CO₂ dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness

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Nattie, Eugene E., and Aihua Li. CO₂ dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness. J Appl Physiol 92: 2119–2130, 2002.—To evaluate the function of widely distributed chemoreceptors during sleep and wakefulness in the rat, we focally stimulate single chemoreceptor sites during naturally occurring sleep-wake cycles by microdialysis of artificial cerebrospinal fluid equilibrated with 25% CO₂. In retrotrapezoid nucleus, this increased ventilation (tidal volume) by 24% only in wakefulness (Li A, Randall M, and Nattie E. J Appl Physiol 87: 910–919, 1999). In caudal medullary raphe, it increased ventilation (frequency) by 15–20% only in sleep (Nattie EE and Li A. J Appl Physiol 90: 1247–1257, 2001). Here, in nucleus tractus solitarius (NTS), focal acidification significantly increased ventilation by 11% in sleep and 7% in wakefulness rostrally (n = 5) and by 16% in sleep and 28% in wakefulness caudally (n = 5). The sleep-wake cycle was unaltered. Dialysis with 5% CO₂ had no effect. Dialysis with 50% CO₂ caudally did not further stimulate ventilation but did disrupt sleep. Central chemoreceptors in the NTS affect breathing in both sleep and wakefulness. The threshold for arousal in caudal NTS is greater than that for the stimulation of breathing.

central chemoreception; arousal; carbon dioxide response; medulla; control of breathing

HYPERCAPNIA AND ACIDOSIS WITHIN the brain stimulate breathing via central chemoreceptors (7, 19, 20, 24–28). These chemoreceptors are located just beneath the ventral medullary surface in the retrotrapezoid nucleus (RTN) and contiguous areas (7, 19, 20, 24–28) and at other sites widely distributed within the brain stem (7, 24–28). These include the regions of the nucleus tractus solitarius (NTS), the locus ceruleus, the midline raphe, the ventral respiratory group, and the fastigial nucleus of the cerebellum. Why are there so many central chemoreceptor sites? As one explanation, we hypothesize that sites differ in their response and physiological role, depending on the state of arousal.

In this paper, we focus on chemoreception in the region of the NTS. The NTS is that part of the respiratory control network commonly labeled the “dorsal respiratory group” (3, 11, 13, 38). It is also involved in many cardiopulmonary reflexes (3, 5, 6, 12, 14, 16, 21, 22, 30–32, 34, 35). Destruction of the NTS in anesthetized cats reduces the respiratory response to systemic hypercapnia, an effect that largely disappears with recovery to consciousness (2). This result suggests that NTS neurons are important in chemosensitivity, at least under anesthesia. Neurons of the NTS studied in vitro exhibit CO₂-dependent changes in membrane potential and firing rate (9, 10), suggesting that NTS neurons can be chemosensitive. With systemic hypercapnia, the expression of the early gene c-fos is increased in the NTS region (17, 36), providing support for the view that the NTS is a site for, or is involved in, central chemoreception. Finally, focal acidification of the NTS region by microinjection of acetazolamide in anesthetized, vagotomized cats and rats increases the amplitude of the integrated phrenic nerve signal (7), indicating that the NTS is a chemoreceptor site that can, by itself, affect breathing.

In this paper, we evaluate the effect of focal acidification of the NTS region on ventilation (VE) during sleep and wakefulness. We use a microdialysis probe (19, 28) to produce a focal acidosis in unanesthetized, unrestrained rats. The probe has a tip with a semipermeable membrane (pores <6,000 Da) of 1-mm length and 240-μm diameter. In prior, similar studies by our laboratory (19, 28), CO₂ was dialyzed into the RTN or medullary raphe of unanesthetized rats. Focal acidification of the RTN increased VE by an effect on tidal volume (VT) in the awake state only; focal acidification of the medullary raphe increased VE by an effect on frequency (f) in sleep only. In this study, we divide the NTS into rostral (rNTS) and caudal (cNTS) portions, showing that focal acidification has a greater effect in cNTS and that, at both NTS loci, VE is stimulated in sleep and wakefulness, with the effect being greater in wakefulness.

METHODS

General Preparation

Animal groups. There are three groups of animals in this report based on the anatomic location of the guide tubes: rNTS (n = 5), cNTS (n = 5), and “wrong place” (n = 4). In addition, there were five rats with indeterminate probe loca-

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tions or with probes mistakenly located within other known chemoreceptor regions. Among the rats with probes located within the general confines of the NTS, the division into rNTS and cNTS was based on whether the probe tip was rostral or caudal to the most rostral aspect of the area postrema. This landmark is easily defined and demarcates the NTS into approximately two halves, as can be seen in Fig. 3.15 in Blessing (4). It is known that the sites of afferent synapses from cardiopulmonary receptors are predominantly in the cNTS (4–6, 12, 14, 21, 22). Four rats with guide tubes located outside the NTS provided a control of focal acidification outside the region of interest. All animals were also treated with 5% CO2 dialysis as an additional control for dialysis without acidification in the region of interest.

Surgery. Nineteen male Harlan Sprague-Dawley rats (300–450 g) were anesthetized with ketamine (100 mg/kg im) and xylazine (20 mg/kg ip). The skull was shaved, and the skin was sterilized with betadine and alcohol. The head was placed into a Kopf stereotaxic holder, and a dialysis guide cannula (0.38 mm OD) with a dummy was implanted into the medulla. The coordinates for probe placement in rNTS were 12.5 mm caudal and 1.0 mm lateral from bregma and 8.0 mm from the medulla. The coordinates for probe placement in rNTS were placed in the abdominal cavity. The animal was allowed to recover before the experiment.

