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Impact of sex on hyperalgesia induced by sleep loss

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ABSTRACT

This study evaluated the impact of sex on the short term consequences of different periods of sleep deprivation and the effect of the respective sleep recovery periods on nociceptive responses. Male and female C57BL/6J mice were assigned to the following groups: paradoxical sleep deprived (PSD) for 72 h, sleep restricted (SR) for 15 days, exposed to respective recovery periods for 24 h, or untreated home-cage controls (CTRL). Mice were submitted to a noxious thermal stimulus to evaluate their nociceptive response after PSD, SR, or recovery periods. Blood was collected for hormonal analysis. The nociceptive response was significantly lower in PSD and SR mice compared to CTRL animals, regardless of the sex. However, SR females had a lower paw withdrawal threshold than males. Sleep recovery was able to restore normal nociceptive sensitivity after PSD in both sexes. The hyperalgesia induced by SR was not reversed by sleep rebound. In females, low concentrations of estradiol were found after SR, and these concentrations continued to decrease after 24 hours of sleep recovery. The PSD male mice exhibited higher concentrations of corticosterone than the CTRL and SR male mice. Corticosterone levels were not affected by SR. Our study revealed that PSD and SR induce hyperalgesia in mice. The SR groups showed marked changes in the nociceptive response, and the females were more sensitive to these alterations. This finding indicates that, although different periods of sleep deprivation change the nociceptive sensitivity in male and female mice, sex could influence hyperalgesia induced by chronic sleep loss.

Introduction

Sleep is a vital function that is related to several physiological systems. This relationship is demonstrated by a significant body of research that describes the impairment of health and well-being after periods of sleep deprivation. For example, a lack of sleep has often been associated with increased pain (Moldofsky, 2001; Smith and Haythornthwaite, 2004; Edwards et al., 2008; Andersen et al., 2009a; Haack et al., 2009; Jones et al., 2009). Total sleep deprivation or selective REM/paradoxical sleep deprivation may have direct effects on nociceptive responses, leading to hyperalgesia in both humans (Onen et al., 2001a; Lautenbacher et al., 2006; Roehrs et al., 2006) and rodents (Onen et al., 2000; Nascimento et al., 2007; Damasceno et al., 2009). Although several studies have demonstrated an association between sleep loss and pain, most of them report the repercussions of acute sleep deprivation. Limited attention has been given to the consequences of chronic sleep reduction. Nevertheless, gradual sleep loss over long periods is becoming increasingly prevalent in modern life, and this circumstance warrants further investigation.

It appears that the distinct changes in multiple physiological processes that result from sleep disruption are sex-dependent (Andersen et al., 2008, 2009b, 2010). This finding indicates that alterations in the nociceptive system induced by sleep loss may be different between males and females. Likewise, some animal studies have identified sex differences in the nociceptive response (DeLeo and Rutkowski, 2000; LaCroix-Fralish et al., 2005; Schütz et al., 2009). However, it is not clear whether sex differences exist in the nociceptive system, or whether these differences are present only in specific painful conditions.

Despite several studies that demonstrate a bidirectional association between sleep and pain and many studies reporting sex differences in the pain responses, there have been limited studies regarding the interaction between sleep loss, pain, and sex differences. Moreover, further investigation is needed on the repercussions of chronic sleep restriction on the nociceptive system. Therefore, the aim of this study was to evaluate the effect of different periods of sleep deprivation on nociceptive responses and to investigate possible sex differences in these responses. We also examined whether sleep recovery periods could reverse alterations in the nociceptive system induced by sleep loss. Finally, we analyzed the effect of sleep deprivation conditions on hormone concentrations (i.e., corticosterone and estradiol).

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Methods

Subjects

Adult male and female C57BL/6J mice aged 90 days were bred in the animal facility of the Centro de Desenvolvimento de Modelos Experimentais (Universidade Federal de São Paulo, Brazil). The animals were housed in standard polypropylene cages in a colony maintained at 22 °C with a 12:12-h light-dark cycle (lights on at 07:00 h). Food and water were available *ad libitum*. The experimental protocol was approved by the University's Ethical Committee for animal experimentation (#707/09).

