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Potential participation of cystatin C in rapid eye movement sleep (REMS) modulation

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Abstract

It has been hypothesized that proteins modulate rapid eye movement sleep (REMS). Studies have shown an increase in the liberation of proteins in the mesencephalic reticular formation of cats during REMS. It has also been determined that protein-synthesis inhibitors diminish REMS and that protease-inhibitors increase this sleep phase. These and other studies support the importance of “di novo” protein molecules in sleep, and in particular, in REMS regulation. In this context, it is important to determine the role of endogenous proteases and their endogenous inhibitors in sleep regulation. In this study, we found that Cystatin C (CC), an endogenous protease inhibitor, diminishes wakefulness and increases REMS. We have also found an increase in CC expression after REMS deprivation and a tendency to decrease after a 2 h period of REMS rebound. We further showed that REMS deprivation increases the expression of Cathepsin H (CH), a protease inhibited by CC. These results suggest that naturally occurring protease-inhibitors enhance REMS, perhaps by facilitating the availability of proteins.
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Several experimental approaches have been used to reveal the mechanisms involved in the generation of the different sleep phases, and hence their function. One of these approaches is to search for sleep inducing molecules. This search began at the turn of the 20th century with the description of Pieron’s hypnotoxin, a hypothetical substance contained in the cerebrospinal fluid (CSF) of animals and even humans, capable of inducing sleep [22]. Presently, there are dozens of described substances with sleep promoting properties that have been identified as neurotransmitters, lipids, neuropeptides, and hormones, among others. Most of these molecules have an effect on non-REMS or delta sleep. However, the molecules that promote REMS are in general peptides or proteins with more or less high molecular weight [4,12].

Drucker-Colin reported an increase in protein concentration in the extracellular *milieu* of the mesencephalic reticular formation (MRF) of cats during REMS periods [9]. In addition, the administration of this fluid to recipient cats increased REMS, whereas the antibodies against this perfusate inhibited this sleep phase [11]. It has also been observed that protein-synthesis inhibitors diminish REMS [10,25], whereas protease inhibitors increase it [27].

After these early studies no further efforts to characterize these or other high molecular weight proteins involved in sleep regulation have been made. However, many sleep-inducing peptides have been described. Strategies such as mRNA differential display or cDNA microarrays [6,7], have been used to identify genes whose expression varies with sleep. Although there is at least one report analyzing changes in proteome expression during sleep deprivation [3], the isolation of proteins has been largely forgotten.

The participation of the endogenous protease inhibitors in sleep regulation is a new concept we would like to introduce. In this line of research, we analyzed the protein amount in the CSF of rats after REMS deprivation as well as in rats after REMS deprivation plus 2 h of sleep rebound. We identified a protein band of approximately 13.5 kD which increases after REMS deprivation. We isolated it and sent it for analysis by liquid chromatography and mass spectrometry (LC MS/MS; Yale Cancer

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Center Mass Spectrometry Resource & W. M. Keck Foundation Biotechnology Resource Laboratory). The protein band was identified as cystatin C (CC). The levels of CC increased after REMS deprivation and had a tendency to decrease after the REMS rebound [13], which is a pattern that concurs with that expected for a sleep inducing factor [4]. CC is an endogenous cystein-protease reversible inhibitor found in several body fluids, including the CSF [5,14]. CC selectively and reversibly inhibits cathepsins B, H, L, and S. These are lysosomal enzymes whose biological function is not thoroughly understood. However, they have been implicated in antigen processing [19], glioma invasiveness [15], and ischemic neuronal death [30], among others. These findings suggest that the excessive expression of the proteases may lead to a dysfunction or structural damage of the organ.

The aim of this study was to determine the effect of the exogenous administration of CC as well as its blockade by antibodies on the sleep-waking cycle. Moreover, we sought to describe CC and CH variations in correlation with the sleep-wake cycle.

Male Wistar rats (250–300 g) were maintained under a controlled dark–light cycle (12:12, lights on at 08:00 a.m.) with food and water *ad libitum.*

Ninety-six rats maintained under the above mentioned controlled conditions were used in this experiment. CSF was extracted by puncturing the *cisterna magna* of anesthetized (pentobarbital) rats at different hours of the day: 9:00, 13:00, 17:00, 21:00, 01:00, and 05:00. Sixteen rats per point were used. Rats were killed and the choroid plexus was isolated.

Sixteen rats were REMS-deprived using the inverted flower pot technique for 48 h. Deprivation began at 09:00 h and finished at 09:00. Eight of them were allowed to sleep for 2 h in control conditions. We also used a control group ($n = 8$). The CSF was extracted at the end of these periods.

