GABAergic Regulation of REM Sleep in Reticularis Pontis Oralis and Caudalis in Rats

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INTRODUCTION
Considerable work has implicated the nucleus reticularis pontis oralis (RPO) in the generation of rapid eye movement sleep (REM) and its related characteristics (reviewed in Siegel 2000). Electrolytic (e.g., Carli and Zanchetti 1965; Gutierrez-Rivas et al. 1978) and chemical lesions (Webster and Jones 1988) that produce extensive damage to RPO have been reported to significantly reduce or eliminate REM, leading to the suggestion that this region is essential for REM generation (reviewed in Siegel 2000). In addition, RPO (or the homologous region in the medial pontine reticular formation) in cats contains sites in which microinjection of the cholinergic agonist, carbachol, induces REM (e.g., Baghdoyan et al. 1984, 1987; Garzon et al. 1998; Vanni-Mercier et al. 1989). In rats, the most effective sites for carbachol induction of REM appear to be located in caudal RPO and bordering nucleus reticularis pontis caudalis (RPC) (Bourgin et al. 1995; Deurveilher et al. 1997).

Less effort has been directed at resolving the role of RPC in generating and regulating REM. In cats, electrolytic lesions of the gigantocellular neurons (found only in RPC) disrupt REM, whereas REM is normal after chemical lesions that destroy only cell bodies (Sastre et al. 1979, 1981). These findings suggest that the lesion-produced effect on REM is due to damage to tracts in RPC and that the gigantocellular neurons are not necessary for REM (Sastre et al. 1979, 1981).

The pontine inhibitory area (PIA) that plays a role in the suppression of postural muscle tone courses through RPO and RPC (Hajnik et al. 2000; Lai and Siegel 1999), and both regions have been implicated in the regulation of muscle tone. A few studies in rats have examined the effect of electrolytic lesions of RPO and RPC in the context of REM without atonia (REM-A) (Mirmiran 1983; Mouret et al. 1967; Sanford et al. 2001a). Lesions in both regions could produce REM without atonia.

A recent series of papers has explored the role of GABA in RPO in the regulation of sleep and wakefulness in cats (Xi et al. 1999, 2001). Unilateral microinjections of both GABA and the GABA_A agonist muscimol (MUS) into RPO, at sites where carbachol induced REM, increased wakefulness, whereas microinjections of the γ-aminobutyric acid-A (GABA_A) antagonist, bicuculline (BIC), produced long-duration REM episodes that could occur without intervening non-REM (NREM) (Xi et al. 1999, 2001). Increases in REM occurred in a dose-dependent manner at concentrations of BIC ≤ 10 mM. However, in some cats, microinjections of higher concentrations produced wakefulness and “hyperextension” (Xi et al. 2001).

The rat is an increasingly important model for examining physiological mechanisms of sleep. However, to our knowledge, there has only been a single study examining the potential influence of GABAergic mechanisms RPO and RPC of rats on sleep and wakefulness. This brief report utilized only one concentration of MUS and one concentration of BIC and implicated GABA in RPO and RPC in regulation of sleep and wakefulness in rats. However, there was no reported alteration in REM, or differences for either drug across injection sites (Camacho-Arroyo et al. 1991). Given the reported increase in REM with the more extensive microinjection studies of BIC into RPO of cats, we examined the effects of various concentrations of MUS and BIC infused into RPO on sleep and
wakefulness. We also performed similar microinjection studies in RPC and compared the effects across regions.

METHODS

The subjects were 17 male Sprague-Dawley rats of approximately 90 days of age at the time of surgery. The animals were given ad libitum access to food and water. The recording room was kept on a 12:12 light:dark cycle with lights on from 0700 to 1900 h and ambient temperature was maintained at 24.5 ± 0.5°C.

