CO₂ dialysis in the medullary raphe of the rat increases ventilation in sleep

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Nattie, Eugene E., and Aihua Li. CO₂ dialysis in the medullary raphe of the rat increases ventilation in sleep. J Appl Physiol 90: 1247–1257, 2001.—Central chemoreceptors are widespread within the brain stem. We hypothesize that function at different sites varies with arousal state. In unanesthetized rats, we produced focal acidification at single sites by means of microdialysis using artificial cerebrospinal fluid equilibrated with 25% CO₂. Tissue acidosis, measured under anesthesia, is equivalent to that observed with 63 Torr end-tidal PCO₂ and is limited to 600μm. Focal acidification of the retrotrapezoid nucleus increased ventilation by 24% only in wakefulness via an increase in tidal volume (Li A, Randall M, and Nattie E. J Appl Physiol 87: 910–919, 1999). In this study of the medullary raphe, the effect of such focal acidification was in sleep (defined by electroencephalographic and electromyographic criteria): ventilation and frequency increased by 15–20% in non-rapid eye movement sleep, and frequency increased by 15% in rapid eye movement sleep. There was no effect in wakefulness. Chemoreception in the medullary raphe appears to be responsive in sleep. Central chemoreceptors at two different locations appear to vary in effectiveness with arousal state. Central chemoreception; arousal; carbon dioxide response; medulla; control of breathing.

CENTRAL CHEMORECEPTORS MEDIATE changes in breathing and blood pressure in response to changes in CO₂ and H⁺ within the brain (16, 20–22). Early work located central chemoreceptors at sites just beneath the ventral medullary surface (16); recent experiments in vitro and in vivo indicate a widespread distribution within the brain stem (3, 5, 14, 15, 20–22, 31). These locations include a superficial region just beneath the surface of the ventral lateral medulla, the region of the nucleus tractus solitarius, the region of the locus ceruleus, the midline raphe, and the region of the ventral respiratory group. Why are central chemoreceptors located at so many sites? As one possible explanation, we hypothesize that differences in response and physiological role of some sites depend on the state of arousal.

We focus on the chemosensitive region lying deep to the ventral medullary surface at the midline, the medullary raphe. Raphe neurons in vitro exhibit CO₂-dependent changes in membrane potential and firing rate (31). In vivo, the firing rate of some medullary raphe neurons is increased by systemic hypercapnia in wakefulness but not in sleep (34). The medullary raphe has known anatomic connections with the respiratory control network (8). Destruction of the medullary raphe in anesthetized and decerebrate piglets reduces substantially the response of the phrenic and hypoglossal nerves to systemic CO₂ stimulation (6). Focal acidification of the raphe by microinjection of acetazolamide in anesthetized, vagotomized animals increases the amplitude of the integrated phrenic nerve signal (3). Stimulation of the raphe electrically or by chemical injection can increase or decrease respiratory output (2, 7, 12). The medullary raphe also is involved in blood pressure regulation (30), control of brown fat metabolism (19), thermoregulation (4), modulation of sensory input and motor output (13), and autonomic integration (17), and it plays a role in the sleep-wake cycle (28, 29). Its overall role in normal autonomic physiology is not fully understood.

We use a microdialysis probe (15) to produce a focal acidosis in the medullary raphe of unanesthetized, unrestrained rats. The probe tip with semipermeable membrane ( pores <6,000 Da) is 1 mm long and 240 μm diameter, with a volume of 45 nl. The guide tube and dialysis probe are made of a rigid, sturdy material, allowing their use in a chronic animal. The goal is to evaluate the effect of focal acidification of the medullary raphe on ventilation during sleep and wakefulness. In a prior, similar study (15), we dialyzed CO₂ into the retrotrapezoid nucleus (RTN) of unanesthetized rats, with sleep determined by behavioral criteria: the rat was considered to be asleep when curled up, motionless, with its eyes closed. We observed that focal acidification of the RTN increased ventilation (V̇E), solely by an effect on tidal volume (VT), in wakefulness only. In this study, we use electroencephalogram (EEG) and electromyogram (EMG) measures to determine sleep and wakefulness objectively and continuously.

METHODS

General Preparation

Surgery. Eight male Sprague-Dawley rats (300–450g) were anesthetized with ketamine (100mg/kg im) and xylazine (20mg/kg ip). The crown of the skull was shaved, and

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the skin was sterilized with povidone-iodine (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 were 11.2 mm caudal and 0 mm lateral from bregma and 10.4–10.6 mm below the dorsal surface. The guide cannula was secured with cranioplasty cement. Three EEG electrodes were screwed into the right side of the skull: the frontal electrode 2 mm anterior to 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 electrode between the frontal and parietal electrodes. For the EMG, a pair of wire electrodes was inserted deep into the neck muscle. The skull wound was sutured. The abdominal surface was shaved, the skin was sterilized, an incision was made through the linea alba, and a sterile telemetry temperature probe (TA-F20, Data Sciences, St. Paul, MN) was placed in the abdominal cavity. The incision was closed, and the animal was allowed to recover for 3–4 days.

