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Research report

GABAergic processes within the median preoptic nucleus promote NREM sleep

Luciana Benedetto^a, Michael H. Chase^{b,c}, Pablo Torterolo^{a,*}^a Department of Physiology, School of Medicine, Universidad de la República, General Flores 2125, 11800 Montevideo, Uruguay^b WebSciences International, 1251 Westwood Blvd, Los Angeles, CA 90024, USA^c UCLA School of Medicine, Los Angeles, CA 90095, USA

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ABSTRACT

GABAergic mechanisms in the preoptic region of the hypothalamus (POA) have been implicated in the generation and maintenance of NREM (quiet) sleep. We recently reported that neurons in the median preoptic nucleus (MnPN) in the POA of the cat are selectively activated during NREM sleep. In the present study, we explored the hypothesis that NREM sleep is controlled by GABAergic mechanisms within the MnPN. Consequently, adult cats were utilized to determine GABA immunoreactivity within the MnPN and to examine the effects on sleep of the microinjection of a GABA_A agonist (muscimol) and a GABA_A antagonist (bicuculline) into this area.

GABAergic neurons were present throughout the MnPN. Compared with control microinjections, after the application of muscimol, the time spent in NREM sleep (59.8 ± 7.5 min) and REM sleep (6.9 ± 4.7 min) decreased compared with control microinjections (103.8 ± 5.2 and 20.2 ± 4.3 min, respectively; $P < 0.005$). In contrast, bicuculline microinjections increased only NREM sleep time (103.0 ± 23.0 vs 77.7 ± 23.7 min; $P < 0.05$).

These results demonstrate that GABAergic processes within the MnPN are involved in the generation and maintenance of sleep, especially NREM sleep.

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1. Introduction

For decades, a variety of studies have demonstrated the importance of the preoptic area of the hypothalamus (POA) in the generation and maintenance of NREM (quiet) sleep [1]. It has also been shown that within the POA, the ventrolateral preoptic nucleus (VLPO) together with the median preoptic nucleus (MnPN) are crucial to this process [2–5].

Neurons within the MnPN of the rat exhibit an increase in the number of cells that are immunoreactive to the Fos protein (an index of neuronal activity) in conjunction with sustained periods of NREM sleep [6]. Unit recordings have confirmed that the MnPN contains a subset of neurons which exhibit an increase in their firing rate during NREM sleep [7]. It has also been demonstrated that the intracerebroventricular administration of sleep factors interleukin-1 or growth hormone-releasing hormone (GHRH) results in an increase in NREM sleep together with an increase in the expression of Fos in MnPN neurons [8,9]. In addition, MnPN neurons project to different regions involved in the generation of sleep and wakefulness, including the dorsal raphe, locus coeruleus, the

perifornical-lateral hypothalamic area (PF/LH), the ventrolateral periaqueductal gray (vlPAG) and the VLPO [10–15]. MnPN neurons also exert inhibitory control over arousal-related cells and excitatory control over sleep-related PF/LH neurons [16]. In this regard, Kumar et al. demonstrated that the inhibition of cells in the MnPN results in the activation of waking-related hypocretinergic and serotonergic neurons [17].

Our previous study in the cat demonstrated that cells in the MnPN are active during NREM sleep [18]. The present research was also conducted in cats in order to determine if GABAergic processes in the MnPN play a role in the generation and maintenance of NREM sleep. Consequently, we first examined the presence of GABAergic cells within the MnPN; then, in chronically instrumented animals, we analyzed the effect on sleep of local microinjections of GABAergic substances.

2. Material and methods

2.1. Animals

Seven adult male cats (*Felis Domesticus*) were used in this study; three were employed to detect GABA immunoreactivity and four were used for intracerebral microinjection studies. The animals were housed with food and water available ad libitum, and they were maintained under normal laboratory conditions (temperature 21–23 °C, 12-h day-night cycle, lights on at 7:00 am). All experimental procedures were conducted in accordance with the “Guide to the Care and Use of Laboratory Animals” (8th edition, National Academy Press, Washington D.C., 2011). An institutional Animal Care and Use Committee approved the experimental

* Corresponding author at: Facultad de Medicina, Universidad de la República, General Flores 2125, 11800 Montevideo, Uruguay. Tel.: +598 2924 34 14x3234.
E-mail address: ptorterolo@fmed.edu.uy (P. Torterolo).

protocol. Adequate measures were taken to minimize pain, discomfort or stress of the animals. In addition, all efforts were made in order to use the minimal number of animals necessary to produce reliable scientific data.

