

THE EFFECTS OF REM SLEEP DEPRIVATION AND REM SLEEP REBOUND ON HYPOCRETIN LEVELS IN MICE BRAINS

Mario Pedrazzoli*, Débora Hipólide, Vânia D'Almeida, Sergio Tufik

Departamento de Psicobiologia - Universidade Federal de Sao Paulo (UNIFESP). Sao Paulo, Brazil

*Correspondence:

Mario Pedrazzoli

Rua Napoleão de Barros, 925

04024-002 - Sao Paulo | SP, Brazil

Phone number: +55 11 55390155 - Fax number: +55 11 55725092

e-mail: pedrazzo@psicobio.epm.br - web site: www.sono.org.br

Received May 19, 2008; accepted November 20, 2008.

ABSTRACT

Background and objective: Hypocretins (orexins) are recently discovered neuropeptides that are produced in a very restricted area of the lateral hypothalamus. These peptides were originally associated with feeding behavior, but have come to be viewed as essential in causing narcolepsy and regulating sleep. Narcolepsy is a sleep disorder associated with REM sleep abnormalities, and it is likely that hypocretins play a major role in regulation of this sleep stage.

Methods: To verify the potential effects of REM sleep deprivation on the levels of hypocretins in the brain, we deprived mice of REM sleep for 96 h and measured the levels of hypocretins in whole brain preparations using an enzyme immunoassay.

Results: Our results showed no difference in the levels of hypocretin 1 and 2 (orexin A and B) in REM sleep-deprived animals or in animals that had a sleep rebound after sleep deprivation.

Conclusions: These results corroborate data in the literature that show that sleep deprivation does not produce significant alteration in hypocretin levels in the mouse brain just after an active period.

Keywords: Hypocretin, Orexin, Sleep, Sleep Deprivation, REM Sleep, Enzyme immunoassay

INTRODUCTION

The discovery of the neuropeptide hypocretin (Hcrt), also named orexin (1,2), has increased the general understanding of the mechanisms of sleep regulation. In 1999 it was reported that canine narcolepsy was caused by a mutation in the hypocretin receptor 2 gene (HCRT_{r2}) (3), and Chemelli et al. also reported that preprohypocretin and Hcrt receptor 2 knockout mice developed narcolepsy (4).

It is now well established that Hcrt plays a major role in sleep regulation and that a mutation in one of the genes within the system or an absence of this peptide in the brain can cause narcolepsy, a sleep disorder related to REM sleep abnormalities (3,5). There are several lines of evidence indicating that Hcrt suppresses

the appearance of REM sleep, whereas the absence or reduction of these peptides or their receptors enhances REM sleep and REM sleep-related phenomena. Hypocretin deficiency is related to abnormal REM sleep in humans and animal models (dog and mouse). Thus, increases in REM sleep or REM sleep-related phenomena are associated with no or low Hcrt in the brain (3-5).

In animal studies, manipulation of the Hcrt system led to alterations in REM sleep. Perfusion of antisense orexin B in the pontine reticular formation in rats increases REM sleep two or threefold (6). In narcoleptic dogs, administration of Hcrt 1 reduced cataplexy and improved the sleep-wake cycle, including a decrease in REM sleep without a change in Slow Wave Sleep (7). However, Fujiki et al. were unable to detect these alterations in narcoleptic dogs (8). In rats, injection of a neurotoxin which acted against hypocretin into the hypothalamic hypocretin neu-

rons led to increased sleepiness, increased REM sleep, and sleep onset of REM periods in these animals, which are all symptoms of narcolepsy (9). In addition, behavioral and physiological studies challenging the hypocretin system have not revealed increases in hypocretin levels above the upper limit of physiological levels that occur during the animals' active phase (10-12).

Considering previous studies, which suggested the participation of Hcrt in REM sleep regulation, and the inability to detect expression above certain levels under any manipulation condition, we examined the effects of REM deprivation and REM sleep rebound on Hcrt 1 and 2 levels in mice brains at the end of the active phase.

EXPERIMENTAL PROCEDURES

Animals and Procedures

Experiments were carried out using locally approved protocols. Three-month-old CJ56B male mice maintained under a light-dark cycle (12h,12h) were deprived of REM sleep for 96 h using the classical platform method (13). After the period of REM deprivation some of the animals were sacrificed (REMd group, n=10) and some were returned to the home cage and allowed to sleep freely for 24 h before being sacrificed (sleep rebound group, n=8). The control group remained in the home cages (n=11). All animals were provided with food and water ad libitum throughout the experimental procedure. After the REM sleep deprivation or sleep rebound procedure, the mice were sacrificed (between 9:00 and 10:00am) by cervical dislocation, and the brains were rapidly removed, frozen over dry ice, and stored at -80°C.

