INDUCTION OF WAKEFULNESS AND INHIBITION OF ACTIVE (REM) SLEEP BY GABAERGIC PROCESSES IN THE NUCLEUS PONTIS ORALIS

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INTRODUCTION

We have recently provided new data that indicate that reticular formation GABAergic inhibitory processes play a role in the generation and/or maintenance of wakefulness as well as being involved in the control of active (REM) sleep (42, 43, 44). These data emanated from studies in chronic cats in which prolonged periods of wakefulness are induced following the injection of GABA and $GABA_A$ agonists within a specific nucleus located in the rostral portion of the pontine reticular tegmentum, the nucleus pontis oralis (NPO) (4, 36, 39). The injection of the corresponding antagonists into the NPO in awake animals resulted in the rapid induction of active sleep, in many cases there being no intercalated episode of quiet (non-REM) sleep.

The objective of the present study was to generate additional, more detailed data regarding the behavioral effects of $GABA_A$ receptor agonists and antagonists and determine whether the functions subserved by the inhibitory processes involving $GABA_A$ receptors are also carried out by $GABA_B$ receptors. These two types of receptors, although responsive to the same ligand, GABA, mediate completely different types of inhibition. $GABA_A$ inhibition is a fast and powerful type of inhibitory process that operates through a postsynaptic increase in chloride conductance; $GABA_B$ inhibition is of a "slower" type mediated by G proteins and a cascade of intracellular second messenger-mediated events (21, 24, 25, 37, 38, 40). Therefore, we were interested in determining whether the electrophysiological responsiveness (via $GABA_A$ inhibition) as well as the metabolic state (via $GABA_B$ inhibition) of reticular neurons should be taken into account when the mechanisms of active sleep and wakefulness are studied.

Accordingly, polygraphic recordings and behavioral observations were obtained before and after $GABA_A$ and $GABA_B$ receptor agonists and antagonists were microinjected, separately, into the NPO in chronic, unanesthetized cats, and the effects of these drugs on the sleep and waking behaviors were then examined. The results of these experiments indicate that wakefulness can be induced by the activation of either of these two types of synaptic receptors and that active sleep occurs when these receptors are blocked. The resultant data suggest that both modalities of GABAergic inhibition of neuronal elements in the NPO play a role in maintaining the state of wakefulness. In addition, active sleep occurs when these

neuronal elements are released from inhibition by the suppression of GABAergic inhibitory processes.

METHODS

Animals and surgical procedures. - Five adult cats were used in the present study. The animals were prepared for monitoring the behavioral states of sleep and wakefulness and for drug administration, as previously described (26, 45). Briefly, under halothane anesthesia, using sterile surgical procedures, the cats were implanted with electrodes for recording the electroencephalogram (EEG), electrooculogram (EOG) and electromyogram (EMG). A Winchester plug, connected to these electrodes, and a chronic head-restraining device were bonded to the calvarium with acrylic cement. A hole 4-5 mm in diameter, which was drilled in the calvarium overlying the cerebellar cortex, was covered with bonewax. This hole provided for subsequent access for a cannula that was used for drug microinjection. Following surgery, antibiotics were administrated both systemically and topically.

After recovery from surgery, all cats were adapted to the recording conditions by placing them in a head-restraining apparatus each day for two weeks. Thereafter, whenever they were placed in the head-restraining apparatus, the animals exhibited spontaneous periods of wakefulness, quiet sleep and active sleep.

Drug administration. - Following the adaptation period, carbachol (0.25 µl, 22 mM in saline) was injected into the rostral pontine reticular formation (L: 2, P: 3, and H: -4; [2]) using a 2 µl Hamilton syringe. The injection syringe was connected to a remote-controlled hydraulic micropositioner so that the animals were not disturbed by the injection procedure. The effective NPO site was defined as the stereotaxic coordinates at which an injection of carbachol induced active sleep with a latency shorter than 4 min. In experimental sessions, all of which were conducted between 10:00 and 16:00 hours, muscimol (0.25 µl, 10 mM in saline), baclofen (0.25 µl, 20 mM in saline), bicuculline (0.25 µl, 1-15 mM in saline), and phaclofen (0.25 µl, 20 mM in saline) were microinjected, unilaterally, into the NPO. In all cats, control solutions of saline (0.25 µl) were injected into the same site that received the injection of drugs. Injections were made more than 30 minutes after the cats were placed in the recording apparatus and after they had exhibited at least one episode of quiet sleep. Drugs were delivered over a period of 1 minute while the animals were in quiet sleep. No injections were carried out during control sessions.

Polygraphic recording and data analysis. - The EEG, EOG and EMG were recorded on a videocassette recorder by means of a PCM recording adapter (Vetter Co., Model 4000) for off-line analysis. Polygraphic recordings, which were divided into 30 sec epochs, were used to construct hypnograms. States of wakefulness (W), quiet sleep (QS) and active sleep (AS) were scored according to standard polygraphic and behavioral criteria (41). The following dependent variables were determined based upon the polygraphic and behavioral observations for each recording session: (1) percentage of time spent in wakefulness, quiet sleep and active sleep during the first hour following the injection as well as throughout the 4-hour recording period; (2) latency to the onset of the first episode of each behavioral state, as measured from the time of the beginning of the injection; (3) number of episodes of each behavioral state per hour (frequency); and (4) duration of the longest episode of each behavioral state. Experimental data are expressed as means ± SEM. The statistical significance of the difference between sample means was evaluated using the two-tailed unpaired Student's t -test and an analysis of variance (ANOVA). The criterion chosen to discard the null hypothesis was $P < 0.05$.

Histological location of injection sites. - At the conclusion of the microinjection experiments, the site of drug injection was marked with 0.5 µl of a 2% solution of Chicago sky blue dye in 0.5 M Na-acetate. The animal was then euthanized with an overdose of Nembutal and perfused

with saline followed by a solution of 10 % formaldehyde. Serial sections of brainstem tissue were examined to verify the injection sites. The histological studies revealed that effective injection sites were located within the NPO (Figure 1).
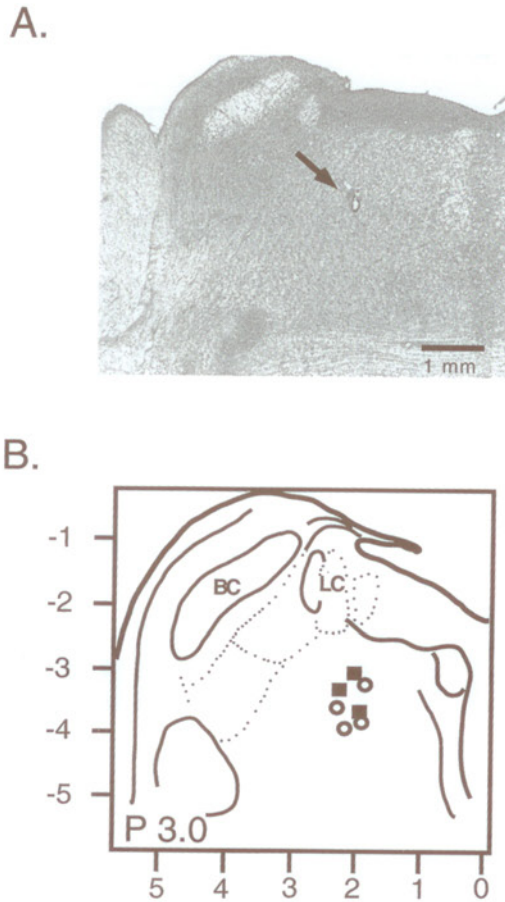

