



Biochemical, biometrical and behavioral changes in male offspring of sleep-deprived mice

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Summary Epidemiological and experimental studies suggest a high prevalence of cognitive impairment and social behavior deficits in adolescents and adults that have experienced prenatal exposure to adverse conditions. This study investigated whether sleep deprivation during the pre-implantation stage of development alters the physiological, behavioral and oxidative metabolic processes in adult male mouse offspring. One group of dams was continuously sleep-deprived using the platform technique from gestational days 0 to 3 (PSD 72). Three additional groups were sleep-deprived by gentle handling for 6 h on gestational days 1 (GH 1), 2 (GH 2) or 3 (GH 3). After sleep deprivation, homocysteine, cysteine, corticosterone, estrogen and progesterone concentrations were measured from the experimental mothers and time-matched controls. The sizes and weights of the male pups were measured at various stages throughout the experiment. At PND 90, behavioral (Activity Box and Elevated Plus Maze) and biochemical parameters were assessed. The dams' plasma progesterone concentrations decreased in the PSD 72 group, and the levels of plasma estradiol increased in GH 2. Corticosterone levels were found to increase after all sleep-deprivation procedures. Homocysteine concentrations increased in the GH 2 but decreased in the PSD 72 group. The offspring of GH 1 mothers exhibited decreased superoxide dismutase activity. Exposure to sleep deprivation had a long-lasting impact on tissue weight; in particular, there was a decrease in hemilateral epididymal fat weight in mature animals from the PSD 72 group. Although some of the alterations observed in the mothers (elevated estrogen and corticosterone levels and decreased progesterone) might have played a role in the permanent alterations in the adult offspring, they were not the main cause. The homocysteine changes detected in the sleep-deprived dams may contribute to redox changes, controlling gene expression and shaping epigenetic development.

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1. Introduction

During pregnancy, two-thirds of women consider their sleep to be abnormal (Pien and Schwab, 2004). These sleep alterations, which begin as early as the first trimester of pregnancy, appear

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to be influenced by pronounced alterations in the levels of reproductive hormones that occur during the gestational period (Lopes et al., 2004; Parry et al., 2006). There is increasing discussion about the risks and consequences related to sleep loss. With increasing numbers of women entering the workplace, any resulting impairments to the quality or duration of their sleep could adversely affect many life events, from safe driving in traffic to social events. Furthermore, hormonal changes associated with a busy modern lifestyle can lead to significant sleep loss, and this, in turn, may have a significant effect during pregnancy. As pregnancies are often not detected before the end of the first month, the inability of the mother to take precautions (including getting adequate sleep) in the initial stages of pregnancy may have several consequences for the offspring.

Although the function of sleep has not been completely elucidated, its homeostatic regulation and the diverse physiological and pathological effects that result from its deprivation or restriction have been well documented in both humans (Penev, 2007; Irwin et al., 2008) and animals (Bergmann et al., 1989; D'Almeida et al., 1998; Frank et al., 1998; Kushida, 2001).

There is evidence that sleep deprivation triggers oxidative processes in various organs (D'Almeida et al., 1997, 1998; Cirelli et al., 2004; Gopalakrishnan et al., 2004; Everson et al., 2005), and the induction of oxidative stress during pregnancy has been already shown to lead to congenital malformations or even fetal death (Wells et al., 2009). In a review article written by Hitchler and Domann (2007), it was suggested that both oxygen and redox conditions may influence the availability of cofactors required by enzymes for the initiation and maintenance of epigenetic events. Furthermore, the redox status is also known to affect the levels of S-adenosyl methionine (SAM, a DNA methyl donor) by changing the activity of SAM synthetase. Previous work from our group demonstrated that sleep deprivation in rats can result in a decrease in homocysteine levels (Hcy; Oliveira et al., 2002); this amino acid participates in a metabolic pathway that is related to congenital malformations (Botto and Yang, 2000; Hague, 2003; Obeid and Herrmann, 2005; Huhta and Hernandez-Robles, 2005) and is the main route for methylation reactions (Castro et al., 2003).

As many congenital malformations are mediated by altered methylation patterns, it is conceivable that if sleep deprivation occurs during the initial period of gestation, metabolic changes in the Hcy pathway could compromise embryonic development. In view of the reported impairments to sleep duration and quality that occur during the early stages of pregnancy (Santiago et al., 2001; Lopes et al., 2004) and the wider societal issue of sleep loss, this finding assumes even greater importance.

In light of this, the aim of this study was to determine whether maternal sleep deprivation occurring during the pre-implantation period could alter embryonic development (by assessing biochemical, behavioral and biometrical parameters) of male offspring, affecting them during adulthood.

2. Materials and methods

2.1. Ethical considerations

The institutional review board (Comissão de Ética em Pesquisa da Universidade Federal de São Paulo) approved this

study and ensured that animal care and evaluations were performed according to ethical standards (CEP: 1225/06). The mice used in this study were treated in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals (Andersen et al., 2004).

2.2. Experimental groups

Three-month-old Swiss mice from the Department of Psychobiology facility were housed in standard polypropylene cages in a temperature-controlled room (23 ± 2 °C) with a 12:12 h light–dark cycle (lights on at 07:00) from birth. They were allowed free access to food and water.

