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ROLE OF HYPOCRETINS AND MCH IN THE CONTROL OF SLEEP AND WAKEFULNESS: FOS AND INTRACEREBRAL MICROINJECTIONS STUDIES IN THE CAT

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ABSTRACT

The posterior and lateral region of the hypothalamus was classically involved in the control of arousal and somatomotor activity. In this area two important groups of neurons reside, both of which use different neurotransmitters: hypocretin and melanin-concentrating hormone (MCH). Both neuronal groups project diffusely throughout the central nervous system, including areas that participate in the generation and maintenance of wakefulness and sleep. In the present report, we review previous studies performed along with Dr. J. Yamuy, Dr. F. R. Morales and Dr. M. H. Chase regarding the hypocretinergic system (Torterolo et al., 2001; Torterolo et al., 2003). In addition, we discuss new findings in relation with the MCHergic system. The data presented strongly suggests that: 1) the hypocretinergic system is not responsible for the generation and/or maintenance of wakefulness *per se*, as it is currently believed. In fact, the hypocretinergic system is likely to be primarily involved in somatomotor functions. 2) The hypocretinergic neurons also play a role during active sleep (AS, also called REM sleep); these neurons are active during this state. 3) The MCHergic system facilitates the generation of AS both at the pontine and medullary reticular formation.

These findings raise critical questions about the function and potential interactions between the hypothalamic MCHergic and hypocretinergic systems in the control of waking and sleep.

Keywords: Hypocretin, orexin, hypothalamus, melanin-concentrating hormone, MCH, sleep, wakefulness

INTRODUCTION

Hypocretin 1 and 2 (also called orexin A and B, respectively) are neuropeptides produced by a group of neurons distributed in the postero-lateral hypothalamic region (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). These neurons have widespread projections throughout the brain and spinal cord (Peyron et al., 1998). Although it is known that a deficit in the hypocretinergic system underlies narcolepsy (Taheri et al., 2002), the physiology of this system is still unclear. It is currently believed that the hypocretinergic neurons are involved in the generation and/or maintenance of wakefulness (Sutcliffe and de Lecea, 2002; Taheri et al., 2002). Supporting this fact, high density of hypocretinergic fibers/terminals is present in waking-related areas, such as the noradrenergic locus coeruleus and the histaminergic tuberomammillar nucleus (Peyron et al., 1998). Hypocretin exerts an excitatory effect over these neurons (Hagan et al., 1999; Huang et al., 2001). Furthermore, intraventricular injection of hypocretin increases the

time spent in wakefulness (Hagan et al., 1999; Nakamura et al., 2000). However, the results presented in this report challenge this hypothesis.

The melanin-concentrating hormone (MCH) is a 19-aminoacid cyclic neuropeptide originally isolated from salmon pituitary glands (Kawauchi et al., 1983). Neurons that employ MCH as a neurotransmitter have been described in the postero-lateral hypothalamus (Bittencourt et al., 1992; Kilduff and De Lecea, 2001). These neurons also have widespread projections; dense MCHergic projections as well as MCH receptors have been found in the waking and sleep-related areas (Bittencourt et al., 1992; Kilduff and De Lecea, 2001).

In the present report, utilizing two different techniques we sought if these systems were involved in the control of sleep and wakefulness. Firstly, we studied the Fos immunoreactivity (as a marker of neuronal activity (Dragunow and Faull, 1989)) in hypocretinergic and MCHergic neurons during these behavioral states. Secondly, we studied the effect of MCH microinjections into critical areas for active sleep (AS) generation: the nucleus pontis oralis (NPO) and the magnocelullar tegmental field of the medulla (MgTF).

MATERIAL AND METHODS

Nineteen adult male cats were used in the present study. Animals were implanted with electrodes for recording waking and sleep parameters (see (Torterolo et al., 2001) for details).