A temperature probe (TA-F20, Data Sciences, St. Paul, MN) was placed in the neck muscles. The pair of wire electrodes was inserted deep into the neck muscles. The frontal electrode 2 mm anterior to the bregma and 2 mm lateral to the midline, the parietal electrode 2 mm anterior to lambda and 2 mm lateral to the midline, and the ground placed between the two. For the electromyogram (EMG), a pair of wire electrodes was inserted deep into the neck muscles. The skull wound was sutured. A sterile temperature-pressure probe (TA-P20, Data Sciences, St. Paul, MN) was placed in the abdominal cavity. The animal was allowed to recover for 3–4 days.

CO2 dialysis solution. The artificial cerebrospinal fluid (aCSF) was equilibrated with 5, 25, or 50% CO2. 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 CO2. The pH of each solution was monitored to ensure that the equilibration was reliable. The dialysis pump was run at a speed of 45 μl/min.

V̇E measurement. The plethysmograph is like those described by Jacky (15) and Pappenheimer (29). The output of the pressure transducer was digitized and sampled at 150 Hz by computer (DataPac 2000 system). The chamber operates at atmospheric pressure with the in and out flow of gas balanced to prevent hyper- or hypobaric conditions. The inflow gas was humidified, and the flow rate was controlled by a flowmeter at 1.4 l/min to prevent rebreathing of exhaled gas (model 7491T, Matheson). The outflow port was connected to the house vacuum system via a flowmeter. A high-resistance “bleed” of the outflow line provided ~100 ml/min of outflow gas to the O2 and CO2 analyzers (Applied Electrochemistry). The plethysmograph was calibrated with 0.3-ml injections.

Oxygen consumption and temperature. Oxygen consumption (V̇O2) was measured by using the Fick principle by calculating the difference in O2 content between inspired and expired gas. V̇O2 = (V̇in × FO2) – (V̇out × FO2), where V̇in is inflow, V̇out is outflow, and FO2 is inspired O2 fraction, and is normalized to ml·g·body wt·h⁻¹. The inflow O2 content was measured at the beginning of each experiment, and the outflow content of O2 was read from the O2 and CO2 sensors constantly during the experiment. A thermometer inside the chamber measured the chamber temperature. Rat body temperature was measured via telemetry from the temperature probe in the peritoneal cavity.

EEG and EMG signals. The signals from the EEG and EMG electrodes were sampled at 150 Hz, filtered at 0.3–50 and 0.1–100 Hz, respectively, and recorded directly on the computer.

Anatomic analysis. At the end of the experiment, the rats were killed, and the medulla was quickly removed, frozen, and then sectioned at 50-μm thickness with a Reichert-Jung cryostat. The sections were counterstained with cresyl violet. We identified anatomic landmarks and the site of dialysis probe placement by using a rat brain atlas (33) for reference. The necessary manipulation of the guide tubes during removal of the brain stem produced tissue disruption in excess of that attributable to simple insertion.

Data analysis. For sleep analysis, we used the EEG and EMG signals; their fast Fourier transform (FFT) analyzed in 3.6-s epochs with delta (0.3–5 Hz), theta (6–9 Hz), and sigma (10–15 Hz) frequency bands, and behavioral observations.

The rats were housed in a room with a light, rest period from 12 AM to 12 PM and a dark, active period from 12 PM to 12 AM. All of the experiments were performed from 9 AM to 4 PM. The state of arousal was defined by using criteria modified from those of Bennington et al. (1) and Trachsel et al. (37). In the awake state, the EEG showed a low-amplitude signal, delta power was low, the ratio of theta to delta power was low, EMG activity was present, and the product of theta and sigma power was low. In non-rapid eye movement (NREM) sleep, the EEG showed a high-amplitude signal, delta power was high, the ratio of theta to delta power was low, the EMG activity was absent or low, and the product of theta and sigma power was moderate to high. In rapid eye movement (REM) sleep, the EEG signal showed low amplitude, delta power was low, the ratio of theta to delta power was high, the EMG activity was absent or low, and the product of theta and sigma power was moderate or high. On occasion, we had to judge the state as indeterminate. Data from indeterminate states are not included.

Our wake state is one of quiet wakefulness, as in active wakefulness the activity of the rat in the plethysmograph prevents reliable measurement of breathing. We applied this analysis to each experiment, as shown in Fig. 1. We determined NREM, REM, and wake periods visually from such records.

For ventilatory measurements, a breath-by-breath analysis was performed with the DataPac system with the pressure deflections and the respiratory cycle time for each breath being determined for 100–300 breaths at defined sleep and wake periods during the experiment. Sighs, sniffing, and recording artifacts were edited from analysis. These data were exported to Sigmaplot 4.0 (Jandel Scientific software), and V̇E per 100 g body wt, f, and V̇E per 100 g body wt were calculated for each breath by using plethysmograph and body temperature for that time period. In our analysis, we were able to obtain two to four defined periods for NREM sleep and wakefulness as a baseline before CO2 exposure. During the 30 min of test dialysis, we obtained data representative of NREM sleep and wakefulness from each animal included in this analysis. We also obtained data in the 20-min recovery period with continued dialysis by using aCSF equilibrated with 5% CO2. REM periods occurred more variably among the animals. REM sleep and ventilatory data are fragmentary and are presented only briefly.

We examined ventilatory data for wakefulness and NREM sleep in all experiments. These data are shown as the averaged absolute values for V̇E, V̇T, and f in the baseline period.
and those obtained as the maximum response to dialysis with 1) 5% CO2-equilibrated aCSF, 2) 25% CO2-equilibrated aCSF, or 3) 50% CO2-equilibrated aCSF. In cases in which data were obtained for a given experimental condition on more than 1 day, these data were averaged so that each rat contributed but one value for each variable in each condition. These absolute values were evaluated statistically by a paired t-test or one-way repeated-measures ANOVA. For example, in rNTS, we compare baseline Ve averaged from two to four measurement periods to the maximum Ve observed during the test dialysis period in which either 5 or 25% CO2 was equilibrated in the dialysate.

