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Cortistatin modulates memory evocation in rats

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Abstract

The neurochemical control of learning depends on several neurotransmitters, hormones, and neuropeptides. Cortistatin is a neuropeptide with sleep-modulating properties that regulates memory consolidation and evocation. Several reports have suggested that learning processes are expressed under diurnal variations; therefore, it seems that the efficiency to solve learning tasks is related to the arousal state. Although we know that cortistatin modulates learning, we do not know whether its effect is subjected to diurnal variations. Hence, we evaluated memory evocation and the sleep–waking cycle along the day. Additionally, we evaluated the effect of cortistatin on motor control and cyclic adenosine monophosphate (cAMP) concentration. Performance of rats was better at 01:00 h than at 13:00 h to solve the Barnes maze. Cortistatin impaired memory evocation, increased rapid-eye-movement (REM) sleep, and decreased wakefulness at 01:00 h, whereas increasing it at 13:00 h. Cortistatin blunts cAMP concentration and impairs motor control at 13:00 h. These results support further a cortistatin modulatory role in the memory process.

Keywords: Cortistatin; Circadian rhythm; Sleep–waking cycle; Memory evocation; cAMP

1. Introduction

The learning process has been conventionally divided for its study in acquisition, consolidation, and evocation. We believe that memory evocation is fundamental to maintain the individual physical and mental integrity, because past events recall is crucial for the display of survival strategies. Studies of the brain structures involved in the regulation of these processes have shown that the hippocampus plays an important role in memory evocation (Riedel et al., 1999; Szapiro et al., 2002). Additionally, several studies support the notion that evocation of memory is not as efficient at every hour of the day. Quite the contrary, it undergoes fluctuations throughout the day (Antoniadis et al., 2000; Chaudhury and Colwell, 2002; Monk, 1994) in tight association to the arousal state. On the other hand, several neurotransmitters regulate memory consolidation and evocation, among them glutamate (Abel and Matthew Lattal, 2001; Szapiro et al., 2002), acetylcholine (Hasselmo et al., 1996; Murai et al., 1995), noradrenaline (Barros et al., 2001; Szapiro et al., 2002), serotonin (Barros et al., 2001; Szapiro et al., 2002), and gamma-amino-butyric-acid (Hasselmo et al., 1996). In addition, neuropeptides (Bennett et al., 1997; Telegdy and Adamik, 2002) and proteins play a role in the modulation of memory consolidation, since protein-synthesis-inhibiting drugs impair it (Vianna et al., 2001). Furthermore, several molecules involved in intracellular signaling also play a role in memory processes, for example the second messenger, cyclic adenosine monophosphate (cAMP), is involved in one of the main signaling pathways of memory consolidation (Mayford and Kandel, 1999; Nguyen and Kandel, 2000; Renger et al., 2000), and memory evocation (Abel and Matthew Lattal, 2001; Szapiro et al., 2002).

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Recently, the neuropeptide cortistatin has been involved in learning processes. Cortistatin is expressed in gamma-amino-butyric acid (GABA)ergic neurons of the cerebral cortex and the hippocampus (de Lecea et al., 1996, 1997). This peptide decreases neuronal excitability in hippocampal slices and in the hippocampus of anesthetized rats. Likewise, it inhibits acetylcholine excitatory actions in the cerebral cortex and the hippocampus (de Lecea et al., 1996). It also impairs memory consolidation in a passive avoidance paradigm, when injected into the hippocampus of freely moving rats (de Lecea et al., 1997) and mice (Flood et al., 1997). Cortistatin also alters the production of cAMP in cultures of hippocampal cells (Sánchez-Alavez et al., 2000).

To further enhance our understanding on cortistatin's role in learning processes, we studied its effects on the performance of trained rats to solve the Barnes circular maze (Barnes, 1979) that rats solve using spatial or procedural strategies. One major focus in our study was to analyze the degree at which circadian rhythms may affect this behavior (Antoniadis et al., 2000; Monk, 1994). Therefore, we evaluated the efficiency of rats to solve the maze throughout the day and based on this observation we selected representative time-points of each phase of the dark–light cycle (13:00 and 01:00 h) to analyze the effect of cortistatin. Likewise, the arousal state was determined by evaluating the sleep–waking cycle of rats and analyzing the effect of cortistatin at both time-points. Motor control was evaluated along the 24 h, and the effect of cortistatin on motor activity was tested at 01:00 and 13:00 h, to determine whether the effect of cortistatin is on mnemonic processes or on locomotion. Furthermore, since cortistatin affects cAMP metabolism (de Lecea et al., 1996; Sánchez-Alavez et al., 2000) we measured hippocampal cAMP generation along the 24 h and determined the effect of cortistatin on the concentration of this molecule at 01:00 and 13:00 h. The overall goal of this study was to document a potential role of cortistatin in the regulation of learning processes and, in particular, of memory evocation.