Fig. 1. - A: *Photomicrograph of a coronal section of the brainstem of a cat depicting the Chicago sky blue stained site of a representative injection of muscimol (arrow).*
   B: *The anatomical location of injection sites (n=7) in the rostral pons.*

The effective injections of both GABA$_A$ and GABA$_B$ agonists and antagonists were all within the NPO. A schematic frontal plane of the cat brainstem is illustrated at level P 3.0. Circles and squares indicate sites where injections were delivered to the left and right side, respectively. BC, Brachium conjunctivum; LC, locus coeruleus.
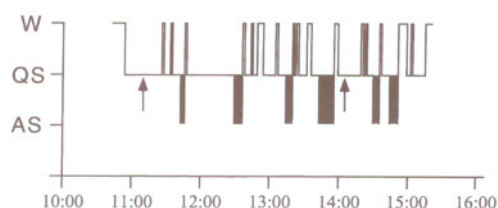
RESULTS

Forty-nine microinjections of drugs were made into the NPO to examine the effects of GABA$_A$ and GABA$_B$ receptor agonists and antagonists on sleep and waking states. In addition, nine control injections of saline were made into the
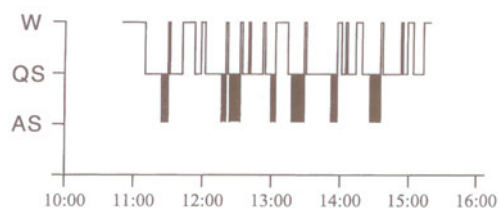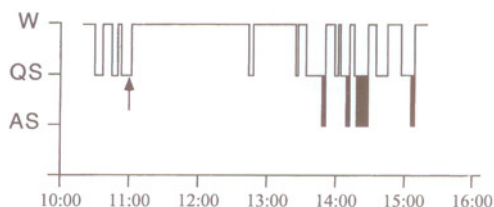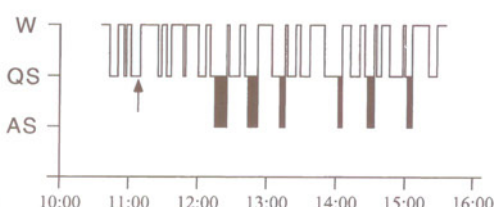
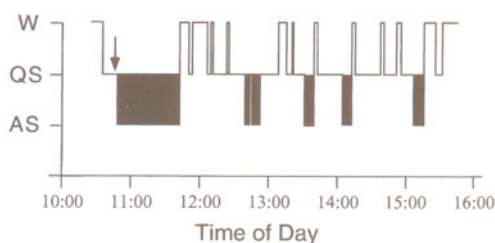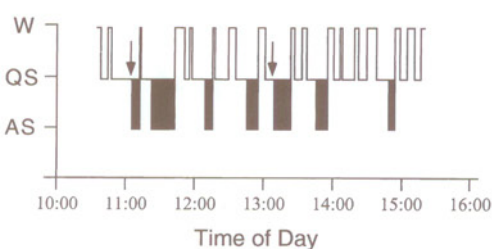Fig. 2. - *Hypnograms of control (A), saline (B), muscimol (C), baclofen (D), bicuculline (E) and phaclofen (F) recording sessions from a representative cat.*

The temporal distribution of behavioral states during a session with two injections of saline (vertical arrows in B) were similar to those observed during control sessions. Note that the injection of muscimol (vertical arrow in C), which was made during a quiet sleep episode, immediately induced wakefulness and blocked the subsequent occurrence of quiet sleep and active sleep for 106 and 141 minutes, respectively. The injection of baclofen (the vertical arrow in D) also immediately induced wakefulness and blocked the subsequent occurrence of active sleep for 67 minutes. In contrast, an injection of bicuculline into the NPO (vertical arrow in E) induced a short-latency, long-duration episode of active sleep. Each of two phaclofen injections into the NPO (two vertical arrows in F) induced, with a short latency, an episode of active sleep. W: Wakefulness, QS: quiet sleep, AS: Active sleep.

same site that received the injections of the preceding drugs. Figure 2 is a series of hypnograms showing the effects of microinjections of muscimol, baclofen, bicuculline and phaclofen in a representative cat. In contrast to saline injections, injections of muscimol and baclofen induced wakefulness, whereas bicuculline
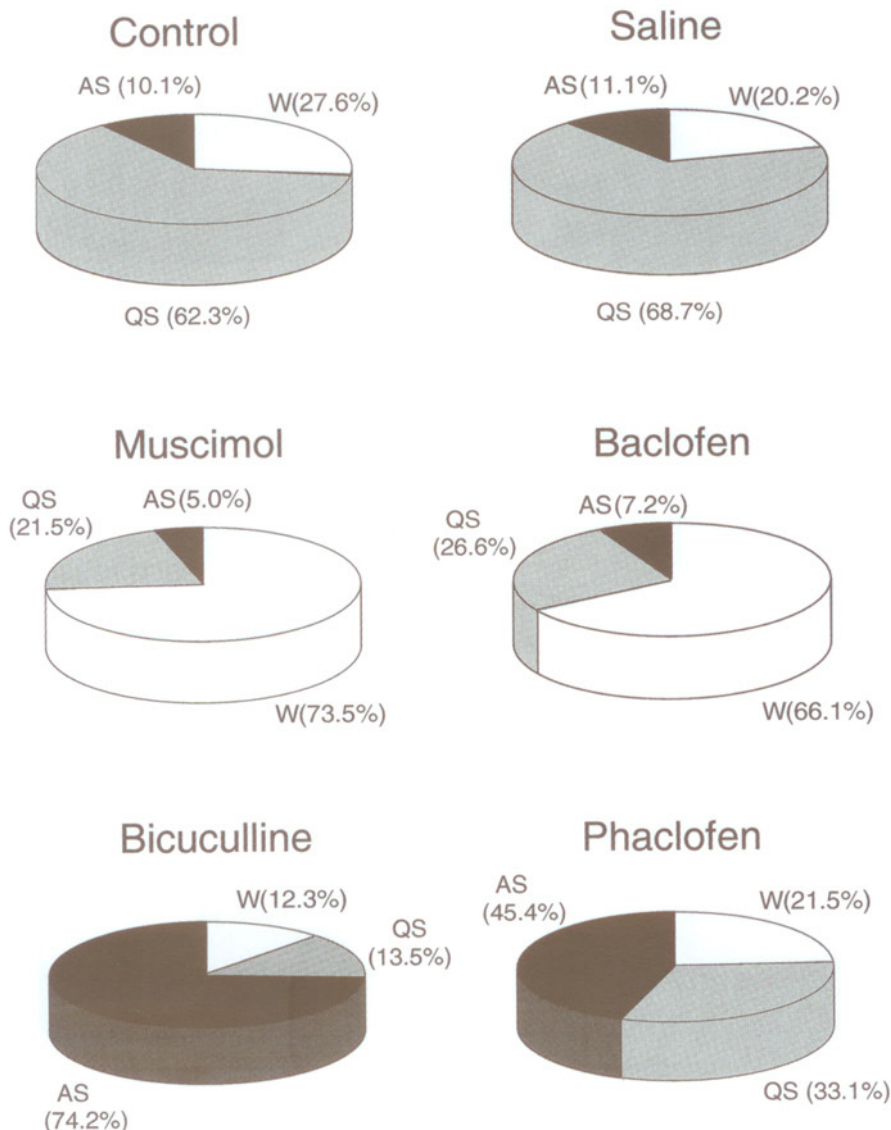
Control

