Regulation of Rapid Eye Movement Sleep in the Freely Moving Rat: Local Microinjection of Serotonin, Norepinephrine, and Adenosine into the Brainstem

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**Study Objectives:** Considerable evidence suggests that rapid eye movement (REM) sleep is induced by glutamatergic activation of cholinergic cells within the pedunculopontine tegmentum (PPT). The aim of this study is to test a popular hypothesis that serotonin, norepinephrine, and adenosine act on PPT cells to regulate REM sleep. This study also tests an alternate hypothesis that serotonin may inhibit REM sleep signs by direct action on the individual REM sleep sign generators.

**Design:** Serotonin, norepinephrine, and adenosine were locally microinjected into the PPT and serotonin was microinjected into the pontine-wave (P-wave) generator (dorsal part of the locus subcoeruleus nucleus) while quantifying the effects on REM sleep and P-wave activity in freely moving rats.

**Setting:** N/A.

**Participants:** N/A.

**Interventions:** N/A.

**Measurements and Results:** Local microinjections of serotonin, norepinephrine, and adenosine into the PPT did not change REM sleep.

**Microinjection of serotonin into the P-wave generator suppressed P-wave activity but not REM sleep.**

**Conclusions:** The present findings provide direct evidence that serotonin, norepinephrine, and adenosine-induced REM sleep suppression in the behaving rat are not mediated by the PPT. The results also provide direct evidence, for the first time, that serotonin suppresses P-wave activity by acting directly on the P-wave generator. These results suggest that the serotonin-induced inhibition of REM sleep in the freely moving rat is probably not mediated through the mesopontine cholinergic cell compartment but, rather, through individual REM sleep sign generators.

**Key Words:** REM sleep; pontine-wave; serotonin; norepinephrine; adenosine; pedunculopontine tegmentum; freely moving rat.

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**INTRODUCTION**

RAPID EYE MOVEMENT (REM) SLEEP IS A SPECIAL BEHAVIORAL STATE identified by the polygraphic signs of activated cortical electroencephalogram (EEG), inactivity of the antigravity muscles, REMs, theta-frequency waves in the hippocampal EEG, and spiky waves in the pontine EEG (P-wave). Evidence from both rat and cat studies over the last 2 decades suggests that each of the events of REM sleep is executed by distinct cell groups in the brain stem. Each of these REM sleep sign-generating neurons is triggered and modulated by the activation of a special group of cells in the cholinergic cell compartment of the pedunculopontine tegmentum (PPT). In support of this statement, anatomic studies have shown that each of these individual REM sleep sign-generating nuclei receives anatomic inputs from the PPT. In a number of studies, it has been shown that the cholinergic activation of individual REM sleep sign-generating nuclei expresses those REM sleep signs independently.

Single-cell recording studies in the rat and cat have shown that the majority of those PPT cells are more active during REM sleep than during slow-wave sleep (SWS). Spontaneous release of acetylcholine in the pontine reticular formation is greater during REM sleep than during waking and SWS. We have recently provided new data that indicate that the activation of cells within the PPT cholinergic cell compartment induces REM sleep. Electrical stimulation of the PPT causes increased acetylcholine release in the pontine reticular formation, indicating that cells within the PPT cholinergic cell compartment are one of the sources of acetylcholine in the pontine reticular formation during REM sleep.

Neurotransmitter-mediated excitation and inhibition of brainstem cells are important processes for the regulation of REM sleep. In recent years, considerable progress has been made in identifying the neurotransmitters and receptors involved in the excitation of PPT cells that induce REM sleep, wakefulness, or both. However, the identification of neurotransmitters involved in the inhibition of PPT cells and suppression of REM sleep in freely moving animals remains incomplete.

Based on single-cell activity patterns in the dorsal raphe (DRN) and locus coeruleus (LC) nuclei and a number of in vivo and in vitro pharmacologic studies, it has been proposed that serotonin and norepinephrine (NE) may inhibit PPT cell activity and in turn block REM sleep. This interpretation receives support from neuroanatomic studies that showed that the PPT receives afferent inputs from the DRN and LC. A microdialysis study in the freely moving cat has shown that the level of extracellular serotonin in the PPT is lowest during REM sleep and highest during wakefulness. The results of this microdialysis study suggest that increased serotonin levels in the PPT suppress REM sleep.

