Original Article

Effects of Paradoxical Sleep Deprivation on MCH and Hypocretin Systems

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Abstract

Melanin-concentrating hormone (MCH) and hypocretins (Hcrt) 1 and 2 are neuropeptides synthesized in the lateral hypothalamic area by neurons that are critical in the regulation of sleep and wakefulness. Their receptors are located in the same cerebral regions, including the frontal cortex and hippocampus. The present study aimed to assess whether 96 hours of paradoxical sleep deprivation alters the functioning of the MCH and hypocretin systems. To do this, in control rats with normal sleep (CTL) and in rats that were deprived of paradoxical sleep (SD), we quantified the following parameters: 1) levels of MCH and hypocretin-1 in the cerebrospinal fluid (CSF); 2) expression of the *prepro-MCH* (*Pmch*) and *prepro-hypocretin* (*Hcrt*) genes in the hypothalamus; 3) expression of the *Mchr1* and *Hcrtr1* genes in the frontal cortex and hippocampus; and 4) expression of the *Hcrtr2* gene in the hippocampus. These measures were performed at 6 Zeitgeber time (ZT) points of the day (ZTs: 0, 4, 8, 12, 16, and 20). In the SD group, we found higher levels of MCH in the CSF at the beginning of the dark phase. In the frontal cortex, sleep deprivation decreased the expression of *Hcrtr1* at ZT0. Moreover, we identified significant differences between the light and dark phases in the expression of *Mchr1* and *Hcrtr1*, but only in the CTL animals. We conclude that there is a day/night modulation in the expression of components of the MCH and hypocretin systems, and this profile is affected by paradoxical sleep deprivation.

Keywords

► hypothalamus
► neuropeptides
► orexin
► circadian rhythm
► REM sleep
► gene expression

Introduction

Melanin-concentrating hormone (MCH) and hypocretins 1 and 2 (Hcrt, also known as orexins) are neuropeptides produced by two groups of neurons primarily located in the posterolateral hypothalamic area. These neurons send diffuse projections throughout the central nervous system.[1–6]

The MCH exerts its effects through two types of G-protein coupled receptors, Mchr1 and Mchr2. Notably, Mchr2 is nonfunctional in rodents.[2,7] On the other hand, Sakurai et al.[8] (1998) identified two hypocretin receptors: Hcrtr1 and Hcrtr2. Both hypocretin and MCH receptors are distributed in approximately the same brain areas in rats, including the frontal cortex and the hippocampus.[9,10]

The MCHergic and hypocretinergic neuronal groups reciprocally regulate sleep and wakefulness;[11–14] the MCH tends to promote sleep, while hypocretins are wake-promoting neuromodulators.[15] Electrophysiological studies[16] have revealed that MCHergic neurons are more active during sleep, particularly in paradoxical sleep (PS), whereas hypocretinergic neurons exhibit higher activity levels during wakefulness. However, recent advances in functional

imaging techniques using genetically-modified mice have revealed the presence of minor groups of active MCHergic neurons during wakefulness and hypocretinergic neurons during PS.[17–19]

Sleep deprivation is a widely recognized health issue, with a prevalence of excessive daytime sleepiness ranging from 9% to 24%. Long-term sleep deprivation can lead to the development of physiological and neurobehavioral problems, reduced quality of life, and increased mortality rates.[20] Furthermore, Naiman[21] (2017) highlighted that specific PS or dream loss constitutes "an unrecognized public health hazard that silently wreaks havoc on our lives, contributing to illness, depression, and a decline in consciousness".

In the present study, we focused on the effect of sleep deprivation, mainly PS deprivation, on the functioning of the MCHergic and hypocretinergic systems. Given the roles of MCH and hypocretin in regulating behavioral states, we hypothesized that PS deprivation could lead to changes in their levels in the cerebrospinal fluid (CSF) and alterations in the gene expression of their precursors and receptors. To test this hypothesis, we euthanized rats at 6 *Zeitgeber* time (ZT) points throughout the day (ZTs: 0, 4, 8, 12, 16, and 20) after 96 hours of PS deprivation (SD group), alongside rats allowed to sleep ad libitum (control group, CTL). In these groups, we analyzed the following parameters: 1) MCH and hypocretin levels in the CSF; 2) the expression of the *Pmch* and *Hcrt* genes in the hypothalamus; 3) the expression of *Mchr1* and *Hcrtr1* in the frontal cortex and hippocampus; and 4) the expression of the *Hcrtr2* gene in the hippocampus.

Materials and Methods

Animals and Housing Conditions
Three-month-old male Wistar rats (weighing between 320 g and 370 g; 96 animals, 8 animals per group) from the Center for the Development of Experimental Models for Medicine and Biology (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia, CEDEME, in Portuguese) were housed at the facility of the Department of Psychobiology at Universidade Federal de São Paulo (UNIFESP).

