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Research report

GABAergic processes within the median preoptic nucleus promote NREM sleep

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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 peptic 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 immunorreactivity 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 immunorreactivity 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

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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 74). 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 ± 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 ± 0.5 μm. 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 ($P < 0.005$,

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