AS (10.1%)   W(27.6%)

QS (62.3%)

Saline

AS(11.1%)   W(20.2%)

QS (68.7%)

Muscimol

QS (21.5%)   AS(5.0%)

W(73.5%)

Baclofen

QS (26.6%)   AS(7.2%)

W(66.1%)

Bicuculline

W(12.3%)

QS (13.5%)

AS (74.2%)

Phaclofen

AS (45.4%)   W(21.5%)

QS (33.1%)

Fig. 3. - *Pie charts presenting the mean percentage of time spent in wakefulness, quiet sleep and active sleep during the first hour following the injection of muscimol (n = 9), baclofen (n = 6), bicuculline (n = 10) and phaclofen (n = 6).*

No injections were carried out during control sessions (n = 12). Compared to the percentages observed following the injection of saline (n = 9), injections of muscimol and baclofen significantly increased the percentage of time spent in wakefulness (muscimol: P<0.01; baclofen: P < 0.05) and significantly decreased the percentage of time spent in quiet sleep (muscimol: P < 0.01; baclofen: P < 0.05). The percentage of active sleep also was significantly reduced following the injection of muscimol (P < 0.05). Injections of bicuculline or phaclofen, on the other hand, significantly increased the percentage of time spent in active sleep (P < 0.01) and decreased the percentage of the time spent in quiet sleep (P < 0.01).

Control

AS (12.2%)   W(37.1%)

QS (50.7%)

Saline

AS(13.3%)   W(33.7%)

QS (53.0%)

Muscimol

QS   AS(5.0%)
(25.2%)

W(69.8%)

Baclofen

AS(11.4%)   W(45.2%)

QS
(43.4%)

Bicuculline

AS   W(19.8%)
(37.7%)

QS (42.5%)

Phaclofen

AS
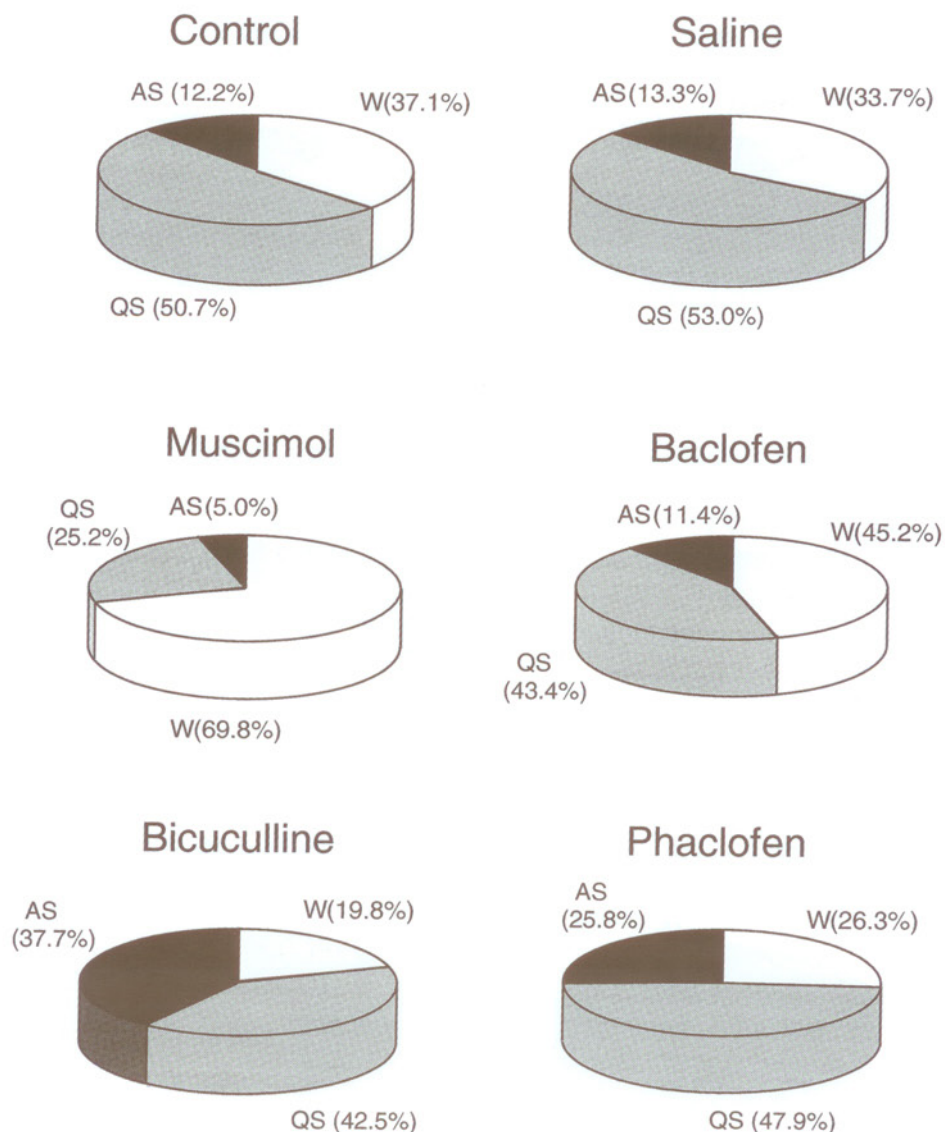(25.8%)   W(26.3%)

QS (47.9%)

Fig. 4. - *Pie charts presenting the mean percentage of time spent in wakefulness, quiet sleep and active sleep during the 4-hour recording period following the injection of drugs.*

No injections were carried out during control sessions (n = 12). Compared to the percentages observed following the injection of saline (n = 9), injections of muscimol significantly increased the percentage of time spent in wakefulness (P < 0.01) and decreased the percentage of time spent in quiet sleep (P < 0.01) and active sleep (P < 0.01). The injection of baclofen only increased the time spent in wakefulness (P < 0.01). In contrast, injections of bicuculline or phaclofen significantly increased the percentage of time spent in active sleep (P < 0.01). However, injections of bicuculline or phaclofen did not produce any significant changes in the percentage of the time spent in wakefulness or quiet sleep during the 4-hour recording period.

and phaclofen induced a behavioral state similar to naturally-occurring active sleep. As shown in Figure 2, both $GABA_A$ and $GABA_B$ receptor agonists and antagonists affected the latency, frequency and duration as well as the amount of time spent in sleep and waking states. The following sections describe, quantitatively, the effects of these drugs on states of wakefulness, quiet sleep and active sleep.