2.2. Detection of GABAergic neurons

2.2.1. Immunohistochemical procedures

Following euthanasia (pentobarbital 50 mg/kg) the animals were perfused with 1 L of heparinized saline followed by 1.5 L of a solution containing 4% paraformaldehyde, 15% saturated picric acid and 0.5% glutaraldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Subsequently, they were perfused with 500 ml of the same solution containing 10% sucrose.

The forebrain was removed and immersed in a post-fixative solution for 24 h, which consisted of 2% paraformaldehyde, 15% saturated picric acid and 10% sucrose in PB. Following post-fixation, the tissue was kept for 3 days in a solution of sucrose (25%) and sodium azide (0.1%) in PB. Thereafter, the forebrain was frozen and serially sectioned at 20 μm using a Reichert–Jung cryostat. The sections were stored in a solution of 0.1% sodium azide in PB-saline (PBS, 0.1 M).

In order to identify GABAergic neurons within the MnPN, polyclonal guinea pig antibodies raised against GABA-keyhole limpet hemocyanin conjugated with glutaraldehyde (NT-108, Protos) were employed. Free-floating sections containing preoptic regions were incubated overnight with the GABA antibody (1:3500) and normal donkey serum (NDS; 3%). Thereafter, the sections were rinsed and incubated for 60 min with biotinylated donkey anti-guinea-pig antibody (1:300) plus NDS. After another rinse, the tissue was incubated in ABC complex (1:200) for 60 min, and exposed to diaminobenzidine and hydrogen peroxide. The same primary antibody and immunohistochemical procedures were employed in previous studies [19–22].

2.2.2. Data analysis

Histological sections were examined with light microscopy. Photomicrographs were taken using a SPOT digital camera mounted on an Olympus BX60 microscope. Images were analyzed using Adobe Photoshop® software.

For each cat, three coronal sections containing the MnPN were analyzed. The shape and distribution of labeled neurons within the MnPN were determined from drawings using a camera lucida attachment and photomicrographs. The MnPN area was defined in a previous report [18].

2.3. Microinjections of GABAergic agents

2.3.1. Surgical procedures

Each cat was prepared for the surgical implantation of electrodes by the administration of xylazine® (2.2 mg/kg, im), atropine (0.04 mg/kg, im) and antibiotics (Tribrissen®, 30 mg/kg, im). Anesthesia was induced with ketamine (15 mg/kg, im); it was maintained with a gas mixture of isoflourane in oxygen (1–3%). The head was positioned in a stereotaxic frame and the skull was exposed. Stainless steel screw electrodes were placed in the frontal and parietal bones to record the electroencephalogram (EEG) and in the orbital portion of the frontal bone to record eye movements (electro-oculogram, EOG). Bipolar electrodes were implanted in both lateral geniculate nuclei in order to monitor ponto-geniculo-occipital (PGO) waves. A Winchester plug, which was connected to these electrodes, and a chronic head-restraining device, were bonded to the skull with acrylic cement. A hole (5 mm in diameter) was drilled in the skull overlying the POA; this hole was maintained clean and sealed with bone-wax until used to provide access for drug administration (GABAergic agents or saline). At the end of these surgical procedures, an analgesic was administered (Buprenex®, 0.01 mg/kg, im). Incision margins were kept clean and a topical antibiotic was administered on a daily basis.

2.3.2. Habituation

After the animals had recovered from the preceding surgical procedures, they were gradually adapted to the recording environment for a period of at least four weeks. This long and slow adaptation period avoided stress during experimental sessions, as evidenced by the animal's quiescent behavior and the fact that they quickly fall asleep.

One of the experimental animals was adapted for 8 weeks, and stable cycles of sleep and wakefulness occurred. Despite the fact that the proportion of sleep states in relation to wakefulness of this animal was lower in comparison with the other experimental animals, we found no reason to exclude it from the experimental data.