In recent years, the rat has become one of the most popular animals used to study the mechanisms of sleep. However, until now, no other published study has targeted the effects of local application of serotonin, NE, or both serotonin and NE into the cholinergic cell compartment of the PPT in the freely moving rat. It is useful to see the effects of local application of serotonin and NE into the PPT cholinergic cell compartment in freely moving rats, especially since it has been found that activation of kainate receptors in the PPT cholinergic cell compartment induces REM sleep. In addition to NE and serotonin, it has been suggested...
suggested that the neuronal metabolite adenosine might be involved in the suppression of REM sleep by inhibiting PPT cells.\textsuperscript{11-19} In order to form a complete understanding of the regulatory mechanisms of REM sleep, it is important to identify neurotransmitters and neuromodulators that are involved in the suppression of spontaneous REM sleep at the level of the PPT cholinergic cell compartment.

The present study was designed to examine the hypothesis that serotonin, NE, adenosine, or a combination thereof may act at the level of the PPT cholinergic cell compartment to suppress REM sleep in the freely moving rat. In this study we have also examined the alternate hypothesis that serotonin may act at the level of individual REM sleep sign generators rather than directly in the PPT. To test this alternate hypothesis, in addition to the PPT injections, serotonin was injected directly into the P-wave generator, the dorsal part of the subcoeruleus nucleus,\textsuperscript{14,15} in the freely moving rat.

METHODS

Subjects and Housing

Experiments were performed on 24 male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250 and 300 grams. The rats were housed individually at 24°C with food and water provided ad libitum with lights on from 7:00 AM to 7:00 PM (light cycle) and off from 7:00 PM to 7:00 AM (dark cycle). The principles for the care and use of laboratory animals in research, as outlined by the National Institutes of Health Publication No. 85-23 (1985) were strictly followed.

Drugs and Vehicle for Microinjections

The drugs used included arterenol bitartrate (NE, mol wt: 319.3), 5-hydroxytryptamine (5-HT) (serotonin, mol wt: 212.7), and adenosine (mol wt: 267.2). All of these drugs were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Each of these drugs was dissolved in 0.9% saline to make the 3 different doses (0.5, 1.5, and 3.0 nmol). This 0.9% saline was also used for the control vehicle microinjection. All of these drugs are hydrophilic. Control saline and drug solutions were freshly prepared under sterile conditions before each use.

These 3 doses of drugs (0.5, 1.5, and 3.0 nmol) were selected based on earlier PPT microinjection studies.\textsuperscript{23,27} In the present study, we expected to see that microinjections of 5-HT or NE would block REM sleep by increasing wakefulness. Accordingly, we selected a dose that is known to induce wakefulness when microinjected into the PPT. In earlier studies, it has been demonstrated that the microinjection of 0.5 nmol of glutamate into the PPT kept rats awake for about 3 hours by eliminating both SWS and REM sleep.\textsuperscript{23,27} Thus, the lowest dose for this study was 0.5 nmol. The middle dose for the PPT microinjections was 1.5 nmol because 1.5 nmol of 5-HT microinjection into the P-wave generator selectively eliminates P-waves for about 4 hours. Since the 1.5-nmol microinjections of 5-HT or NE into the PPT did not change wakefulness or sleep, we doubled this dose to 3.0 nmol and used it as the highest dose for this study.

