Sleep is differently modulated by basal forebrain GABA_A and GABA_B receptors

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Manfridi, Alfredo, Dario Brambilla, and Mauro Mancia. Sleep is differently modulated by basal forebrain GABA_A and GABA_B receptors. Am J Physiol Regulatory Integrative Comp Physiol 281: R170–R175, 2001.—There is evidence that GABA plays a major role in sleep regulation. GABA_A receptor agonists and different compounds interacting with the GABA_A receptor complex, such as barbiturates and benzodiazepines, can interfere with the sleep/wake cycle. On the other hand, there is very little information about the possible role of GABA_B receptors in sleep modulation. The nucleus basalis of Meynert (NBM), a cholinergic area in the basal forebrain, plays a pivotal role in the modulation of sleep and wakefulness, and both GABA_A and GABA_B receptors have been described within the NBM. This study used unilateral infusions in the NBM to determine the effects of 3-hydroxy-5-aminomethylisoxazole hydrobromide (muscimol hydrobromide), a GABA_A receptor subtype agonist) and 5-aminomethylisoxazole hydrobromide (muscimol hydrobromide), a GABA_A receptor subtype agonist) on sleep parameters in freely moving rats by means of polygraphic recordings. Muscimol (0.5 nmol) and baclofen (0.7 nmol) induced an increase in slow-wave sleep and an inhibition of wakefulness. Muscimol, but not baclofen, also caused a decrease in desynchronized sleep parameters. The results reported here indicate that 1) the NBM activation of both GABA_A and GABA_B receptors influences the sleep/wake cycle, and 2) GABA_B receptors not GABA_A receptors are important for desynchronized sleep modulation, suggesting that the two GABAergic receptors play different roles in sleep modulation.

GABA, the most abundant inhibitory neurotransmitter in the central nervous system, interacts with two different receptor subtypes, namely GABA_A and GABA_B. Both cause inhibition, but they are coupled to different ionic mechanisms; activation of the GABA_A receptor complex increases membrane conductance for chloride ions, producing postsynaptic inhibition (for review, see Ref. 37). GABA_B receptors operate through second messengers, causing increased outward K^+ conductance or decreased Ca^{2+} conductance (for review, see Ref. 9).

The basal forebrain is involved in the control and maintenance of arousal and sleep states (for reviews, see Refs. 38, 40). The nucleus basalis of Meynert (NBM), a cholinergic nucleus that provides the major extrinsic cholinergic innervation of the neocortex (20), lies in the caudal part of the basal forebrain. Recent data have highlighted the importance of NBM in sleep and wakefulness modulation in cats (2), dogs (28), and rats (25, 26). GABAergic projections to the NBM arising from the ventral striatum and the nucleus accumbens (42) as well as from the amygdala (33) have been described. Moreover, the GABAergic neurons in the NBM outnumber cholinergic neurons by 2:1 (13). Many of these NBM GABAergic cells are locally projecting interneurons, but a subpopulation of long projecting GABAergic neurons has also been described (14).

Many experimental studies have demonstrated that local injections of GABA_A agonists into the basal forebrain affect cortical cholinergic activity by reducing cortical acetylcholine turnover (39), acetylcholine release (41), and high-affinity choline uptake (6). In agreement with an electrophysiological study in which projecting cells from the NBM, putative cholinergic neurons, were inhibited by GABA (17), these data strongly suggest that the activity of NBM cholinergic neurons is controlled by a GABAergic input.