2. Materials and methods

2.1. Subjects

Forty-three male Wistar rats (250–350 g), were implanted with a guide cannula aimed at the right lateral ventricle (A=0.8; L=1.5; V=3.6). Additionally, 24 male Wistar rats (250–350 g) were implanted with a set of electrodes for conventional sleep recordings and a guide cannula aimed at the lateral ventricle. Rats were anesthetized with Halothane (2–5%). After surgery, all rats were housed individually and maintained under constant temperature and a controlled light–dark cycle (22±1 °C, and 12/12 light–dark cycle, lights on at 08:00 h). Water and food (Rat Chow, Purina) were available ad libitum. One week was allowed for recovery. At the day of the experiment, all rats were injected with either vehicle (saline, 5 μl) or cortistatin (500 ng/5 μl) (Phoenix Pharmaceuticals, CA). Rats were intracerebroventricularly (i.c.v.) injected with the aid of a KD Scientific pump at a rate of 1 μl/min through an injector inserted into the guide cannula. The used dose of cortistatin has been shown to affect memory processes in other studies (Sánchez-Alavez et al., 2000). All efforts were made to minimize animal suffering and to reduce the number of animals used in this study. Guidelines and requirements stipulated by the ethics committee of our Faculty of Medicine at the UNAM were fully fulfilled.

2.2. Barnes circular maze

The Barnes maze is a validated test often used for the assessment of spatial memory in rodents. We used a modification of the one originally described by Barnes (Barnes, 1979). Our maze consists of a 150-cm-diameter and 3.5-cm-thick circular wooden disc, 90 cm high. Forty holes, 7-cm diameter, were equidistantly located around the perimeter and centered 5 cm from it. A black wooden escape tunnel (10×10×30 cm) was placed beneath one hole, selected randomly for each rat. The maze is placed in a 4×4 m room with white-light illumination and white walls, which has several spatial cues. One speaker to deliver a buzzing sound (90 dB) is located 1 m above the center of the maze affixed to the room's ceiling. A video camera was used to videotape the task for its off-line analysis. The buzzing sound and ambient light were used as aversive stimuli.

2.3. Barnes maze solution

To solve this maze, rats start by looking for the escape tunnel randomly and then switch to one of two strategies—spatial or sequential—according to the training progress. In the spatial strategy, rats seem to use cues located in the room to orient themselves in the maze and to elect the right direction toward the escape tunnel. In this situation, the cues are the room's door, the video system and several pictures on one of the walls. Rats often make the mistake of exploring one or two holes adjacent to the target one. However, we still consider that they are using a spatial strategy. In the sequential strategy, rats choose one hole, which can even be the farthest one (20 holes) from the target and, from there, they start exploring every single hole sequentially until they reach the escape tunnel. This means that the spatial cues are not taken into account by the rats to find the target. In addition to the strategy, we also recorded the number of non-target holes explored (errors), the number of visits to previously explored holes (perseverances), and the total time to solve the maze.

2.4. Training

Forty-two rats were trained to solve the Barnes maze during four successive days at 09:00 h. Each session (one

everyday) consisted of four trials, for a total of 16 trials in 4 days. On the first trial, rats were placed into the escape tunnel for 1 min. At the end of this time, rats were placed on the center of the Barnes maze within a cylindrical opaque chamber and a buzzing sound (90 dB) was delivered. Rats remained in this chamber for 10 s, and then were set free to explore the maze and find the escape tunnel. The trial ended once the rat entered the escape tunnel or once 4 min had elapsed. When the rat entered into the escape tunnel the buzzer was turned off and the rat was allowed to stay in the tunnel for 1 min. The escape tunnel was always located beneath a located in the same spatial position although the hole changed since the Barnes maze was rotated. The spatial location was selected randomly for each rat at the beginning of training.

2.5. Test

Upon completion of the initial training, 10 rats were evaluated at different time points of the day—09:00, 13:00, 17:00, 21:00, 01:00, 05:00 h—for three consecutive days to determine whether their performance would differ as a result of diurnal variations. It is noteworthy to mention that during the dark phase of the cycle, rats were evaluated with lights on, this way we offered the rats the opportunity to use spatial cues at any time. The rest of the rats were divided into two groups and received (i.c.v.) either saline (n=18) or cortistatin (n=14) 30 min prior to one trial session during the light phase (13:00 h) and one trial session during the dark phase (01:00 h). We used these time points because they are representative of each phase of the light–dark cycle (see Fig. 2).