Behavioral and immunohistochemistry procedures for Fos studies

In order to study Fos immunoreactivity during the waking-sleep cycle, the animals spent one to two hours before euthanasia (time needed for Fos proteins to reach an optimal concentration (Dragunow and Faull, 1989)) either in quiet wakefulness (QW, n = 3), active wakefulness with motor activity (AW with M, n = 3), active wakefulness without motor activity (AW without M, n = 3), quiet sleep (QS, n = 4) or AS induced by carbachol (AS-carbachol, n = 3) (for details in the procedures, see (Torterolo et al., 2001; Torterolo et al., 2003)).

Animals were sacrificed with sodium pentobarbital (60 mg/kg) and perfused for immunohistochemistry. The brain was frozen and serially sectioned at 20 µm. Single immunostaining procedures were performed to identify MCH or hypocretin-2 (Hcrt-2). Double immunohistochemistry was performed for Fos and Hcrt-2, Fos and MCH as well as MCH and Hcrt-2 (see (Torterolo et al., 2001) for details). In order to identify MCH immunoreactivity, sections were incubated with rabbit MCH antibody (Phoenix Pharmaceuticals, 1:1500). ABC complex and diaminobenzidine-H202 reaction was

employed as detecting agents. Pyronin-Y counterstaining was utilized to identify the cytoarchitecture of the analyzed areas.

Sections were analyzed by light microscopy; photomicrographs were obtained using a SPOT digital camera. The distribution of immunolabeled neurons was determined by drawings using a camera lucida.

Hypocretinergic and MCHergic neurons with Fos immunoreactivity (Hcrt+Fos+ and MCH+ Fos+) were analyzed in two representative coronal sections selected for each cat at the tuberal level of the hypothalamus (A 10 to 11 (Berman and Jones, 1982)). The number of neurons per section is presented as the mean ± S.E.M. The statistical significance of cell counts was assessed using ANOVA-Fisher tests. The criterion chosen to discard the null hypothesis was $P < 0.05$.

Microinjection studies

Preliminary intracerebral microinjection studies were performed in three cats. In 2 cats, series of MCH and control microinjections were performed in the NPO, in the site where carbachol generated AS (P -2 to -4; L 1.5 to 3; H -3.5 to -5 (Berman, 1968)). During experimental sessions the animals were maintained in a head-restraining device with continuous monitoring of their behavioral states. MCH (1.5 µg in 0.25 µl of saline) was applied unilaterally using a Hamilton syringe over a period of 2 minutes. Saline (0.25 µl) and sham microinjections were performed as control (see (Torterolo et al., 2002) for details). Using the same protocol, series of MCH (1.5 µg) and control microinjections were performed in the MgTF (P -8 to -9; L 1 to 2; H -7 to -8) of one cat. Sleep parameters were analyzed during five hours. The statistical significance of the results was assessed using the two-tailed unpaired Student's *t*-test. The criterion used to discard the null hypothesis was $P < 0.05$.

RESULTS

Characteristics of immunostained neurons

As it is shown in Figure 1, MCHergic and hypocretinergic neurons were intermingled in the lateral hypothalamus of the cat. Examples of hypocretin and Fos double-labeled neurons are exhibited in Figure 2.