Surgical Procedures

Treatment of the animals and surgical procedures were in accordance with an approved institutional animal welfare protocol (#00-196). Efforts were made to minimize the number of animals and their suffering. Rats were anesthetized with pentobarbital (40 mg/kg., intraperitoneal), placed in the stereotaxic apparatus, and secured using blunt redundant ear bars.\textsuperscript{51} With the use of sterile procedures, cortical electroencephalogram (EEG), dorsal neck muscle electromyogram, electrooculogram, hippocampal EEG (to record theta wave), and pontine EEG (to record P-wave) recording electrodes were chronically implanted, as described elsewhere.\textsuperscript{23,27} In addition to these electrodes, stainless steel guide tubes (26-gauge) with an equal length stylet inside were stereotaxically implanted 2 mm above the PPT (bilateral, N = 18 rats) or P-wave generator (unilateral, N = 6 rats), as described previously.\textsuperscript{21,24}

Habituation for the Microinjection and Polygraphic Recordings

During recovery, habituation, and free-moving recording periods, all rats were housed under a 12:12-hour light:dark cycle with free access to food and water. For about 10 days, after a postsurgical recovery period of 3 to 7 days, and also for about a week immediately before the surgery, all rats were handled by the experimenter for about 15 minutes (between 9:30 and 10:00 AM) to habituate animals for the head plug connection and microinjection procedures. During these habituation days, rats were also habituated to a sound-attenuated recording cage (2.5 X 1.5 X 1.5 ft) and free-moving polygraphic recording conditions for 10 days (between 10:00 AM and 4:00 PM). To minimize the possible stress due to the microinjection procedure, all microinjections were done while animals were freely moving in the recording cage, as described previously.\textsuperscript{23,24}

Intracerebral Microinjections and Experimental Design

During experimental recording sessions, animals were connected to the polygraphic recording system 15 minutes before a microinjection into the PPT or P-wave generator. The microinjection system consisted of a 32-gauge stainless steel injector cannula with a 26-gauge collar that extended 2.0 mm beyond the implanted guide tube. The collar was connected to a 1.0-µl motor-driven Hamilton microsyringe with PE 20 tubing. While the animal was connected to the recording system, the stylet was removed and a control vehicle-filled (100-nl volume of 0.9% saline) or drug-filled (100-nl volume) injector was introduced through the guide tube for the injection. One minute after the insertion of the injector cannula, 100 nl of control saline or drug was microinjected over a 60-second period. The cannula was gently withdrawn 2 minutes after the injection, and the stylet was reintroduced inside the guide tube. Animals were free to move around the cage with the cannula in place during the microinjections. Due to the extended tubing and the cannula, the injections could be made while the animals were moving around. Immediately after completion of the microinjection procedure, polygraphic variables were recorded continuously for a session of 6 hours (between 10:00 AM and 4:00 PM) when rats would normally be sleeping.\textsuperscript{32}

During each recording session, only 1 site received a single microinjection of control vehicle (control saline) or any 1 of the 3 doses (0.5, 1.5, and 3.0 nmol) of drug in random order. Eighteen of those PPT guide tube-implanted rats were divided randomly into 3 groups (6 rats per group). Three different drugs (serotonin, NE, and adenosine) were tested in 3 different groups. Each of these rats received a total of 4 microinjections (control vehicle and 0.5, 1.5, and 3.0 nmol of drug) in random order in 4 different experiment sessions. Two of these 4 injections were in the right and the other 2 in the left PPT. Each of the 6 P-wave generator guide tube-implanted rats received 1 control saline and one 1.5-nmol dose of serotonin in random order and in 2 different experiment sessions. Each of these experiment sessions was separated by at least 3 days. At the end of all experimental sessions and before perfusion, with the use of the same injector used for control saline or drugs, 100 nl of black ink was microinjected 1 mm dorsal to each injection site for localizing injection sites as described earlier.\textsuperscript{23,27}

Determination of Behavioral States and Data Analysis

For the purpose of determining possible effects on sleep and wakefulness, 3 behavioral states were distinguished based on the visual scoring of polygraphic records as described earlier.\textsuperscript{23} The behavioral states of wakefulness (W), SWS, and REM sleep were scored in successive 10-second epochs. The polygraphic measures provided the following dependent variables that are quantified for each trial: (1) percentage of recording time spent in W, SWS, and REM sleep; (2) latencies to onset of the first episode of REM sleep; (3) total number of REM sleep episodes; (4) mean duration of REM sleep episode; and (5) P-wave den-
sity (waves/minute) in REM sleep. The effects of different treatments on the above-mentioned variables were statistically analyzed (1 and 2-way ANOVA and posthoc tests) with the use of StatView statistical software (Abacus Concepts, Berkeley, CA). Individual variable specific statistics are presented in the results section.