All animals were kept on a 12:12-h light-dark cycle (lights on at 7 a.m.) under controlled temperature conditions (21–24°C), with free access to food and water.

Ethical Statement
Animal care and use procedures were performed by trained personnel and conducted following the guide "Animal Models as tools in Ethical Biomedical Research" (UNIFESP, 2010).[56]

Appropriate measures were taken to minimize the pain, discomfort, and stress of the animals. All efforts were made to use the minimal number of animals necessary to produce reliable scientific data. The Ethics Committee at UNIFESP approved this experimental protocol (CEUA: 8359230215). All animals had access to water and food ad libitum during the whole experiment.

Control Groups
The light-dark cycle is an essential environmental clue that entrains the animals' circadian rhythms. Thus, it is referred to as the "time giver" or *Zeitgeber* in German.[22] The experimental room lights were turned on at 7:00 a.m. (*Zeitgeber* time zero, or ZT0) and turned off at 7:00 p.m. (ZT12).

The CTL animals were placed in their home cages (4 to 5 rats per cage) in the same room as the SD animals. Since any experimental manipulation may cause some level of stress, we chose to use cage-control animals, considering that Machado et al.[23] (2004) found that even wide platform-control could cause sleep deprivation.

As shown in ►**Figure 1**, 6 subgroups (8 animals per group) were created according to the ZT when euthanasia was performed: CTLZT0 (7 a.m.), CTLZT4 (11:00 a.m.), CTLZT8
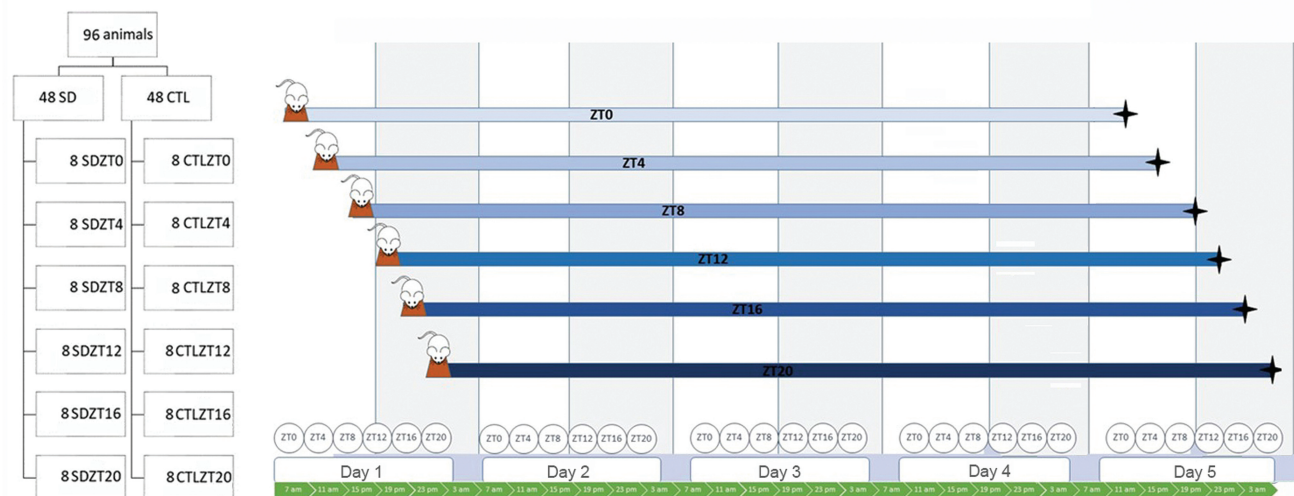
Fig. 1 *Experimental design.* A total of 96 male Wistar rats were divided into two groups: control (CTL) and paradoxical sleep deprivation (SD). Each of these groups was subdivided into 6 subgroups (*Zeitgeber times, Zts*: 0, 4, 8, 12, 16, and 20). The SD experiments initiated at different ZTs and brains were collected for real-time polymerase chain reaction (RT-PCR) analysis after 96 hours. Notes: The gray bars represent the dark phase, and white spaces represent the light phase. + time of euthanasia.

(3:00 p.m.), CTLZT12 (7:00 p.m.), CTLZT16 (11:00 p.m.) and CTLZT20 (3:00 a.m.).

Paradoxical Sleep Deprivation Groups

For the SD group, we created 6 subgroups of animals (with 8 animals each). The SD experiments were initiated at the same ZTs as described for the CTL group (SDZT0, SDZT4, SDZT8, SDZT12, SDZT16, and SDZT20) (►Fig. 1). After 96 hours, the animals in each group were euthanized, along with the CTL animals of the same ZT group. Euthanasia was performed in another room, and animals in both groups were randomly assigned for this procedure.