We also show the data as the maximum percent change in each state, comparing the maximum value during the 30-min test dialysis period with the mean of the baseline control values in that state. Use of the percent change allows normalization and statistical comparison of responses occurring among different days. For example, in rNTS, we compare the percent change in Ve produced by dialysis of 25% CO2-equilibrated aCSF during the 30-min test period on 1 day to that with dialysis of 5% CO2-equilibrated aCSF during the 30-min test period on another day. This comparison is done with a repeated-measures ANOVA with post hoc tests performed when significant differences were found.

The results for V02 and body temperature during 25 or 5% CO2 tests were compared by ANOVA.

Experimental protocol. We dialyzed each rat with 5 and 25% CO2, with cNTS rats also being treated with 50% CO2, by using both morning and afternoon measurement periods. The rat was judged to be awake or asleep by the criteria...
were calculated for 100–V

NREM sleep and wakefulness. In this example, f and
dialysis with aCSF equilibrated with 25% CO2. Dialy-
baseline condition and during and after 30-min test
and NREM sleep (open circles) periods chosen in the
CO2 or maintained at 5% CO2, and measurements were
40 min. The dialysis solution was then changed to 25 or 50%
matization, baseline measurements were made over the next
were performed with the rat breathing room air. After accli-
acclimate. Dialysis with 5% CO2-equilibrated aCSF began
when the rat was placed into the chamber and continued
through the entire experimental period. All experiments
were with the rat breathing room air. After acclimat-
ization, baseline measurements were made over the next
40 min. The dialysis solution was then changed to 25 or 50%
CO2 or maintained at 5% CO2, and measurements were
taken over the 30-min test period. The dialysis tube and
cannula dead space is taken into account along with the
exchange membrane. After 30 min, the dialysis solution was
changed to 5% CO2 or maintained at 5% CO2, and measure-
ments were again made at 10–20 min.

RESULTS

Typical Experiment

Figure 1 shows, for a typical experiment, the EEG
and EMG signals, the FFT-derived parameters, and
calculated VT, f, and V\(E\) for wakefulness (solid circles)
and NREM sleep (open circles) periods chosen in the
baseline condition and during and after 30-min test
dialysis with aCSF equilibrated with 25% CO2. Dialy-
sis with aCSF equilibrated with 5% CO2 occurred from
beginning to end except for the 30-min test period
marked at the bottom. The power spectrum records
show nicely the typical sleep cycling of the rat with
periods of high-delta power (NREM sleep) interspersed
with periods of REM and wakefulness. V\(E\), VT, and f
were calculated for 100–300 breaths in periods of
NREM sleep and wakefulness. In this example, f and
V\(E\) were increased during 25% CO2 dialysis in the NTS
in both wakefulness and NREM sleep, with VT increasing
only in the awake state. V\(E\), VT, and f then returned
to baseline levels after the 25% CO2 dialysis period.
The location of the dialysis probe in this example was
in the cNTS.

Table 1. Percentage of time in NREM sleep or in
wakefulness during the entire experimental period
in the rNTS and cNTS groups

<table>
<thead>
<tr>
<th>CO(2) Dialysis</th>
<th>rNTS (n = 5)</th>
<th>cNTS (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NREM</td>
<td>AW</td>
</tr>
<tr>
<td>5%</td>
<td>53 ± 4</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>25%</td>
<td>50 ± 3</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>50%</td>
<td>43 ± 5</td>
<td>54 ± 6</td>
</tr>
</tbody>
</table>

Values are mean ± SE in %; n, no. of animals. Data are paired for
sleep or wakefulness. rNTS and cNTS, rostral and caudal nucleus
tractus solitarius, respectively; NREM, non-rapid eye movement;
AW, awake.

outlined above. We analyzed all awake and NREM data in
each experiment, regardless of the amount of time the rat
was in each state. This allowed a paired comparison of effects
but assumed that any sleep-wake effect would be present,
regardless of the amount of time spent in sleep or the depth
of sleep.

At the start of an experiment, the rat was gently held
while the dummy cannula was removed and the dialysis
probe inserted into the guide tube. Then the rat was placed
into the plethysmograph chamber and allowed 30–40 min to
acclimate. Dialysis with 5% CO2-equilibrated aCSF began
when the rat was placed into the chamber and continued
through the entire experimental period. All experiments
were performed with the rat breathing room air. After accli-
matization, baseline measurements were made over the next
40 min. The dialysis solution was then changed to 25 or 50%
CO2 or maintained at 5% CO2, and measurements were
taken over the 30-min test period. The dialysis tube and
cannula dead space is taken into account along with the
exchange membrane. After 30 min, the dialysis solution was
changed to 5% CO2 or maintained at 5% CO2, and measure-
ments were again made at 10–20 min.

Figure 1 shows, for a typical experiment, the EEG
and EMG signals, the FFT-derived parameters, and
calculated VT, f, and V\(E\) for wakefulness (solid circles)
and NREM sleep (open circles) periods chosen in the
baseline condition and during and after 30-min test
dialysis with aCSF equilibrated with 25% CO2. Dialy-
sis with aCSF equilibrated with 5% CO2 occurred from
beginning to end except for the 30-min test period
marked at the bottom. The power spectrum records
show nicely the typical sleep cycling of the rat with
periods of high-delta power (NREM sleep) interspersed
with periods of REM and wakefulness. V\(E\), VT, and f
were calculated for 100–300 breaths in periods of
NREM sleep and wakefulness. In this example, f and
V\(E\) were increased during 25% CO2 dialysis in the NTS
in both wakefulness and NREM sleep, with VT increasing
only in the awake state. V\(E\), VT, and f then returned
to baseline levels after the 25% CO2 dialysis period.
The location of the dialysis probe in this example was
in the cNTS.

