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Paradoxical sleep deprivation activates hypothalamic nuclei that regulate food intake and stress response

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Summary A large body of evidence has shown that prolonged paradoxical sleep deprivation (PSD) results in hypothalamic–pituitary–adrenal (HPA) axis activation, and in loss of body weight despite an apparent increase of food intake, reflecting increased energy expenditure. The flowerpot technique for PSD is an efficient paradigm for investigating the relationships among metabolic regulation and stress response. The purpose of the present study was to examine the mechanisms involved in the effects of 96 h of PSD on metabolism regulation, feeding behaviour and stress response by studying corticotrophin-releasing hormone (CRH) and orexin (ORX) immunoreactivity in specific hypothalamic nuclei. Once-daily assessments of body weight, twice-daily measurements of (spillage-corrected) food intake, and once-daily determinations of plasma adrenocorticotrophic hormone (ACTH) and corticosterone were made throughout PSD or at corresponding times in control rats (CTL). Immunoreactivity for CRH in the paraventricular nucleus of the hypothalamus and for ORX in the hypothalamic lateral area was evaluated at the end of the experimental period. PSD resulted in increased diurnal, but not nocturnal, food intake, producing no significant changes in global food intake. PSD augmented the immunoreactivity for CRH and plasma ACTH and corticosterone levels, characterizing activation of the HPA axis. PSD also markedly increased the ORX immunoreactivity. The average plasma level of corticosterone correlated negatively with body weight gain throughout PSD. These results indicate that augmented ORX and CRH immunoreactivity in specific hypothalamic nuclei may underlie some of the metabolic changes consistently described in PSD.

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1. Introduction

Studies on the effects of sleep deprivation on food intake in rats have consistently shown a peculiar syndrome character-

ized by hyperphagia and loss of body weight—effects not dependent on the sleep deprivation method used (Bhanot et al., 1989; Elomaa, 1985; Kushida et al., 1989). Body weight loss takes place even in sleep-deprived rats fed high-calorie diets (Everson and Wehr, 1993; Koban et al., 2008; Suchecki et al., 2003). Numerous studies have addressed the mechanisms involved in such a major energy imbalance. On the one hand, total sleep or paradoxical sleep deprivation (PSD)

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reduces anabolic hormones such as leptin (Everson and Crowley, 2004; Koban and Swinson, 2005), testosterone (Andersen et al., 2005), and insulin (Hipolide et al., 2006). On the other hand, sleep deprivation increases sympathetic (Andersen et al., 2005; Meerlo et al., 2008) and hypothalamic–pituitary–adrenal (HPA) axis activity (Fadda and Fratta, 1997; Koban et al., 2006; Suchecki et al., 1998; Suchecki et al., 2002), increasing catabolic hormones such as corticosterone and adrenaline. Hyperphagia, in turn, can be explained by a progressive increase of neuropeptide Y (NPY) expression in the hypothalamic arcuate nucleus from 5 to 20 days of PS deprivation in addition to reduction of proopiomelanocortin, a well-known anorexic peptide (Koban et al., 2006). Recently, the same group used a high energy-containing liquid diet and reported a direct correlation between expression of NPY in the arcuate nucleus and food intake (Koban et al., 2008).

Orexin (ORX) is a recently identified orexigenic neuropeptide implicated in the regulation of food intake and energy metabolism (Bernardis and Bellinger, 1996; Date et al., 1999). This peptide is synthesized by neurons located in the lateral hypothalamic area (LHA), which project to waking-related areas, such as the locus coeruleus, dorsal raphe, and tuberomammillary nuclei indicating that ORX is involved in the regulation of waking (Hagan et al., 1999; Williams et al., 2004). Corticotropin releasing factor (CRH), in turn, is synthesized in neurons of the paraventricular nucleus of the hypothalamus (PVN) and orchestrates the neuroendocrine stress response, participates in metabolic pathways known to increase energy expenditure, decreases food intake (Woods et al., 1998) and, like ORX, also promotes waking (Chang and Opp, 2001; Sanford et al., 2008). CRH mRNA expression increases progressively from five to 20 days of PSD (Koban et al., 2006), whereas ACTH and corticosterone (CORT) levels are reported to be increased earlier—already by one day of sleep deprivation (Andersen et al., 2005; Suchecki et al., 1998).

Taken together, these data justify the investigation of ORX and CRH activities within the initial four days of PSD in rats. We sought to investigate ORX and CRH immunoreactivities (ORX-IR and CRH-IR) in the LHA and PVN, respectively, at the end of this period. In addition, we also evaluated food intake corrected for spillage, and daily ACTH and CORT plasma concentrations within the course of PSD.