2.3.3. Experimental sessions and drug administration

All experimental sessions were initiated at 9 am in a temperature-controlled room (21–23 °C) where recording sessions of 4 h were carried out after drug administration. All animals had free access to water and food until the beginning of each recording session. During all sessions, the animals' head was held in a stereotaxic position by a chronic head-holder. The EEG, EOG, PGO and electromyogram (EMG, via superficial electrodes which were placed on the skin above the neck muscles) were recorded and analyzed using an AC-amplifier and the Spike2 software (CED, Cambridge, UK).

After five consecutive stable baseline sessions (with $a < 10\%$ fluctuation in sleep and waking parameters), experimental microinjections were initiated. For this purpose, at the beginning of each recording sessions, the GABA_A antagonist bicuculline

methobromide (0.1 mM, Sigma–Aldrich, St Louis, MO, USA), the GABA_A agonist muscimol hydrobromide (25 mM, Sigma–Aldrich) or sterile saline were microinjected into the MnPN (AP, 14.5; L, 0.0; H, –0.8 mm; according to [23]); the volume of the solutions was 0.2 μl . Microinjections were performed using a microsyringe (1 μl , Hamilton Co, Reno, USA) over a period of two minutes; the microsyringe was left at its target for a subsequent period of a minute.

Microinjections of physiological saline, or recordings that were carried out when no drugs were injected (sham experiments), were used as controls. During sham we performed the same procedures as in the microinjections experiments; however, the tip of the microsyringe was placed on the brain surface, avoiding trauma to the area of interest [24,25]. Because sham and saline microinjection experiments yielded the same results, these data were pooled. Microinjections of muscimol or bicuculline (three per animal) were intercalated with control microinjections (nine per animal, 3 saline and 6 sham) on different experimental days. In two animals, bicuculline and muscimol studies were conducted in the same animal, but in different experimental series that were temporarily separated by approximately two months (each series had its own control microinjections). In another two animals, only bicuculline or muscimol studies were performed. Following the end of the experimental series, euthanasia was performed with pentobarbital (50 mg/kg) and the animals perfused with fixatives for histological studies.

2.3.4. Data analysis

The states of sleep and wakefulness were determined on the basis of polygraphic records that were divided into 10-s epochs, which were analyzed according to standard criteria for determining states of sleep and wakefulness [26]. Artifact-free EEG traces of different time-windows were subjected to a fast Fourier transform (FFT), and power spectrum histograms were obtained.

The total time spent in wakefulness, NREM and REM sleep as well as the duration, frequency, number of episodes and sleep latencies were analyzed.

2.3.5. Statistics

All values are presented as means \pm S.E.M. (standard error). For statistical analysis, the data obtained from controls and drug microinjections were averaged for each animal. Then, the statistical significance of the difference between controls versus drug effects was evaluated utilizing a two-tailed paired Student's *t*-test. The criterion used to discard the null hypotheses was $P < 0.05$.

3. Results

3.1. GABA immunohistochemistry

Fig. 1A is a representative schematic coronal section of the POA, which highlights the MnPN. As depicted in Fig. 1, GABA immunoreactivity was present within MnPN neurons. These neurons were stained dark brown (Fig. 1B); most were oval in shape with an average major diameter of $17.0 \pm 0.5 \mu\text{m}$. GABAergic neurons were distributed throughout this nucleus (Fig. 1C). Using the primary antibody against GABA, the visualization of GABAergic fibers in the POA was scarce.

3.2. Microinjections studies

Bicuculline, muscimol and saline microinjections into the MnPN did not alter the patterns of EEG and EMG activity during sleep and wakefulness. In fact, slow waves and sleep spindles with similar characteristics were present during NREM sleep after the microinjections of saline, bicuculline or muscimol (Fig. 2). These data are supported by power spectrum histograms; note in the representative examples exhibited in Fig. 2 that power within the delta (0.5–3 Hz) and sigma (spindles frequency; approximately 9–15 Hz) frequency bands was similar.

Sleep parameters changed following the microinjection of bicuculline (0.1 mM, 0.2 μl) into the MnPN. The time spent in NREM sleep increased 32.6% compared with controls ($P < 0.05$, Table 1). The time spent in wakefulness (*W*) decreased approximately 16% in relation to control values ($P < 0.05$, Table 1); this effect was most pronounced during the last three hours of recording (Figs. 3A and 4). No significant differences were found in the time spent in REM sleep, in episodes durations and frequencies, as well as in NREM and REM sleep latencies (Fig. 4 and Table 1).