Screw electrodes were implanted in the skull for recording the cortical electroencephalogram (EEG). The EEG electrodes were placed contralaterally (AP 1.0, ML 1.0; AP −4.5, ML 3.0). A reference screw electrode was placed over the frontal sinus. Stainless steel wire electrodes were implanted in the dorsal neck musculature for recording the electromyogram (EMG). Leads from the recording electrodes were routed to a nine-pin miniature plug that mated to one attached to a recording cable. Guide cannulae (26 ga.) for microinjections were bilaterally implanted with their tips aimed 1.0 mm above RPO (AP −9.3, ML ±1.1, DV −6.5) or RPC (AP −10.5, ML ±1.5, DV −6.8). The coordinates were taken from the atlas of Kruger et al. (1995), which is more accurate for brain stem structures in Sprague-Dawley rats. The recording plug and cannulae were affixed to the skull with dental acrylic and anchor screws.

The surgical procedures were performed stereotactically under aseptic conditions. The rats were anesthetized with isoflurane (5% induction; 2% maintenance). Buprenorphine (0.5 mg/kg) was administered for potential postoperative pain. The rats were allowed a minimum of 14 days to recover prior to beginning the experiment. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by Eastern Virginia Medical School’s Animal Care and Use Committee (Protocol No. 99-022).

Each rat was studied in its home cage, which was placed into a sound-attenuating chamber during recording sessions. For electrophysiological recording, a lightweight, shielded recording cable was connected to the plug on the rat’s head. The cable was attached to a commutator that permitted free movement of the rat within its cage.

The animals were habituated to the handling procedures and recording chamber over the course of six recording sessions prior to receiving any drug microinjections. In the first two sessions, the rats were connected to the cable and left undisturbed for 6 h. During the next two sessions, the animals received prerecording handling, including placement of the microinjection cannulae, just as they would be for the experimental sessions, and afterward they were connected to the cable for 6 h. For the last two sessions, control microinjections of saline were administered, followed by 6-h polygraphic recordings. The second saline recording was used as the baseline control in this study. Following habituation and baseline sessions, the rats received microinjections of the various concentrations of each drug and a last saline alone control in a counterbalanced order. All recording sessions were separated by a minimum of 4 days.

For microinjections, injection cannulae (33 ga.), which projected 1 mm beyond the tip of the guide cannulae, were secured in place within the guide cannulae. The microinjection cannulae were connected to lengths of polyethylene tubing that in turn were connected to 1.0 μl Hamilton syringes. The injection cannulae and tubing were prefilled with the solution to be injected.

MUS (muscimol hydrobromide, 5-aminomethyl-3-hydroxyisoxazole) and BIC (l2-bicuculline methiodide) were obtained from Sigma-Aldrich, St. Louis, MO. The solutions were prepared in 0.9% physiological saline, which served as the vehicle. Following habituation and control recordings with microinjections of saline alone (0.2 μl), microinjections of MUS (200, 1,000.0 μM) and BIC (0.056, 0.333, 1.0, 1,000.0, and 10,000.0 μM) were made prior to the start of the recording procedure. The volume of all microinjections was 0.2 μl. A second control saline microinjection was counterbalanced with the drug injections. The solutions were slowly infused over 3 min (0.07 μl/min), and the injection cannulae were allowed to stay in place for 1.0 min after the microinjection was finished. After receiving the drug or saline injections, the rats were placed in the sleep recording chambers and connected to the cables as described above.

In addition, at the end of the study a higher concentration of BIC (10,000.0 μM) was microinjected into RPO in nine animals and into RPC in four animals. Polygraphic studies were not conducted in these animals because of the behavioral effects produced at this concentration. The behavior exhibited by these animals, and others that showed overt behavioral reactions at lower dosages, was observed and recorded.

Studies were conducted on each test day between 1100 and 1700 h. EEG and EMG output from the polygraphs (Grass model 12) was routed to an A/D board, digitized at 128 Hz, and stored to file using a custom program.

Trained observers visually determined wakefulness, NREM, and REM from digitized records using standard electrographic criteria (Sanford et al. 1995, 1998, 2001b). Wakefulness was scored based on the presence of low-voltage, fast EEG; high-amplitude, tonic EMG level, and phasic EMG bursts that could be associated with gross body movements. NREM was scored based on the presence of spindles interspersed with slow waves, lower muscle tone, and no gross body movements or EEG desynchronization. For scoring REM, onset was noted immediately following the last slow spindle of NREM that occurred in conjunction with decreasing or fully relaxed muscle tone. Afterward, REM was scored continuously during the presence of low voltage, fast EEG, θ-rhythm, and muscle atonia.