Experimental and clinical evidence indicates that GABA_B receptors play a major role in sleep regulation. Several compounds interfering with the sleep-wake cycle, including barbiturates and benzodiazepines, are agonists to the different binding sites of the GABA_A receptor complex (37). Benzodiazepines shorten sleep latency, increase slow-wave sleep (SWS), and inhibit desynchronized sleep (DS) (18). Furthermore, it has been shown that in rats the peripheral administration of muscimol, a GABA_A receptor agonist, increased the time spent in SWS and DS (18), and local injections of muscimol in the posterior hypothalamus (23) and in the periaqueductal gray (36) of cats can modify normal SWS and/or DS. Despite these observations, the effects of GABA_A receptors on sleep regulation are not yet fully understood. Moreover, not just GABA_A but also GABA_B receptors are distributed throughout the NBM of the rat (8). However, data about NBM modulation by GABA_B receptors and about the effects of GABA_B receptors on sleep are very scarce (10, 11, 22).

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Taking into consideration the presence of GABAergic afferents and of GABA_A and GABA_B receptors in the NBM and the importance of this nucleus in sleep and wakefulness modulation, this study was designed to assess how GABAergic manipulations of the rat NBM affected sleep and wakefulness parameters. We tested the hypothesis that both GABA_A and GABA_B receptors in the NBM play a significant role on sleep/wakefulness behavior by in vivo electroencephalographic (EEG) recordings of naturally sleeping-waking rats that had previously been injected intra-NBM with muscimol and baclofen.

MATERIALS AND METHODS

Animals and recording apparatus. The experiments were conducted in compliance with international laws and policies for the care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals [DHEW Publication No.(NIH) 85–23, Revised 1985, Office of Science and Health Reports, DHHS/NIH, Bethesda, MD 20205]; European Community Council Directive 86/609, OJ L 358,1, Dec. 12, 1987). Male albino rats (CD-COBS, Charles River, Calco, Italy; 250–300 g) were anesthetized (pentobarbital sodium 40 mg/kg + chloral hydrate 180 mg/kg ip), positioned in a stereotaxic apparatus (David Kopf, Tujunga, CA), and surgically provided with EEG and nuchal electromyographic (EMG) electrodes to monitor states of sleep and wakefulness. A stainless steel guide cannula (length 1.5 cm; outer diameter 0.5 mm) with an indwelling stylet was stereotaxically implanted unilaterally, with its tip placed 2 mm above the NBM to minimize cellular damage on the injection site [Fig. 1: 1.4 mm posterior to bregma, 2.4 mm lateral from the midline, and 5 mm below the surface of the dura mater; coordinates according to Paxinos and Watson (33a)]. The EEG and EMG electrodes were connected through insulated leads to an integrated circuit socket attached to the skull (tetether and slip ring). The experiments were started after 2–3 days of habituation to the cable. The animals were individually housed on a 12:12-h light-dark cycle (lights on 0900) at 23 ± 1°C and had free access to food and water.

The drugs were administered through a stainless steel needle inserted into the guide cannula and connected by polyethylene tubing to a 0.5-µl Hamilton microsyringe. The needle extended 2 mm past the tip of the guide cannula, its tip resting in the NBM. Polygraphic recordings (obtained by means of a Grass model 7 polygraph) were visually scored in 30-s epochs subdivided into wakefulness (W), SWS, and DS. The following parameters were taken into account: the percentage of time spent in each phase (W, SWS, and DS), SWS latency (defined as the interval between the microinjection and the appearance of the first SWS episode), and DS latency (defined as the interval between the first SWS episode and the appearance of the first DS episode). Finally, because changes in the percentage of time spent in DS may only be due to a general effect on total sleep time, the time spent in DS as a percentage of total sleep (DS/total sleep) was also considered, as this parameter can highlight a specific effect on DS.

On completion of the experiments, an overdose of chloral hydrate was administered, and dye (50 nl Pontamine sky blue 1%) was microinjected in the site of the microinjections; the brains were then removed and fixed. The position of the dye spot was reconstructed on 40-µm-thick, frozen, neutral red- or cresyl violet-stained sections (Fig. 1).