**Histologic Localization of Injection Sites**

At the conclusion of the microinjection experiments, rats were deeply reanesthetized with pentobarbital (60 mg/kg, intraperitoneal) and perfused transcardially with heparinized cold phosphate buffer (0.1 M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and processed for the histologic localization of injection sites as described earlier.25,27

**RESULTS**

A total of 72 microinjections were made in 36 PPT injection sites (Figure 1). These 36 PPT injection sites were equally distributed for the microinjections of serotonin, NE, and adenosine (12 sites for each chemical). In those 12 sites, 18 microinjections were 3 different doses (0.5, 1.5, and 3.0 nmol, N = 6 injections per dose) of chemical and 6 microinjections were control saline. Based on histologic identification and earlier pharmacologic studies, all of these microinjection sites were within the glutamate-induced REM sleep-generation site of the cholinergic cell generator sites received 1 control saline and one 1.5-nmol dose of serotonin. Based on our earlier mapping studies, all of these microinjection sites were within the limits of P-wave generator in the dorsal part of the subcoeruleus nucleus.14,50 All of these 6 P-wave microinjection or recording sites and 6 contralateral recording sites exhibited good-quality P-waves during REM sleep, confirming that these microinjection and recording sites were within the P-wave generator.

**Rapid Eye Movement Sleep After Serotonin Microinjection into the PPT**

The percentage of time spent in REM sleep after serotonin microinjection of control saline (N = 6 injections) and after each of the 3 doses (0.5, 1.5, and 3.0 nmol; N = 6 injections for each dose) of serotonin are presented in Figure 2. Behavioral and polygraphic signs of REM sleep after serotonin application resembled those signs during REM sleep after control saline microinjections throughout the 3-hour periods of recordings. Two-way ANOVA revealed no significant effect of treatment and or treatment X time interactions. In addition, 3 different types of posthoc analyses (Scheffe F-test, Dunnett t-test, Fisher PLSD) were done and did not show any significant difference in the percentage of REM sleep after 3 different doses of serotonin compared to after control saline microinjections into the PPT. Posthoc Scheffe F-test between control saline microinjection and different doses of serotonin microinjections into the PPT showed no significant changes in the latency of the first episode of REM sleep after injection, total number of REM sleep episodes for the 3-hour period of recordings, and mean duration of REM sleep episodes (Figure 2). These results demonstrate that the microinjection of serotonin into the PPT did not cause any changes in REM sleep. We have also analyzed the P-wave density after microinjections of serotonin and control saline into the PPT. The REM sleep P-wave density after serotonin microinjection (41.53±6.7) and after control saline microinjection (40.32±7.5) was not significantly different (Scheffe F-test and Dunnett t-test). Similarly, none of these 3 serotonin doses caused any significant changes (2-way ANOVA and Scheffe F-test) in the total percentage of W and SWS for the 3-hour period of recordings.

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**Figure 1**—Location of microinjection sites in the pedunculopontine tegmentum (left side) and dorsal part of nucleus subcoeruleus (P-wave generator; right side). Schematic coronal sections through the brainstem are illustrated at levels AP: -1.20, AP: -0.70, AP: -0.30, and AP: -0.08 in reference to stereotaxic anteroposterior “0” (labeled at right top). The locations of serotonin (filled circles; in the PPT, N = 12 and P-wave generator, N = 6), norepinephrine (stars in the PPT, N = 12), and adenosine (empty circles in the PPT, N = 12) microinjections. Abbreviations of anatomic terms: Aq, aqueduct; ATg, CG, central gray; CnF, cuneiform nucleus; DpMe, deep mesencephalic nucleus; DR, dorsal raphe nucleus; IC, inferior colliculus; IC, locus coeruleus; LDT, laterodorsal tegmental; LL, lateral lemniscus; LSO, lateral superior olive; m5, motor root trigeminal nucleus; mcp, midcerebellar peduncle; Me5, mesencephalic trigeminal tract nuclei; Ml, pontine reticular nucleus, oral; PAC, peri-aqueductal gray; PB, parabrachial nucleus; PPT, pedunculopontine tegmental nucleus; scp, superior cerebellar peduncle; SptC, subcoeruleus nucleus; VTe, ventral tegmental nucleus. Scale bar: 1 mm.

