Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat

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Nattie, Eugene, and Aihua Li. Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat. J Appl Physiol 89: 153–162, 2000.—Under anesthesia, inactivation of the retrotrapezoid nucleus (RTN) region markedly inhibits breathing and chemoreception. In conscious rats, we dialedyzed muscimol for 30 min to inhibit neurons of the RTN region reversibly. Dialysis of artificial cerebrospinal fluid had no effect. Muscimol (1 or 10 mM) significantly decreased tidal volume (VT) (by 16–17%) within 15 min. VT remained decreased for 50 min or more, with recovery by 90 min. Ventilation (VE) decreased significantly (by 15–20%) within 15 min and then returned to baseline within 40 min as a result of an increase in frequency. This, we suggest, is a compensatory physiological response to the reduced VT. Oxygen consumption was unchanged. In response to 7% CO₂ in the 1 mM group, absolute VE and change in VE were significantly reduced (by 19–22%). In the 10 mM group, the response to dialysis included a time-related increase in frequency and decrease in body temperature, which may reflect greater spread of muscimol. In the awake rat, the RTN region provides a portion of the tonic drive to breathe, as well as a portion of the response to hypercapnia.

control of breathing; GABAₐ receptors; rostral ventrolateral medulla; central chemoreceptors; hypoventilation

THE ROSTRAL VENTROLATERAL MEDULLA (RVLM) contains neurons involved in the control of breathing. Acidic stimulation of the medullary surface at the RVLM increased respiratory output (see Refs. 4, 17, 18), and surface cooling there produced apnea and a severely curtailed CO₂ sensitivity in anesthetized animals (4). Focal cooling by a small probe (5) showed the most sensitive site to include the retrotrapezoid nucleus (RTN), parapyramidal neurons of the medullary raphe, and juxtafacial portions of the nucleus paragigantocellularis lateralis (PGCL). The neurons of the RTN, as described by retrograde tracing experiments (26), lie ventral to the facial nucleus, extending for a small distance rostral and caudal to this structure. Our operative definition of the RTN region includes the RTN proper together with adjacent parapyramidal neurons and neurons within the juxtafagal PGCL.

In anesthetized cats and rats, focal acidification in the RTN region stimulated respiratory output (6, 15, 16), whereas focal destruction by neurotoxin injection (17–19, 21) substantially reduced phrenic nerve activity and the response to systemic hypercapnia. The importance of these two functions of the RTN region in more physiological conditions, i.e., in the absence of anesthesia, is less certain. This paper represents the second in a series of studies from our lab designed to test the hypothesis that neurons in the RTN region provide a tonic drive to breathe and are an integral part of the central chemoreceptor system in the conscious mammal. In the first study, we produced unilateral and focal destruction of a portion of the RTN region by neurotoxin, injected under anesthesia, in the rat (1). After 2 days of recovery, the rats were studied in the waking state over 3 wk. These animals, with histologically proven RTN lesions, breathed normally at rest but had a 39% reduction in the response to systemic hypercapnia, a substantial effect but less dramatic than that observed under anesthesia.

Other investigators have studied the role of neurons in the RVLM in awake mammals. Bilateral lesions produced by electrocoagulation in the cat RVLM, in an area that included the RTN region, resulted in hypoventilation during wakefulness and a markedly diminished response to hypercapnia (25). In the unanesthetized goat (10, 21), focal cooling of the RVLM, including the RTN region, decreased ventilation (VE) and the response to hypercapnia in the waking state, but the effects were small compared with those observed with cooling of the same location under anesthesia. In both studies, the size of the region affected was likely to be substantially larger than in our rat neurotoxin study.

The purpose of this study is to evaluate the role of neurons in the RTN region in the control of breathing in the awake state by focally, acutely, and reversibly altering their function. The model is an unanesthetized rat with the GABAₐ receptor agonist muscimol applied to the RTN region by microdialysis. Our rationale is that the use of muscimol to inhibit RTN neurons in the awake rat may allow us to uncover their function by avoiding less specific and more widespread effects produced by neuronal destruction or cooling. The hypothesis is that the RTN provides a tonic stimulation of breathing in wakefulness and the full ventilatory re-
response to systemic hypercapnia requires the integrity of the RTN.

