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## Orexin activation precedes increased NPY expression, hyperphagia, and metabolic changes in response to sleep deprivation

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**Martins PJ, Marques MS, Tufik S, D'Almeida V.** Orexin activation precedes increased NPY expression, hyperphagia, and metabolic changes in response to sleep deprivation. *Am J Physiol Endocrinol Metab* 298: E726–E734, 2010. First published January 5, 2010; doi:10.1152/ajpendo.00660.2009.—Several pieces of evidence support that sleep duration plays a role in body weight control. Nevertheless, it has been assumed that, after the identification of orexins (hypocretins), the molecular basis of the interaction between sleep and energy homeostasis has been provided. However, no study has verified the relationship between neuropeptide Y (NPY) and orexin changes during hyperphagia induced by sleep deprivation. In the current study we aimed to establish the time course of changes in metabolite, endocrine, and hypothalamic neuropeptide expression of Wistar rats sleep deprived by the platform method for a distinct period (from 24 to 96 h) or sleep restricted for 21 days (SR-21d). Despite changes in the stress hormones, we found no changes in food intake and body weight in the SR-21d group. However, sleep-deprived rats had a 25–35% increase in their food intake from 72 h accompanied by slight weight loss. Such changes were associated with increased hypothalamus mRNA levels of prepro-orexin (PPO) at 24 h followed by NPY at 48 h of sleep deprivation. Conversely, sleep recovery reduced the expression of both PPO and NPY, which rapidly brought the animals to a hypophagic condition. Our data also support that sleep deprivation rapidly increases energy expenditure and therefore leads to a negative energy balance and a reduction in liver glycogen and serum triacylglycerol levels despite the hyperphagia. Interestingly, such changes were associated with increased serum levels of glucagon, corticosterone, and norepinephrine, but no effects on leptin, insulin, or ghrelin were observed. In conclusion, orexin activation accounts for the myriad changes induced by sleep deprivation, especially the hyperphagia induced under stress and a negative energy balance.

neuropeptide Y; leptin; glucagon; insulin; liver glycogen; triacylglycerol

SEVERAL PIECES OF EVIDENCE support that sleep duration plays a role in body weight control (4, 15, 56). Everson et al. (10), Kushida et al. (24), and Rechtschaffen et al. (46), using an animal model, characterized a syndrome induced in response to sleep deprivation especially composed of a progressive increase in food intake. Interestingly, Spiegel et al. (55) has shown that humans who are sleep restricted for 2 days (sleeping 4 h/night) were hungrier and developed a craving for food with a high carbohydrate content. Later, the hyperphagic behavior during sleep deprivation was attributed to an increase and decrease in the hypothalamic expression of neuropeptide Y (NPY) and proopiomelanocortin (POMC), respectively (20). Supporting such changes in these neuropeptides, earlier studies

in different species have found a reduction in circulating levels of leptin in response to sleep loss (11, 22, 55, 56). Although the described changes in the neuropeptides and leptin levels of sleep-deprived animals agreed with the current model of body weight control (48), the main concern of this explanation for hypothalamic neuropeptide changes induced by sleep deprivation is the large weight loss at the time in which leptin was measured. Leptin is released by adipocytes in response to changes in the energy balance, providing the brain information about energy (fat) stores (17). Recently, we showed that chow-fed rats present with an impaired food intake during the early days of sleep deprivation, which leads to large weight loss (30, 31). In addition, the weight loss observed in sleep-deprived rats on the chow diet was accounted for mainly by a reduction of adipose tissue (39). Therefore, it is unknown whether reduced leptin levels are the major regulator of the hypothalamic neuropeptide changes and, consequently, the hyperphagia during sleep deprivation since the previous studies assessed the leptin levels only after days of reduced weight (11, 22).

The previous descriptions of increased food intake and energy expenditure in response to sleep deprivation (3, 22), as well as the fasting-feeding cycle modulation of sleep allocation (37), reinforce the interconnection between the energy homeostasis process and sleep control. However, only after the identification of hypothalamic neuropeptides named orexins (or hypocretins) was the molecular basis of the interaction between sleep and energy homeostasis control provided (57). Initially, the orexins (orexin A and orexin B) were recognized as feeding behavior regulators due to their production in the lateral hypothalamus (previously known as the feeding center) and their pharmacological activity (9, 47). Later, the orexins were shown to have a role in the maintenance of wakefulness, as demonstrated by the association of orexin signaling deficiencies and the sleep disorder narcolepsy (6, 26). Thereafter, evidence that fasting and sleep deprivation increase both NPY and the orexin precursor, prepro-orexin (PPO), mRNA levels in the hypothalamus of rats (20, 45, 47) supported the interaction of these peptides in the control of feeding and sleep behaviors. Although it has been hypothesized that the increased appetite observed during the sleep deprivation is a condition that might involve orexin activation (19, 20), no study has verified the relationship between NPY and orexin changes in regard to the hyperphagia induced by the sleep deprivation. Knowing that in the hypothalamus there are several hormonal and nutrient signals from the periphery that are sensed and integrated to allocate the appropriate behavior and physiological response to maintain the energy homeostasis (16, 48), the present study was conducted to establish the time course of changes in metabolite, endocrine, and hypothalamic neuropeptide expression associated with hyperphagia induced by sleep deprivation.

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## METHODS

**Animals, housing conditions, and ethical care.** Male Wistar rats from a colony maintained by the Psychobiology Department of the Federal University of São Paulo were employed. These animals were derived from the Charles River Laboratories (Wilmington, MA) foundation colony. During the experiment, all animals were kept on a 12:12-h light-dark cycle (lights on at 0700) under controlled temperature (21–24°C) conditions and with free access to food and water. Animal care and use procedures were carried out by trained personnel (FELASA Category C) and conducted in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals*. The experimental protocol was approved by the Ethics Committee of the Federal University of São Paulo [Comitê de Ética em Pesquisa (CEP; Research Ethical Committee) no. 0064/01].

