

## Are endogenous sex hormones related to DNA damage in paradoxically sleep-deprived female rats?

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### ABSTRACT

The aim of this investigation was to evaluate overall DNA damage induced by experimental paradoxical sleep deprivation (PSD) in estrous-cycling and ovariectomized female rats to examine possible hormonal involvement during DNA damage. Intact rats in different phases of the estrous cycle (proestrus, estrus, and diestrus) or ovariectomized female Wistar rats were subjected to PSD by the single platform technique for 96 h or were maintained for the equivalent period as controls in home-cages. After this period, peripheral blood and tissues (brain, liver, and heart) were collected to evaluate genetic damage using the single cell gel (comet) assay. The results showed that PSD caused extensive genotoxic effects in brain cells, as evident by increased DNA migration rates in rats exposed to PSD for 96 h when compared to negative control. This was observed for all phases of the estrous cycle indistinctly. In ovariectomized rats, PSD also led to DNA damage in brain cells. No significant statistically differences were detected in peripheral blood, the liver or heart for all groups analyzed. In conclusion, our data are consistent with the notion that genetic damage in the form of DNA breakage in brain cells induced by sleep deprivation overrides the effects related to endogenous female sex hormones.

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### Introduction

Disturbances in the sleep-wake cycle and sleep deprivation are driven by the demands and opportunities of modern life. Currently, most sleep deprivation occurs in the REM/paradoxical phase, which occurs during the last half of the total sleep session. It is in this phase that most restorative processes take place. Indeed, our recent study demonstrated that, in 2007, 26.7% of 1042 individuals surveyed complained of early awakening (unpublished data).

There are several approaches that may be used to understand the functional properties of paradoxical sleep. A common procedure involves sleep deprivation in several species and subsequent observation of behavioral and physiological changes. Thus, most studies of sleep deprivation focus on paradoxical sleep deprivation (PSD). However, the consequences of sleep disturbances/sleep deprivation have been documented essentially only in men. In sleep surveys, women report considerably more sleep problems than men (Lindberg et al., 1997; Silva et al., 2008; Arber et al., 2009). In rodents, several behavioral, hormonal and neurochemical changes have been found

after periods of sleep deprivation in males (Andersen et al., 2002; Frussa-Filho et al., 2004; Ruiz et al., 2007; Fukushiro et al., 2007; Perry et al., 2008; Dash et al., 2009; Alvarenga et al., 2009a,b). Although data from female animals are lacking, a recent study found that female rats submitted to PSD for 96 h in the diestrus phase had their estrous cycles disrupted during the recovery period as demonstrated by persistent diestrus (Antunes et al., 2006).

In recent years, increasing attention has been given to genetic changes caused by sleep loss in different sleep-loss contexts (Cirelli et al., 2004, 2006; Terao et al., 2006; Guindalini et al., 2009). For example, PSD for 96 h can induce molecular changes in the brain such as those genes related to metabolic processes, response to stimulus (including stress and inflammation), circadian/sleep cycles, regulation of cell proliferation and signaling pathways (Guindalini et al., 2009). Moreover, brain tissue showed extensive genotoxic damage in PSD rats, in a time-dependent fashion, as the effect was more pronounced in the 96 h (Andersen et al., 2009). Collectively, these findings provide a unique set of alterations that might be specific related to regulation of paradoxical sleep phase and sleep homeostasis processes, as well as to the biological basis of sleep disorders.

If PSD disrupts the estrous cycle (Antunes et al., 2006) and both sexes display distinct sleep recovery patterns as a result of PSD (Andersen et al., 2008), selective sleep deprivation may promote

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genomic instability in the brain as well as other organs due to hormonal fluctuations during the estrous cycle. Such a study would contribute to our understanding of the potential consequences associated with sleep deprivation on the neuroendocrine profile and reproduction functions of females. The single cell gel (comet) assay is a rapid, simple and reliable biochemical method for evaluating DNA damage in mammalian cells (Tice et al., 2000). The extent of the comet is correlated with increased DNA damage.

