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AMYGDALOID CONTROL OF ALERTING AND BEHAVIORAL AROUSAL IN RATS: INVOLVEMENT OF SEROTONERGIC MECHANISMS

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INTRODUCTION

Studies of the regulation of rapid eye movement sleep (REM) have focused almost exclusively on the brainstem caudal to the midbrain. Impetus for the interest in the caudal brainstem, particularly the pons, came from the early work demonstrating that following brainstem transections through the caudal midbrain, cats still experienced periodic collapses of muscle tone associated with twitches and pontine waves (30, 74). However, there were suggestions that the forebrain might play a role (51).

Two observations stimulated Morrison and Reiner (50) to propose that the initiation of REM in *intact* animals might well require interactions among fore- and hindbrain mechanisms: *a*) Jouvet (31) had reported that decerebrate cats could be induced to enter a REM-like state by a number of stimuli not normally sleep-promoting, such as passing a stomach tube, inserting a rectal probe, opening the mouth, and passively flexing and extending joints, a finding later confirmed by Hobson (22). Consequently, Morrison and Reiner (50) suggested that the brainstem, lacking modulation by rostral structures, might be in an unstable, supersensitive state. *b*) In the normal cat, homeostatic mechanisms usually regulated by the hypothalamus are suppressed during REM (52), and the same could be argued for the decerebrate cat. Clearly, a central reorganization in the hypothalamus and/or other rostral structures must take place at or before the transition from non-REM to REM. Given the decerebrate cat's abnormal propensity to enter REM, Morrison and Reiner (50) suggested that midbrain transection serves as a substitute for the suppression of forebrain control that precedes natural REM.

These ideas did not deny the important role played in REM by the caudal brainstem but implied that full understanding of the mechanisms initiating and maintaining REM would require a more global outlook. Indeed, recent investigations have revealed interesting effects of forebrain manipulations on REM. Baghdoyan

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et al. (2) demonstrated that the simultaneous infusion of carbachol into cholinoceptive regions of the basal forebrain and the pons significantly reduced the ability of the latter to generate a REM-like state. Further, studies by Sakai and colleagues (57) have revealed that the posterior hypothalamus influences the generation of REM via descending neural pathways: ibotenic acid lesions induced a 300% increase in REM during the first post-injection day and released the same reflex-induced episodes seen after decerebration. Moreover, Marini *et al.* (42) reported a selective enhancement of REM for several days (more episodes rather than lengthened episodes) following bilateral ibotenic acid lesions of the thalamic nucleus, centralis lateralis.

We chose to study the amygdala, a limbic structure involved in evaluating and producing behavioral responses to emotionally significant stimuli (1, 18, 37, 38). The amygdala has reciprocal connections with the cholinergic basal forebrain (15, 19, 20, 78) and with pontine regions involved in both alerting behaviors and REM (3, 36, 46, 61, 63). In waking, these connections position the amygdala to modulate cortical arousal and brainstem alerting processes based on stimulus relevance and on emotional expectations of stimulus importance. In REM, this connectivity suggests that the amygdala may have a role in modulating the endogenously-generated activity that resembles that of alert waking, e.g., low amplitude, high frequency waves on the electroencephalogram (EEG), hippocampal theta, and increased brain temperature (48).

As a first step in examining the role of the amygdala in modulating alerting and REM sleep phenomena, we have looked at the effect of locally manipulating serotonin (5-HT) in the amygdala. The major serotonergic innervation of the amygdala comes from the dorsal raphe nucleus (DRN) with additional input from the median raphe nucleus (MRN) (12). The state-dependent neural activity of the DRN is well-documented (8, 41, 45, 72), and 5-HT has a role in the regulation of REM although the exact mechanism(s) of action is not known.

Current thought holds that 5-HT originating in the DRN may have an inhibitory role in the generation of REM and one of the state's signature features, ponto-geniculo-occipital (PGO) waves, presumably via direct projections to the pontine generator region (64). However, a recent study (23) found relatively few serotonergic synapses on cholinergic neurons in the pedunculopontine and laterodorsal tegmentum (PPT/LDT). Further, the researchers observed that the synapses were asymmetrical, a specialization most often associated with excitation, not inhibition. Our own work, using microinjection techniques to apply serotonergic drugs locally into PPT, has yielded equivocal results (56, 59, 60), except for a significant reduction in the number of successful REM entrances after microinjections of the 5-HT_{1A} agonist, 8-OH-DPAT (59).

In this study, we microinjected 5-HT and the broad spectrum 5-HT antagonist, methysergide, locally into the amygdala and examined the effect on behavioral state and REM phenomena in freely moving rats. In order to compare directly our behavioral results with the distribution of 5-HT receptors, we measured, in a separate group of rats, the binding of [^{3}H]CN-IMI ([^{3}H]cyanoimipramine) to 5-HT uptake sites located presynaptically as a measure of 5-HT innervation.

MATERIAL AND METHODS

Subjects.

The subjects were 19 male Sprague-Dawley rats. The rats were maintained under 12 hr light/dark conditions and given *ad lib* access to food and water. Some rats served in more than one experiment.

Surgical procedures.

All surgical procedures were performed stereotaxically under aseptic conditions. Ketamine (85 mg/kg) and Xylazine (15 mg/kg) were administered intraperitoneally for anesthesia.

The tips of bipolar stainless steel (0.25 mm) electrodes were stereotaxically placed bilaterally in the vicinity of the locus coeruleus (P 0.3 (intra-aural zero), ML ± 1.0, DV 7.0) to record the pontine component of PGO waves (13, 32, 33, 43, 44). The electroencephalogram (EEG) was recorded from screw electrodes implanted in the skull. The electromyogram (EMG) was recorded by stainless steel wire electrodes implanted in neck muscles. Guide cannulae (26 ga.) for microinjections were bilaterally implanted with their tips aimed 1.0 mm above the amygdala (A 6.7 (intra-aural zero), ML ± 4, DV 7.0). Leads from the recording electrodes were routed to a nine pin miniature plug that mates to one attached to a recording cable. The recording plug, cannulae and stimulating electrodes were affixed to the skull with dental acrylic and anchor screws.

Drug injection regimen for 5-HT.

The rats (N=11) were allowed to exhibit two complete sleep cycles after which 5-HT (1.5 mM) or saline was microinjected into the amygdala during REM or NREM. Microinjections were administered at a rate of 0.1 µl/min and were terminated after one minute or a change of state, whichever came first. Microinjections in different sleep episodes were separated by at least 30 min, and no more than 0.5 µl was injected in any one six-hour recording period. Seven days elapsed between successive experimental conditions.

Drug injection regimen for methysergide.

The rats (N=10) were allowed to exhibit two complete sleep cycles after which 0.4 µl saline alone or methysergide (1.0 mM solution) was injected during non-REM (NREM). Microinjections were given at a rate of 0.1 µl/min. All drug injections were administered in a counterbalanced order. Seven days elapsed between successive doses.

Microinjection procedures.

The experiments used remotely controlled microinjections so that drugs could be administered in NREM and REM. Injection cannulae (30 ga.) were fitted to 60 cm lengths of PE 20 tubing. The tubing and cannulae were filled with the solution to be injected and the free end of the tubing was attached to a 1.0 µl Hamilton syringe. An air bubble was allowed to form in the line so that fluid movement could be verified. For electrophysiological recording the cable was attached to the plug on the rat's head. The injection cannulae were secured in place in the guide cannulae. The rat was then allowed free movement inside the recording cage. When the rat was in the desired state, the microinjections were delivered using a custom-made syringe pump with a remote control feature. The pump was quiet and did not awaken the rats.