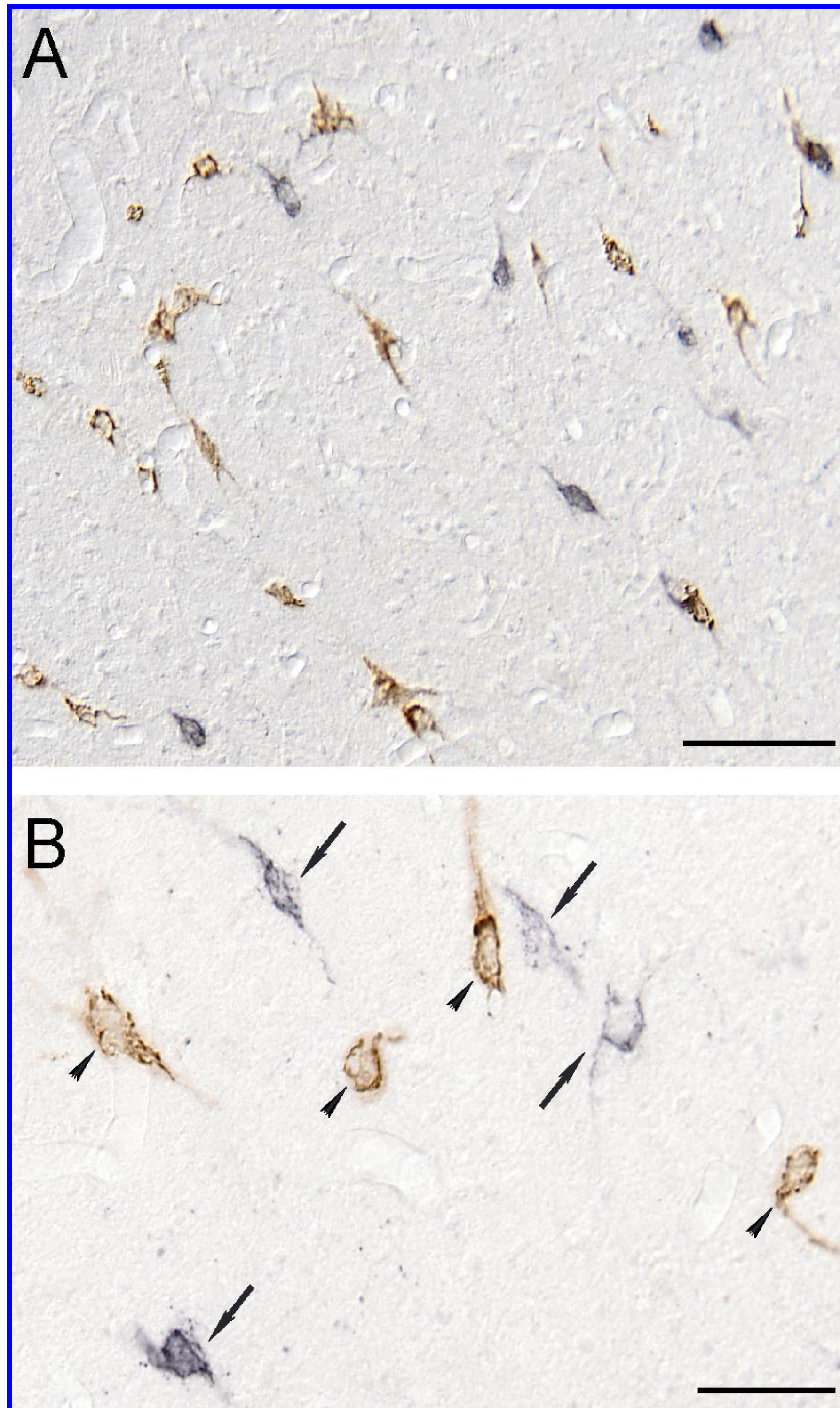

Figure 1. MCHergic and hypocretinergic cells are intermingled in the lateral hypothalamus of the cat. A-B. Photomicrographs of the lateral hypothalamic area of the cat. The sections were immunostained for MCH (in brown, arrowheads) and hypocretin (in black, arrows). Sections were processed utilizing the ABC method and the DAB-H_2O_2 reaction to detect peroxidase activity. This reaction was enhanced with nickel to label the hypocretinergic cells. Calibration bars: A, 100 μm; B, 50 μm.