Paradoxical Sleep Deprivation Procedure

We applied the modified multiple platform method to deprive the animals of PS for 96 hours. It consists of placing 8 rats on 12 narrow circular platforms (diameter = 6.5 cm) that were introduced inside a tiled tank (measuring $143 \times 414 \times 30$ cm).[18] This tank was filled with water up to 1 cm below the upper surface of the platforms.

One day before the experimental protocol, the animals were allowed to adapt to the procedure for one hour. In the present study, sleep recordings were not conducted. However, we took into consideration the results of a previous study[23] from our laboratory, which demonstrated that the multiple platform method led to the complete absence of PS and a moderate decrease (31%) in slow-wave sleep. All animals had access to water and food ad libitum during the SD procedure.

Cerebrospinal Fluid Collection

At the end of the experimental protocol (►Fig. 1), the SD and CTL animals were placed in a glass chamber (measuring $30 \times 20 \times 20$ cm) containing halothane until they showed signs of anesthesia, such as lack of tail reflex. The animals were then placed in a stereotaxic apparatus for CSF collection from the cisterna magna using a syringe connected to a 1-mL insulin-like needle. The CSF limpid aliquots (ranging from 100 μl to 150 μl) were frozen immediately in dry ice and stored at -80°C until used; CSF samples with blood were discarded.

Euthanasia and Tissue Collection

The SD and CTL animals were euthanized at the end of the experimental protocol (►Fig. 1). Their brains were removed, and the hypothalamus, hippocampus, and frontal cortex were dissected, collected, and immediately frozen. We chose to analyze the cortex and hippocampus because these two areas are active during PS and are involved with essential functions attributed to sleep.[24,25]

Quantification of MCH and Hypocretin in the CSF

The CSF levels of MCH and hypocretin were measured using the commercially-available enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Burlingame, CA, United States) according to the manufacturer's instructions.

These EIA kits were designed to detect MCH or hypocretin in a range from 0.45 ng/mL to 8.1 ng/mL based on the principle of "competitive" EIA. Our sample levels were within the linear range of the kit's standard curve, meaning that the results we obtained had high reproducibility and reliability. Measurements of 50 μL of CSF were performed for each sample. The samples were incubated in a 96-well immunoplate with 25 μL of rabbit anti-MCH or anti-hypocretin antibodies (available with the kit); 25 μL of biotinylated MCH or hypocretin were introduced, and the samples were incubated at room temperature for 2 hours under agitation. The immunoplates were washed 4 times with 350 μL of assay buffer. Subsequently, 100 μL of streptavidin horseradish peroxidase (SAHRP) were applied, and the samples were incubated at room temperature for 1 hour under agitation. After incubation, the immunoplates were washed 4 times with 200 μL of assay buffer. Next, 100 μL of the colorimetric substrate solution were applied, and the samples were incubated at room temperature for 1 hour under agitation. The reaction was terminated by adding 100 μL of stop solution (hydrochloric acid 2 mol/L, 2N HCl).

Quantifications were made based on the color intensity of each well. Yellow intensity is directly proportional to the concentration of biotinylated–SAHRP but inversely proportional to the samples' peptide concentration. This is due to the competitive binding of biotinylated peptides to the standard peptide or of sample peptides to the primary antibody.

Unknown sample concentrations were determined by extrapolation to a standard curve at 450 nm of wavelength using a SpectraMax M2 fluorometer (Molecular Devices LLC, San Jose, CA, United States). Readings were corrected using blanks.

Gene Expression Analyses

The harvested tissues were stored in sterile microtubes at -80°C, and RNA was extracted using the Brazol reagent (LGC Biotecnologia, Cotia, SP, Brazil). The quantity and quality of the extracted RNA were measured using the GeneQuant Pro (Amersham Pharmacia Biotech, Uppsala, Sweden) RNA/DNA calculator. Total RNA (1 ug) was used to synthesize the complementary DNA (cDNA) using ImProm-II Reverse Transcriptase (Promega, Madison, WI, United States).

A diluted cDNA sample was used as a template for real-time polymerase chain reaction (PCR) amplification using 2X Maxima SYBR GREEN/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, United States), and the respective primers for *Pmch*, *Hcrt*, *Mchr1*, *Hcrtr1*, *Hcrtr2*, *Beta-actin*, and *Hprt1* (the last two as reference genes) were used according to tissue specificity (see ►Table 1). Amplification and detection were performed using an Applied Biosystems Step One Plus Real-Time PCR system (Thermo Scientific). A two-step cycling protocol was used.

Target mRNA levels were normalized for each well to endogenous controls *Beta-actin* (NCBI GenBank accession number: beta-actin mRNA, NM_031144) and *Hprt1* (NCBI GenBank accession number: Hprt1 mRNA, NM_012583.2). Forward and reverse primers for the targets were used at the final concentration of 10 pmol. The PCR products were

Table 1 Primer sequences for *Pmch*, *Hcrt*, *Mchr1*, *Hcrtr1*, *Hcrt2*, *Actb*, and *Hprt1*.