2. Methods

2.1. Animals

Male Wistar rats (3–4 months of age) were bred and raised in the animal facility of the Department of Psychobiology of Universidade Federal de São Paulo (UNIFESP), housed in groups of four in plastic cages from weaning until the onset of the deprivation procedure. The animals were habituated to the sleep deprivation room for two weeks and handled every day for a week before the beginning of the experiment. Throughout the study, the animals were maintained under controlled temperature (21 ± 2 °C) and a 12 h/12 h light/dark cycle (lights on at 7:00 h). The Ethics Committee in Research of UNIFESP approved all procedures (CEP # 0638/05).

2.2. Groups

Rats were randomly distributed in two main groups: PSD and control (CTL). PSD was induced by the single platform technique. The animals were individually maintained for 96 h in water chambers (22.0 cm long, 22.0 cm wide, and 35 cm high), for proper assessment of food intake and spillage. Within the chambers, PSD rats were placed onto a 7.0 cm diameter platform immersed in water up to 1.0 cm below the platform upper surface. Whenever rats on the platforms lapse into paradoxical sleep, they lose muscle tone, make facial contact with or fall into the surrounding water, awaken, and the cycle begins again (Cohen and Dement, 1965). CTL rats were kept in a similar chamber filled with sawdust bedding instead of water. This was done because previous studies from our group have shown that rats placed onto large immersed platforms (14.0 cm) to purportedly control for PSD-related stress are actually also deprived of paradoxical sleep, albeit to a lesser extent (Machado et al., 2004; Suchecki et al., 2000).

All rats were habituated to their experimental environment for 1 h/day for the two consecutive days preceding the onset of the study. This routine is adopted so that each PSD animal is trained to balance on the platform to avoid excessive falling in the water, unless there is a decrease in muscle tone due to sleep onset. CTL and PSD chambers were cleaned twice a day, throughout the experimental period, at the same time of food intake assessment (see below).

2.3. Experimental procedure

Animals were weighed immediately before being placed in the chambers and at 8:00 h thereafter. At 8:00 h and at 17:00 h, pellet leftovers were removed and weighed, and the food container was refilled a predetermined amount of chow (150 g). At the same time-points, food crumbs spilled in the water were separated from faeces, dried overnight (at 50 °C) and weighed as reported in detail by Martins et al. (2006). Actual nocturnal and diurnal food intake was estimated at these respective time-points by subtracting the amounts of spilled food and pellet leftovers from 150 g.

2.4. Blood sampling

During the week preceding the onset of experiments, the animals were daily handled to get habituated to blood sampling. Blood samples of approximately 300 μ l were collected from a small cut on the tail tip at 8:00 h before PSD (basal levels) and throughout the experiment. The tail skin was cleaned with an antiseptic solution before and after each sampling, and a neomycin-containing ointment (Nebacetin, Bayer ®) was applied topically to prevent water contact and local infection.

Blood samples were collected in pre-cooled Eppendorf tubes containing 0.05 ml of EDTA (60 mg/ml), centrifuged immediately at 2300 rpm at 4 °C for 20 min, and the resulting plasma samples were frozen in clean Eppendorf tubes at -20 °C for later determination of plasma ACTH and CORT. ACTH was assayed by a sequential chemiluminescence immunometric method (DPC Immulite, Los Angeles, CA, USA). The sensitivity of the method is 9 pg/ml, and intra- and interassay variations are 9.6% and 9.4%, respectively. CORT levels were

measured in duplicate by a double antibody radioimmunoassay method specific for rats and mice, using a commercial kit (ICN Biomedicals, Costa Mesa, CA, USA), according to the original protocol modified by Thiruvikraman et al. (1997). The sensitivity of the assay is 1.25 ng/ml and the intra- and interassay variations are respectively 7.1% and 6.5%, as informed by the manufacturer.

2.5. Tissue preparation

At the end of 96 h, PSD and CTL animals were anesthetized with halothane and kept under mechanical ventilation. They were fully heparinized before transcardiac perfusion-fixation with 0.9% NaCl (at 10 ml/min for 30–60 s) and phosphate-buffered 4.0% formaldehyde (at 10 ml/min for 10–15 min) freshly depolymerised from paraformaldehyde salt. The brain tissue was allowed to fix in situ for 24 h prior to removal from skull to achieve full formaldehyde binding, then removed and kept in the same fixative solution (phosphate-buffered formaldehyde 1%) at 4 °C until being processed for immunohistochemistry. Three to four 1-in-5 series of 30 µm-thick coronal sections were then cut with a vibrating microtome (VT1000; Leica, Heidelberg, Germany) through the rostro-caudal axis and collected in Tris buffer for immediate immunohistochemical staining.