Statistical significance was determined through one-way analysis of variance (ANOVA) and a Student–Newman–Keuls post-hoc test. Significance was accepted at $P<0.05$.

2.6. Sleep

For sleep recordings, the rats were acclimated to the recording conditions for 24 h before sleep was recorded. Rats were i.c.v. injected with saline and then recordings were made at same time points of the day—09:00, 13:00, 17:00, 21:00, 01:00, 05:00 h—to determine the sleep–waking cycle, the recordings lasted for 4 h. Once the diurnal

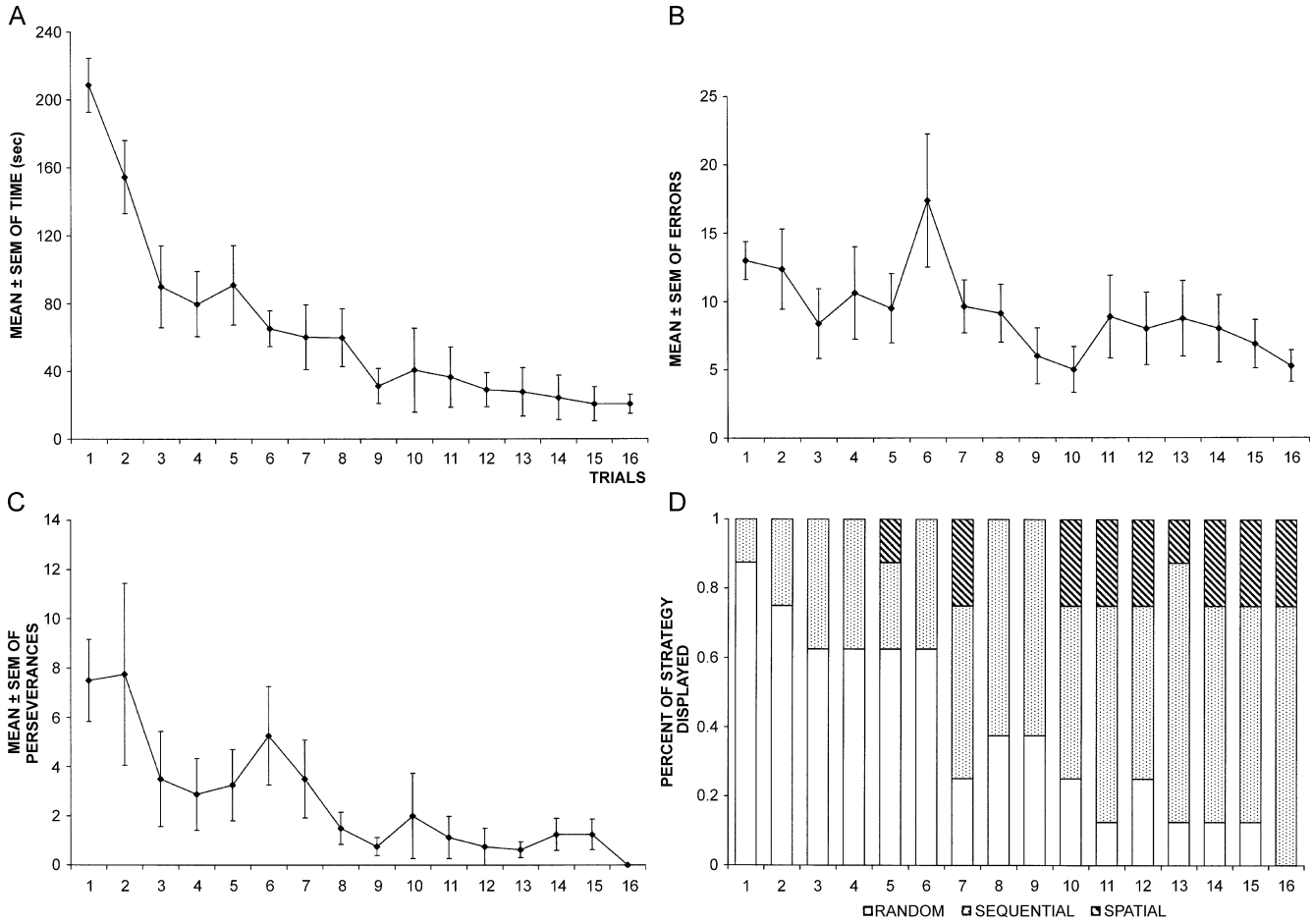

Fig. 1. Illustration of the rat's performance in the Barnes maze along 16 trials. (A) Learning curve, (B) errors, (C) perseverances and (D) strategies. As it can be seen at the end of the training, rats solve the Barnes maze with great proficiency: time, errors and perseverances decreased. All rats used one of the two (spatial or sequential) strategies at the end of the training period. At this time, we considered that the rats were trained to solve the maze ($P<0.05$).

variations of the sleep–waking cycle were determined, we used the two representative time points of the light–dark cycle (01:00 and 13:00 h) to analyze the effect of cortistatin. Sleep recordings were scored visually and analyzed with the HYPNOS program.