To determine the regularity of two estrous cycles, female mice were submitted to a vaginal smear two weeks prior to commencing the experimental procedures. Male mice were placed in the females' home cages for mating at 03:00 h, before being withdrawn at 07:00 h. Gestational day 0 (GD 0) was designated when either a vaginal plug or sperm was observed. After pregnancy verification, the animals were assigned to one of five groups: Platform sleep deprivation from GD 0 to GD 3 (PSD 72), gentle handling on gestational day 1 (GH 1), gentle handling on gestational day 2 (GH 2), gentle handling on gestational day 3 (GH 3), and a control group (CT) in which the dams were allowed to sleep normally throughout pregnancy. Subsequently, animals were allowed to maintain pregnancy normally in the Department of Psychobiology facility until delivery. Another set of animals was used to study certain biochemical parameters of the dams during the pre-implantation period. They were assigned to one of four experimental groups (as described before) or the respective control group for each day of the pregnancies. After the experimental procedures, the animals were euthanized and plasma concentrations of homocysteine, cysteine, corticosterone, progesterone and 17β -estradiol were measured.

2.3. Sleep deprivation

Sleep deprivation was carried out using the platform technique according to Silva et al. (2004). The gentle handling protocol was performed (from 10:00 to 16:00 h) by either touching the animals with a brush or gently moving their cages whenever they closed their eyes.

After sleep deprivation, the females of each group were housed in separate cages (2–4 animals from each group *per* cage), and about three days before birth they were housed individually. As one of the aims of this study was to evaluate certain behavioral parameters, and because it is known that early manipulation of the offspring leads to behavior alterations, we counted animals without removing them from their mothers.

2.4. Offspring biometric evaluation

On post-natal day (PND) 3, the animals were weighed and the ratio between male and female pups (sex ratio) was evaluated. At PND 30, the animals were weaned and the male pups were housed in groups with a maximum number of 18 *per* cage, until they were 3 months of age (female pups were used in another experiment, data not shown). During this

period, measurements of their weight and their naso-anal length (NAL) were made on a monthly basis. These measures were used to calculate the Lee index [$\sqrt[3]{\text{weight (g)} \times \text{NAL (cm)} \times 1000}$]. Only the male offspring were used in this study.

2.5. Offspring behavioral evaluation

At PND 90, the animals were submitted to behavioral evaluation in the Elevated Plus Maze and in locomotor Activity Box.

2.6. Elevated Plus Maze

To evaluate their levels of anxiety, the animals were tested in the Elevated Plus Maze. This maze, which was elevated 50 cm from the ground and surrounded by a 15 cm-high wall, consisted of two open arms [50 cm \times 10 cm] that were directly opposing and two enclosed arms [50 cm \times 10 cm]. Individual trials lasted for 5 min each. At the beginning of each trial, the animals were placed at the center of the maze, inside a box with 12 cm \times 12 cm dimensions. The maze was cleaned with 70% [v/v] ethanol solution after each trial. The time spent in the open arms and the total number of entries into each of the four arms was recorded. In this test, anxiety was measured as a function of decreased open-arm exploration.

2.7. Locomotor Activity Box

The Opto-Varimex locomotor activity cages [Columbus Instruments, Columbus, OH] consisted of 20 cm \times 30 cm \times 40 cm cages that were surrounded with photoelectrical horizontal detection sensors. The locomotor activity of the rats was assessed by the instrument every 5 min for the entire 30 min duration of the test.

2.8. Offspring evaluation

Ninety-one-day-old controls and sleep-deprived male offspring were euthanized by decapitation between 08:00 and 11:00 h, and their blood was collected in either heparin- or EDTA-coated tubes. Measurements of homocysteine and cysteine concentrations and antioxidant activities of superoxide dismutase and catalase were made. Erythrocytes and plasma were separated by centrifugation, and erythrocytes were processed and stored at -80°C for antioxidant enzyme analysis. Plasma from the EDTA-coated tubes was used for the measurements of homocysteine and cysteine.

After blood collection, the heart, liver, gastrocnemius muscle and hemi-lateral epididymal fat were dissected and weighed.

2.9. Hormonal and biochemical parameters

Total plasma Hcy and cysteine (Cys) concentrations were measured using High Performance Liquid Chromatography with fluorimetric detection and isocratic elution, according to Oliveira et al. (2002).

Serum corticosterone was assayed using the ImmuChem Double Antibody RIA kit (MP Biomedicals, Orangeburg, NY, USA). Plasma progesterone and 17β -estradiol were assayed using ImmuChem Coated Tube RIA Kits (MP Biomedicals, Costa Mesa, CA, USA).

2.10. Antioxidant enzymes

The blood that was collected in heparin tubes was centrifuged at 3000 rpm/10 min/ 4°C to separate the red blood cells, and these were then washed to obtain a hemolysate. Subsequently, spectrophotometric assays of the catalase (CAT) and superoxide dismutase (SOD) activity in this hemolysate were performed according to Adamo (1989) Adamo et al. (1989) and McCord and Fridovich (1969), respectively. Results were expressed as U (units)/mg Hb (hemoglobin).

