Effects of unilateral lesions of retrotrapezoid nucleus on breathing in awake rats

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Akilesh, Manjapra R., Matthew Kamper, Aihua Li, and Eugene E. Nattie. Effects of unilateral lesions of retrotrapezoid nucleus on breathing in awake rats. J. Appl. Physiol. 82(2): 469–479, 1997.—In anesthetized rats, unilateral retrotrapezoid nucleus (RTN) lesions markedly decreased baseline phrenic activity and the response to CO2 (E. E. Nattie and A. Li. Respir. Physiol. 97: 63–77, 1994). Here we evaluate the effects of such lesions on resting breathing and on the response to hypercapnia and hypoxia in unanesthetized awake rats. We made unilateral injections [24 ± 7 (SE) nl] of ibotenic acid (IA; 50 mM), an excitatory amino acid neurotoxin, in the RTN region (n = 7) located by stereotaxic coordinates and by field potentials induced by facial nerve stimulation. Controls (n = 6) received RTN injections (80 ± 30 nl) of mock cerebrospinal fluid. A second control consisted of four animals with IA injections (24 ± 12 nl) outside the RTN region. Injected fluorescent beads allowed anatomic identification of lesion location. Using whole body plethysmography, we measured ventilation in the awake state during room air, 7% CO2 in air, and 10% O2 breathing before and for 3 wk after the RTN injections. There was no statistically significant effect of the IA injections on resting room air breathing in the lesion group compared with the control groups. We observed no apnea. The response to 7% CO2 in the lesion group compared with the control groups was significantly decreased, by 39% on average, for the final portion of the 3-wk study period. There was no lesion effect on the ventilatory response to 10% O2. In this unanesthetized model, other areas suppressed by anesthesia, e.g., the reticular activating system, hypothalamus, and perhaps the central retrotrapezoid nucleus, may provide tonic input to the respiratory centers that counters the loss of RTN activity.

ventrolateral medulla; control of breathing; central chemoreceptors; carbon dioxide sensitivity; hypoxia; unanesthetized rats

NEURONS ACCESSIBLE from the surface of the rostral ventrolateral medulla (RVLM) have been shown to be quite important in the control of breathing in anesthetized and reduced preparations (5, 6, 17, 20, 21). For example, surface cooling at the intermediate (Schläfke's) surface area results in apnea and a virtual absence of any CO2 sensitivity (5, 20). The use of a cooling probe (6) localized the anatomic regions involved to those lying beneath the caudal part of the rostral (Mitchell's) chemosensitive area and rostral half of Schläfke's area including the parapyramidal neurons (14), the retrotrapezoid nucleus (RTN) (8, 25, 26, 31), portions of paragigantocellularis lateralis (35), and dendrites from the more dorsal retrowratal nucleus (32).

A major structure in this RVLM region is the RTN. Pearce et al. (31) defined the RTN region in the rat as extending from the rostral border of the nucleus ambiguous to the rostral border of the facial nucleus, lying within a few hundred micrometers of the ventrolateral medulla (VLM) surface, and bounded laterally by the spinotrigeminal tract and medially by the pyramidal tract. The neurons in the RTN have anatomic (31, 34) and physiological connections (8, 31) to the more dorsally located respiratory neuron pools. RTN neurons discharge tonically or phasically with the respiratory rhythm (8), and both types increase their firing rate with increased systemic CO2. Microinjections of acetazolamide in the RTN region produce a focal acidosis that, in turn, increases phrenic nerve output, indicating the presence of central chemoreception within this region (7). Central chemoreceptors are present at many other sites as well (3, 23). Unilateral chemical or electrolytic lesions of the RTN region in anesthetized or decerebrate cats (24, 25) and in anesthetized rats (26) decrease baseline phrenic nerve output, often to apnea, and markedly decrease the ventilatory response to breathing CO2. These effects were most impressive in the anesthetized preparations and less so in the decerebrate animals. These observations suggest the presence of an important tonic drive, of RTN origin, to the more dorsally located respiratory neuron groups. But the strength of this drive may be dependent on the state of arousal of the animal.

In goats with preimplanted VLM surface-cooling devices, Forster and colleagues (13, 28, 29) observed that bilateral cooling of an area on the VLM surface, which includes portions of the rostral and intermediate areas, caused sustained apnea under anesthesia. However, cooling the same areas in the same animals when they were unanesthetized caused only a modest attenuation of breathing. These observations are similar to the reduced effectiveness of RTN lesions in decerebrate vs. anesthetized cats (25) and support the idea that the arousal state may importantly influence the effectiveness of RTN lesions. In contrast, Schläfke et al. (33) produced bilateral coagulation of the intermediate areas in cats, which, when subsequently studied under unanesthetized conditions, demonstrated hypoventilation at rest, breathing air, and an almost complete loss of the ventilatory response to breathing CO2.