**METHODS**

Sprague-Dawley rats weighing 300–400 g were anesthetized with intramuscular ketamine (100 mg/kg) and intraperitoneal xylazine (20 mg/kg). With the rat in a Kopf stereotaxic device, a guide tube of 0.38 mm outside diameter filled with a dummy cannula (CMA/11, Acton, MA) was inserted 10.6–10.8 mm below the dorsal surface of the skull at coordinates 2.2 mm caudal to lambda and 1.8 mm lateral from the midline (24). The guide tube was secured to the skull with cranioplast cement, and the wound was sutured.

A sterile telemetric temperature probe (TA-F20, Data Sciences, St. Paul, MN) was placed unattached in the abdominal cavity. The animal was allowed to recover for 3–4 days. In four rats, an arterial catheter was implanted into the femoral artery and thread subcutaneously, exiting at the back of the neck. It was connected via saline-filled tubing to a transducer for measurement of blood pressure.

On each experimental day, the rat was weighed, the dummy cannula was removed, and the dialysis probe was inserted into the guide tube. The active dialysis site, which protruded 1 mm beyond the guide tube tip, was 1 mm in length and 0.24 mm in diameter, with membrane pores limiting movement to molecules under 6,000 Da. Animals were placed into a Pappenheimer-style plethysmograph chamber (7, 23) and allowed 30–40 min to acclimate. We measured $V_{\text{E}}$, oxygen consumption ($V\dot{O}_2$), and body temperature ($T_b$). After acclimation, three sets of data while the animals were breathing room air and one during an initial CO$_2$ response were obtained over 50 min with the dialysis probe in place but without flow through it. The dialysis pump was then turned on with artificial cerebrospinal fluid (aCSF) flowing at a rate of 4.0 μl/min. The composition of the aCSF was (in mM) 152 sodium, 3.0 potassium, 2.1 magnesium, 2.2 calcium, 131 chloride, and 26 bicarbonate. The calcium was added after the aCSF was warmed to 37°C and equilibrated with 5% CO$_2$. Air-breathing measurements were repeated over 10 min, and then the dialysate was switched to 1) aCSF, 2) 1 mM muscimol in aCSF, or 3) 10 mM muscimol in aCSF. The time at which the new dialysate reached the tissue (washout of the dead space accounted for) is determined as $t = 0$ in our analysis. After 30 min, the dialysate was changed to aCSF, which was continued for the final 60 min. Measurements were taken over a 20- to 30-s period at 0, 5, 15, 40, 50, 60, and 90 min. We collected data for up to 190 min, but we focused our formal statistical analysis and report on only the results to 90 min. The reason for this is an unexpected and large decrease in $T_b$ observed in the 10 mM muscimol group beginning at 90 min. The entire experimental period was 2.5 h.

We added fast green dye to the dialysate to facilitate visualization of the dialysate outflow and to allow a histological test for membrane leak. In addition, we examined the membrane just after each dialysis. We monitored the outflow rate semiquantitatively by visualization of the drops of dialysate coming out of the outflow tubing. Animals in this report did not have leaks in the dialysis membrane during the experiment.

There were three experimental groups. Group 1 ($n = 7$ trials in 6 rats) was dialyzed during the 30-min treatment period with aCSF used as the vehicle. Group 2 ($n = 6$ trials in 5 rats) was dialyzed with 1 mM muscimol in aCSF and Group 3 ($n = 5$ trials in 4 rats) with 10 mM muscimol. Eight animals were used overall. One received all three treatments, one received one treatment, and all others received two of the three treatments. The rats were studied in a state that we refer to as quiet wakefulness. The experiments were performed between 9 AM and 4 PM. The rats were housed on a midnight-to-noon circadian light-dark cycle, so we collected data during the end of the quiet/sleep (light) circadian period and the beginning of the active/wake (dark) circadian period. This yielded reproducible data representing quiet wakefulness. If we waited until later in the active/wake period, the rats were more prone to sniffing, grooming, and active movement such that the ventilatory data were more variable and difficult to obtain. The rats were under constant observation during the experiment and were studied in subdued light.

For the CO$_2$ response, the inspired CO$_2$ was increased by switching the inflow gas to a mixture with 7% CO$_2$ and 93% air, which slowly increased the inspired CO$_2$ value over 8–9 min, as measured in the outflow gas. We measured ventilatory variables from 8 to 9 and from 9 to 10 min, as shown in Figs. 2–4. These values were essentially the same at the two measurement periods. CO$_2$ responses were measured before, during, and just after dialysis treatment to evaluate the effects of muscimol on the response to increased CO$_2$.