CO2 dialysis solution. The artificial cerebrospinal fluid (aCSF) was equilibrated with 5 or 25% 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.

Ve measurement. The plethysmograph chamber used in these experiments is similar to the setup described by Jacky (10) and Pappenheimer (24). The analog output of the pressure transducer was converted to a digital signal and directly sampled at 150 Hz by computer using the DataPac 2000 system. The data were also recorded on tape using a digital system (model 3000A, Vetter). The animal chamber operates at atmospheric pressure, with the inflow and outflow of inspired gases balanced to prevent hyper- or hypobaric conditions in the box. The inflow gas was humidified, and the flow rate was controlled by a flowmeter (model 7491T, Matheson). The outflow port was connected to the in-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 flow rate through the plethysmograph was maintained at ≥1.4 l/min to prevent CO2 rebreathing. The plethysmograph was calibrated with 0.3-ml injections.

O2 consumption, CO2 production, and temperature measurement. O2 consumption (VO2) and CO2 production (VCO2) were measured using the Fick principle by calculating the difference in O2 or CO2 content between inspired and expired gas [VO2 = (V in × FI O2) − (V out × FO2)], where V in and V out represent inflow and outflow flow rate, FI O2 is fraction of inflow O2, and FO2 is fraction of outflow O2, and normalized to milliliters per gram of body weight per hour. The inflow O2 content was measured at the beginning of each experiment, and the outflow content of O2 and CO2 was read from the O2- and CO2 sensors constantly during the experiment. A thermometer inside the chamber measured the chamber temperature. Rat body temperature (Tb) was measured using the analog output 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 with backup on tape.

Anatomic analysis. At the end of the experiment, the rats were killed and the medulla was quickly removed and frozen and then cut into sections (50 μm thick) with a Reichert-Jung cryostat. The sections were counterstained with cresyl violet.

We identified anatomic landmarks and the site of dialysis probe placement using a rat brain atlas (25) for reference. The guide tubes were removed postmortem but before brain stem removal and sectioning, which required manipulation and produced tissue disruption. This procedure facilitated the anatomic verification of guide tube and probe tip location but also increased the volume of tissue disruption compared with that produced by simple insertion.

Data analysis. For sleep analysis we used the raw EEG and EMG signals, the fast Fourier transform of the EEG signal analyzed in 3.6-s epochs using delta (0.3–5 Hz), theta (6–9 Hz), and sigma (10–17 Hz) frequency bands, and behavioral observations. The rats were housed in a room with midnight to noon being the light or rest period and noon to midnight being the dark or active period. All the experiments were performed between 9 AM and noon, during the end of their rest period, or between noon and 4 PM, during the beginning of their active period. The state of arousal was defined using criteria modified from those of Bennington et al. (1) and Trachsel et al. (33). The rat was judged to be awake when 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. The rat was judged to be in non-rapid eye movement (NREM) sleep when 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. The rat was judged to be in rapid eye movement (REM) sleep when 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 sigma and theta power was moderate or high. When the rat was resting quietly on all four paws with eyes open or closed, it was sometimes difficult to distinguish light sleep from quiet wakefulness. On occasion, we had to judge the state as indeterminate. Data from indeterminate states are not included. We applied this analysis to each experiment, as shown in Fig. 1. We determined NREM sleep, REM sleep, and wakefulness visually from such records.

For ventilatory measurements, a breath-by-breath analysis was performed using the DataPac III system, with the pressure deflections and the respiratory cycle time for each breath being determined for ≥100, and up to 300, breaths at defined sleep and wakefulness periods during the experiment. Sighs, sniffing, and recording artifacts were edited from analysis. These data were exported to Sigmaplot 4.0 (Jandel Scientific), and VT per 100 g body wt, frequency (f), and Ve per 100 g body wt were calculated for each breath using plethysmograph temperature and Tb for that time period. In our initial analysis, we grouped the experiments on the basis of the amount of time during the experiment the rat was asleep: ≥50% defining “sleep” and ≤50% defining “awake.” For NREM sleep and wakefulness, we were able to obtain three to four defined periods before CO2 exposure and two periods after exposure. During the 30 min of test dialysis, we obtained NREM sleep and wakefulness data representative of the first, second, and third 10-min periods. For our second analysis, we examined ventilatory data in each state in all experiments. Here we calculated the maximum percent change in each state comparing the maximum value during the 30-min test dialysis period with a baseline control value in that state. Our REM sleep data are more fragmentary, in that REM sleep periods in the rat are brief.