The present study was undertaken to observe in the unanesthetized rat the effects of a specific unilateral RTN lesion on the ventilatory response to breathing room air, 7% CO2 in air, and 10% O2. We hypothesize that in awake and intact animals, the effects of RTN lesions would still be apparent but would be of smaller magnitude.

METHODS

General protocol. The study was approved by the animal ethics committee at Dartmouth Medical School. Male Sprague-
Dawley rats, weighing between 290 and 415 g at the beginning of the study, were housed in the animal resource center on a 12:12-h light-dark cycle and given food and water ad libitum. Each animal underwent four sets of ventilatory measurements while breathing room air, 7% CO\textsubscript{2} in air, and 10% O\textsubscript{2} before placement of the lesion and then one to two sets of measurements on alternate days after the lesion, for a total period of 21 days. On the day after the final set of awake measurements, the rat was anesthetized with ketamine (100 mg/kg im) and xylazine (15 mg/kg ip), and a final set of ventilatory measurement was made under anesthesia. The brain was then perfusion fixed in situ and the brain stem was removed, frozen, and sectioned. The treated and control groups were handled in identical ways except for the differing injectates.

Surgery. After the four sets of preoperative ventilatory measurements were obtained, the animal underwent the unilateral RTN injection either with ibotenic acid (IA) or mock cerebrospinal fluid (mCSF), both injectates containing fluorescent beads (Polysciences) for subsequent identification of injection location. The animals were anesthetized with ketamine (100 mg/kg im) and xylazine (15 mg/kg ip), and the crown and left cheek were shaved, and the left mandibular branch of the facial nerve was exposed and placed on a bipolar stimulating electrode. The animal was placed in a stereotactic frame (Kopf) on a heating pad to maintain the body temperature. The crown was exposed through a 1-cm midline incision, and a burr hole was drilled on the skull at a point 3 mm caudal and 2 mm left lateral to lambda. A tungsten recording microelectrode (0.005-in. diameter, FHA) was threaded through a glass micropipette with a tip diameter of ~10 µm and glued together. The pipette was primed with either IA or mCSF and fluorescent beads. Injection volumes of 25 nl were made by using a Picospritzer (General Valve), on the basis of the droplet diameter measurements, under a microscope before pipette insertion. This primed glass micropipette was slowly advanced through the burr hole to a point 10.5 mm ventral to the skull surface where the pipette tip should be just dorsal to the facial nucleus. To verify this and guide the pipette through the facial nucleus, the potentials evoked by peripheral stimulation of the facial nerve was exposed and placed on a bipolar stimulating electrode. The animal was then placed in a stereotactic frame (Kopf) on a heating pad to maintain the body temperature. The crown was exposed through a 1-cm midline incision, and a burr hole was drilled on the skull at a point 3 mm caudal and 2 mm left lateral to lambda.

After the apparent ideal injection location was identified, the pipette was advanced ventrally from the facial nucleus, the field potentials would decrease in size, and the tip would enter the RTN region. Normally, the depth of the pipette tip was ~10.8 mm from the skull surface. This method is an adaptation of the one used by Brown and Guyenet. (4). Once the apparent ideal injection location was identified and after a stabilization period of 10 min, four ~50-nl aliquots were injected, with 5-min intervals between the injections. We did not measure the injection volumes during the injection period but used injection parameters measured in vitro. Fifteen minutes later, the pipette was removed, the skin was sutured, and the animal was placed in a Plexiglas box with 100% O\textsubscript{2} flowing until the animal recovered from the anesthesia. The animal was given food and water ad libitum during the postoperative period. The mortality associated with the surgery was ~33% irrespective of the injectates and may be a reflection of the difficulty of the procedure itself as well as the effects of RTN lesions in animals still under anesthesia. During preliminary experiments, the mortality was even higher, but with experience and the simple postoperative use of exposure to a high O\textsubscript{2} concentration, we were able to reduce it. No animals had any wound sepsis.