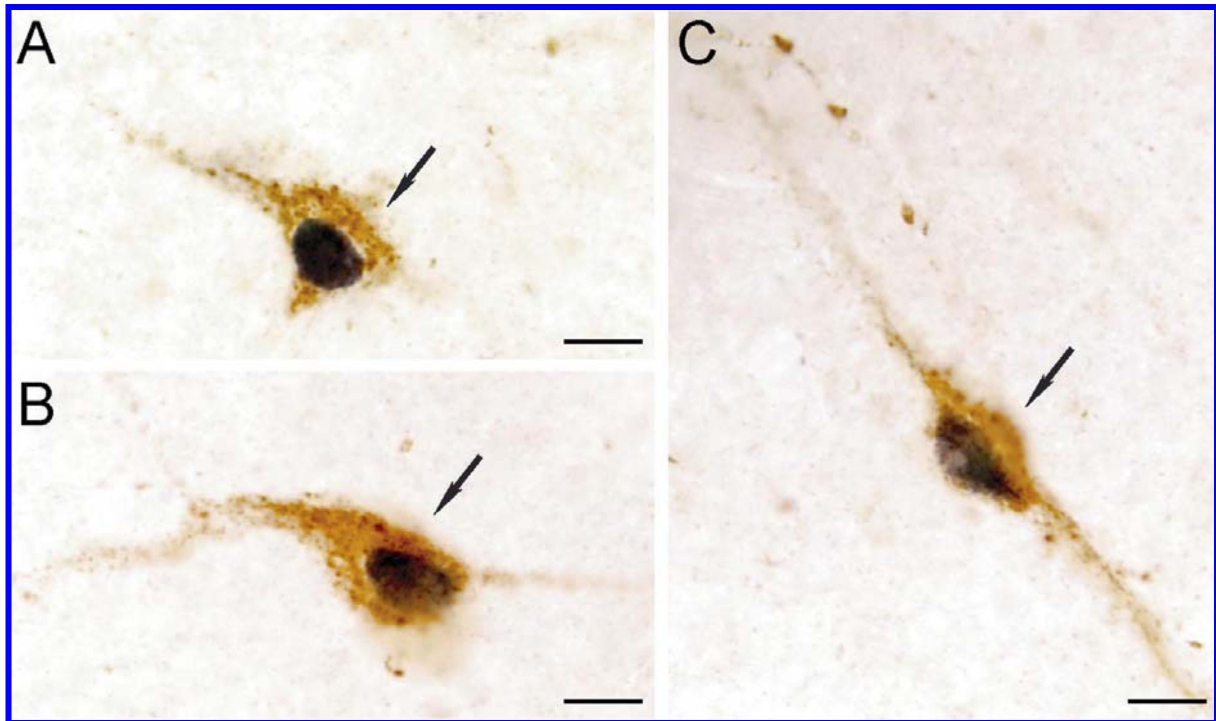

Figure 2. A-C. Photomicrographs illustrating examples of Fos immunoreactive hypocretinergic neurons (arrows) from the lateral area of the hypothalamus. Hypocretin immunoreaction is stained in brown. Fos immunoreactivity, which is black, is restricted to the nuclei. Sections were taken from an AS-carbachol animal and processed employing the ABC method and the DAB-H_2O_2 reaction to detect peroxidase activity. This reaction was enhanced with nickel to label the Fos protein. Calibration bars: A-C 10 μm.

Fos in hypocretinergic neurons

Fos immunoreactivity in hypocretinergic neurons (Hcrt+ Fos+ neurons) was observed in all the analyzed groups of animals. The number of Fos immunoreactive hypocretinergic neurons was higher during AW-with M (184 ± 19.6) compared with the others conditions (P < 0.0001). No significant differences were found among AW-without M (5.7 ± 2.5), QW (3.0 ± 1.9), and QS (1.6 ± 1.0). In addition, the number of Hcrt+ Fos+ was higher during AS-carbachol (60.3 ± 11.0) compared with QW, AW without M and QS (P < 0.01).

Figure 3 exhibits examples of the distribution of the Hcrt+ Fos+ neurons during AW with M, QW and AS-carbachol. From the total number of hypocretinergic neurons, 79% and 34% were immunoreactive for Fos during AW-with M and AS-carbachol, respectively. A small percentage of Hcrt+ Fos+ neurons were observed during AW-without M (2%), QW (2%) and QS (1%).