The results for Ve, VT, f, VO2, VCO2, and Tb during 25 or 5% CO2 tests were compared by two-way repeated-measures

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paired ANOVA with treatment and time as factors (SigmaStat, Jandel Scientific) or by paired t-test. Post hoc tests were performed when significant differences were found.

Experimental Protocol

We dialyzed each of eight rats with 5 and 25% CO₂ during morning and afternoon measurement periods when the rats were expected to be asleep or awake, respectively. Ultimately, the rat was judged to be awake or asleep by the criteria outlined above. Rats were not always predominantly awake or asleep during the time period in the circadian cycle of greater likelihood for each state, so our initial paired experimental design did not work out in practice. As a result, we analyze our results using two approaches. In the first

Fig. 1. A typical experiment with dialysis of artificial cerebrospinal fluid (aCSF) equilibrated with 5% CO₂ during the entire period before and after the period from 0 to 30 min during which the dialysate was equilibrated with 25% CO₂. Top trace: raw electroencephalogram signal; 2nd trace: raw electromyogram signal. Traces labeled delta, theta/delta, and sigma + theta represent the fast Fourier transform parameters used in our sleep analysis: delta power (0.3–5 Hz); theta (6–9 Hz)/delta; and sigma (10–17 Hz) + theta. Respiratory frequency, Ve, ventilation. Wakefulness, NREM sleep, REM sleep. Arousal state is first defined using electroencephalogram and electromyogram criteria. Then, within a period of a single arousal state (wakefulness, NREM sleep, or REM sleep), 100–300 breaths were analyzed, and single-breath values of f, tidal volume (VT), and Ve were calculated and averaged. For this example, we show data for all 3 states. During the 30-min dialysis with 25% CO₂-equilibrated aCSF, f and Ve increase in NREM and REM sleep. Dialysis with 25% CO₂-equilibrated aCSF did not disrupt the normal sleep cycling.
approach, we grouped responses to 5 and 25% CO₂ during wakefulness or sleep as defined by our EEG and EMG criteria. If the rat was asleep for most (>50%) of the entire experiment and for most of the 30-min test dialysis period, the experiment was called a sleep experiment, and ventilatory data were analyzed only in the NREM sleep periods. If the rat was awake for most of the entire experiment and for the 30-min test dialysis period, the experiment was called an awake experiment, and ventilatory data were analyzed only in the periods of wakefulness. Some rats were dialyzed with 5 or 25% CO₂ more than once. Once for data analysis and statistics, multiple runs were averaged, so that each rat contributed one set of data in any condition. Data analysis was paired; e.g., responses to 25 and 5% CO₂ dialysis in rats of the sleep group were compared using data obtained from each of six rats. Insofar as the rats that slept for most of the experimental period tended to have NREM sleep periods with more consistently increased delta power and those that were awake for most of the experimental period had the opposite, this approach allowed us to clearly separate the effects in sleep from the effects in wakefulness.

In the second approach, we analyzed all wakefulness, NREM sleep, and REM sleep data in each experiment regardless of the amount of time the rat was awake or asleep. This allowed a paired comparison of effects but assumed that any sleep/wakefulness effect will be present regardless of the amount of time spent in sleep or the depth of sleep. In some experiments, there may be no periods of sleep or wakefulness. The change in f, VT, and V˙E was calculated using a single maximum value obtained in any state during the 30-min dialysis period and a value obtained in that same state during the preceding control period.

The rats would initially be weighed and then gently held while the dummy cannula was removed and the dialysis probe was inserted into the guide tube. The animals were placed into a 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 in the room air-breathing condition. Baseline measurements were made over the next 40 min. The dialysis solution was then changed to 25% CO₂ or maintained at 5% CO₂, and measurements were taken over 30 min. The dialysis tube and cannula dead space is taken into account along with the dialysis fluid flow rate, so that time 0 is the estimated time at which the test solution reaches the exchange membrane. After 30 min, the dialysis solution was changed to 5% CO₂ or maintained at 5% CO₂, and measurements were again made over 40 min.

RESULTS

Typical Experiment

Figure 1 shows, for a typical experiment, the raw EEG and EMG signals, the fast Fourier transform-derived parameters, and calculated f and V˙E for the state-specific periods chosen before, during, and after dialysis with aCSF equilibrated with 25% CO₂. Dialysis with aCSF equilibrated with 5% CO₂ occurs from beginning to end, except for the 30-min test period. This experiment was judged to be in the sleep group, inasmuch as in 50% of the total experiment and in 77% of the 30-min 25% CO₂ dialysis period, the rat was in NREM sleep. The rat was in REM sleep for 7.4% of the total period and for 6.5% of the test dialysis period. The rat was awake for 43% of the total period, much of this in the initial portion of the experiment, and for 16.7% of the test dialysis period.