2.11. Statistical methods

Maternal homocysteine, cysteine, corticosterone, 17β -estradiol and progesterone levels were compared using the one-way analysis of variance (ANOVA) test for differences between the pregnant CT groups; when necessary, this was followed by the Tukey test. For a comparison of the experimental groups with their respective CT groups, the Mann–Whitney *U* test was used. To analyze the loss of pregnancy after sleep deprivation, Pearson's Chi-square test was used. The sex ratio, litter sizes, homocysteine concentrations, cysteine concentrations, SOD, CAT and the results from the anxiety tests of the pups were analyzed using a one-way ANOVA test. To test for differences between each experimental group and the CT groups, Dunnett's test was also used when necessary. The biometrical parameters and the results of the locomotion test were analyzed using repeated measures ANOVA; when necessary, the Dunnett's test was also used. On occasions when the data did not show a normal distribution, it was converted into ranks before analysis. Data were analyzed using the statistical software SAS System for Windows (Statistical Analysis System, V 8.02, SAS Institute Inc., 1999–2001, Cary, NC). The results are presented as mean \pm S.E.M., and the level of significance was considered to be $p < 0.05$.

3. Results

3.1. Homocysteine and cysteine concentrations in dams

Fig. 1A shows the plasma homocysteine concentrations in dams. The ANOVA test did not detect any differences in the concentration of this amino acid between the control groups for each of the three days of pregnancy (ANOVA: $p = 0.843$). When comparing each control group with its respective sleep-deprivation group, we found an increase in the plasma concentration of Hcy in the GH 3 group (Mann–Whitney: $p = 0.029$), and a decrease in the PSD 72 group (Mann–Whitney: $p = 0.028$). Moreover, we did not find any differences in plasma Cys concentrations (ANOVA: $p = 0.721$; Fig. 1B).

3.2. Hormonal levels in sleep-deprived dams

The plasma concentrations of progesterone in control animals increased after GD 1 ($p < 0.05$; Fig. 2A). However, compared to the time-matched controls, sleep deprivation caused a reduction in progesterone concentrations in the PSD 72 group ($p = 0.014$). Although there were no differences in the concentration of 17β -estradiol on different gestational

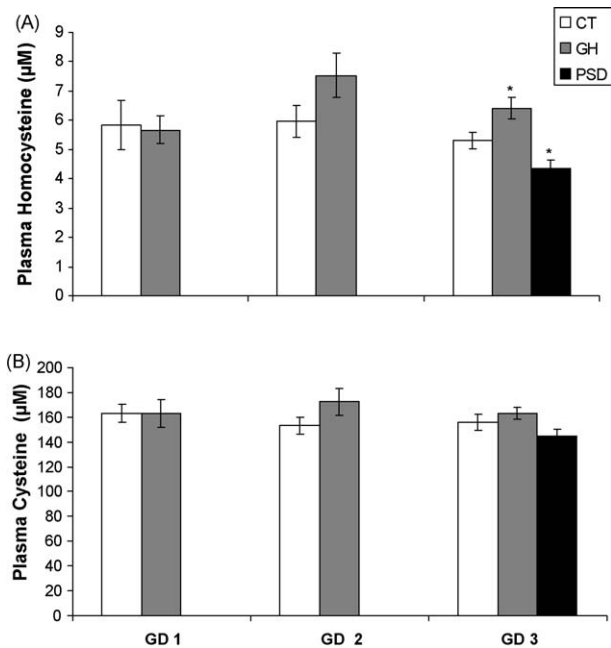


Fig. 1 Plasma homocysteine (A) and cysteine (B) concentrations of pregnant mice after various experimental procedures ($n = 5-8$). CT = control; GH = gentle handling; PSD = platform sleep deprivation; GD = gestational day. Values are presented as mean \pm S.E.M. *Different from the respective CT group ($p < 0.05$).

days (ANOVA: $p = 0.419$), the levels of estradiol in group GH 2 animals were significantly higher than in control group animals (Mann–Whitney; $p = 0.02$; Fig. 2B). Corticosterone concentrations increased after sleep deprivation (Mann–Whitney; $p < 0.05$); this effect was not dependent on the gestation day (ANOVA: $p = 0.405$; Fig. 2C).

3.3. Pregnancy maintenance after sleep deprivation

After sleep deprivation, we found a loss in the maintenance of pregnancy in all experimental groups. The Chi-square test indicated a significant difference in pregnancy maintenance between sleep-deprived and control animals ($p = 0.002$; Fig. 3).

3.4. Litter size and offspring sex ratio

The offspring were counted at PND 3, and an evaluation of the sex ratio was carried out. We found no statistical differences in either the litter size (ANOVA: $p = 0.65$) or the sex ratio (ANOVA: $p = 0.731$) of the animals that were included in each experimental group (Table 1).