Therefore, to study the effects of PSD on DNA damage in female rats, we determined the role of endogenous sexual hormones in ovariectomized (OVX) and intact rats during three different phases of the estrous cycle. Secondly, we also examined hormonal levels in these rats.

## Materials and methods

### Animals

Three-month-old female Wistar rats were bred and raised in the animal facility of the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) of Universidade Federal de São Paulo (UNIFESP). The animals were housed in a colony maintained at a controlled temperature ( $22^{\circ} \pm 1^{\circ} \text{C}$ ) using a 12 h light-dark cycle (lights on from 0700 to 1900 h) and allowed free access to food and water inside standard polypropylene cages. The experimental protocol was approved by the Ethical Committee of UNIFESP (#09/0066).

### Ovariectomy surgical procedures

Animals were anesthetized with diazepam (4 mg/kg, i.p.) and ketamine (75 mg/kg, i.p.). A group of female rats was submitted to ovariectomy (OVX) by removing the glands through one abdominal incision while under anesthesia. This model is effective for investigating behavioral changes in sex-hormone deficient contexts. Marked endothelial dysfunction and intimal thickening also occur following menopause. After 30 days of surgery, the animals were submitted to experimental procedures. The main importance of utilizing ovariectomized females after 30 days of surgery was to avoid any hormonal influence originating from their reproductive cycle (Monteiro et al., 2008; de Fraga et al., 2009).

### Vaginal cytology

Vaginal smears were conducted in order to determine the phase of the estrous cycle, and all samples were taken between 0800 and 0900 h. Changes in vaginal epithelial cell morphology were used to indicate the phase of the estrous cycle in terms of the occurrence of the following three cell types in the vaginal smears: leukocytes, cornified cells, and nucleated epithelial cells. Proestrus (P) was characterized by many nucleated epithelial cells and a few leukocytes, Estrus (E) by many cornified cells and no leukocytes, and Diestrus (D) by the presence of few nucleated epithelial cells and many leukocytes. All animals were smeared daily, and the rats that had two regular cycles were selected. Female rats were randomly assigned into a paradoxical sleep-deprived group or home-cage control (CTRL) group ( $n = 5\text{--}7/\text{group}$ ).

### Paradoxical sleep deprivation (PSD)

The experimental groups were submitted to a single platform sleep deprivation method. The single platform-on-water (flower pot) method is extensively used for depriving paradoxical sleep. We elected to use the single platform method in order to examine the possible alterations in body weight in each female rat that could occur during the sleep deprivation period. This technique involves placing the animal on a narrow circular platform (10 cm in height and 6.5 cm

in diameter) placed inside a chamber (23 X 23 X 35 cm) filled with water to within 1 cm of their upper surface over a period of 96 h. At the onset of each paradoxical sleep episode, the animal experiences a loss of muscle tone and falls into the water, thus being awakened. The narrow platform procedure causes complete and selective loss of PS during all 4 days. Therefore, our aim in choosing this technique was to evaluate the changes that occur in the rat cerebral cortex that would reflect a predominant suppression of paradoxical sleep over 4 days. Food and water were provided *ad libitum* by placing chow pellets and water bottles on a grid located on top of the chamber. The basis of the feeder was adapted with a plate to prevent pieces of chow from falling into the water. The water in the chamber was changed daily throughout the PSD period. Control rats were placed inside the water chamber, but, instead of water, the chamber was filled with sawdust.

### Groups and experimental design

Fourteen days prior to the experimental period, the intact rats were smeared daily and the animals that had two regular cycles were selected. After the determination of estrous phase, female rats were randomly distributed into the following groups: home-cage control groups (CTRL-D, CTRL-P and CTRL-E) or sleep-deprived groups (PSD-D, PSD-P and PSD-E). We also included OVX groups (CTRL and PSD).