The plethysmograph chamber used in these experiments is similar to that described by Pappenheimer (23). The analog output of the pressure transducer was recorded on a strip chart recorder (Honeywell, model 1200) and Vetter Digital system (model 3000A). Inflow of humidified gas and outflow are balanced by using a flowmeter (Matheson, model 7491T) and a vacuum system. The flow rate through the plethysmograph was at or above 1.4 l/min, with 100 ml/min of outflow gas going to the O$_2$ and CO$_2$ analyzer (Applied Electrochemistry, model SA-3). The temperature in the chamber was measured before each measurement period. Rat $T_b$ was measured continuously via the analog output from the telemetric temperature probe in the abdomen. The plethysmograph chamber was calibrated each day with 0.3 ml of air injected at approximately the rate of inspiration. Calibrations are performed with the rat replaced by an equally sized inert object, which allows a more accurate and reliable calibration, and with the rat in the chamber, which evaluates the chamber leak rate.

Analog respiratory data were digitized (DataPac III), and a breath-by-breath analysis was performed with the pressure deflections and the respiratory cycle time for each breath determined over a 20- to 30-s time period (DataPac III). $V_r$ (per 100 g of body wt), $V_e$, and $V_{te}$ (per 100 g body wt) were calculated (SigmaPlot IV). $V_{O2}$ was determined by the difference in outflow oxygen (determined by the O$_2$ sensor) vs. inflow oxygen with a constant flow rate of 1.4 μl/min.

The results for $V_e$, $V_r$, and $T_b$ in room air or breathing 7% CO$_2$ were compared within each experimental group by one-way repeated-measures ANOVA and among the experimental groups by a two-way ANOVA with treatment (aCSF, 1 mM muscimol, 10 mM muscimol) and time as factors (Sigmaplot, Jandel Scientific software; SYSTAT). In some cases, the Friedman repeated-measures ANOVA on ranks was required. Post hoc tests (Dunnett’s, Student-Newman-Keuls) were performed when significant differences were found. The air-breathing ventilatory data were evaluated in two ways. First, the absolute values of $V_e$, $V_r$, and $f$ in the 12 air-breathing measurement periods were compared over the entire 2.5-h experiment. Second, these data are expressed as percent of baseline (%baseline), which was determined as the mean of three sets of measurements made over 10 min before the onset of dialysis. This was done because we were unable to use the same animals reliably for

$\text{CO}_2$ response were obtained over 50 min with the dialysis animals were breathing room air and one during an initial added after the aCSF was warmed to 37°C and equilibrated calcium, 131 chloride, and 26 bicarbonate. The calcium was flowing at a rate of 4.0 μl/min. The entire experimental period was 2.5 h. The plethysmograph chamber was calibrated each day with 0.3 ml of air injected at approximately the rate of inspiration. Calibrations are performed with the rat replaced by an equally sized inert object, which allows a more accurate and reliable calibration, and with the rat in the chamber, which evaluates the chamber leak rate. Analog respiratory data were digitized (DataPac III), and a breath-by-breath analysis was performed with the pressure deflections and the respiratory cycle time for each breath determined over a 20- to 30-s time period (DataPac III). $V_r$ (per 100 g of body wt), $f$, and $V_e$ (per 100 g body wt) were calculated (SigmaPlot IV). $V_{O2}$ was determined by the difference in outflow oxygen (determined by the O$_2$ sensor) vs. inflow oxygen with a constant flow rate of 1.4 μl/min.

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all three dialysis tests and we wanted to normalize for differences in initial values among the groups.

The CO₂ breathing data are evaluated in two ways. First, the absolute values of \( \dot{V} \), \( V_T \), and \( f \) are compared (one-way ANOVA) for the three CO₂ tests before, during, and after each dialysis treatment (aCSF, 1 mM, 10 mM muscimol). Second, the change in these values, calculated as the CO₂-stimulated value at each CO₂ challenge minus the average of the control values just before and after, are compared for the three CO₂ tests before, during, and after dialysis treatment (one-way ANOVA). After the last 7% CO₂ reading, the inspired gas is switched to air, and the plethysmograph is opened briefly to allow the high CO₂ to escape. On closure of the plethysmograph, another ~10 min are allowed to pass before the initial post-CO₂ stimulation values are taken.