**Sleep deprivation procedure.** The sleep deprivation procedure used was the classical platform method, which consists of placing one animal on top of a narrow platform (6.5 cm in diameter) surrounded by water in a 23 × 23 × 35 cm container [for details, see Martins et al. (30)]. Previous data from our laboratory show that the platform method results in a complete reduction of paradoxical sleep and a 37% decrease in slow-wave sleep (27). All animals were allowed to adapt to the platform for 30–40 min for 3 consecutive days, and sleep deprivation was started at 0800 after 1 day of washout. The control group animals were put individually into the same container as the sleep-deprived animals, but the water was substituted by wood paring. This substitution was also performed during the rebound period in the recovered group.

**Experimental protocol.** Seven days before the experiment, male rats were housed individually and adapted to consume a balanced liquid diet. The liquid diet (cat. no. F1268; Bio-Serv, Frenchtown, NJ) was delivered by liquid diet feeding tubes (cat. no. 9011; Bio-Serv) and was accessible during all experiments from wall feeders. The liquid diet differs only slightly from chow, and its caloric profile was 20.8% of the calories derived from protein, 11.9% from fat, and 67.3% from carbohydrates, providing a total of 1.0 kcal/ml. Thereafter, seven groups were established. In four of the groups, the rats were continuously sleep deprived for 24 (SD-24h), 48 (SD-48h), 72 (SD-72h), and 96 h (SD-96h), whereas another group of rats were kept under the control condition (CT). The last two groups were composed of rats that were 96-h sleep deprived and permitted to sleep for 24 h (RB-24h) and rats that were sleep deprived daily from 4 PM to 10 AM (sleep ad libitum from 10 AM to 4 PM) over 21 days before sample harvesting (SR-21d), as described previously elsewhere (28). The samples from the control group were harvested on the same day as the RB-24h. Body weight and food intake were recorded daily between 0700 and 0800. Animals of each group were euthanized randomly by decapitation between 0800 and 0900 for tissue sample collection.

**Analytical procedures.** Blood aliquots were collected in tubes containing prechilled disodium fluoride, EDTA, and either heparin or no anticoagulant (Becton-Dickinson). Tubes were centrifuged at 4°C for 10 min at 3,000 rpm to extract plasma and serum aliquots, respectively. A set of serum aliquots were stored at –80°C for insulin, glucagon, leptin, adiponectin, ghrelin (Linco Research, St. Louis, MO), and corticosterone measurements (ICN-Biomedical, Orangeburg, NY) using RIA kits. Immediately after collection, plasma and another set of serum aliquots were used for glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triacylglycerol determination using colorimetric automated procedures (ADVIA 1650; Bayer Diagnostics) routinely performed in clinical laboratories. To measure ketone bodies, deproteinized supernatants were obtained by collecting blood aliquots in perchloric acid (1 M) and then centrifuged at 4°C for 20 min at 3,400 rpm. Deproteinized supernatants were used to determine acetoacetate and β-hydroxybutyrate concentrations spectrophotometrically (5) immediately after the collection. Liver samples were rapidly excised, weighed, and maintained in potassium hydrox-

ide (30%) solution until glycogen storage was measured by sulphuric acid anthrone reaction (49) on the same day of euthanization.

**Tissue preparation and real-time PCR.** The hypothalamus was dissected on the basis of anatomic landmarks. Briefly, the brains were placed ventral side up, and the whole hypothalamus was scooped out with a dissecting microspatula using the optic chiasm as the anterior boundary, the medial mammillary body as the posterior boundary, and the lateral edge of the hypothalamus at the border of the pyriform lobes as the lateral boundary. Following the dissections, Trizol reagent (Invitrogen, Carlsbad, CA) was added to the hypothalamus and total RNA isolated according to the manufacturer's protocol. An agarose gel (1%) electrophoresis was done to evaluate the integrity of the molecule. After DNase (Promega) treatment, total RNA was reverse transcribed using ImProm-IITM Reverse Transcriptase (Promega). Diluted cDNA were added to 2× SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and the respective primers for PPO, NPY, POMC, agouti-related protein (AgRP), and melanin-concentrating hormone (MCH) (Table 1). The expression of target genes was normalized using α1-tubulin as the endogenous control. Quantitative real-time PCR assays were performed using 96-well plates sealed with optical quality film (Applied Biosystems) on an Opticon-2 Real-Time PCR Detection System (MJ Research; incorporated by Bio-Rad Laboratories). The analysis of relative expression was performed using a standard dilution curve-based method for relative real-time PCR data processing performed on a Microsoft Excel spreadsheet (25). Results were expressed in arbitrary units.

**Statistical analysis.** Results are presented as means ± SE. A two-way ANOVA for repeated measurements was performed to analyze the effects of sleep loss on body weight and food intake pre- and postdistinct sleep deprivation periods. For the metabolites, hormones, and gene expression parameters, we performed a one-way ANOVA. All ANOVAs were followed by Duncan's multiple range tests and with an α-value set at 0.05.

## RESULTS

In Fig. 1, we present the changes in the body weight of rats sleep deprived for different periods of time. As described previously, there was only a slight weight loss after 48 h of sleep deprivation, which remained reduced after 24 h of recovery [ANOVA interaction factor  $F_{(6,60)} = 9.32$ ,  $P < 0.0001$ ].

