Carbachol Injections Into the Ventral Pontine Reticular Formation Activate Locus Coeruleus Cells in Urethane-Anesthetized Rats

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Study Objectives: Two pontine reticular regions are implicated in cholinergic triggering of rapid eye movement (REM) sleep: the dorsomedial tegmental region and the ventral nucleus pontis oralis. We previously determined that, in urethane-anesthetized rats, microinjections of a cholinergic agonist, carbachol, into the dorsal region produce REM sleep-like effects comprising cortical activation, hippocampal theta rhythm, suppression of hypoglossal (XII) nerve activity, and silencing of pontine noradrenergic neurons. Our goal was to determine whether carbachol injections into the ventral nucleus pontis oralis elicit comparable effects.

Design: Recording of cortical electroencephalogram, hippocampal activity, XII nerve activity, and discharge of noradrenergic cells of the locus coeruleus.

Setting: Basic neurophysiologic research laboratory.

Participants and Interventions: Urethane-anesthetized, paralyzed, and artificially ventilated or nonparalyzed and spontaneously breathing rats with microinjections of carbachol (10 nL, 10 mM) into the ventral nucleus pontis oralis.

Measurements and Results: In artificially ventilated rats, carbachol injections repeatedly elicited cortical activation and hippocampal theta rhythm. Concomitantly, the activity of locus coeruleus neurons increased from 2.0 per second ± 0.4 (SE) to 2.6 per second ± 0.4 (P < .05, n = 8), as did XII nerve activity (by 42.5% ± 8.8%; P < .01). In spontaneously breathing animals, carbachol similarly activated the cortical electroencephalogram and hippocampal activity, whereas XII nerve activity was reduced by 6.7% ± 2.5% (P < .05) together with increased ventilation, as indicated by reduced end-expiratory CO₂.

Conclusion: Carbachol injections into the ventral nucleus pontis oralis activate, rather than silence, noradrenergic locus coeruleus neurons. This is not compatible with the state of REM sleep.

Abbreviations: EEG, electroencephalogram; EMG, electromyogram; GC, genioglossal; LC, locus coeruleus; REM, rapid eye movements; SEM, standard error of the mean; TP, tracheal pressure; XII, hypoglossal

Key Words: Arousal, cortical activation, hypoglossal motoneurons, hippocampus, pons, REM sleep, theta rhythm.

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INTRODUCTION

THE RAPID EYE MOVEMENT (REM) STAGE OF SLEEP IS CHARACTERIZED BY CORTICAL AND HIPPOCAMPAL ACTIVATION, RAPID EYE MOVEMENTS, SILENCING OF BRAINSTEM AMINERGIC NEURONS, AND POSTURAL ATONIA. Cholinergic activation plays an important role in the generation of REM sleep, since pontine microinjections of cholinergic agonists into the pontine reticular formation trigger or enhance a rapid eye movements sleep-like state, whereas antagonists suppress REM sleep, and endogenous acetylcholine release is increased in the pons in association with REM sleep. The localization of the site or sites at which REM sleep-like phenomena are elicited most effectively is important for advancing our understanding of the neurochemistry and neurophysiology of this stage of sleep.

Microinjection studies in cats point to a discrete region in the dorsal pontine tegmentum as the site for the cholinergic trigger-

Disclosure Statement

Drs. Fenik, Ogawa, Davies, and Kubin have indicated no financial conflicts of interest.

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Thus, the localization of pontine sites at which carbachol increases REM sleep in rats is uncertain. The goal of the present study was to better define the electrophysiologic changes elicited by carbachol from the ventral pontine reticular formation. To assess the relationship between REM sleep and distinct electrophysiologic effects elicited from the ventral pontine reticular formation, we investigated whether such injections inhibit the activity of noradrenergic neurons of the locus coeruleus, a cellular behavior typical of REM sleep.\(^{32-34}\) To ensure stable single-cell recordings and satisfactory precision of the injections, the study was performed in urethane-anesthetized rats, an experimental model in which carbachol injections into the dorsal pontine reticular formation inhibit pontine noradrenergic neurons and evoke other signs of REM sleep.\(^{7,18,19}\) We found that, in contrast to the effects of dorsal injections, ventral injections increase locus coeruleus cell and XII nerve activity.