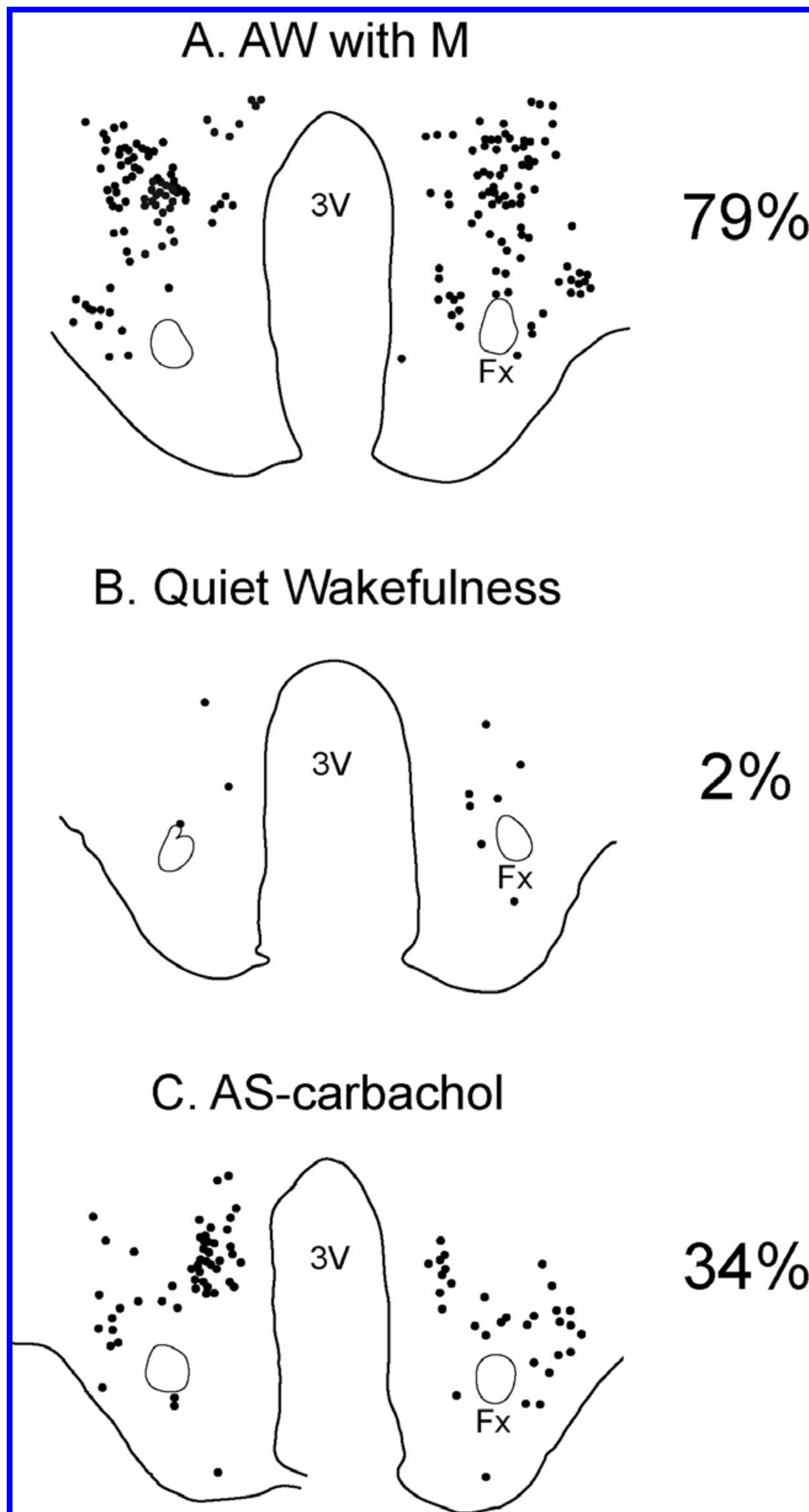

Figure 3. Distribution of Fos and hypocretinergic neurons during active wakefulness with motor activity (AW with M), quiet wakefulness and AS-carbachol. The camera lucida drawing shows the distribution of Hcrt+ Fos+ neurons. Each mark indicates one labeled neuron. The percentage of Hcrt+ Fos+ neurons from the total number of hypocretinergic neurons is shown. 3V, third ventricle; Fx, fornix.

Fos in MCHergic neurons

In spite of the presence of Fos+ neurons intermingled with the MCHergic cells, we found that MCHergic neurons did not exhibit Fos immunoreactivity in any of the conditions studied.