*Microinjections of muscimol and baclofen induced wakefulness.*

Microinjections of either muscimol (10 mM, n = 9) or baclofen (20 mM, n = 6) into the NPO during quiet sleep induced, with a short latency, wakefulness. The effects of muscimol and baclofen on the percentage of time that the cats spent in sleep and waking states during the first hour following the injection are presented in Figure 3. Compared to the percentage observed following saline injections (n = 9), injections of muscimol significantly increased the time spent in wakefulness (263.9%, P < 0.01) and significantly reduced the time spent in active sleep (55.0%, P < 0.05) and quiet sleep (68.7%, P < 0.01). Baclofen also significantly increased the time spent in wakefulness (227.2%, P < 0.05), and reduced the time spent in quiet sleep (61.3%, P < 0.05).

In order to determine the effect of injections of muscimol and baclofen for a longer period of time, an examination was made of the percentages of time spent in sleep and waking states for a 4-hour recording session following each injection. These results are summarized in Figure 4. Following the injection of muscimol, the changes in the percentage of time spent in wakefulness, quiet and active sleep during the 4-hr recording period were similar to those observed during the first hour, which indicates a long duration for the effect of muscimol. However, following the injection of baclofen, the changes in the time spent in either wakefulness or sleep during the 4-hour recording period were not statistically significant compared with control data (Figure 4). These results indicate that the response to baclofen was limited to the first hour following the injection.

The effects of microinjections of muscimol and baclofen were also assessed by measuring the latency to the onset of the first episode of wakefulness and active sleep following the injection, which was performed during quiet sleep (Figure 5). The mean latency of muscimol and baclofen-induced wakefulness was 4.0 ± 0.8 minutes and 3.9 ± 0.8 minutes, respectively. These values were significantly shorter compared with saline-injected controls (saline: 16.2 ± 2.8 minutes, n = 9). Injections of muscimol and baclofen also significantly increased the mean latency to active sleep (muscimol: 120.5 ± 20.2 minutes, n = 9; baclofen: 78.9 ± 15.9 minutes, n = 6; saline: 40.7 ± 4.1 minutes, n = 9).

The effects of muscimol and baclofen injections into the NPO on the frequency of sleep and waking states are shown in Figure 6. It should be noted that, due to the long duration of wakefulness induced by the injection of muscimol, the number of episodes of wakefulness, quiet sleep and active sleep was greatly reduced during the 4-hour recording period. Therefore, the frequency of these three behavioral states following the injection of muscimol was significantly less compared to the
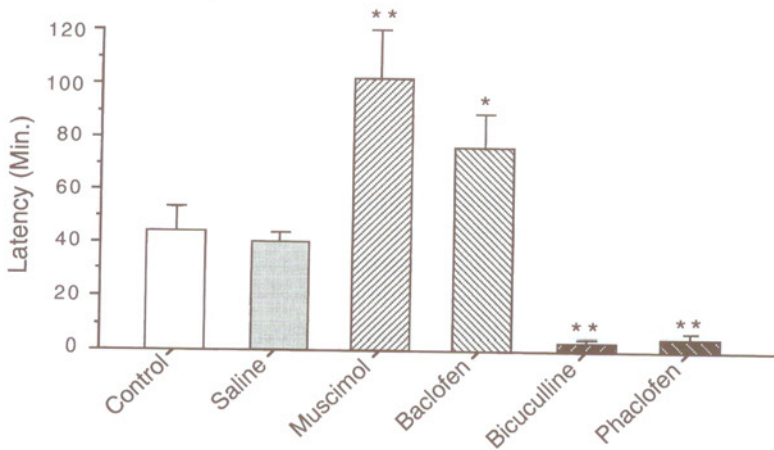
A. Wakefulness
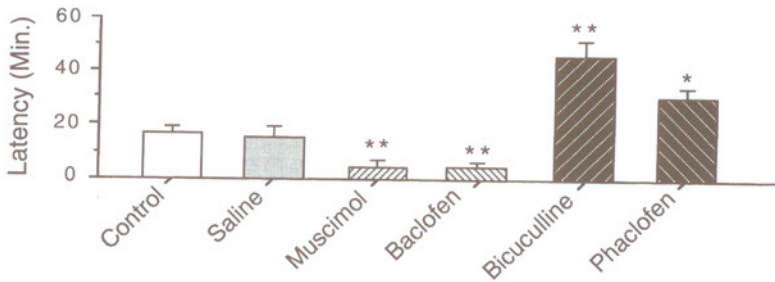
B. Active Sleep

Fig. 5. - *Effects of muscimol, baclofen, bicuculline and phaclofen on the latency of wakefulness and active sleep.*

These graphs show the latency to the onset of the first episode of wakefulness and active sleep either following injections or during control sessions. Each bar represents the mean latency; error bars indicate the SEM of each population. Injections of muscimol and baclofen significantly decreased the mean latency of wakefulness and increased the mean latency of active sleep. Injections of phaclofen or bicuculline, on the other hand, significantly increased the mean latency of wakefulness and reduced the mean latency of active sleep. Asterisks indicate the levels of statistical significance of the difference between means: *P < 0.05, **P < 0.01.

frequency of occurrence following saline injections (wakefulness: 2.2 ± 0.2 epi-sode/hour [muscimol] *vs.* 4.0 ± 0.3 episode/hour [saline], P < 0.01; active sleep: 0.6 ± 0.2 episode/hour [muscimol] *vs.* 1.1 ± 0.2 episode/hour [saline], P < 0.05; quiet sleep: 2.2 ± 0.2 episode/hour [muscimol] *vs.* 4.0 ± 0.3 episode/hour [saline], P < 0.01). However, baclofen injections did not produce any significant change in the frequency of these states.
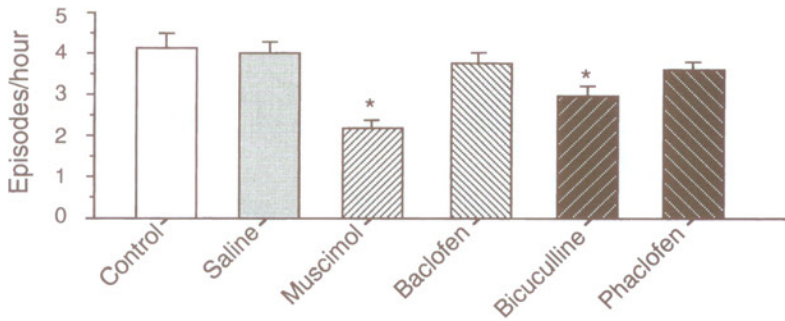
The effect on the duration of the longest episodes of each behavioral state was examined following the injection of muscimol or baclofen (Figure 7). Both muscimol