Paradoxical sleep deprivation (PSD)

Mice were placed inside cages (38 × 31 × 17 cm, 5 mice per cage) containing 13 circular platforms (3.5 cm in diameter) with water 1 cm above the platform surface for 72 h. At the onset of each paradoxical sleep episode, the animals experienced a loss of muscle tonus and fell into the water, causing them to awaken. The water in the cage was changed daily, and food and water were available *ad libitum* throughout the PSD period in the form of pellets and water bottles that were placed on a grid located on top of the tank. We recently demonstrated that this protocol can suppress paradoxical sleep during all periods and can significantly compromise slow-wave sleep in adult mice (Zager et al., 2009). A control (CRTL) group was maintained in cages that contained sawdust and the same number of animals as the treated groups.

Sleep restriction (SR)

Mice were submitted to a modified multiple platform method (Zager et al., 2009) as described above (5 mice per cage containing 13 circular platforms) for 21 h (beginning at 13:00 h) and for 15 days (SR period). After each 21 h sleep deprivation period, the mice were allowed to sleep for 3 h (sleep opportunity beginning at 10:00 h).

Hot plate test

Immediately after PSD, SR, recovery, or the equivalent period in CTRL mice, individual animals were placed on a hot plate (Ugo Basile, Biological Research Apparatus Company, Italy) maintained at 50 ± 0.1 °C for the evaluation of nociceptive sensitivity. The latency to withdraw the paw (lick of either a hindpaw or forepaw or jumping off) to avoid thermal nociception was measured (in seconds), at which point the mouse was immediately removed from the hot plate. A latency period of 90 sec was defined as complete analgesia and used as the cut-off time for mice that did not respond (Bolles and Fanselow, 1982). The test was conducted between 08:00 and 10:00 h.

Experimental design

Male and female mice were randomly assigned to CTRL (n = 10), PSD (n = 10 males and 9 females), SR (n = 9 males and 8 females), and recovery period groups. The recovery period groups were sleep-deprived or sleep-restricted and then returned to home cages and allowed to have undisturbed, spontaneous sleep for 24 h. These groups were named PSD-R (n = 10 males and 8 females) and SR-R (n = 9). The CTRL mice were maintained in separate cages in the same room as the experimental mice during the procedures and were euthanized on the same day as the other groups. By housing all of the groups in the same room, we controlled for the environmental conditions. After residing in the water tanks (PSD and SR groups) or home-cages (CTRL, PSD-R, and SR-R groups), the mice were brought individually to an adjacent room for pain behavior assessment (Fig. 1). Throughout the experimental protocol, the females were submitted to vaginal smear cytology to determine their estrous cycle phases (Caligioni, 2009). During the process of running the PSD and SR protocols, some animals were euthanized because they failed to remain on the top of the platforms and developed hypothermia. As a result, the number of animals in each group was different.

Hormonal analysis

At the end of the hot plate test, all animals were brought individually to an adjacent room and decapitated between 10:00 and