The inverted flower pot was a small acrylic platform (diameter: 6 cm; height: 6 cm) surrounded by water 1 cm deep. Rats were provided with food and water at all times during the sleep deprivation period. Due to the shallow level of the water they could walk across the cage as they pleased. When the animals entered into REMS, which is characterized by loss of postural tone, they fell from the platform and woke up [8].

All rats were anesthetized with pentobarbital, and CSF was extracted from the *cisterna magna* (as mentioned above). CSF was centrifuged at $4000 \times g$, 20 min, at 4 °C. Pellets were discarded and supernatants were maintained at −20 °C until further use.

Rats were anesthetized with pentobarbital, and decapitated. The brains were extracted and choroid plexuses were dissected. The choroid plexuses of three rats were pooled homogenized in tris-sucrose buffer with proteases inhibitors centrifuged at $600 \times g$, 10 min, 4 °C. Supernatant was centrifuged at $39{,}000 \times g$, 15 min, 4 °C. Total proteins were quantified by Lowry's method.

To assess CC or CH protein expression, homogenized choroid plexuses or CSF were run in 17% analytical SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described elsewhere [16]. Briefly, tissue homogenates (50 μg of protein) were mixed 1:1 with sample Laemmli buffer and heated (95 °C, 5 min) prior to loading on a 0.75 mm thick gel. Samples were subjected to electrophoresis (150 V, 2 H) and gels were transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, U. K.) at 100 V for 1 h at 4 °C. The membrane was incubated with 3% PBS-tween, 10% nonfat dry milk, and 2% goat normal serum for 30 min at room temperature (rt), followed by incubation with anti-CC (Upstate Biotechnology) or anti-CH (Santa Cruz Biotechnology) 1:2000 overnight at 4 °C. The blot was washed with PBS-tween (3 times, 5 min/each), then incubated 1 h at rt in a 1:2000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate, and developed with diaminebenzidine (0.5 mg/ml in PBS plus 0.3 μl/ml 30% H_2O_2). Density of bands was analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, California).

Fourteen male Wistar rats (250–350 g) were used for the sleep recordings. They were implanted under anesthesia with a set of electrodes that included three stainless steel screw electrodes, one implanted on the frontal bone which was used to ground the animal, and the other two on the hippocampus ($P = 4.0$, $L = 2.5$, $V = 2.5$) to record electroencephalographic activity. Two additional wire electrodes were placed on the neck muscles to record electromyographic activity. One stainless steel cannula was also implanted aimed at the right lateral ventricle ($P = 0.8$, $L = 1.5$, $V = 3.8$) and was used to administer the drugs.

One week after the surgery, control rats were acclimatized to the recording conditions for 24 h. In the first day of the experiment, they received an ICV administration of saline (4 ul) at 10:00 am with the aid of a Hamilton microsyringe controlled by an infusion pump at 1 ul/min and then connected to the recording system. All rats were recorded for 12 h using amplifiers (Neurotop MM 3116 K de NIHON KOHDEN) and software was used to collect and analyze the data (ICELUS program. University of Michigan Programming by g system. Dr. Mark Opp).

The next day rats were ICV injected with either: Human CC (ABCAM; 0.7 pmol/4 ul; $n = 6$) or Anti-CC (Upstate Biotechnology; 105 ng/4 ul; $n = 8$) at 10:00 am, and recorded for 12 h. The sleep-waking cycle was evaluated by computing the total time spent in waking, non-REMS (nREMS) and REMS. Latency to the onset of the first nREMS and REMS was also evaluated, as well as mean duration and frequency of these periods.

Results are reported as mean values ± SEM. Significant differences were obtained by using a one-way ANOVA test and a Tuckey HSD, as *post hoc* test, considering $p < 0.05$ statistically significant.

We analyzed the diurnal variation of CC in the CSF. Cystatin C polyclonal antibody recognized one band of approximately 13.5 kDa, as well as another band with higher molecular weight, which we identified as pro-CC, the precursor peptide (Fig. 1). It has been reported that pro-CC is 20 amino acids longer than CC [1] and the anti-CC employed recognizes both CC and its precursor peptide, as stated by the supplier (Upstate biotechnology). Densitometry was obtained for these bands and a statistical analysis was made. We found a statistical difference between 09:00 with respect to all the other hours analyzed for CC as well as for pro-CC (Fig. 1). We also analyzed CH in choroid plexus and we did not find variation across the day (data not shown).

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