The experiments were consecutively replicated in four subgroups of PSD and CLT rats (two CTL and two PSD animals at a time) to avoid circadian influences due to having an excessive number of rats to be perfusion-fixed, with consequent heterogeneous timing for CRH-IR and ORX-IR and large intra-group variability. To avoid further circadian influences on CRH-IR and ORX-IR that could affect inter-group comparison, perfusion procedures (lasting approximately 15 min per rat) began at 09:00 h, and were performed alternating one PSD and one CTL rat.

2.6. Primary antibodies

The following primary antibodies were used to study the expression of the respective hypothalamic peptides: Orexin-A (Chemicon International, rabbit polyclonal antibody Cat # AB3098; 1:3000); CRH (Advanced Targeting Systems, rabbit polyclonal antibody Cat # AB-02; 1:20,000). GFAP antibody was used for internal control (rabbit polyclonal antibody against glial fibrillary acidic protein, DAKO Cat # M0761; 1:3000).

2.7. Immunohistochemistry

The protocol was established based on preliminary essays on antibody titration and incubation conditions. A few sections from the same hypothalamic level were incubated with GFAP (internal control) or incubated with the diluent solution without any primary antibody (negative control).

Tissue sections were processed for free-floating immunohistochemistry according to the streptavidin-biotin-peroxidase method. Briefly, sections were incubated in 1% H₂O₂ for 30 min for endogenous peroxidase quenching, followed by permeabilization with Tris–Triton 0.1% solution, and then incubated in 1% BSA for 30 min for blocking of unspecific binding. Sections were then incubated with primary antibodies against GFAP, ORX or CRH diluted in Tris HCl 0.1 M, pH 7.4

containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 for 48 h at 4 °C. After rinsing, the tissue was incubated for 10 min at room temperature in secondary antibody for 10 min at room temperature (from DAKO LSAB™ Kit, a labelled streptavidin-biotin reagent system, which includes a biotinylated secondary antibody plus horseradish-labelled streptavidin-biotin reagents). After rinsing in Tris buffer, the complex was visualized with DAKO liquid 3,3'-diaminobenzidine chromogen solution. The tissue was transferred to buffer solution to stop the reaction, rinsed again in distilled water and mounted onto silanized glass slides. The sections were left to dry overnight, dehydrated through graded alcohols, cleared in xylene and coverslipped.

Peptide immunoreactivity was quantified by visual counting of cells at a 10x magnification by an investigator (MLG) blind to identification of the treatment group. By using immunohistochemical staining, labeled neurons were easily identified as brown neuronal bodies. All labeled (staining light to dark brown) neuronal bodies in both hemispheres of each section were counted and averaged for total immunoreactive cells. Three to four sections (per structure/brain along the rostro-caudal axis) were counted and averaged for each rat. Values obtained are expressed as number of positively stained neurons ± SEM of 6 animals/group.

Digital photomicrographs were taken through a Nikon Eclipse E600 upright microscope equipped with Plan Apo objectives and connected to a Dell workstation computer through the PixeLink digital camera. Color photomicrographs were transformed into black and white TIFF images with 300 pixels resolution with the aid of Adobe Photoshop 7 software. Adobe Photoshop was also used for figure composition (Figs. 3 and 4).

2.8. Statistical analysis

Changes in chow intake were analyzed by a 2-way repeated-measures ANOVA performed with the GLM (General Linear Models) procedure, with Group (PSD and CTL), Day (Day 1, Day 2, Day 3, Day 4) and Period (morning, evening) as main factors. Differences in nocturnal or diurnal food intakes between PSD and CTL groups were analyzed by Student's *t*-test. Body weight variation and hormone levels were analyzed by a 2-way repeated-measures ANOVA with Group as the main factor and Day as the repeated measure (Basal, D1, D2, D3 and D4). Newman–Keuls post hoc analysis was performed whenever required. Correlations between average corticosterone secretion and mean weight variation, between ORX-IR and mean weight variation, and between neuropeptide immunoreactivity and average hormone levels were studied using Pearson's correlation test. The number of immunoreactive cells was analyzed by the Student's *t*-test. The level of significance was established at $p < 0.05$.