Table 1. Primer sequences used in quantitative PCR reactions

Gene	Primers
α1-Tubulin	
Sense	5'-TACCCCTCGCATCCACTTCCCT-3'
Antisense	5'-CGCTTGGTCTTGATGGTGGCA-3'
NPY	
Sense	5'-GGGACACTACATCAATCTCATC-3'
Antisense	5'-AAGGGTCTTCAAGCCTTGTTT-3'
POMC	
Sense	5'-CGAGATTCTGTACAGTCCGTC-3'
Antisense	5'-TTCATCTCCGTTGCTGGAAACAC-3'
PPO	
Sense	5'-AGAAGACGTTCTTCTGCGGT-3'
Antisense	5'-CGTGGTTACCGTTGGCTGAA-3'
AgRP	
Sense	5'-CTGCTGCAGAAGGCAGAACG-3'
Antisense	5'-GAAGAAGCGGCAGTAGCACGG-3'
MCH	
Sense	5'-TTCCCAGCTGAGAATGGAGTTCAG-3'
Antisense	5'-GCCAGATACACCTGAGCATGTC-3'

NPY, neuropeptide Y; POMC, proopiomelanocortin; PPO, prepro-orexin; AgRP, agouti-related protein; MCH, melanin-concentrating hormone.

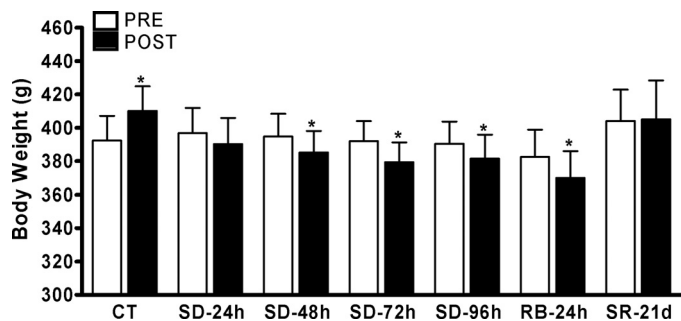


Fig. 1. Body weight (means  $\pm$  SE) of rats pre- (PRE; open bars) and post-sleep deprivation (POST; closed bars) for 24 (SD-24h), 48 (SD-48h), 72 (SD-72h), and 96 h (SD-96h); 24-h rebound (RB-24h), 21 days of sleep restriction (SR-21d), and control (CT) rats. \*Different from respective pre-sleep deprivation using Duncan's test,  $P < 0.05$ .

On the other hand, the body weight of the control group animals increased during this period (Fig. 1).

Surprisingly, earlier than described previously by Everson et al. (10), Kushida et al. (24), Rechtschaffen et al. (46), and Koban and colleagues (20–22), we found that rats fed a liquid diet had a 25–35% increase in their food intake after 72 and 96 h of sleep deprivation [ANOVA interaction factor  $F_{(6,60)} = 8.80$ ,  $P < 0.0001$ ]. During the recovery period the rats consumed significantly less liquid diet compared with presleep deprivation consumption (Fig. 2). Interestingly, our protocol of 21 days of sleep restriction did not change body weight or food intake (Figs. 1 and 2).

We assessed the gene expression of hypothalamic neuropeptides involved in the control of both food intake and sleep. Despite the fact that there was no change in the expression of AgRP [ $F_{(6,60)} = 1.34$ ,  $P = 0.25$ ], the expression of NPY was increased from 48 up to 96 h of sleep deprivation, supporting the observed changes in the food intake, whereas recovery and sleep-restricted groups were similar to the control animals [ $F_{(6,60)} = 4.85$ ,  $P < 0.001$ ] (Fig. 3, A and B). As expected, PPO gene expression was increased from 24 up to 96 h of sleep deprivation, but it was reduced to the control levels after the recovery period [ $F_{(6,60)} = 5.36$ ,  $P < 0.0001$ ; Fig. 3C]. Interestingly, not only did chronically sleep-restricted rats have a higher expression of PPO, but MCH was also increased [ $F_{(6,60)} = 2.33$ ,  $P = 0.042$ ; Fig. 3D], and POMC gene expression was reduced [ $F_{(6,60)} = 3.30$ ,  $P = 0.043$ ; Fig. 3E].

Despite a slight reduction in insulin levels [ $F_{(6,60)} = 3.07$ ,  $P = 0.01$ ; Fig. 4] after 96 h of sleep deprivation there were no statistical differences compared with the control group. There was also no effect on the serum levels of leptin [ $F_{(6,60)} = 1.70$ ,  $P = 0.13$ ] or ghrelin [ $F_{(6,60)} = 1.81$ ,  $P < 0.11$ ] after different periods of sleep deprivation or after 21 days of sleep restriction (Fig. 4, A, C, and D). However, we found that continuous sleep deprivation had an increasing effect on serum glucagon levels, which were increased significantly in the 48- to 96-h sleep deprivation groups [ $F_{(6,60)} = 6.29$ ,  $P < 0.0001$ ], but the levels were similar to the control group after 24 h of recovery (Fig. 4B). Adiponectin levels were also changed [ $F_{(6,60)} = 3.10$ ,  $P < 0.01$ ], showing a significant reduction after 72 h of sleep deprivation (Fig. 4E).

Stress response hormones were also distinctly modified by the sleep disturbance protocols. Regarding home cage control, corticosterone [ $F_{(6,60)} = 10.99$ ,  $P < 0.001$ ] levels were in-

creased significantly from 24 h and norepinephrine levels [ $F_{(6,60)} = 6.25$ ,  $P < 0.001$ ] during 48–96 h of sleep deprivation, but 24 h of recovery was sufficient to reverse the changes in both hormones (Fig. 5, A and B). The sleep-restricted group showed no changes in serum glucagon levels; however, corticosterone, epinephrine [ $F_{(6,60)} = 4.56$ ,  $P < 0.01$ ], and norepinephrine levels were increased (Fig. 5, A–C).

In accord with our previous observations, sleep deprivation reduced liver glycogen stores. In the present experiment we found that, during 24–96 h of continuous sleep deprivation, the liver glycogen content was reduced [ $F_{(6,60)} = 6.33$ ,  $P < 0.0001$ ]. Nevertheless, after 24 h of recovery, the liver glycogen store was still lower than that observed in the control group, although 21 days of sleep restriction did not change the levels (Fig. 6).