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 sleep and wakefulness. In the sleep group with 25% CO₂ dialysis, there were 7.4 ± 0.5 (SE) NREM sleep periods per total experiment with a mean duration of 7.7 ± 0.4 min. During the 30-min test dialysis period, there were 3.2 ± 0.2 NREM sleep periods with a 6.9 ± 0.4 min average duration. In the sleep group with 5% CO₂ dialysis, there were 7.0 ± 0.5 NREM sleep periods per total experiment with an average duration of 7.2 ± 0.6 min. During the 30-min test dialysis period, there were 3.4 ± 0.2 NREM sleep periods with a 5.9 ± 0.6 min average duration. Dialysis with 25% CO₂ did not affect sleep cycling.

V˙E, Vr, and f were calculated for 100–300 breaths in periods of NREM sleep and wakefulness. For grouped data, each experiment was placed into the sleep or the awake group on the basis of the percentage of time in the whole experiment and of the 30-min dialysis period in NREM sleep or wakefulness, and we report ventilatory data only in that state. For this typical example, we have calculated ventilatory results in all three states. In wakefulness, f and V˙E were unaffected by 25% CO₂ dialysis in the medullary raphe. In NREM sleep, baseline V˙E was similar to that in wakefulness and f was lower. With 25% CO₂ dialysis, V˙E and f increased during the first half of the dialysis period and then returned to baseline levels toward the end of the 25% CO₂ dialysis period. This recovery of V˙E and f during 25% CO₂ dialysis was not the usual response, as can be seen in the grouped data discussed below. In REM sleep, baseline V˙E was lower and f was higher than in NREM sleep or wakefulness. With 25% CO₂ dialysis, V˙E and f increased.

Sleep

Analysis of the percentage of total time in NREM sleep and wakefulness for our rats, with the animals grouped by whether the measurements were obtained in the late sleep period (9 AM to noon) or the early active period (noon to 4 PM), shows that, for 22 runs in 8 rats obtained before noon, they are, on average, awake for 52 ± 7% (SE) of the time and in NREM sleep for 42 ± 6% of the time. For the experiments after noon (18 runs in 8 rats), the rats were awake for 47 ± 5% of the time and in NREM sleep for 45 ± 4% of the time. Thus these rats showed, as a group, approximately equal distributions of NREM sleep and wakefulness whether they were measured before or after noon.

Initially, we grouped our data on the criterion of the state in which the rat spent the most time during the entire experiment and the 30-min test dialysis period. Rats that were mostly in NREM sleep during the experiment, whether before or after noon, were grouped as sleep. Rats that were mostly awake during the experimental period, whether before or after noon, were grouped as awake. Table 1 shows the percentage
of time in each state during the entire experiment and during the 30-min test dialysis period for animals paired according to predominant sleep or wakefulness. Six rats contributed paired data in sleep; six (not the same rats) contributed paired data in wakefulness. The sleep group, on average, spent 55–57% of the total experiment time and 65% of the 30-min dialysis test period in NREM sleep. The awake group spent 67–73% of the total experiment time and 65% of the 30-min dialysis test period awake.

Ventilatory Responses to 25% CO₂ Dialysis in the Medullary Raphe: Initial Approach

Figure 2 shows the $\dot{V}E$, $V_T$, and $f$ responses of the sleep group to test period dialysis with 5 or 25% CO₂. The results are paired and plotted in 10-min bins. For example, ventilatory data obtained in NREM sleep during the 10-min period before the onset of dialysis are grouped as the 25-min time point. $\dot{V}E$ is significantly greater with 25% CO₂ dialysis. Two-way repeated-measures paired ANOVA using treatment (25 vs. 5% CO₂ dialysis) and time showed a significant interactive effect ($P < 0.03$), with a Bonferroni post hoc comparison showing differences between treatments at 10 min ($P < 0.005$), 20 min ($P < 0.01$), and 30 min ($P < 0.01$). Frequency is significantly greater with 25% CO₂ dialysis. Two-way repeated-measures paired ANOVA using treatment (25 vs. 5% CO₂ dialysis) and time showed a significant interactive effect ($P < 0.001$), with Bonferroni post hoc comparison showing differences between treatments at 10, 20, and 30 min ($P < 0.005$). $V_T$ was not significantly affected by 25 or 5% CO₂ dialysis in sleep. For these analyses, the four initial baseline values were combined into a single value for each animal.