At the conclusion of the experiments, the animals were killed, and the medulla was removed and placed in 4% paraformaldehyde. They were frozen then sectioned at 50-μm thickness with a Reichert-Jung cryostat. Sections were counterstained with cresyl violet, and with the assistance of a rat brain atlas, we identified anatomical landmarks and the site of dialysis probe placement (24). The guide tubes were removed postmortem but before brain stem removal and sectioning. To remove them required manipulation and produced tissue disruption. This facilitated the anatomical verification of guide tube and probe tip location but also increased the volume of tissue disruption compared with that produced by simple insertion. In two additional rats, a 30-min period of dialysis with a fluorescein dye of molecular weight (332) similar to that of muscimol (195) was followed by a 15-min dialysis of aCSF before death. The brain stems of these animals were frozen immediately, sectioned, and viewed with a fluorescence microscope, with the appropriate images digitized via camera and computer. This approach, used in an earlier paper (7), allowed an approximate evaluation of the spread of the muscimol into the brain stem during dialysis.

### RESULTS

Figure 1 shows digitized tracings of plethysmograph pressure for representative sections of breathing during a typical experiment with 1 mM muscimol in the test-period dialysate. Note that \( V_T \) is decreased after muscimol exposure in the RTN region while the animal is breathing air or increased CO₂.

The mean ± SE absolute values of \( \dot{V} \), \( V_T \), and \( f \) over the entire 2.5-h experiment are shown in Figs. 2–4 for dialysis with aCSF and 1 mM or 10 mM muscimol, respectively. These same data are expressed as %baseline in Fig. 5.

**Unilateral RTN dialysis with aCSF: Effects on \( \dot{V} \)**

There was remarkably little change in \( \dot{V} \), \( V_T \), and \( f \) over the 2.5-h experiment, including the period of no dialysis during the first 50 min and the period of aCSF dialysis over the next 100 min (Fig. 2). One-way repeated-measures ANOVA showed no significant changes in \( \dot{V} \), \( f \), or \( V_T \). When analyzed as %baseline (Fig. 5), none of the three ventilatory variables showed any significant change over the 30-min aCSF test dialysis and the 60-min aCSF dialysis recovery period.

**Unilateral RTN dialysis with 1 mM and 10 mM muscimol: Effects on \( \dot{V} \)**

Absolute \( \dot{V} \) analyzed for all data over the 2.5-h experiment was significantly lower in the 1-mM treatment group compared with the aCSF group (Figs. 2–4; two-way ANOVA; \( F = 15.976; df = 2; P < 0.001 \); Dunnett’s post hoc comparison; \( P < 0.05 \) for aCSF vs. 1 mM muscimol; there was no effect of time or treatment \( \times \) time). The absolute values of \( V_T \) or \( f \) for all data did not differ among the groups because the initial absolute values of these variables differed.
Analysis of absolute values within groups did show significant effects. One-way repeated-measures ANOVA for the 1-mM muscimol treatment group showed a significant decrease in VT (F = 2.318; df = 11; P = 0.02) and VE (F = 2.369; df = 11; P = 0.018) with no effect on f. One-way repeated-measures ANOVA for the 10-mM muscimol treatment group showed a significant decrease in VT (F = 5.745; df = 11; P < 0.001), no effect on V˙E, and a significant increase in f (Friedman repeated-measures ANOVA on ranks; χ² = 23.087; df = 11; P = 0.017). Post hoc analysis to pinpoint the exact times at which these effects were significant within each group suffered from the variability of absolute values within treatment groups.