**Figure 2**—Rapid eye movement (REM) sleep after microinjections of 3 different doses of serotonin (5-HT) and control saline into the pedunculopontine tegmentum (PPT). Bars in the upper panel represent percentages (means and SEM) of REM sleep during each of the 3-hour periods after injection of control saline (N = 6), 0.5 nmol of 5-HT (N = 6), 1.5 nmol of 5-HT (N = 6), and 3.0 nmol 5-HT (N = 6). In the lower panel, bars represent means and SEM for the latency (in minutes, lower left panel), number of episodes (middle panel), and duration (in seconds, right panel) of REM sleep following control saline and 3 different doses of 5-HT microinjections into the PPT. Note that the REM sleep values for the percentage, latency, number, and duration after microinjections of 3 different doses of 5-HT are not significantly different (Scheffe F-test and Dunnett t-test) than after control saline microinjection into the PPT.
Rapid Eye Movement Sleep After Adenosine Microinjection into the PPT

The percentage of time spent in REM sleep for the first 3 hours after microinjections of control saline (N = 6 injections) and 3 different doses (0.5, 1.5, and 3.0 nmol; N = 6 injections for each dose) of NE were comparable between control saline injection and the different doses of NE microinjection into the PPT (2-way ANOVA and Scheffe F-test). Behavioral and polygraphic signs of REM sleep after NE application resembled those signs during REM sleep after control saline microinjections throughout the 3-hour recording period. Similar to the serotonin microinjections, compared to the control saline injection, none of these 3 different doses of NE microinjections into the PPT caused any significant changes in the total percentage of REM sleep (2-way ANOVA and Scheffe F-test), latency to the first episode of REM sleep (Scheffe F-test), total number of REM sleep episodes (Scheffe F-test), and mean duration of REM sleep episodes during the 3 hours of recordings (Scheffe F-test) (Figure 3). These results demonstrate that NE microinjection into the PPT was unable to cause any changes to REM sleep in the freely moving rat. The total percentages of W and SWS values after all 3 doses of NE were comparable with the total percentages of W and SWS values after control saline microinjection (2-way ANOVA and Scheffe F-test).

Rapid Eye Movement Sleep After Adenosine Microinjection into the PPT

Two-way ANOVA and Scheffe F-tests showed that after any 1 of the 3 doses (0.5, 1.5, and 3.0 nmol; N = 6 injections for each dose) of adenosine microinjections into the PPT, the total percentage of REM sleep was not significantly different than after control saline (N = 6 injections) microinjection into the PPT. Statistical analysis (Scheffe F-test) between any 1 of the 3 different doses of adenosine and control saline microinjections into the PPT showed no significant difference in the latency of the first episode of REM sleep after injection, total number of REM sleep episodes for the 3-hour period of recordings, and mean duration of REM sleep episodes (Figure 4). These results demonstrate that adenosine microinjection into the PPT was unable to cause any changes to REM sleep. The total percentages of W and SWS after injections between control saline and different doses of adenosine are comparable (2-way ANOVA and Scheffe F-test).

Pontine Wave Density and REM Sleep After Serotonin Microinjection into the P-wave Generator