**METHODS**

**Animal Preparation and Recording Procedures**

Experiments were performed on 13 adult male Sprague-Dawley rats (body weight: 418 g ± 15 [SEM]) obtained from Charles River Laboratories, Wilmington, MA. The procedures for anesthesia, surgery, and recording followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

The animals were anesthetized with halothane (2%) followed by urethane (1 g/kg, intraperitoneal supplemented by 50 mg intravenously administered injections at approximately 1-hour intervals). The trachea was intubated, and a femoral artery and vein canulated for arterial blood pressure monitoring and fluid injections, respectively. To record the XII nerve electrophysiology, one XII nerve was cut peripherally, freed from the surrounding tissue, and placed in a cuff-type recording electrode.\(^{33}\) Both vagi were cut in the neck to enhance XII nerve activity and make it independent of lung-volume feedback. The animal was placed in a stereotaxic head holder, two openings made in the caudal medial aspect of the right parietal bone, and the dura was removed for inserting a carbachol-containing pipette and hippocampal recording electrode. Another opening was made in the interparietal bone for inserting a recording electrode into the locus coeruleus. For monitoring the cortical electroencephalogram (EEG), two screws were attached to the skull—one in the frontal bone 2 mm anterior and 2 mm to the left, and one into the parietal bone 3 mm posterior and 2 mm to the left, of the bregma. To record hippocampal activity, two Teflon-insulated platinum wires (0.002 inch, A-M System, Carlsborg, WA), with tips separated by 0.8 mm, were placed into the CA1 region, 3.7 mm posterior to and 2.2 mm to the right of the bregma and 2.4 mm below the cortical surface. The position of this electrode was adjusted to maximize the amplitude of the theta rhythm elicited by a strong hindlimb pinch. Genioglossal muscle electromyogram (EMG) was recorded with a pair of twisted wires inserted into the muscle.\(^{31}\)

The animals either breathed spontaneously or were paralyzed by pancuronium bromide (2 mg/kg administered intravenously, supplemented with 1 mg/kg injections as needed) and artificially ventilated at 50 to 70 lung inflations per minute. In both conditions, the inspired air was enriched with oxygen (final O\(_2\) concentration 30%-60%). Before and following paralysis, the level of anesthesia was assessed by continuously monitoring the cortical EEG, arterial blood pressure, and XII nerve activity and intermittently applying a pinch to the tail or hindlimb. A regular respiratory rhythm, steady XII nerve electroneurogram and blood pressure, and only transient changes in cortical and hippocampal signals elicited by pinching indicated that the animal was adequately anesthetized. The rectal temperature was maintained at 36 °C to 37°C by a heating pad. The end-expiratory CO\(_2\) was monitored (Columbus Instruments capnograph, Columbus, OH). Following neuromuscular paralysis, the ventilatory parameters were adjusted to maintain a steady respiratory modulation of XII nerve activity at a level similar to that prior to paralysis. No pressor drugs were used, and the mean arterial blood pressure was 65.7 ± 2.8 (SEM) mm Hg.

**Electrophysiologic Recordings**

The activity of locus coeruleus cells was recorded using glass pipettes filled with 0.5 M Na acetate and 2% Pontamine sky blue dye (ICN Biomedicals, Inc., Aurora, OH), having tip diameters of 2.5 to 3.0 µm (resistance 4-5 MΩ at 1 kHz) and held in a hydraulic manipulator (Haer, Brunswick, ME). The following criteria were used to identify locus coeruleus neurons:\(^{35,34,36}\) (1) location 1.2 mm lateral to the midline and 5.3 to 6.3 mm below the cerebellar surface; (2) low (0.5-5 Hz) and steady firing rate; (3) action potentials of long duration (> 0.8 milliseconds), with a notch on their falling slope and a prolonged afterpotential; and (4) transient excitation followed by a short silent period in response to a hindlimb pinch. In our earlier studies and preliminary experiments for this study, we also determined that all cells meeting these criteria and subsequently localized within the locus coeruleus were silenced by the adrenergic α2 receptor agonist, clonidine (2-4 µg, administered intravenously)\(^{38,19}\). Since the systemic clonidine effect is long lasting and could interfere with the effects of carbachol, the clonidine test was not used in this study. The recording sites were marked by the iontophoretic deposition of Pontamine sky blue (5 µA, 10 minutes, tip negative). Only the cells classified as noradrenergic using the above criteria and subsequently histologically localized within the locus coeruleus were included in the study.

