressful stimuli that could underlie sex differences and gender disparities in sleep dysfunction. In the current study the presence or absence of restraint stress was a determining factor in the ability of reproductive hormones to influence sleep. That is, restraint stress produced a sex difference in REMS amount that was not observed in controls, but it did not substantially alter NREMS. Therefore, not only was the sex difference in the REMS response to restraint stress altered by hormone manipulations, but when examined in the context of basal REMS the converse was also apparent. That is, the ability of sex and reproductive hormones to influence sleep appears to be sensitive to stress. Therefore, the interaction of stress with sleep regulatory mechanisms is a potential determinant of sex differences in sleep dysfunction and associated co-morbidities. Since sex differences in homeostatic responses to sleep loss appear to be more complicated and not dependent on gonadal sex (Paul et al., 2006), genetic and or phenotypic sex may also mediate some of these responses and must eventually be examined to fully understand the nature of sex differences in sleep dysfunction.

Experimental Procedures

Animals

Adult female and male mice (C57BL/6J, 6–8 weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained on a 14-hour light:10-hour dark (14L:10D) schedule throughout the study. We chose this LD cycle to replicate results from preliminary

studies that were performed with mice from our animal facilities that were maintained on LD 14:10 for breeding purposes. Food and water were available ad libitum, and animals were housed for at least two weeks prior to experimental use. All protocols and procedures were approved by the Northwestern University Animal Care and Use Committee. All experiments were conducted in accord with accepted standards of humane animal care, as outlined in the Ethical Guidelines.

Recording-Implant Surgery

Male (n = 8) and female (n = 8) mice with intact gonads were anesthetized with an intraperitoneal injection of a drug cocktail containing ketaset (80 mg/kg) and xylazine (8 mg/kg) and implanted with electroencephalograph (EEG) and electromyograph (EMG) electrodes for polysomnographic recording of sleep-wake states. Two stainless-steel recording screws (Small Parts Inc. Miami Lakes, FL) were positioned contralaterally to each other on the skull surface. The first was located 1 mm anterior to Bregma and 0.5 mm right of the central suture, whereas the second was located 0.5 mm posterior to Lambda and 1 mm left of the central suture. EMG activity was monitored using stainless-steel, Teflon-coated wires inserted bilaterally into the nuchal muscle. Once the EEG and EMG wires were in place, they were attached to a prefabricated head implant, containing a 1 × 4 pin grid array and attached to the skull using cyanoacrylamide adhesive. In order to control for pain, over the next 36 hours mice were given Buprenex (buprenorphine; 2 mg/kg subcutaneously) once every 12 hours. All procedures were performed on a heating pad and mice were allowed to recover for at least 14 days before being transferred to sleep recording chambers.

EEG/EMG Recordings

Following recovery, the intact animals were placed in a sleep-recording chamber and connected to a lightweight rotating tether system (Plastics One, Roanoke, VA), which enabled complete freedom of movement throughout the cage. Except for the recording tether, conditions in the recording chamber were identical to those in the home cage. Mice were allowed a minimum of seven additional days to acclimate to the tether. Recording of EEG and EMG waveforms began at zeitgeber time (ZT) 0 (light onset in 14:10 LD). Waveforms were collected using Multisleep (Actimetrics, Evanston, IL), a software system designed specifically for gathering and analyzing rodent sleep data.

Restraint Stress

During restraint stress the animals remained attached to the sleep recording tether and were inserted into 50 ml polypropylene conical centrifuge tubes with holes added for breathing and a polypropylene plunger with a hole cut for the tail was placed behind them to restrict rostro-caudal movement. Animals remained in their recording cages and were observed to verify that they were immobile during the restraint stress. Following restraint stress, animals were removed from the tubes and remained in their recording cages for 16 hours of continuous recording of recovery sleep. All mice in the study remained awake during the restraint stress and handling procedures. The stage of estrous was not controlled in females because of findings that the estrous cycle has little effect on sleep amount in mice (Koehl et al., 2003). Controls were kept awake by a gentle handling procedure (previously described in Paul et al., 2006).

Gonadectomies

Separate groups of naïve mice were anesthetized, gonadectomized and implanted with EEG/EMG recording electrodes. In female mice (n = 8), an incision was made dorsally through the skin layer and muscle just above the location of the ovary. The ovary was expressed to the surface and ligated using a silk suture. The ovary was removed, the uterine horn was returned to the cavity, and the muscle and skin layers were sutured. In male mice (n = 8), a vertical

incision was made at the midline of the abdomen, and fatty and connective tissue were removed to expose the inner sacs that encase the testes. A second incision was made through each casing, and the testes, fat pad and epididymes were expressed to the outside and removed. The area was cleaned, and the incisions were closed using silk suture. Immediately following GDX while still under anesthesia, mice were implanted with recording electrodes (see above) and allowed to recover for at least 14 days. After recovery, mice were returned to sleep recording chambers and allowed to acclimate for seven additional days after which they underwent the 3-day restraint stress protocol.

Hormone Replacement

After the restraint stress protocol, the GDX animals were removed from the sleep recording chambers and returned to their home cages for at least one week after which they were implanted with 60-day continuous release hormone pellets (Innovative Research, Sarasota, FL). Animals were anesthetized by inhalation of isoflurane (0.2 ml) in a transparent cylindrical induction chamber (10" × 6" diam) a small incision was made dorsally and slightly lateral to the nape of the neck approximately 3 mm caudal to the ear. Each hormone pellet (1/8" diameter) was subcutaneously implanted and the incision was sealed with silk suture. Female mice were implanted with 17 β -estradiol (1,3,5-Estratriene-3,17 β -diol; .072mg total; n = 8) and male mice were implanted with testosterone (17 β -hydroxyandrost-4-en-3-one; 12.5 mg total; n = 7). After surgery animals were allowed at least seven days of recovery, returned to the sleep recording chambers and allowed at least another seven days of acclimation. The mice then underwent the 3-day restraint stress protocol. It is important to note that HR mice were not naïve to restraint, and therefore some habituation to the procedure may have occurred.