Given the variability of the initial absolute VT and f values, we repeated the analyses using the data expressed as %baseline as shown in Fig. 5. Baseline was defined as the average of the three sets of data obtained just before t = 0, the onset of dialysis treatment. Both doses of muscimol produced significant effects on VT. Two-way ANOVA of the data in all three groups over the time period of the experiment showed a significant effect for treatment (F = 6.824; df = 2; P = 0.002), with post hoc analysis showing significance of the 1 mM and 10 mM results compared with aCSF (Student-Newman-Keuls test; P < 0.05). There was no effect of time nor was there an interactive effect. One-way repeated-measures ANOVA within the 1 mM group showed a significant effect (F = 2.445; df = 6; P = 0.048) at 15 and 50 min (Dunnett’s test; P < 0.05), with mean ± SE values that were 86 ± 4 and 83 ± 7% of baseline, respectively. Friedman repeated-measures ANOVA within the 10 mM group showed a significant effect (χ² = 18.343; df = 6; P = 0.005) at 5, 15, 40, 50,
Ve decreased initially during dialysis with both the 1 mM and 10 mM doses to values of 81.9 ± 7 and 79.7 ± 7% of baseline, respectively, at 5 min and to values of 84.8 ± 2 and 83.2 ± 7% of baseline, respectively, at 15 min. Two-way ANOVA of the %baseline data evaluating the effects of treatment and time shows a significant effect of 1 mM treatment vs. aCSF ($F = 3.229; \text{df} = 2; P = 0.044$) at 5 and 15 min (Dunnett’s test; $P < 0.05$). One-way repeated-measures ANOVA on the 1 mM results shows a significant effect ($F = 3.204; \text{df} = 6; P = 0.015$) at 5 and 15 min (Dunnett’s test; $P < 0.05$). One way repeated-measures ANOVA on 10 mM results showed a significant effect ($F = 3.960; \text{df} = 6; P = 0.007$), but post hoc analysis shows this to be the value

60, and 90 min (Dunnett’s test; $P < 0.05$), with respective median values, expressed as %baseline, of 93, 84, 75, 78, 79, and 91. Recovery from the 30-min test dialysis took place by 60–90 min in the 1 mM group and was approaching completion at 90 min in the 10 mM group. Unilateral muscimol dialysis into the RTN region decreased VT in the awake rat.

The effects on f were somewhat different. Two-way ANOVA, constructed as for the VT analysis above, showed a significant effect for treatment ($F = 6.674; \text{df} = 2; P = 0.002$) and for time ($F = 2.493; \text{df} = 6; P = 0.027$) with no interactive effect. However, post hoc evaluation showed this to be significant for the comparison between aCSF and the 10 mM dose ($P < 0.05$; Dunnett’s test). Repeated-measures one-way ANOVA of the data in the 1-mM group showed no significant effect. In the 10 mM dose group, f was increased significantly ($F = 2.559; \text{df} = 6; P = 0.046$). Muscimol dialysis into the RTN region produced an increase in f that was noted just after the cessation of dialysis and likely began during the dialysis and that reached significance in the 10 mM group.

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Dialysis with aCSF, 1 mM, and 10 mM muscimol: Effects on response to CO₂. Dialysis with the 1 mM or 10 mM dose of muscimol had no significant effect on VO₂ (Fig. 6). The 1 mM and 10 mM doses had significant effects on Tb (one-way repeated-measures ANOVA; P < 0.005) at 60 and 90 min for each dose (Dunnett’s test; P < 0.05). In the 10 mM dose group, Tb continued to decrease after 90 min to values of 36°C at 180 min (data not shown). Mean arterial blood pressure was unaffected by the 1 mM dose (Fig. 6; four trials in three rats). Blood pressure measured in a single animal dialyzed with 10 mM muscimol was unaffected.

Dialysis with aCSF, 1 mM, and 10 mM muscimol: Effects on response to CO₂. In the aCSF group (Fig. 2), neither the absolute values of CO₂-stimulated Ve, VT, and f nor the change in these values, comparing CO₂-stimulated with the average of pre- and post-CO₂ baseline values, differed significantly among the three dialysis tests.

In the 1 mM muscimol dialysis group (Fig. 3), absolute Ve with CO₂ stimulation was significantly decreased both during and after muscimol dialysis (one-way repeated-measures ANOVA; F = 22.87; df = 2; P < 0.001; P < 0.05, post hoc Dunnett’s test). Mean ± SE values of absolute Ve (expressed as ml·min⁻¹·100 g⁻¹) were 250 ± 15 before muscimol; 202 ± 22 during muscimol; and 202 ± 20 after muscimol. This represents an average decrease of −19%. The change in Ve was also significantly decreased (F = 12.765; df = 2; P = 0.002; P < 0.05, post hoc Dunnett’s test) both during and after muscimol from 171 ± 15 ml·min⁻¹·100 g⁻¹ to 133 ± 20 and 134 ± 17, respectively, an average decrease of −22%.