3. Results

3.1. Assessment of peripheral metabolism

3.1.1. Body weight variation (Fig. 1A)

ANOVA revealed main effects of Group ($F_{1,45} = 20.75$; $p < 0.001$) and an interaction between Group and Day ($F_{3,42} = 3.36$; $p < 0.03$). Post-hoc analysis showed that whereas CTL rats did not significantly change their body

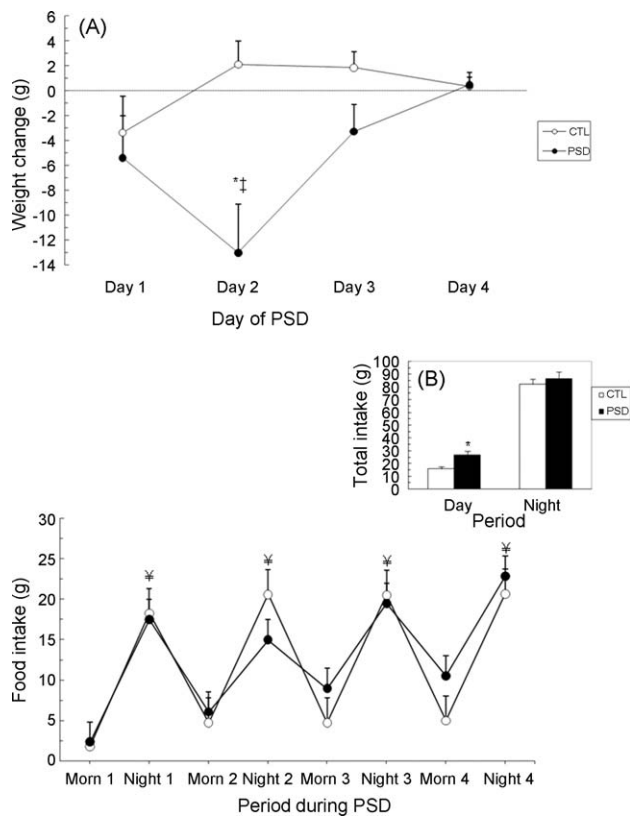


Figure 1 (A) Body weight change (g) of control (CTL; $n = 8$) and paradoxical sleep deprived (PSD; $n = 8$) groups during 4 days of PSD. Body weight was measured daily at 8:00 h and variation was calculated by the equation [(current weight – previous weight)/previous weight \times 100]. (B) Actual food intakes of CTL and PSD rats were measured twice a day at 8:00 h (estimated nocturnal intake) and at 17:00 h (estimated diurnal intake) after subtraction of the residues collected from the water in the deprivation chamber. The insert shows the total amounts of diurnal and nocturnal food intake. Data are presented as mean \pm SEM. * = significantly different from CTL group at the same time point ($p < 0.05$); † = significantly different from the other days, within the same group ($p < 0.05$); ‡ = significantly different from corresponding diurnal food intake ($p < 0.05$).

weights, PSD rats exhibited body weight loss on Day 2, which differed from Days 1, 3 and 4 and from CTL rats on Day 2 ($p < 0.05$) (Fig. 1).

3.1.2. Food Intake (Fig. 1B)

ANOVA showed an interaction between Group and Day ($F_{1,14} = 7.23$; $p < 0.001$). Analysis of this interaction revealed that CTL rats ate significantly more on Days 2 and 3 than on Day 1 ($p < 0.05$) and that PSD rats ate significantly more on Days 3 and 4 than on Days 1 and 2 ($p < 0.001$). In addition, there was an interaction between Day and Period ($F_{3,42} = 3.06$; $p < 0.04$). Analysis of this interaction showed that nocturnal food intake was significantly higher than diurnal chow ingestion ($p < 0.001$) for both groups; diurnal food intake was significantly augmented on Days 2, 3 and 4, compared to Day 1 ($p < 0.001$), but nocturnal food intake on Days 3 and 4 was significantly higher than that on Days 1 and 2 ($p < 0.001$). Total (after 96 h) diurnal food intake was significantly greater for PSD