### Sample collection

At the end of the PSD period, all groups were brought to an adjacent room and decapitated. Blood samples were collected and stored individually. The control rats were decapitated along with the PSD groups for each estrous cycle phase. Blood was collected in glass tubes and centrifuged at 3018.4g for 15 min at room temperature for serum and at  $4^{\circ} \text{C}$  to obtain plasma. Intra-assay coefficients of variations are given in parentheses. The serum testosterone (7.7%) and progesterone (6.5%) were measured by chemiluminescent enzyme immunoassay (Advia Centaur, Bayer Corporation, Tarrytown, NY, USA). Plasma corticosterone (7.1%) concentrations were assayed by a double antibody radioimmunoassay method specific for rats and mice using a commercial kit (MP Biomedicals, Orangeburg, New York). The sensitivity of the assay was 0.25 ng/ml. Central fragments from heart, liver and brain (prefrontal cortex) were collected and minced in 0.9% NaCl. The supernatant was removed, and the cellular suspensions ( $\sim 10 \mu\text{l}$ ) were used for the single cell gel (comet) assay.

### Single cell gel (comet) assay

The protocol used for peripheral blood, brain, heart, and liver cells followed the guidelines outlined by Sasaki et al. (2002) with some modifications. The topographical prefrontal cortex region was chosen taking into consideration previous studies published by our group (Alvarenga et al., 2009a,b; Andersen et al., 2009; Guindalini et al., 2009). Briefly, a volume of 5  $\mu\text{l}$  of peripheral blood was added to 120  $\mu\text{l}$  of 0.5% low-melting-point agarose at  $37^{\circ} \text{C}$ , layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. Similarly, the supernatants (cellular suspension, 10  $\mu\text{l}$ ) of the organ samples were added to 120  $\mu\text{l}$  of 0.5% low-melting-point agarose at  $37^{\circ} \text{C}$ , layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed, and slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer (pH > 13) for 20 min and then electrophoresed for another 20 min, at 0.7 V/cm, 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5), fixed in absolute ethanol and stored until analysis on a fluorescent microscope at 400 $\times$  magnifica-

tion. Independent positive controls using cells from peripheral blood, brain (prefrontal cortex), heart and liver of the negative control group were treated *in vitro* with 10 µg/mL MMS (methylmethanesulfonate) for 30 min at 37 °C, in order to ensure the reproducibility and sensitivity of assay as established in previous studies conducted by our group investigating noxious activities of chemicals or biological agents in multiple organs of rats (Ribeiro et al., 2004; Grassi et al., 2007; Andersen et al., 2009).

#### Genotoxicity data analysis

A total of 50 randomly captured comets per animal (25 cells from each slide) (Hartmann et al., 2003) were examined blindly by one expert observer at 400× magnification using a fluorescent microscope (Olympus) connected to a black and white camera and to an image analysis system (Comet Assay II, Perceptive Instruments, Suffolk, Haverhill, UK) calibrated previously according to manufacturer's instructions. The computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail, and damaged cells have a tail and look like a comet. To measure DNA damage, two image analysis system parameters were considered: tail intensity (% migrated DNA) and tail moment (the product of the tail length and the fraction of DNA in the comet tail) (Hartmann et al., 2003). Since none of the groups showed significant differences between these parameters, we presented the tail moment for the results.

#### Statistical methods

Since tail moment data are expressed in arbitrary units, these values were evaluated statistically with the Kruskal–Wallis non-parametric test followed by the *post-hoc* Dunn's test. Values are expressed as mean ± SD. Hormonal data were analyzed using a two-way ANOVA test followed by a Duncan test for comparison between groups and phases. Body weight was analyzed by Student's *t*-test for independent samples. The level of significance was set at 5%. Data are presented in the figures and text as means ± SD.