A. Wakefulness
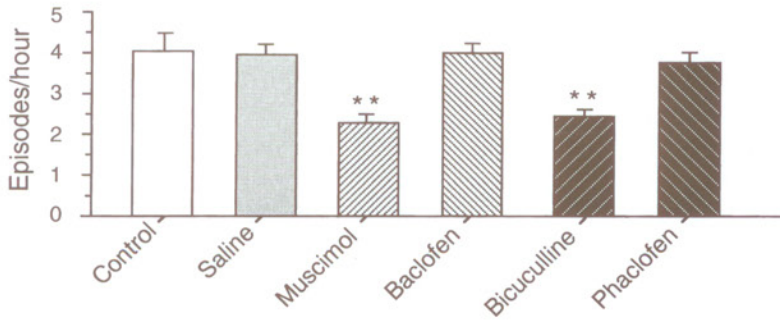
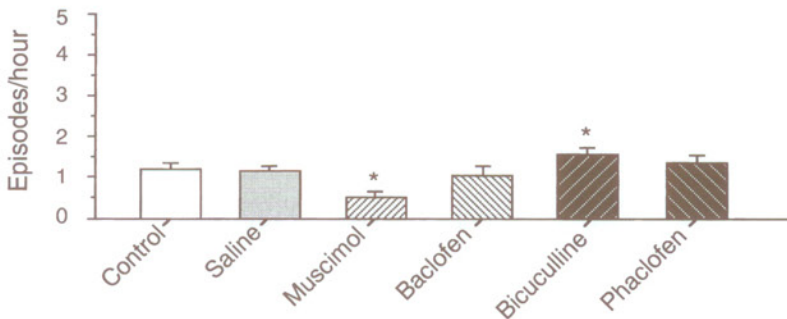## B. Quiet Sleep

C. Active Sleep

Fig. 6. - *Effects of muscimol, baclofen, bicuculline and phaclofen on the frequency of wakefulness, quiet sleep and active sleep.*

These graphs present the number of episodes of behavioral states per hour (frequency) following injections and during control sessions. Each bar represents the mean frequency; error bars indicate the SEM of each population. Injections of muscimol significantly reduced the frequency of all three behavioral states. Injections of bicuculline, on the other hand, significantly increased the frequency of active sleep. Injections of either baclofen or phaclofen had no effect on the frequency of any of these behavioral states. Asterisks indicate the levels of statistical significance of the difference between means: *P < 0.05, **P < 0.01.

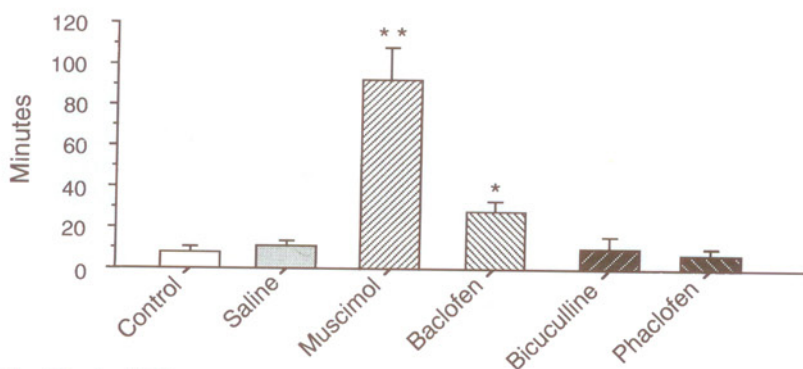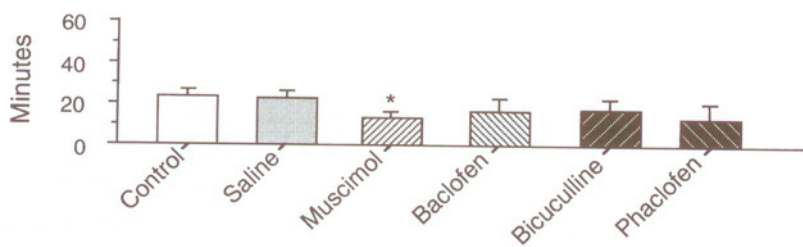## A. Wakefulness

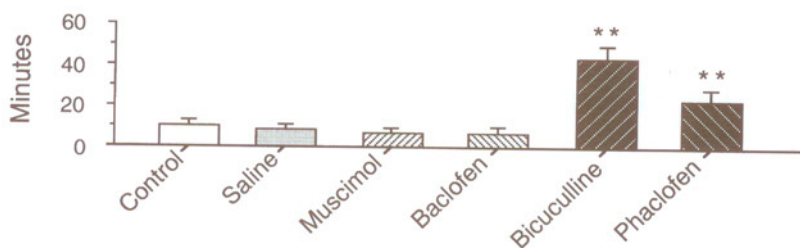## B. Quiet Sleep

C. Active Sleep

Fig. 7. - *Effect of muscimol, baclofen, bicuculline and phaclofen on the duration of wakefulness, quiet sleep and active sleep.*

These graphs show the duration of the longest episodes of wakefulness, quiet sleep and active sleep following injections and during control sessions. Each bar represents the mean duration; error bars indicate the SEM of each population. Injections of muscimol and baclofen significantly increased the mean duration of wakefulness. Injections of bicuculline and phaclofen, on the other hand, significantly increased the mean duration of active sleep. Asterisks indicate the levels of statistical significance of the difference between means: *$P < 0.05$, **$P < 0.01$.

and baclofen significantly increased the duration of wakefulness. These data are consistent with the above findings that muscimol and baclofen increased the time spent in wakefulness, and reduced the latency to wakefulness. The mean duration of muscimol-induced wakefulness, however, was significant longer than that of baclofen-induced wakefulness (muscimol: 93.9 ± 13.2 minutes vs. baclofen: 28.4

± 5.6 minutes, P < 0.01). The mean duration of quiet sleep was significantly reduced following the injection of muscimol (muscimol: 12.9 ± 2.3 minutes vs. saline: 22.9 ± 4.0 minutes).

Four control injections, which were placed in a region outside and posterior to the NPO (P: 4.3 ± 0.2 mm; Berman 1968), did not induce wakefulness. These injections also did not produce statistically significant changes in either the percentage of time spent in sleep and waking states or their latency, duration or frequency compared with those observed following the injection of saline into the NPO.

Bicuculline and phaclofen produce a prolonged active sleep.

Both bicuculline methiodide (n = 6) and bicuculline-free base (n = 4) were used in the present study to test the possibility that bicuculline methiodide was acting in a non-specific fashion (6). There was no statistical difference between the data obtained from these two groups, which indicates that the bicuculline-induced responses were due to the effects of blocking $GABA_A$ receptors. Consequently, the data from both groups were combined for analysis.

The effects of bicuculline (10 mM, n = 10) and phaclofen (20 mM, n = 6) injections on the percentage of time spent in sleep and waking states were examined during the first hour following the injection as well as throughout the 4-hour recording period (Figure 3 and Figure 4). During the first hour following the injection of bicuculline, the cats spent most of their time in active sleep. The increase in the percentage of time spent in active sleep following the injection of bicuculline was statistically significant compared to that following the injection of saline (568.5%, P < 0.01; Figure 3). This increase was accompanied by significant decreases of 80.3% and 38.9% in the time spent in either quiet sleep or wakefulness (P < 0.01 and P < 0.05, respectively). Phaclofen injections also significantly increased the time spent in active sleep (309.0%, P < 0.01) and reduced the time spent in quiet sleep (51.8%, P < 0.01) during the first post-hour injection.