Likely due to glycogen breakdown, no effects of sleep deprivation on circulating glucose [ $F_{(6,60)} = 1.09$ ,  $P = 0.42$ ] or ketone body levels [ $F_{(6,60)} = 0.60$ ,  $P = 0.72$ ] were found (Fig. 7, A and B). ANOVA revealed the effects of different sleep deprivation periods on the total cholesterol [ $F_{(6,60)} = 2.67$ ,  $P = 0.02$ ], HDL cholesterol [ $F_{(6,60)} = 3.50$ ,  $P < 0.01$ ], and triacylglycerol [ $F_{(6,60)} = 4.53$ ,  $P < 0.001$ ] levels (Fig. 7, C–E). However, post hoc comparison revealed only that triacylglycerol levels were reduced compared with the control group during 48–96 h of continuous sleep deprivation (Fig. 7E). Therefore, no effects of sleep deprivation or restriction on the blood levels of total cholesterol and HDL cholesterol were found.

## DISCUSSION

In this study, we have shown that the hyperphagia induced during the sleep deprivation of rats on a liquid diet starts earlier than described previously for rats on a chow diet deprived by disk-over-water (10, 24) or platform (22, 30) use. This difference is likely due to an easier access of food. Consequently, unlike rats on only a chow diet, a slight reduction in body weight was noticed during 48–96 h of sleep deprivation (Fig. 1). Of even greater importance is that during the recovery period the sleep rebound blunted the hunger, and despite lower body weight the food intake was reduced to a level below that which was recorded before (pre) sleep deprivation (Figs. 1 and 2). Surprisingly, no effects on food intake or body weight were observed in the rats that were sleep restricted for 21 days (Figs. 1 and 2). Together, these data suggest that, rather than a counterregulation response to weight loss, hyperphagia in rats

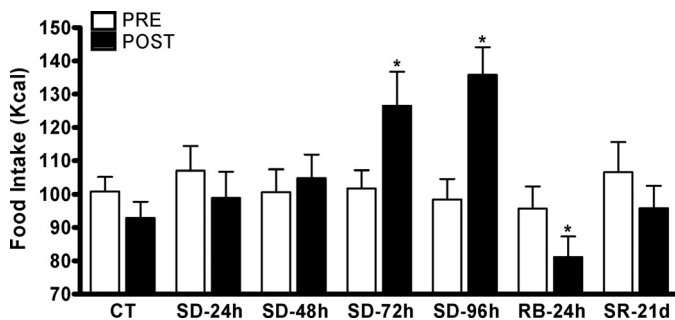


Fig. 2. Food intake (means  $\pm$  SE) by rats pre- (open bars) and post-sleep deprivation (closed bars) for SD-24h, SD-48h, SD-72h, and SD-96h; RB-24h, SR-21d, and CT rats. \*Different from respective pre-sleep deprivation using Duncan's test,  $P < 0.05$ .

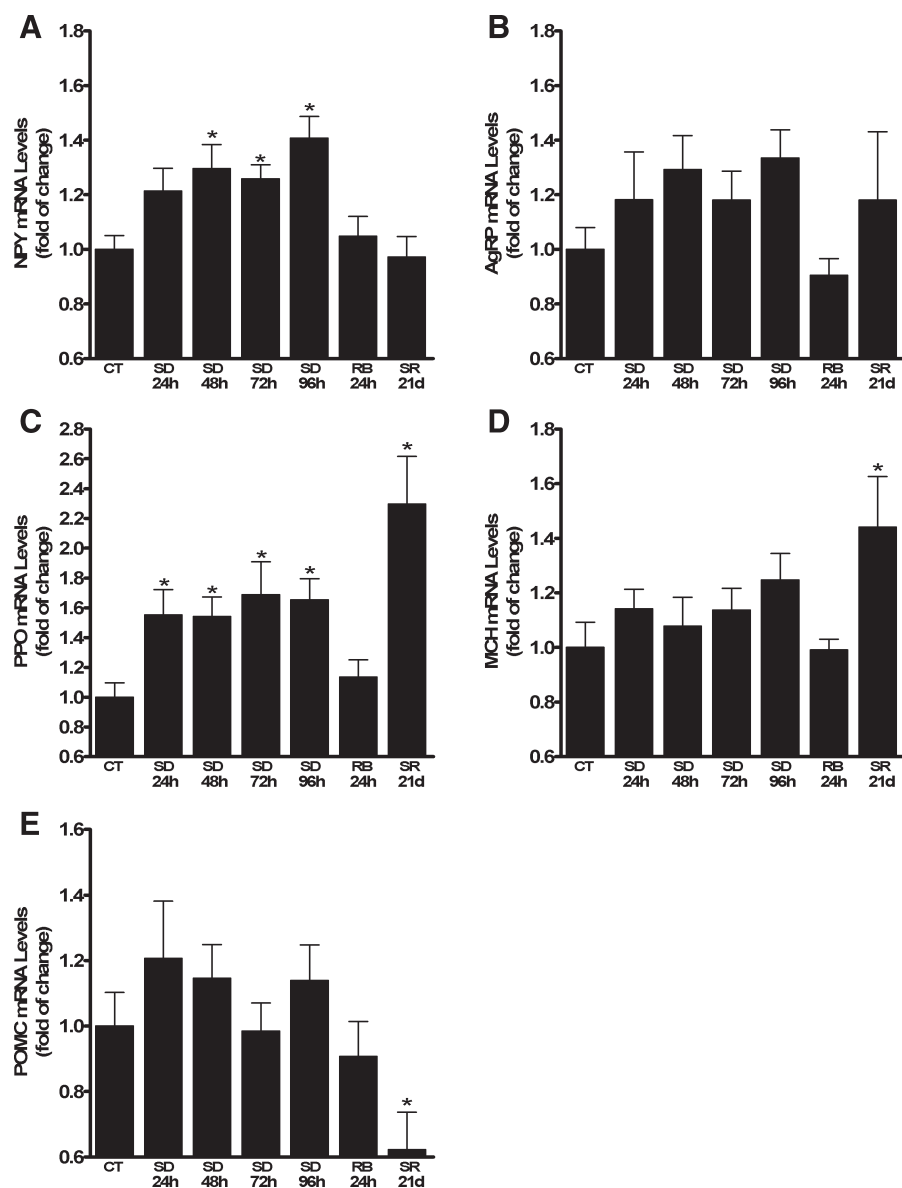


Fig. 3. Hypothalamic mRNA levels (means  $\pm$  SE) of neuropeptide Y (NPY; A), agouti-related peptide (AgRP; B), prepro-orexin (PPO; C), melanin-concentrating hormone (MCH; D), and proopiomelanocortin (POMC; E) after SD-24h, SD-48h, SD-72h, and SD-96h; RB-24h, SR-21d, and CT rats. \*Different from control group using Duncan's test,  $P < 0.05$ .