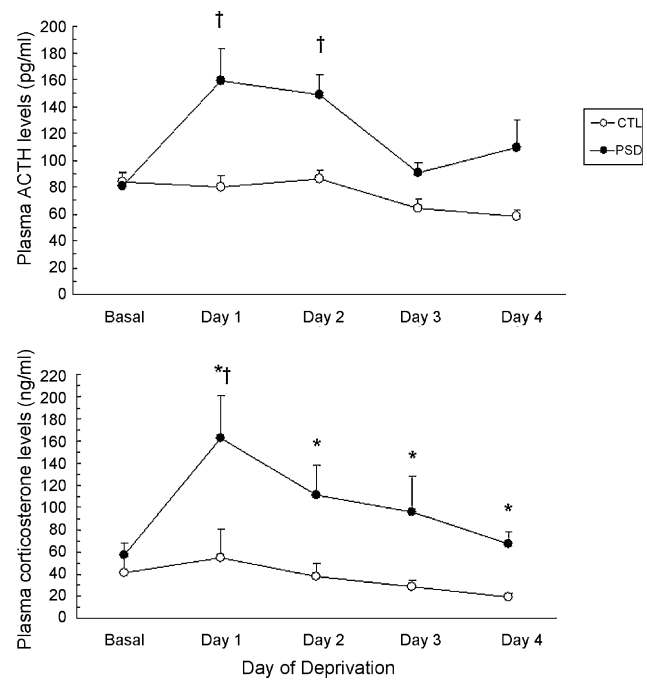


Figure 2 (A) Adrenocorticotrophic hormone (ACTH) plasma levels (pg/ml) of control (CTL, $n = 8$) and paradoxical sleep deprived rats (PSD, $n = 8$). (B) Corticosterone (CORT) plasma levels (ng/ml) of CTL and PSD rats. Blood samples were collected every day at 8:00 h. Data are presented as mean \pm SEM. * = significantly different from CTL group on the same day ($p < 0.05$), † = significantly different from levels found on the other days ($p < 0.05$).

than for CTL rats (26.51 ± 9.9 g versus 15.88 ± 5.1 g respectively; $t = 2.69$; $p < 0.02$), whereas total nocturnal food intake was similar between the groups (82.12 ± 12.2 g versus 86.49 ± 15.5 g, respectively; $t = -0.625$; $p < 0.54$).

3.2. Hormone levels

3.2.1. ACTH levels (Fig. 2A)

Main effects of Group ($F_{1,14} = 15.00$; $p < 0.005$), Day ($F_{4,56} = 6.57$; $p < 0.001$) and an interaction ($F_{4,56} = 3.84$; $p < 0.01$) were revealed. CTL rats did not show any change in ACTH secretion throughout the experimental period, whereas ACTH levels of PSD rats were higher on Days 1 and 2 of sleep deprivation than basal levels and those found on Days 3 and 4 ($p < 0.02$) (Fig. 2).

3.2.2. CORT levels (Fig. 2B)

ANOVA revealed main effects of Group ($F_{1,14} = 10.48$; $p < 0.01$) and Day ($F_{4,56} = 4.06$; $p < 0.001$). *Post hoc* analysis showed that PSD rats exhibited higher CORT levels than CTL animals throughout the four-day period of experiment, and that CORT levels in PSD rats were highest on Day 1 ($p < 0.03$).

3.3. Immunohistochemistry

3.3.1. ORX-IR cells (Fig. 3)

PSD increased the number of ORX immunoreactive cells in the LHA above control values [168.98 ± 8.3 ($n = 6$) versus 109.21 ± 4.9 ($n = 8$) respectively; $t = 6.84$; $p < 0.0001$] (Fig. 3).

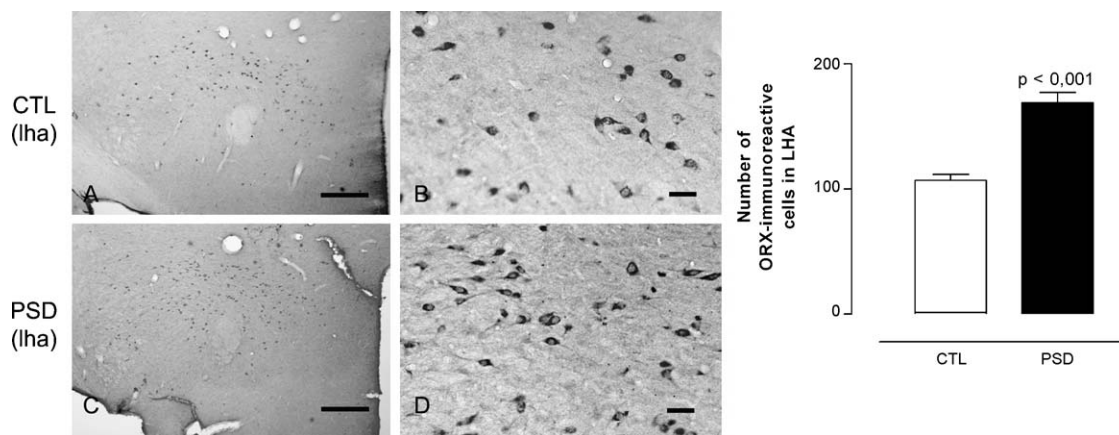


Figure 3 Representative microphotographs of orexin (ORX) immunoreactivity. A and B: ORX immunoreactivity in the lateral hypothalamic area (LHA) of representative CTL rat; C and D: ORX immunoreactivity in LHA of representative PSD rat. Scale bars represent 250 μm in A and C; and 25 μm in B and D. The graphic shows the mean values \pm SEM of immunoreactive cells counted in the LHA of CTL ($N = 6$) and PSD ($N = 6$) groups.

3.3.2. CRH-IR cells (Fig. 4)

Likewise, PSD augmented CRH-IR in the PVN above control values [51.03 ± 3.2 ($n = 8$) versus 29.78 ± 3.4 ($n = 7$) respectively; $t = 2.96$; $p < 0.001$] (Fig. 4).