Following the microinjections of muscimol, the time spent in NREM sleep was reduced 42% compared with controls ($P < 0.005$,

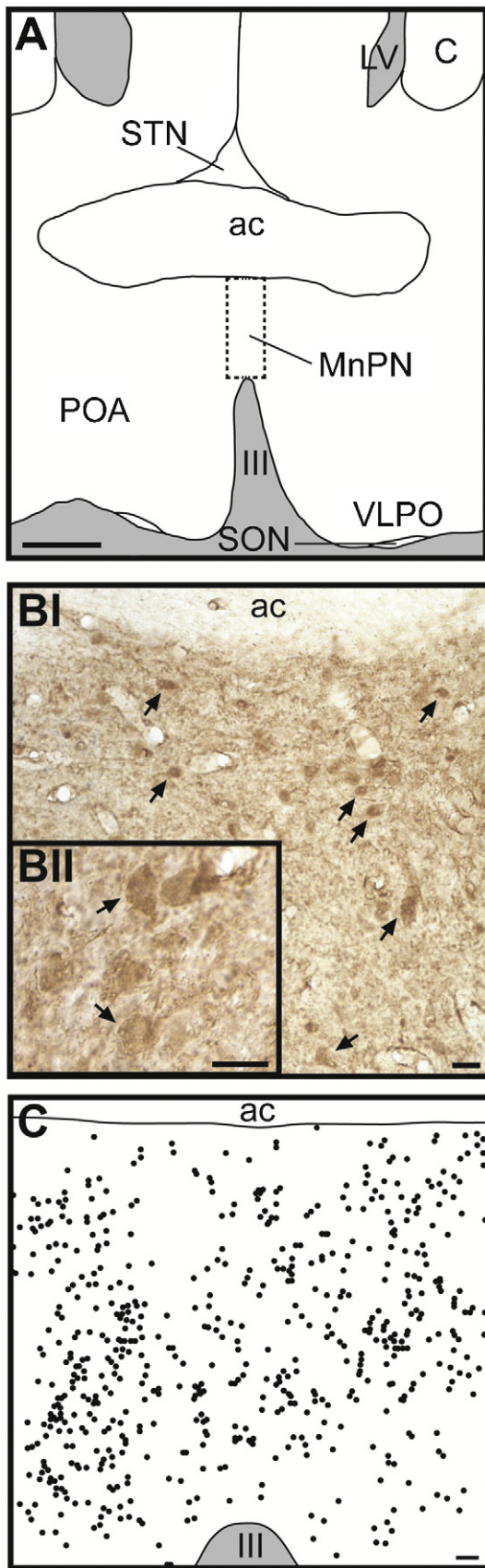


Fig. 1. (A) Schematic of the localization of the median preoptic nucleus (MnPN) in a coronal section of the hypothalamus. (B) Representative photomicrography showing GABAergic neurons at the level of the MnPN, which were identified by their brown-stained cytoplasm. BI and BII represent GABAergic neurons at different magnifications. Arrows indicate cell bodies immunoreactive to GABA. (C) Camera lucida

Table 1
Waking and sleep before and after the microinjection of bicuculline into the MnPN.

	Control	Bicuculline
Wakefulness		
Time (minutes)	146.4 ± 31.1	122.4 ± 28.0*
Number of episodes	63.1 ± 21.5	76.7 ± 20.9
Episode duration (minutes)	3.0 ± 1.1	1.9 ± 0.5
Frequency (episodes/hour)	15.8 ± 5.4	19.2 ± 5.2
NREM sleep		
Time (minutes)	77.7 ± 23.7	103.0 ± 23.0*
Number of episodes	62.9 ± 22.0	77.2 ± 21.3
Episode duration (minutes)	1.5 ± 0.7	1.7 ± 0.7
Frequency (episodes/hour)	15.7 ± 5.5	19.3 ± 5.3
Latency (minutes)	9.5 ± 4.9	3.6 ± 1.1
REM sleep		
Time (minutes)	15.9 ± 7.7	14.6 ± 5.3
Number of episodes	3.4 ± 1.6	3.7 ± 1.6
Episode duration (minutes)	3.4 ± 1.2	3.8 ± 0.5
Frequency (episodes/hour)	0.9 ± 0.4	0.9 ± 0.4
Latency (minutes)	111.2 ± 64.9	116.9 ± 62.8

Mean ± standard error. *, $P < 0.05$, two tailed paired Student *t*-test. Data was obtained from 3 animals. Each animal received 9 controls and 3 bicuculline microinjections.