Cortical, hippocampal, locus coeruleus cell, genioglossal EMG, and XII nerve activity were amplified (N101, Neurolog System; Digitimer, Hertfordshire, England or P-5 Grass; Quincy, MA), with filter bandwidths set at 1 to 100 and 3 to 8 Hz for the cortical and hippocampal signals, respectively, and 30 to 2500 Hz for the remaining signals. XII nerve and GG EMG activity were full-wave rectified and passed through a moving average circuit (time constant 100 milliseconds; MA-821 RSP; CWE, Inc., Ardmore, PA) to obtain a smooth record of changes in activity with the central respiratory rhythm. These signals, together with an event marker, arterial blood pressure, tracheal pressure and end-expiratory CO\(_2\), were monitored on a chart recorder (TA-11; Gould Instruments, Valley View, OH), and recorded on a digital tape recorder (C-DAT; Cygnus Technology, Delaware Water Gap, PA).

**Microinjections**

A glass pipette (A-M System, Carlsborg, WA, internal diameter 0.25 mm, tip diameter 20-30 µm) filled with 10 mM carbachol chloride (carbachol, Sigma, St. Louis, MO) and 2%
Pontamine sky blue in 0.9% NaCl was inserted into the ventral pontine reticular formation to a depth of 8.6 to 9.5 mm from the cortical surface using an approach established in earlier studies.\textsuperscript{19,31,37} Ten nanoliters of carbachol (18.3 ng) were injected over 10 to 20 seconds by applying pressure to the fluid in the pipette while the movement of the meniscus was monitored with a calibrated microscope. To control for the effect of Pontamine, in preliminary experiments, carbachol was injected without the dye in the same pontine region. These injections evoked identical effects on hippocampal activity, EEG, and XII nerve activity as those with Pontamine described in this report.

**Experimental Protocol and Data Analysis**

Seven experiments were performed in paralyzed animals to assess the effects of ventral pontine carbachol injections on the behavior of locus coeruleus cells. Once steady, single-cell, extracellular activity of putative locus coeruleus cells was achieved, the cell was first tested with a hindlimb pinch and then the carbachol microinjections were made. To ensure repeatability of the carbachol responses, sufficient time (at least 36 minutes and typically 1 hour) was allowed between injections. With such intervals, carbachol elicits similar responses from the ventral pontine reticular formation without any sign of adaptation.\textsuperscript{31} An injection of carbachol was considered effective if at least one of the recorded signals (usually XII nerve activity) changed within 10 minutes after the injection and the response lasted at least 2 minutes. For the effective carbachol injections, locus coeruleus cell activity was averaged over 60-second segments of records centered around the point of maximal effect. These measurements were compared with the activity obtained from 60-second control periods preceding the injection and 60-second segment after the recovery from the effect of carbachol. Changes in the magnitude of XII nerve activity and central respiratory rate were assessed during the same periods from the moving averages of the XII nerve electromyogram. The difference between the peak amplitude during the central inspiratory phase of the respiratory cycle and the level of the signal when no activity was present (during a part of the central expiratory period) was used as the measure of the magnitude of the motor output from the XII nerve. The central respiratory rate was determined from the frequency of respiratory bursts in XII nerve activity. The latencies of the responses to carbachol were determined from the injection onset to the first noticeable change in XII nerve activity, and their durations, from the onset of the change to the point of 50% recovery.