The measures examined were total REM (min), total NREM (min), total sleep (REM + NREM), REM percentage (total REM/total sleep * 100), number and average duration (min) of REM episodes, and REM latency (min). The data were analyzed with analysis of variance (ANOVA) procedures for repeated measures. When appropriate, planned comparisons were performed using Bonferroni corrected t-test.

On completion of the experiment, the rats were overdosed with pentobarbital sodium (150 mg/kg ip) and perfused intracardially with 0.9% saline and 10% formalin. The brains were processed to determine cannula and electrode placements. For this purpose 40-μm slices were made through the areas of interest with a cryostat, and the sections were stained with cresyl violet. Injections sites in RPO, RPC, or outside either region were verified by comparing sections to those in the stereotaxic atlas of Kruger et al. (1995). Only rats that had verified locations in RPO or RPC were included in the analyses.

RESULTS

Sleep parameters after microinjections into RPO

In the 6-h recording period after microinjection of BIC into RPO, there was a concentration-dependent increase in REM parameters that reached significance at the 1.0 μM concentration (Fig. 1, left). Microinjection of the 1.0 μM concentration of BIC produced a significant increase in total REM and REM percentage. This was accompanied by an increase in mean REM episode duration that did not reach Bonferroni-corrected significance. There also were no significant changes in REM episode number, REM latency, total NREM, or total sleep.

In the analysis of the entire 6-h recording period (Fig. 1, right), microinjection of 1.000.0 μM MUS into RPO produced significant decreases in total REM, number of REM episodes, and REM percentage. There was no significant alteration in mean REM episode duration or REM latency. Total sleep was decreased at the 1.000.0 μM concentration, though these changes did not reach a Bonferroni-corrected level of significance. None of the changes at the 200.0 μM concentration of MUS were significant.

The total REM and total NREM data were also analyzed in...
2-h blocks across the recording period. The enhancement of REM produced by microinjections of 1.0 μM BIC began in the first 2-h and became significantly elevated during the second 2-h block (Fig. 2). There were no significant changes in total NREM. The changes in REM produced by 1,000.0 μM MUS occurred in the last 4-h of recording, though none of the reductions for 2-h blocks reached Bonferroni-corrected significance (Fig. 3). In addition, reductions in total REM produced by the 200.0 μM concentration of MUS did not reach Bonferroni-corrected significance.

Sleep parameters after microinjections into RPC

Sleep parameters after microinjection of BIC into RPC are presented in the left panel of Fig. 1. There were no significant alterations in sleep at any of the dosages tested.

Microinjection of the 200.0 μM concentration of MUS into RPC produced a decrease in the number of REM episodes and decreases in total REM and REM percentage that did not reach Bonferroni-corrected significance (Fig. 1, right). Microinjections of the higher 1,000.0 μM concentration of MUS did produce significant decreases in total REM, number of REM episodes, and REM percentage, as well as a nonsignificant decrease in mean REM episode duration.

In the analysis of 2-h blocks, there were no significant changes in REM with either concentration of BIC (Fig. 2), and neither MUS (Fig. 3) nor BIC produced significant alterations in total NREM in any block. The reduction in REM produced by 1,000.0 μM MUS microinjected into RPC was significant in all blocks (Fig. 3). The smaller reduction produced by 200.0 μM MUS was significant during the second 2-h block.

Behavioral effects of BIC microinjections

Behavioral reactions, predominantly characterized by circling and by spontaneous escape-like behaviors, were observed...
in some rats after receiving a microinjection of 1,000.0 μM BIC. Overt behaviors were observed in three of nine rats microinjected into RPO and three of eight rats microinjected into RPC. No overt behavioral effects were observed in the remaining rats.

Because a subset of rats at the 1,000.0 μM concentration of BIC exhibited behavioral reactions, we separately examined the sleep records of those showing behavior and those not showing behaviors. Figure 4 presents comparisons of sleep parameters between saline and BIC injection into RPO and RPC in rats that showed (BR) and did not show (Non-BR) overt behavioral reactions. With microinjections of 1,000.0 μM BIC into RPO, rats that did not show an overt behavioral reaction to the drug exhibited increased REM and decreased NREM, whereas those that did show overt behaviors exhibited decreased REM. In contrast, after microinjection of 1,000.0 μM BIC into RPC, neither “behaving” nor “nonbehaving” rats showed alterations in sleep.