3.5. Biometric measurements

At PND 3, body weight increased in animals of the GH 1 and GH 3 groups, and decreased in the GH 2 group (Dunnett: $p < 0.05$; Fig. 4). Throughout the development period, the animals did not show any other alterations in body weight (ANOVA: $p = 0.119$; Fig. 5A); however, there was a decrease in

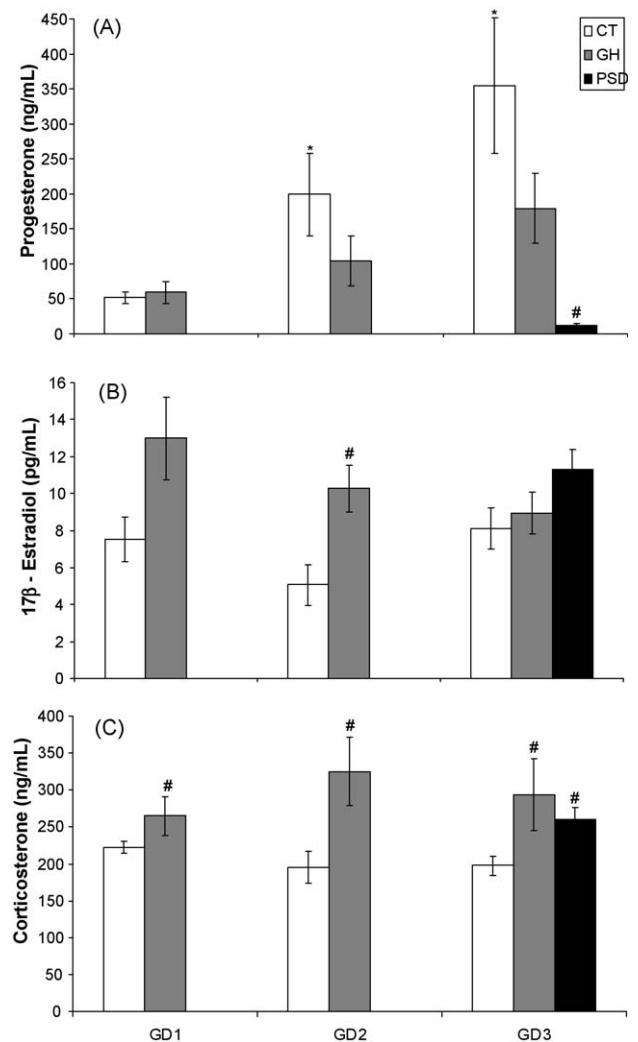


Figure 2 Plasma progesterone (A), 17 β -estradiol and corticosterone (C) after sleep deprivation during pregnancy ($n = 4-8$). CT = control; GH = gentle handling; PSD = platform sleep deprivation; GD = gestational day. Values are presented as mean \pm S.E.M. *Different from the GD 1 control group ($p < 0.05$); #different from the respective GD control group ($p < 0.05$).

the NAL on GH 1 (Dunnett: $p < 0.05$; Fig. 5B) that persisted until PND 30 (Dunnett: $p < 0.05$) and a decline in the Lee index (Fig. 5C) and was maintained until PND 17 (Dunnett: $p < 0.05$). The increase in NAL that occurred at PND 90 of the control group animals did not occur in the animals of the GH 1 group (Dunnett: $p < 0.05$). Compared to the CT group, there was an increase in the NAL of the GH 2 group (Dunnett: $p < 0.05$).

3.6. Organs

As demonstrated in Table 2, animals from the PSD 72 group had significantly less epididymal fat than CT group animals (Dunnett: $p < 0.05$). A similar result was obtained when epididymal weight was expressed as a percentage of total body mass (Dunnett: $p < 0.05$). No other statistical differences were observed between the groups (ANOVA: $p > 0.05$).

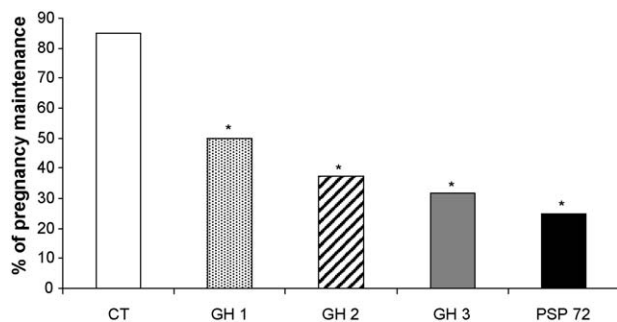


Figure 3 Maintenance of pregnancy after the experimental procedures ($n = 16-22$). CT = control; GH = gentle handling; PSD = platform sleep deprivation. Values are presented as percentage. *Different from the CT group ($p = 0.002$).

3.7. Behavioral measurements

We did not find any changes in the anxiety parameter of the Elevated Plus Maze test, i.e., the time spent in the open arms (ANOVA: $p = 0.138$); however, we did observe changes in locomotion between the PSD 72 animals and the CT group animals during this test (Dunnett: $p < 0.05$). Despite the alterations to locomotion in the Elevated Plus Maze test, there were no significant differences in locomotion between the experimental groups and the CT group when the Activity Box test was used (ANOVA: $p = 0.247$; Table 3).