can be a consequence of activating the feeding centers by prolonged wakefulness.

Indeed, Koban et al. (20) have shown that sleep deprivation increased the expression of the most potent orexigenic peptide, NPY, and reduced the anorexigenic peptide POMC in the hypothalamus of rats. Such changes have been attributed to a reduction in the leptin levels observed after 5 or more days of sleep deprivation (11, 22). Nevertheless, long-term sleep deprivation of rats on a chow diet induces large weight loss, especially through the reduction of adipose tissue (39), which can account for the reduced serum leptin levels. In such cases, increased NPY and the consequent hyperphagia should be a response to weight loss rather than an effect of the sleep deprivation itself. However, more recently, Koban et al. (21) have shown that rats receiving Ensure, a human liquid diet, developed hyperphagia after 6 days of sleep deprivation, and body weight changes were greatly minimized. Therefore, if the body weight of rats on a liquid diet was only slightly modified by sleep deprivation, it is possible that the changes in the serum

leptin levels might also be different from those reported for rats on chow diet. Unfortunately, the leptin levels were not reported in the previous studies of rats sleep deprived on a liquid diet (14, 21). However, in the present study we show that through 96 h of sleep deprivation the weight loss of rats on a liquid diet was  $<4\%$ , and there were no significant changes in the serum levels of insulin, leptin, and ghrelin (Fig. 4, A, C, and D, respectively). The absence of changes in serum leptin levels is reinforced by no changes in white adipose tissue leptin mRNA levels (data not shown). Therefore, since the sleep deprivation of rats on a liquid diet leads to increasing food intake (Fig. 2) and NPY hypothalamic expression (Fig. 3A) but no changes in the major hormones associated with the control of its expression, including leptin (Fig. 4), we believe that other factors linked directly to the sleep deprivation should be determinant for this type of hyperphagia.

The expression of NPY in the hypothalamus occurs especially in a population of neurons of the arcuate nucleus and is colocalized with another orexigenic peptide, AgRP. We veri-

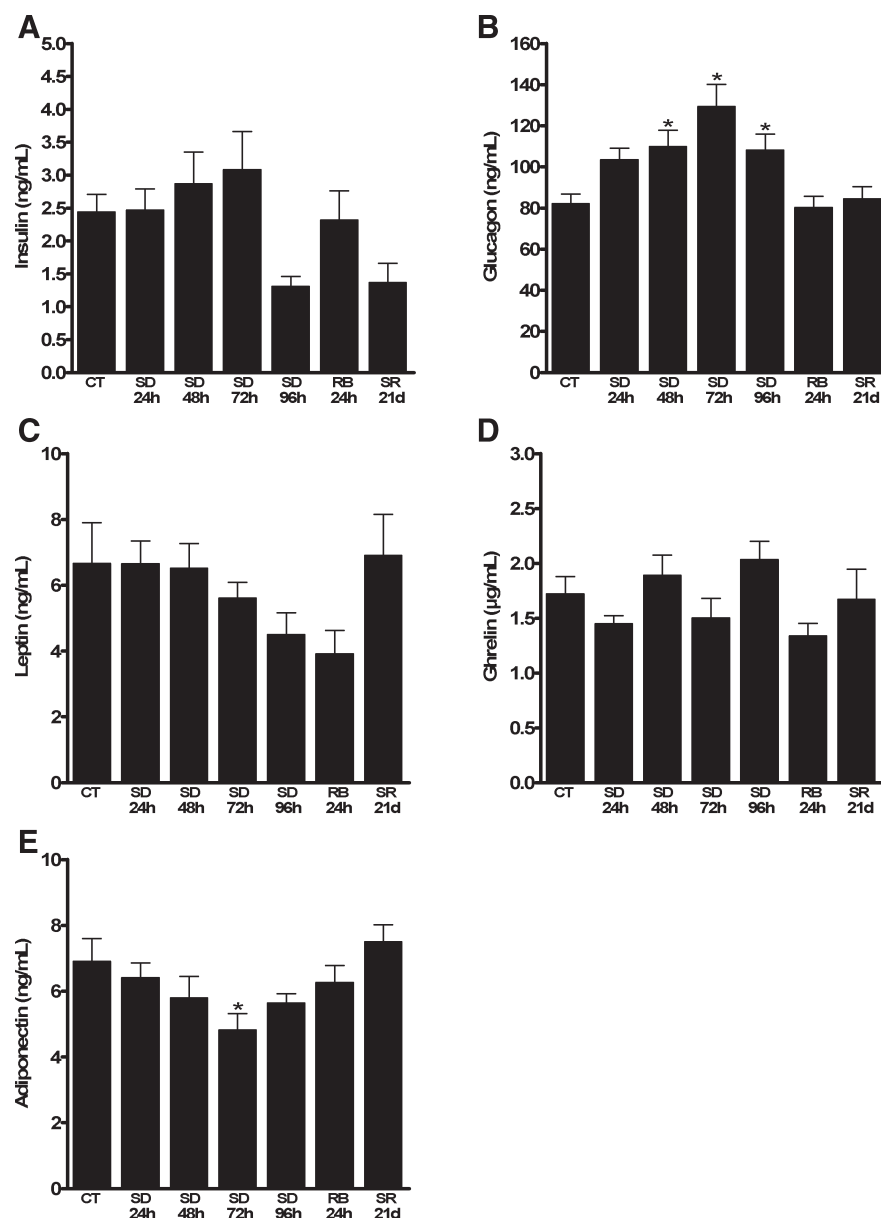


Fig. 4. Serum levels (means  $\pm$  SE) of insulin (A), glucagon (B), leptin (C), ghrelin (D), and adiponectin (E) after SD-24h, SD-48h, SD-72h, and SD-96h; RB-24h, SR-21d, and CT rats. \*Different from control group using Duncan's test,  $P < 0.05$ .