Ventilatory measurements. Ventilation was measured by using a whole body plethysmograph (2). In brief, the animal was placed in an acrylic box of ~7 liters volume. This box was connected to a similar reference box by a narrow tube and a differential pressure transducer (Validyne). The respiratory gas mixture was delivered via a humidifier, and the animal was continuously exposed to the mixture unless ventilatory measurements were being made. When measurements were made, the inlet and outlet of the main box were closed and the pressure transducer signal was recorded on a chart recorder (MFE). As the animal inhales, it warms and humidifies the inspired air, which causes an increase in the chamber pressure. The reverse happens when the animal exhales. The tidal volume (VT) was calculated from the magnitude of the pressure oscillations by comparing the pressure change induced by injecting a known volume of air and use of the gas laws. The breathing frequency was read directly by counting breaths on the record. The barometric pressure was recorded daily, the chamber temperature and the time in between the change of gas mixtures, and the core temperature of the animal at the beginning and end of each experimental period.

After body weight and core (rectal) temperature were recorded, the rat was placed in the box with room air flowing through. The animal would typically explore the surroundings, groom itself, and then, finally, settle down. The measurements while the animal was breathing room air were made with the animal quiet but awake. Then a mixture of 7% CO\textsubscript{2} in air was introduced for 15 min, and measurements were taken. After the box was flushed with room air for 10 min, 10% O\textsubscript{2} in nitrogen was introduced for 15 min and measurements were taken. The animals were exposed to air, 7% CO\textsubscript{2} in air, and 10% O\textsubscript{2} in a chamber with room air flowing through, with a 10-min washout time in between the change of gas mixtures. No animal had ventilation measured more than twice per day. All measurements were made during the daytime. Body temperature measured at the beginning was used for room air calculations, that measured at the end for calculating the ventilation while the animal was breathing hypoxia, and the midvalue between was taken for calculating the ventilation while the animal was breathing 7% CO\textsubscript{2} in air.

Anatomic analysis. At the end of the study period, including ventilation measurements under anaesthesia, the brain was fixed in situ by serially perfusing through the left ventricle of the heart 0.1 mM phosphate-buffered saline at a pH of 7.3 and then 4% paraformaldehyde in phosphate-buffered saline. The brain stem was removed, frozen in dry ice, and cut at 50-µm thickness in a cryostat (Cryocut 1800) at ~20°C. The adjacent sections were placed alternately onto gelatinized glass slides. From unstained slides, injection location was identified by seeing the fluorescent beads under the fluorescence microscope. The other set of slides was stained with cresyl violet for detailed histological study of tissue destruction and reactive gliosis and for anatomic localization of the fluorescent beads. We used the shape of neurons as described by Ellenberger and Feldman (Fig 3C in Ref. 9) to characterize RTN neurons. These were counted in the RTN region, that area lying ventral to the facial nucleus, in lesioned and in nonlesioned sections from the same animal. The volume of injection was calculated as follows: the total number of sections that contained fluorescent beads was counted and multiplied by the section thickness (50 µm) to...
determine the rostral-caudal length of the injection. The section with the largest cross-sectional area of beads was taken as the center of injection. This area was measured by using a computer image-analysis system (Image Pro). The injection volume was calculated by using the measured area and height and a simple geometric model of two adjoining circular cones. In the stained slides, the area corresponding to the location of maximal fluorescent beads and adjacent areas were examined in detail looking for gliosis, swelling, and neuronal destruction. Videocamera images of these areas were digitized with computer software (Image Pro).

RESULTS

We present results from 17 animals that completed the entire study duration and had adequate anatomic analysis to determine the injection site. The treated group consisted of seven rats, the mCSF injection control, six rats, and the control with IA injection into non-RTN regions, four rats. These three groups had initial mean body weights of $334 \pm 6$ (SE), $364 \pm 13$, and $357 \pm 3$ g, respectively. In the first few days after surgery, all rats lost weight but then gained weight, with group weights at the end of the experiment being $377 \pm 6$, $396 \pm 16$, and $389 \pm 6$ g, respectively. The initial mean presurgery body temperatures for the three groups were $36.9 \pm 0.3$ (SE), $37.3 \pm 0.3$, and $37.3 \pm 0.1$ °C, respectively. During the 21-day course of measurements, mean body temperature of each group did not vary significantly from these initial presurgery values.

In the calculation of the volume of injection, there was a discrepancy between the original injection volume estimate made on the basis of injection parameters measured in vitro and the volume calculated from measurements of the anatomic distribution of the fluorescent beads. We had estimated that we would inject 200 nl. The mean volume observed via anatomic analysis in the IA RTN-injection group (n = 7) was $24.4 \pm 7$ (SE) nl; for the mCSF control injection group (n = 6) it was $79.8 \pm 30$ nl; and for the non-RTN IA-injection control group (n = 4), $24.1 \pm 11.8$ nl.