Table 2
Waking and sleep before and after the microinjection of muscimol into the MnPN.

	Control	Muscimol
Wakefulness		
Time (minutes)	116.0 ± 8.8	173.3 ± 11.8**
Number of episodes	64.5 ± 14.4	51.3 ± 10.2
Episode duration (minutes)	2.2 ± 0.7	4.1 ± 1.2*
Frequency (episodes/hour)	16.1 ± 3.6	12.8 ± 2.5
NREM sleep		
Time (minutes)	103.8 ± 5.2	59.8 ± 7.5**
Number of episodes	65.1 ± 14.7	51.2 ± 10.3
Episode duration (minutes)	1.8 ± 0.42	1.3 ± 0.1
Frequency (episodes/hour)	16.3 ± 3.7	12.8 ± 2.6
Latency (minutes)	3.9 ± 0.3	5.9 ± 2.4
REM sleep		
Time (minutes)	20.2 ± 4.3	6.9 ± 4.7**
Number of episodes	4.5 ± 0.8	2.1 ± 1.2*
Episode duration (minutes)	4.9 ± 0.4	2.4 ± 0.7*
Frequency (episodes/hour)	1.1 ± 0.2	0.5 ± 0.3*
Latency (minutes)	68.4 ± 24.9	101.3 ± 40.2

Mean ± standard error. *, $P < 0.05$; **, $P < 0.005$, two-tailed paired Student *t*-test. Data was obtained from 3 animals. Each animal received 9 controls and 3 bicuculline microinjections.

Table 2). This effect was most pronounced during the second and third hour of recording (Figs. 3B and 5). NREM sleep latency was not modified. As shown in Figs. 3B and 5, REM sleep was also affected by muscimol microinjections, which resulted in a reduction of 66% compared with controls ($P < 0.005$, Table 2). The decrease in REM sleep was due to a decrease in both the frequency and duration of the episodes ($P < 0.05$, Table 2). The latency to REM sleep was not affected.

The time spent in *W* increased 49% after muscimol microinjections compared with controls ($P < 0.005$); this increase was due to an increase in episode duration ($P < 0.05$, Table 2). This increase in the time spent in *W* was evident and statistically significant principally during the second and third hour of recording (Figs. 3B and 5).

drawing of a representative coronal section of the MnPN (AP level: +14.5 according to [23]). Each dot represents a GABA immunoreactive cell body. ac, anterior commissure; C, caudate nucleus; LV, lateral ventricle; POA, preoptic area; SON, supraoptic nucleus; STN, triangular septal nucleus VLPO; ventrolateral preoptic nucleus; III, third ventricle. Calibration bars: A, 1 mm; BI and BII, 10 μ m; C, 100 μ m.

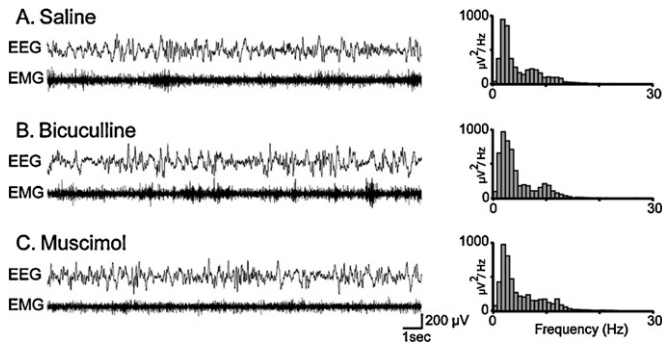


Fig. 2. Representative examples of raw recordings and power spectra of the frontal-parietal EEG during NREM sleep following GABAergic agents or saline microinjections within the MnPN. Note that the EEG recordings and power spectral histograms during NREM after the three different treatments are similar.