Hormone Assays

After sleep-wake recording, mice were returned to their home cages and were allowed seven days of recovery. On day 8, mice were decapitated and trunk blood was collected in heparinized tubes, stored at 4°C overnight, and centrifuged the following day at 1,500 g for 20 min at 8°C, and the serum was stored at -80°C until assayed. Levels of testosterone and luteinizing hormone (an indicator of estradiol levels Bronson, 1976) were determined using radioimmunoassay (RIA). Hormone levels in pellet-implanted females (luteinizing hormone: 2.4 ± 0.2 ng/ml;) and males (testosterone: 0.12 ± 0.02 ng/ml) were in the physiological range. A subset of GDX mice that received hormone and placebo pellets (n = 6 in each group) were euthanized immediately following stress and CORT levels were determined using RIA. These mice were not implanted with recording electrodes and did not undergo sleep-wake recording.

Sleep Scoring and Data Analysis

After collection, all waveforms were classified into 10-second epochs of wake (low-voltage, high-frequency EEG; high-amplitude EMG), NREMS (high-voltage, mixed-frequency EEG; low-amplitude EMG) or REMS (low-voltage EEG with a predominance of theta activity (6–10 Hz); very low amplitude EMG).

Statistics

Sleep data were analyzed using MANOVAs to detect between- (sex and hormone status (GDX/HR was run as a repeated measure)) and within- (treatment and phase) factor differences. A repeated measures ANOVA was used to detect the effect of time (averaged into 2-hour intervals) on experimental variables. Posthoc analysis was conducted using Student t-tests to follow-up variance of main effects and interactions. Within subject variables were paired and all t-statistics were reported as the absolute value. CORT levels were analyzed using and ANOVAs and posthoc analysis was conducted using a Tukey test. Significance was set at $P < .05$ for all analyses.

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Abbreviations

HPA	hypothalamic-pituitary-adrenal
REMS	rapid eye movement sleep
NREMS	non-rapid eye movement sleep
GDX	gonadectomy
HR	hormone replacement
CORT	corticosterone
ZT	zeitgeber time
LD	light:dark

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Females

● Restrained
○ Control

Figure 1.

Percentage of REMS in (a) intact, (b) GDX, and (c) HR females after 1 hour of restraint stress or control (handled). Each of the groups has eight mice except for GDX females in which there are seven mice. Line graphs on the left depict REMS during the 16-hour recovery period (beginning at ZT 8) averaged into 2-hour intervals. The shaded areas represent the dark phase. Bar graphs on the right depict recovery REMS averaged into the total 16-hour recovery period, 6 hours of light-phase recovery, and 10 hours of dark-phase recovery. Following stress, GDX increased and HR reduced dark-phase REMS amount in females. For each data point the error bars represent mean $\pm$ SEM of sleep amount in all mice in the designated group. Significance was ascribed at $*P < .05$ for all statistical analyses.

Figure 2.

Percentage of REMS in (a) intact, (b) GDX, and (c) HR males after 1 hour of restraint stress or control (handled). Each of the groups has eight mice except for HR males in which there are seven mice. Line graphs on the left depict REMS during the 16-hour recovery period (beginning at ZT 8) averaged into 2-hour intervals. The shaded areas represent the dark phase. Bar graphs on the right depict recovery REMS averaged into the total 16-hour recovery period, 6 hours of light-phase recovery, and 10 hours of dark-phase recovery. Following stress, GDX reduced and HR increased dark-phase REMS amount in males. For each data point the error bars represent mean $\pm$ SEM of sleep amount in all mice in the designated group. Significance was ascribed at $*P < .05$ for all statistical analyses.

Figure 3.

Percentage of NREMS after 1 hour of restraint or control (handled) in intact, GDX, HR female and male mice. There were eight female and eight male mice in each condition except for GDX females and HR males who each had seven mice. The 16-hour recovery period is averaged into 2-hour intervals. The shaded areas represent the dark phase and the arrow below the graphs indicates the termination of restraint and onset of recovery (ZT 8). GDX and HR did not cause substantial differences in the NREMS response to restraint stress. For each data point the error bars represent mean $\pm$ SEM of sleep amount in all mice in the designated group. Significance was ascribed at $*P < .05$ for all statistical analyses.

Figure 4.

The sex difference in the REMS response to restraint stress was eliminated by GDX and restored by HR. Bar graphs depict REMS expressed as the difference between restraint stress and control (restraint stress - control) in minutes during the (a) light and (b) dark phases in females and males. There were eight mice in each group except for GDX females and HR males each of which had seven mice. For each data point the error bars represent mean $\pm$ SEM of sleep amount in all mice in the designated group. Significance was ascribed at $*P < .05$ for all statistical analyses (posthoc Tukey test).

Figure 5.

Serum CORT levels after restraint stress in (A) intact, GDX, and HR mice, and (B) females and males. The same data appear in both graphs for ease of comparison between groups. There were six female and six male mice in each condition. Serum samples were obtained from a separate cohort of animals immediately following 1-hour of restraint. For each data point the error bars represent mean $\pm$ SEM of CORT levels in all mice in the designated group. Significance was ascribed at $*P < .05$ for all statistical analyses (posthoc Tukey test).