Statistical significance was assessed by means of one-way analysis of variance (ANOVA) and post-hoc Duncan test. Significance was accepted at $P<0.05$.

2.7. cAMP

A total of 30 rats without manipulation were used in this part of our study to determine the hippocampal concentration of cAMP. Eighteen of them were killed by decapitation and the hippocampus was collected immediately. Rats were killed along the 24 h at the same time-points used for the Barnes maze and sleep recordings (three rats per point). The tissue was frozen and stored at −20 °C for less than a week until analyzed. The last 12 rats were killed at 13:00 and 01:00 h, six rats per point, the hippocampus was collected and incubated for 1 h with saline or cortistatin; thereafter, the tissue was stored at −20 °C until used. For cAMP determination, the tissue was thawed and homogenized with trichloroacetic acid (6% w/v), then centrifuged at 2000×*g*/15 min and the soluble phase was collected and washed with a diethyl-ether saturated solution. The final volume was lyophilized at −60 °C. cAMP was measured with the enzyme-immunoassay Amersham Kit (Amersham Pharmacia Biotech, NJ), following the manufacturer's instruction. Samples or standards are added to individual wells of a camp immunoassay plate followed by 100 μl of anti-cAMP immunoglobuline G (IgG). After 2 h of incubation al 4 °C, the plates are thoroughly washed and bound IgG is measured via 1 h incubation with an anti-IgG peroxidase conjugated secondary antibody (50 μl per well). The enzymatic activity was measured by incubating with 150 μl of 3,3′,5,5′, tetramethylbenzidine at room temperature. Reaction is quantified in an ELISA reader at 450 nm and the cAMP concentration was determined as the inverse of B/Bo percentage expressed in fmol. The optical density (OD) was converted into percentage of binding, using the following percentage equation: %B/Bo=OD sample of NSB/OD of Bo-OD of NSB×100, where NSB is the non-specific binding and Bo the binding without cAMP. Once a standard calibration curve depicting %B/Bo was obtained, the concentration of cAMP was derived from it.

Statistical significance was determined using one-way ANOVA and a Student–Newman–Keuls post-hoc test. Significance was accepted at $P<0.05$.

2.8. Drucker beam

This apparatus consists of five wooden beams of different widths (24, 18, 12, 6, and 3 mm), 2 m long, placed on two pedestals that allow a 15° inclination Drucker-Colín and García-Hernández, 1991.

2.9. Training

In this task, a rat is trained to walk on the 2 m long, 3 mm wide, wooden beam, in order to reach its home-cage. To get to its cage, the rat must be motivated and must have a fine control of balance and posture. This training is accomplished by sequential trials on successively thinner beams. Once the animals performed this task in less than a minute, we considered the rat to be trained and we proceeded to evaluate the diurnal variation of its performance.

2.10. Test

Rats were divided into two groups: saline (n=6 rats) and cortistatin (n=5 rats). Their performance on the Drucker beam was evaluated for 3 days at the same time-points used for the other procedures. Once the diurnal variations in this task were determined, we evaluated the performance at 13:00 and 01:00 h under saline or cortistatin, injected 30 min before each test.

Statistical significance was determined through one-way ANOVA and a Student–Newman–Keuls post-hoc test. Significance was accepted at $P<0.05$.

3. Results

Once all the animals were trained for 16 trials (Fig. 1), they solved the Barnes maze with great proficiency, consistently requiring less than 40 s to solve the Barnes maze after the tenth session (Fig. 1A). Likewise, the errors (Fig. 1B) and perseverances (Fig. 1C) decreased consistently, and the strategy employed was 70% sequential and 30% spatial at the end of the training (Fig. 1D).