Peptide extraction

Frozen brains were weighed and boiled in 2 ml of Milli-Q water in 24-well plates for 10 min. After cooling on ice, the plate was spun briefly and acetic acid and HCl were added to each well to a final concentration of 1 M and 20 mM, respectively. Each brain was homogenized at 40,000 rpm for 30 s. Then, 2 ml of peptide extraction buffer (1 M acetic acid and 20 mM HCl) were added to each brain, followed by centrifugation at 5500 rpm for 20 min. The supernatants were aliquoted into 96-well plates and stored at -80°C.

Enzyme immunoassay (EIA)

The principle of the assay is based on a competitive EIA protocol as described by Lin et al. (14). Briefly a 96-well plate is pre-coated with secondary antibody and non-specific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the peptide specific antibody, while the Fab fragment of the peptide-specific antibody will be competitively bound to both biotinylated peptide and targeted peptide in the samples. The biotinylated peptide interacts with the enzyme, which causes the substrate to produce a colored solution. The intensity of the color is inversely proportional to the amount of peptide in the sample. Hypocretin levels were determined using a standard curve (range 0.1–40 ng/ml) prepared using a synthetic peptide (Phoenix Pharmaceuticals, Belmont, CA, USA), which was run with each plate.

RESULTS

Our data did not show any significant differences in the levels of hypocretin 1 or 2 in the brains of mice deprived of REM sleep for either 96 h or 24 h of rebound sleep after REM sleep deprivation (Fig 1).

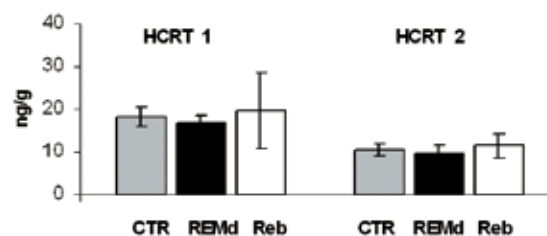


Figure 1: Hypocretin 1 and 2 levels in control (CTR), REM sleep deprived (REMd) and Rebound (Reb) groups. The data are mean±SD.

DISCUSSION

The regulation of REM sleep by hypocretins is a significant issue, as narcolepsy, a sleep disorder related to REM sleep abnormalities, is associated with a lack of hypocretins in the brain (5). This fact suggests that hypocretins are likely to have a role in normal REM sleep regulation, and attempts to understand this involvement are highly relevant.

In the present study, we sought to investigate the association between hypocretins and REM sleep. To do so, we used the paradigm of REM sleep deprivation, in which we did not allow the animals to enter this phase of sleep, to examine the effects of a lack of REM sleep on the hypocretin system. We also investigated the effects of REM sleep rebound on hypocretin levels to verify the effects of excessive REM sleep on hypocretin levels.

We observed that after 96 h of REM sleep deprivation, there was no change in the levels of either hypocretin 1 or hypocretin 2 compared to control animals. We also did not detect any changes in hypocretin levels after rebound sleep, a period of 24 h of free sleep following 96 h of REM sleep deprivation.

These results showed a similar pattern of response to REM sleep deprivation, which were observed at the same time of the day using rats as subjects and cerebrospinal fluid (CSF) instead of brain tissue. In a previous study, we did not observe an increase in the levels of hypocretin 1 in the CSF following REM sleep deprivation at the end of a period of wakefulness, despite a marked increase of levels in the middle of the sleep episode, a period when levels are supposed to be low (10).

A similar response to REM sleep deprivation was found when we analyzed levels of preprohypocretin and hypocretin receptors 1 and 2 mRNA in rats at the same time of the day. In addition, we detected no alteration in mRNA levels of the preprohypocretin and hypocretin receptors in rats sacrificed at the end of the active period, although some increases in hypocretin receptor 1 levels (mainly in the amygdala and hypothalamus) and decreases in the

hypocretin 2 receptor were observed after the sleep rebound (15). Studies in nocturnal rodents and diurnal squirrel monkeys have shown high CSF hypocretin levels in the active period, low levels during sleep, and increased levels after sleep deprivation only in the rest phase, when the Hcrt levels are lower, suggesting there is a ceiling effect (10,12,16-18).

Lin et al. (14) developed the EIA methodology to measure brain hypocretin in mice. They found significant differences among several inbred mice strains displaying the high sensitivity of the assay. However, when investigating hypocretin responses in regular circadian variations or different light exposure conditions, they did not find the expected dark/light or activity/rest fluctuations observed in other CSF studies (10,12,19). These results from Lin et al. (14) suggest that total brain level measurements are not a good approach to assess the Hcrt function in the brain, as they may reflect the sum of opposite regional effects. It is possible that the present results showing no variation of Hcrt after REM sleep deprivation in mice could also reflect the use of total brain homogenates.