3.8. Antioxidant enzymes of the adult offspring

Although there were differences in the CAT activity of erythrocyte within experimental groups (ANOVA: $p < 0.01$), there were no differences between the experimental and control groups, as shown in Fig. 6. Compared to the control groups, there was a significant decrease in erythrocyte SOD activity in the GH 1 group (Dunnett: $p < 0.05$).

3.9. Homocysteine and cysteine concentrations in adult offspring

Although there were differences in the concentrations of cysteine within experimental groups (ANOVA: $p = 0.006$), there were no significant differences between experimental and control groups. There were no differences in the concentrations of homocysteine between the groups (ANOVA: $p = 0.968$; Table 4).

4. Discussion

Many studies on prenatal manipulation and its consequences to the offspring have measured behavioral (Meek et al.,

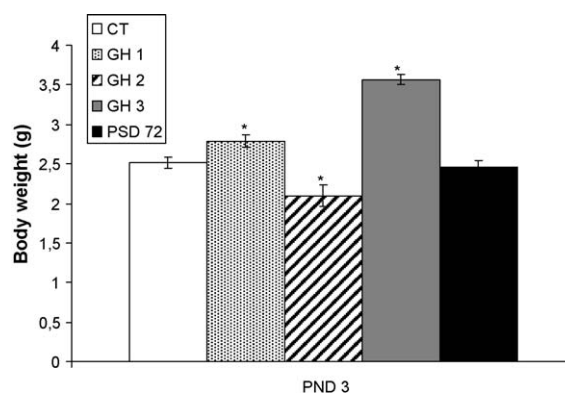


Figure 4 Body weight of mice after sleep deprivation of their mothers on PND 3 ($n = 11-15$). CT = control; GH = gentle handling; PSD = platform sleep deprivation. Values are presented as mean \pm S.E.M. *Different from the CT group ($p < 0.05$).

2006), biometric (Mueller and Bale, 2006) or biochemical parameters (Kapoor et al., 2006; Budge et al., 2007). The aim of this study was to assess the consequences of sleep deprivation (total or paradoxical) that occurred during the pre-implantation stages in pregnant mice on the male offspring. We discovered alterations in the body weight at PND 3, in the naso-anal length, the Lee Index, the amount of epididymal fat, and levels of antioxidant enzymes in the adult offspring from sleep-deprived mothers.

In a study from Nishina et al. (1996), it was shown that the first alteration to sleep occurs during the fifth day of a rat's pregnancy; this change consisted of a decrease in the duration of episodes of slow-wave sleep. Therefore, we believe that the manipulations that we performed in this study were not influenced by the basal sleep patterns at the pre-implantation developmental stage.

Previously, we demonstrated that there were lower plasma concentrations of homocysteine in rats that were subjected to sleep deprivation by the platform technique than in control group animals (Oliveira et al., 2002). During pregnancy, the methionine pathway is crucial to embryonic development; it participates in many processes, including both DNA and RNA synthesis, DNA methylation, and antioxidant defense (Stipanuk, 2004). Moreover, alterations in this pathway have been shown to be widely related to congenital malformations (Botto and Yang, 2000). In our study, we observed opposing changes to the plasma levels of Hcy, which varied according to the nature of the sleep deprivation that was induced during pregnancy. There was an increase in plasma Hcy levels in the GH 3 group and a decrease in plasma Hcy levels in the PSD 72 group.

These opposite responses may be due to the method of sleep deprivation that was applied. In the first method, the animals experienced total sleep deprivation. Silva et al.

Table 1 Litter size and sex ratio after sleep deprivation at different pregnancy times.

Group	CT	GH 1	GH 2	GH 3	PSD 72	p value (ANOVA)
Litter size	10.29 \pm 0.57	9.38 \pm 0.56	9.83 \pm 0.56	9.29 \pm 0.78	8.75 \pm 0.75	0.650
Sex ratio	1.24 \pm 0.18	3.40 \pm 1.48	2.03 \pm 0.70	1.20 \pm 0.32	0.98 \pm 0.18	0.731

CT = control; GH = gentle handling; PSD ($N = 4-17$). Values are presented as mean \pm S.E.M.