## Results

#### DNA damage

In this study, we evaluated genetic damage induced by sleep deprivation associated with ovariectomy *in vivo* in different target organs. Extensive genotoxic effects were observed in brain cells, in which an increased DNA migration rate was detected in rats submitted to PSD compared to negative control. This was observed for all phases of the estrous cycle indistinctly. In ovariectomized rats, the observations were made with significant statistical differences evident compared to control ( $p < 0.05$ ). However, no significant statistical differences were detected among all phases of the estrous cycle, including the ovariectomized groups ( $p > 0.05$ ). The findings are summarized in Fig. 1.

No significant differences in DNA damage were found in the peripheral blood cells of female rats submitted to PSD for 96 h during all phases of the estrous cycle as well as ovariectomized rats when compared to negative controls (CTRL) (Fig. 2A). No genotoxicity was found in the liver in any experimental group compared to the respective control group (Fig. 2B), and no statistically significant genotoxicity was found in heart cells. These findings are summarized in Fig. 1C.

Rat blood, liver, brain and heart cells were further assayed with MMS to ensure the sensitivity of the assay. Indeed, significant sensitivity was observed when compared to negative controls ( $p < 0.05$ ).

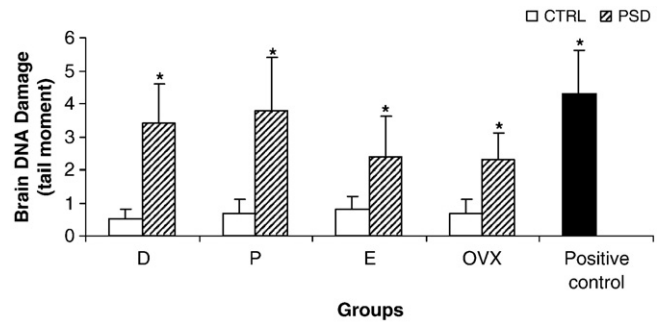


Fig. 1. DNA damage expressed as the mean tail moment in rat brain (prefrontal cortex) cells following paradoxical sleep deprivation (PSD) at the three phases of the estrous cycle and in ovariectomized rats. Values are expressed as mean ± SD. \* $p < 0.05$  as compared to negative control. CTRL—control group; PSD—paradoxical sleep deprived group. Proestrus (P); Estrus (E); and Diestrus (D) phases of estrous cycling ( $n = 5–7$ /group).

#### Hormonal analysis

##### Progesterone

In the CTRL groups, progesterone [ $F_{(1,35)} = 4.43$ ;  $p < 0.04$ ] reached its highest concentrations in the CTRL-P and CTRL-E females, as compared to the CTRL-OVX group ( $p < 0.01$  and  $p < 0.05$  respectively),