There was a statistically significant increase in the time spent in active sleep during the 4-hour recording period following injections of bicuculline (183.5%, P < 0.01; Figure 4). The changes in the time spent in either quiet sleep or wakefulness during the 4-hour recording period, however, were not statistically significant (Figure 4). During the 4-hour recording period following the injection of phaclofen there was also a significant increase of in the time spent in active sleep (94.0%, P < 0.05), but there were no statistically significant changes in the time spent in quiet sleep or wakefulness. The above data indicate that the effects of both bicuculline and phaclofen were mainly due to changes that occurred during the first hour following the injection.

Microinjections of bicuculline and phaclofen induced an active sleep with a short latency (Figure 5). The mean latency of bicuculline and phaclofen-induced active sleep was 2.5 ± 0.5 and 3.1 ± 0.5 minutes, respectively. This mean latency was significantly shorter than the mean latency of active sleep following the injection of saline (saline: 40.7 ± 4.1 minutes, P < 0.01). Injections of bicuculline

or phaclofen also significantly increased the mean latency of wakefulness (bicuculline: 41.2 ± 5.2 minutes vs. saline: 16.2 ± 2.8 minutes, P < 0.01; phaclofen: 30.8 ± 4.5 minutes vs. saline: 16.2 ± 2.8 minutes, P < 0.05).

Changes in the frequency of sleep and waking states following bicuculline and phaclofen injections into the NPO are shown in Figure 6. Following the injection of bicuculline, the frequency of active sleep was significantly increased during the subsequent 4-hour recording period (bicuculline: 1.6 ± 0.1 episode/hour vs. saline: 1.1± 0.1 episode/hour, P < 0.05). On the other hand, the frequency of both wakefulness and quiet sleep was significantly reduced following the injection of bicuculline (wakefulness: 2.5 ± 0.2 episode/hour [bicuculline] vs. 4.0 ± 0.3 episode/hour [saline], P < 0.01; quiet sleep: 2.9 ± 0.2 episode/hour [bicuculline] vs. 4.0 ± 0.3 episode/hour [saline], P < 0.05). However, there were no significant changes in the frequencies of wakefulness, quiet sleep or active sleep following the injection of phaclofen compared with those following the injection of saline.

Microinjections of bicuculline and phaclofen induced a long duration episode of active sleep. The mean duration of the longest episode of active sleep following the injection of bicuculline and phaclofen was 41.7 ± 4.8 minutes and 22.7 ± 5.3 minutes, respectively (Figure 7). These episodes were significantly longer than the episodes of active sleep that occurred following the injection of saline (saline: 9.6 ± 0.5 minutes, P < 0.01). However, the mean duration of the longest episodes of quiet sleep and wakefulness following the injection of bicuculline or phaclofen were not significantly different from that following the injection of saline.

Bicuculline-induced active sleep is dose dependent.

Bicuculline was injected into the NPO at different concentrations, ranging from 1 mM to 15 mM. Figure 8A demonstrates that microinjections of bicuculline into the NPO produced a dose-dependent increase in the percentage of active sleep observed during the first hour following the injection. During this time period, the injection of 1 mM of bicuculline increased the percentage of time spent in active sleep by 55.2% compared with that following the injection of saline. The percentage of time spent in active sleep increased steadily with increasing concentrations of bicuculline and reached the highest value with a concentration of 10 mM. Injections of higher concentrations (≥ 15 mM) of bicuculline, on the other hand, did not further increase the percentage of time spent in active sleep. In some experiments (n = 3), these high concentrations of bicuculline induced a state of wakefulness accompanied by hyperexcitation.

Increasing the dosage of bicuculline also produced a dose-dependent decrease in the latency to the onset of the first episode of active sleep (Figure 8B). Following the injection of the lowest dosage of bicuculline (1 mM), the mean latency to active sleep was 11.6 ± 5.1 minutes, which was significantly shorter than that observed following saline injection (saline: 40.7 ± 4.1 minutes, P < 0.05). The mean latency to active sleep decreased sharply following the injection of a 2 mM solution of bicuculline. The 10 mM dosage of bicuculline produced the shortest latency to active sleep.
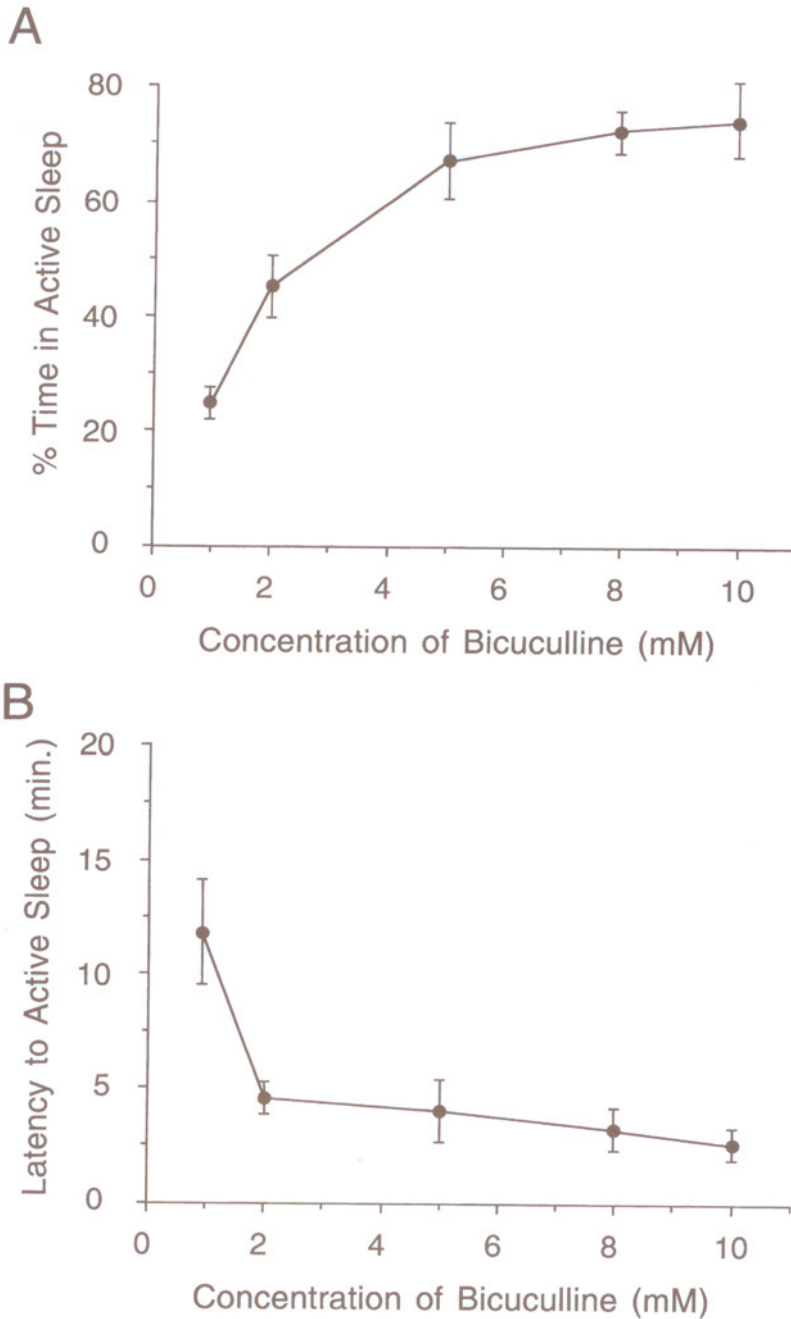
Fig. 8. - *Dose-response effects of bicuculline on the mean percentage of time spent in active sleep (A) and the latency of active sleep (B) during the first hour after microinjection.*