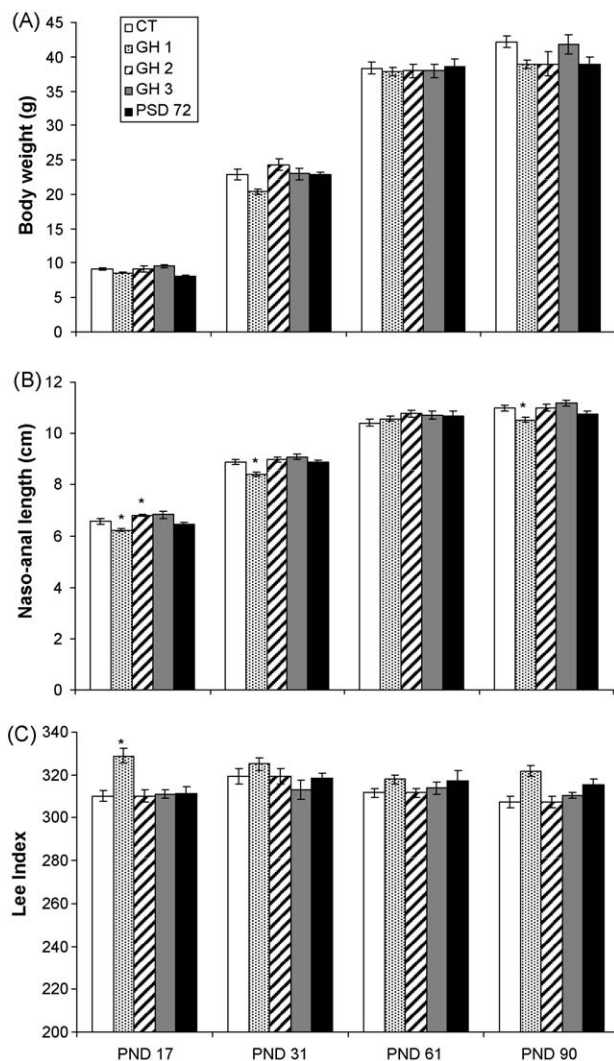


Figure 5 Body weight (A), naso-anal length (B) and Lee index (C) throughout the life of mice that were the offspring of mothers that experienced sleep deprivation at different times in their pregnancy ($n = 7-15$). PND = post-natal day; CT = control; GH = gentle handling; PSD = platform sleep deprivation. Values are presented as mean \pm S.E.M. *Different from the CT group ($p < 0.05$).

(2004) reported that following total sleep deprivation (using the multiple platforms method), there were reductions in both slow-wave sleep (up to 80%) and paradoxical sleep (95%). Other salient factors might include the duration of sleep deprivation or their response to the stressful situation. It is known that during sleep deprivation there is an augmented expenditure of energy, and that long periods of sleep deprivation result in an energy deficit (Bergmann et al., 1989; Hipólido et al., 2006; Martins et al., 2006). Thus, the energy balance associated with the procedure should be taken into consideration. Moreover, if there is already augmented energy expenditure during pregnancy, this competition could lead to long-term consequences. As it is an intermediate metabolite of methionine pathways, alterations in the level of Hcy will be associated with either hyper- or hypomethylation of the DNA. Any such changes to the epigenetic status could lead to long-term alterations in the expression of genes. Studies have shown that high levels of plasma Hcy are associated with global DNA hypomethylation (Chiang et al., 2009; Ingrosso and Perna, 2009). Therefore, our finding of low homocysteine levels in the offspring of some groups of sleep-deprived mice (PSD 72) could indicate a state of global hypermethylation. This might result in the suppression of gene expression, which could affect fetal programming. These changes could conceivably be responsible for the persistence of long-term effects, such as the substantial reduction in epididymal fat that was observed in mice of the PSD 72 group. Further studies will be required to test this hypothesis.

We have also discovered that prevalence of lost pregnancies varied according to the day on which sleep deprivation was performed. Prior to our study, Pigareva (2006) described the sleep-deprivation-induced loss of pregnancy. In her study, rats were sleep-deprived by gentle handling during both day and night (when the rat's activity is maximal) for three consecutive days during the first week of pregnancy. The author reported that loss of pregnancy occurred only when the animals were sleep-deprived during the day; this suggested that the effect was mainly due to the sleep deprivation and was not related to other factors that were inherent to the deprivation protocol (such as stress and increased movement). Pigareva's finding that the loss of pregnancy was not directly caused by the stress associated with sleep deprivation, allowed us to discount the possibility that the

Table 2 Organs weight of mice after sleep deprivation of their mothers at different pregnancy time in grams (g) and organ weight percent (%) of total body mass.

Group	CT	GH 1	GH 2	GH 3	PSD 72	p value (ANOVA)
Heart (g)	0.20 \pm 0.01	0.22 \pm 0.01	0.18 \pm 0.01	0.21 \pm 0.01	0.18 \pm 0.01	0.112
Liver (g)	1.99 \pm 0.05	2.17 \pm 0.12	2.07 \pm 0.10	2.22 \pm 0.09	2.14 \pm 0.10	0.263
Fat (g)	0.38 \pm 0.03	0.36 \pm 0.04	0.45 \pm 0.06	0.34 \pm 0.02	0.23 ^a \pm 0.02	0.024
Gastrocnemius (g)	0.24 \pm 0.01	0.21 \pm 0.03	0.23 \pm 0.01	0.23 \pm 0.01	0.24 \pm 0.02	0.816
Heart (%)	0.50 \pm 0.03	0.50 \pm 0.06	0.47 \pm 0.02	0.48 \pm 0.02	0.46 \pm 0.01	0.747
^a Liver (%)	4.99 \pm 0.13	5.00 \pm 0.09	5.17 \pm 0.14	5.18 \pm 0.14	5.41 \pm 0.22	0.552
Fat (%)	0.93 \pm 0.07	0.84 \pm 0.08	1.09 \pm 0.11	0.79 \pm 0.06	0.59 \pm 0.06	0.009
Gastrocnemius (%)	0.60 \pm 0.03	0.49 \pm 0.08	0.58 \pm 0.03	0.53 \pm 0.03	0.61 \pm 0.03	0.214

($n = 5-14$) CT = control; GH = gentle handling; PSD = platform sleep deprivation. Values are presented as mean \pm S.E.M.