Figure 3 shows responses of $\dot{V}E$, $V_T$, and $f$ responses of the awake group to test period dialysis with 5 or 25% CO₂. Two-way repeated-measures paired ANOVA using treatment (25 vs. 5% CO₂ dialysis) and time showed no significant effects. Similar analysis for data during wakefulness also showed no significant effects of treatment or time. Comparison of sleep group data with awake group data in either treatment showed no significant differences in $\dot{V}O_2$, $VCO_2$, but $T_b$ was significantly lower in sleep ($P < 0.01$, by 2-way ANOVA). The difference in mean values was between 0.3 and 0.4°C.

Ventilatory Responses to 25% CO₂ Dialysis in the Medullary Raphe: Second Approach

In the second approach, we did not group the experiments by amount of sleep or wakefulness but utilized,
as much as possible, data in wakefulness, NREM sleep, and REM sleep in each experiment. We expressed the maximum increase in f and V˙E in each state during the 30-min test dialysis period as a percentage of the closest pretest dialysis baseline period in that same state.

The NREM sleep and wakefulness f data are shown in Fig. 6 as a function of the percentage of total experimental time spent in sleep (NREM and REM). When the observations during the 30-min test dialysis period are made in wakefulness, there is no effect of 25% CO2 dialysis regardless of the amount of time during the experiment the rat was asleep. In marked contrast, when the observations of f are made during NREM sleep, there is an effect of 25% CO2 dialysis, and the degree of this effect is greater the more time the rat was asleep during the experiment. Linear regression analysis shows $y = (0.41 \pm 0.21)x - 4.1$ ($r = 0.53$) for 25% CO2 dialysis and $y = - (0.04 \pm 0.08)x + 3.9$ ($r = 0.2$) for 5% CO2 dialysis. Here again, the slope for the 5% CO2 dialysis data lies outside the 95% confidence interval for the slope of the 25% CO2 dialysis data. In this analysis as well, focal acidosis of the medullary raphe increases f and V˙E in sleep, and the effect is greater if more of the experimental time is spent in sleep.

In a comparison of REM sleep periods during the 25% CO2 exposure with REM sleep periods in the prior baseline period with 5% CO2 dialysis ($n = 5$ rats), f increased by $15 \pm 4.5\%$, Vt decreased by $3 \pm 5\%$, and V˙E increased by $5.1 \pm 5.9\%$. In rats with continuous 5% CO2 dialysis, comparing REM sleep data in the 30-min test period with prior baseline REM sleep periods ($n = 6$ rats), we observed a $2.5 \pm 2.7\%$ increase in f, a $1 \pm 0.6$ CO2 dialysis response lies outside the 95% confidence interval for the 25% CO2 dialysis results. Paired t-test comparison of the maximum percent increase in f during sleep in the 25% CO2 dialysis data vs. that observed during wakefulness shows $P < 0.01$. A similar analysis of the ventilation data shows $y = (0.41 \pm 0.21)x - 4.1$ ($r = 0.53$) for 25% CO2 dialysis and $y = - (0.04 \pm 0.08)x + 3.9$ ($r = 0.2$) for 5% CO2 dialysis. Here again, the slope for the 5% CO2 dialysis data lies outside the 95% confidence interval for the slope of the 25% CO2 dialysis data. In this analysis as well, focal acidosis of the medullary raphe increases f and V˙E in sleep, and the effect is greater if more of the experimental time is spent in sleep.

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3.5% change in $V_T$, and a $3 \pm 3\%$ change in $V_E$. The increase in $f$ during 25% CO$_2$ dialysis compared with 5% CO$_2$ for the remaining experimental time. Values are means $\pm$ SE for 6 rats in each group.

**Anatomy**

In Fig. 7, we show for each of the eight rats the cross section of the medulla that contains the greatest area of tissue disruption caused by the guide tube and dialysis probe. The top two cross sections represent actual stained sections of typical results for more rostral and caudal probe placements. The schematic sections below each stained section show the location of the probes in the other six rats grouped into the rostral and caudal sections. This rostral and caudal grouping is for ease of presentation; all probe sites were within the rostral-to-caudal confines of the facial nucleus.