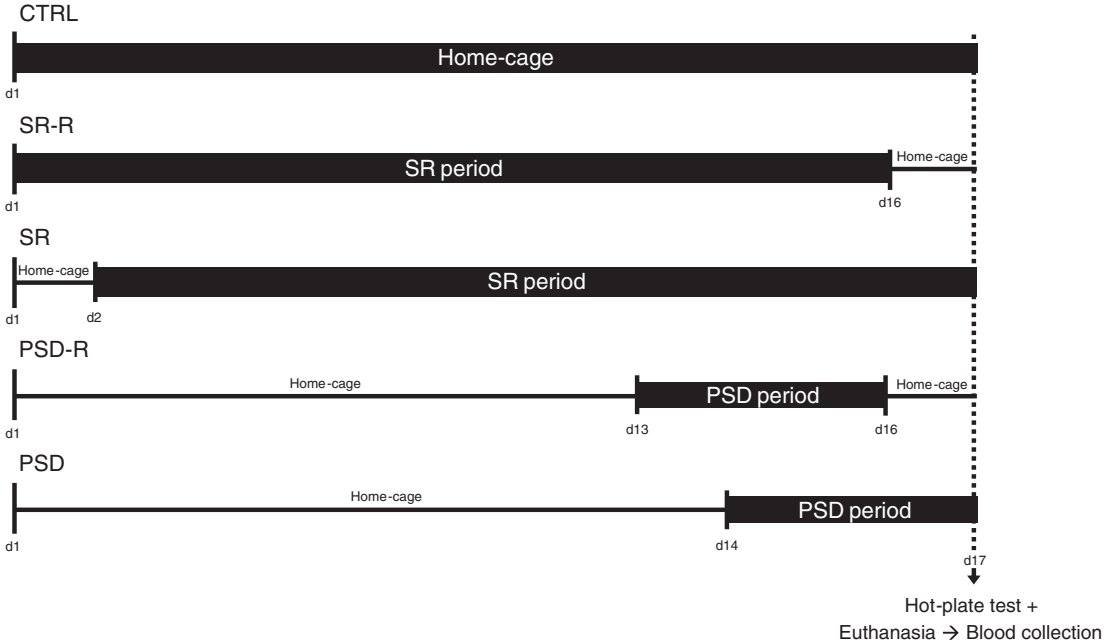

Fig. 1. The experimental design is illustrated. Abbreviations are as follows: CTRL, control; SR, sleep restriction; SR-R, sleep restriction + sleep recovery; PSD, paradoxical sleep deprivation; PSD-R, paradoxical sleep deprivation + sleep recovery; d, day of the experimental protocol.

12:00 h. Blood was collected in glass tubes and centrifuged (3,500 rpm/15 min) at 4 °C to obtain samples of plasma. Intra-assay coefficients of variation are given in parentheses. Plasma corticosterone concentrations (7.1%) were assayed using a double antibody radioimmunoassay method specific for rats and mice using a commercial kit (MP Biomedicals, USA). The sensitivity of the assay was 0.25 ng/ml. The radioimmunoassay technique was used to determine the concentrations of estradiol (7.6%, MP Biomedicals, USA), and the minimum detection limit was 10 pg/ml.

Statistical analysis

The results of the nociceptive response tests and the hormonal concentrations showed a non-normal distribution. Therefore, the data were analyzed using the non-parametric Kruskal-Wallis test. *Post hoc* comparisons were performed using the Games-Howell test when necessary. The body weight gain data were evaluated by an analysis of variance (ANOVA) followed by Tukey's test for comparisons between the groups. The results are expressed as medians±SEM. The level of significance was set at $p<0.05$.

Results

Effects of sleep loss on body weight

As expected, the CTRL groups had a gain in body weight during the experimental period. Acute and chronic sleep loss significantly affected body weight. Both the PSD ($p<0.05$) and SR ($p<0.005$) groups showed significant body weight loss compared to CTRL mice, as revealed by a one-way ANOVA [$F_{(5,50)}=14.359$; $p<0.001$] followed by Tukey's test (Fig. 2A). The body weight remained reduced in the PSD-R ($p<0.001$) and SR-R ($p<0.001$) males compared to CTRL group (one-way ANOVA [$F_{(5,50)}=14.261$; $p<0.001$]). In addition, the PSD-R ($p<0.05$) and SR-R ($p<0.001$) males showed significant body weight reductions compared to PSD-R and SR-R female groups, respectively (see Fig. 2B). These data demonstrate that 24 h of sleep rebound was not enough to body weight regain in PSD-R and SR-R male groups.

Effects of sleep loss on pain sensitivity

A lack of sleep induced significant hyperalgesia in both male and female mice (Kruskal-Wallis [$H(5)=36.525$; $p<0.001$]). The PSD ($p<0.001$), and SR ($p<0.001$) groups showed a significant increase in the nociceptive response compared to the CTRL groups (Fig. 3A). For example, the PSD group showed a reduction in paw withdrawal latency of 43% in males and 55% in females, whereas the SR group exhibited a decrease of 38% and 57% in males and females, respectively, compared to the CTRL animals. The SR females exhibited shorter paw withdrawal latencies than the SR males (15.3 *vs.* 22.6 sec; $p<0.01$).