The present results suggest that the ceiling effect observed in rat CSF hypocretin levels after REM sleep deprivation just after the wakefulness period can also be observed in mouse brains. Another possible explanation is that intracellular stores of hypocretin are not necessarily regulated in the same way as extracellular levels. In CSF hypocretin studies, the release varies with time of the day (10-12), whereas in the mouse brain the content did not (14). It is possible that the ceiling effect might only apply to CSF and extracellular levels. Dissociation between release and storage is well established for many other neurotransmitters.

In addition, we would like to emphasize that one should be very careful when using the whole brain to verify Hcrt levels under behavioral or physiological manipulations. We suggest that further studies using REM sleep deprivation or other manipulations should focus on more specific brain regions, such as the hypothalamus and brainstem, since Hcrt variations in these brain areas during the sleep/wake cycle have already been reported (19).

ACKNOWLEDGMENTS

We thank FAPESP (grant # 98/14303-3) and AFIP for the financial support. We thank Drs Emmanuel Mignot and Ling Lin from Stanford University for Hcrt1 and Hcrt2 measurements.

REFERENCES

1. de Lecea L, Kilduff T, Peyron C, Gao XB, Foye PE, Danielson PE, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci* 1998;95:322-7.
2. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G-protein coupled receptors that regulate feeding behavior. *Cell* 1998;92:573-85.
3. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 1999;98:365-76.

4. Chemelli RM, Willie JT, Sinton C, Elmquist J, Scammell T, Lee C, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 1999;98:437-51.
5. Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 2000;6:991-7.
6. Thakkar MM, Ramesh V, Cape EG, Winston S, Strecker RE, McCarley RW. REM sleep enhancement and behavioral cataplexy following orexin (hypocretin)-II receptor antisense perfusion in the pontine reticular formation. *Sleep Res Online* 1999;2:112-20.
7. John J, Wu MF, Siegel JM. Systemic administration of hypocretin-1 reduces cataplexy and normalizes sleep and waking durations in narcoleptic dogs. *Sleep Res Online* 2000;3:23-8.
8. Fujiki N, Yoshida Y, Ripley B, Mignot E, Nishino S. Effects of IV and ICV hypocretin-1 (orexin A) in hypocretin receptor-2 gene mutated narcoleptic dogs and IV hypocretin-1 replacement therapy in a hypocretin-ligand-deficient narcoleptic dog. *Sleep* 2003;26:953-9.
9. Gerashchenko D, Blanco-Centurion C, Greco MA, Shiromani PJ. Effects of lateral hypothalamic lesion with the neurotoxin hypocretin-2-saporin on sleep in Long-Evans rats. *Neuroscience* 2003;116:223-35.
10. Pedrazzoli M, D'Almeida V, Martins PJ, Machado RB, Ling L, Nishino S, et al. Increased hypocretin-1 levels in cerebrospinal fluid after REM sleep deprivation. *Brain Res* 2004;995:1-6.
11. Martins PJ, D'Almeida V, Pedrazzoli M, Lin L, Mignot E, Tufik S. Increased hypocretin-1 (orexin-a) levels in cerebrospinal fluid of rats after short-term forced activity. *Regul Pept* 2004;117:155-8.
12. Yoshida Y, Fujiki N, Nakajima T, Ripley B, Matsumura H, Yoneda H, et al. Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. *Eur J Neurosci* 2001;14:1075-81.
13. Cohen HB, Dement WC. Sleep: changes in threshold to electroconvulsive shock in rats after deprivation of "paradoxical" phase. *Science* 1965;150:1318-9.
14. Lin L, Wisor J, Shiba T, Taheri S, Yanai K, Wurts S, et al. Measurement of hypocretin/orexin content in the mouse brain using an enzyme immunoassay: the effect of circadian time, age and genetic background. *Peptides* 2002;23:2203-11.
15. D'Almeida V, Hipolide DC, Raymond R, Barlowc K.B.L, Parkesc J, Pedrazzoli M, et al. Opposite effects of sleep rebound on orexin OX1R and OX2R receptor expression in rat brain. *Brain Res Mol Brain Res* 2005;136:148-57.
16. Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, et al. Fos expression in orexin neurons varies with behavioral state. *J Neurosci* 2001;21:1656-62.
17. Porkka-Heiskanen T, Alanko L, Kalinchuk A, Heiskanen S, Stenberg D. The effect of age on prepro-orexin gene expression and contents of orexin A and B in the rat brain. *Neurobiol Aging* 2004;25:231-8.
18. Zeitzer J, Buckmaster CL, Parker KJ, Hauck CM, Lyons DM, Mignot E. Circadian and homeostatic regulation of hypocretin: implications for consolidation of wake. *J Neuroscience* 2003;23:3555-60.
19. Taheri S, Sunter D, Dakin C, Moyes S, Seal L, Gardiner J, et al. Diurnal variation in orexin A immunoreactivity and prepro-orexin mRNA in the rat central nervous system. *Neurosci Lett* 2000;279:109-12.