All five dosages of bicuculline were injected in 0.25 μl of saline. Data were obtained from 18 injections. Each point presents the mean for each dose of bicuculline; error bars indicate the SEM.

DISCUSSION

We began our examination of GABAergic effects on the reticular formation with questions about the physiological role of GABAergic terminals in the NPO and their possible contribution to the modulation of sleep; serendipitously, we found that GABA produced prolonged periods of wakefulness when it was injected into the NPO of the sleeping cat. These new experimental findings allowed us to extend our hypotheses regarding the reticular formation control of sleep and wakefulness. Prior to these results, we assumed that in order for wakefulness to be maintained, excitatory activity in the reticular activating system was both necessary and sufficient. However, this hypothesis could not explain why NPO neurons are also innervated by a rich plexus of GABAergic inhibitory fibers and short-axoned GABAergic neurons are distributed in and in the vicinity of the NPO (9, 17, 27). Furthermore, both $GABA_A$ and $GABA_B$ receptors, and GABA transaminase have been identified in the NPO, which indicates the presence of GABAergic synaptic transmission in this area (5, 12, 28). Thus, prior to our recent experiments, the functions subserved by inhibitory innervation of neurons in the NPO and by GABAergic processes in this area were unknown.

Effects of GABAergic agonists and antagonists on wakefulness and active sleep.
The present data demonstrate that microinjections into the NPO of the $GABA_A$ and $GABA_B$ receptor agonists, muscimol and baclofen, induced wakefulness. On the other hand, injections of the $GABA_A$ and $GABA_B$ antagonists, bicuculline and phaclofen, produced active sleep. These data clearly indicate that brainstem GABAergic processes are critical for the generation and maintenance of wakefulness and that they also play a role in the control of active sleep.

While both $GABA_A$ and $GABA_B$ receptor subtypes are clearly involved in these state-controlling processes, the present quantitative analyses indicate differences between the effects of $GABA_A$ and $GABA_B$ receptor agonists and antagonists on the states of wakeful and active sleep. The effects of the $GABA_A$ receptor agonists and antagonists were stronger than those of the $GABA_B$ receptor agonists and antagonists. Therefore, we suggest that GABAergic processes acting on $GABA_A$ receptors may play a more important role in the control of wakefulness and active sleep than those mediated by $GABA_B$ receptors. This is consistent with the fact that synaptic inhibition mediated by $GABA_A$ receptors are relatively more prominent in the central nervous (21, 25, 37, 38).

Postsynaptic inhibition mediated by $GABA_A$ receptors has both electrophysiological and pharmacological differences from that mediated by $GABA_B$ receptors. For example, binding of GABA or $GABA_A$ agonists to $GABA_A$ receptors opens Cl⁻ channels. This inhibition is characterized by a relatively large amplitude, a short-latency and a fast time course. On the other hand, inhibition mediated by $GABA_B$ receptors, which are coupled to Ca^{2+} and K^+ channels via G proteins and second messengers, is slow with a long time course (21, 24, 25, 37, 38). These differences reflect the fact that $GABA_A$-mediated inhibition is primarily a *shunting* type of

inhibition, which would strongly inhibit NPO target neurons, whereas GABA$_B$-mediated inhibition would tend to suppress the activity of these neurons by opening of K$^+$ channels and by diminishing intracellular Ca^{2+}. Therefore, our data indicate that not only the electrophysiological state of NPO cells is important, but that modulation of intracellular second messengers and Ca^{2+} levels is also involved in the control of sleep and waking states.

Effects of GABAergic agonists and antagonists on quiet sleep.

GABA has been postulated to have a role in sleep, particularly vis-à-vis hypnotics such as the barbiturates and benzodiazepines, which are agonistic modulators of GABA$_A$ receptors (37, 40). These compounds, when administered systemically, shorten sleep latency, increase the percentage of the time spent in quiet sleep and inhibit active sleep in a variety of mammalian species (3, 7, 10, 18, 19, 29).

In the present study, microinjections into the NPO of both GABA agonists and antagonists, especially GABA$_A$ agonists and antagonists, produced a reduction in the percentage of time spent in quiet sleep. However, it is unlikely that the changes in quiet sleep observed following the injections of GABA agonists and antagonists in this study are due to a primary, direct effect of these substances on neurons within the NPO because the injection of muscimol or baclofen during quiet sleep (the present study), and during either naturally-occurring active sleep or a carbachol-induced active sleep-like state (unpublished data), always induced wakefulness. The injection of bicuculline or phaclofen during wakefulness, on the other hand, was capable of immediately inducing active sleep without a preceding period of quiet sleep. In addition, following injections of GABA agonists and antagonists, most of the time was spent in either wakefulness or active sleep, respectively; consequently, less time was spent in quiet sleep.

Site specificity.

The behavioral responses to microinjections of both GABA$_A$ and GABA$_B$ agonists and antagonists in our experiments can properly be attributed to the effect of these substances on neurons within the NPO, and not to the diffusion of these substances to other structures for the following reasons. First, the study of Martin (22) demonstrated that the average maximal radius of drug diffusion from an intracerebral injection of muscimol with the volume of 1.0 µl reaches approximately 1.7 mm during the first 20 minutes. In the present study, an injection volume of only 0.25 µl was employed. The radius of drug diffusion, therefore, should be less than 1.0 mm after 20 min. Second, microinjections of muscimol and bicuculline, which were targeted at a region about 1 to 2 mm posterior to the NPO in the present study, did not induce wakefulness or active sleep, respectively. Third, Nitz and Siegel (30, 31) recently reported that GABA release increases during active sleep in the locus coeruleus and the dorsal raphe nucleus, which are adjacent to the NPO. In addition, microinjections of muscimol into the dorsal raphe increased active sleep, on the other hand, microinjections of the GABA antagonist, picrotoxin, blocked active sleep (30, 31). This result is opposite to that observed in the present

experiments, when muscimol was injected into the NPO. Therefore, it is evident that it is only in the NPO that GABA promotes wakefulness, while GABA antagonists injected into this structure selectively induce active sleep.