Table 1 shows the percentage of time spent in
NREM or awake states during the 30-min test dialy-
sis period in the rNTS and cNTS groups. There was no
significant difference in rNTS when each state was
compared in 5 vs. 25% dialysis (paired t-test). Dialysis
with 25% CO2, compared with 5% CO2 control, did not
affect sleep-wake periods in rNTS. This remained true
cNTS. The amount of time during the 30-min test
period in NREM sleep or in wakefulness did not differ
significantly between 25 and 5% control dialysis. How-
over, in cNTS during the 30-min dialysis with 50%
CO2, the amount of time in NREM sleep decreased
significantly, and the amount of time in wakefulness
increased significantly compared with the 5% dialysis
(P < 0.05; one-way repeated-measures ANOVA with

Sleep

Table 1 shows the percentage of time in NREM sleep
and wakefulness during the entire protocol for both the
5 and 25% CO2 dialysis experiments in rNTS and for 5,
25, and 50% dialysis experiments in the cNTS. The
average percentage of time spent in each state for the
entire experimental period did not differ significantly
when 5 vs. 25% dialysis in rNTS (paired t-test) or 5, 25,
and 50% dialysis in cNTS (one-way repeated-measures ANOVA) were compared.

Figure 2 shows the average percentage of time spent
in NREM or awake states during the 30-min test dialy-
sis period in the rNTS and cNTS groups. There was no
significant difference in rNTS when each state was
compared in 5 vs. 25% dialysis (paired t-test). Dialysis
with 25% CO2, compared with 5% CO2 control, did not
affect sleep-wake periods in rNTS. However, in cNTS during the 30-min dialysis with 50%
CO2, the amount of time in NREM sleep decreased
significantly, and the amount of time in wakefulness
increased significantly compared with the 5% dialysis
(P < 0.05; see text for details).

Fig. 2. Distribution of sleep and wakefulness during the 30-min test
dialysis period. Data are from rostral nucleus tractus solitarius
(rNTS; n = 5; A) and caudal NTS (cNTS; n = 5; B) groups. Left bars:
NREM sleep; right bars: awake. The percentage of the 30-min period
is shown for 5% CO2 control (open bars) and 25 and 50% CO2 test
(hatched bars). Mean ± SE values are represented. *Significantly
different from control, i.e., the 5% CO2 dialysis data, P < 0.05 (see
text for details).
post hoc Tukey test). Dialysis with 50% CO₂ in the cNTS was likely to awaken the rat. Dialysis with 25% CO₂ was not, although there appeared to be a trend toward less sleep and more wakefulness that did not reach statistical significance.

In terms of the number and duration of NREM periods, in rNTS with 5% CO₂ dialysis, there were 7.3 ± 0.8 (SE) NREM periods per total experiment with a mean duration of 6.5 ± 0.5 min. With 25% CO₂ dialysis, there were 8.9 ± 0.3 NREM periods per total experiment with an average duration of 6.0 ± 0.3 min. During the 30-min test dialysis period with 5% CO₂ dialysis, there were 2.6 ± 0.5 NREM periods with an 8.8 ± 2.7 min average duration, whereas, with 25% CO₂ dialysis period, there were 2.3 ± 0.2 NREM periods with a 6.0 ± 0.6 min average duration. None of these differences between 5 and 25% CO₂ dialysis were statistically. During the 30-min test period with 5% CO₂ dialysis, there were 3.6 ± 0.6 NREM periods with an average duration of 5.2 ± 1.1 min average duration, and, with 50% CO₂ dialysis, there were 1.4 ± 0.5 NREM periods with an average duration of 5.2 ± 2.5 min. There were significantly fewer NREM episodes during the 30-min test period in the 50% CO₂ dialysis group compared with the 5% CO₂ dialysis groups (P < 0.05; one-way repeated-measures ANOVA; Tukey post hoc test). There was no significant effect on the duration of NREM episodes during the 30-min dialysis period, nor did dialysis with 25% CO₂ affect either the number or duration of NREM episodes significantly.

Among those rats that exhibited any REM sleep, in rNTS this accounted for 8–10% of the total experiment and of the 30-min test period. In cNTS, REM accounted for 3–4% of the total experiment and of the 30-min test period. There was no obvious effect of dialysis with CO₂ on the amount of REM sleep in the total experiment or in the 30-min test period.

Ventilatory Responses to 5% CO₂ Dialysis in the NTS

In these control experiments, the aCSF equilibrated with 5% CO₂ was dialyzed for the entire experiment, including the 30-min test period. Table 2 shows mean (±SE) values of Vₑ, Vₜ, and f obtained during the baseline period and during the test period in NREM sleep and wakefulness in rNTS and cNTS. There was no significant effect on these variables in either sleep or wakefulness by dialysis of aCSF equilibrated with 5% CO₂.

Ventilatory Responses to 25% CO₂ Dialysis in the NTS

Figures 3 (rNTS) and 4 (cNTS) show mean (±SE) absolute values of Vₑ, Vₜ, and f obtained during the baseline period (solid bars) and the maximum obtained during the test period (hatched bars). In these experiments, the test period dialysate was aCSF equilibrated with 25 or 50% CO₂, whereas, during the baseline and recovery periods, the dialysate was aCSF equilibrated with 5% CO₂. In the rNTS (Fig. 3) during NREM sleep, maximum Vₑ during the period of NTS focal acidification was significantly greater than baseline (P < 0.02; paired t-test). Vₜ and f were increased as well, but these effects were not significant (P = 0.09; paired t-test). In the rNTS during wakefulness, maximum Vₑ during the period of focal acidification was significant greater than baseline (P < 0.01; paired t-test). Vₜ was also significantly increased (P < 0.03), as was f (P < 0.02).