In another 6 rats, we determined the effects of ventral pontine carbachol injections on the cortical, hippocampal and XII nerve activity (but not locus coeruleus cells) before and during neuromuscular paralysis. In these experiments, carbachol injections were first made while the animal was breathing spontaneously. Following an injection that caused the characteristic changes in the EEG and hippocampal theta rhythm, the carbachol pipette was left in place, the animal paralyzed, and a second injection performed with the animal artificially ventilated at a constant rate and volume. In this series, the second injection was done at least 1 hour after the first (average 78.3 minutes ± 5.3 (SEM), range 60-99 minutes).

**Histology**

At the conclusion of the experiment, the animal was deeply anesthetized with urethane administered intravenously (2 g/kg), and decapitated. The brainstem was removed, fixed in 10% phosphate-buffered formalin, and cut on a vibratome into 100-μm transverse sections. The sections containing the Pontamine blue dye marks were serially mounted and stained with Neutral red.

**Statistical Analysis**

Following verification that the data were normally distributed, paired, two-tailed Student t tests, one-way repeated measures analyses of variance or Pearson product moment correlations were used for statistical analysis (SigmaStat; Jandel, Inc., San Rafael, CA). The variability of the means was characterized by the standard error (SEM). Differences were considered significant when $P < .05$.

**RESULTS**

**Locus Coeruleus Cells are Activated Following Carbachol Injections Into the Ventral Pontine Reticular Formation**

In 7 paralyzed and artificially ventilated animals, one or more carbachol injections were made at 12 sites within the ventral region of the nucleus pontis oralis. Injections in 8 of these sites were effective, and in 4, there was no effect (filled and open circles, respectively, in Figure 1). The activities of 10 locus coeruleus cells were recorded during these injections: 6 cells during effective injections; 2 during ineffective injections; and 2 during injections into 2 sites, 1 effective and 1 ineffective. Thus, the locus coeruleus cell activity was recorded during 8 effective and 4 ineffective carbachol injections into ventral pontine sites.

During all effective injections, XII nerve activity increased by 42.5% ± 8.8% (range 21%-88%, $P < .01$, $n = 7$). In one case not included in the average, a low level of XII nerve activity prior to injection did not allow for an accurate determination of the relative increase of XII nerve activity, but excitation clearly occurred following carbachol. This case is illustrated in Figure 2. Following 6 out of the 8 effective injections, the increase of XII nerve activity coincided with the appearance of the hippocampal theta rhythm (3.5-4.5 Hz in urethane-anesthetized rats\textsuperscript{26}) and activation of the cortical EEG (Figure 2, compare the two top traces in B and C). The respiratory rate increased from 42.4 per minute ± 2.7 to 47.5 per minute ± 3.3 ($P < .05$, $n = 7$), or by 12.1% ± 4.3% of control (range: 6%-34%). The responses started 3.1 minutes ± 0.4 (range: 1-5 minutes, $n = 8$) after the onset of the injection, and their duration was 15.7 minutes ± 3.9 (range: 9-37 minutes, $n = 7$). There were no significant changes of the arterial blood pressure during these responses.

The activity of all 8 locus coeruleus cells studied during the effective injections increased (Figure 2E), whereas the activity of the 4 cells that were recorded during ineffective injections did not change. The increases coincided with the increases of XII nerve activity. Panels A-C of Figure 2 show an example of a locus coeruleus cell recorded during an effective injection, and panel D shows the recording site for this cell. The average locus coeruleus cell activity increased from 2.0 per second ± 0.4 ($n = 8$) before carbachol to 2.6 per second ± 0.4 ($n = 8$) during the responses, and then recovered to 2.1 per second ± 0.5 ($n = 6$) later (Figure 2E). This effect was statistically significant when tested by one-way repeated-measures analysis of variance ($F_{2,7} = 8.94$, $P < .01$). The relative increase in locus coeruleus cell discharge was

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by 34.7% ± 10% of control (range: 6%-83%). In the tests in which carbachol evoked no changes in the EEG, hippocampal, or XII nerve activity, the discharge frequency of the 4 locus coeruleus cells studied also did not change (2.2 per second ± 0.7 before and 2.0 per second ± 0.5 after carbachol injection; \( P = .3, n = 4 \)).