Two procedures were used to control for the possibility of drug diffusion prior to injection. First, each drug injection was matched with an appropriate saline injection. Second, the rats were allowed to have two sleep cycles prior to microinjecting either drug or saline. Comparing pre-injection recordings from saline and drug conditions allowed us to determine whether there was an effect of diffusion on any particular day. If the pre-injection recordings did not differ, comparisons between saline alone and drug conditions were made using the post-injection recordings with assurance that the drug was administered at the desired time.

Polygraph recording.

Six-hour polygraph studies were conducted. For recording, the rats were connected to a lightweight shielded cable routed to a Grass Model 79 polygraph equipped with 7P58 amplifiers. The amplified signals from the two PGO channels were sent into an A/D converter and processed by DataWave, Experimenter's Workbench software. PGO waves were detected using a threshold crossing criterion, and their amplitudes were stored for further analyses. The electrophysiological data and video record of each rat's behavior were stored on videotape using a Telefactor Modac-1 recorder.

Scoring behavioral state.

Waking, NREM and REM were determined by trained observers from the paper record produced by the Grass polygraph using standard EEG and EMG criteria and PGO wave activity in conjunction with the videotape record of behavior. The record of behavioral state was synchronized to the computer file containing PGO wave information. This allowed PGO wave amplitude and frequency to be determined for specific states, and it allowed episodes of waking with movement artifact to be discarded from the analysis.

Behavioral arousal was scored when changes in electrophysiological signals indicated an abrupt change from NREM or REM to waking and/or there was an immediate change from a sleeping posture to activity.

Analyses of behavioral state.

The sleep/wake cycle under pre-injection and post-injection conditions was examined. Saline and drug pre-injection records were compared to determine whether behavioral state had been altered prior to drug injection. Saline and drug post-injection records were examined to detect alterations in sleep and waking states due to specific drug effects. The measures used were: total recording period (TRP), time spent asleep (TSA), sleep efficiency (TSA/TRP), NREM percentage (NREM time/TSA), REM percentage (REM time/TSA), number of REM episodes and mean duration of REM episodes.

Autoradiographic studies.

Preparation of tissue sections. A group of control rats (Sprague-Dawley, 90 day old, 300 gm) was decapitated and their brains were frozen on powdered dry ice and stored at -70°C until sectioned. Coronal sections (20 µm) were cut on a cryostat at -15°C, at the level of Paxinos and Watson (53) plates 49 and 52, thaw-mounted onto gelatin-coated slides (2 sections/slide) and desiccated overnight at 4°C. These slide-mounted sections were then stored at -70°C until assayed.

[^{3}H]CN-IMI binding to uptake sites for 5-HT. The binding of [^{3}H]CN-IMI to uptake sites for 5-HT was measured by autoradiography using the method of Kovachich *et al.* (34). Sections were incubated with 0.3 nM [^{3}H]CN-IMI (New England Nuclear) in Tris buffer (50 mM Tris, 150 mM NaCl), pH 7.4, for 24 hr at 4°C. Non-specific binding, defined using 5 µM sertraline, was less than 10% of total binding. Following incubation, sections were washed in cold buffer for 60 min, dipped into cold water and dried. Sections were loaded into cassettes for 2-3 weeks depending on the regions of the brain.

Quantitation of autoradiograms. All films were developed using Kodak GBX developer (3 min), dipped in water, and fixed in Kodak GBX fixer (6 min) at room temperature. The autoradiograms were analyzed on a DUMAS (Drexel Unix-based Microcomputer Imager Analysis System) densitometer using the Brain software package (14). Plastic embedded tritium standards (American Radiolabelled Chemicals, St Louis, MO) calibrated using brain-mash sections were used for quantitation (16, 17). Binding of [^{3}H]CN-IMI in the amygdala was compared to that in DRN, a major 5-HT cell body area (34).

Histological procedures for rats studied in behavioral experiments.

The rats used in the behavioral experiments were overdosed with sodium pentobarbital (50-100 mg/100 gm intraperitoneally) and perfused intracardially with 9% saline and 10% formalin.

Evans blue dye of equal volume to the experimental microinjections was infused to assist in locating the injection site. The brains were processed to determine cannula and electrode placements. For this purpose the brains were embedded in celloidin; 40 μm slices were made through the areas of interest, and the sections were stained with cresyl violet.

RESULTS

1. *Microinjections of 5-HT.*

Alterations in behavioral state. - Microinjections of 5-HT during REM significantly decreased the latency to a change of state to either waking or NREM compared to saline, $t(10) = 2.44$, $p < .035$ (Fig. 1A). This is demonstrated polygraphically in Figure 2. Microinjections of 5-HT during NREM also significantly decreased the latency to a change of state to either waking or REM compared to saline, $t(10) = 2.49$, $p < .032$.

Whereas microinjections of 5-HT had significant effects in both REM and NREM, the relative effect on behavioral state and the time course of the effect varied considerably by state. Microinjections of 5-HT during REM resulted in

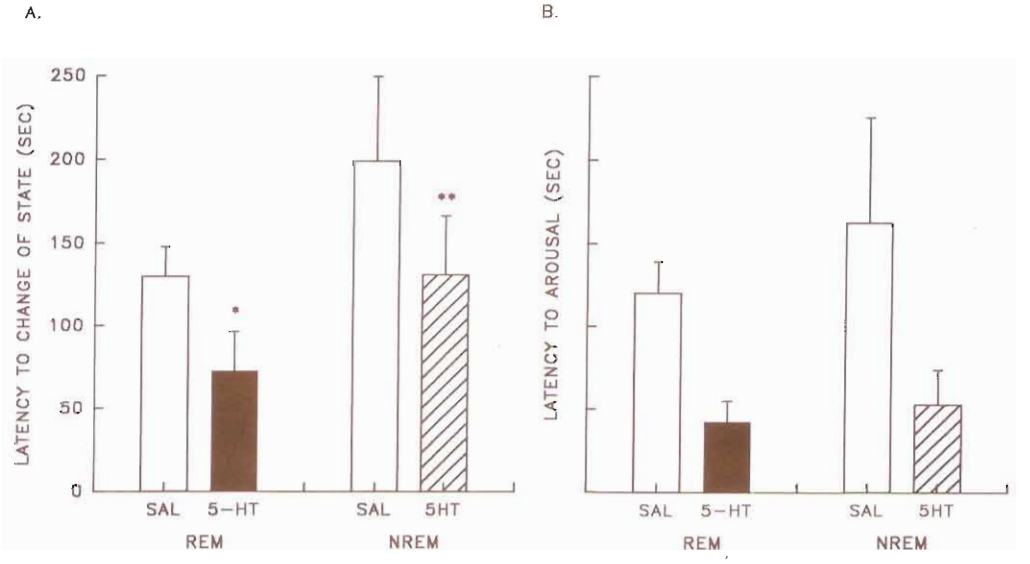

Fig. 1 - *A. Mean latencies to change of state after microinjections of saline or 5-HT.* 5-HT produced shortened latencies when injected in both REM and NREM although the effect was much more pronounced in REM.

B. Mean latencies to arousal after injections of saline or 5-HT during REM and NREM. No analyses were conducted on these data because not all animals aroused. Vertical bars indicate standard error of the mean.

behavioral arousal on 65% of the trials compared to 46% for saline in REM, and 38% for saline in NREM and 42% for 5-HT in NREM. The latencies to behavioral arousal were generally shorter after 5-HT (Fig. 1B). These data were not analyzed statistically because behavioral arousal was not observed in some rats in all conditions.