In the cNTS (Fig. 4) during NREM sleep, maximum Vₑ during the period of focal acidification compared with the previous baseline period was significant greater than baseline for both 25 and 50% CO₂ (P < 0.01; paired t-test comparing 25 or 50% to baseline values for that experiment). Vₜ was significantly increased for both 25 and 50% (P < 0.01; paired t-test), as was f (P < 0.02). In the cNTS during wakefulness, maximum Vₑ during the period of focal acidification was significantly greater than baseline for both 25 and 50% (P < 0.01; paired t-test). Vₜ was significantly increased for both 25 and 50% (P < 0.02; paired t-test).

Table 2. Control data for Vₑ, Vₜ, and in NREM sleep and wakefulness with 5% CO₂ in aCSF during both the baseline and the 30-min test period

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
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<th>Test</th>
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<tbody>
<tr>
<td></td>
<td>Vₑ</td>
<td>Vₜ</td>
<td>f</td>
<td>Vₑ</td>
</tr>
<tr>
<td>rNTS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Awake</td>
<td>82.1 ± 4.1</td>
<td>1.05 ± 0.03</td>
<td>81 ± 5</td>
<td>81.7 ± 4.0</td>
</tr>
<tr>
<td>NREM</td>
<td>75.6 ± 3.7</td>
<td>0.99 ± 0.01</td>
<td>76 ± 4</td>
<td>76.4 ± 3.7</td>
</tr>
<tr>
<td>cNTS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Awake</td>
<td>78.9 ± 4.7</td>
<td>1.03 ± 0.03</td>
<td>81 ± 6</td>
<td>77.2 ± 4.9</td>
</tr>
<tr>
<td>NREM</td>
<td>73.4 ± 4.8</td>
<td>0.98 ± 0.02</td>
<td>76 ± 7</td>
<td>72.9 ± 5.3</td>
</tr>
</tbody>
</table>

Values are means ± SE in ml-100 g⁻¹·min⁻¹ for ventilation (Vₑ), ml/100 g for tidal volume (Vₜ), and breaths/min for frequency (f); n = 5 animals in both groups. Baseline values are shown for 2–4 baseline measurement periods, and maximum value observed in the test period is shown.
The f was significantly increased for 50% \( (P < 0.01; \text{paired } t\text{-test}) \), but the normality test failed in the 25% \( \text{CO}_2 \) group, and use of a Wilcoxon signed-rank test failed to show significance \( (P = 0.06) \). In both cases, rNTS and cNTS, the ventilatory variables, returned to baseline by 20 min after cessation of dialysis with 25 or 50% \( \text{CO}_2 \) (data not shown).

REM periods were present in both baseline and 25% \( \text{CO}_2 \) test period dialysis in four of five rNTS rats and two of five cNTS rats. In the two cNTS cases, there was no increase in \( \dot{V}_E \) with 25% \( \text{CO}_2 \) dialysis compared with baseline. In the four rNTS cases, \( \dot{V}_E \) during 25% \( \text{CO}_2 \) dialysis compared with baseline increased by 7.7, 14.6, 14.7, and 24.7%, respectively.

Comparison of Normalized Responses to 25% \( \text{CO}_2 \) Dialysis in the NTS

Figure 5 compares the percent change in \( \dot{V}_E \) obtained from experiments with 5% \( \text{CO}_2 \) dialysis during the test period (open bars) to the percent change obtained from experiments with 25 or 50% \( \text{CO}_2 \) dialysis during the test period (hatched bars). This normalization allows comparisons among data obtained on different days and gives quantitative estimates of the responses. In rNTS during NREM sleep, the mean maximum increase in \( \dot{V}_E \) with 25% \( \text{CO}_2 \) dialysis is 11.1%, which is significantly greater than that observed with 5% \( \text{CO}_2 \) dialysis \( (P < 0.01; \text{paired } t\text{-test}) \). The mean maximum increase in \( \dot{V}_T \) (data not shown) is 11.6%, which is significantly greater than that observed with 5% \( \text{CO}_2 \) dialysis \( (P < 0.01; \text{paired } t\text{-test}) \), and the mean maximum increase in f (data not shown) is 7.2%, which is not significantly different from the value observed with 5% \( \text{CO}_2 \) dialysis. In rNTS during wakefulness, the mean maximum increase in \( \dot{V}_E \) with 25% \( \text{CO}_2 \) dialysis is 6.9%, which is significantly greater than that ob-

Fig. 3. Absolute values of \( V_T \) (A), f (B), and \( \dot{V}_E \) (C) for rNTS during sleep and wakefulness. Baseline with 5% \( \text{CO}_2 \) dialysis before the 30-min test period (solid bars) and maximum response during the 30-min test period with 25% \( \text{CO}_2 \) dialysis (hatched bars) data are shown as means \( \pm \text{SE} \) \( (n = 5) \). *Significantly different from control, i.e., the 5% \( \text{CO}_2 \) dialysis data (see text for details).