XII Nerve Activity Decreases Following Ventral Pontine Carbachol Injections in Spontaneously Breathing Animals

Our locus coeruleus cell recordings in paralyzed rats indicated that ventral pontine carbachol injections had excitatory effects at the cortical, hippocampal, central respiratory, XII motoneuronal, and noradrenergic cellular levels. However, in an earlier study, similarly placed injections in nonparalyzed, spontaneously breathing rats reduced the genioglossal EMG. To determine whether this difference is related to the mode of ventilation (spontaneous vs artificial), rather than to some other differences in the protocol or recording conditions, we performed experiments in 6 rats in which XII nerve, cortical, and hippocampal activities were recorded during ventral pontine carbachol injections placed at the same site first prior to, and then following, neuromuscular paraly-

![Figure 1](image-url.com)
sis. These injections were placed at similar locations to those in the part of the study with locus coeruleus cells (filled triangles in Figure 1).

When the rats breathed spontaneously, carbachol induced hippocampal and cortical activations identical to those observed in our paralyzed animals (Figure 3A-B). The average latency and duration of these responses were 2.4 minutes ± 0.5 (n = 6) and 24.9 minutes ± 3.9 (n = 5), respectively (in 1 animal, the response to carbachol lasted more than 30 minutes, and urethane was intravenously administered prior to the occurrence of a full recovery). However, in contrast to the effects in paralyzed animals, XII nerve activity decreased by 8.1% ± 2.4% of control (range: 1.4%-15%, P < .05, n = 6), and the simultaneously recorded activity of the genioglossal EMG decreased by 24.9% ± 6.1% of control (range: 8%-52%, P < .01, n = 6). Concurrently, there was evidence that ventilation increased. The tracheal pressure swings increased from 5.7 cm H₂O ± 0.3 before to 6.1 cm H₂O ± 0.3 during the response (P < .05, n = 6), and this was associated with a decreased end-expiratory CO₂, from 8.4% ± 0.9% to 7.8% ± 0.8% (P < .01, n = 6, absolute changes in individual animals were 0.3%-1.2%). The reduced CO₂ level may have contributed to the observed depression of XII nerve and genioglossal EMG activity. However, neither the relative XII nerve nor genioglossal EMG decreases were positively correlated with the CO₂ decrease (r = .03, P = .52, and r = .06, P = .13, respectively; Pearson product moment correlation). The absence of the correlation might be due to the fact that the depressant influence of reduced CO₂ interacted with the activating effect of carbachol on the XII nerve and genioglossal activity.

In each animal, a second carbachol injection was placed at the same ventral pontine site after the animal was paralyzed and artificially ventilated. These injections evoked changes in the hippocampal activity and cortical EEG similar to those elicited prior to paralysis (cf. Figure 3B and D). Both the latency and durations of the responses were also similar (2.9 minutes ± 0.6 (n = 6) and 21.0 minutes ± 4.7 (n = 5), respectively). As in the paralyzed animals used in the part of the study with locus coeruleus cells, XII nerve activity increased (by 21.4% ± 6.1% above the precarba-

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**Figure 2**—Example of an excitatory response elicited by carbachol (10 nL, 10 mM) in a paralyzed and artificially ventilated rat. Panel A shows that hippocampal (Hipp) and cortical (electroencephalogram [EEG]) activations occur simultaneously and parallel to the increase in locus coeruleus (LC) cell activity (firing rate) and increased magnitude of moving average of hypoglossal (XII) nerve activity (bottom). In this recording, XII nerve activity was nearly absent prior to the carbachol injection and then appeared after carbachol. At the end of the response, there were several bouts of partial recovery from the effect of carbachol, and during each all signals varied synchronously in a manner consistent with the pattern of the initial response to carbachol. Calibration bars also apply to panels B and C, which show expanded portions of the record in A prior to and after carbachol injections, as indicated by the arrows. In C, note a regular theta rhythm in the hippocampal signal. D: iontophoretically marked recording site for the LC cell illustrated in A-C. Me5 refers to mesencephalic trigeminal nucleus. E: the individual firing rates of the 8 LC cells studied prior to carbachol injections, at the time of a maximal increase of XII nerve activity following carbachol and after recovery.
DISCUSSION