Effects of sleep recovery on pain sensitivity

As shown in Fig. 3B, sleep recovery for 24 h was able to restore nociceptive sensitivity to control levels in the PSD mice. The paw withdrawal latency was still shorter in the SR males ($p<0.05$) and females ($p<0.01$) when compared with the respective CTRL group even after 24 h of sleep recovery (Kruskal-Wallis [$H(5)=25.245$;

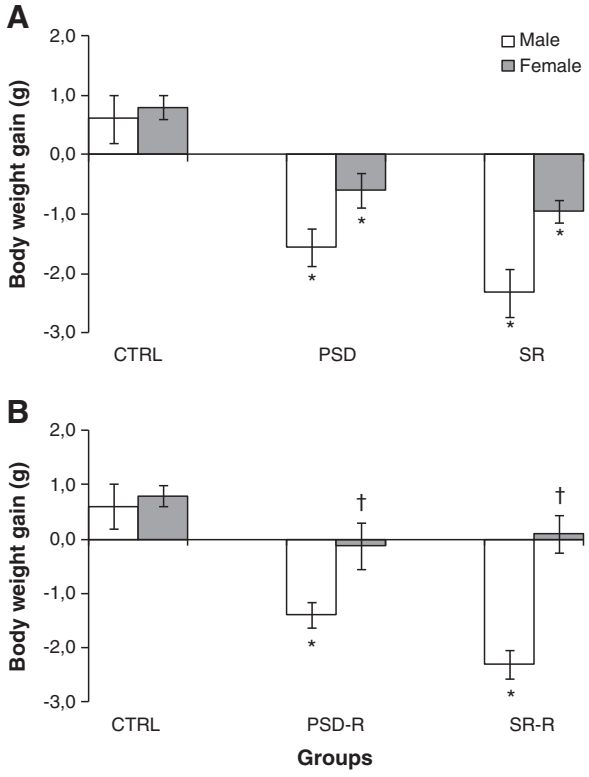

Fig. 2. The effects of sleep loss on body weight gain in control (CTRL, n=10), paradoxical sleep deprived (PSD, n=10 males and 9 females), sleep restricted (SR, n=9 males and 8 females) (panel A) and sleep recovery period (PSD-R, n=10 males and 8 females, and SR-R, n=9) (panel B) male and female mice are shown. The data are shown as means±SEM. * *vs.* respective CTRL group; † *vs.* other sex, same treatment. $p<0.05$.

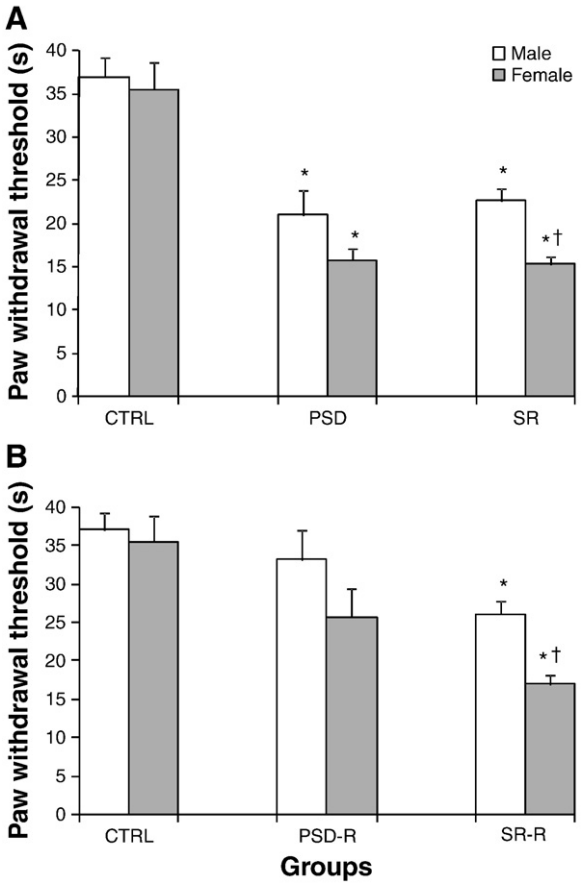

Fig. 3. The effects of sleep loss on the paw withdrawal threshold in response to thermal noxious stimulus in control (CTRL, n=10), paradoxical sleep deprived (PSD, n=10 males and 9 females) and sleep restricted (SR, n=9 males and 8 females) male and female mice (panel A) and after sleep recovery periods (PSD-R, n=10 males and 8 females, and SR-R, n=9) (panel B) are shown. The data are shown as means±SEM. * *vs.* respective CTRL group; † *vs.* other sex, same treatment. $p<0.05$.