Fig. 4. Absolute values of \( V_T \) (A), f (B), and \( \dot{V}_E \) (C) for cNTS during sleep and wakefulness. Baseline with 5% \( \text{CO}_2 \) dialysis before the 30-min test period (solid bars) and maximum responses during the 30-min test period with 25 or 50% \( \text{CO}_2 \) dialysis (hatched bars) data are shown as means \( \pm \text{SE} \) \( (n = 5) \). *Significantly different from control, i.e., the 5% \( \text{CO}_2 \) dialysis data (see text for details).
In cNTS during wakefulness, the mean maximum increase in \( \dot{V}_E \) is 28.4% with 25% CO\(_2\) dialysis and 23.4% with 50% CO\(_2\) dialysis, which are significantly greater than that observed with 5% CO\(_2\) dialysis (\( P < 0.005\); one-way repeated-measures ANOVA, Tukey post hoc test, \( P < 0.05\)) but not different from each other. The mean maximum increase in \( \dot{V}_T \) (data not shown) is 9.3% for 25% CO\(_2\) dialysis and 16.9% for 50% CO\(_2\) dialysis. These values are significantly greater than that observed with 5% CO\(_2\) dialysis (\( P < 0.03\); Friedman rank test with repeated measures; Dunnett post hoc test, \( P < 0.05\)) but not different from each other. The mean maximum increase in \( f \) is 22.8% with 25% CO\(_2\) dialysis and 12.5% with 50% CO\(_2\) dialysis. These responses are significantly different from that observed with 5% CO\(_2\) dialysis (\( P < 0.05\); one-way repeated-measures ANOVA), which Student-Newman-Keuls post hoc test shows to be present only for comparison of 5 and 25% CO\(_2\) dialysis.

To ask whether the responses to focal dialysis with CO\(_2\) are greater at the cNTS and in wakefulness, we performed a two-way ANOVA with location (rNTS vs. cNTS) and arousal state (sleep vs. awake) as factors, looking only at the response to 25% CO\(_2\) dialysis. For \( \dot{V}_E \), this ANOVA showed a significant effect of location (\( P < 0.01\)) and a significant interactive effect of location and arousal state (\( P = 0.015\)). Post hoc comparison with Bonferroni correction showed that the effect in the cNTS was significantly greater than that in rNTS during wakefulness (\( P < 0.05\)) but not during sleep. For \( \dot{V}_T \), this ANOVA showed only a significant effect of location: cNTS differed from rNTS (\( P < 0.05\)). For \( f \), this ANOVA showed only a significant effect of location: cNTS differed from rNTS (\( P < 0.03\)). We conclude that 25% CO\(_2\) dialysis in cNTS has a greater effect than in rNTS on \( \dot{V}_E \), \( \dot{V}_T \), and \( f \) and that, for \( \dot{V}_E \), this cNTS effect is greater in wakefulness than in sleep.

\( \dot{V}_O_2 \) and Body Temperature

In the rNTS, the initial mean body temperature values for the 5 and 25% CO\(_2\) dialysis experiments were 38.0 ± 0.01 and 38.3 ± 0.2°C, whereas in cNTS they were 37.9 ± 0.02 and 38.4 ± 0.2°C, and these values did not change significantly during the experiment. The temperature data were obtained as 5-min averages, making it difficult to detect sleep-related changes reliably.

In the rNTS, the initial mean \( \dot{V}_O_2 \) values for the 5 and 25% CO\(_2\) dialysis experiments were 1.0 ± 0.01 and 1.0 ± 0.01 ml·g\(^{-1}\)·h\(^{-1}\), whereas, in the cNTS, they were 0.98 ± 0.02 and 1.04 ± 0.02 ml·g\(^{-1}\)·h\(^{-1}\), and these values did not change significantly during the experiment. The time resolution of our ability to measure \( \dot{V}_O_2 \) is limited, as we utilized the Fick principle applied to plethysmograph inflow and outflow, which occurs slowly relative to the changes in sleep and wake state.
Anatomy

In Fig. 6, we show, for each of the 10 rats in the rNTS and cNTS groups, the cross section of the medulla that contains the greatest area of tissue disruption caused by the guide tube tip and dialysis probe. The three sections on the left show the locations in the five rNTS experiments; those on the right show the locations in the five cNTS experiments. The top left and the middle right show actual stained sections of typical results for rNTS and cNTS probe placements, respectively. The schematic sections show the location of the probes in the other rNTS and cNTS rats. In Fig. 7, we show the locations of the guide tube tip and dialysis probe in four rats in which the sites were judged not to be in the NTS region. For three of these cases (top two and bottom left), the probe location was well rostral to the NTS; for one (bottom right), the location was deep into the medulla. These rats had similar baseline and maximum values during the CO₂ dialysis test period in NREM and wakefulness, whether treated with 5 or 25% CO₂ dialysis.

DISCUSSION

Methods

We produced focal acidification within a single chemoreceptor site in an unanesthetized rat by using microdialysis of aCSF equilibrated with CO₂ [see Natie and Li (28) for additional discussion of technique]. A high flow rate through the dialysis probe, 45 µl/min, and a high-CO₂ concentration, 25%, are needed to deliver CO₂ to the tissue in sufficient amounts to produce the breathing response. Lower flow rates at this CO₂ level do not result in stimulated breathing, nor do high-flow rates that use, as a control, aCSF equilibrated with 5% CO₂ affect breathing, metabolic rate, or temperature (Ref. 19; this study).

Initially, the need for such a high concentration of CO₂ may seem surprising. We have measured tissue pH at the tip of the dialysis probe in the RTN of the unanesthetized rat during dialysis at this flow rate with aCSF equilibrated with 25% CO₂. The output of the tissue pH electrode in the RTN region changed by ~4 mV (unpublished observations). Exposure to 7% inspired CO₂ changed the output of the tissue pH electrode at the same site by ~9 mV (unpublished observations). Thus focal dialysis with 25% CO₂ delivered by high flow rates results in tissue acidification that is about one-half that observed with 7% CO₂ inhalation. This constitutes a mild-to-moderate stimulus intensity.