Lin et al. (20) reported that the injection of muscimol into the ventrolateral part of the posterior hypothalamus induces a significant increase in both active and quiet sleep and Sastre et al. (35) found that muscimol, when injected into the periaqueductal gray, promotes active sleep. However, we found that muscimol induced wakefulness when applied within the NPO.

The location of GABAergic cell bodies.

The preceding data indicate the importance of GABAergic neurotransmission in the NPO in the control of wakefulness and active sleep, but they do not directly address the question of the location of the responsible GABAergic cell bodies. With regard to the location of the somas of these GABAergic neurons, there are two possibilities: (1) local GABAergic interneurons are located within the NPO; or (2) GABAergic neurons are located outside the NPO and project to this area. In support of the first hypothesis, short-axoned GABAergic neurons have been found to be distributed in and in the vicinity of the NPO (9, 17). On the other hand, anatomical data also indicate that there exists a large number of GABAergic neurons whose axons project over long distances in the CNS (27). We therefore consider that a critical next step in our understanding of the role of the NPO is the identification of the population of GABArergic neurons whose activity promotes wakefulness.

Neuronal mechanisms of the pontine GABAergic system in the control of behavioral states.

We hypothesize that when the GABAergic system that innervates the NPO is activated, it functions to eliminate sleep and produce wakefulness. When the activity of this GABAergic system is decreased physiologically or its inhibitory actions on the NPO cells are blocked by the injection of either $GABA_A$ or $GABA_B$ antagonists, the state of active sleep occurs. Therefore, we suggest that there is a "gating" mechanism within the NPO. This "gating" mechanism apparently operates in such a way that wakefulness is induced and maintained when the activity of GABAergic synaptic transmission in the NPO is dominant, so that the activity of neurons in the NPO, whose discharge is selectively related to active sleep (AS-ON neurons), is tonically inhibited. Thus, active sleep occurs when this pattern of GABAergic synaptic transmission is suppressed. Recent findings of Sakai and Koyama (34), demonstrating that AS-ON cells in the pons are under tonic GABAergic inhibitory control during wakefulness, support this interpretation of the present data.

The neuronal mechanisms and the interactions of different neurotransmitter systems and brain regions that are involved in the production and regulation of the behavioral states of sleep and wakefulness are the focus of current investigations in many laboratories (15, 33, 39). In this respect, the present data raise questions regarding the manner in which the proposed GABAergic system might interact

with excitatory neurotransmitter systems in this region, particularly the pontine cholinergic system, which has been shown to be critically involved in the generation of active sleep (1, 4, 11, 15, 33, 39). In this regard, we suggest that pontine GABAergic processes are responsible for the generation and maintenance of wakefulness as well as functioning as part of the mechanisms that control active sleep.

Because this volume commemorates the contribution made by Dr. Nathaniel Kleitman to sleep research, we wanted to address one of the principal questions that was of interest to him. Accordingly, we present the following discussion in the spirit of continuing to investigate the concepts and hypotheses that Kleitman promulgated.

Chapter 36 of Kleitman's book *Sleep and Wakefulness* (16) begins as follows, "Which came first—the hen or the egg? In the alternation of sleep and wakefulness, which of the two states interrupts the other?" (page 363). These questions appear to be founded on the underlying assumption that one of these states is either dominant and/or a drive for its occurrence is continuously present. The other state would occur when there is an interruption of this dominant state. We suggest that Kleitman's formulation is correct and that sleep is the state that is "interrupted", i.e., that wakefulness is achieved because sleep is inhibited. In this regard, our present data indicate that a GABAergic inhibitory system within the NPO interrupts sleep, thus allowing wakefulness to arise and to be maintained.

Following Kleitman's line of questioning, we then asked a second question: what would be the state of an animal if the activity of our putative GABAergic "waking" system is "interrupted"? To answer this question, we blocked GABA postsynaptic receptors in the NPO. Under these conditions, active sleep was elicited. Thus, we conclude that there is an ongoing, persistent, spontaneously active drive to promote active sleep, even in an awake animal. This drive is so powerful that wakefulness occurs only when active sleep processes are inhibited (i.e., by GABAergic inhibition).

Another approach to answering the question posed by Kleitman is to ask what state an animal might be in if all of the specific needs for its health and well being were satisfied. That is, if the animal was not hungry, did not feel an urge to procreate, was neither too hot nor too cold, did not fear predators, etc. Would such an animal be awake or asleep; and if it slept, would its sleep be quiet or active?

Such an environment appears to exist for each mammal; it occurs *in utero* during the animal's ontogenetic development. During this period, the predominant state is that of active sleep (32). Later, wakefulness and quiet sleep arise and sleep and wakefulness alternate due to circadian influences. Now let us theoretically eliminate all circadian influences, and place an adult animal in the "*in utero*" type of environment. We believe that in this hypothetical situation active sleep would predominate.

Thus, the present data, which indicates that active sleep must be suppressed in order for wakefulness to occur, suggests that active sleep may indeed be the dominant behavioral state, essentially replacing both the hen and the egg in Kleitman's question regarding behavioral states, and providing an answer to "which came first". We suggest that the answer to this question is active sleep.

We recognize that the ideas presented above are based upon a limited amount of data, and that these data are being considered only within the context of Kleitman's theoretical interests in sleep and wakefulness. Certainly, there are other interpretations of our data and other structures and mechanisms that would need to be taken into account before the issue of "which came first" could be addressed in a formal manner. Therefore, the preceding should be considered as reflecting concepts that are appropriate for discussion within the context of this volume, but that they will require a great deal more data and analyses before being presented for formal debate and comment.

SUMMARY

The present study was undertaken to explore the role of brainstem GABAergic processes in the control of the behavioral states of sleep and wakefulness, and to compare the effects of $GABA_A$ agonists and antagonists with those of $GABA_B$ agonists and antagonists on these behavioral states. Accordingly, the following drugs were microinjected into the nucleus pontis oralis (NPO) in chronic, unanesthetized cats: muscimol ($GABA_A$ agonist), bicuculline ($GABA_A$ antagonist), baclofen ($GABA_B$ agonist) and phaclofen ($GABA_B$ antagonist). The percentage, latency, frequency and duration of each behavioral state were measured in order to quantify the effects of these microinjections on wakefulness and sleep.

Microinjections of either muscimol or baclofen immediately induced wakefulness. There was a significant increase in the duration and the percentage of time spent in wakefulness as well as an increase in the latency to active (REM) sleep. These changes were accompanied by a decrease in the percentage of time spent in active and quiet sleep. In contrast, injections of bicuculline or phaclofen produced active sleep. The percentage of time spent in active sleep and the frequency of active sleep increased while the percentage of time spent in wakefulness and the latency to active sleep was significantly reduced. The effects of $GABA_A$ receptor agonists and antagonists on wakefulness and active sleep were comparable, but stronger than those of $GABA_B$ receptor agonists and antagonists. These data indicate that pontine GABAergic processes acting on both $GABA_A$ and $GABA_B$ receptors play a critical role in generating and maintaining wakefulness and in controlling the occurrence of state of active sleep.

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