4. Discussion

In the present study we demonstrated the presence of GABAergic neurons in the MnPN of the cat. Furthermore, utilizing GABAergic agents that interact with the GABA_A receptor, we determined that the inhibition of GABAergic processes within the MnPN promotes wakefulness, while disinhibition produces NREM sleep.

4.1. Technical considerations

The cat is a classic species that has been employed in sleep research for decades; it exhibits long lasting and consolidated states

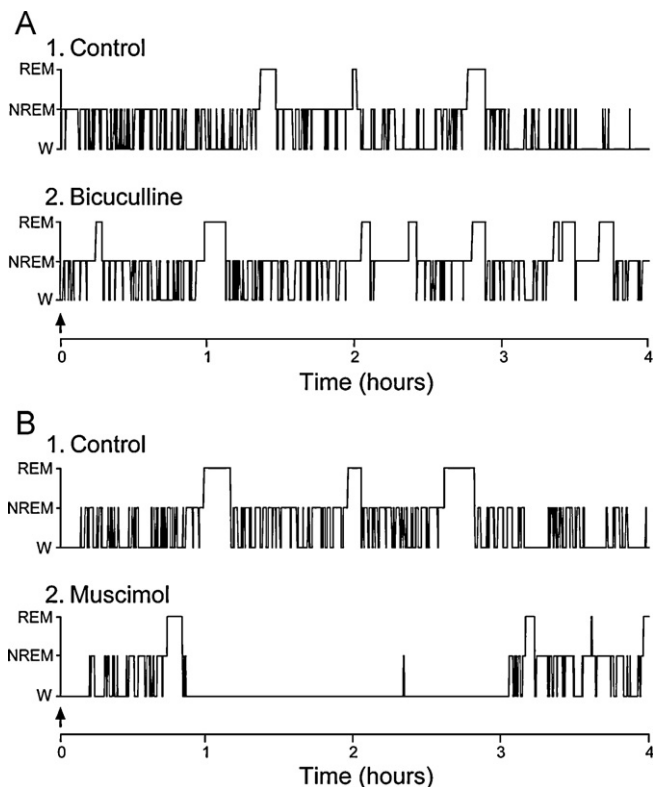


Fig. 3. Representative hypnograms of two animals after bicuculline (A) and muscimol (B) microinjections. Arrows indicate the time of the microinjections. Bicuculline (GABA_A antagonist; 0.1 mM, 0.2 μl) that was microinjected into the MnPN resulted in an increase in the total amount of sleep and a decrease in wakefulness. The microinjection of muscimol (GABA_A agonist; 25 mM, 0.2 μl) produced a marked decrease in the time spent in sleep, mainly during the second and third hour of recording. Control microinjections are shown in A1 and B1.

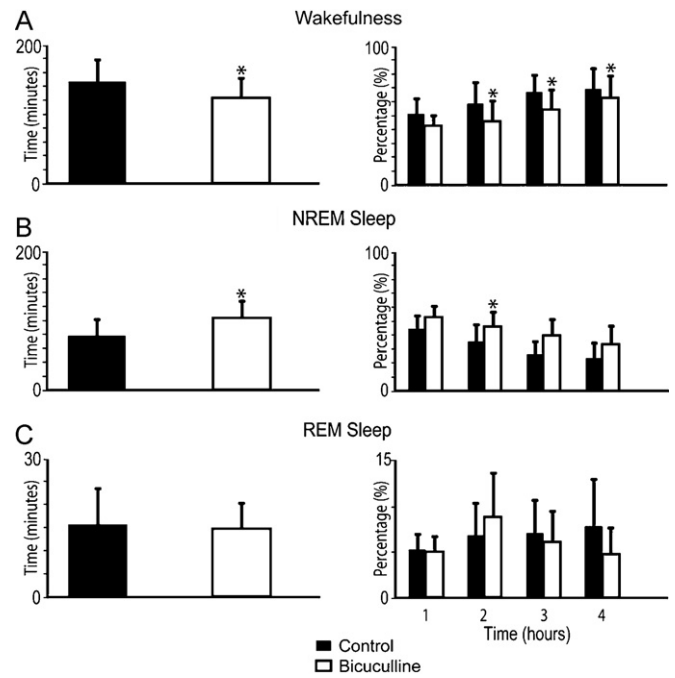


Fig. 4. Effects on sleep of bicuculline microinjections into the MnPN. The graphic charts show the mean time spent during the total time of the recording (left column) and the mean percentage time in blocks of one hour (right column) during wakefulness, NREM and REM sleep after bicuculline or control microinjections. Group mean differences are indicated by asterisks (*, $P < 0.05$; two-tailed paired Student t -test).