Our main finding is that, in anaesthetized, paralyzed, and artificially ventilated rats, carbachol injections into a discrete site near the ventral margin of the nucleus pontis oralis increased the activity of both noradrenergic locus coeruleus cells and XII motoneurons. The increase was accompanied by cortical EEG activation, the appearance of hippocampal theta rhythm, and increased respiratory rate. Consistent with earlier suggestions, some components of this activation (EEG, hippocampus, respiratory rate) were compatible with REM sleep. However, the changes in locus coeruleus cell and XII motoneuronal activity were opposite to those typical of REM sleep. This difference suggests that the role of cholinergic activation within the ventral pontine reticular formation is not related to REM sleep.

Activation of Locus Coeruleus Cells and XII Motoneurons by Ventral Pontine Carbachol Injections

In agreement with earlier studies in urethane-anesthetized rats, we found that carbachol injections into the ventral pontine reticular formation elicited hippocampal theta rhythm and cortical activation. Since this is typical of REM sleep, and given extensive evidence that pontine carbachol injections trigger or enhance a REM sleep-like state in chronically instrumented behaving cats and rats, it is understandable that the cortical and hippocampal activation produced by carbachol from pontine sites is interpreted as a REM sleep-like effect. Also supporting the interpretation that carbachol injections into the ventral pontine reticular formation elicit a REM sleep-like effect.

Figure 3—A within-animal comparison of the effects of ventral pontine carbachol injections at the same site during spontaneous breathing (A and B) and after neuromuscular paralysis and artificial ventilation (C and D). The carbachol-induced activation of the cortical electroencephalogram (EEG) and hippocampal (Hipp) theta rhythm are similar under both conditions. However, the peak inspiratory hypoglossal (XII) nerve activity and genioglossal electromyogram (GG EMG), both represented by the moving average of these signals, are reduced following carbachol during spontaneous breathing, whereas XII nerve activity increased after carbachol injection when the animal was paralyzed and artificially ventilated. The increased tracheal pressure (TP) swings and the reduced end-expiratory CO₂ after carbachol injection during spontaneous breathing indicate an increased respiratory effort and ventilation. In the bottom records, TP and end-expiratory CO₂ were kept constant by artificial ventilation. Under these conditions, an increase of XII nerve activity occurred, which reflects the primary central effect of ventral pontine carbachol on XII motoneurons. The amplification of XII nerve activity is the same in all 4 panels.

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state are the experiments in chronically instrumented behaving cats in which carbachol injections into the ventral nucleus pontis oralis produced EEG and hippocampal activation. However, in some of those studies, a “dissociated” state, comprising cortical desynchronization and motor activation, occurred. Thus, the behavioral state produced by some of these injections was not unambiguously REM sleep-like. On the other hand, carbachol injections into the ventral pontine reticular formation in spontaneously breathing, urethane-anesthetized rats elicited cortical and hippocampal activation accompanied by an accelerated respiratory rate and decreased genioglossal EMG, two additional phenomena characteristic of REM sleep. Together, these data lent support for the interpretation that cholinergic activation within the ventral nucleus pontis oralis contributes to the generation of REM sleep. However, considering that both REM sleep and wakefulness are associated with cholinergic, cortical, hippocampal, and respiratory activation, one must interpret the effects of pontine carbachol in studies under anesthesia and in behaving animals in which a limited number of parameters are monitored with caution, as the distinction between REM sleep-like and wakefulness-like effects may not be clear.