Upon completion of this training, we evaluated the diurnal variations of the performance to solve the Barnes maze. The results showed that, during the dark phase of the cycle, at 21:00, 01:00 and 05:00 h, rats were able to shorten the time spent to solve the maze to about 11 s during the 3 days of testing (results of the third day are shown in Fig. 2A), whereas during the light phase of the cycle (09:00, 13:00 and 17:00 h), the rats needed 30 s and they included random searching to solve the maze. Saline injected at 01:00 and 13:00 h produced no changes in this pattern. In contrast, cortistatin increased significantly the time to solve the maze at these two time-points (13:00 saline: 25±2; cortistatin: 38±6, 01:00 saline: 10±1; cortistatin: 30±5) (Fig. 2A). Although cortistatin increased spatial strategy expression (from 20% to 40%) while reducing sequential strategy (from 50% to 30%) at 13:00, rats increased their time to solve the Barnes maze. The delay at 01:00 h resulted from deterioration of the sequential strategy (from 60% to 20%) associated with an increase (40%) in random searching (Fig. 2B), thereby increasing the number of errors (Fig. 2C) and perseverances (Fig. 2D).

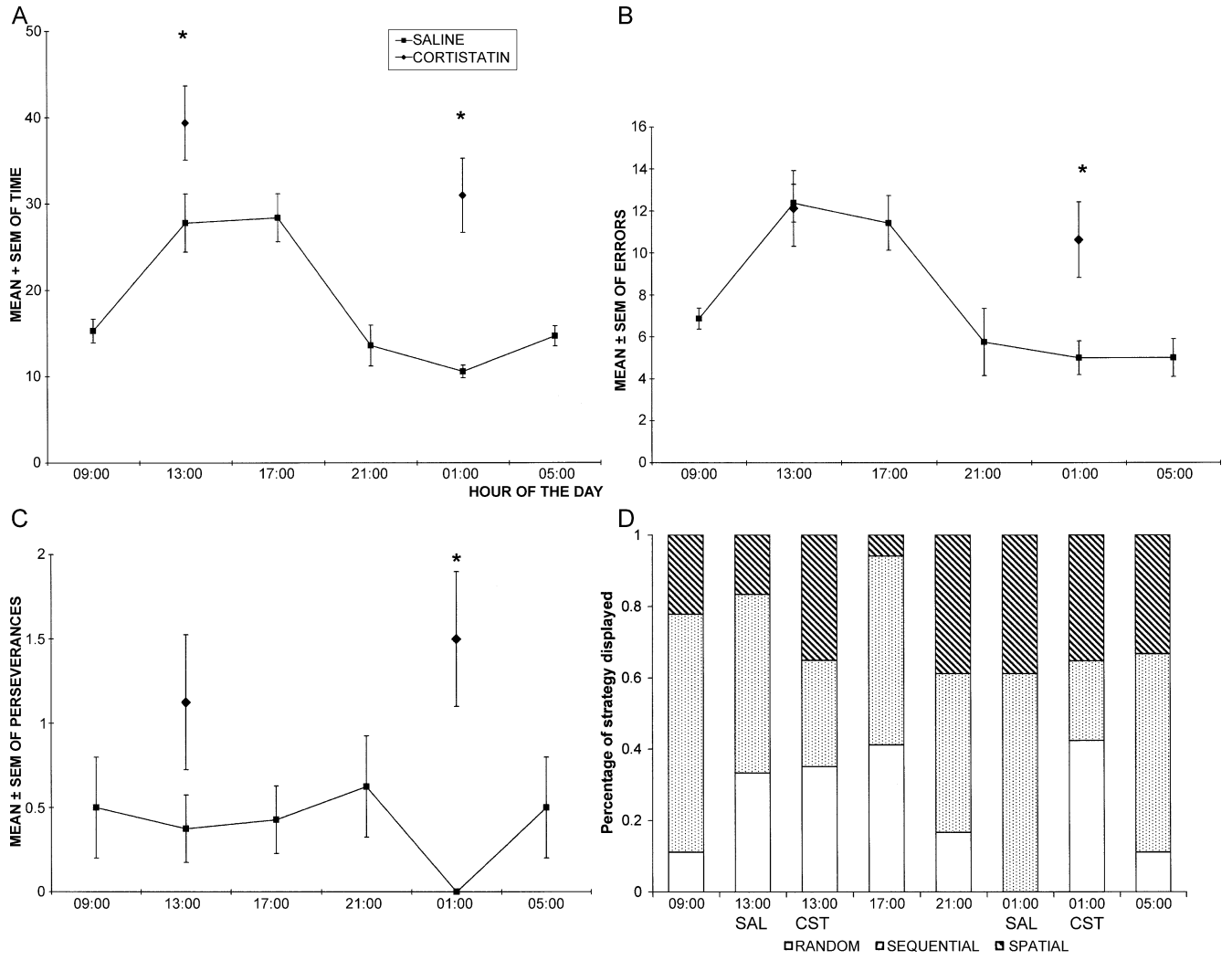

Fig. 2. Changes induced by the light–dark cycle and cortistatin on the rat's ability to solve Barnes maze. (A) Learning curve, (B) errors, (C) perseverances and (D) strategies. These results indicate that the performance of the rats