Primer	Forward	Reverse	Reference sequence
Pmch	ATCGGTTGTTCCTTCTCTGGA	TGCTTGGAGCCTGTGTTCTTTGTG	NM_012625.1
Hcrt	AGGACTAGGACAGGGATAGAAG	CGCAGAGCTAGAGCCATATC	NM_013179.2
Mchr1	GCAAAGGCACCTGACAATTC	CAGGGTAGCCTTGGGTTTAAT	NM_031758.1
Hcrtr1	CCTTAAAAGAGTGTTTGGGATG	AGTTGTAGATGATAGGGTTGGC	NM_013064.1
Hcrtr2	GCTGTCGCTGCTGAGATAAA	CATTGAGGATGCTGATTGGTAGA	NM_013074.1
Actb	GACGGTCAGGTCATCACTATC	AGAGGTCTTTACGGATGTCAAC	NM_031144.3
Hprt1	GCTGACCTGCTGGATTACAT	CCCGTTGACTGGTCATTACA	NM_012583.2

subjected to a heat dissociation protocol (a gradual increase of temperature from 60° C to 95° C) for melting curve analyses.

Relative Gene Expression

The relative gene expression was calculated using the comparative Ct (2-ΔCt) method.[26] The PCR amplification sign of the target gene transcript in one group submitted to the experimental protocol was compared to the transcript of the reference gene's amplification sign. This analysis was used to compare the differences among experimental groups at different ZTs.

Statistical Analysis

Relative gene expression values were reported as mean ± standard error of the mean (SEM) values. For both study groups, a generalized linear model (GLM) with gamma distribution was used to determine the effect of ZT, SD, and the interaction (group *vs.* ZT). When necessary, post-hoc analyses were performed using the Sidak test. The level of significance was set at $p \leq 0.05$. Data analyses were conducted using the IBM SPSS Statistics for Windows (IBM Corp, Armonk, NY, United States) software, version 21.0, and graphs were made using GraphPa Prism 7.0 (Graph Pad Software, San Diego, CA, United States).

Cosinor Analysis

When the GLM test showed a significant difference between experimental groups, we performed the cosinor analysis to test the rhythmicity in a 24-hour period. This method consists of a periodic regression analysis that adjusts a cosine function to the temporal series values, and detects if the adjusted curve shows a statistically significant oscillation or not. This analysis was carried out using the El Temps software (Dr. A. Diez-Noguera, University of Barcelona, Barcelona, Catalonia, Spain).