Not detecting any change in REM sleep after PPT microinjections of serotonin, NE, and adenosine, we microinjected a 1.5-nmol dose of serotonin directly into the P-wave generator. In control recordings of REM sleep, after unilateral saline microinjection into the P-wave generator, P-waves were present in both the injected and the contralateral hemispheres (Figure 5A). However, unilateral microinjection of serotonin into the P-wave generator decreased P-wave counts in the ipsilateral P-wave generator during REM sleep (Figure 5B). In order to quantify the serotonin microinjection-induced reduction of P-waves, both ipsilateral and contralateral P-wave densities during REM sleep were quantified as waves per minute for the entire 6-hour period of each recording session. After unilateral control saline microinjection into the P-wave generator, P-wave densities in the ipsilateral P-wave generator (42.5±6.2) and contralateral P-wave generator (42.2±6.3) were not significantly different (2-way ANOVA). Since during the control injection-recording day, REM sleep P-wave densities in the ipsilateral and contralateral P-wave generators were comparable, P-wave densities from these 2 generators were combined to express a control P-wave density. Finally, P-wave densities in six 1-hour periods (repeated measure) were compared between 3 different treatment groups (1, control saline; 2, serotonin-treated P-wave generator; and 3, serotonin-treated contralateral P-wave generator). Two-way ANOVA indicated a significant main effect of treatment [F(2,15) = 39.10, P = 0.0001] and a significant treatment X time interaction [F(2,5) = 4.33, P = 0.0659]. Posthoc analysis (Scheffe F-test) revealed that the P-wave densities in the serotonin-treated P-wave gen-
results of this study also provide direct evidence contrary to the indirect evidence that has fueled the popular theory that NE and adenosine suppress REM sleep by direct inhibition of the brainstem cholinergic system.

During the last 3 decades, many studies have demonstrated that the systemic application of both serotonin and NE agonists suppress REM sleep in humans as well as in animals. It is commonly believed that these aminergic neurotransmitters act on the PPT and laterodorsal tegmentum (LDT) to suppress REM sleep. Indeed, in vitro electrophysiologic recording studies in the brain slice have shown that serotonin and NE inhibit PPT/LDT cells. In support of these in vitro studies, 1 study in the behaving rat has shown that local microinjection of serotonin into the LDT reduces the mean duration of REM sleep episode. On the other hand, iontophoretic application of serotonin into the LDT/PPT during REM sleep does not change the firing rate of REM-on cells. The result of this iontophoretic study makes it less plausible that serotonin has a direct effect on PPT/LDT cell activity in relationship to the regulation of REM sleep. Consistent with the iontophoretic study, in another microinjection study in the behaving cat, it was shown that microinjection of serotonergic drugs in the PPT did not change REM sleep or pontogeniculooccipital waves. The results of the present study also demonstrated that microinjection of serotonin into the PPT of the freely moving rat does not change REM sleep. Consistent with the present result, a microdialysis experiment in the rat showed that acetylcholine release in the thalamus, 1 of the major targets of the PPT, is higher during W than SWS and is essentially the same as during REM sleep. This evidence suggests that during W, when serotonin and NE release are maximum, the level of acetylcholine release from the PPT is

**DISCUSSION**

The principal findings of this study are that (1) local microinjection of serotonin directly into the P-wave generator suppresses P-wave activity during REM sleep, (2) microinjection of serotonin into the cholinergic cell compartment of the PPT does not produce any change in REM sleep, and (3) microinjections of NE and adenosine into the cholinergic cell compartment of the PPT do not produce any change in REM sleep. These results provide the first direct evidence to indicate that serotonergic inhibition of REM sleep may occur at the level of individual REM sleep sign generators rather than at the PPT in freely moving rats. The results of this study also provide direct evidence contrary to the indirect evidence that serotonin suppresses REM sleep by direct inhibition of the brainstem cholinergic system.

Unilateral microinjections of serotonin into the P-wave generator did not produce any noticeable gross behavioral changes. The percentages of REM sleep values after unilateral serotonin microinjection into the P-wave generator were not significantly different (2-way ANOVA and Scheffe F-test) than after unilateral control saline microinjections into the P-wave generator (Figure 6). These results indicate that local microinjection of serotonin into the P-wave generator was unable to change the total amount of REM sleep.

**Figure 5**—Sample polygraphic recordings of rapid eye movement (REM) sleep after a single unilateral microinjection of control saline (A) or 1.5 nmol dose of serotonin (B) into the P-wave generator. Note the qualitative similarity in both records showing characteristic electrographic signs of REM sleep. In spite of qualitative similarity, P-waves are less frequent after serotonin injection (B) as compared to after control saline injection (A). Also note that the decrease in number of P-waves is much greater in the brain hemisphere ipsilateral to the injection site (PonR) compared to the contralateral site (PonL). Time scale = 5 seconds. EEG, electroencephalogram; EMG, electromyogram; EOG, electromyogram.