3.4. Correlations (Fig. 5)

An inverse correlation between total weight gain and overall CORT secretion throughout the PSD period was observed ($r = -0.50$; $N = 16$; Fig. 5). The immunoreactivity of both neuropeptides was positively correlated with the average ACTH ($r = 0.61$; $N = 13$ for ORX versus ACTH, and $r = 0.69$; $N = 13$ for CRH versus ACTH) and CORT release ($r = 0.58$; $N = 13$ for ORX versus CORT, and $r = 0.59$; $N = 13$ for CRH versus CORT).

4. Discussion

The present study showed that (1) PSD resulted in loss of body weight, with increased diurnal food intake, (2) plasma CORT

levels were higher in PSD than in CTL rats throughout the experimental period, (3) PSD increased ORX-IR and CRH-IR in LHA and PVN, respectively, and (4) there was a negative correlation between overall CORT secretion and body weight gain.

These results confirm the well-known syndrome induced by PSD, namely, loss of body weight despite of increased food intake (Everson and Crowley, 2004; Hanlon et al., 2005; Hipolide et al., 2006; Koban et al., 2006, 2008; Koban and Swinson, 2005; Kushida et al., 1989; Suchecki et al., 2003; Suchecki and Tufik, 2000), although some authors reported no differences in the latter variable (Everson and Wehr, 1993; Martins et al., 2006). Contradictory results regarding food intake in PSD may be related to disregarding of food spillage and lack of differentiation between nocturnal and diurnal food intakes. In the present study, assessments of food intake performed twice a day revealed that PSD rats show a somewhat blunted feeding pattern, with increased diurnal food intake whilst CTL rats exhibited a quite stable difference between the diurnal and nocturnal periods. Our results

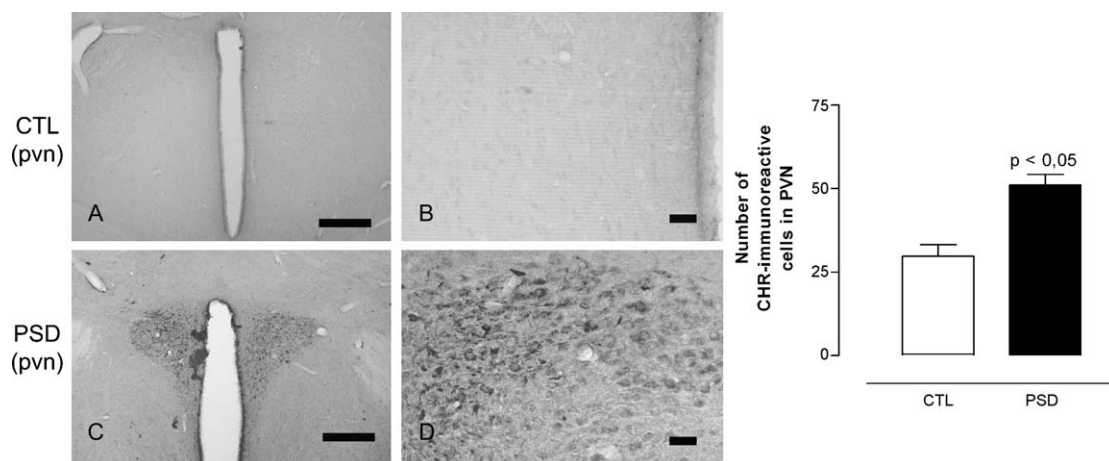


Figure 4 Representative microphotographs of corticotropin-releasing hormone (CRH) immunoreactivity. A and B: CRH immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) of a representative CTL rat; C and D: CRH immunoreactivity in the PVN of a representative rat of the PSD group. Scale bars represent 250 μm in A and C; and 25 μm in B and D. The graphic shows the mean values \pm SEM of immunoreactive cells counted in the LHA of CTL ($N = 6$) and PSD ($N = 6$) groups.

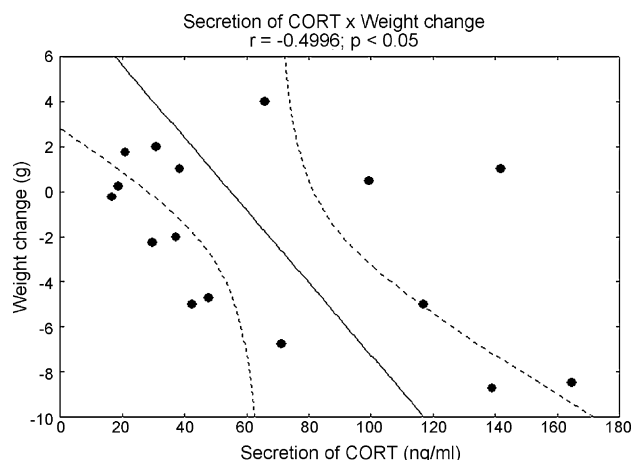


Figure 5 Correlation between body weight change and corticosterone secretion during the 96 h period of PSD. This correlation included control (CTL) and paradoxical sleep deprived (PSD) animals ($N = 16$).