Confounding the interpretation of carbachol-injection studies in rats is the fact that relatively large volumes or concentrations of the drug were used in most studies. For example, Vertes et al injected 1 to 10 μg of carbachol in 100 or 200 nL of saline, Bourgin et al injected carbachol of various concentrations in a volume of 50 nL, and Marks and Birabil injected muscarinic agonists in a volume of 60 nL. The resulting anatomic distributions of the effective sites are imprecise and suggest that REM sleep can be enhanced, or at least distinct REM sleep-like phenomena elicited, from relatively wide regions of the pontine reticular formation. If so, this would be in contrast to most studies in cats that point to a relatively discrete site located in the dorsal nucleus pontis oralis or the sub-locus coeruleus region as most effective for triggering of a REM sleep-like state, with injections into other pontine regions having no effects or eliciting various dissociated states. In addition, in behaving rats, pontine carbachol injections enhance REM sleep, but they also often reduce slow-wave sleep and enhance wakefulness. The latter may be due to the use of relatively large doses of carbachol that produce excessive stimulation at the injection sites or a spread of the drug into wakefulness-promoting regions, or both. Thus, the studies with pontine carbachol injections in rats suggest that both wakefulness and REM sleep can be activated, although it is not known whether the injection site, the dose, the behavioral state of the animal, or a combination thereof determines the outcome of a given injection. For example, data from cats suggest that the effect of pontine carbachol injection depends on the behavioral state of the animal at the time of injection.

Importantly, in the two studies in behaving rats that used carbachol volumes and concentrations comparable to those in our study, injections into the region referred to in the atlas of Paxinos and Watson as sub-locus coeruleus-α strongly enhanced REM sleep and triggered pontogeniculooccipital waves, a major sign of REM sleep. Consistent with this, we previously reported that, in urethane-anesthetized, paralyzed, and artificially ventilated rats, small doses of carbachol (18.3 ng in 10 nL) injected into the dorso medio-lateral pontine area located just ventrolateral to the ventral part of the laterodorsal tegmental nucleus and just anterior to sub-locus coeruleus-α can repeatedly trigger short episodes comprising activation of the cortical EEG, the appearance of hippocampal theta rhythm, a profound suppression of XII motoneuronal activity, and silencing of pontine noradrenergic neurons (both locus coeruleus and A5). Those earlier findings in urethane-anesthetized and paralyzed rats provide a suitable reference for the interpretation of our present study in which, using the same rat preparation, we characterized the nature of the effects of carbachol injections into the ventral pontine reticular formation.

While the effects elicited in urethane-anesthetized, paralyzed, and artificially ventilated rats from the dorsal pontine region occur with a profound suppression of XII nerve activity (by 70%-90%) and silencing of pontine noradrenergic neurons, this study, we found that ventral carbachol injections increased locus coeruleus cell and XII nerve activity. In our experiments, the average baseline activity of LC cells was 2.0 per second ± 0.4, similar to their activity reported during quiet waking (1.45 per second ± 0.14) or active waking (2.15 per second ± 0.16) in behaving rats. Following both dorsal and ventral injections, the effects often begin in less than 2 minutes, but the excitatory responses elicited from the ventral sites have a relatively long duration (9-37 minutes), whereas the episodes elicited from the dorsal sites last only 3 to 4 minutes. Thus, while carbachol injections into both dorsal and ventral pontine regions produce cortical and hippocampal activation, their effects on noradrenergic locus coeruleus cell and XII nerve activity are opposite, and the durations of the effects produced from the two sites also differ. Thus, the dorsal and ventral region appear to contain functionally distinct cholinoreceptive pontine sites.

The ventral pontine region investigated in this study is located near the junction of the nucleus pontis oralis, reticulotegmental nucleus, and B9 group of serotonergic neurons and is rich in muscarinic cholinergic receptors. The in vivo effects of carbachol on cells located in the ventral nucleus pontis oralis are unknown, whereas, in vitro, both excitatory and inhibitory responses to cholinergic agonists have been described in cells of this region. Serotonergic and other cells present in this area project to many brain regions, including those that control sleep-wake behavior and activate corticoseptohippocampal networks. The site also receives afferents from other brain regions implicated in the generation of both REM sleep and wakefulness, including cholinergic projections from pedunculopontine and laterodorsal tegmental nuclei.