**Figure 6**—Rapid eye movement (REM) sleep and P-wave density after a single unilateral microinjection of control saline or 1.5 nmol dose of serotonin (5-HT) into the P-wave generator. Bars in the upper panel show hourly percentages of REM sleep (mean and SEM) during 6-hour periods after injection of control saline (empty bars, N = 6) and 1.5-nmol dose of serotonin (diagonally striped bars, N = 6). Note that the percentages of REM sleep after microinjections of 5-HT are not significantly different than after microinjections of control saline (Scheffe F-test). Bars in the lower panel show hourly density of REM sleep P-waves (mean and SEM). Empty bars represent P-wave density values after microinjection of control saline. Diagonally striped and gray bars represent P-wave density after microinjections of 5-HT into the P-wave generators ipsilateral and contralateral to the 5-HT injection sites, respectively. Note the significant reduction of P-wave density in the 5-HT-microinjected (ipsilateral, diagonally striped bars) P-wave generator. Posthoc Scheffe F-tests: asterisk represents the comparison with control saline. *P < 0.05; ***P < 0.001.
similar to that during REM sleep, when serotonin and NE release are at a minimum. Thus, the release of serotonin and NE in the PPT does not appear to influence the activity of PPT cells. The fact that the direct application of serotonin into the PPT did not change REM sleep is consistent with the recent observation that there is an absence of 5-HT$_2$ receptors in the cholinergic cells of the PPT.62

Our study also indicates that, like serotonin, NE might not be acting directly on the PPT to suppress REM sleep in the freely moving rat. In the behaving cat and in adult guinea-pig brain slices, NE excites PPT cells.56 The excitatory effect of NE on the PPT does not block REM sleep. This hypothesis that NE suppresses REM sleep by inhibiting PPT cells. Since no other study has tested sleep effects of NE microinjection into the PPT of the behaving rat, our results could not be compared.

A number of single-cell recording studies have reported that the neurons within the LC and DRN are most active during W, considerably less active during SWS, and completely inactive during REM sleep.5,23,54 Extracellular levels of serotonin in all brain regions investigated so far seem to follow the firing pattern of the raphe neurons, being highest during W, lower during SWS, and lowest during REM sleep.5,23,54 In the PPT, in addition to the typical REM-on cells, a group of cells was first shown in the cat to be more active during the cortical EEG-activated states of W and REM sleep compared to during SWS.6,14 Later, this type of PPT cell was formally labeled as the Wake-REM-on cell.2 More recently, for the first time, these Wake-REM-on cells were also recorded in the freely moving rat.55 Based on single-cell activity patterns and pharmacologic responses to glutamate microinjections, the PPT has been considered to be involved in the induction of both W and REM sleep.22,23,54 Since the brain levels of 5-HT are highest during W, and in theory 5-HT is also involved in the inhibition of REM sleep-generating cells, the PPT cells should remain silent during W. On the contrary, a majority of those PPT cells (Wake-REM-on type) in cats and rats are more active during W than during REM sleep.2,19 These observations from single-cell recording studies indicate that spontaneously released 5-HT and activities of the LC and DRN cells are not linked to the ongoing cellular activity in the PPT.54 Thus, 5-HT and NE release within the PPT may not be involved in the regulation of REM sleep. In the present study, the results indicating that microinjections of 5-HT and NE did not change REM sleep are compatible with the above suggestion that 5-HT and NE release within the PPT may not be involved in the regulation of W or REM sleep.