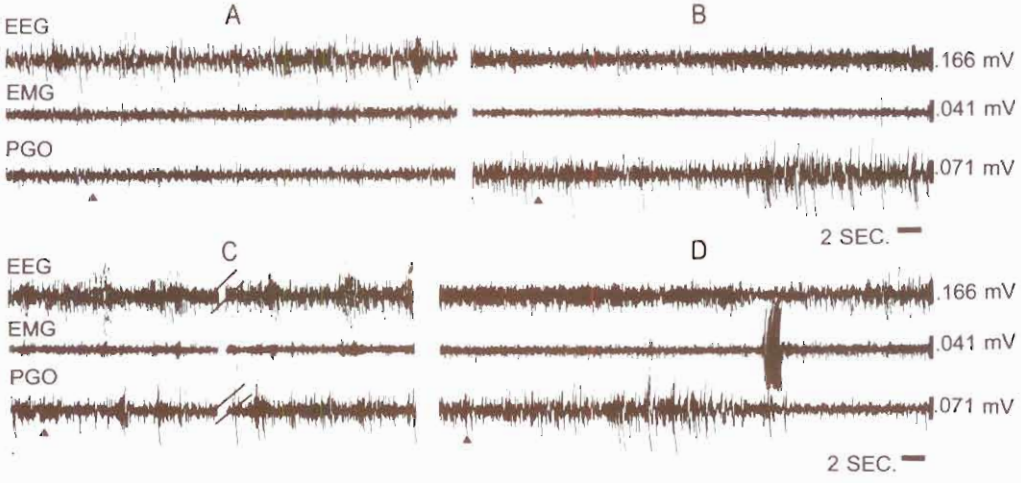

Fig. 2. - *Excerpts from polygraph recordings after microinjection into the amygdala of saline (A: NREM; B: REM) and 5-HT (C: NREM; D: REM).*

Note the abrupt arousal after microinjection of 5-HT into the amygdala during REM (D). In contrast, microinjections of 5-HT during NREM produced arousal less frequently and did not prevent the entrance into REM (C). Note the arousal from REM (D) produced by 5-HT injected into the amygdala. The break displayed in C represents 10 min of continuous recording. The solid triangles indicate microinjection onset.

The relative effectiveness of microinjections in producing a change of state from REM and NREM at different post-injection latencies was analyzed using the Kolmogorov-Smirnov test for cumulative distribution functions. The time course for microinjections of 5-HT in REM differed significantly from saline in REM ($p < .028$), saline in NREM ($p < .005$) and 5-HT in NREM ($p < .028$). There were no significant differences between 5-HT and saline injections in NREM or between the two saline conditions. For example, 62% of microinjections of 5-HT during REM resulted in a change of state within 60 sec compared to 15% after saline (Fig. 3). In contrast, 29% of microinjections of 5-HT during NREM resulted in a change of state within 60 sec compared to 25% after saline (Fig. 3).

Sleep architecture. - Sleep architecture over the entire 6-hour recording period was analyzed using dependent *t*-test procedures for planned comparisons. There were no significant differences between 5-HT and saline in any of the measures we examined (Table 1).

Table 1. - *Sleep architecture after microinjections of saline or 5-HT into the amygdala.*

	Saline		5-HT	
	Mean	SD	Mean	SD
Total REM	48.5	13.4	50.3	13.8
Sleep efficiency	61.1	10.3	63.6	8.5
REM %	13.6	3.7	14.2	3.7
REM duration	2.6	0.8	2.6	0.3
REM count	19.0	4.2	19.5	5.6

PGO waves. - PGO waves were not analyzed due to the short durations of many of the REM episodes after microinjections of 5-HT. There were not enough episodes of sufficient duration to allow samples adequate for statistical purposes.

2. *Microinjections of Methysergide.*

Sleep achitecture. - The effects of microinjections of methysergide on sleep architecture are presented in Table 2. Comparisons were made with dependent *t*-test procedures for planned comparisons. Methysergide significantly increased, $t(9) = 3.22$, $p < .011$, sleep efficiency, but no other significant changes were observed in the sleep architecture parameters we measured.

Table 2. - *Sleep architecture after microinjections of saline or methysergide into the amygdala.*

	Saline		Methysergide	
	Mean	SD	Mean	SD
Total REM	49.7	17.6	49.6	16.9
Sleep efficiency	71.5	8.0	80.0	5.0*
REM %	25.7	7.3	25.0	6.0
REM duration	3.1	0.8	2.9	0.4
REM count	17.1	6.7	16.6	4.3

*p < .011.

PGO waves. - Usable PGO wave recordings were obtained in 9 of the 10 rats microinjected with methysergide. Pre-injection saline and pre-injection methysergide conditions for waking, NREM and REM were compared using dependent *t*-tests. The post-injection data were analyzed for each behavioral state with a 2 (Drug Condition) X 4 (Recording Period: 30 mins post-injection, 31-60 minutes post-injection and remaining time) within subjects ANOVA.

There were no significant differences between PGO wave frequency in the pre-injection condition for any state. The Drug Condition main effects were significant for waking and NREM. Methysergide significantly increased overall PGO wave rate in waking ($M = 6.0 \pm 0.9$ vs $M = 3.4 \pm 0.8$), $F(1, 8) = 5.61$, $p < .045$, and NREM ($M = 6.5 \pm 1.1$ vs $M = 2.7 \pm 0.3$), $F(1, 8) = 5.79$, $p < .043$, compared to saline. The time course of the effect of methysergide on PGO waves in NREM is

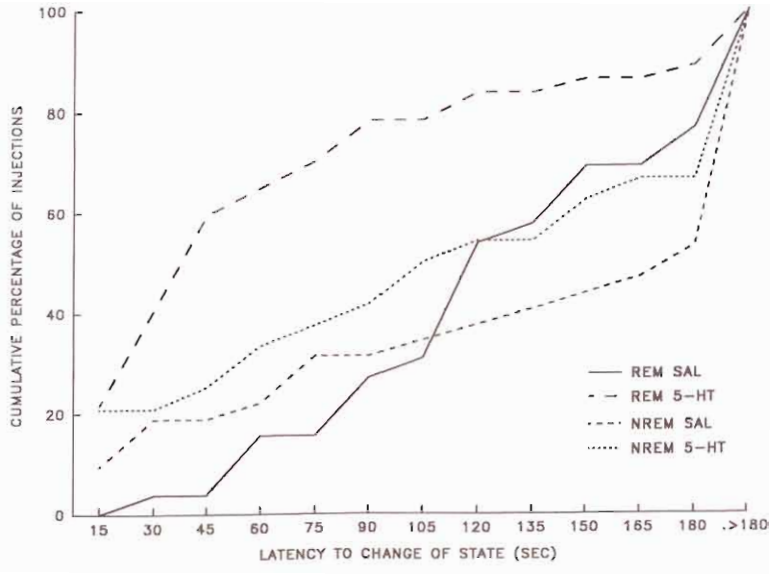

Fig. 3. - *Percentage of injections in REM and NREM producing a change of state graphed cumulatively across time demonstrating the dramatic effect of 5-HT microinjections in REM compared to 5-HT injections in NREM or saline injections in either REM or NREM.*

shown in Figure 4. Figure 5 presents a polygraphic recording demonstrating the increase in PGO waves in NREM after methysergide. No significant drug effects were observed on PGO wave frequency in REM (M = 14.8 ± 2.2 vs M = 11.9 ±