is better in the dark phase of the cycle. Cortistatin impairs rat's proficiency to solve the Barnes maze at the two time-points tested, 13:00 and 01:00 h. Errors and perseverances were significantly affected at 01:00. In both situations, cortistatin impaired the sequential strategy ($P<0.5$).

cAMP concentration also fluctuated along the 24 h. Its highest concentration was detected in the hippocampus at 01:00, with another small peak at 13:00 h (Fig. 3). Cortistatin blunted the production of cAMP at both time-points (Fig. 3).

At the dark phase of the cycle, these nocturnal animals spent more time awake as compared to the light phase of the cycle, during which they spent more time in REM sleep. At 01:00 am, cortistatin decreased waking and increased REM sleep significantly, whereas at 13:00 h cortistatin reduced REM sleep with no modification in waking (Fig. 4).

Regarding motor control and equilibrium, the variation profile in the Drucker beam performance observed along the 24 h was similar to that of the Barnes maze performance. The performance was more efficient since the rats solved it in less time at 21:00, 01:00, and 05:00 h than at 13:00, 17:00, and 21:00 h (Fig. 5). Unlike saline, cortistatin

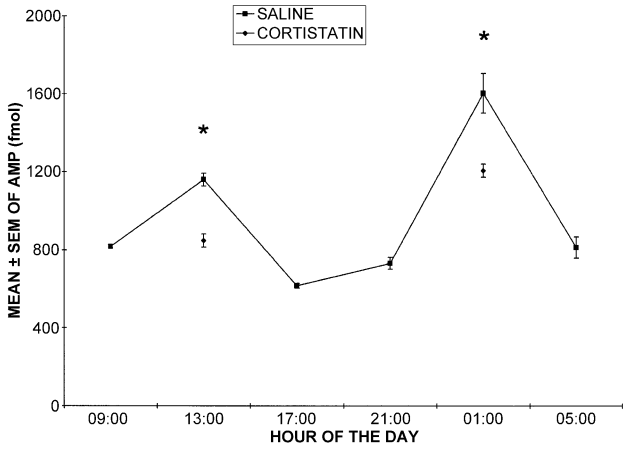

Fig. 3. cAMP concentration in the hippocampus and its changes induced by the light–dark cycle and cortistatin. Cortistatin blunted the concentration of cAMP at both time-points: 13:00 and 01:00 h ($P<0.5$).