One advantage of microinjection studies in anesthetized animals is that small volumes, of the order of 1 to 10 nL, can be injected reliably, allowing for a satisfactory spatial resolution of the effective sites. In addition, by using paralyzed and artificially ventilated animals, one can separate primary central effects from secondary reflex changes. Here we observed that the primary effects of cholinergic stimulation within the ventral pontine reticular formation include activation of pontine noradrenergic neurons and XII motoneurons, changes opposite to those occurring during REM sleep. Since the cortical and hippocampal effects of these injections were compatible with both REM sleep and wakefulness or arousal, we cannot exclude that, during natural REM sleep, the dorsal pontine site discussed above and the ventral site investigated in the present study are both activated, their concurrent activation contributing to distinct aspects of REM sleep. If so, our finding may help explain the occurrence of various REM sleep-like dissociated states in response to pharmacologic activation of cholinergic receptors located in distinct pontine regions.
ternatively, the 2 sites may have different functions and control 2 distinct behavioral states, such as REM sleep and active wakefulness.

Opposite Effects of Ventral Pontine Carbachol Injections on XII Motoneurons in Nonparalyzed and Paralyzed Animals

The goal of the second part of our study was to reconcile our present results in anesthetized, paralyzed, and artificially ventilated rats with those previously obtained in urethane-anesthetized, nonparalyzed, spontaneously breathing rats. In both studies, 10 to 40 nL of 10 mM carbachol injected into the ventral pontine region adjacent to the ventral tegmental nucleus elicited cortical and hippocampal activation and accelerated the respiratory rate. However, in contrast to the carbachol effects in paralyzed rats (this study), in spontaneously breathing rats, the respiratory-modulated activity of XII motoneurons was reduced. By performing injections at the same site prior to and after neuromuscular paralysis, we confirmed that in spontaneously breathing rats, genioglossal EMG and XII nerve activity are suppressed by ventral pontine carbachol injections. The magnitude of the suppression of genioglossal EMG observed in the present study (8%-28%) was smaller than that reported previously (74%), and the duration was slightly shorter (24.9 minutes vs 28.6), both differences likely due to the smaller volumes of carbachol used in the present study (10 nL vs 20-40 nL).

The depressant effects of ventral pontine carbachol injections on XII motoneurons that occur in spontaneously breathing but not artificially ventilated rats may, at least in part, be secondary to increased ventilation and the resulting decrease in end-expiratory CO₂ following carbachol injections. In spontaneously breathing animals, this alone would decrease XII nerve activity by reducing the inspiratory drive to XII motoneurons. However, the magnitude of the depression was not proportional to the end-expiratory CO₂ decrease, suggesting that additional mechanisms related to neuromuscular paralysis were involved. Since most reflex effects from cranial afferents to XII motoneurons are inhibitory, it is conceivable that, together with activation of respiratory effort produced by the ventral pontine carbachol injections in spontaneously breathing animals, peripheral receptors are activated whose reflex effect on XII motoneurons is inhibitory. If we then consider that neuromuscular paralysis suppresses transmission in the reflex pathways to XII motoneurons, it is not surprising that reflex-driven decrements in XII motoneuronal activity may occur following ventral pontine carbachol injections in spontaneously breathing rats. In contrast, when the animals are paralyzed and artificially ventilated, secondary effects of CO₂ and other reflexes are eliminated, allowing for a full expression of the central effect.

In conclusion, we found that the central effects of carbachol injections into the ventral nucleus pontis oralis on noradrenergic neurons of the locus coeruleus and XII motoneurons are excitatory and, as such, oppose to those occurring during natural REM sleep or following carbachol injections into the dorsal pontine reticular formation in urethane-anesthetized rats. These excitatory effects from ventral pontine sites may be unrelated to REM sleep or may represent an excitatory component of REM sleep in motoneurons (such as the asynchronous muscle twitches during natural REM sleep). The role of cholinergic activation within the ventral pontine region in behavioral control needs to be further assessed in chronically instrumented behaving rats. We also found that the secondary effects of ventral pontine carbachol injections that occur in nonparalyzed animals may mask the primary central effects. The differences related to the presence or absence of neuromuscular paralysis that we found in anesthetized rats highlight the need for careful monitoring of the motor, respiratory, and central noradrenergic activity in future behavior studies of this ventral pontine region.

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