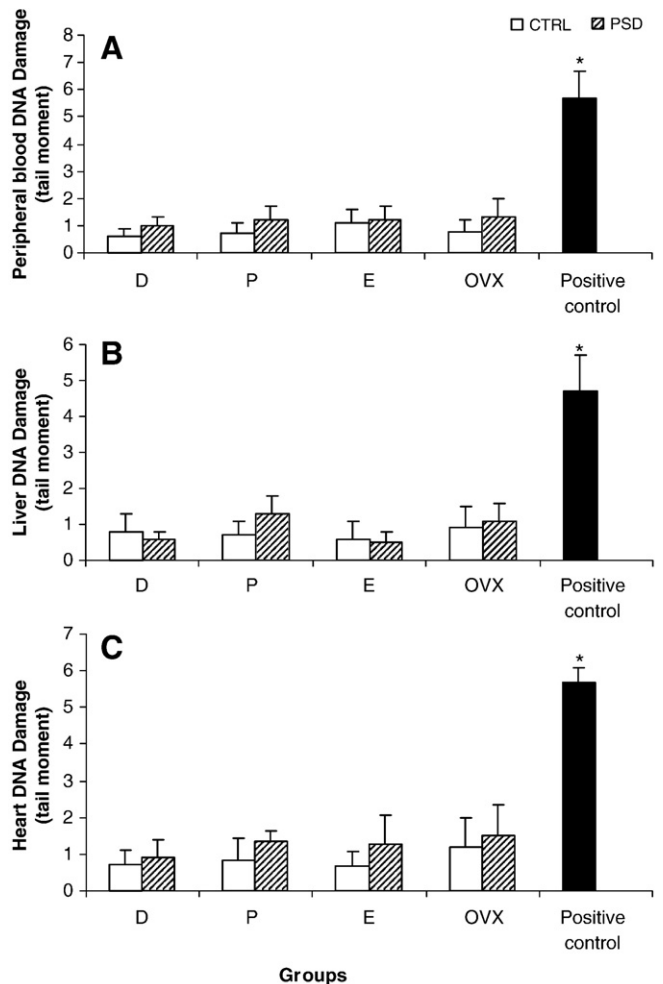


Fig. 2. DNA damage expressed as the mean tail moment in peripheral blood (A), liver (B) and heart (C) cells following paradoxical sleep deprivation (PSD) at the three phases of the estrous cycle and in ovariectomized rats. Values are expressed as mean ± SD. \* $p < 0.05$  as compared to negative control. CTRL—control group; PSD—paradoxical sleep deprived group. Proestrus (P); Estrus (E); and Diestrus (D) phases of estrous cycling. ( $n = 5–7$ /group).

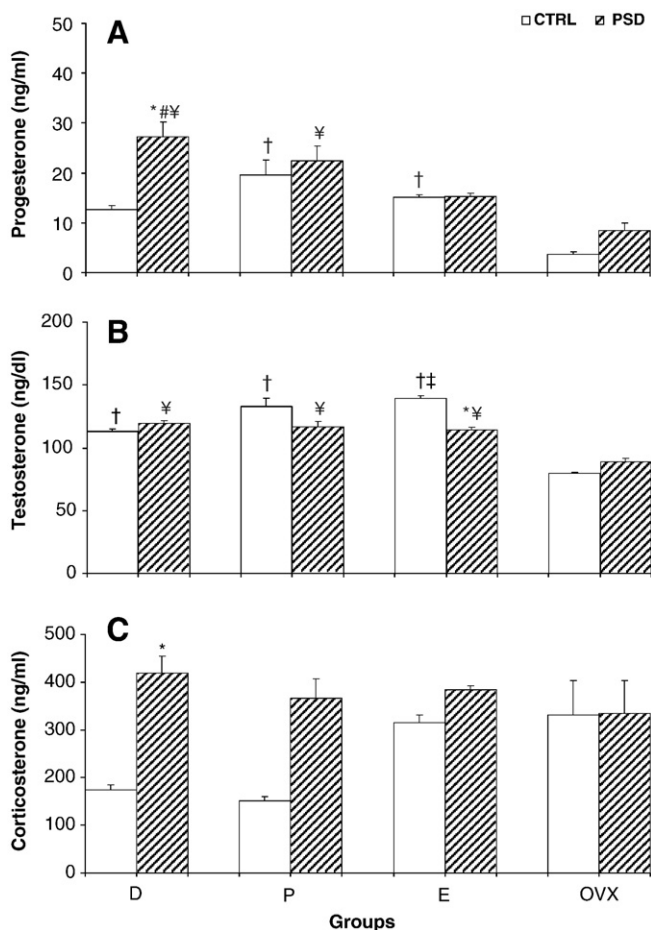
as shown in Fig. 3A. Progesterone concentrations were increased in PSD-D females when compared to the respective CTRL, PSD-E and PSD-OVX groups ( $p < 0.01$ ,  $p < 0.05$  and  $p < 0.01$  respectively), and the PSD-P presented higher progesterone levels than PSD-OVX ( $p < 0.02$ ).