The locations of the injections are shown in Figs. 1 (IA), 2 (mCSF), and 3 (IA at non-RTN sites). We show the location and size of the largest area of fluorescence observed in the serial sections examined in each animal. We have reproduced the exact size (to scale) of the area of fluorescence because of the variation observed in injection size. These mark the injection centers but do not tell us the size or volume of the region with neuronal dysfunction. Four IA injections (Fig. 1) were clearly centered within the region ventral and ventromedial to the facial nucleus. Three others were centered, respectively, 100, 150, and 350 µm caudal to the caudalmost aspect of the facial nucleus, sites within the described RTN region in rat (31). The distribution of the mCSF control injections (Fig. 2) was similar to that of the IA injections. Three were centered ventral to the facial nucleus, one was centered in the ventrolateral aspect of the facial nucleus itself, one was lateral to the nucleus, and one was ventromedial to the facial nerve lying just rostral to the facial nucleus. The center of four IA injections that had no effect on breathing (Fig. 3) were (in the figure) well rostral to the facial nucleus and nerve (A), medial to the facial nucleus and deep relative to the VLM surface (B), lateral to the facial nucleus and deep to the VLM surface (C), and just at the VLM surface adjacent to the pyramidal tract at 100 µm caudal to the caudal aspect of the facial nucleus, i.e., within the RTN region (D). This injection center, shown in Fig. 3D, associated with no effect on breathing, is close to those shown on Fig. 1, F and G, which did have significant effects on breathing.

Fig. 1. A-G: partial medullary cross section for each of 7 rats in ibotenic acid (IA) injection group that represents section with largest area of fluorescent beads for that injection. Darkened regions, computer-reconstructed images of actual area of fluorescence. Nos., distance from bregma (26). Cross sections are reconstituted from those in atlas of Paxinos and Watson (26). VII, Facial nucleus; P, pyramidal tract; AMB, nucleus ambiguous; IO, inferior olivary nucleus. Bar, 1 mm.
It was clear from microscopic observation of injection locations that tissue disruption extended beyond the region of visible fluorescent beads. Tissue edema and gliosis were visible in most cases at and around the IA injection center, although some control injections also resulted in focal gliosis. A representation of the RTN region in a control nonlesioned side of the medulla is shown in Fig. 4A. Note, ventral to the facial nucleus, the presence of numerous fusiform-shaped neurons that appear similar in morphology to those described in Ellenberger and Feldman (9) as RTN neurons. Figure 4B shows the opposite lesioned side from this same animal. No such RTN neurons are visible in the lesioned area, and it appears to be swollen. For the seven IA lesion rats, the mean number of such neurons in the RTN region was 54 ± 7 (SE) in the control nonlesioned side and 35 ± 5 in the lesioned side, respectively (P < 0.01, Student’s paired t-test).

The mean (±SE) absolute values for minute ventilation breathing room air and 7% CO₂ for the mCSF control and IA lesion groups are shown on Fig. 5. The results are plotted vs. the day, on average, that the measurements were obtained. After the final measurement day in the awake condition, the results obtained under general anesthesia are shown on the same plots. Presurgery mean values in the IA injection and mCSF control injection groups were, respectively, for room air ventilation (n = 28 and 26, respectively), 268 ± 7 and 253 ± 10 (SE) ml/min; for room air VT, 2.65 ± 0.01 and 2.47 ± 0.10 ml; and for room air frequency, 102 ± 3 and 103 ± 3 breaths/min. For 7% CO₂ exposure, ventilation presurgery in the two groups was 616 ± 21 and 570 ± 26 ml/min, VT was 3.87 ± 0.08 and 3.80 ± 0.13 ml, and frequency was 159 ± 3 and 149 ± 3 breaths/min, respectively. For presurgery hypoxic stimulation, ventilation was 419 ± 17 and 468 ± 25 ml/min, VT was 3.16 ± 0.05 and 3.19 ± 0.10 ml, and frequency was 132 ± 5 and 145 ± 5 breaths/min, respectively.