$p<0.001$]). The SR-R females also had lower paw withdrawal thresholds than the SR-R males (17 *vs.* 26.1 sec; $p<0.05$).

Hormone concentrations

The corticosterone concentration values are presented in Table 1. The PSD mice exhibited higher concentrations of corticosterone than the CTRL and SR groups. The Kruskal-Wallis test ($H(5)=22.781$; $p<0.05$) followed by Games-Howell *post hoc* test demonstrated that the PSD males showed significant differences compared to the CTRL group (136.4 *vs.* 28.4 ng/ml; $p<0.05$). Moreover, in the recovery groups the levels of corticosterone in the SR-R males were statistically lower than those in the SR-R females (51 *vs.* 32.2 ng/ml; $p<0.05$), as revealed by Kruskal-Wallis test ([$H(5)=25.23$; $p<0.05$]). Because the SR mice showed sex differences in their nociceptive responses, we elected to examine the estradiol concentrations only in the female mice that were submitted to the SR protocol. Low concentrations of estradiol were observed after SR (-21% in relation to CTRL), and the estradiol concentrations were decreased after 24 h of sleep recovery relative to the CTRL and SR groups (Kruskal-Wallis [$H(2)=11.112$; $p<0.005$]), as shown in Fig. 4 ($p<0.01$). It is important to note that all females submitted to SR, regardless of their estrous cycle phase at the start of the protocol, showed a constant diestrus during the SR period. In contrast, the CTRL and PSD females displayed regular estrous cycles. Several aliquots of plasma were excluded from the hormonal analysis due to refrigeration problems. Therefore, the number of animals used in the hormonal analysis was lower than that for the pain behavior analysis.

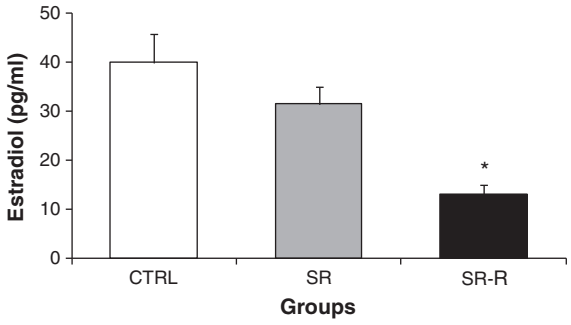

Fig. 4. Mean±SEM concentrations of plasma estradiol in control (CTRL, n=9), sleep-restricted (SR, n=6) and recovery period (SR-R, n=5) female mice groups are shown. * *vs.* CTRL and SR. $p<0.05$.

Discussion

The present findings reveal that acute and chronic experimental sleep loss were both able to produce marked alterations in the nociceptive sensitivity in male and female mice. Our findings demonstrated sex differences in nociceptive responses during chronic SR, with females exhibiting a lower paw withdrawal threshold during a hot plate test. Moreover, an investigation of the positive effects of sleep recovery on the modulation of the nociceptive response indicated that 24 h of sleep reversed the hyperalgesia produced by PSD. In contrast, the same period of sleep recovery was not able to restore normal nociceptive sensitivity in the SR mice.

An increase in nociceptive sensitivity after sleep deprivation has been reported in animal studies (Hicks et al., 1978, 1979; Onen et al., 2000, 2001b; Nascimento et al., 2007; Wei et al., 2008; Andersen et al., 2009a; Damasceno et al., 2009). However, these studies were conducted primarily in rats. Only one study evaluated the nociceptive response in mice, and it found no significant change in the thermal threshold for the hot plate test after 48 h of PSD (Asakura et al., 1992). Our study is the first to demonstrate that male and female mice submitted to acute and chronic sleep deprivation show hyperalgesia.