Drugs. The drugs used were muscimol hydrobromide (3-hydroxy-5-aminomethylisoxazole hydrobromide), a GABA_A receptor agonist, and baclofen (β-(aminomethyl)-4-chlorobenzenepropanoic acid), a GABA_B receptor agonist. All the drugs were purchased from Research Biochemicals International (Natick, MA). The substances were dissolved in 0.9% saline, and the solutions were adjusted to pH 7 with NaOH.

Experimental protocol. A total of 16 rats was used. Three were discarded because of inappropriate placement of the cannulas. Five rats were used in pilot experiments and injected with different doses of baclofen and muscimol. The remaining eight were recorded after administration of vehicle or test substances, so each served as its own control. All eight animals received three microinjections: one vehicle, one muscimol, and one baclofen. Experiments were randomly scheduled with intervals of at least 3 days between them, and none of the animals received the same treatment twice. Experiments were done at 10:00 AM, during the light phase of the light-dark cycle, when rats are mostly asleep. The rats were picked up and held throughout the needle insertion and injection. Immediately after insertion of the needle, the substances were injected unilaterally in a constant volume of...
100 nl in 1 min. After the microinjections, the needle was left in place for 1 min. During the 5-h experiment, each animal's behavior was studied with the help of a closed-circuit video camera.

**Statistical analysis.** Experimental variables were analyzed using repeated-measures ANOVA, with Tukey’s test for post hoc comparisons. The values are given as means ± SE.

**RESULTS**

ANOVA revealed a significant treatment effect \([F = 7.31; \text{df}: 2,14; P = 0.006]\) of GABA_A and GABA_B receptor stimulation within the NBM on the percentage of time spent in W during the 5-h recording. The control value (vehicle) was 29.8 ± 2.1. Muscimol (0.5 nmol, corresponding to 100 ng), a specific agonist to the GABA_A receptor subtype, and baclofen (0.7 nmol, corresponding to 150 ng), a specific agonist to the GABA_B receptor subtype, significantly reduced the time spent in W to 24.3 ± 1.6 and 21.7 ± 0.8, respectively. ANOVA also indicated a significant effect \((F = 16.6; \text{df}: 2,14; P = 0.002)\) of GABA_A and GABA_B receptor stimulation within the NBM on the percentage of time spent in SWS. The control value (vehicle) was 59 ± 1.9. Muscimol and baclofen both increased the time spent in SWS: to 68.4 ± 1.8 and 65.5 ± 0.9, respectively. Finally, ANOVA revealed a significant treatment effect \((F = 15.5; \text{df}: 2,14; P = 0.000)\) of GABA_A and GABA_B receptor stimulation within the NBM on the percentage of time spent in SWS. The control value (vehicle) was 11.2 ± 0.7. Muscimol reduced the time spent in SWS: 7.3 ± 1. Baclofen had no effect on this parameter (12.8 ± 0.9).

Figure 2 shows the hourly totals, to underline the time course and the maximum effects of the drugs injected. Statistical analysis revealed significant effects of muscimol and baclofen on the first 2 h of recording: SWS was increased and W decreased. Muscimol had a significant effect on DS from the 1st to the 3rd hour of recording.

Figure 3 shows the drugs’ effects on SWS and DS latencies and on the percentage of time spent in DS vs. total sleep. ANOVA revealed a significant treatment effect \((F = 11.8; \text{df}: 2,14; P = 0.001)\) on SWS latency. The control value (vehicle) was 20.5 ± 2.7 min. Muscimol and baclofen reduced the SWS latency to 7.3 ± 1.5 and 12.3 ± 1.8 min, respectively. ANOVA also found a significant treatment effect \((F = 10.3; \text{df}: 2,14; P = 0.001)\) on DS latency. The control value (vehicle) was 27.8 ± 4.8 min. Muscimol increased this to 101.6 ± 20.7 min. Baclofen had no significant effect on this parameter (37.3 ± 3.3 min). Finally, ANOVA showed a significant treatment effect \((F = 19; \text{df}: 2,14; P = 0.000)\) on the percentage of time spent in DS vs. total sleep (DS/total sleep), an important parameter to highlight specific effects on DS. The control value was 15.8 ± 0.9. Muscimol reduced this to 9.6 ± 1.3. Baclofen had no effect on this parameter (16.5 ± 1.2).