**DISCUSSION**

**Methods**

Using microdialysis of aCSF equilibrated with 25% CO$_2$, we produce focal acidification within a single chemoreceptor site in an unanesthetized rat. The microdialysis probe tip has dimensions of $1 \text{ mm} \times 0.24 \mu \text{m}$; volume is 45 nl. Tissue damage and gliosis are present in the region surrounding the probe tip location (Fig. 7). We allow 3–4 days of recovery from surgery before conducting an experiment. In our experience, the rat is eating and gaining weight by this time, and we diminish the likelihood of probe or EEG/EMG connector damage and, perhaps, of greater tissue

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**Fig. 6.** Maximum percent increase in $f$, determined by comparison of values in any state during the 30-min test dialysis period with values in the same state during the baseline period just before this test dialysis period as a function of percentage of total experimental time in sleep (NREM and REM). Top: NREM sleep with 25% (●) and 5% (○) CO$_2$ dialysis. Bottom: wakefulness with 25% (●) and 5% (○) CO$_2$ dialysis.
reaction to the presence of the probe by conducting experiments at this time. We dialyze at a high flow rate, 45 \( \mu \text{L/min} \), which we have found necessary to deliver sufficient amounts of highly diffusible CO\(_2\) to the tissue to produce the focal acidification. This flow rate does not, by itself, produce changes in breathing or metabolism or in the sleep-wakefulness cycle, as is evident in the responses to control experiments with 3 h of dialysis with 5% CO\(_2\) in the dialysate. The tissue reaction does not seem to interfere with the ability of this dialysis approach to acidify the tissue. We have evaluated in anesthetized rats the intensity and spread of the focal pH change induced by this method (15). At the probe, the pH change is similar to that observed with an increase in end-tidal PCO\(_2\) to 63 Torr; with increasing distance from the probe, the pH change lessens, such that there is no detectable change at 600 \( \mu \text{m} \). We are, at present, measuring arterial and medullary tissue pH in the unanesthetized rat during exposure to microdialysis-induced focal CO\(_2\) and to systemic increases in CO\(_2\). Preliminary data suggest that in the unanesthetized rat the degree of pH change is less.

In our initial study of focal CO\(_2\) application in the RTN region, we relied on behavioral criteria to distinguish sleep from wakefulness. In the present study, we use EEG and EMG data to determine continuously the sleep/wakefulness state of the rat. Our observations indicate that when the rat is curled up, motionless with its eyes closed and its head tucked between the forepaws, it is usually in NREM sleep, as judged in our prior study (15). However, our present observations, similar to those of others (33), indicate that the rat arousal state cycles continuously, with wakefulness, NREM sleep, and REM sleep having different frequencies and durations depending on the time in the light/dark circadian period. Constant recordings of EEG and EMG information give access to greater amounts of usable data during any experiment and allow reliable determination of periods of quiet wakefulness (see below). Other improvements in experimental design include continuous dialysis with aCSF through the entire experiment. This adds an important control for any effects of dialysis per se. We also analyze 100–300 breaths for each defined sleep/wakefulness state. This large sample of breathing in any state minimizes breath selection bias and gives a better overall estimate of breathing.

We report only data obtained from rats with the dialysis guide tubes placed within the rostral aspect of the medullary raphe, as proven by postmortem anatomic analysis. Our probe locations are constrained to within the rostral-to-caudal dimension of the facial nucleus. We are affecting only a portion of the medullary raphe, but it is a portion that is known to have connections to other brain stem sites involved with the control of breathing (2, 3, 7, 12), that contains chemosensitive neurons (31), and that expresses chemosensitivity (2).

**Sleep/Wakefulness States**

We altered the circadian light/dark period to facilitate these experiments. Our hope was to make measurements in sleep from 9 AM to noon, which would be the end of the rat’s imposed circadian sleep (light) period, and in wakefulness from noon to 4 PM, which would be the beginning of the imposed circadian active (dark) period. We observed approximately equal amounts of NREM sleep and wakefulness during our before-noon and after-noon experiments. Although this obviated our ideal design, the result is not surprising, given the published data on rat diurnal sleep-wakefulness cycling; i.e., at the end of the sleep period and beginning of the active period, there is a merging of the frequency of the NREM sleep and wakefulness states (Fig. 2) (33). Measurements obtained continuously in rats over 24-h periods (33) indicate that, early in the
Medullary Raphe and Breathing

Earlier studies involving electrical or glutamate stimulation of the medullary raphe reported excitation (2, 7, 12) and inhibition of breathing (12, 18). Thus the raphe is known to have the capability of altering $V_E$.

Chemoreception in the raphe has been studied in vivo and in vitro. Veasey et al. (34) reported that six neurons in the medullary raphe of unanesthetized cats increased their firing rate with systemic hypercapnia. In four of these neurons, responses to hypercapnia in NREM sleep were absent. These workers hypothesized that these raphe cells function as “gain setters” for motor neurons, here involved with breathing. Dreshaj et al. (6) disrupted the function of rather large volumes of the medullary raphe in decerebrate or anesthetized piglets by making up to five 300-nl injections of the local anesthetic lidocaine or the excitatory amino acid neurotoxin ibotenic acid. These were presumably placed in the rostral-to-caudal axis; no detailed anatomy of the entire region affected is available. Nevertheless, these experiments resulted in profound reductions in the response of ventilatory output to increased systemic CO$_2$. The implication is that the medullary raphe has an important role in central chemoreception. These studies do not distinguish between an effect directly on chemoreceptors themselves and an effect via a reduction in a required excitatory activity emanating from the raphe to the respiratory network, which is permissive for the full chemoreceptor response.