We interpret the need for high-dialysate flow and CO₂ concentration to reflect the ease by which tissue blood flow can remove CO₂ added from this focal source. Tissue pH data obtained during dialysis under anesthesia support this interpretation (19). The pH change at the probe tip during 25% CO₂ dialysis was like that observed with an increase in end-tidal CO₂ to

Fig. 6. Anatomic locations of the tip of the dialysis probe site in the 5 rats in each of the rNTS (left) and cNTS (right) groups. Two sections are digitized images of actual stained sections (top left and middle right) showing the site in 2 rats. The darkened area within the solid rectangle shows the damaged tissue. These 2 are chosen as representative of rNTS and cNTS groups. The other sections are schematic images modified from a rat atlas (33) showing the probe tip location, represented by the shaded ellipses, of the other 4 rNTS (left) and cNTS (right) rats. LPGi, nucleus paragangiocellularis lateralis; AMB, nucleus ambiguous. Nos. are millimeters caudal to bregma. Scale bar, 1 mm.
63 mmHg, a much greater focal acidosis than that observed in the unanesthetized rat. In anesthesia, the cerebral blood flow response to focal hypercapnia would be less, thereby accounting for the greater focal acidosis.

In the unanesthetized rat, we do not know the degree of spread of the focal pH change within the tissue during dialysis. However, under anesthesia (19), with increasing distance from the probe, the pH change lessens, such that there is no detectable change at 550 μm. In the unanesthetized animal, we expect the region of pH change to be even more circumscribed. The absence of any effect on breathing of dialysis in our four wrong-place guide tube placement animals also supports the focal nature of the stimulus. We conclude that dialysis with 25% CO₂ in the unanesthetized rat produces a focal, mild-to-moderate tissue acidosis useful for the goals of the experiment.

The presence of the guide tube and microdialysis probe inevitably produces damage in the tissue. The probe tip itself has a relatively small volume of 49 nl, which is an acceptable size for microinjection experiments. However, the guide tube is present in the tissue from the dorsal surface down into the NTS region. Remarkably, this has little effect. Rats eat and gain weight within 1–2 days after the surgery, with very few animals developing infection or inflammation. There is a tissue reaction to the presence of the guide tube, which, in practice, does not seem to interfere with the ability of this dialysis approach to acidify the tissue.

Our rats, like those of others (37), have continuous cycles with awake, NREM, and REM periods. We recorded EEG and EMG information continuously to allow reliable determination of the state of arousal, and we analyzed breathing variables in a state-determined manner. We obtained a large sample of breathing in any state, 100–300 breaths, which minimizes breath selection bias and gives a better overall estimate of breathing.

We report results from rats with the dialysis guide tubes placed within the rNTS or cNTS as determined by anatomic analysis, and we grouped the data according to this rostral-caudal division. The rostral aspect of the area postrema is the landmark chosen to separate rNTS from cNTS. Figure 3.15 in Blessing (4) shows, in summary form, the results of many studies that examined the locations of afferent endings in the NTS from different sources as well as the sites, which, when stimulated, affect various autonomic functions. In general, cardiopulmonary afferents and function are located more caudally in the NTS; gustatory and gastrointestinal afferents, more rostrally. This arbitrary separation of guide tube locations is useful, as it uncovers quantitatively different responses based on location. The response of Ve to the focal acidification is significantly greater in cNTS than in rNTS, and this effect was greatest in the awake state.

Sleep-wake States

We made our measurements from 9 AM to noon, the end of the rat’s imposed circadian light (sleep) period, and from noon to 4 PM, the beginning of the imposed circadian dark (active) period. In our prior study (28),
we observed approximately equal amounts of NREM sleep and of wakefulness during both our AM and PM experiments. As noted, this result is not unexpected in that, at the end of the diurnal sleep period and the beginning of the active period, there is a merging of the frequency of NREM and awake periods (see Fig. 2; Ref. 37). During the hours just before and after the switchover time (noon in our case), periods of wakefulness increase and high-delta power episodes are less robust. Early in the wakefulness period, the rats are not as active as they are later. This facilitates our measurement of breathing, which requires minimal movement within the plethysmograph. The state of wakefulness in our studies necessarily represents quiet wakefulness. We observed periods of quiet wakefulness and NREM sleep that occurred with a frequency and duration similar to previous reports for the rat obtained with 24-h monitoring (37).

Initially during an exposure to 5–7% inspired CO₂, sleeping rats invariably awaken (unpublished observations) as do sleeping newborn piglets (8). This arousal is likely due to stimulation of peripheral and central chemoreceptors. It is of interest that focal acidiﬁcation in the medullary raphe (28) and the region of the NTS (this study) by microdialysis of aCSF equilibrated with 25% CO₂ does not awaken the rat even though Vₑ is stimulated. This lack of arousal may also be present with focal acidiﬁcation of the RTN region (19), although in that study we judged arousal state by using behavioral criteria, which could have overlooked changes in state detectable by EEG and EMG measures. A surprising and interesting ﬁnding of this study then is that microdialysis with 50% CO₂ in the cNTS does awaken or arouse the rats. This stands in contrast to the relative absence of such an arousal effect during dialysis with 25% CO₂. Because the response of Vₑ is not different during 25 and 50% CO₂ dialysis (see below), we conclude that the arousal threshold associated with focal acidiﬁcation of the cNTS is higher than the threshold required for stimulation of breathing. We do not know the degree to which the tissue pH in cNTS is more acidic with 50 vs. 25% CO₂ dialysis in the unanesthetized rat. In our prior tissue pH study of the RTN conducted with the animals under anesthesia (19), dialysis with 50% CO₂ produced about a doubling of the pH change at the probe tip compared with 25% CO₂ dialysis. The spread of the tissue pH change with 25 and 50% CO₂ dialysis was very similar, suggesting that the effect we observe here with 50% CO₂ producing arousal is due to greater stimulation within the same region of acidiﬁcation, not to a larger region being affected.