As a final step in the experiment, we microinjected the dosage of BIC that has been reported to be maximally effective in producing REM in cats (Xi et al. 1999). Microinjections of 10,000.0 μM BIC consistently produced prolonged wakefulness as well as circling and spontaneous escape-like behaviors in virtually all of the animals we tested. Rats microinjected with this concentration of BIC into RPO exhibited circling and escape-like behaviors, and we were unable to fully complete the microinjections in one of these animals due to the immediate reaction it had to this concentration of drug. Only one rat microinjected into RPO had no overt behavioral response at the 10,000.0 μM dose.

In the animals microinjected into RPC with 10,000.0 μM BIC, similar circling and escape-like behaviors were observed. These animals also did not fully support themselves quadrupedally. Polygraphic studies were not attempted in any of the animals at this concentration due to the hyper-excited reactions the animals had.

**Histology**

Figure 5 presents line drawings illustrating cannulae placement in all animals receiving microinjections of MUS or BIC.
All injection sites were either in RPO or RPC, though there were variations in placement along the rostrocaudal extent of each nucleus. Injection sites in rats that showed overt behaviors after injection of higher concentrations of BIC, and those that did not are differentially indicated in Fig. 5. However, there were no obvious differences in injection sites that could explain whether overt behavioral effects were observed.

**DISCUSSION**

The present data indicate that GABAergic mechanisms in both RPO and RPC of rats are involved in the inhibitory regulation of REM sleep. However, there were significant differences in the effects of GABAergic agents in the two areas. Microinjections of MUS into both RPO and RPC suppressed REM. In contrast, microinjections of concentrations of BIC ≤1,000.0 μM into RPO enhanced REM, but produced no significant effects on sleep when microinjected into RPC. Higher concentrations of BIC injected into both regions produced circling and escape-like behavior when microinjected.

Xi et al. (1999) suggested that GABAergic regulation of RPO might arise either from local interneurons or from external neurons that send axons into RPO. Recent data support the idea that local GABAergic neurons are involved and that RPO, not RPC, is the critical region for the generation of REM. Maloney et al. (2000) found that while the overall numbers of Fos-positive neurons increased in RPO in association with a deprivation-induced REM rebound, the numbers of GABAergic neurons expressing Fos were decreased in association with REM rebound. In contrast, GABAergic neurons expressing Fos in RPC were increased in association with normal and rebound REM. Maloney et al. (2000) suggested that GABAergic neurons in RPO may be active during NREM and inactive during REM. This would provide local GABAergic inhibition of RPO “REM-on” neurons during NREM and wake and would disinhibit them during REM. This hypothesis is consistent with our finding that BIC in RPO enhanced REM and suggests that the mechanism of action could be via antagonizing local GABAergic inhibitory neurons.

RPO begins at the rostral edge of the pons and is continuous with RPC. Together, they form two divisions of the core of the pontine reticular formation (reviewed in Siegel 2000).
though the demarcation between divisions is not distinct, RPC contains giant cells, whereas RPO does not (Siegel 2000). We relied on histological verification to determine whether the injection site was in RPO or RPC. However, given the fact that these regions are adjacent, there is always the possibility that drug diffused from one region into the other. The similar effects of MUS on REM when microinjected into either region suggests this.

Lesions placed in both RPO (e.g., Carli and Zanchetti 1965; Gutierrez-Rivas et al. 1978; Webster and Jones 1988) and RPC (Sastre et al. 1979, 1981) have been found to disrupt REM, though disruptions in REM due to lesions in RPC may result from damage to fibers of passage (Sastre et al. 1979, 1981). The distinct lack of effect of BIC (1,000.0 μM and less) on sleep when microinjected into RPC compared with the REM promoting effect BIC had in RPO also indicates that there may be considerable regional specificity for neurons actually involved in the generation of REM.