**DISCUSSION**

In this study, the stimulation of GABA receptors in the NBM significantly increased the amount of time spent in SWS and SWS latency and reduced the amount of time spent in W, despite the fact that the GABAergic agonists muscimol and baclofen were administered at the beginning of the light period, a time of day when the incidence of spontaneous sleep is already very high in this species. This confirms previous reports of the pivotal role of the NBM in SWS modulation (for reviews, see Refs. 38, 40).

There is substantial evidence that NBM cholinergic neurons promote cortical arousal (7, 31, 40). Previous in vivo studies have shown that GABA provides an inhibitory input to the NBM cholinergic neurons (6). Furthermore, an in vitro study showed that cholinergic NBM neurons were inhibited by GABA and by the GABA_A agonist muscimol (16). On the basis of these
data, we can put forward the hypothesis that muscimol injected in the NBM may influence sleep through a direct effect on cholinergic neurons by activating GABA<sub>A</sub> receptors.

On the other hand, the NBM has approximately equal proportions of cholinergic, GABAergic, and non-GABAergic-noncholinergic neurons; cholinergic and noncholinergic neurons are codistributed and intermingled (13). A substantial proportion of the GABAergic and nonGABAergic-noncholinergic cells is cortically projecting neurons (14). Considering the high expression of the GABA<sub>A</sub> receptor on noncholinergic neurons (15), muscimol may possibly have a direct effect on GABAergic and/or nonGABAergic-noncholinergic neurons. In the in vitro study performed by Khateb and colleagues (16) found that the GABA<sub>B</sub> agonist baclofen had no effects on cholinergic NBM neurons. Thus the baclofen-induced increase in SWS and decrease in W observed in the present study are unlikely to be due to a direct effect on cholinergic NBM neurons. It is possible to hypothesize that GABA<sub>B</sub> receptors may indirectly inhibit cholinergic activity by inhibiting excitatory glutamatergic afferents to NBM cholinergic cells (10). However, considering that the precise location of the GABA<sub>B</sub> receptors in the NBM is still not known, baclofen could also act on any noncholinergic neuron in the NBM.

In addition to the effects on SWS and W, the present study also found that muscimol had striking effects on DS. The amount of time spent in DS decreased and DS latency highly increased. These results seem to be a direct consequence of GABA<sub>A</sub> receptor stimulation, because NBM microinjections of baclofen had no effects on DS.

Very little information is available on how NBM affects DS. It has been shown that NBM injections of cholinergic (2, 26, 28) and glutamatergic (25) agonists can affect DS. These findings, together with the present results, raise the question of how the NBM interacts with the brain stem areas that are well known to play a pivotal role in DS control (21).

The locus ceruleus (LC) noradrenergic neurons and the dorsal raphe (DR) serotonergic neurons cease firing during DS (27, 35). Several lines of evidence suggest that the cessation of activity of these neurons is permissive to the induction of DS (1). Inhibition of these neurons seems to be responsible for their reduced firing, and it has recently been suggested that DR and LC neurons are actively inhibited by a GABAergic population during DS (29, 30). In the NBM, a subpopulation of GABAergic neurons that give rise to descending projections to the brain stem has been described (13, 14). Moreover, GABAergic neurons projecting to both LC and DR neurons have been located in different brain areas, including the ventral pallidum/substantia innominata complex (12, 24, 34), a region partially corresponding to the NBM. If GABAergic neurons in the NBM are part of a caudally projecting inhibitory system acting on the LC and DR, muscimol is likely to reduce DS by inhibiting these NBM GABAergic projecting neurons. The inhibition of this inhibitory system would prevent the cessation of activity of LC and DR neurons that is permissive to the induction of DS.