Results

MCH and Hypocretin in the CSF

We did not observe, in any ZT, significant differences between the CTL and SD groups, neither in MCH nor in Hcrt CSF levels (►**Fig. 2**). We also compared the Hcrt and MCH CSF levels of both groups in six ZTs. Considering the MCH levels, the GLM test showed an effect of the interaction (group*ZT; $p = 0.015$). While in the CTL group there was no statistically significant difference regarding the ZTs, in the SD group, we observed higher levels of MCH in the CSF at ZT12 compared to ZTs 4 and 8 (Wald = 33.274; gl = 11; $p = 0.000$). On the other hand, there were no differences in Hcrt CSF levels among ZTs in either group.
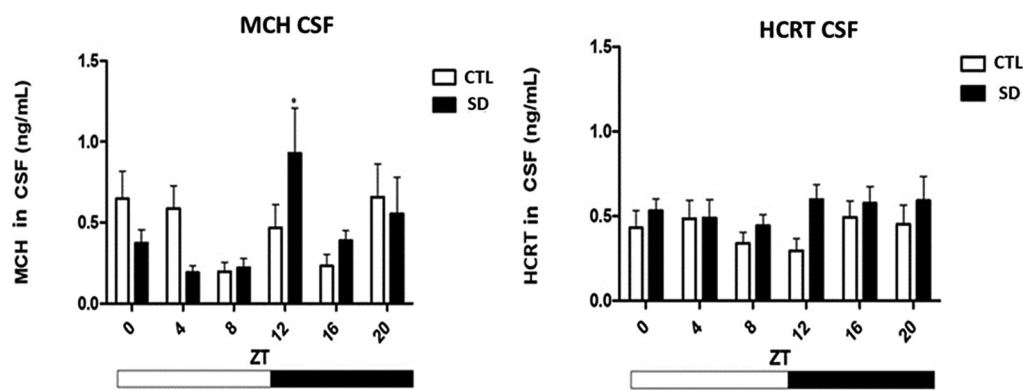
Fig. 2 *MCH and hypocretin levels in the cerebrospinal fluid (CSF) of rats*. This figure shows MCH and hypocretin CSF levels (ng/mL) of rats in the CTL and SD groups at 6 ZTs (0, 4, 8, 12, 16, and 20). Notes: * indicates statistically significant differences in the SD group in the comparison of ZTs 4 and 8; $p \leq 0.05$. The horizontal white bar indicates lights on, and the horizontal dark bar indicates lights off. Values expressed as mean ± standard error of the mean (SEM); N = 5-8.
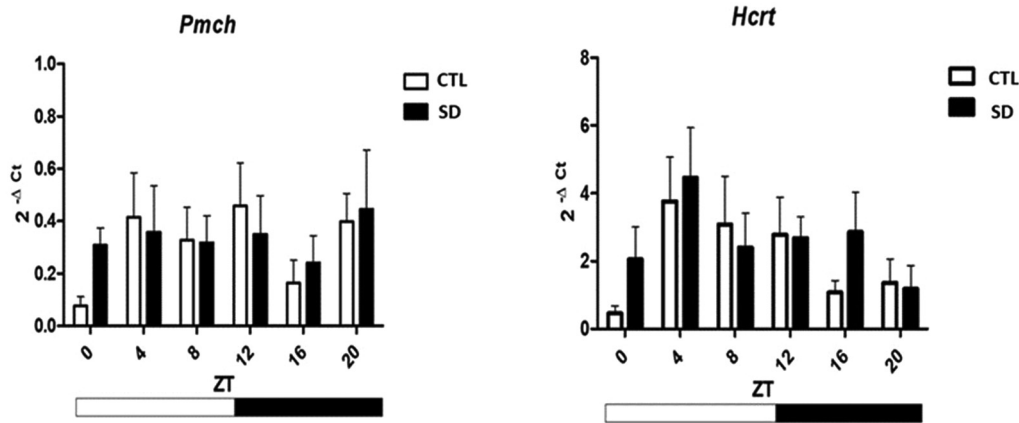
Fig. 3 *Relative gene expression of* Pmch *and* Hcrt *in the hypothalamus of rats*. This figure shows the relative gene expression ($2^{-\Delta Ct}$) of *Pmch* and *Hcrt* in the hypothalamus of rats in the CTL and SD groups at 6 ZTs (0, 4, 8, 12, 16, and 20). Notes: The horizontal white bar indicates lights on, and the horizontal dark bar indicates lights off. Values expressed as mean $\pm$ SEM; N = 5-8.

Gene Expression

Hypothalamus

For the same ZTs, we did not find any statistically significant differences between the SD and CLT groups, neither in the expression of the *Pmch* and *Hcrt* genes (►**Fig. 3**). Furthermore, we did not detect diurnal variation in the expression of these genes in either group.

Frontal Cortex

We did not find any statistically significant differences in *Mchr1* gene expression in the rats' frontal cortex in the CTL and SD groups (►**Fig. 4**). However, we noticed a statistically significant difference in the interaction (group *ZT; $p = 0.008$) in the expression of *Mchr1* gene. In turn, in the CTL group, we observed higher levels of gene expression at ZT4 compared to ZT8 (Wald $= 26.665$; gl $= 11$; $p = 0.005$), while no differences were found in the SD group among ZTs.

Concerning *Hcrtr1* gene expression, we observed higher levels of it in CTL-ZT0 when compared to SD-ZT0 ($p = 0.025$). Moreover, there was a statistically significant difference in the interaction group*ZT ($p = 0.002$), and, in the CTL group,

the gene expression levels were lower at ZT8 when compared to ZTs 0 and 12 (Wald $= 40.021$; gl $= 11$; $p = 0.000$).

Hippocampus

We analyzed *Mchr1, Hcrtr1, and Hcrtr2* gene expression in the rats' hippocampus in both groups (►**Fig. 5**). We did not find any statistically significant differences between the groups in any of these genes. Furthermore, no significant diurnal variation in the expression of these genes was observed in either group.

Cosinor Analysis

The cosinor analysis showed no statistically significant differences considering MCH CSF levels in either group. This lack of differences was also observed for *Mchr1, Hcrtr1* and *Hcrtr2* expression in the frontal cortex and hippocampus in both groups.