Microinjections of MCH into the NPO

In two cats, the NPO was localized by carbachol microinjections (0.2 µl, 4 µg/µl). In these animals carbachol generated AS with a latency of 4 and 5 minutes, and a duration of 78 and 46 minutes, respectively. Into the same sites, MCH and control microinjections were performed. Because sleep parameters were similar after saline or sham microinjections, these results were pooled as "sham/saline". MCH microinjections (1.5 µg) produced an increase in the AS percentage. During the first recording hour, the amount of time spent in AS increased 70% in comparison to "sham/saline" level (20.5 ± 2.6 Vs. 12.0 ± 1.6, $P < 0.01$). This increment in AS time was still statistically significant when the analysis was performed for the total recording time (23.6 ± 0.9 Vs. 18.7 ± 1.0, $P < 0.005$). In addition, MCH decreased the AS latency to 25% of "sham/saline" values (6.2 ± 1.4 Vs. 24.3 ± 2.6, $P < 0.001$).

Microinjections of MCH into the MgTF

MCH microinjections into the MgTF increased the percentage of AS during the first recording hour, in comparison with "sham-saline" microinjections (18.7 ± 2.3 Vs. 10.2 ± 4.2, $P < 0.05$). MCH did not modify the AS latency.

DISCUSSION

Since studies performed in the early 20th century it is known that the hypothalamus is critical for the control of behavioral states (von Economo, 1930). Different waking and sleep related neuronal groups (i.e., tuberomamillar histaminergic neurons, hypocretinergic neurons, ventrolateral preoptic GABAergic-gallaninergic neurons) with extensive interactions among them have been described in the hypothalamus (Lin et al., 1988; Sherin et al., 1996; Peyron et al., 1998; Sherin et al., 1998). Hypocretin and MCH-containing neurons are intermingled in the lateral hypothalamus. MCHergic neurons have axosomatic, axodendritic and somatosomatic contact with hypocretinergic neurons and both neuronal types have considerable overlapping in their axonal projections to waking and sleep-related areas (Bittencourt et al., 1992; Peyron et al., 1998; Bayer et al., 2002; Guan et al., 2002); this anatomical relationship suggests an important functional interaction among these systems.

In order to test the hypothesis that both the hypocretinergic and MCHergic systems are involved in the control of wakefulness and sleep, we have performed two functional approaches: Fos and intracerebral microinjections studies.

Fos studies: Hypocretin and motor activity

The present data demonstrate that hypocretinergic neurons become active during aroused (alert) wakefulness, but just when the animal is moving. In the absence of motor activity during aroused wakefulness, quiet wakefulness or quiet sleep, the hypocretinergic system is not activated to any significant extent. Others evidences support the hypothesis that the hypocretinergic system is primarily involved in somatomotor functions: 1) Intraventricular microinjections of hypocretin produce an increase in locomotor activity that is suppressed by dopamine antagonists (Nakamura et al., 2000). 2) Hypocretin levels in the cerebro-spinal fluid (CSF) and in areas such as the perifornical hypothalamus are positively correlated with motor activation (Kiyashchenko et al., 2002; Wu et al., 2002; Martins et al., 2004). 3) Discharge rate of units in the perifornical hypothalamic area increases in association with heightened muscle activity (Alam et al., 2002). 4) Hypocretinergic terminals lie in close apposition to hypoglossal, trigeminal and ventral horn motoneurons; direct application of hypocretin onto intracellularly recorded lumbar motoneurons results in depolarization of their membrane potential, a decrease in input resistance and sustained discharge (van den Pol, 1999; Fung et al., 2001; Yamuy et al., 2001). It is interesting to note that in the Restless Leg Syndrome, which is characterized by irresistible leg movements, there is an increase in hypocretin CSF levels (Allen et al., 2002). Therefore, the hypocretinergic system is well positioned to initiate, maintain and facilitate motor activity by operating directly on motoneurons and/or by modifying the activity of supraspinal systems that are involved in motor functions.