There was no significant effect of IA lesions in the RTN region on room air ventilation (one-way analysis of variance (ANOVA) of IA group alone; two-way ANOVA of IA and control groups evaluating treatment and
Fig. 4. Montage of computer-derived images of region ventral to facial nucleus, at −100 µm caudal to its most rostral pole, for control (nonlesioned side; A) and lesioned side (B) of same animal. Note presence in A of small fusiform-shaped neurons (arrows) lying ventral to large distinctive facial neurons, i.e., in retrotrapezoid nucleus (RTN) region. Pyramidal tract lies just to right of area shown. In B, these neurons are absent, and region ventral to facial nucleus is swollen. Pyramidal tract lies just to left of area shown. Center of fluorescent bead distribution for this IA injection was 100 µm rostral, and it lay at lateral aspect of RTN region (see Fig. 1A). Portion of pipette tract is in right middle aspect of B. Bar, 100 µm.
time. Mean Vt and frequency during room air breathing also did not differ between the two groups (data not shown). After anesthesia, minute ventilation breathing room air was lower in the IA lesion group, but this difference was not significant (unpaired t-test).

The mean (±SE) absolute values for minute ventilation breathing 7% CO₂ in the IA and control groups are also shown on Fig. 5. In the control group, ventilation on 7% CO₂ increased significantly during the time course of the experiment (P < 0.01; one-way ANOVA; significant differences at 11.5, 15.5, and 19.5 days, respectively; post hoc analysis at each time), whereas in the IA RTN lesion group, ventilation on 7% CO₂ decreased significantly (P < 0.02; one-way ANOVA; significant differences at all measurement times; post hoc analysis). A two-way ANOVA comparing lesion vs. controls with time showed a significant difference (P < 0.01) that was present from day 8 on (post hoc analysis). Mean absolute Vt (data not shown) was also significantly decreased (P < 0.001; two-way ANOVA) at measurement day 11.5 on (post hoc analysis). Mean absolute frequency (data not shown) was decreased (P < 0.04; two-way ANOVA), but this difference was significant only at measurement day 11.5 (post hoc analysis). When animals were under anesthesia, minute ventilation, Vt, and frequency were lower in the IA-treated group, but the differences were not significant (unpaired t-test).

The mean changes in minute ventilation breathing 7% CO₂ vs. room air, calculated for each animal, are shown for the IA lesion and control groups in Fig. 6, and those for Vt and frequency are shown in Fig. 7. The change in ventilation was significantly less in the treated group (P < 0.001; two-way ANOVA) from measurement day 5 on (post hoc analysis). At day 19.5, the average change in ventilation was 39% smaller in the treated than in the control group. After anesthesia, the change in minute ventilation was less in the treated group by 52%, although it did not reach significance (unpaired t-test). This decrease in CO₂ sensitivity after RTN lesions was mostly the result of an effect on Vt (Fig. 7). The change in Vt in the lesion group was significantly less in the RTN lesion group (P < 0.001; two-way ANOVA) from measurement day 5 on (post hoc analysis). At day 19.5, the change in Vt in the lesion group was 38% of that in the control group. After anesthesia, the mean change in Vt was less in the lesion group by 46% (P = 0.06; unpaired t-test). There was a significantly smaller change in frequency in the lesion group (P < 0.04; two-way ANOVA), but the effect was small and significant only at the day 19.5 measurement period (P < 0.02; post hoc analysis). After anesthesia, there was no significant difference in the change in frequency between the two groups (two-way ANOVA).

The change in minute ventilation, Vt, and frequency in response to 10% O₂ breathing did not differ between the lesion and the control injection group while animals were awake or under anesthesia (Fig. 8).

DISCUSSION

Methods. The animals that form the basis of the data reported in this paper were all in good health during the entire experimental period. In preliminary experiments, mortality during RTN lesion placement was high presumably because of the acute effects of RTN lesions in a deeply anesthetized animal. We tried numerous measures to improve survival but found that two seemed of primary importance: 1) placing the lesion in as timely a manner as possible so that...
recovery occurred as the effects of the anesthetic agents were wearing off and 2) exposing the animal to 100% O₂ during the recovery period. It was also difficult to reliably place the pipette tip into the small RTN region. The addition of the technique, described by Brown and Guyenet (4), that allows measurement of facial nucleus field potentials produced by stimulation of the facial nerve added greatly to our success rate. Some variability remains in injection size and location. Figures 1–3 show the locations, for all injections reported here, of the cross section with the largest bead area and the precise size of that area. The injection sites for the IA and mCSF injection groups are in the RTN region, as defined in the rat by Pearce et al. (31). The four IA injections that had no effects on breathing (Fig. 3) are in the vicinity of the RTN region.