More direct studies of chemoreception in the medullary raphe involve focal acidification by the microinjection of acetazolamide, an inhibitor of carbonic anhydrase (3). That respiratory output is increased after many, but not all, such injections is interpreted to indicate the presence within the medullary raphe of chemoreceptors, in that the animals, on ventilators, have normal CO$_2$ levels and pH at other brain sites. Studies of medullary slice preparations that include the raphe and of isolated neurons from the medullary raphe have described neurons that can be excited, or inhibited, by CO$_2$, providing evidence of putative chemoreceptive neuronal functions in the raphe (31).

Raphe Acidification Increases Breathing Frequency in Sleep

The major finding of this study is that focal acidification of the medullary raphe via microdialysis of CO$_2$-laden aCSF increases $V_E$ in the unanesthetized rat during sleep but not during wakefulness. In NREM sleep, there is a robust increase in $V_E$, which occurs via an increase in $f$ (15–20%). In REM sleep, $f$ increases by 15%, but a small decrease in $V_T$ obviates any increase in $V_E$.

We used two approaches for data analysis. In the first, experiments were grouped by the amount of time during the experiment and during the 30-min test dialysis period that the rat was asleep: >50% defining the sleep group and <50% defining the awake group. In each group, responses to 25% or 5% CO$_2$ dialysis were paired. This approach maximizes the amount of data available in sleep and wakefulness, allowing a detailed time course analysis as shown in Figs. 2–4. In this approach, we did not examine wakefulness data in the sleep group and vice versa, thereby losing some information.

In our second approach, we analyzed data in wakefulness, NREM sleep, and REM sleep in each experiment. We measured the peak $f$ and $V_E$ response during the 30-min test dialysis period compared with the $f$ and $V_E$ in the same arousal state before the test dialysis period. This allowed a paired analysis in each state of the maximum response to focal acidosis in the raphe but did not allow any analysis of the time course of the response, since the amount of NREM and REM sleep in the experiments with predominant wakefulness was small and, in some cases, absent, and vice versa. This analysis did uncover the surprising relationship, shown on Fig. 6, between the degree of the focal acidification effect on breathing and the total amount of experimental time in sleep.

We explain this relationship by the depth of NREM sleep. By inspection, in rats with less total experimental time in sleep, the depth of NREM sleep, on average, is smaller. To estimate this, we averaged for each animal the percent increase in delta power in all NREM periods in each experiment compared with the delta power during wakefulness in each case. We then
averaged these data for the two groups. In the sleep group, the average increase in delta power was 243 ± 22% (n = 6); in the awake group, it was 192 ± 6% (P < 0.05, by t-test). We suggest that the degree of the effect of focal acidification in the medullary raphe on breathing in sleep depends on the depth of sleep.

In a prior experiment with focal acidification of the same region of the medullary raphe produced by microinjection of acetazolamide in anesthetized, vagotomized rats (3), an increase in the amplitude of the integrated phrenic nerve activity was observed without a change in f. It is difficult to compare this experiment with the present experiment in the unanesthetized rat, inasmuch as the vagus nerve was sectioned and systemic PCO$_2$ was maintained at a constant normal value.

We conclude that the major effect of focal acidification of the medullary raphe in the unanesthetized rat is an increase in VE in sleep that occurs via an increase in f. In contrast, Veasey et al. (34) reported that a small number of neurons in the medullary raphe increased their firing rate with systemic hypercapnia, but only in wakefulness. It may be that these workers recorded from neurons more directly attuned to the motor act of breathing, neurons that were not chemosensitive. It may also be that our focal acidosis stimulates nonserotonergic raphe neurons, although the in vitro work of Richerson (31) showing specific pH-sensitive responses of raphe neurons argues against this.

In respect to central chemoreception, we suggest that each of many locations for central chemoreceptors has a specific role that depends on arousal state and stimulus intensity. The overall sensitivity of the system to a small increase in CO$_2$ systemically, such that all sites are stimulated, is quite large. Inhalation of 7% CO$_2$ in the unanesthetized rat increases VE by ≥200% (15). Focal acidification of the RTN or the medullary raphe increases VE by 15–24% with a predominant effect in one arousal state for either site. It seems reasonable to hypothesize (22) that the fine overall sensitivity of the system to increased systemic CO$_2$ requires stimulation of multiple chemoreceptor sites and that some sites operate more importantly in wakefulness, others in sleep. Thus the contribution of the raphe would be to increase f in sleep.