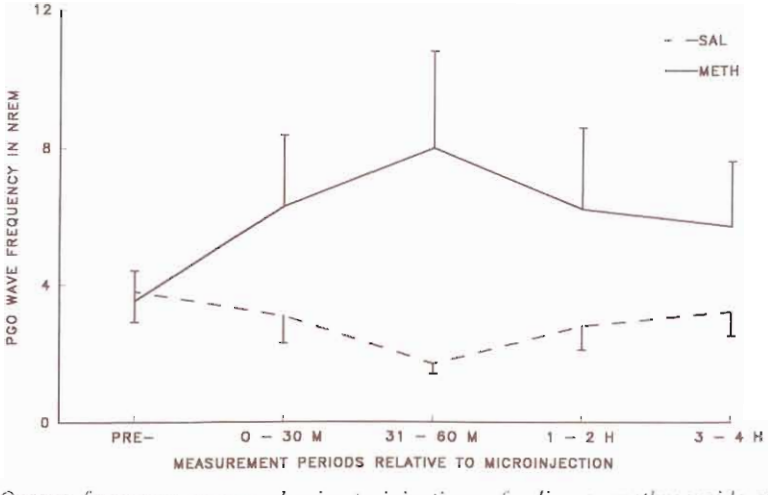

Fig. 4. - *PGO wave frequency measured prior to injections of saline or methysergide and at 4 post-injection intervals in NREM.*

Vertical bars indicate standard error of the mean.

1.7). Neither the Recording Period main effects nor the Drug Condition X Recording Period interactions reached significance in any of the analyses.

No significant change in PGO wave amplitude was observed in any state.

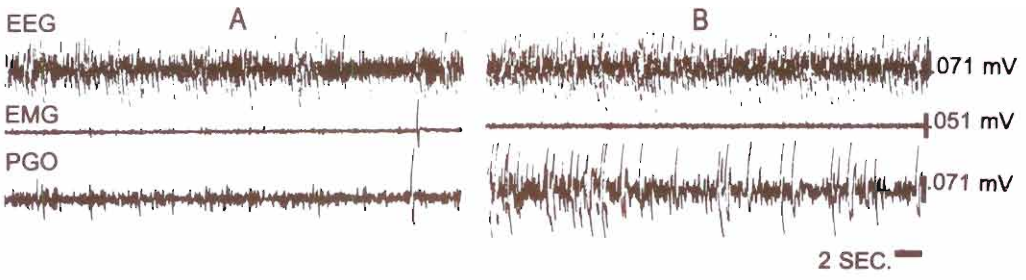

Fig. 5. - *Polygraph record showing NREM after saline (A) and after methysergide (B) microinjected into the amygdala.*

Both excerpts are records from the same animal at approximately 12 min after injection. Note the striking increase in PGO wave frequency after methysergide (B), though the effect was most dramatic in this rat.

3. *Binding studies.*

The results of the binding studies are summarized in Table 3. Quantitative autoradiographic analysis of [^{3}H]CN-IMI binding to 5-HT uptake sites indicated moderate innervation in the central, medial and lateral nuclei with relatively greater innervation in the basolateral nucleus.

Table 3. - *Binding to (3H)CN-IMI in the amygdala. Mean and SEM are indicated.*

Region			(^{3}H)CN-IMI binding (fmol/mg protein)
Basolateral	N.	4	1300 ± 31
Central	N.	4	300 ± 14
Lateral	N.	4	550 ± 23
Medial	N.	4	736 ± 28

4. *Histology*

Figure 6 presents representative photomicrographs of electrode placements in the brainstem that successfully recorded PGO waves (A) and cannula placements bilaterally in the amygdala (B). Figure 7 presents line drawings encompassing the histological location of the injection sites in the amygdala for all animals microinjected in either the 5-HT (A) and/or the methysergide (B) protocols.

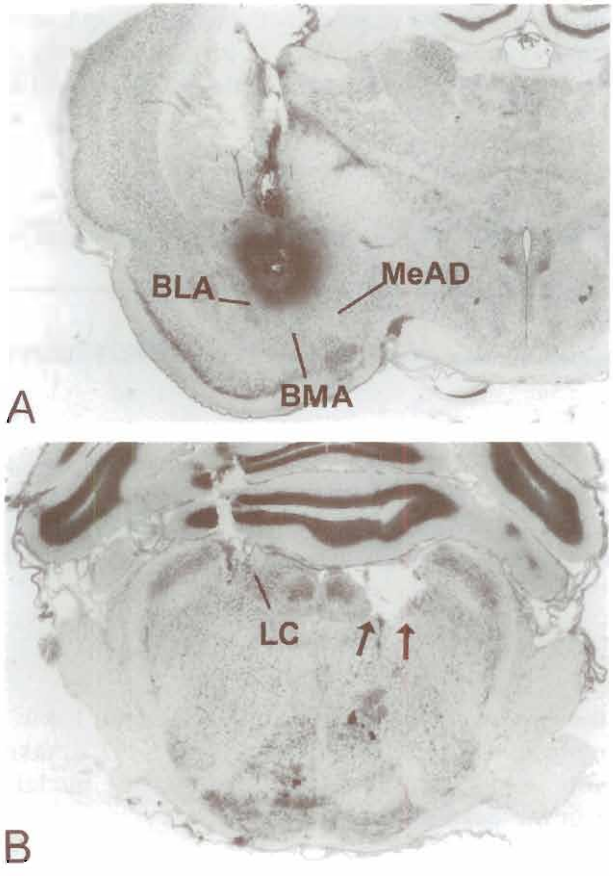

Fig. 6. - *Photomicrograph of a representative cannula placement in the amygdala (A) and recording electrode placement in the pons (B).*

Arrows indicate individual tips of bipolar recording electrode. BLA, basolateral amygdaloid nucleus, anterior; BMA, basomedial amygdaloid nucleus, anterior; LC, locus coeruleus; MeAD, medial amygdaloid nucleus, anterodorsal.

DISCUSSION

Role of amygdala in sleep and behavioral arousal.

The results demonstrate that the amygdala is involved in the modulation of behavioral state and that 5-HT has a role in this modulation. Microinjecting 5-HT into the amygdala during NREM or REM produced short-latency changes of state either to another sleep state or to behavioral arousal, with the effect being more pronounced after injections during REM. Serotonin did not significantly alter overall sleep architecture. Microinjecting methysergide, a broad spectrum 5-HT antagonist, during NREM increased the relative amount of sleep (sleep efficiency) without significantly altering other measures of sleep architecture.