The injection volumes, measured by geometric analysis of fluorescent bead distribution, were much smaller than we anticipated on the basis of the injection parameters used. This difference is most likely due to loss of injection volume and fluorescent beads into the subarachnoid space when the pipette tip emerged beyond the ventral surface before or during the injection or to blockage of the pipette tip by tissue or blood. The group that, by chance, had the larger injection volumes on average is the control group. Thus the observed effects in the IA injection group cannot be explained by larger injection volumes of this group relative to controls. In past experiments, with pipette tips placed within a few hundred micrometers of the ventral surface by direct observation via a ventral approach, we found excellent agreement between the injection volume expected by use of in vitro calibrated injection parameters and that observed by using analysis of fluorescent bead distribution (3, 26). This suggests that the lack of such agreement in the present study is, in part, due to placing long pipettes into the ventral medulla from a dorsal approach. Such a long pipette insertion makes it more likely that the tip may become partially obstructed by tissue or blood clogging it. The RTN location near the surface of the VLM and quite distant from the dorsal pipette insertion site increases the likelihood that the tip might protrude through the ventral surface and break during the search for the RTN.

The fluorescent bead distribution at the injection sites shows that the medullary injections spread preferentially in the rostral-to-caudal direction. In prior work with 10-nl injections, the mean rostral-to-caudal injection length was 666 µm; the diameter at the largest cross section was 270 µm (26). In this study, the mean injection length and the diameter of the largest injection cross section were, for the IA group, 1,500 and 440 µm; for the mCSF control group, 1,000 and 930 µm; and for the non-RTN IA group, 800 and 554 µm, respectively. This injection shape with preferential spread in the rostral-to-caudal plane may reflect the architecture of the RVLM.

The use of the neurotoxin IA deserves some comment. Kainic acid, a similar excitatory amino acid toxin, can result in lesions at sites distant from the injection (18). These “distant” lesions develop over days to weeks, and such an effect could interfere with the...
chronic study design of these experiments. IA was chosen because it has been reported that it does not result in such distant lesions (11, 12). Like kainic acid, it destroys neuronal soma, preferentially sparing axons.

We had hoped, by using perfusion fixation of the brain and examination of every other 50-µm thick section, to measure the region of tissue damage by using gliosis and absent neurons as criteria. This proved to be difficult. At and around most IA injection sites identified by the presence of fluorescent beads, there was evidence of edema and gliosis, but control mCSF injections also resulted in gliosis, making this criterion nonspecific. It was clear that the region of tissue disruption was more extensive than the region of visible fluorescent beads. Ideally, we would be able to identify RTN respiratory neurons by some anatomic criterion and then evaluate their numbers. At present, we cannot do this. But, in this study, we did identify RTN neurons with the shape of those described by Ellenberger and Feldman (9). Using this simple and relatively nonspecific criterion, we did show that the numbers of this type of neuron in the region ventral to the facial nucleus were diminished in the lesioned side. These data indicate loss of these cell types. It is also possible that the region of neuronal disruption extends into other adjacent neuroanatomically designated groups like the parapyramidal cells, the nucleus paragigantocellularis lateralis, and the subretrofacial and retrofacial nuclei. In the future, other anatomic techniques will be needed to prove whether cells in these groups have been affected. It is likely that they have, and it will prove to be a challenge to attribute the functional effects to the appropriate neurons.

The major measured variable in this study was ventilation, and the technique used was that of whole body plethysmography (2). The advantage of this approach is the totally unencumbered nature of the measurements. Others have had prior experience with this technique (27), and we are aware as well of its difficulties and pitfalls (16). Our presurgery control data for the IA and mCSF groups agree with past published values for the awake rat breathing room air, 7% CO₂, and 10% O₂ (2, 16). In room air breathing, presurgery control measurements in the mCSF and IA injection groups for VT values, expressed as milliliters per kilogram body weight, were 6.79 and 7.93, respectively, similar to the values published by others of 6.87 (16) and 6.67 (2). Our frequency data were 103 and 102 min compared with 103 for Lai et al. (16) and 102 with the terminology of Smith et al. (34), who identified, by means of retrograde anatomic tracing, neurons in this region that projected to the ventral and dorsal respiratory groups. In the rat, this RTN region is defined as extending from the rostral aspect of the nucleus ambiguus to the rostral border of the facial nucleus, lying within a few hundred micrometers of the VLM surface, and bounded laterally by the spinotrigeminal tract and medially by the pyramidal tract (31). Lesions in other nearby regions, e.g., the subretrofacial (1) and retrofacial region (22) and a specific portion of the ventral respiratory group (19), have also been associated with apnea in anesthetized animal preparations. These studies led to the idea that an important tonic input or drive to other respiratory neurons originated within the RVLM, including the RTN region. Elimination of this input could result in apnea. However, these preparations were made with animals under general anesthesia, a known depressant of breathing, and they often were subject to vagotomy and to denervation or ablation of the carotid body, a source of tonic stimulation to respiratory neurons.