Fos studies: Hypocretin and active sleep

An increase in the number of Hcrt+ Fos+ neurons was observed during AS-carbachol. This cholinergically-induced state resembles natural AS (Kubin, 2001). This finding is in agreement with an increase in the hypocretin-1 release detected in the hypothalamus and basal forebrain during AS (Kiyashchenko et al., 2002). In addition, a subpopulation of unidentified neurons of the posterior and lateral hypothalamus increase their firing rate during AS as well as during both AS and wakefulness (Vanni-Mercier et al., 1984; Szymusiak et al., 1989; Steininger et al., 1999; Alam et al., 2002). The hypocretinergic activation during AS may not be disconnected with motor activity; it is known that although motor output is inhibited at the motoneuron level, the supraspinal motor systems are very active during AS (Nakamura et al., 1978; Chase and Morales, 1983; Chase and Morales, 2000).

Fos studies: MCH.

Unexpectedly and in sharp contrast with the hypocretinergic neurons, we did not see Fos immunoreaction in the MCHergic neurons in any of the analyzed conditions. *In vitro* recordings have demonstrated that while hypocretinergic neurons are in an intrinsic state of membrane depolarization which promotes their spontaneous activity, MCHergic neurons are hyperpolarized and inactive in resting state (Eggermann et al., 2003). This result suggests that the activation of MCHergic neurons is more restricted, and probably these neurons are not active throughout the selected conditions; this fact can limit the expression of *c-fos* mRNA and the synthesis of the Fos protein (Hughes and Dragunow, 1995). However, a recent study in rats has shown an increase in Fos immunoreactivity in the MCHergic neurons during AS (Verret et al., 2003). The different species used in each case, as well as the difference between AS induction (rebound Vs. carbachol microinjections) could explain the disagreement with our results.

Microinjection studies: MCH and active sleep.

The NPO is a critical area for the generation and maintenance of AS; microinjections of cholinergic agonists produce a long-duration AS-like state while GABA agonists applied to this area suppress AS and generate wakefulness (Kubin, 2001; Xi et al., 2001). In order to test the function of MCH in this area we microinjected this peptide with a previously utilized dose (Rossi et al., 1999; Verret et al., 2003). MCH microinjections increased the time the animals spent in AS, and decreased the AS latency. This effect was similar to the one obtained with hypocretin-1 or hypocretin-2 (Xi et al., 2002). At the level of the MgTF, preliminary results showed that MCH facilitates the generation of AS; the MgTF is a critical area for AS generation, as well as for the production of AS atonia (Sakai, 1988; Morales et al., 1999). In agreement with these results, MCH applied into the cerebral ventricle of the rats produced an increase in AS (Verret et al., 2003). The presence of MCHergic fibers/terminals as well as the presence of one of the already described MCHergic receptors (MCHR-1) in both the NPO and MgTF, suggests that the observed effect is due to the modulation of local neurons (Chambers et al., 1999; Hervieu et al., 2000; Sailer et al., 2001; Saito et al., 2001).

Although the role of the MCHergic system in sleep is still elusive, the following facts suggest that this neuronal system is involved in the control of sleep: 1) MCHergic neurons strongly innervate waking and sleep-related areas. 2) Although no Fos immunoreactivity was observed in MCHergic neurons in the cat, the number of MCH+ Fos+ neurons increased with AS in the rat. 3) Intracerebral (the present study) and intraventricular microinjection studies (Verret et al., 2003) showed that MCH facilitates the generation of AS. However, we consider that unit recordings of identified MCHergic

neurons as well as microdialysis studies are needed to clarify the role of these neurons in relation to wakefulness and sleep.

CONCLUSIONS

In the present report we studied the hypocretinergic and the MCHergic systems in relation to the sleep-waking cycle. Our data demonstrate that the hypocretinergic system is no responsible for the maintenance of wakefulness *per se*; on the contrary, this system is likely to be primarily involved in somatomotor functions. In addition, the finding that a significant portion of hypocretinergic neurons are activated during AS, indicates that these cells may participate in a still unknown process that occurs during this behavior.

The facilitation of AS generation following microinjection of MCH into both the NPO and the MgTF suggests that the MCHergic system is involved in the control of AS.

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