**Medullary Raphe and Sleep**

The medullary raphe seems to be involved in the sleep-wakefulness cycle, but its precise role is uncertain (28, 29, 32). Raphe neurons exhibit their greatest firing rate during wakefulness, a lower firing rate in NREM sleep, and a very low or absent firing rate in REM sleep (11, 34). It is suggested that the sleep-related decrease in firing rate disinhibits mesopontine cholinergic neurons that are active in REM sleep, that the raphe neurons are part of the brain stem mechanisms of sleep regulation (32). The source of the decrease in raphe firing rates in sleep is unknown. The rat breathes at a slower frequency in NREM sleep than in wakefulness, and in NREM sleep, raphe neurons fire at slower rates. It has been suggested that the sleep-related changes in firing rate of raphe neurons affect upper airway caliber, inasmuch as there are projections from the raphe to hypoglossal motor neurons, and direct dialysis of serotonin (5-hydroxytryptamine, 5-HT) on hypoglossal motor neurons increases genioglossus muscle activity, which would increase upper airway caliber (9). Thus one may presume that decreased firing in the raphe could result in less 5-HT release and less excitation with reduced upper airway motor tone (35), which occurs in sleep.

How do our findings fit into this picture? We know from the in vitro experiments of Richerson (31) that medullary raphe neurons can be excited, or inhibited, by CO$_2$. Our data indicate that, in the intact, unanesthetized rat, focal medullary raphe acidification increases f predominantly and that this occurs in NREM and REM sleep but not in wakefulness. We would like to know whether raphe neurons increase their firing rate with focal CO$_2$ stimulation in vivo in NREM and REM sleep and in wakefulness. It seems possible that, in wakefulness, these neurons, which are already firing, cannot be further stimulated. During NREM and REM sleep, when their in vivo firing rates are lower, focal CO$_2$ can have a greater effect. We hypothesize that focal CO$_2$ increases the raphe neuron firing rate in NREM and REM sleep. The focal CO$_2$ stimulation of the raphe converts its input to the respiratory premotor and motor neurons to input similar to that in wakefulness; that is, focal acidification changes raphe firing to be more similar to that in wakefulness. The result is a stimulation of 5-HT release at hypoglossal and other respiratory neurons, which increases f. The rat remains asleep, but breathing becomes similar to that in wakefulness. An analogous interpretation was made by Berner et al. (4) in their studies of raphe inactivation and thermoregulation. With lidocaine injection into the raphe, responses to cold stress originating in the periphery or centrally in the hypothalamus were inhibited. They observed decreases in VO$_2$ and muscle EMG activity similar to those observed with spontaneous entry into sleep from wakefulness. It was as if the raphe inactivation by lidocaine initiated thermoregulatory responses associated with sleep. In our case, raphe activation by CO$_2$ initiated respiratory responses associated with wakefulness.

If medullary raphe stimulation by focal CO$_2$ application in sleep increases f by the removal of a normal inhibition of raphe neurons in this state, we would expect the resultant f to be similar to that observed in wakefulness. Examination of our paired data provides some support for this conclusion, inasmuch as the f values with CO$_2$ stimulation of the raphe in sleep are only slightly greater than those observed in wakefulness.

**Physiological Roles of the Medullary Raphe**

Two general theories for raphe function, possibly relevant to our study, have been proposed. Jacobs and Fornal (11) observed that in a wide variety of behavioral circumstances the firing rate of raphe neurons is
closely correlated with motor activity, especially repetitive motor activity, such as breathing. They suggest that the raphe serves to facilitate motor activity, with sensory processing being suppressed. Auxiliary to this modulatory function is a coordination of autonomic function. Lovick (17) proposed that the raphe serves to modulate existing activity in autonomic neural control systems and that this modulation is state dependent.

If the raphe serves as a modulatory system for somatic motor and autonomic control, then why should it exhibit chemosensitivity? We note that another brain stem region that may serve mainly in a modulatory capacity, the locus ceruleus, also exhibits chemosensitivity in vitro and in vivo (5, 23, 27). These two monoamine systems may work in concert to set the gain of motor output/sensory processing and autonomic control in different states of alertness and arousal. CO2 may serve as a fundamental chemical signal to these two sets of neurons to determine their level of activity, to affect the proposed gain function, and to coordinate autonomic processes. The ventilatory chemoreceptor aspects of raphe and locus ceruleus neurons may be adjunct to this broader CO2-dependent function. Dr. Jing Shi performed many of the experiments reported here; Eva Liu and Joshua Osborne performed early versions of the experiment. This research was supported by National Heart, Lung, and Blood Institute Grant HL-28066.

REFERENCES