As an initial step in evaluating possible roles of anesthesia and carotid body input to the observed effects of RTN lesions in anesthetized animals, some have studied the effect of RTN lesions in a decerebrate cat before and after carotid ablation (25). In this preparation, in the absence of any depressant effect of general anesthesia, unilateral RTN lesions decreased the amplitude of the integrated phrenic nerve activity (PNA), but the effect developed more slowly and was less pronounced, in general, than in the anesthetized cat. Carotid ablation near the end of the experiment further decreased PNA. These observations suggested that the role of RTN neurons in the control of breathing depended on the state of arousal of the animal and that the RTN provided a tonic input similar to that coming from the carotid body.

Two other investigators have examined effects of VLM cooling and lesions on animals in a fully awake experimental state. In cats, Schlafke et al. (33) produced bilateral intermediate area coagulation and found that resting room air breathing was markedly diminished. These cats also demonstrated very little re-
response to increased CO₂ after coagulation. The extent of damaged tissue in the medulla of these cats is uncertain.

More recent studies by Forster et al. (13) and colleagues (28, 29) have examined the effects of reversible VLM cooling in an unanesthetized goat. The cooling probes used were large, covering –4 × 4 mm for each probe on each side of the VLM surface, but the goat medulla is also large compared with that of the rat and the cat. Preliminary experiments had shown the depth of cool tissue to be 1–2 mm from the VLM surface during the time period used for observation. The results differed from those of Schlälke et al. (33). VLM surface cooling at the caudal portion of the rostral (Mitchell’s) area and the rostral portion of Schlälke’s area had the most dramatic effects (13, 28). When the goats were awake, cooling decreased ventilation at rest by ~50%, with no apnea observed. With animals under anesthesia, the decrease in ventilatory output was much more dramatic, with frequent apneas in each animal tested. In the chronic goat model, the effects of cooling the VLM surface depended on whether the goat was awake or anesthetized. We have no clear explanation as to why the coagulation of the intermediate area in the cat produced more dramatic effects than did cooling of the VLM surface in the chronic goat model, although axons of passage will have been destroyed by the coagulation. The coagulation probes were 1.3 mm in diameter and were placed on the ventral medullary surface. Also, the blood supply to the medulla originates at the ventral surface, and it seems possible that coagulation might have effected blood flow to larger regions of the medulla than were affected by the cooling.

We wanted to produce, with an animal under anesthesia, a unilateral lesion limited to the region of the RTN and then allow the animal to recover while we made observations of breathing in the animal in the unanesthetized state during the following 3 wk. In prior studies in the anesthetized animal, unilateral injections of kainic acid of 10-nl volume and 5 mM concentration were sufficient to result in large reductions in PNA and in the response to CO₂ (24, 25). Preliminary studies showed no effect of such injected volumes and concentrations when the animal was studied while awake. By trial and error we found that, with the use of IA, we needed injection volumes >10 nl and 50 mM concentrations to observe any effect.

We were able to make such injections in the RTN region successfully. The resultant effects on breathing room air while at rest were unexpected. There was no evidence of apnea, and there was no significant effect on minute ventilation in the lesion group. While there might have been effects on metabolic rate or on dead space ventilation that confounded the evaluation of resting eupneic ventilatory output solely by the measure of minute ventilation, the absence of any effect was consistent and surprising. We had previously suggested that the RTN is a source of tonic stimulatory input to respiratory neurons on the basis of the decreases in resting baseline respiratory output observed in anesthetized or decerebrate animals after unilateral RTN lesions. Forster et al. (13) found in unanesthetized goats a similar effect after cooling VLM surface areas and also concluded that the RTN, and adjacent regions, provide a tonic drive. The discrete unilateral RTN lesions in this study show no such effect in the unanesthetized rat. Our experiment differs from those of Schlälke et al. (33), Forster et al. (13), and their colleagues (28, 29) in that our lesions were circumscribed and unilateral and likely affected a region much smaller than that affected by the bilateral cooling probes or by the bilateral coagulation (13, 28, 29, 33). It appears that it will be important to quantify reliably the size of these regions of neuronal disruption.