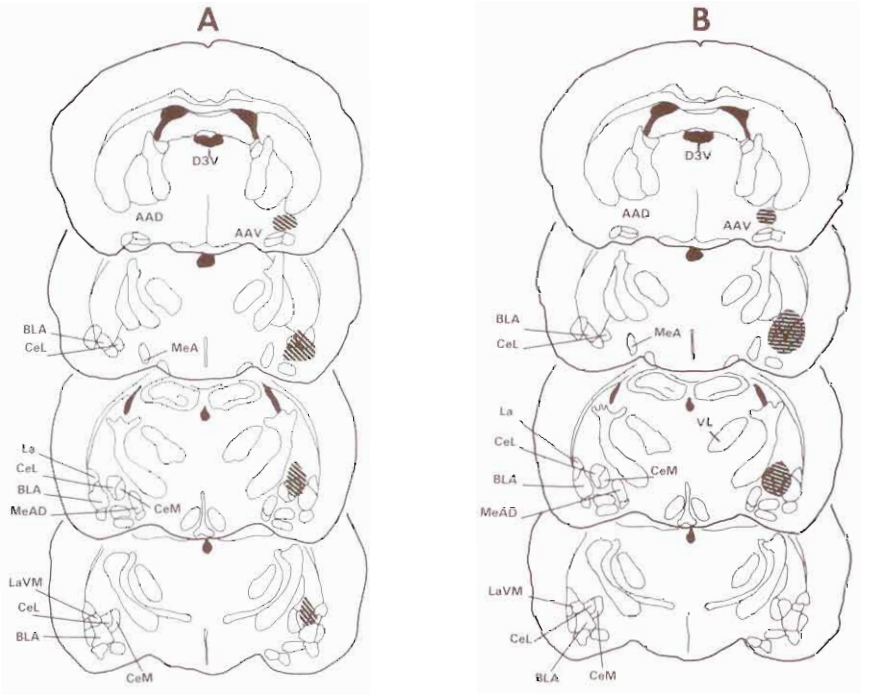

Fig. 7. - *Line drawing illustrating the microinjection sites (cross-hatched region) for all rats studied with 5-HT (A) and methysergide (B).*

Note that some rats were used in both studies. AAD, anterior amygdaloid area, dorsal; AAV, anterior amygdaloid area, ventral; CeL, central amygdaloid nucleus, lateral; CeM, central amygdaloid nucleus, medial; D3V, dorsal third ventricle; La, lateral amygdaloid nucleus; LaVM, lateral amygdaloid nucleus, ventromedial; MeA, medial amygdaloid nucleus, anterior. Plates adapted from Paxinos and Watson (53).

The current results differ from those we have obtained after microinjecting serotonergic drugs into the brainstem. Microinjecting 5-HT into the vicinity of LDT in rats reduced REM episode duration and inhibited the overall amount of REM (25). Similarly, microinjections of the 5-HT_{1A} agonist, 8-OH-DPAT, into PPT of cats reduced the number of successfully maintained REM episodes without reducing attempts to enter REM (59). In the present study, microinjections of 5-HT into the amygdala during NREM did not prevent subsequent entrances into REM, but microinjections during NREM and REM did shorten episode duration, often dramatically. It is possible that the effect of the microinjection during REM may mimic to some degree processes that naturally occur in the amygdala because the DRN resumes firing near the termination of REM (72). Such an increase could also accompany behavioral arousal from NREM where DRN firing occurs, but is reduced compared to that of waking (8, 41, 45, 72). Together, our findings of different effects from manipulations of 5-HT in forebrain and hindbrain regions suggest that REM initiation and maintenance may be modulated by 5-HT acting in different brain regions.

Amygdaloid modulation of alerting.

PGO wave activity appears to be related to attentional and informational processes. In cats, the largest amplitude elicited PGO waves in waking occur during alerting situations or in response to novel stimuli (5, 58). These behavioral observations are bolstered by cellular studies indicating that activity in the pontine PGO wave-generator region blocks the synchronized mode of thalamic spindle generation, thereby facilitating information transfer to the cortex (66); and the demonstration that carbachol activation of cells in PPT produces hippocampal theta rhythm (73). We have proposed that the presence of a PGO wave reflects the registration of a stimulus by the brain, and that wave amplitude reflects the level of activation so that stimuli that elicit alerting responses such as orienting also elicit higher amplitude PGO waves (58).

The amygdala is anatomically situated to modulate brainstem alerting mechanisms (3, 36, 46, 61, 63) based on stimulus characteristics. It may have a role in attentional processes (15). Further, the amygdala plays a role in investing sensory events with emotional significance (18, 27, 76) and in evaluating the emotional significance of stimuli and initiating appropriate responses (1, 18, 37, 38). The amygdala also is critical to the formation and expression of conditioned fear (11).

Several lines of evidence suggest a functional connectivity between the amygdala and PGO wave generator regions. Electrical stimulation of the central nucleus of the amygdala increases PGO wave frequency during REM in cats (6). Additionally, PGO waves can be recorded from the basolateral amygdala temporally later than PGO waves recorded from LGB during REM in cats (7). Single-unit activity in the lateral amygdala (4) and the central nucleus (55) increases during REM. This increase in unit activity may be related to the role of the amygdala in PGO waves (4). Because cells in the amygdala respond to novel and significant stimuli affecting different sensory modalities (38), the amygdala might provide a mechanism by which stimuli with emotional attributes could influence alerting responses.

We found that the local microinjection of methysergide into the amygdala increased PGO wave frequency in waking and NREM, possibly by antagonizing serotonergic input from DRN. The failure of methysergide to increase PGO waves in REM is not surprising considering the normally suppressed firing of the DRN in REM (8, 41, 45, 72). Thus, one would not expect that antagonizing 5-HT in the amygdala would have the same effect in REM, when 5-HT already would be greatly reduced or absent. In contrast, Calvo *et al.* (6) provided exogenous stimulation of the central nucleus which in turn increased PGO wave activity during REM.

We were unable to determine statistically the effects of 5-HT on PGO waves because there were not enough REM episodes of sufficient duration under the drug condition to obtain an adequate representation of PGO wave activity. Anecdotally, in some of the longer REM episodes there did appear to be a suppression of PGO wave activity. However, because there were few of these episodes and because PGO waves can be a low probability event in rats, this evidence is suggestive at best.

The present results regarding PGO waves contrast with those we have found after microinjecting serotonergic drugs into the brainstem PGO-generator region. In cats we have been unable to inhibit PGO waves without altering behavioral state using microinjections into PPT of 5-HT agonists (59). Similarly, we have been unable to release them consistently with microinjections of 5-HT antagonists (56). In rats, we were not able to alter the responsivity of auditory-elicited PGO waves with microinjections of the 5-HT_{1A} agonist, 8-OH-DPAT, into PPT, and we concurrently found PPT to have moderate 5-HT innervation but few 5-HT_{1A} receptor sites (60). By comparison, in LDT, where 5-HT has been demonstrated to hyperpolarize "burst" neurons *in vitro* (40), there is a relatively extensive 5-HT innervation and a moderate number of 5-HT_{1A} receptor sites (60). It has also been demonstrated that 5-HT_2 receptor sites are closely associated with cholinergic cells in LDT/PPT (47). Our laboratory currently is examining how manipulation of serotonergic mechanisms in this region affects PGO wave generation in rats.

Behavioral arousal vs. alerting.

The seemingly contradictory effects of 5-HT on mechanisms of behavioral arousal and alerting deserves comment. One would expect alerting and behavioral arousal to be linked, yet 5-HT in the amygdala increased the tendency to behavioral arousal, whereas a 5-HT antagonist increased an indicator of alerting, the PGO wave. The paradoxical nature of REM suggests that alerting and behavioral arousal can be distinguished. This is best demonstrated in cats with the atonia of REM eliminated by pontine lesions. These cats can behaviorally orient to external stimuli without awakening during REM without atonia (49). An aberration of the linkage may be involved in narcolepsy, which can be produced by stimuli with emotional content (21).