#### Testosterone

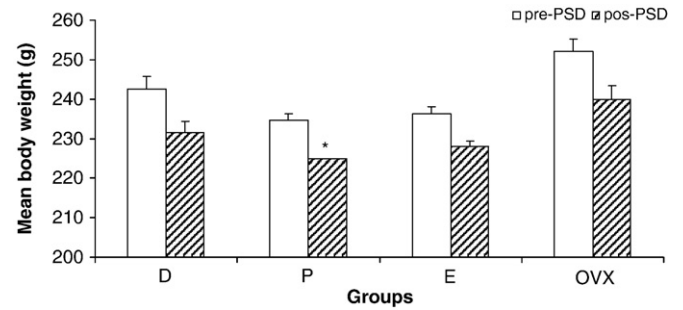
The ANOVA test for testosterone concentrations [ $F_{(1,37)} = 1.44$ ;  $p > 0.05$ ] was significantly higher in the CTRL-D, CTRL-P and CTRL-E groups as compared to the CTRL-OVX group ( $p < 0.001$ ). However, the increase observed in the CTRL-E group was higher than that in the CTRL-D group ( $p < 0.02$ ). The Duncan test indicated a significant increase in testosterone concentrations in sleep deprivation groups D, P and E in comparison to the OVX group ( $p < 0.01$ ,  $p < 0.01$  and  $p < 0.01$ , respectively), as shown in Fig. 3B. Interestingly, the PSD-E group presented a significant reduction in testosterone levels compared to the respective control group ( $p < 0.01$ ).

#### Corticosterone

Corticosterone values [ $F_{(1,31)} = 5.29$ ;  $p < 0.02$ ] are presented in Fig. 3C. PSD-D rats presented a significant increase in corticosterone levels in relation to their respective control group ( $p < 0.05$ ). There were no other statistically significant group differences.



**Fig. 3.** Mean  $\pm$  SD concentrations of serum progesterone (in ng/ml, panel A), testosterone (in ng/dl, panel B) and plasma corticosterone (in ng/ml) in control (CTRL) and paradoxical sleep-deprived (PSD) rats at the three phases of the estrous cycle and in ovariectomized rats. (A) \*Different from the respective CTRL group; †different from PSD-estrus; ‡different from PSD-OVX; ††different from CTRL-OVX. (B) †Different from the respective CTRL group; ‡different from PSD-OVX; ††different from CTRL-OVX; ‡‡different from CTRL-D. (C) \*Different from the respective CTRL group. ( $n = 5-7$ /group).



**Fig. 4.** Mean body weight (mean  $\pm$  SD) of rats maintained as control (CTRL) or submitted to paradoxical sleep-deprived (PSD) at the three phases of the estrous cycle and in ovariectomized rats. \*Different from the respective CTRL group. ( $n = 5-7$ /group).

#### Body weight

The Fig. 4 depicts the body weight of the groups. Student's *t*-test for independent samples showed that only proestrus group had a significantly decrease in weight after the PSD period ( $p < 0.01$ ). No animal died unexpectedly during this experiment.

#### Discussion

The major finding of the present study was that DNA damage occurs in brain cells independent of the phase of the estrous cycle. The comet assay in our experimental conditions failed to detect the presence of DNA damage in peripheral blood cells of female rats submitted to PSD for 96 h. An earlier study conducted by our group revealed that male rats submitted to PSD for the same period develop DNA breakages in peripheral blood cells (Andersen et al., 2009).

The relationship between female sex hormones and genotoxicity and/or mutagenicity remains unclear. Some studies have also pointed out increased frequencies of micronuclei, structural chromosome aberrations, and sister chromatid exchange induced by estradiol in human lymphocytes *in vitro* (Dhillon et al., 1994). Furthermore, discordant results described the effect of physiological hormonal oscillation on DNA integrity. For example, a higher level of DNA damage was found at the time of ovulation compared to the early follicular phase in women who were not taking oral contraceptives (Kapiszewska et al., 2005). Conversely, Bajpayee et al. (2005) evaluated DNA damage using the single cell gel (comet) assay in blood lymphocytes of 18 normal healthy and nonsmoking women and reported an absence of genotoxic lesions related to the hormonal cycle. In the same way, data obtained from our research group suggest a lack of genotoxicity induced by physiological levels of female sex hormones as assessed by the alkaline comet assay *in vivo* and *in vitro* (Braz and Salvadori 2007a,b). Men who suffer from sleep apnea, and consequently exposed to sleep deprivation, have higher basal levels of DNA damage and are more sensitive to the effects of the DNA-damaging agents (Kontogianni et al., 2007).