Effects on response to 7% CO₂. As discussed above, cooling of the VLM surface and lesions of the RTN in the anesthetized animal result in essentially absent responses to CO₂. Schlälke et al. (33) described marked reductions in CO₂ sensitivity measured in the unanesthetized cat after bilateral intermediate area coagulation. Forster et al. (13) also found strong effects on the CO₂ response with VLM surface cooling at the intermediate and rostral areas in unanesthetized goats. After the initial 10 s of the cooling response, there was a greater effect of VLM cooling on hypercapnic and eucapnic breathing than on breathing in exercise or hypoxia, suggesting that the regions cooled contained central chemoreceptors or were involved in processing or modulating central chemoreceptor information. In fact, the greatest quantitative effect of this VLM surface cooling in the unanesthetized goat was on the hypercapnic response.

In the present study, with more circumscribed and unilateral RTN lesions, the effect on the response to 7% CO₂ was the only significant finding. On average, this response was decreased by 39% as measured during the final (third) week of the protocol. The fact that both of these other studies (13, 33), conducted in unanesthetized animals but with larger medullary regions of neuronal disruption, and our study, with a relatively discrete region of neuronal disruption, describe significant effects on the response to increased CO₂ suggests an important role of the neurons within the RTN region of the RVLM on the central chemoreceptor response.

Recent evidence has supported the idea that central chemoreceptors have a widespread distribution within the brain stem (3, 7, 23). Using 1-nl injections of the carbonic anhydrase inhibitor acetazolamide, we have been able to produce focal medullary regions of acidosis that are associated with stimulation of PNA (7). The region of acidosis is within 300–400 μm of the center of the acetazolamide injection. This provides us with a probe to look for locations of central chemoreception. To date, responsive locations include the region of the locus ceruleus (7); the dorsal respiratory group (7); the ventral respiratory group (23); the midline caudal raphe (3); and just beneath the VLM surface at the rostral, intermediate, and caudal topographical chemosensitive areas, including the RTN region (7). In the anesthetized animal, RTN lesions can almost abolish central chemosensitivity; in the unanesthetized rat,
RTN lesions produce only a 39% decrease in the response to hypercapnia. It may be that anesthesia diminishes central chemoreception at non-RTN sites preferentially, explaining the large reduction in central chemosensitivity in the anesthetized animal with RTN lesions. In the unanesthetized animal, destruction of the RTN removes one source of central chemosensitivity, but the remaining widespread central chemosensitive sites are still operative.

An alternative explanation involves the interaction of multiple afferent inputs from vagal and carotid body sources that may be necessary for normal central chemosensitivity. In the anesthetized animal experiments, the vagus nerves are cut and high inspired O2 fractions are used that will minimize carotid body afferent input. In this situation, RTN neuronal disruption could be more effective in both decreasing resting baseline ventilatory output and in decreasing the ventilatory response to hypercapnia. In support of this possibility are observations in the unanesthetized goat that show greater decreases in ventilation with VLM cooling after denervation of the carotid bodies. Also, in decerebrate animals with RTN lesions, subsequent peripheral chemodenervation further decreased phrenic activity, suggesting an additive effect of peripheral chemodenervation and RTN lesions (25).

Effects on response to 10% O2. Cooling of relatively large areas of the VLM surface also decreased the ventilatory response to hypoxia in the unanesthetized goat (13), at least over the initial 10 s of the cooling. This was attributed to the loss of some non-chemoreceptor-mediated tonic facilitation, which then affected ventilatory responses to hypercapnia, hypoxia, and exercise. In our rat experiments, we observed no effect of unilateral RTN lesions on the response to 10% O2. Again, the smaller region of neuronal disruption in our experiment may account for the lack of an effect like that reported in the unanesthetized goat.

RTN and human arcuate nucleus homology? A region of the rostral VLM has recently been identified as being abnormal in some victims of sudden infant death syndrome (10, 15). Hypoplasia of the arcuate nucleus and decreased muscarinic receptor binding have been reported in a subset of victims of sudden infant death syndrome. The precise anatomic homology between human arcuate neurons and the RTN is unknown, but it appears possible that these two groups of neurons serve similar functions. The effects in the unanesthetized rat of bilateral RTN lesions and of interactions of the effects of such lesions with arousal state and with the degree of various afferent input appear to be worthy of further study, especially given the possible homology between RTN and the human arcuate nucleus.

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