Discussion

With the aim of exploring the effect of the PS deprivation on the physiology of the MCHergic and hypocretinergic
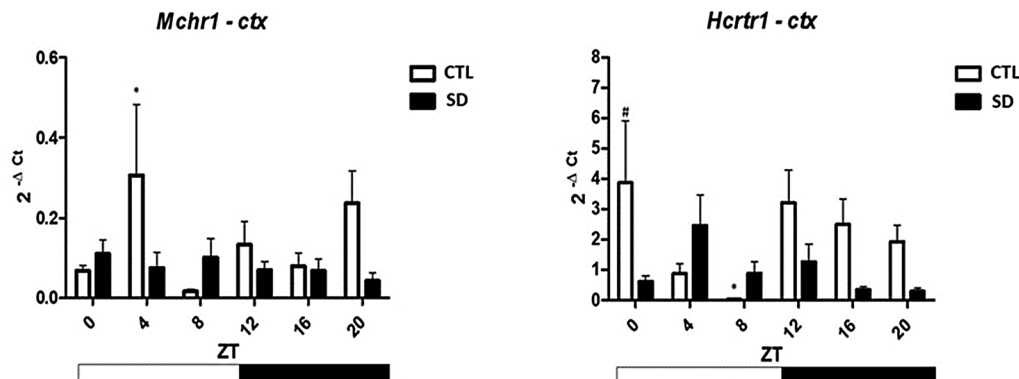
Fig. 4 *Relative gene expression of* Mchr1 *and* Hcrtr1 *in the frontal cortex of rats*. This figure shows the relative gene expression ($2^{-\Delta Ct}$) of *Mchr1* and *Hcrtr1* in the frontal cortex of rats in the CTL and SD) groups at 6 ZTs (0, 4, 8, 12, 16, and 20). Notes: *indicates a statistically significant difference in the CTL group in comparison to ZT8 (*Mchr1*) and in the CTL group in the comparison between ZTs 0 and 12 (*Hcrtr1*); # indicates a statistically significant difference between CTL and SD groups in the same ZT (*Hcrtr1*); $p \leq 0.05$. The horizontal white bar indicates lights on, and the horizontal dark bar indicates lights off. Values expressed as mean $\pm$ SEM N = 5-8.
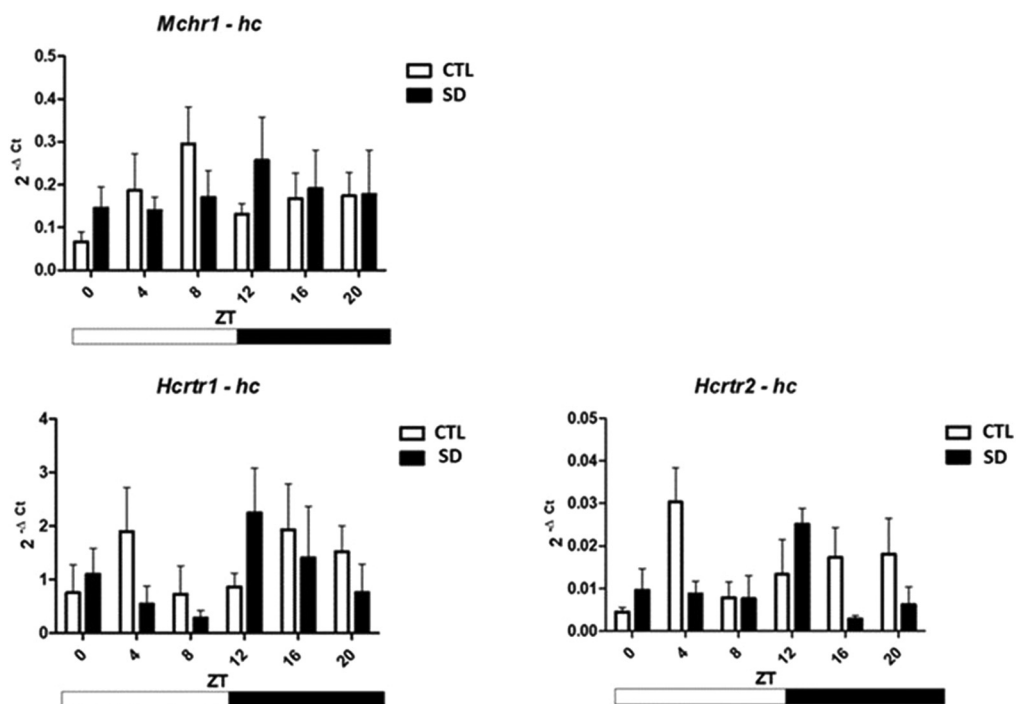
Fig. 5 *Relative gene expression of* Mchr1, Hcrtr1, *and* Hcrtr2 *in the hippocampus of rats.* This figure shows the relative gene expression ($2^{-\Delta Ct}$) of *Mchr1*, *Hcrtr1*, and *Hcrtr2* in the hippocampus of rats in the CTL and SD) groups at 6 ZTs (0, 4, 8, 12, 16, and 20). Notes: The horizontal white bar indicates lights on, and the horizontal dark bar indicates lights off. Values expressed as mean ± SEM; N = 5- 8.

systems, we analyzed MCH and Hcrt CSF levels, the expression of the *Pmch* and *Hcrt* genes, as well as the expression MCH and hypocretin receptors in the frontal cortex and hippocampus. We found that the physiology of both systems is affected in a subtle way, either by the lack of PS and by the time of the day (ZT).