The ability of sleep recovery to reverse the increase in nociceptive sensitivity induced by sleep loss is still controversial. Hicks et al. (1979) showed that hyperalgesia in female Sprague-Dawley rats persisted until 96 h after the end of PSD. However, Onen's study reported that the alterations in the nociceptive response in male Wistar rats induced by 72 h of PSD returned to baseline levels after 24 h of sleep rebound (Onen et al., 2000). More recently, our group demonstrated that 96 h of PSD decreased the paw withdrawal threshold for the hot plate test in male Wistar rats, and that this effect persisted after 24 h of sleep recovery (Nascimento et al., 2007). Our current study found that the hyperalgesia induced by 72 h of PSD was reversed by sleep recovery. It is interesting to note that 24 h of sleep rebound was not sufficient to return the nociceptive response to control baseline values in the SR mice. The fact that the nociceptive sensitivity remained altered after sleep recovery suggests that gradual sleep loss over long periods causes more marked alterations in the nociceptive system than acute sleep deprivation. Altogether, it seems that variations in the duration of sleep deprivation, the type of noxious stimulus, and the sex and strain of the animal could explain some of the discrepancies in the literature.

The mechanisms through which sleep loss can induce hyperalgesia are not fully understood. It has been reported that some brain structures and neurotransmitter systems that regulate the sleep-wake cycle are also involved in the control of the nociceptive response (Bannister et al., 2009; Watson et al., 2010). Sleep deprivation has been shown to produce changes in various neurotransmitter systems, such as the monoaminergic system (mainly the serotonergic raphe nuclei, the noradrenergic locus coeruleus, and dopaminergic neurons), which are involved in the control of pain and sleep (Bannister et al., 2009; Ohayon, 2009). These alterations in the neurotransmitter levels can lead to changes in neurotransmitter receptor function (Longordo et al., 2009). For example, the capacity of serotonin (5-HT) to inhibit or facilitate the nociceptive response depends on the receptor subtype that is expressed in the sensory pathway. It is known that the spinal 5-HT_{1A} receptors modulate antinociceptive actions, and that the 5-HT_{2C} and 5-HT_3 receptors mediate pronociceptive actions (Bannister et al., 2009). However, both the intrathecal administration of selective 5-HT_{1A} or 5-HT_{2C} receptor antagonists into the lumbar level of the spinal cord reduced nociceptive hypersensitivity induced by PSD in rats (Wei et al., 2008). This finding suggests that the continuous stimulation of the wake-promoting system during sleep deprivation may alter the receptor function and contribute to maintenance of hyperalgesia. Although additional studies are needed to elucidate possible central mechanisms and neurotransmitter systems through which sleep loss could alter nociceptive sensitivity, it is speculated that REM/paradoxical sleep is essential for the integrity of the nociceptive system.

Table 1
The effects of acute and chronic sleep loss on concentrations of plasma corticosterone (ng/ml) are shown. The data are expressed as medians±SEM.

	Groups				
	CTRL	PSD	SR	PSD-R	SR-R
Male	28.43 (±0.9)	136.47 (±39.4)[a]	34.34 (±1.2)	28.19 (±0.44)	33.22 (±1.7)
Female	35.96 (±2.2)	164.86 (±57.7)	121.1 (±46.5)	67.84 (±14.2)	51 (±3.8)[b]

CTRL: control (n=8 males and 9 females); PSD: paradoxical sleep deprivation (n=10 males and 9 females); SR: sleep restriction (n=5 males and 8 females); PSD-R: paradoxical sleep deprivation + recovery (n=10 males and 8 females); SR-R: sleep restriction + recovery (n=5 males and 9 females). [a] *vs.* respective CTRL group; [b] *vs.* SR-R male. $p<0.05$.

The relationship between sex and nociception is complex. Whereas some studies have demonstrated that female rodents show a greater nociceptive response to noxious stimuli (Mogil et al., 2000; Tall and Crisp, 2004; Li et al., 2009), others have found no sex differences in nociception (Greenspan et al., 2007; Leo et al., 2008). For example, in a series of tests of thermal and mechanical acute nociception in female and male mice of four strains, including C57BL/6J (which was used in the present study), the authors failed to find significant differences in nociception between the sexes (Leo et al., 2008). Consistent with these results, our findings did not show sex differences in pain behavior within the CTRL group or after PSD. In contrast, when the animals were submitted to 15 days of SR, the females exhibited lower nociceptive thresholds. This indicates that sex may influence the nociceptive response during chronic sleep deprivation.