In our experiments, baclofen, unlike muscimol, did not have any specific effects on DS. Very few studies have dealt with the role of GABA<sub>B</sub> receptors in sleep behavior. An in vivo study showed that a GABA<sub>B</sub> antagonist, CGP-35348, peripherally injected into rats increased W, whereas SWS decreased; DS increased only after a high dose of CGP-35348 (11). To find out whether in our experimental conditions a higher dose of baclofen had any effects on DS parameters, we
injected four rats with a double dose of baclofen (300 ng). The EEG showed an asymmetry: the injected side became hypersynchronous, whereas the controlateral side maintained a normal appearance with desynchronized and synchronized patterns (data not shown). Therefore, it seems that if GABA\textsubscript{B} receptors do have a role in DS modulation, their effect is due neither to an interaction with the NBM (present data) nor to an interaction with the periaqueductal gray area, where it has been shown that muscimol increases DS but baclofen has no effect (36).

In vivo studies demonstrated that peripherally administered muscimol and midazolam, a benzodiazepine, had very different effects on sleep: muscimol increased SWS and DS amount but had no effect on SWS latency. Midazolam increased SWS and reduced the amount of DS and the SWS latency (18). In the present study, muscimol injected into the NBM increased SWS but reduced the SWS latency and the amount of DS. The effects of NBM-injected muscimol seem similar to those of peripherally administered midazolam but different from peripherally administered muscimol. This might reflect the different routes of administration in the two studies. However, it has also been shown that peripherally injected tiagabine, a GABA uptake inhibitor, had an inhibitory effect on DS (19), and this is in line with the findings described in the present paper.

The possibility of the effects on sleep/wake parameters described here being due to motor impairments can be ruled out. GABA agonists injected into the caudal part of the ventral pallidum/substantia innominata complex, corresponding to the NBM, had no effects on locomotor activity (4, 10), and in this study we did not observe any motor syndrome.

The GABA agonists in the present study were injected in a volume of 100 nl. It has been estimated that for a muscimol injection of 500 nl, the maximal average radius of the drug diffusion should be 1.2 mm after 20 min (36). Considering the much smaller volume injected in this study and considering also the size of the NBM, our results are unlikely to be due to the drugs spreading into areas close to the NBM. This is supported by the different results in the three rats discarded from the experimental protocol because of inappropriate placement of the cannula (data not shown).

In one of these three, the cannula was placed by mistake in the ventrolateral thalamic nuclei. The EEG recorded after muscimol and baclofen injections was highly synchronized in both hemispheres even when the rat was awake and exploring or feeding. Contemporaneous presence of behavioral waking and synchronized EEG was never observed after GABAergic agonist injections into the NBM. In the other two discarded rats, the cannulas were implanted too rostrally, in the anterior part of the ventral pallidum. In these rats, the EEG recorded after GABAergic agonist injections showed an increase in W and a decrease in SWS and DS, the opposite of what we observed after GABAergic agonist injections into the NBM. These results are in agreement with data showing that muscimol injected into the rostral part of the ventral pallidum/substantia innominata complex enhanced motor activity (3, 5) and perfusion of the medial septum, a basal forebrain cholinergic nucleus next to the NBM and to the ventral pallidum, and reduced SWS while increasing cortical arousal and locomotor activity (32).

In conclusion, the main findings of this study indicate that exogenously administered GABAergic agonists acting on both GABA\textsubscript{A} and GABA\textsubscript{B} receptors in the NBM exert a modulatory influence on sleep. These results confirm and extend previous reports that both GABA\textsubscript{A} and GABA\textsubscript{B} receptors are involved in sleep-wake modulation. Moreover, the different effects of muscimol and baclofen on DS suggest that in the NBM only the GABA\textsubscript{A} receptors play a role in DS modulation.

REFERENCES