The NTS and Chemoreception

The NTS is a major site of afferent integration of autonomic reﬂexes (4–6, 12, 14, 15, 18, 21–23, 30–32, 34, 35). Gastrointestinal and gustatory reﬂexes synapse in the rostral half, and cardiovascular and respiratory functions in the caudal half [see Fig. 3.15 in Blessing (4)]. In the cNTS, cardiovascular neurons can receive afferent input from convergent sources (21, 22, 30–32). Integrative function of NTS neurons can be modulated by inﬂuences that include GABAergic interneurons, GABAergic input from other sites including the rostral ventrolateral medulla (18), and peptide modulators like angiotensin II (30–32) and substance P (23, 30–32, 34).

With respect to breathing, the NTS or dorsal respiratory group contains neurons with direct efferent connections to respiratory motoneurons (3, 11, 38). cNTS neurons at or just below the level of the area postrema are an initial synapse site for afferents, with information regarding lung volume and carotid body chemoreception. Chemoreception in the NTS has been studied in vivo and in vitro. Individual cNTS neurons in brain stem slices showed clear excitation in hypercapnia (9, 10). Whether these neurons are involved in respiratory chemoreception is unknown, because their functional output is not measured. Studies in vivo used the technique of focal acidiﬁcation produced by 1-nl injections of acetazolamide in anesthetized cats and rats with control of systemic CO₂ by ventilator (7). Increases in phrenic nerve activity produced by such injections indicated the presence of functional chemoreception in the region of the NTS. Systemic hypercapnia increased c-fos expression in the NTS (17, 36), and bilateral lesions of the NTS produced by injection of the neurotoxin kainic acid reduce breathing at rest and in response to systemic hypercapnia when studied with the animals under anesthesia (2). In the awake state, these abnormalities are less obvious, being present in some but not all animals and to a lesser degree (2).

In summary, these studies support the idea that central chemoreception is present in the NTS region. Given the many synapses present and the central role of the NTS in reﬂex integration, it seems possible that the chemoreceptive process may involve synaptic transmission, as well as the demonstrated cell-speciﬁc effects of CO₂ (9, 10).

NTS Acidification Increases Breathing in Sleep and Wakefulness

The major ﬁnding of this study is that focal acidiﬁcation in the region of the NTS increases Vₑ via VT and in both NREM sleep and wakefulness. This indicates a direct role of the NTS in central chemoreception. Overall, this effect is greater in cNTS than in rNTS, with this difference being highly signiﬁcant in the awake state. This rostral-caudal difference may be due to the presence of a greater number of chemoreceptor neurons located more caudally or, possibly, to the spread of CO₂ into the cNTS region from the more rostrally located dialysis sites. In this case, the response would be less as, with spread, more CO₂ would be cleared by local blood ﬂow, and the stimulus intensity at more distant caudal sites would be less. We cannot rule out this latter explanation, although, as discussed above, we believe that the spread of tissue acidiﬁcation with dialysis in the unanesthetized animal is quite small. In that afferents for cardiopulmo-
nary reflexes predominantly synapse in cNTS, this would seem to be a more likely site for focally located chemoreception, especially if the sensing mechanism involves synaptic events.

We expected that the ventilatory response to 50% CO₂ dialysis would be greater than with 25%. As discussed above, from tissue pH data obtained in anesthesia, we would expect a greater degree of acidosis. It is possible that the degree of the system response to focal acidosis is tempered by the coexisting hypocapnia, which would inhibit other chemoreceptor sites. One can imagine a state wherein greater stimulation of a single site may be balanced by the accompanying hypocapnia, such that overall Vₑ does not increase.

Comparison of RTN, Raphe, and NTS Central Chemoreceptor Function

We suggest that each of many locations for central chemoreception has a specific role that depends on arousal state. The overall sensitivity of the system to a small increase in CO₂ systemically, such that all sites are stimulated, is quite high. Inhalation of 7% CO₂ in the unanesthetized rat increases Vₑ by ≥200% (28). Focal acidification of the RTN (19, 28) or the medullary raphe increases Vₑ by 15–24% with a predominant effect in one arousal state for either site, whereas focal acidification of the cNTS with 25% CO₂ increases Vₑ by 15.8% in NREM sleep and by 28.4% in wakefulness. These responses to site-specific focal acidification are likely to be underestimates, as the systemic hypocapnia associated with the increase in breathing will inhibit other chemoreceptor sites. We hypothesized (25–27) that the high overall sensitivity of the system to increased systemic CO₂ requires stimulation of multiple chemoreceptor sites and that different clusters of sites operate in wakefulness than in sleep. Our data so far support this hypothesis. In NREM sleep, the medullary raphe would increase f, and the cNTS would increase Vₑ and f. In wakefulness, the RTN would increase Vₑ, and the cNTS would increase Vₑ and f. Also, a greater stimulus intensity in the cNTS tends to cause arousal.

A picture is emerging of many central chemoreceptor sites, each of which, when stimulated, contributes a small response that will add to the large response produced by stimulation of all sites. Each site may also contribute to arousal but, as judged by the findings shown here in cNTS, only when exposed to a greater stimulus intensity. It is also possible that some sites may not cause arousal, even with exposure to a greater stimulus intensity, although this seems unlikely. The threshold for arousal appears to be greater than for stimulation of breathing. When the system is stimulated below the arousal threshold, each site contributes differently to the ventilatory response, depending on whether the brain is awake or asleep. With greater stimulus intensity, the dependence of these contributions on arousal state may change. We hypothesize that, with low-stimulus intensities, a set of central chemoreceptor sites will together produce a ventilatory response without an associated arousal. With greater stimulation, the outputs of these sites will change, other sites will join in, the ventilatory response will be enhanced, and arousal will be more likely to occur.

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