Given that the brain is considered to be the main organ affected by sleep deprivation, we carefully evaluated the prefrontal cortex of female rats subjected to the PSD protocols under different phases of the estrous cycle. Our results demonstrated extensive genotoxic damage in rats exposed to PSD for 96 h. The phase of the estrous cycle did not interfere with the comet assay results, and this was also true for the ovariectomy group. This result is in agreement with our previous observations that more dramatic changes occur after 4 days of PSD (Andersen et al., 2003, 2005a, 2009; Zager et al., 2007), and it suggests that long periods of PSD may lead to long-term effects that may become increasingly harmful. Taken together, our results suggest that paradoxical sleep loss can induce genetic damage in prefrontal cortex cells of female rats, independent of the phase of the estrous cycle. Our data confirm the critical importance of prefrontal cortex during sleep.

As corticosterone concentrations were increased after PSD, as expected and previously reported (Andersen et al., 2004a,b; Zager et al., 2007), some could argue the stress involved in this platform technique. This methodology results in a total suppression of paradoxical sleep in female rats (Andersen et al., 2008). However, we acknowledge that sleep deprivation is an inherently stressful procedure, thus it may not be possible to completely extricate sleep deprivation effects from general stress effects. Although the basal concentrations of corticosterone could be deemed as somewhat elevated, this only emphasizes the notion that all rats used in our experiments were manipulated in the same manner. The values found here reflect the hormone concentrations of the rat strain investigated as well as the effects of the experimental conditions that the rats experienced. The increase in corticosterone during PSD showed that the rats were adequately responsive to this condition. Yet, several aspects in our findings argue against the possibility that non-specific stress *per se* could account for our observations (Andersen et al., 2004a,b) in which the PSD group differed from the other groups investigated.

Recently, Chang et al. (2008) argued that sleep deprivation predisposes the liver to oxidative stress and phospholipid damage, therefore leading to injury of genetic material. Nevertheless, our results did not detect DNA breakage in liver cells for all female groups investigated. Such findings are in line with our previous published data (Andersen et al., 2009). The time course of the effects of sex hormones on the liver associated with sleep deprivation remains unknown, since the development of genetic damage in target cells depends not only on the initial levels of induced DNA damage and its repair but also on other contributing factors, including the production of reactive metabolites, their distribution, and their effects on cell proliferation. Nevertheless, no single test is capable of detecting all genotoxic agents.

To further elucidate the possible outcomes of sleep deprivation on the cardiovascular system, we evaluated genotoxicity in the heart. Loss of sleep induces elevations in circulating levels of cholesterol (Andersen et al., 2004b), stress-related hormones (Andersen et al., 2004a) and catecholamines (Irwin et al., 1999, 2003; Andersen et al., 2005a,b), with an increase in blood pressure and heart rate (Kato et al., 2000). Furthermore, habitual sleep loss and insomnia are markers of subclinical heart disease and are independent predictors of cardiovascular disease risk (Mallon et al., 2002). Our results show that neither acute sleep deprivation nor endogenous female sexual hormones exert any detectable genotoxic activity in the heart. As these findings are novel and the underlying mechanisms are still unknown, it is difficult to satisfactorily explain these observations.

In conclusion, our results reveal that sleep deprivation exerts genetic damage in the form of DNA breakage in brain cells in female rats, regardless the estrous phase. Since DNA damage is an important step for events leading to genomic instability that can later promote the development of degenerative diseases, this study represents an initial evaluation of the potential health risks associated with sleep deprivation.

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