In this study, the fact that the microinjection of 5-HT into the PPT did not block REM sleep is a negative result compared to an earlier microinjection study.56 Is it possible that the microinjection of 5-HT into the PPT might have diffused into the LC, the DRN, or both? Hypothetically, diffusion of these drugs into the LC or the DRN may have neutralized their behavioral action. For the following reasons, we believe that this is not likely to be the explanation for our apparent negative results. First, to study the extent of diffusion in the rat brainstem, an earlier study has shown that a single microinjection of radioactive choline (78.0 nmol choline in 100 nl of saline) into the pontine reticular formation diffused almost 1 mm from the injection site.28 Since the diffusion of solute (for this study, radioactive choline and for our study, 5-HT) depends equally on the total volume of solvent and the concentration of solute, it is unlikely that the maximum concentration (3.0 nmol) of drugs used in the present study would diffuse more than 0.5 mm. Indeed, functional diffusion studies have shown that similar concentrations and volumes of drugs in the PPT diffuse only 0.2 to 0.3 mm away from the center of the injection site (see histologic confirmation).25,27 In the present study, injection sites in the PPT were almost 2.0 mm away from the LC and DRN. Secondly, in the present study we have also demonstrated that the microinjection of 5-HT (1.5 nmol/100 nl) into the P-wave generator suppresses P-wave activity for about 4 hours. The P-wave generator is much closer than the PPT is to the LC and DRN. If 100 nl of 5-HT diffused more than 1 mm, we would not have seen suppression of P-wave activity after 5-HT microinjections into the P-wave generator. Thus, we are confident that our 5-HT microinjections into the PPT did not diffuse to the LC or the DRN. On the other hand, with microinjection studies in the LDT, injected materials are most likely to diffuse into the LC, DRN, and the 4th ventricle because the LDT is less than 0.5 mm away from these areas.

Another transmitter, adenosine, is also shown to be involved in the induction of SWS.70,71 More recently, adenosine has been suggested to be involved in the regulation of REM sleep by inhibiting PPT/LDT cells.48,50 In the cat, application of adenosine into the LDT has been shown to decrease REM sleep.84 However, in the present study, we did not see any effect in the sleep-wake parameters after application of adenosine into the PPT. The conclusion that adenosine does not appear to inhibit LDT/PPT cells in the rat is also indicated by another study in a slice preparation.85 These seemingly conflicting results of increased REM sleep in the cat and no sleep effect in the rat after application of adenosine into the LDT/PPT might be explained by species difference. In addition to species difference between the study in the cat50 and the present study, different effects may be due to dose. In the earlier study, a highly concentrated (300 µmol) adenosine solution was continuously perfused for 2 hours (1.0 µL/min) into the LDT. Given the amount of time, concentration, and proximity to the aqueduct, it is possible that the adenosine might have diffused into the aqueduct. Thus, the sleep-wake effects of adenosine in the cat study may have another site of action. We believe that adenosine is involved in the regulation of the sleep-wake cycle by its action in brain areas other than the PPT. Indeed, it has already been suggested that adenosine is involved in the regulation of the sleep-wake cycle by acting on the basal forebrain and preoptic area.49,81,83,84

During W and SWS, the executive neuronal population of the REM sleep-generating network in the brainstem appears to be held in inhibitory restraint by serotonin released from the DRN.2,54 Three lines of experimental evidence support this statement. First, the firing rates of the serotonergic neurons in the DRN decrease at sleep onset, decline progressively in NREM sleep, and nearly cease in REM sleep.10,31,65,84 Second, shutting off the DRN by localized cooling,28 eliminating its inputs to the P-wave generator by cutting its afferent fibers,59 or counteracting release or availability of serotonin in the brain by pharmacologic manipulations85,86 all lead to release of P-waves in all behavioral states including REM sleep. Third, serotonin-increasing pharmacologic manipulations56 and electrical stimulation of the DRN59 suppress P-waves. The sum of this evidence suggests that serotonin may act at the level of the P-wave generator to suppress this REM sleep sign. Consistent with the indirect evidence mentioned above, in this study we have demonstrated that the local microinjection of serotonin into the P-wave generator suppresses P-wave activity but not REM sleep. These results provide the first direct evidence that serotonin inhibits the P-wave generator. Consistent with our results, a recent immunohistochemical receptor-mapping study in the rat has demonstrated 5-HT$_1$ receptors to be abundant on the neurons within the P-wave generator.87 In summary, the results of the present study demonstrate that the serotonergic inhibition of REM sleep in the freely moving rat might be mediated in part through the inhibition of individual REM sleep sign generators rather than through the direct inhibition of the PPT.

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