corroborate previous observations obtained without control for food spillage (Elomaa, 1981; Suchecki et al., 2003). Collectively, these data confirm the idea that PSD increases energy expenditure (Bergmann et al., 1989; Bhanot et al., 1989; Everson and Wehr, 1993; Hipolide et al., 2006). Adding to this notion is the finding that PSD progressively increases oxygen consumption and expression of uncoupling protein-1 in brown adipose tissue, which is involved in non-shivering thermogenesis (Koban and Swinson, 2005).

The increased motivation for feeding is convincingly explained by augmented NPY expression in the arcuate nucleus (Koban et al., 2006). Nonetheless, because ORX has been shown to be involved in waking, energy balance and activation of the HPA axis (Scammell, 2001), it seems to be a natural candidate to mediate some of the PSD-induced changes. Previous evidence consistently indicates that ORX-IR would be increased after PSD deprivation. First, leptin, which is secreted by adipocytes, signals energy stores to the brain, and represents the main peripheral inhibitor of ORX and NPY neurons (Klok et al., 2007), is reduced in rats sleep deprived by the platform technique (Koban and Swinson, 2005) or by the disk-over-water method (Everson and Crowley, 2004). This reduction is quite independent of the energy content of the diet and is reversed by 24 h of sleep recovery (Martins et al., 2007). Second, a previous study from our laboratory showed that either 6 h or 96 h of PSD lead to increased levels of ORX in the rat cerebrospinal fluid (Pedrazzoli et al., 2004), which very likely indicates increased activity of central orexinergic neurons. These neurons are stimulated by agonists of the glutamate ionotropic receptor (Li et al., 2002) and inhibited by high doses of dopamine and by agonists of the D_2 dopamine receptor (Alberto et al., 2006). The fact that mid-term (24 h long) sleep deprivation results in increased levels of glutamate, at least in the cortex (Bettendorff et al., 1996), combined with reduced dopaminergic transmission, which is reflected by increased postsynaptic D_2 receptor density in brain areas receiving dopaminergic afferences immediately after 96 h of PSD (Nunes Junior et al., 1994), may underlie the changes observed in orexinergic activity.

That prolonged sleep deprivation or sleep restriction is stressful to rats appears to be unquestionable. The activation of the HPA axis takes place along the entire axis, as reflected either by reduction of the number of CRH receptors in the pituitary (Fadda and Fratta, 1997), by increased mRNA expression in the PVN (Koban et al., 2006) or by augmented CRH-IR (present study). The activation of the HPA axis by ORX (Kuru et al., 2000; Moreno et al., 2005) may represent an additional mechanism for this outcome, besides stress. As a result of CRH neuronal stimulation, plasma ACTH and CORT elevate significantly above basal levels (Martinez-Gonzalez et al., 2004; Meerlo et al., 2002; Suchecki et al., 2002). Interestingly, in the present study we followed ACTH and CORT plasma concentrations in the same rats throughout the PSD period and were able to show that these hormones peaked on PSD day 1, and then trended downward but remained higher than that of CTL rats. This is in contrast with a previous study that showed persistent elevation of ACTH and CORT levels throughout PSD (Andersen et al., 2005). A possible explanation for this discrepancy may be found in the different methodologies used for blood sampling. In the latter, different groups of animals were sacrificed each day, whereas in the present study the same animals were handled for weighing and blood sampling daily, and daily handling may interfere with secretion of stress hormones. Recent data from our laboratory showed that increased levels of CORT are already detectable by 6 h of sleep deprivation (unpublished data). Thus, it seems likely that early activation of the HPA axis, leading to increased levels of glucocorticoids, sets in motion metabolic changes maintained throughout the sleep deprivation period, as seen in the present study, although a major role for the sympathetic nervous system cannot be ruled out (Pilcher et al., 1990). The idea that glucocorticoids may be involved in body weight change is supported by two sets of data. First, in this study we observed a negative correlation between overall plasma CORT secretion and body weight gain throughout the sleep deprivation period, indicating that the higher the CORT production, the less the animals gained weight. Second, we have recently reported that treatment of rats with metyrapone, an inhibitor of corticosterone synthesis, during PSD, prevents body weight loss (Tiba et al., 2008), suggesting that CORT plays an important role in regulation of body weight.