Xi et al. (2001) have suggested that activation of the GABAAergic system in RPO eliminates sleep and promotes wakefulness. In their proposal, a “gating” mechanism produces wakefulness when GABAAergic tone in RPO is high and allows REM when GABAAergic tone is low. In support of this idea, they found that microinjection of 10.0 mM MUS into RPO in cats significantly increased wakefulness at the expense of REM and NREM, whereas 10.0 mM BIC enhanced REM without significant alterations in wakefulness or NREM. In rats, we found that lower concentrations of both MUS and BIC could produce significant reduction and enhancements in REM, respectively, without producing observable alterations in NREM. This suggests that with the proper concentrations, GABAAergic drugs in RPO may specifically alter REM and wakefulness without affecting NREM, though concentrations high enough to produce prolonged wakefulness may necessarily be associated with a decrease in NREM.

Microinjections of BIC into RPO of cats induce a pronounced, short-latency increase in REM that can last up to 1 h (Xi et al. 1999), and a similar effect may be observed in guinea pigs (Torterolo et al. 2002). The present results in rats support the general suggestion, based on this work, that GABAAergic regulation of RPO is important for the generation of REM. However, we did not see such a dramatic immediate increase in REM in rats. Instead, there was an overall increase in REM across the 6-h recording period that was greatest in the third and fourth hours after the injection. Similar species differences have also been observed in the effects of the cholinergic agonist, carbachol, in inducing REM. Microinjections of carbachol into RPO in cats (Garzon et al. 1998; Vanni-Mercier et al. 1989) and guinea pigs (Torterolo et al. 2002) produced a similar prolonged increase in REM. In contrast, microinjections of carbachol into rats may produce lesser magnitude increases in REM that occur with longer latency, with more frequent episodes than fewer prolonged episodes (reviewed in Kubit 2001). Indeed, in rats, both episode duration and episode number were only slightly elevated after microinjections of BIC into RPO, though there was a 35% increase in total REM across the recording period at the 1.0 μM concentration.

It should be noted that some of the differences among studies could be due to methodological differences. We microinjected BIC and MUS bilaterally prior to beginning the recording period. Studies microinjecting carbachol into the pons to induce REM typically use unilateral microinjections in cats (e.g., Baghdoyan et al. 1984, 1987; Garzon et al. 1998; Vanni-Mercier et al. 1989) and rats (e.g., Bourgin et al. 1995, 1999; Marks and Birabil 1998). Marks and Birabil (1998) found that unilateral microinjection of carbachol induced REM when microinjected into RPO in rats, but that REM did not vary from normal after bilateral application. Interestingly, the previous brief report using microinjections of MUS and BIC in rats utilized unilateral microinjections given prior to recording and did not find an effect on REM (Camacho-Arroyo et al. 1991). Studies conducted in cats examining the effects of GABAAergic drugs in RPO utilized unilateral microinjections, though these were also given during NREM in head-fixed animals (Xi et al. 1999, 2001). Thus the possibility exists that unilateral microinjection of BIC given during NREM could produce an immediate induction of REM in rats similar to that seen in cats. Indeed, the REM promoting effects we saw with BIC in RPO are relatively modest in comparison to those recently reported to be produced by application of BIC into the subcoeruleus/sublaterodorsal region. Higher concentrations of BIC (8–10 mM) applied unilaterally to this region by microiontophoresis in head fixed (Boissard et al. 2002) and by microinjection in freely moving (Pollack and Mistlberger 2003) rats produced significant reductions in REM latency and increases in REM amounts.