MCHergic System

As a first step, we analyzed the MCH levels in the CSF. Surprisingly, we did not find differences between the groups. In this regard, it is important to note that the CSF concentration of MCH does not directly reflect the hypothalamus's content of MCH, because the cerebral ventricles only receive input from approximately one-third of MCH-producing neurons,[27] and neuroactive substance concentrations in the CSF vary among different ventricular regions.[28]

We also analyzed the diurnal variation of the MCH in the CSF. Interestingly, although the cosinor analysis did not confirm a diurnal rhythm, we found a subtle diurnal variation in the MCH levels that reach significance only during SD. The MCH concentration in the CSF of SD animals was higher at ZT12 (beginning of the dark phase) than at ZT4 and ZT8 (during the light phase). These results are in accordance with those of a previous work[29] from our group, which showed that MCH CSF levels were higher at ZT0 in relation to ZT8 in SD animals. Moreover, using immunohistochemistry, Gerics et al.[30] (2017) demonstrated that hypothalamic MCH is higher at the end of the sleep period (ZT12). We can hypothesize that the higher concentration of MCH at ZT12 in the SD group is related to a higher release to the ventricular system, due to the increased sleep pressure after a long period of forced wakefulness during the natural sleep phase. Because MCH CSF levels increase in advance of feeding at night, and MCHergic neurons that project to the ventricle system increase their activity during feeding,[27] another possible explanation is that the activity of these neurons is enhanced during SD. In this regard, there is an increase in feeding behavior with sleep loss.[31] It is also possible that this diurnal variation during SD may be due to changes in the function of the suprachiasmatic nucleus (SCN), which is the master circadian oscillator of the brain.[22,32] In this regard, it is known that total SD promotes functional changes in the SCN.[33]

There are only a few studies in the literature regarding the diurnal variation of MCH levels in the CSF. Pelluru et al.[34] (2013) measured MCH CSF levels in rats at four time-points of the day, finding that MCH concentration is higher at noon than at midnight or at the end of the dark phase. Our results showed that, although there were variations in MCH CSF levels throughout the day in the CTL group, these differences do not reach statistically significant values. This corroborates the results of our previous study,[29] which showed no difference in MCH CSF levels when comparing CTL animals euthanized at ZT0 versus ZT8. It is important to note that Pelluru et al.[34] used Long-Evans strain rats, which might explain the differences from our results, considering the physical and behavioral differences between Long-Evans and Wistar strains.[35,36] In addition, we performed the assessments at six time-points of the day rather than four, as in the study by Pelluru et al.,[34] which may have led to differences in the results due to the temporal sampling differences.

As a second step, we analyzed in both groups the expression of the *Pmch* and *Mchr1* genes in the frontal cortex and

hippocampus. Unexpectedly, we did not find significant differences between the groups.

Regarding the diurnal variation in gene expression, we did not find a clear diurnal rhythm in the expression of *Pmch* in the controls, which was in accordance with previous findings.[37,38] Neither did we found diurnal rhythm in the expression of *Pmch* in SD rats, in spite of the fact that, in a previous study by our group,[29] we found that the expression at ZT8 was greater than at ZT0 in SD animals.

In the same report,[29] we described a greater *Mchr1* expression in the hippocampus at ZT8 compared to ZT0 in the controls. Although in the present study we observed the same tendency, the results did not reach statistical significance.

In the present study, we detected higher levels of *Mchr1* gene expression in the frontal cortex of CTL animals at ZT4 when compared to ZT8. The ZT4 sample corresponds to the first four hours of the light phase. Hence, there is a higher expression of the MCH receptor when animals concentrate most of their sleep. Anyway, we must consider that mature protein formation involves mRNA translation, prepro-protein generation, and the posttranslational modification of the protein.[39] Hence, it is expected that the temporal peak of gene expression does not coincide with the increase in protein presence, in this case, MCHR-1. Since the variation found in CTL animals was lost in the SD group, we can hypothesize that SD interferes with the physiological profile of *Mchr1* gene expression.

Hypocretinergic System

Previous studies[40–42] showed that Hcrt levels in the CSF are higher during total SD and PS deprivation in relation to control conditions. During SD, it is likely that hypocretinergic neuronal activity is augmented to maintain vigilance states.[43,44] One study[42] performed by our group used a PS deprivation protocol of 96 hours, and demonstrated that Hcrt levels in SD rats are higher in relation to the CTL at ZT8, but not at ZT0. We could not reproduce this result in the present study, probably due to the high number of subgroups we had. However, although statistical significance was not reached, the Hcrt levels were higher during SD at most of the ZTs evaluated. Interestingly, the mean difference between the groups was maximal at the end of the resting phase (SD: 0.6 ± 0.21 ng/uL; CTL: 0.3 ± 0.17 ng/uL).