Based on numerous studies in the literature, we expected 5-HT to inhibit the generation of the PGO wave, a sign of alerting (5, 58), but the most obvious effect appeared to be on behavioral arousal. Studies of unit activity in DRN and MRN suggest a possible resolution to this puzzle. The activity of some DRN and MRN neurons is suppressed during, and for several seconds following, orienting to visual and auditory stimuli (26). The typical orienting response of cats is to turn the head or rotate the ear pinna toward the source of the stimulus. At this time, all other overt movement is usually suppressed. The suppression of DRN unit activity also often accompanies the appearance of PGO waves (8, 41, 45, 72). Thus, it may be that 5-HT inputs into the amygdala may act as a trigger to resume other behavioral activity after the pause in that behavior during orienting. Hence, during a period of spontaneous PGO wave occurrence, i.e., REM, the effect of exogenous 5-HT on behavioral arousal could be regarded as an extension or exaggeration of its usual role in waking.

Anatomical basis of amygdaloid modulation of behavioral state.

The amygdala has anatomical connections that provide a possible substrate for control of behavioral state. The central nucleus projects to several brainstem regions implicated in the generation of REM and PGO waves. A major pathway

originating in the central nucleus and projecting to the lateral parabrachial region of the pontomesencephalic tegmentum has been demonstrated in several species including rats (3, 36, 46, 61) and cats (36, 71). This region has recently been demonstrated to have an important role in the generation of REM and PGO waves. Microinjections of the cholinergic agonist carbachol into the parabrachial region of cats produces increased REM and PGO waves that persist over a period of several days (9, 10). In rats, reciprocal projections have been demonstrated between the amygdala and PPT, LDT and the locus coeruleus (29, 54, 63, 75). In cats, these areas are implicated in the control of REM and PGO wave generation (65); and similar reciprocal projections with the amygdala have been demonstrated (24, 71).

Connections of the amygdala to other forebrain regions may also be important. It projects to and receives input from cholinergic regions in the basal forebrain in rats (15, 19, 20, 78) which, in turn, has reciprocal connections with the brainstem reticular formation including PPT/LDT (28, 29, 39, 62, 67, 77). The basal forebrain of cats contains neurons that have increased firing in REM and alert wakefulness, as well as a group of neurons that increase firing at sleep onset and during NREM (69, 70). Cells with similar state-related firing patterns have also been found in the anterior hypothalamic and medial preoptic areas in rats (35). The existence of these cells suggests that the basal forebrain contains neurons that may promote sleep as well as neurons that are involved in cortical activation (68, 70). Given the demonstrated role the basal forebrain plays in arousal, the reciprocal connections between the amygdala and basal forebrain may well be involved in the amygdaloid modulation of behavioral arousal that we observed.

Site of action for 5-HT.

The results of our autoradiographic studies suggest that the basolateral nucleus has relatively denser serotonergic innervation than the central, medial and lateral nuclei. Studies using immunocytochemical techniques have also demonstrated relatively heavy 5-HT innervation in the basolateral, lateral and medial nuclei and in the medial edge of the central nucleus, with much lower density in the lateral central nucleus (12). Although there were variations in actual injection sites, given the volume we injected, there could easily have been diffusion into regions with the greatest serotonergic innervation, e.g., the basolateral nucleus. Further work will be required to determine the precise site(s) of action of 5-HT in the amygdala.

Conclusion.

The present study indicates that serotonergic mechanisms in the amygdala have a role in modulating alerting and behavioral arousal. The amygdala provides a potential mechanism by which emotion may influence alerting behaviors and arousal state. These findings suggest that one should not focus entirely on the brainstem in attempting to delineate the mechanisms underlying the control of REM sleep. Taken together, our results and those examining serotonergic mechanisms in the brainstem suggest that forebrain and brainstem regions may work together to regulate REM initiation and maintenance.

SUMMARY

The role of 5-HT mechanisms in the amygdala in the modulation of sleep and arousal states and PGO waves was examined. Studies of the amygdala suggest that it provides a neural mechanism by which emotionally-relevant or significant stimuli may influence behavioral state and alerting mechanisms. The amygdala projects massively (via the central nucleus) into brainstem regions involved in alerting and in the generation of REM and PGO waves. Serotonergic innervation of the amygdala comes from DRN and to a lesser degree MRN. Microinjections of 5-HT into the amygdala produced short-latency changes of state from NREM and REM with the effect being relatively greater in REM. Microinjections of the 5-HT antagonist, methysergide, increased sleep efficiency and increased PGO wave frequency in waking and NREM. These results demonstrate an important role for the amygdala in the control of behavioral state and alerting mechanisms and suggest that 5-HT exerts some of its regulatory effects via an influence on forebrain regions.

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REFERENCES

1. Amaral, D. G., Price, J. L., Pitkanen, A. and Carmichael, S. T. Anatomical organization of the primate amygdaloid complex. Pp. 1-66. In: Aggleton, J. P. (Ed.), *The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction.* Wiley-Liss Inc., New York, 1992.
2. Baghdoyan, H. A., Spotts, J. L. and Snyder, S. G. Simultaneous pontine and basal forebrain microinjections of carbachol suppress REM sleep. *J. Neurosci.*, **13**: 229-242, 1993.
3. Bernard, J. F., Alden, M. and Besson, J. M. The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: A phaseolus vulgaris leucoagglutinin (PHA-L) study in rat. *J. Comp. Neurol.*, **329**: 201-229, 1993.
4. Bordi, F., LeDoux, J. E., Clugnet, M. C. and Pavlides, C. Single-unit activity in the lateral nucleus of the amygdala and overlying areas of the striatum in freely behaving rats: Rates, discharge patterns, and responses to acoustic stimuli. *Behav. Neurosci.*, **107**: 757-769, 1993.
5. Bowker, R. M. Waking PGO waves: Largest amplitudes occur only during alerting reactions not high arousal levels. Pp. 179-199. In: Bandler, R. M. (Ed.), *Modulation of Sensorimotor Activity during Alterations in Behavioral States.* Alan R. Liss, Inc., New York, 1984.
6. Calvo, J. M., Badillo, S., Morales-Ramirez, M. and Palacios-Salas, P. The role of the temporal lobe amygdala in ponto-geniculo-occipital activity and sleep organization in cats. *Brain Res.*, **403**: 22-30, 1987.