^a Different from the CT group ($p < 0.05$).

Table 3 Behavioral parameters of mice after sleep deprivation of their mothers at different times in pregnancy.

Group	CT	GH 1	GH 2	GH 3	PSD 72	p value (ANOVA)
Elevated Plus Maze						
Time spent in the open arm	13.5 ± 2.61	21.18 ± 5.58	10.64 ± 2.66	6.54 ± 1.26	18.32 ± 4.19	0.138
Total entries in the arms	8.85 ± 0.86	12.56 ± 1.09	8.15 ± 1.24	10.33 ± 1.67	13.70 ^a ± 1.81	0.011
Activity Box						
5 min	883.8 ± 99.7	849.8 ± 79.1	871.3 ± 60.9	801.1 ± 19.7	732.4 ± 63.3	0.247
10 min	823.2 ± 75.2	873.0 ± 102.3	817.7 ± 47.4	770.0 ± 23.9	723.3 ± 44.5	
15 min	785.8 ± 73.0	784.8 ± 97.8	801.0 ± 48.6	699.1 ± 28.9	596.9 ± 54.4	
20 min	740.9 ± 60.7	741.7 ± 90.5	812.8 ± 58.5	720.2 ± 27.5	629.3 ± 18.0	
25 min	661.0 ± 66.3	768.8 ± 111.1	726.5 ± 81.7	667.5 ± 35.3	641.7 ± 33.0	
30 min	640.6 ± 69.9	735.8 ± 89.1	731.2 ± 93.4	575.1 ± 64.9	639.0 ± 60.7	

(n = 9–20) CT = control; GH = gentle handling; PSD = platform sleep deprivation. Values are presented as mean ± S.E.M.

^a Different from the CT group (p < 0.05).

high maternal concentrations of plasma corticosterone from both sleep-deprivation techniques could have influenced pregnancy maintenance in our study.

However, there is still a concern about the circadian variation of plasma corticosterone concentration. Studies have reported that its concentration peak occurs at the beginning of the rats' activity period (the dark period), and that it is mediated by the rhythms of adrenocorticotrophic and hypothalamic hormones. (Engeland and Arnhold, 2005). Therefore, it is possible that the induction of sleep deprivation during different periods of the circadian rhythm (Pigareva, 2006), could either change the intensity of the response

to glucocorticoids or that the circadian variations could mask the responses seen by the author.

It is known that estrogen and progesterone play crucial roles in regulating uterine receptivity for blastocyst attachment (Yoshinaga, 1988; Groothuis et al., 2007). Thus, the pregnancy loss seen in the PSD 72 and GH 2 groups could be caused by either the decrease in progesterone or the increase in 17β-estradiol. However, since we did not find a significant decrease in the plasma progesterone or 17β-estradiol concentrations in the other experimental groups, it is possible that other mechanisms could contribute to our findings. In view of the parameters that we measured in our experiments, it was difficult to explain why we found this high rate of pregnancy loss in our experimental groups.

Although we have found an increase in pregnancy loss after sleep deprivation, there were no differences in either the number of pups born, or in the sex ratio of the offspring. Previously, studies have reported that the offspring sex ratio can be influenced by various types of prenatal manipulation, including restraint stress (Mueller and Bale, 2006), temperature stress (Wells et al., 2009), poor nutrition (Wells et al., 2009) and exposure to pollution (Abe et al., unpublished observation). Regarding the litter size (a parameter that has not been widely considered in the existing literature), Mueller and Bale (2006) demonstrated that this was not affected by restraint stress during three different stages in pregnancy (first, second and third week).

When measuring the body weight of the offspring at day three, we observed that although all mice from the gentle handling groups were different from the CT group mice, the changes were not linear. Groups GH 1 and GH 3 had higher body weights than the CT group, and the GH 2 had lower body weights than the CT group. This is the first evidence that the effects from a specific manipulation (sleep deprivation) vary according to the day of pregnancy on which it is performed. When body weights were analyzed throughout development, those differences disappeared. Interestingly, despite the absence of a difference in the biometrical parameters, we found that mice in the PSD 72 group has almost half the epididymal fat weight of mice from the CT group at 3 months. Moreover, if we expressed this parameter as a percentage of total body weight, this difference was maintained; this proved that the reduction in fat was not a result of low initial body mass. It is known that stressful situations during