fied that NPY mRNA levels were elevated significantly from 48 to 96 h of sleep deprivation (Fig. 3A), but despite its colocalization, no significant changes in AgRP expression were found (Fig. 3B). Interestingly, the arcuate nucleus receives projections from the lateral hypothalamus area, where two other peptides, the orexins and MCH, which are linked to food intake and sleep control, are produced (43). We found that mRNA levels of the peptide precursor of orexins, PPO, were higher during 24–96 h of sleep deprivation (Fig. 3C), whereas no change in MCH was observed (Fig. 3D). The temporal organization of these events suggests that increased orexinergic activity during sleep deprivation activates arcuate NPY neurons, consequently leading to the hyperphagia observed from 72 h of sleep deprivation. There is numerous evidence to support such a hypothesis of interaction between orexins and NPY on the hyperphagia induced by sleep deprivation, including the fact that arcuate injection of orexins increases food intake (35). Also, orexin neurons project densely into the

arcuate nucleus (36), and both orexin receptors (OX1R and OX2R) are also expressed in this nucleus (8). In addition, central administration of orexin increases *Fos* expression (61) and electrical activity (59) of the arcuate NPY neurons. Furthermore, the orexigenic effects of intracerebroventricular (icv) administration of orexin-A can be inhibited partly by a NPY-Y1 receptor antagonist (18).

In rodents, the orexinergic system seems to respond quickly to modifications in the sleep-wake cycle, increasing both the concentration of orexin-A in cerebrospinal fluid and hypothalamic mRNA levels after 6–8 h of sleep deprivation (42, 45). Furthermore, 24 h of sleep recovery reduced the higher cerebrospinal fluid levels of orexin-A observed after 96 h of sleep deprivation to levels below that of the control animals (42). On the other hand, a single or daily icv administration of orexin only increases food intake during the first hours postadministration and has no effect on 24-h food intake (50, 62). Together, these data suggest that orexin has a short-term effect,

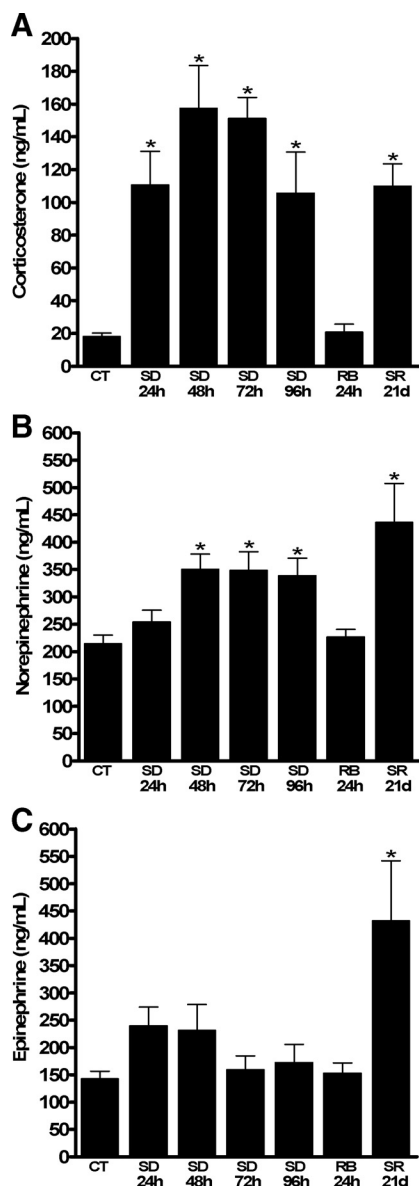


Fig. 5. Serum levels (means  $\pm$  SE) of corticosterone (A), norepinephrine (B), and epinephrine (C) after SD-24h, SD-48h, SD-72h, and SD-96h; RB-24h, SR-21d, and CT rats. \*Different from control group Duncan's test,  $P < 0.05$ .

and so a tonic action of the orexins sustained by the continuous sleep deprivation is required to consequently increase NPY expression and 24-h food intake. This could explain the absence of changes in NPY expression and food intake of sleep-restricted rats despite changes in PPO gene expression (Fig. 3, A and C). It is important to notice that the effects of chronic sleep restriction were assessed at 1–2 h before the beginning of the sleep window (supposed to occur from 10 AM to 4 PM). Therefore, a higher PPO expression is expected due to the previous extended period of wakefulness. Yet this was not enough time to increase NPY expression, which seems to require 48 h of sleep deprivation to be increased (Fig. 3A). In accord with that, we also observed that sleep had a powerful effect to reverse not only PPO but also NPY expression (Fig. 3, A and C), which rapidly brought the animals from a hyperphagic to hypophagic condition (Fig. 2). It is possible that