Although we observed some habituation to handling and sampling procedures, plasma ACTH and CORT levels remained elevated in PSD rats. The fact that CRH-IR in the PVN was higher in these animals indicates that the HPA axis was activated as a whole. Whether habituation to the procedure was also reflected on CRH neurons is not possible to establish, but this might indeed have been the case, for although there was a difference between PSD and CTL rats in the number of CRH immunoreactive cells, the magnitude was not remarkable, which might indicate that the CORT negative feedback mechanism is effective. Interestingly, a progressive increase in CRH mRNA expression was reported previously in rats, at 5, 10, 15 and 20 days of sleep deprivation (Koban et al., 2006), indicating that at some point, the negative feedback mechanism is impaired by this prolonged stressor. Nonetheless, to the best of our knowledge, this is the first time in which the whole HPA axis is investigated in the same animal under PSD.

Finally, it is worth mentioning the bidirectional relationship between ORX and the HPA axis. ORX stimulates the HPA axis both at the central and peripheral levels, and the presence of orexin-1 and 2 receptors has been reported in the PVN, median eminence, anterior pituitary and adrenal cortex and medulla (Moreno et al., 2005; Spinazzi et al., 2006). The positive correlation between ORX-IR and plasma ACTH and CORT levels found in the present study strengthens the importance of this neuropeptide for the regulation of stress response. ORX synthesis, in turn, is regulated by glucocorticoids, since adrenalectomy results in decreased expression of pre-pro-orexin, which is re-established by dexamethasone administration (Stricker-Krongrad and Beck, 2002). Moreover, the orexigenic effect of this peptide is greatly reduced by adrenalectomy (ADX) and is increased by replacement with high doses of CORT. Therefore, glucocorticoids appear to regulate the activity of orexinergic neurons under stressful situations (Ford et al., 2005).

An integrative hypothesis of the sequence of events taking place during PSD is shown in Fig. 6. In response to PSD there is a prompt activation of the HPA axis, reflected by high plasma levels of ACTH and CORT already on the first day of sleep deprivation. Increased CORT production leads to lipolysis and loss of fat mass which, in turn, results in low leptin levels. The consequence of low leptin levels is the weakening of the inhibitory regulation on ORX neurons, leading to increased

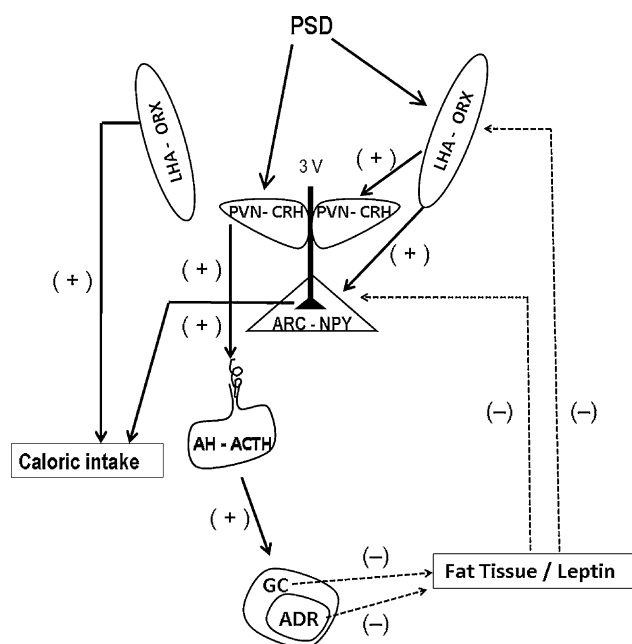


Figure 6 Schematic drawing of an integrative hypothesis of the present results. Solid arrows indicate stimulatory effects, and dashed arrows inhibitory effects. Increased secretion of corticosterone (GC) promotes lipolysis, thus reducing the strength of leptin inhibitory activity on orexin (ORX) and neuropeptide Y (NPY) neurons. With increased activity of these orexigenic neuropeptides, stimulation for food intake ensues. In addition, ORX stimulates the HPA axis, sustaining the release of corticosterone. LHA, lateral hypothalamic area; PVN, paraventricular nucleus; CRH, corticotropin-releasing hormone; ARC, arcuate nucleus; AH, adenohypophysis; ADR, adrenalin; 3V, 3rd ventricle.

immunoreactivity of the neuropeptide. The increase of ORX activity within the LHA stimulates food intake directly or *via* NPY (Yamanaka et al., 2000), and induces HPA axis activity. This situation is likely to be maintained for as long as PSD is imposed to the animal, as has been shown in 20-day sleep deprived rats (Koban et al., 2006; Koban et al., 2008).

Role of the funding sources

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Conflict of interest

The authors declare that they have no conflict of interests.

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