7. CALVO, J. M. and FERNANDEZ-GUARDIOLA, A. Phasic activity of the basolateral amygdala, cingulate gyrus and hippocampus during REM sleep in the cat. *Sleep*, **7**: 202-210, 1984.
8. CESPUGLIO, R., FARADJI, H., GOMEZ, M. E. and JOUVET, M. Single unit recordings in the nuclei raphe dorsalis and magnus during the sleep-waking cycle of semi-chronic prepared cats. *Neurosci. Lett.*, **24**: 133-138, 1981.
9. DATTA, S., CALVO, J. M., QUATTROCHI, J. J. and HOBSON, J. A. Long-term enhancement of REM sleep following cholinergic stimulation. *NeuroRep.*, **2**: 619-622, 1991.
10. DATTA, S., CALVO, J. M., QUATTROCHI, J. J. and HOBSON, J. A. Cholinergic microstimulation of the peribrachial nucleus in the cat. I. Immediate and prolonged increases in pontogeniculo-occipital waves. *Arch. Ital. Biol.*, **130**: 263-284, 1992.
11. DAVIS, M. The role of the amygdala in fear and anxiety. *Ann. Rev. Neurosci.*, **15**: 353-375, 1992.
12. FALLON, J. H. and CIOFI, P. Distribution of monoamines within the amygdala. Pp. 97-114. In: AGGLETON, J. P. (Ed.), *The Amygdala: Neurobiological Aspects of Emotion, Memory and Mental Dysfunction*. Wiley-Liss Inc, New York, 1992.
13. FARBER, J., MARKS, G. A. and ROFFWARG, H. P. Rapid eye movement sleep PGO-type waves are present in the dorsal pons of the albino rat. *Science*, **209**: 615-617, 1980.
14. FEINGOLD, E., SESHADRI, S. B. and TRETIAK, O. Hardware and software design considerations in engineering an image processing workstation: Autoradiographic analysis with DUMAS and the BRAIN autoradiographic analysis software package. Pp. 175-203. In: MCEACHRON, D. L. (Ed.), *Experimental Biology and Medicine: Functional Mapping in Biology and Medicine*. Karger, Basel, 1986.
15. GALLAGHER, M. and HOLLAND, P. C. The amygdala complex: Multiple roles in associative learning and attention. *Proc. Natl. Acad. Sci.*, **91**: 11771-11776, 1994.
16. GEARY, W. A. and WOOTEN, G. F. Quantitative film autoradiography of opiate agonists and antagonists. *J. Pharmacol. Exp. Ther.*, **225**: 234-240, 1983.
17. GEARY, W. A. and WOOTEN, G. F. Quantitative film autoradiography for tritium: Methodological considerations. *Brain Res.*, **337**: 99-108, 1985.
18. GESCHWIND, N. The disconnection syndromes in animals and man. *Brain*, **88**: 237-294, 1965.
19. GROVE, E. A. Neural associations of the substantia innominata in the rat: Afferent connections. *J. Comp. Neurol.*, **277**: 315-346, 1988.
20. GROVE, E. A. Efferent connections of the substantia innominata in the rat. *J. Comp. Neurol.*, **277**: 347-364, 1988.
21. GUILLEMINAULT, C. Narcolepsy syndrome. Pp. 338-346. In: KRYGER, M. H., ROTH, T., and DEMENT, W. C. (Eds.), *Principles and Practice of Sleep Medicine*. W. B. Saunders, Philadelphia, 1989.
22. HOBSON, J. A. The effects of cortical and muscular activity during sleep and waking in the cat. *EEG Clin. Neurophysiol.*, **19**: 41-62, 1965.
23. HONDA, T. and SEMBA, K. Serotonergic synaptic input to cholinergic neurons in the rat mesopontine tegmentum. *Brain Res.*, **647**: 299-306, 1994.
24. HOPKINS, D. A. and HOLSTEGE, G. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. *Exp. Brain Res.*, **32**: 529-547, 1978.
25. HORNER, R. L., SANFORD, L. D., PACK, A. I. and MORRISON, A. R. Microinjection of serotonin in the vicinity of the laterodorsal tegmental (LDT) nucleus inhibits REM sleep. *Neurosci. Abstr.*, **21**: 180, 1995.
26. JACOBS, B. L. and AZMITIA, E. C. Structure and function of the brain serotonin system. *Physiol. Rev.*, **72**: 165-229, 1992.
27. JONES, B. and MISHKIN, M. Limbic lesions and the problem of stimulus-reinforcement associations. *Exp. Neurol.*, 362-377, 1972.

28. JONES, B. E. and BEAUDET, A. Distribution of acetylcholine and catecholamine neurons in the cat brainstem: A choline acetyltransferase and tyrosine hydroxylase immunohistochemical study. *J. Comp. Neurol.*, **261**: 15-32, 1987.
29. JONES, B. E. and YANG, T. The efferent projections from reticular formation and the locus coeruleus studied by anterograde and retrograde axonal transport in the rat. *J. Comp. Neurol.*, **242**: 56-92, 1985.
30. JOUVET, M. Recherches sur les structures nerveuses et les mécanismes responsables des différentes phases du sommeil physiologique. *Arch. Ital. Biol.*, **100**: 125-206, 1962.
31. JOUVET, M. Cataplexie et sommeil paradoxal reflexes chez le chat pontine. *C. R. Soc. Biol. Paris*, **159**: 383-387, 1964.
32. KAUFMAN, L. S. PGO waves in rats in the non-paradoxical sleep states. *Brain Res.*, **276**: 73-80, 1983.
33. KAUFMAN, L. S. and MORRISON, A. R. Spontaneous and elicited PGO spikes in rats. *Brain Res.*, **214**: 61-72, 1981.
34. KOVACHICH, G. B., ARONSON, C. E., BRUNSWICK, D. J. and FRAZER, A. Quantitative autoradiography of serotonin uptake sites in rat brain using 3H-cyanoimipramine. *Brain Res.*, **454**: 78-88, 1988.
35. KOYAMA, Y. and HAYAISHI, O. Firing of neurons in the preoptic/anterior hypothalamic areas in rat: Its possible involvement in slow wave sleep and paradoxical sleep. *Neurosci. Res.*, **19**: 31-38, 1994.
36. KRETTEK, J. E. and PRICE, J. L. Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. *J. Comp. Neurol.*, **178**: 225-254, 1978.
37. LEDOUX, J. E. Emotion. Pp. 419-459. In: PLUM, F. (Ed.), *Handbook of Physiology. The Nervous System. Vol. 5. Higher Functions of the Brain.* American Physiological Society, Bethesda, 1987.
38. LEDOUX, J. E. Emotion and the amygdala. Pp. 339-351. In: AGGLETON, J. E. (Ed.), *The Amygdala: Neurobiological Aspects of Emotion, Memory and Mental Dysfunction.* Wiley-Liss, Inc., New York, 1992.
39. LOSIER, B. J. and SEMBA, K. Dual projections of single cholinergic and aminergic brainstem neurons to the thalamus and basal forebrain in the rat. *Brain Res.*, **604**: 41-52, 1993.
40. LUEBKE, J. I., GREENE, R. W., SEMBA, K., KAMONDI, A., MCCARLEY, R. W. and REINER, P. B. Serotonin hyperpolarizes cholinergic low-threshold burst neurons in the rat laterodorsal tegmental nucleus in vitro. *Proc. Natl. Acad. Sci.*, **89**: 743-747, 1992.
41. LYDIC, R., MCCARLEY, R. W. and HOBSON, J. A. Serotonin neurons and sleep. I. Time course of dorsal raphe discharge, PGO waves and behavioral state. *Arch. Ital. Biol.*, **126**: 1-28, 1987.
42. MARINI, G., GRITTI, I. and MANCIA, M. The role of some thalamic nuclei in sleep mechanisms: Evidence from chemical lesions in the cat. Pp. 171-198. In: MANCIA, M. and MARINI, G. (Eds.), *The Diencephalon and Sleep.* Raven Press, New York, 1990.
43. MARKS, G. A., FARBER, G. and ROFFWARG, H. P. Metencephalic localization of ponto-geniculo-occipital waves in the albino rat. *Exp. Neurol.*, **69**: 667-677, 1980.
44. MARKS, G. A., FARBER, G., RUBENSTEIN, M. and ROFFWARG, H. P. The demonstration of ponto-geniculo-occipital waves in the albino rat. *Exp. Neurol.*, **69**: 648-666, 1980.
45. MCGINTY, D. J. and HARPER, R. M. Dorsal raphe neurons: Depression of firing during sleep in cats. *Brain Res.*, **101**: 569-575, 1976.
46. MOGA, M. M. and GRAY, T. S. Evidence for corticotropin-releasing factor, neurotensin, and somatostatin in the neural pathway from the central nucleus of the amygdala to the parabrachial nucleus. *J. Comp. Neurol.*, **241**: 275-284, 1985.