the hypophagia observed during the sleep recovery is due to an increased sleep time after the forced sleep loss, also referred to as a sleep rebound. Using our protocol of sleep deprivation, it has been shown that following 96 h of sleep deprivation the awake time of rats was reduced, whereas the sleep time was increased during the initial 24 h of recovery, especially due to an increase in paradoxical sleep (27). Since the animals were free to express both feeding and sleep behaviors during the recovery period, it is possible that during the recovery period the sleep mechanisms overcome the hunger mechanisms, consequently reducing food intake. However, if that was the case, we would expect that the NPY expression be kept high, if not higher, after a hypophagic sleep recovery period. Hence, we assume that the continuous orexin activation induced by the sleep deprivation increases NPY expression, whereas periods of sleep prevented or reversed it. Modirrousta et al. (32) have proposed that orexin neurons may act to sustain waking during sleep deprivation, whereas MCH neurons may act to promote sleep following sustained waking. Therefore, the increased expression of PPO and MCH observed in the sleep-restricted animals (Fig. 3, C and D, respectively) may be explained by the recurring cycle of extended waking/consolidated sleep experienced during the 21 days (28). Indeed, orexin action in the arcuate nucleus seems to not only increase NPY neuron activity but also reduce that of POMC neurons (35). In our experiment, the expression of POMC did not change through the 96 h of sleep deprivation, but it was reduced after 21 days of sleep restriction. Our data agree with the previous observations that the inhibitory effect of POMC on arcuate expression occurred only at 20 days of sleep deprivation (20). However, 21 days of sleep restriction failed to induce hyperphagia, which suggests that reduction of POMC expression is not required for the hyperphagia associated with sleep deprivation. One could argue that the sleep restriction protocol did not reduce sleep, but it has been shown previously that this protocol reduces total sleep time, especially paradoxical sleep, throughout the 21 days of restriction, whereas a sleep debit was reinforced by the sleep rebound during 48 h of recovery (29).

Increased glucocorticoid profiles are known to be present in subjects submitted to experimental sleep loss (53, 54). Likewise, sleep loss has been associated with higher levels of ACTH and glucocorticoids in rats (2, 3, 39). Following the observed changes in PPO mRNA during the sleep deprivation, we observed that corticosterone and norepinephrine levels were increased at 24 and 48 h of sleep deprivation, respectively (Fig. 5, A and B). Indeed, previous studies have described

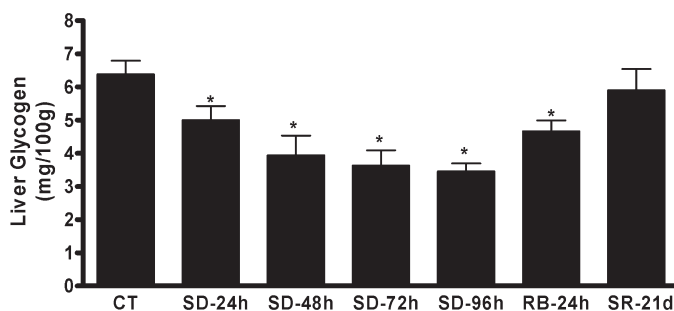


Fig. 6. Liver glycogen concentration (means  $\pm$  SE) after SD-24h, SD-48h, SD-72h, and SD-96h; RB-24h, SR-21d, and CT rats. \*Different from control group using Duncan's test,  $P < 0.05$ .

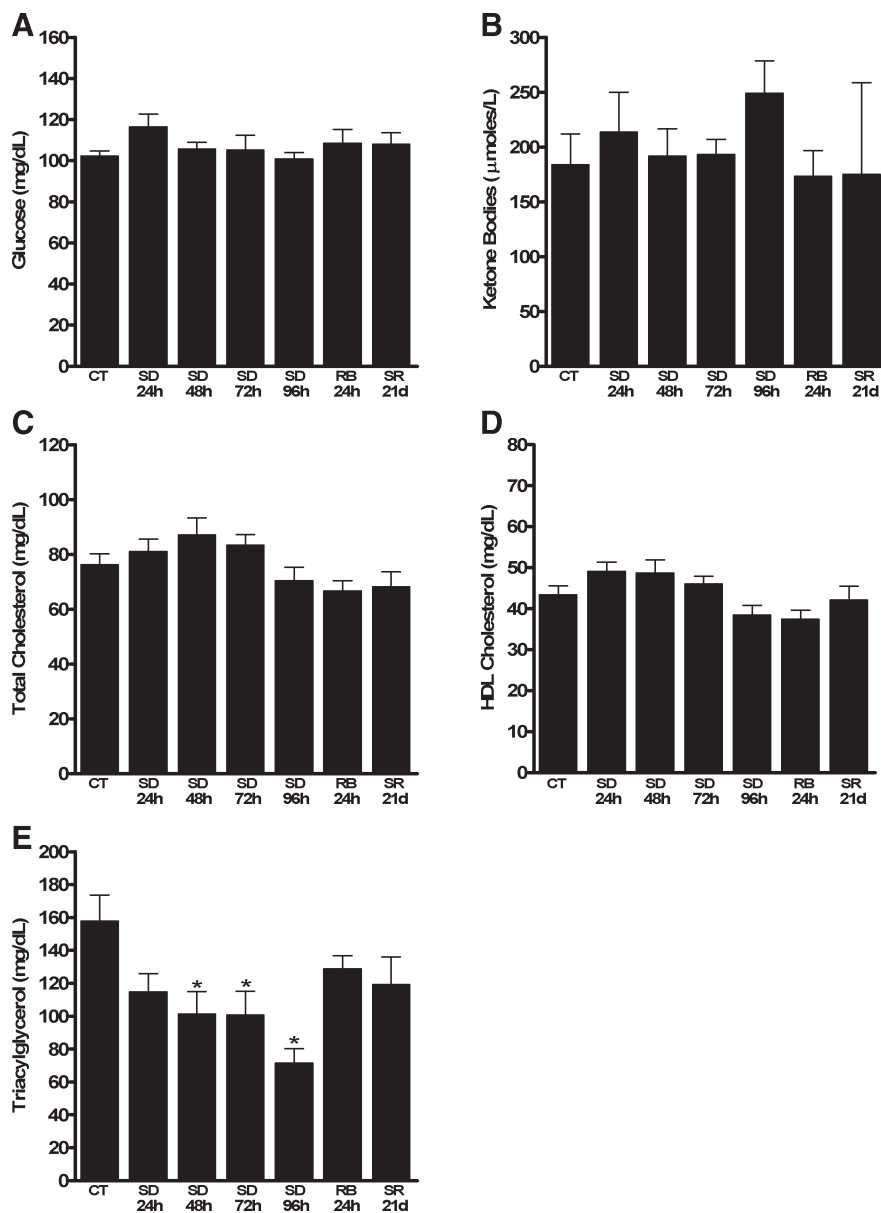


Fig. 7. Blood levels (means  $\pm$  SE) of glucose (A), ketone bodies (B), total cholesterol (C), high density lipoprotein (HDL) cholesterol (D), and triacylglycerol (E) after SD-24h, SD-48h, SD-72h, and SD-96h; RB-24h, SR-21d, and CT rats. \*Different from control group using Duncan's test,  $P < 0.05$ .