The SR method mimics the human lifestyle of restricted sleep durations and prolonged wakefulness, a condition that is progressively increasing in current society. In our study, the SR group showed marked changes in the nociceptive response, with females being more susceptible than males to the negative effects of chronic sleep loss. It is reasonable to assume that these alterations in the nociceptive system could be related to changes in estradiol levels. Females exhibited low endogenous concentrations of estradiol after the SR protocol, and this decrease was more accentuated in SR females after 24 h of sleep rebound. This result may indicate a possible long-lasting impairment of the gonadal hormones that results from sleep loss. Estrogens have been suggested to play a role in the nociceptive system (Craft et al., 2004; Craft, 2007). However, the exact role of estradiol is still controversial. The results of animal studies have suggested that high concentrations of estradiol are both pronociceptive (Lu et al., 2009) and antinociceptive (Kramer and Bellinger, 2009; Aloisi et al., 2010). In this context, low estradiol concentrations could be related to sex differences in the nociceptive response and in the hyperalgesic effects of chronic sleep loss.

It is important to note that SR females had their estrous cycles disrupted. We previously demonstrated that estrous cyclicity is markedly affected after PSD in rats (Antunes et al., 2006). In that study, only the females that started PSD during the diestrus phase showed an anestrous period (constant diestrus phase) during sleep recovery. In the present study, all of the female mice submitted to SR exhibited an anestrous phase throughout the chronic SR protocol. These findings indicate that SR may profoundly affect ovarian hormone release and modulate physiological and behavioral processes related to estrous cyclicity.

Evidence suggests that prolonged stress can decrease the nociceptive threshold and exacerbate some painful conditions (Alexander et al., 2009). Thus, the question arises as to whether the alterations we observed in pain thresholds were caused by PSD/SR or by the stress inherent in our methodology. It is well established that the PSD method induces increases in corticosterone (Andersen et al., 2004, 2005). In fact, our results showed that corticosterone levels changed after 72 h of PSD. We acknowledge that sleep deprivation is an inherently stressful procedure, so it may not be possible to completely extricate the sleep deprivation effects from the general stress effects. Nevertheless, several aspects of our findings argue against the possibility that non-specific stress could account for our observations. Interestingly, the SR mice showed no significant increases in corticosterone, whereas they showed marked changes in nociceptive sensitivity. Of note, our previous study demonstrated that the SR method did not affect corticosterone levels in rats (Zager et al., 2007; Andersen et al., 2009b). It seems that there is an adaptation of the hypothalamic-pituitary-adrenal response in animals that are submitted to chronic stress. To our knowledge, this is the first time that the influence of 15-day SR on the corticosterone levels of mice has been reported. The lack of increased levels of corticosterone after partial sleep loss suggests that the mice were able to cope with the stress inherent in the procedure. Because the animals showed hyperalgesia after being exposed to SR, whereas their corticosterone levels remained unchanged, these results suggest that the changes in pain sensitivity were due to sleep loss rather than to stress.

In recent years, there has been increasing evidence regarding the health dangers caused by inadequate sleep. Among the many impacts of sleep deprivation on health and well-being, alterations in pain sensitivity have received considerable attention. Given the close associations between acute and chronic pain and sleep disturbance, and the substantially greater prevalence of many pain conditions in women, it is critical to understand how changes in sleep patterns, like PSD and SR, can alter pain and the interaction of these changes with gonadal hormones. Furthermore, it is crucial to understand the roles of the nervous, endocrine, and immune systems in the sex differences in hyperalgesia induced by sleep loss. Our findings revealed that chronic SR changes the nociceptive response in mice, and that females were more affected by these alterations. This knowledge is essential for a better understanding of the role of sleep in the nociceptive system and how sleep loss could modulate sex differences in nociception.

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