of NREM and REM sleep as well as wakefulness [26]. Since its brain size is large compared with many other species, such as mouse or rat, intracerebral microinjections can be more readily restricted to the site of interest, which is particularly important when dealing with a small nucleus such as the MnPN [23]. Nevertheless, bicuculline or muscimol could have diffused outside the MnPN and

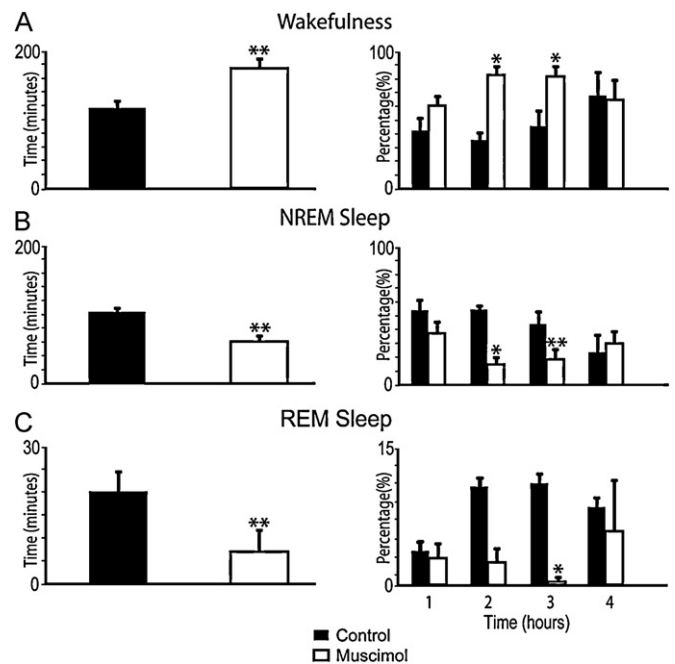


Fig. 5. Effects on sleep of muscimol microinjections into the MnPN. The mean time spent during the entire recording period (left column) and the mean percentage of time in blocks of one hour (right column) during wakefulness, NREM and REM sleep after muscimol or control microinjections. Group mean differences are indicated by asterisks (*, $P < 0.05$; **, $P < 0.005$; two-tailed paired Student t -test).

affected the activity of adjacent neurons within the POA. However, the local microinjection of 0.5 μ l of muscimol in the cat has a maximum effective diffusion radius of 1.2 mm [27]. Thus, the administration of 0.2 μ l would not be expected to exceed 0.5 mm. It is also well established that the diffusion rate of a drug depends on several variables such as its diffusion coefficient, molecular weight, the characteristics of the brain region where the injections is performed, etc. [28]. The molecular weight of bicuculline (509.3 g/mol) is higher than that muscimol (195 g/mol), which results in a comparative smaller diffusion radius. Therefore, it is unlikely that either bicuculline or muscimol reached the VLPO, the other critical sleep-related area of the POA, which is at a distance of 2–3 mm from the MnPN [23].

As in previous studies, several microinjections were carried out in the same animal [24,25,29]. We assume that the integrity of neurons in the nucleus was not been critically altered due to the fact that sham sessions were intercalated between microinjections of either saline or drug, and the results of sham microinjections were similar to baseline recordings and to saline microinjections; it is known that lesions in the MnPN affect the time spent in sleep and wakefulness [30].

It is important to point out that in spite of the long adaptation period and the animals' quiescent behavior in the recording set up, the cat that only received the bicuculline microinjections had a small amount of sleep during control experiments; therefore, we cannot discard the possibility that stress contributed to these results. Nevertheless, bicuculline increased NREM sleep time (57.5 ± 6.6 vs 30.6 ± 6.3 min, $P=0.02$) and decreased its latency (3.0 ± 1.3 vs 17.9 ± 2.9 , $P=0.004$; two-tailed unpaired *t*-test) in comparison to control microinjections in this animal. The low amount of control sleep of this animal increased the variability when the entire population of animals was analyzed; however, after we made a paired analysis, the effects of bicuculline were still evident.