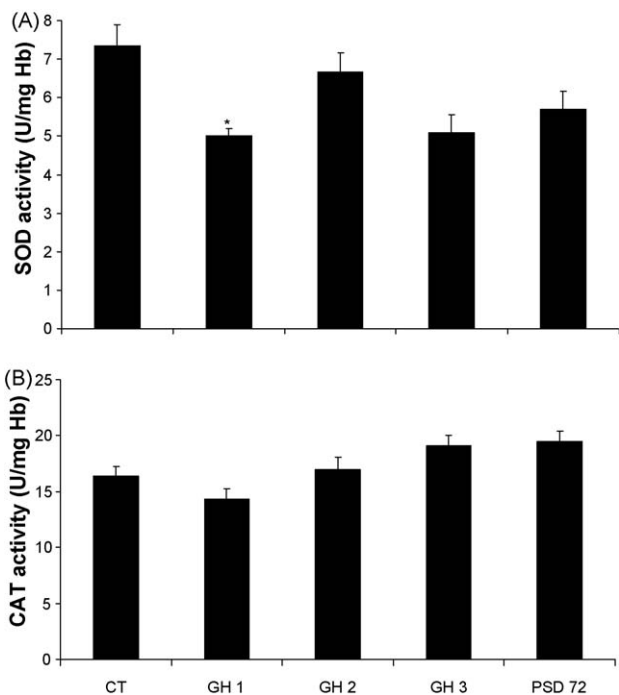


Figure 6 Erythrocyte activity of antioxidant enzymes SOD (A) and catalase (B) in the offspring of mice whose mothers were subjected to sleep deprivation at different times in pregnancy (n = 8–24). CT = control; GH = gentle handling; PSD = platform sleep deprivation. Values are presented as mean ± S.E.M. *Different from the CT group (p < 0.05).

Table 4 Plasma homocysteine and cysteine of mice after sleep deprivation of their mothers at different pregnancy time.

Group	CT	GH 1	GH 2	GH 3	PSD 72	p value (ANOVA)
Hcy (μM)	2.37 \pm 0.10	2.35 \pm 0.10	2.31 \pm 0.09	2.24 \pm 0.03	2.39 \pm 0.10	0.968
Cys (μM)	182.23 \pm 13.15	149.84 \pm 9.29	168.31 \pm 8.75	220.73 \pm 9.36	184.78 \pm 14.05	0.006

CT = control; GH = gentle handling; PSD = platform sleep deprivation; Hcy = homocysteine; Cys = cysteine. Values are presented as mean \pm S.E.M.

pregnancy can lead to changes in body weight at birth and, depending on the nature and duration of the stress, these could persist into adulthood (Vallé et al., 1995). We have not found any studies that analyze the NAL and Lee index of the offspring of rodents that were subjected to prenatal manipulations.

Previous studies have shown that there is an increase in locomotion after sleep deprivation that is induced by the multiple platforms technique, and that these alterations may result from changes to the dopaminergic system (Keller et al., 1984), via dopamine increases in the striatum of rats (Ghosh et al., 1976). Whether the development of hypersensitivity in postsynaptic dopaminergic receptors is a major contributor to these effects remains to be determined. Thus, interaction between the dopaminergic system and other inhibitory systems may play an important role in the effects of sleep deprivation. Based on these results, we sought to determine whether alterations to the dopaminergic system could be perpetuated in the offspring. However, we were unable to identify locomotor changes in the experimental groups compared to the CT group. There were also no differences in measures of anxiety. Previous studies have shown that alterations in the behavior of offspring depend on both the type of stress that is induced and on the time at which the stress is applied during pregnancy (Suchecki and Palermo Neto, 1991; Estanislau and Morato, 2005; Barros et al., 2006; Zueno et al., 2008); the alterations found in these studies were a result of manipulations performed during the third week of pregnancy.

Regarding the biochemical parameters of the offspring, we did not find any alterations in either plasma Hcy and Cys concentrations or erythrocyte CAT activity between the sleep-deprived and CT groups. When we analyzed the activity of SOD, we discovered a decrease in its activity in GH 1 group mice.

The balance between the production and the removal of reactive species is known to change during development (Mavelli et al., 1982; Hitchler and Domann, 2007). The level of expression of antioxidant enzymes is also known to vary during development (Harvey et al., 1995). Moreover, it is known that there are increases in the mitochondrial activity of the placenta due to an increase in energy demand and high rates of multiplication; this could increase reactive oxygen species (ROS) production (Rodriguez et al., 2000; Kim et al., 2005). Hodge et al. (2005) demonstrated that the control of gene expression via the reductive/oxidative (redox) regulation of transcription represents an important aspect of cellular function; for example, the excess levels of ROS (as detected in many tumor types) are associated with lower levels of antioxidant enzymes (by methylation of the gene promoter), whereas overexpression of SOD resulted in a reduction of cell proliferation (Cullen et al., 2003). Our

results suggested that the decrease in SOD activity could lead to an imbalance of the defense against ROS, and that this low activity could be caused by gene reduced expression due to hypermethylation. If this hypothesis is correct, these findings provide further proof that pre-implantation manipulations can influence fetal programming and have long-lasting consequences.

In conclusion, this study describes long-lasting alterations to the offspring of dams that were exposed to sleep deprivation during early stages of pregnancy. These alterations appeared to be more directly related to sleep deprivation than to other stress factors associated with the methods used. The long-lasting changes to the offspring of sleep-deprived mice may be caused by alterations in fetal programming induced by differential methylation. This epigenetic contribution of normal sleep to development suggests new avenues of research into understanding the long-lasting effects of sleep on an animal's offspring.

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Conflict of interest

The authors declare that they have no competing interests.

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