Absolute VT was decreased by 1 mM muscimol (F = 4.629; df = 2; P = 0.038) both during and after muscimol (P < 0.05, Dunnett’s test) from 1.55 ± 0.02 ml/100 g to 1.41 ± 0.06 and 1.41 ± 0.05, respectively, an average decrease of −9%. However, the change in VT produced by the CO₂ stimulus was not significantly different among the three measurement periods. The absolute f was significantly decreased (F = 10.241; df = 2; P = 0.004) both during and after (P < 0.05; Dunnett’s test) 1 mM muscimol dialysis from 157 ± 10 min⁻¹ to 141 ± 9 and 140 ± 9 min⁻¹, respectively, an average decrease of −10%. The change in f produced by the CO₂ stimulus was not significantly different among the three measurement periods.

In the 10 mM muscimol dialysis group (Fig. 4), absolute CO₂-stimulated Ve was not significantly affected, nor was the change in Ve. Absolute VT was decreased by muscimol (one-way repeated-measures ANOVA; F = 4.528; df = 2; P = 0.048) and after muscimol (P < 0.05, Dunnett’s test) from 1.52 ± 0.2 ml/100 g to 1.24 ± 0.06 ml/100 g, an average decrease of −18%. The change in VT produced by the CO₂ stimulus was not significantly different among the three measurement periods. The absolute f was not significantly affected, nor was the change in f produced by the CO₂ stimulus.

Anatomical location of dialysis probes. Figure 7 shows computer-modified, digitized ×2 images of cresyl violet-stained medullary cross sections at the approximate center of the tissue disruption at the site of the dialysis probe tip for animals that received the 1 mM muscimol. In each case, the probe tip was in the region of the RTN. The results (not shown) are similar for the other animals. The image at bottom right shows the cross section of the medulla taken from a separate rat that shows the greatest area of fluorescence after 30-min dialysis of fluorescein and 15 min of aCSF washout. In two such rats, the volume of tissue fluorescein distribution was 724 and 703 nl, respectively. The average rostral-to-caudal spread was 900 μm, and the average radius of the cross section with largest fluorescence area was 650 μm. Figure 8 shows three medullary cross sections from one rat taken at different rostral-caudal levels to show the path of the guide tube, which traverses the medulla in a plane different from that of the sections. At bottom right at higher magnification, the site of the probe tip is shown. Note gliosis and thickening of meninges.
DISCUSSION

Limitations of technique. Microdialysis is a useful technique to deliver neuroactive substances to a focal region of the medulla of the unanesthetized rat. We have used this approach successfully for thyrotropin-releasing hormone (TRH) and CO₂ in the RTN region (7, 16). One concern about the technique is the degree of spread of the dialyzed substance within the tissue. For CO₂, we measured tissue pH at different distances from the dialysis probe. For TRH and muscimol, it is more difficult to measure the exact tissue region affected. However, we can estimate this region by using data from the literature and from our own studies of the spread via dialysis of fluorescein, which has a molecular weight similar to muscimol and TRH.

From the literature, it appears that a considerable concentration difference of neuroactive substance is required between the inside and outside of the dialysis probe. Measures of tissue delivery of glutamate (2) dialyzed at 10–1,000 mM and 2.0 μl/min for 30 min showed a 35% delivery. For our 1 mM muscimol dialysis, this would result in a maximum concentration of 0.35 mM outside the probe. A drug dialysis study using two probes, one for delivery, the other for collection and measurement, showed a similar finding: steady-state concentrations 1.5 mm from the delivery probe that were 10–100 times lower than in the dialysate (8).

As reported in our initial dialysis paper (7), the use of our fluorescein marker with 1 mM concentration in the probe showed an average (n = 5) rostral-to-caudal spread of 870 μm and an average radius at the cross section with the largest area of 555 μm. The estimated volume of distribution was 590 nl, which is 49% of the approximate volume of the RTN region (1.2 ml). The results from similar dialysis of fluorescein in two additional rats in this study are similar: 713 nl, 900 μm rostral-to-caudal spread, and 650 μm average radius of cross section with largest area. From these data, we estimate that, with dialysis of 1 mM muscimol, the tissue distribution is likely to be predominantly within the RTN region and the concentration at the probe is likely to be 0.35 mM or less.