We did not observe a significant diurnal rhythm in the Hcrt CSF levels in either group. However, a day/night rhythmicity has been previously described in the hypocretinergic system, with a Hcrt CSF peak at the end of the active phase and lower levels at the end of the light phase.[41,45,46] Similarly, Deboer et al.[40] (2004) observed circadian rhythmicity in Hcrt CSF levels under constant light conditions. This rhythmicity is lost following SCN lesion, showing that the SCN exerts control over the circadian rhythmicity of the hypocretinergic system.

We did not find any significant differences between the groups in terms of the *Hcrt* gene expression. Regarding the diurnal variation in the expression of this gene, the present work showed a clear peak at ZT4 and a nadir at ZT0 in both groups; however, these results did not reach statistical significance. Previous data are contradictory; while Taheri et al.[47] (2000) found a diurnal variation for *Hcrt* gene expression in the hypothalamus with a nadir at 19:00, this profile was not found in mice by Stutz et al.[37] (2007) or by Wang et al.[38] (2017). Furthermore, another study[48] evaluated *Hcrt* expression in the hypothalamus (paraventricular nuclei, dorsomedial nuclei, arcuate nuclei, and lateral area) of Sprague Dawley rats and found no difference in gene expression between day and night.

There was a decrease in *Hcrtr1* gene expression in the frontal cortex in SD rats compared to the controls only at ZT0 (end of active phase), when Hcrt neuronal activity is maximal.[49] In contrast, SD did not affect the expression of Hcrt receptors (neither type 1 nor 2) in the hippocampus, confirming the results obtained by our group using autoradiography.[50]

During prolonged SD, desensitization can occur in an adaptive process that regulates the number and function of neurotransmitter receptors. Hence, sleep loss can lead to the impairment of neurotransmission involved in vigilance maintenance with consequences for its stability.[51] The frontal cortex is a susceptible region regarding sleep. During slow-wave sleep, the electroencephalogram presents its highest voltage and slowest brain wave oscillations in the frontal cortex compared to other cortical areas, and SD alters frontal cortex functions.[24] This may explain why we observed differences in the gene expression of Hcrt and MCH receptors only in the frontal cortex, but not in the hippocampus.

Our results show that, in CTL rats, the expression of *Hcrtr1* in the frontal cortex is maximal at ZT0 and ZT12 and minimal at ZT8, which corresponds to the middle of the resting (light) phase. This partially agrees with the results found by Wang et al.,[38] who showed that the *Hcrtr1* acrophase occurs at ZT15 in the rats' perifornical area. Interestingly, ►**Figure 4** shows that this diurnal rhythm is lost during SD.

Technical Considerations

The present study has a limitation regarding the sample size, which may have influenced the results. We used eight animals per group, but some samples were discarded because of different technical problems (such as blood in some CSF samples). Hence, the analyses were performed with an N = 5-8, which limited the statistical power when we compared 6 groups. This limitation also affected the cosinor analysis.

Sleep deprivation, either total and partial, is a stressful factor, leading to increased plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone in the experimental animals.[31,52] Although we have used the modified multiple platform method, which reduces stress by allowing more significant locomotion and social interaction among animals,[53] some residual effects may be present. The MCHergic system is involved in stress response, and intracerebroventricular injections of this peptide cause an increase in plasmatic corticosterone levels and produce anxiety-like behavior in mice.[54] Thus, we cannot discard the possibility that some of the effects described in the SD animals may be related to the stress response.

Furthermore, another fact to consider is that when measuring the *pMCH* and *Hcrt*, we used the whole hypothalamus

for the analysis. Hence, this technical issue diluted the concentration of *pMCH* and *Hcrt*, whose neurons are located mainly in the posterolateral hypothalamus.[1,55]

Conclusion

The CSF levels of MCH and Hcrt present non-significant diurnal variations in control conditions. Interestingly, when animals were deprived of PS, the MCH levels were higher at the beginning of the dark phase. The expression of the *Pmch* and *Hcrt* genes in the rat hypothalamus did not present a clear diurnal rhythm and was not altered by 96 hours of PS deprivation. In contrast, we observed a diurnal variation in the expression of *Mchr1* and *Hcrtr1* in CTL animals in the frontal cortex, which was lost when animals were deprived of PS. Sleep deprivation also decreased the expression of *Hcrtr1* at ZT0. In summary, we found subtle day/night variations in different components of the MCHergic and hypocretinergic systems, and this daily profile was affected, although in a minor way, by PS deprivation.

Data Availability
The data that support the findings of the present study are available from the corresponding author upon reasonable request.

Funding

Conflict of Interests
The authors have no conflict of interests to declare.

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