The concentration of MUS and BIC utilized to produce wakefulness and REM, respectively, in cats and guinea pigs was 10 mM for each drug. This is considerably higher than the concentrations we found effective in rats. Because of the REM-promoting effects reported for higher concentrations of BIC in other species, we did try a higher (10 mM) concentration of BIC in a number of the animals we studied. However, we were unable to conduct polygraphic studies because of the behavioral effects this high concentration produced in most of the animals. These included escape behaviors and prolonged episodes of circling when BIC was microinjected into RPO (8 of 9 rats). Xi et al. (1999) reported that higher concentrations of BIC (>15 mM) also produced increased wakefulness and hyper-excitability in cats when microinjected into RPO. In the four rats microinjected into RPC, we observed circling, agitation, and wakefulness and possibly suppressed muscle tone as observed from their lack of quadrapedal support. Previous work has implicated RPC in the types of overt behaviors that we observed, e.g., intra-cranial stimulation in the ventral region of RPC produced high levels of flight behavior (St-Laurent 1988). However, the induction of these behaviors is not specific to RPC. Application of BIC and other GABA A antagonists produced flight behavior when applied to various regions of the brain including the central gray (Schmitt et al. 1985), inferior colliculus (Brandão et al. 1988), medial (Schmitt et al. 1985), and posterior (Shekhar and DiMicco 1987) hypothalamus and peribrachial region of the pons (Sanford et al. 1998). Similar behavior has been reported in association with REM-enhancing dosages of BIC microinjected into the dorsal subcoeruleus region of rats (Pollack and Mistlberger 2003).

There is the possibility that the behavioral effects at high dosages of GABAAergic drugs may be due to nonspecific effects of the drugs. Because of its widespread distribution and universal inhibitory action, the application of GABAAergic drugs to almost any site in the brain will have behavioral consequences,
and the effects of GABAergic agonists and antagonists may be, respectively, similar to any drug producing inhibition or excitation (Paredes and Agmo 1992).

Microinjections of a 1,000.0 \( \mu M \) concentration of BIC into both RPO and RPC in a subset of rats produced behavioral effects similar to those we saw at the higher concentration. However, only microinjections into RPO produced significant differences in sleep and behavior. Nonbehaving rats showed increases in REM, whereas rats exhibiting injection-induced behaviors during wakefulness showed decreases in REM parameters. There was no observed difference in sleep parameters between behaving and nonbehaving rats that received microinjections into RPC. The small number of animals that showed overt behavioral reactions at this concentration does not allow unequivocal conclusions to be drawn. However, the results do follow the proposal of Chase and his colleagues (e.g., Torterolo et al. 2002; Xi et al. 1999) that RPO contains neurons that are involved in generating REM and wakefulness, and that these are regulated, at least in part, by GABAergic mechanisms.

A recent preliminary study has found that infusion of BIC via reverse microdialysis produces a concentration-dependent increase in acetylcholine (ACh) in the medial pontine reticular formation in cats (Baghdoyan et al. 2002). The increase was blocked by co-infusion of MUS, which had no effect on ACh when infused alone. By comparison, the failure of BIC, at any concentration we examined in RPC, to enhance REM suggests that neurons in this region play a less significant role in the actual generation of REM than do neurons in RPO. This suggestion is in line with studies demonstrating that several compounds applied to RPO promote REM, including carbachol (e.g., Baghdoyan et al. 1984, 1987; Garzon et al. 1998; Marks and Birabil 1998; Vanni-Mercier et al. 1989), vasoactive intestinal peptide (Bourgin et al. 1997, 1999), and an adenosinergic agonist (Marks and Birabil 1998).

RPO and RPC contain neurons involved in producing the atonia of REM (Hajnik et al. 2000; Lai and Siegel 1999). Electrolytic lesions of RPO and RPC in rats can produce REM-A (Mirmiran 1983; Mouret et al. 1967; Sanford et al. 2001a). Relatively small unilateral lesions were sufficient to eliminate the atonia of REM (Sanford et al. 2001a), but larger bilateral lesions in either RPO or RPC could lead to behavioral release during REM-A (Mirmiran 1983; Mouret et al. 1967; Sanford et al. 2001a). Although GABAergic inhibition of neurons in both of these areas suppressed REM, we did not observe alterations of muscle tone during REM in any of the animals studied. This suggests that fibers of passage in these regions may need to be damaged to eliminate the atonia of REM, although damage to fibers of passage may not be sufficient to produce the full behavioral release of REM-A.

In conclusion, the present results demonstrate that the GABAergic agonist, MUS, in both RPO and RPC, can suppress REM, and that the antagonist, BIC, in RPO, but not RPC, can enhance REM. The results support the idea that GABAergic regulation of RPO is involved in the generation of REM and wakefulness. Following previous studies, the results suggest that RPO may play a smaller role in the generation of REM, though GABAergic mechanisms in this region can strongly inhibit REM.

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DISCLOSURES

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REFERENCES