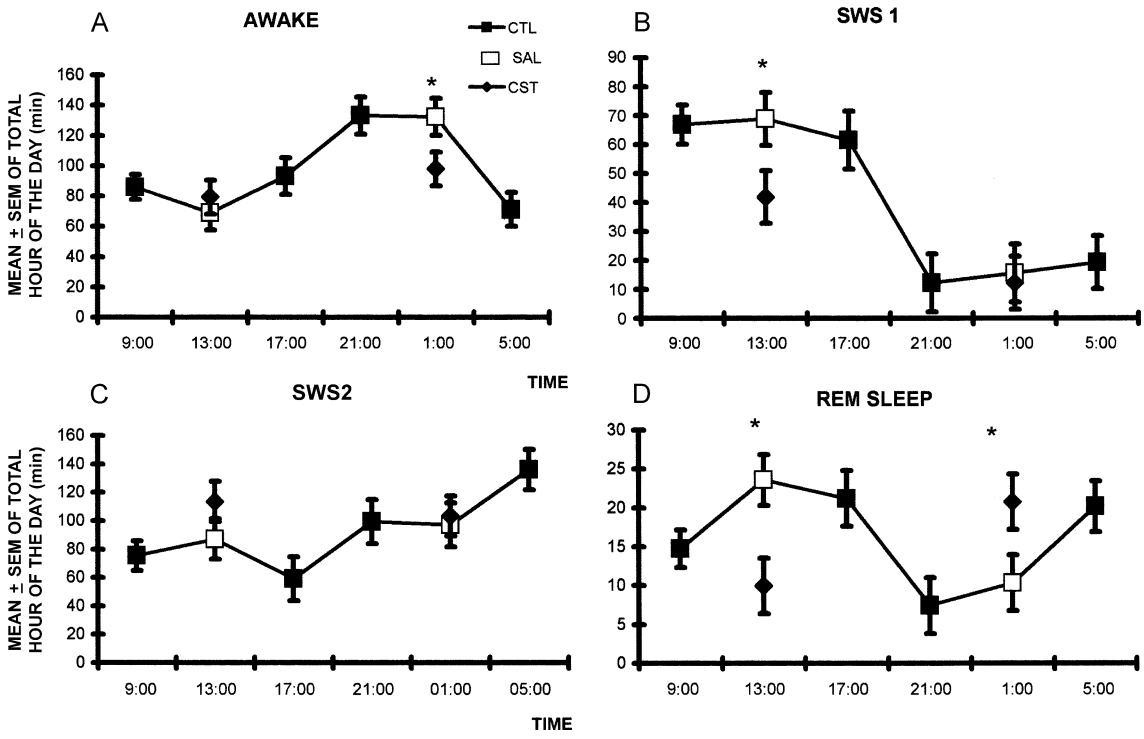

Fig. 4. Sleep–waking cycle and the effects of cortistatin at two representative time-points of the cycle (01:00 and 13:00 h). (A) Waking, (B) slow wave sleep 1, (C) slow wave sleep 2, and (D) rapid-eye-movement (REM) sleep ($P<0.05$).

deteriorated this performance at 13:00 h, but had no significant effect at 01:00 am (Fig. 5).

4. Discussion

Our data reveal that rats trained to solve the Barnes maze perform with great proficiency along the light–dark cycle, yet, their performance was better during the dark phase, since the rats needed less time and selected a strategy to solve the maze. We interpret these results as indicating that the brain capacity might not be changing along the day but the way the brain solves the problem. It has been previously suggested that evocation efficiency changes throughout the day, as assessed using several tasks (Antoniadis et al., 2000; Chaudhury and Colwell, 2002; Monk, 1994). For example, Monk suggests that the efficiency to solve memory tasks in humans follows a circadian rhythm, related to the arousal state, since the execution in the day is better than during the nighttime (Monk et al., 1997). Moreover, other indicators of cognitive functions, such as the neuroelectric component P300, have diurnal variations. Higuchi et al. (2000) have shown that the latency of the P300 is correlated positively with sleepiness and negatively with attention level in humans. Additionally, we found that although the rats solve with great proficiency the maze throughout the cycle, the strategy employed is different in both phases of the cycle. During the light cycle, the rats used 50% of sequential and 20% of spatial strategies, whereas during the dark cycle the rats used 60% of sequential and 40% spatial strategies. The rat's success in locating the escape tunnel is commonly believed to depend on the ability to make and use a spatial representation of the environment derived from the visual extramaze cues surrounding the maze. This ability is often referred as allocentric navigation or spatial strategy and is believed to depend on the hippocampus. Although the rats generally show a high tendency to use allocentric navigation to solve spatial mazes, proficient performance can be achieved using alternative strategies that are independent from the use of extramaze cues, which has been named sequential strategy and is referred to as egocentric navigation, and involves to caudate–putamen nucleus. Hence,

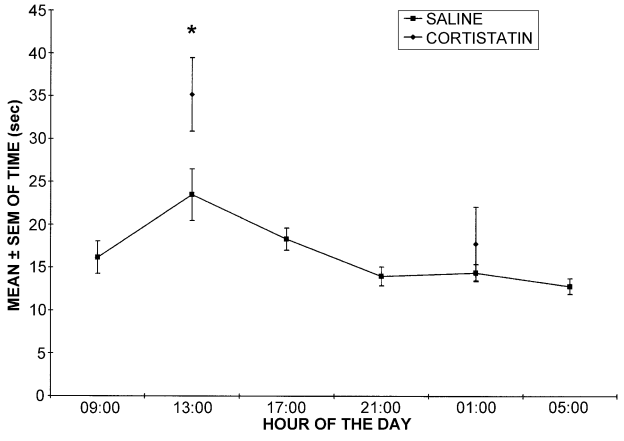

Fig. 5. The performance in the Drucker beam and the changes induced by the light–dark cycle and cortistatin at 13:00 and 01:00 h. Cortistatin deteriorated significantly the execution at 13:00 h ($P<0.5$).

both types of strategies can be used depending on the situation, for example the presence or absence of environmental light.

These results support the notion that the function of several brain structures follow a diurnal rhythm. For example, the release of hippocampal acetylcholine, an important neurotransmitter in cognitive processes, shows a circadian variation. At the start of the dark (active) period of nocturnal animals, hippocampal acetylcholine levels are increased and this increase is associated to enhanced hippocampal activity.