increased corticotropin-releasing hormone (CRH) expression in the paraventricular nucleus (PVN) of sleep-deprived rats (12, 20). Several lines of evidence support that orexinergic activation mediates sympathetic and hypothalamus-pituitary-adrenal axis activation during sleep deprivation. Administration of orexins into the cerebral ventricles quickly increased plasma ACTH and subsequently corticosterone levels in a dose-response fashion (13, 23). Such effects were explained by the activation of the PVN, as demonstrated by increased *c-fos* (23) and CRH mRNA levels (1). Intriguingly, the effect of orexins on the hypothalamus-pituitary-adrenal axis is independent of its effects on NPY neurons (34). Therefore, except for no changes in NPY expression after 21 days of sleep restriction, rats showed the highest PPO expression and increases not only in norepinephrine but also epinephrine levels (Fig. 3, B and C). These response profiles also agree with the previous observation that icv injections of orexin increased plasma levels of norepinephrine and epinephrine at lower and higher doses, respectively (52).

Such sympathetic activation in response to high orexin activity might also be related to other metabolic and hormonal changes observed during the sleep deprivation. Spiegel and colleagues (53, 54) have shown elevations of the sympathovagal balance in response to sleep loss. In addition, increased sympathetic activity of the splenic nerve is classically associated with increased release of glucagon by the  $\alpha$ -cells of the pancreas (58). This is in accord with the observation that glucagon concentration was increased in animals deprived from 48 to 96 h but was not changed in animals sleep restricted for 21 days (Fig. 4B). Glucocorticoids and catecholamines also have important effects on glycogen metabolism; however, liver glycogen (Fig. 6) was not changed in sleep-restricted animals despite their higher levels of corticosterone, norepinephrine, and epinephrine (Fig. 5). Therefore, it is likely that the glycogen reductions described in the present study (Fig. 6) were directed by increases in glucagon levels induced by sleep deprivation (Fig. 4B). In addition, such changes in the gluc-

gon levels are consistent with the observed changes of NPY expression in the hypothalamus (Fig. 3A). Together, these data suggest that the tonic action of orexins on arcuate NPY neurons might lead to increased glucagon secretion and reduced liver glycogen. In agreement with such an idea, previous studies have shown that icv injection of either NPY or orexin-A increases circulating glucagon levels (40, 41). However, additional experiments are needed to fully prove this hypothesis.

In opposition to other orexigenic peptides, the orexins increase energy expenditure (50), which seems to occur through thermogenesis and/or locomotor activity. Intra-arcuate injections of orexin-A in anesthetized rats increased oxygen consumption ( $\dot{V}O_2$ ) and body temperature (60). On the other hand, daily intra-PVN injection of orexin-A increased locomotor activity with no change in food intake (38). In agreement with these orexin effects, besides hyperphagia, weight loss and increased energy expenditure have been shown in rats undergoing prolonged sleep deprivation by different procedures (3, 22). This high-energy expenditure has been explained by the increased activity of uncoupling proteins in the liver, skeletal muscle, and brown adipose tissue (7, 22). Additionally, it is well known that sympathetic activation under higher energy demand is responsible for increased fatty acid contribution as an energy source. For instance, activation of the ventromedial nucleus accelerates the turnover of triacylglycerols, which increases the production of heat through uncoupling proteins in brown adipose tissue (51). In the same way, icv injection of orexin increased body temperature and the firing rate of the sympathetic nerves to the interscapular brown adipose tissue but was blunted after the ventromedial nucleus of the hypothalamus was damaged (33). In this sense, although we found no changes in the blood levels of glucose, ketone bodies, and cholesterol lipoproteins, there was a progressive reduction in serum triacylglycerols during the sleep deprivation (Fig. 7), suggesting that sleep deprivation quickly increases energy expenditure and fatty acid utilization likely though a thermogenic process.

Interestingly, opposed metabolic repercussions have been observed in narcoleptic patients with cataplexy, which were associated with impaired orexin function by lower cerebrospinal fluid orexin-1 levels compared with a well-matched control group with idiopathic hypersomnia. Narcoleptic patients, despite consuming 15% less calories daily, had higher body and fat mass as well as circulating levels of leptin and triacylglycerols, among other metabolic abnormalities. Altogether, these data suggest that the implication of the orexinergic system on metabolism seems to go beyond the food intake and energy expenditure and may involve specific actions on glucose and lipid metabolism control (44). In summary, based on our results, 3 days of continuous sleep deprivation are sufficient to trigger hyperphagia in rats fed a liquid diet. Although several parameters, such as food composition/palatability and accessibility, are important to establish how much food is consumed during the sleep deprivation, it is most likely that the daily amount of sleep lost determines the onset of hyperphagia. However, this mechanism seems to be complex and requires a tonic action of the orexins on NPY neurons that is likely to overcome the anorectic effects of CRH that can also be activated by the orexins during sleep deprivation. Our present data suggest that orexin activation explains a myriad of changes induced by sleep deprivation, especially hyperphagia under stress and the negative energy balance.

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## DISCLOSURES

The authors have nothing to disclose.

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