47. MORILAK, D. A. and CIARANELLO, R. D. 5-HT_2 receptor immunoreactivity on cholinergic neurons of the pontomesencephalic tegmentum shown by double immunofluorescence. *Brain Res.*, **627**: 49-54, 1993.
48. MORRISON, A. R. Brainstem regulation of behavior during sleep and wakefulness. Pp. 91-131. In: SPRAGUE, J. M. and EPSTEIN, A. W. (Eds.), *Progress in Psychobiology and Physiological Psychology. Vol. 8.* Academic Press, New York, 1979.
49. MORRISON, A. R., SANFORD, L. D., BALL, W. A., MANN, G. L. and ROSS, R. J. Stimulus elicited behavior in rapid eye movement sleep without atonia. *Behav. Neurosci.*, **109**: 972-979, 1995.
50. MORRISON, A. R. and REINER, P. B. A dissection of paradoxical sleep. Pp. 97-110. In: MCGINTY, D. J., DRUCKER-COLIN, R., MORRISON, A. R., and PARMEGGIANI, P. L. (Eds.), *Brain Mechanisms of Sleep.* Raven Press, New York, 1985.
51. PARMEGGIANI, P. L. Telencephalo-diencephalic aspects of sleep mechanisms. *Brain Res.*, **7**: 350-359, 1968.
52. PARMEGGIANI, P. L. Temperature regulation during sleep: a study in homeostasis. Pp. 97-143. In: OREM, J. and BARNES, C. D. (Eds.), *Physiology in Sleep.* Academic Press, New York, 1980.
53. PAXINOS, G. and WATSON, C. *The Rat Brain.* Academic Press, San Diego, XXVI-236 pp., 1986.
54. PETROV, T., KRUKOFF, T. L. and JHAMANDAS, J. H. Branching projections of catecholaminergic brainstem neurons to the paraventricular hypothalamic nucleus and the central nucleus of the amygdala in the rat. *Brain Res.*, **609**: 81-92, 1993.
55. REICH, H. Modulation of unit activity in the amygdala of unrestrained cats during the sleep-waking cycle. *Neurosci. Lett.*, **35**: 209-214, 1983.
56. ROSS, R. J., SANFORD, L. D., MANN, G. L., BRANDOM, D. J. and MORRISON, A. R. Role of serotonin in pontine control of rapid eye movement sleep (REM): lack of evidence for $5HT_2$ receptor mechanism. *Neurosci. Abstr.*, **19**: 592, 1993.
57. SAKAI, K., MANSARI, M. E., LIN, J. S., ZHANG, G. and VANNI-MERCIER, G. The posterior hypothalamus in the regulation of wakefulness and paradoxical sleep. Pp. 171-198. In: MANCIA, M. and MARINI, G. (Eds.), *The Diencephalon and Sleep.* Raven Press, New York, 1990.
58. SANFORD, L. D., MORRISON, A. R., BALL, W. A., ROSS, R. J. and MANN, G. L. The amplitude of elicited PGO waves: a correlate of orienting. *EEG Clin. Neurophysiol.*, **86**: 438-445, 1993.
59. SANFORD, L. D., ROSS, R. J., SEGGOS, A. E., MORRISON, A. R., BALL, W. A. and MANN, G. L. Central administration of two 5-HT receptor agonists: effect on REM sleep initiation and PGO waves. *Pharmacol. Biochem. Behav.*, **49**: 93-100, 1994.
60. SANFORD, L. D., TEJANI-BUTT, S. M., ROSS, R. J. and MORRISON, A. R. Elicited PGO waves in rats: Lack of 5-HT_{1A} inhibition in putative pontine generator region. *Pharmacol. Biochem. Behav.*, **53**: in press, 1995.
61. SAPER, C. B. and LOEWY, A. D. Efferent connections of the parabrachial nucleus in the rat. *Brain Res.*, **197**: 291-317, 1980.
62. SATOH, T. and FIBIGER, H. C. Cholinergic neurons of the laterodorsal tegmental nucleus: Efferent and afferent connections. *J. Comp. Neurol.*, **253**: 277-302, 1986.
63. SEMBA, K. and FIBIGER, H.C. Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: A retro- and antero-grade transport and immunohistochemical study. *J. Comp. Neurol.*, **323**: 387-410, 1992.
64. SIMON, R. P., GERSHON, M. D. and BROOKS, D. C. The role of raphe nuclei in the regulation of ponto-geniculo-occipital wave activity. *Brain Res.*, **58**: 313-330, 1973.
65. STERIADE, M. and MCCARLEY, R. W. *Brainstem Control of Wakefulness and Sleep.* Plenum Press, New York, XV-499 pp., 1990.

66. STERIADE, M., PARE, D., BOUHASSIRA, M., DESCHENES, M. and OAKSON, G. Phasic activation of lateral geniculate and perigeniculate thalamic neurons during sleep with ponto-geniculo-occipital waves. *J. Neurosci.*, **9**: 2215-2229, 1989.
67. SWANSON, L. W., MOGENSON, G. J., GERFEN, C. R. and ROBINSON, P. Evidence for a projection from the lateral preoptic area and substantia innominata to the "mesencephalic locomotor region" in the rat. *Brain Res.*, **295**: 161-178, 1984.
68. SZYMUSIAK, R. S. Magnocellular nuclei of the basal forebrain: Substrates of sleep and arousal regulation. *Sleep*, **6**: 478-500, 1995.
69. SZYMUSIAK, R. S. and MCGINTY, D. Sleep-related neuronal discharge in the basal forebrain of cats. *Brain Res.*, **370**: 82-92, 1986.
70. SZYMUSIAK, R. S. and MCGINTY, D. Sleep-waking discharge of basal forebrain projection neurons in cats. *Brain Res. Bull.*, **22**: 423-430, 1989.
71. TAKEUCHI, Y., MEGIRIAN, D. and HOPKINS, D. A. Reciprocal connections between the amygdala and parabrachial nuclei: ultrastructural demonstration by degeneration and axonal transport of horseradish peroxidase in the cat. *Brain Res.*, **239**: 583-588, 1982.
72. TRULSON, M. E. and JACOBS, B. L. Raphe unit activity in freely moving cats: Correlation with level of behavioral arousal. *Brain Res.*, **163**: 135-150, 1979.
73. VERTES, R. P., COLOM, L. V., FORTIN, W. J. and BLAND, B. H. Brainstem sites for the carbachol elicitation of the hippocampal theta rhythm in the rat. *Brain Res.*, **96**: 419-429, 1993.
74. VILLABLANCA, J. Behavioral and polygraphic study of "sleep" and "wakefulness" in chronic decerebrate cats. *EEG Clin. Neurophysiol.*, **21**: 562-577, 1966.
75. WALLACE, D. M., MAGNUSON, D. J. and GRAY, T. S. The amygdalo-brainstem pathway: Selective innervation of dopaminergic, noradrenergic and adrenergic cells in the rat. *Neurosci. Lett.*, **97**: 252-258, 1989.
76. WEISKRANTZ, C. Behavioral changes associated with ablation of the amygdaloid complex in monkeys. *J. Comp. Physiol. Psychol.*, **49**: 381-391, 1956.
77. WILSON, P. M. A photographic perspective on the origin, form, course and relations of the acetylcholinesterase-containing fibers of the dorsal tegmental pathways in the rat brain. *Brain Res. Rev.*, **10**: 85-118, 1985.
78. ZABORSKY, L., LERANTH, C. and HEIMER, C. Ultrastructural evidence of amygdalofugal axons terminating on cholinergic cells of the rostral forebrain. *Neurosci. Lett.*, **52**: 219-225, 1984.