Dialysis with 10 mM muscimol most certainly affected a larger region. The average volume for dialysis with fluorescein at 10 mM was 1,580 nl (7), 2.7 times greater than with 1 mM. After 90 min in the 10-mM muscimol group, Tb decreased to values of 36°C and f and Ve increased in temporal association with this hypothermia, suggesting that spread of the muscimol to adjacent regions may have occurred. In a single experiment, we dialyzed the lower 1 mM muscimol dose directly into the medullary raphe and observed a similar large fall in Tb, which, in this case, occurred immediately with the onset of dialysis. We hypothesize...
that the fall in $T_b$ and the associated increase in $f$ reflect events involving muscimol actions within the medullary raphe. We have chosen to limit our data presentation to the time period before this large drop in $T_b$.

Effects of muscimol dialysis in the RTN. The major findings in this study are that dialysis of muscimol at the 1 mM dose into the RTN region of the conscious rat decreases $V_T$ and $V_E$ reversibly without affecting $V_O^2$ and $T_b$, at least within the period of the dialysis and immediately thereafter. Dialysis of muscimol at the 10 mM dose also significantly decreases $V_T$. The results are complicated by the observation that $f$ increases within, and shortly after, the period of muscimol dialysis in both the 1 mM and 10 mM experiments, being larger and reaching statistical significance in the latter. The net effect on $V_E$ is complex, with an initial decrease that is predominantly the result of the strong muscimol effect on $V_T$, followed by a return to baseline within minutes of cessation of muscimol dialysis, reflecting the delayed increase in $f$.

The response to $CO_2$ is also altered in a similar manner, an effect most clearly seen in the 1 mM results. Both the absolute level of $CO_2$-stimulated $V_E$ and the $CO_2$-induced change in $V_E$ were significantly decreased by 19–22%, suggesting an effect on the central chemoreceptor system. Both $V_T$ and $f$ were decreased in absolute values, although their change was not significantly affected. In the 10 mM results, the response of absolute $V_T$ was significantly reduced, but $V_E$ was unaffected because of the increase in $f$ that occurred during the experiment.

We suggest that this increase in $f$ represents two processes. First, there is an attempt by the animal to maintain normal $V_E$ in the presence of a powerful inhibition of $V_T$ with constant metabolic rate. The mechanism for this response is not known but could involve immediate responses at non-RTN chemoreceptor sites to decreases in $PCO_2$ associated with the initial hyperventilation. Second, the increase in $f$ could reflect spread of muscimol into adjacent regions at which a nonchemoreceptor effect may arise. As mentioned above, in the 10 mM fluorescein experiments, there was a greater tissue volume affected, and, in the 10 mM muscimol experiments, delayed changes in $T_b$ and $f$ were observed.

We interpret our experiments to support the hypothesis that the RTN provides a tonic drive to respiratory control neurons, even in the awake unanesthetized state. In the conscious rat, this seems to predominantly affect $V_T$. There is anatomical evidence for $GABA_A$ receptors in this region (29). And, in anesthetized animals, muscimol or GABA applied to the brain surfaces by cisternal injection or ventriculocisternal perfusion inhibits respiratory output (13, 27). Application directly to the VLM surface in anesthetized animals also decreases respiratory output and blood pressure (12, 14, 28). Direct injection of muscimol (200 nl; 5–10 mM) into the ventral medulla in anesthetized cats can decrease blood pressure without effects on breathing, indicating an anatomical specificity of sites for effects on breathing and blood pressure (11).

Comparison of RTN chemoreceptor stimulation and muscimol inhibition. Focal stimulation of the RTN region by microdialysis of $CO_2$ in the awake rat increases $V_E$ by 18% entirely as a result of an increase in $V_T$. The stimulus intensity at the center of the focal region of acidosis was like that caused by inhalation of 9% $CO_2$, and the region of acidosis was limited to within 550 $\mu$m of the probe in the anesthetized rat (16). In the present experiment, inhibition of RTN neurons in the awake rat by microdialysis of muscimol (1 mM) decreases the response to systemic hypercapnia by 19–22%. These separate results indicate that neurons in this region provide a...
portion of the CO$_2$-related drive to breathe in the awake rat.