4.2. GABAergic neurons of the MnPN

We were able to identify the cell bodies of GABA-immunoreactive neurons within the MnPN. To the best of our knowledge, this is the first description of the presence of GABAergic neurons in the MnPN of the cat, although neurons immunoreactive to GABA has been found in different subareas of the POA [31]. This result is also in accordance with several studies in rodents where GABAergic neurons have been observed within the MnPN [2,3,32,33].

4.3. Role of the MnPN in the generation of sleep

The MnPN is located dorsal and ventral to the anterior commissure, at the midpoint along the lamina terminalis [34–36]. In relation to its putative role in sleep, we have previously reported that the number of Fos+ neurons within the MnPN of the cat (but not in the VLPO) increases during NREM sleep and is positively correlated with the time spent in this state [18]. Neurons in the MnPN of the rat, but not in the VLPO, exhibit an increase in Fos protein immunoreactivity during NREM sleep induced by interleukin-1 or by a warm environment [6,8]. The preceding data, together with our demonstration that the microinjection of bicuculline into the MnPN increases NREM sleep, suggest that this nucleus plays a role in the generation of this sleep state.

The administration of the GABA_A agonist muscimol into the MnPN increased wakefulness; this effect was produced by a decrease in both NREM and REM sleep. Suntsova et al. found that most neurons in the MnPN of the rat exhibit an elevated firing rate during both NREM and REM sleep [7]. Similar proportions of sleep active (31.9%) and waking active (33.0%) neurons, together

with many waking/REM sleep active neurons (23.4%), have been recorded in the MnPN of the mice [37]. These data suggest that the MnPN may have a role not only in NREM but also in REM sleep. On the other hand, Baker et al. found that interleukin-1, which increased the number of Fos+ neurons in the MnPN, also increased NREM sleep despite the suppression of REM sleep [8]; these authors suggested that the MnPN is involved in the generation of NREM sleep, but not REM sleep. Our previous results in cats also indicate a strong relationship between the activity of MnPN neurons and the generation and maintenance of NREM, but not REM sleep [18]. In a recent study, Hsieh et al., [14] have shown that MnPN neurons that project towards the vPAG, that has been involved in the control of sleep [25], are active during NREM but not during REM sleep. Therefore, the reduction of REM sleep by muscimol microinjections could be just a consequence of the transient suppression of NREM sleep [38]. Therefore, we consider that new data are needed in order to understand the role of the MnPN in REM sleep.

Suntsova et al. [16] studied the functional role of projections from the MnPN to neurons located in the perifornical and lateral hypothalamic region (PF/LH), which is an area where peptidergic neurons that produce hypocretins and melanin concentrating hormone (MCH) are located [39,40]. Neurons that are active during wakefulness within the PF/LH, which are likely hypocretinergic [41–43], are inhibited by electrical or chemical stimulation of the MnPN. On the other hand, sleep-active neurons that are likely MCHergic [44] are activated [16]. In addition, perfusion of muscimol into the MnPN of the rat suppresses sleep and increases the activity of most wake-on neurons; a reduction in the firing rate of the majority of the sleep-active neurons within the PF/LH was also observed [16]. These data suggest that different groups of neurons in the MnPN promote sleep by regulation of sleep and waking systems.

In hour-by-hour analyses, we observed a delay in the sleep promoting effect of bicuculline as well as in the awakening effect of muscimol (Figs. 3–5). In order to interpret these results, it is important to take into account the fact that the MnPN has been shown to have homeostatic functions with respect to sleep regulation. According to Gvilia, activation of MnPN GABAergic neurons in rats is principally related to sleep pressure and not to sleep amount [45]. Therefore, one possible explanation for the delayed response is that, following the administration of bicuculline, MnPN neuronal activity may increase the pressure to sleep, but not generates it directly. Our working hypothesis is that the MnPN regulates sleep pressure by facilitating the sleep-promoting functions of the VLPO; in order to test this hypothesis, a detailed study of the functional interactions between the MnPN and the VLPO is required.

5. Conclusions

The MnPN of the cat is involved in the generation of NREM sleep. GABA acting through GABA_A receptors within this nucleus plays a critical role in this process.

Conflict of interest

None.

Acknowledgments

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