Additionally, we found that cortistatin modulated this proficiency at 01:00 and 13:00 h. In both situations, cortistatin impaired the sequential strategy (procedural memory), since we observed that the effect of cortistatin on the election of strategy had not diurnal variations. Random searching remained unchanged at 13:00 h, but the use of spatial strategy increased; therefore, a reduction in time to solve the Barnes maze would be expected. However, an effect on motor control should be taken into account, since cortistatin impaired motor skills at this time-point. Since spatial strategy was improved, it can be inferred that this type of memory was not affected by the motor impairment. During the dark phase, this peptide seems to interfere more directly with memory evocation, since it reduced the sequential strategy and increased random searching, thereby increasing the number of errors and perseverances. These results suggest that its effect was restricted to procedural memory, while spatial memory remained unaffected. Cortistatin effect on memory evocation has been suggested previously by other studies (Flood et al., 1997; Sánchez-Alavez et al., 2000). However, we have now shown that cortistatin rather modulates than impairs memory evocation.

Likewise, cAMP production in the hippocampus depicted diurnal variations, showing the highest peak at 01:00 h, in accordance with the best performance of the rat on the Barnes maze, whereas the lowest concentration during the light phase is consistent with the worst performance. These results are consistent with reports that suggest that activation of the cAMP pathway is important for memory evocation (Abel and Matthew Lattal, 2001; Barros et al., 2003). Cortistatin tested at 01:00 and 13:00 h blunted production of cAMP with similar efficacy at both time-points. cAMP is expected to foster the rat's proficiency in this task (Renger et al., 2000), therefore its reduction was consistent with worsening of this performance. However, the rats that received the i.c.v. administration of cortistatin and solved the Barnes maze revealed that the sequential strategy was affected but not the spatial one. Cortistatin's effect on procedural memory may be a consequence of affecting the caudate–putamen nucleus rather than the hippocampus. However, he have to prove this hypothesis by administrating cortistatin into the hippocampus as into the caudate–putamen.

The Drucker beam task reveals that the required motor-equilibrium control behavior also underwent diurnal variations, providing additional support to the observation that motivated behaviors are expressed as diurnal variations (Antoniadis et al., 2000; Monk, 1994; Monk et al., 1997). Performance in this task was also depressed by cortistatin at 13:00 h but not at 01:00 h. This effect suggests that cortistatin might interfere with motor control during the light phase, but not with motivation since rats performed more slowly than control rats but successfully. An effect on motor control may interfere with the performance of any task, such as the Barnes maze. Therefore, it is very likely that this action of cortistatin is contributing to the deterioration of the performance in the Barnes maze during the light phase. However, during the dark phase, cortistatin did not affect locomotion, yet the effect on the performance in the Barnes maze persisted, suggesting independent effects.

The results of the sleep–waking cycle reproduce previous reports. At the dark phase of the cycle (active period) of these nocturnal animals, they exhibit more time awake and less REM sleep phase as compared to the light phase. During this phase, rats solved the Barnes maze and Drucker beam tasks with the best performance. de Lecea et al. (1996) have previously reported that cortistatin regulates the sleep–waking cycle, increasing the no-REM sleep. However, we explored this effect only during the light phase. In this study, we found that, during the dark phase, cortistatin increases REM sleep and decreases waking. In contrast, during the light phase, cortistatin decreases REM sleep.

We do not know which is the action mechanism of cortistatin, however, we know that cortistatin interacts with several neurotransmission systems, such as GABA and acetylcholine in the hippocampus. We believe that cortistatin facilitates GABA action, and it has been reported that GABA induces amnesia when infused into the hippocampus (Hasselmo et al., 1996). On the other hand, cortistatin inhibits acetylcholine's action in the hippocampus (de Lecea et al., 1996). This neurotransmitter is involved in memory evocation, since cholinergic antagonists impair memory evocation (Szapiro et al., 2002). Probably, cortistatin affects cholinergic neurotransmission in the hippocampus and, in this way, modulates memory processes. However, we do not discard the possibility that cortistatin affects other modulatory systems in the brain, involved in memory processes, such as noradrenaline, serotonin, and endorphins (Abel and Matthew Lattal, 2001; Barros et al., 2001; Silva et al., 1998; Szapiro et al., 2002). Additionally, it is possible that its effect is a consequence of an interaction with other cerebral structures, such as the cerebral cortex, the nucleus striatum, through interactions with somatostatin receptors that are widely distributed along the brain or with interactions with its own recently described receptors (Robas et al., 2003).

Acknowledgments

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