The presence of other chemoreceptor sites, both centrally (3, 6, 15–18, 20) and in the periphery, and the use of an animal model with a “closed-loop” control system complicate the quantitative analysis of these data. As noted above, inhibition of V$_E$ by muscimol without change in metabolic rate would increase CO$_2$, which can stimulate non-RTN chemoreceptors. The final level would reflect a balance of muscimol-induced inhibition in the RTN region and CO$_2$ excitation at other chemoreceptor locations. Similarly, stimulation of V$_E$ by focal CO$_2$ dialysis in the RTN may lower CO$_2$ levels at other chemoreceptor locations, again tempering the quantitative nature of the response. Thus the use of a conscious rat with a closed-loop control system to examine focal effects in the RTN may disguise the overall importance of neurons in the RTN region in the control of breathing. However, this type of experiment demonstrates the significance of having many central chemoreceptor locations; they can temper or modulate responses that might arise at a single location. Further, we can postulate that the large sensitivity of the respiratory control system to small changes in brain interstitial fluid pH, when studied in the unanesthetized state (9), requires that many chemoreceptor locations simultaneously stimulated. The presence of many chemoreceptor sites, each with moderate sensitivity, would provide stability in that no single site can easily bring about a large change in V$_E$ (this concept arises from discussions with John Remmers). When all sites are stimulated, high sensitivity occurs as a result of an additive, or multiplicative, interaction. Here, with inhibition of one site, the RTN, the system response quickly corrects the initial hypoventilation.

Effects of dialysis of TRH in the RTN: Comparison to muscimol. In a study similar to this one, we dialyzed TRH into the RTN of conscious rats (7). Both 1 mM and 10 mM doses produced an increase in V$_E$, T$_R$, V$_O_2$, and arousal, but the effects were greater and longer lasting with the 10-mM dose. Our interpretation was that the RTN may be involved in the integration of signals, which can match changes in V$_E$ to changes in metabolic rate. The observed stimulation of breathing was appropriate for the observed increase in metabolism. For muscimol, the decrease in V$_E$ occurs without a change in metabolic rate, suggesting the presence of hypoventilation, a disruption of the link between V$_E$ and metabolism.

What is the quantitative importance of the RTN in the control of breathing? Why did bilateral coagulation of the RVLM (25) in cats and bilateral cooling of the RVLM in conscious goats (10) show greater effects on breathing both at rest and in response to hypercapnia than our lesion (1) and muscimol (this study) experiments? How can we compare responses to neurotoxin lesions of the RTN region in conscious rats with responses to reversible inhibition of the RTN by muscimol?

For the first issue, there are clear differences in approaches. Different species are involved, but, perhaps more importantly, the volume of tissue affected by bilateral coagulation (25) or cooling (10) is certainly much greater than in our unilateral lesion (1) and muscimol dialysis experiments. Destruction or cooling of more RTN neurons (including contiguous RVLM neurons) seems likely to have a greater effect on breathing and CO$_2$ sensitivity.

For the second issue, the neurotoxin-induced lesions had no effect on resting breathing but decreased the response to 7% CO$_2$ by 39% (1), whereas 1 mM muscimol dialysis decreased V$_E$ by 15–20% and the response to 7% CO$_2$ by 19–22%. For the lesion experiments, we used cell counts to estimate that 35% of one RTN region was destroyed; for the muscimol dialysis we used fluorescein dialysis to estimate that up to 49% of the RTN was affected (7). The RTN tissue volumes affected by neurotoxin lesion and muscimol dialysis are similar. Why is the CO$_2$ much more reduced in the lesion experiment and the resting V$_E$ reduced only in the muscimol dialysis experiment? The greatest difference in the two protocols is in the timing and reversibility of the RTN disruption. Muscimol has an effect in 5 min that is reversible within 60 min of cessation of dialysis. The lesions (1) were produced under anesthesia and are permanent, and their effects were not studied until 48 h after their production. It is possible that some type of neuronal plasticity or adaptation occurred in the 2 days of recovery allowed in that protocol. It is also possible that there were small differences in the anatomical nature of the neurons affected in the two studies, differences that we cannot, at present, detect with the anatomical techniques used.

Conclusions and physiological significance. We conclude that the RTN provides an important excitatory input to neurons that determine the VT and the overall level of V$_E$ in the awake resting state and that the RTN is involved in the response to systemic hypercapnia during wakefulness.

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