Ventilatory effects of gap junction blockade in the RTN in awake rats

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Ventilatory effects of gap junction blockade in the RTN in awake rats. Am J Physiol Regul Integr Comp Physiol 287: R1407–R1418, 2004. First published August 12, 2004; doi:10.1152/ajpregu.00404.2004.—We tested the hypothesis that gap junctions in the RTN also modify the ventilatory response to CO2 when focally perfused within the retrotrapezoid nucleus (RTN). We tested this hypothesis by measuring minute ventilation (Vₑ), tidal volume (Vₜ), and respiratory frequency (Fᵣ) responses to increasing concentrations of inspired CO₂ (FICO₂ = 0–8%) in rats during wakefulness. We confirmed that the RTN was chemosensitive by perfusing the RTN unilaterally with either acetazolamide (AZ; 10 μM) or hypercapnic artificial cerebrospinal fluid equilibrated with 50% CO₂ (pH ~6.5). Focal perfusion of AZ or hypercapnic aCSF increased Vₑ, Vₜ, and Fᵣ during exposure to room air. Carbenoxolone (300 μM) focally perfused into the RTN decreased Vₑ and Vₜ in animals <11 wk of age, but Vₑ and Vₜ were increased in animals >12 wk of age. Glycyrrhizic acid, a congener of carbenoxolone, did not change Vₑ, Vₜ, or Fᵣ when focally perfused into the RTN. Carbenoxolone binds to the mineralocorticoid receptor, but spironolactone (10 μM) did not block the disinhibition of Vₑ or Vₜ in older animals when combined with carbenoxolone. Thus the RTN is a CO₂ chemosensory site in all ages tested, but the function of gap junctions in the chemosensory process varies substantially among animals of different ages: gap junctions amplify the ventilatory response to CO₂ in younger animals, but appear to inhibit the ventilatory response to CO₂ in older animals.

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Chemical synaptic transmission is the predominant form of intercellular signaling in the adult vertebrate central nervous system. However, gap junctions form low resistance “electrical synapses” through which electrical current or small molecules may pass (23, 30). Electrical coupling between neurons was thought to occur primarily in the central nervous system during early development in vertebrates and in phylogenetically more primitive animals (42). However, electrical coupling via gap junctions appears to be important in a variety of mammalian central nervous system functions even in older animals (6, 13, 16, 22, 24, 46, 55, 59). Diffusion of signal molecules through these intercellular communications seems to coordinate processes such as embryogenesis, growth, differentiation, and cellular responses to injury (30, 47, 57, 60). In addition, electrical coupling among neurons permits rapid and synchronous responses to stimuli.

Connexins (Cx) are the proteins from which gap junctions are formed. Immunohistochemical staining demonstrated the presence of Cx26, Cx32, and Cx36 in multiple regions of the brain stem involved in respiratory control (62, 64). Of the respiratory-related sites in which gap junctions are expressed, the pre-Bötzinger complex, locus ceruleus (LC), retrotrapezoid nucleus (RTN) in the ventrolateral medulla, and nucleus of the solitary tract (NTS) are sensitive to CO₂ (9, 44, 63). Therefore, gap junctions may play a role in respiratory control in older animals. We recently examined the effect of carbenoxolone, which blocks gap junctions, on ventilation and ventilatory responses to CO₂. After we focally perfused the NTS unilaterally with carbenoxolone (300 μM), the ventilatory response to hypercapnia was reduced in awake rats 7–10 wk of age. However, focal perfusion of carbenoxolone did not alter ventilation or the ventilatory response to CO₂ in animals >10 wk of age (46).

Gap junctions are also found in the RTN. Therefore, we tested the hypothesis that gap junctions in the RTN also modify the ventilatory response to CO₂. In an initial set of studies, we established that the RTN was CO₂ sensitive by focally perfusing acetazolamide (AZ) or hypercapnic artificial cerebrospinal fluid (aCSF) into small regions within the RTN. We also examined the ventilatory response to CO₂ before and after focal perfusion of carbenoxolone into the RTN in rats aged 7–19 wk. We found that carbenoxolone diminished the response to CO₂ in younger animals (aged 7–10 wk), but the ventilatory response to CO₂ was enhanced after unilateral perfusion of carbenoxolone in older rats (aged 13–19 wk).

METHODS

The St. Lawrence University Animal Review Committee approved this study. Adult Sprague-Dawley rats of either sex aged 7–19 wk and weighing between 250 and 350 g were used for this study. Animals were kept on a 12:12-h light/dark cycle and provided food and water ad libitum. Surgery. Rats were anesthetized with xylazine (6 mg/kg ip) followed by ketamine-HCl (70 mg/kg im). Each animal was secured in a stereotaxic apparatus (Kopf Instruments), a midline incision was made, a hole was drilled in the skull 11.5 mm caudal to bregma and 2.2 mm from midline (left side), and a push-pull guide cannula (Plastics One, Roanoke, VA; ID 0.29 mm, OD 0.56 mm) was inserted through the burr hole. Two additional holes were drilled into the skull to place anchor screws to hold the cannula. The guide cannula and the anchor screws were attached to the skull with cyanoacrylate (Plastics One). The scalp was sutured together up to the border of the cement cap. A dummy cannula was screwed into the guide cannula to maintain patency of the lumen during the recovery period and be-
tween experiments. The dummy cannula extended 0.3 mm beyond the end of the guide cannula. Approximately 1 wk after surgery, ventilatory measurements were made in each animal. In preliminary experiments (data not shown), ventilation was unaffected by the surgery or by perfusion of the RTN with aCSF alone.

**Solutions and solution delivery.** aCSF contained the following salts (in mM): 124 NaCl, 3 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 26 NaHCO₃, and 10 glucose. Before delivery, aCSF was equilibrated with 95% O₂ and 5% CO₂, warmed to 37°C, and loaded into 3-ml plastic syringes. The pH of all solutions was 7.48. Solutions were delivered to the RTN using a push-pull syringe pump (World Precision Instruments, Sarasota, FL). Flow was directed through polyethylene tubing that was connected to an inner delivery cannula (Plastics One; ID 0.1 mm, OD 0.2 mm). Before perfusate delivery, the line and inner cannula were flushed with aCSF or aCSF plus the drug to be tested, and the solution was perfused throughout each experiment.

After the dead space of the inner cannula was filled, the cannula was screwed into the guide cannula, and the return vacuum line was connected. Like the dummy cannula, the inner delivery cannula extended 0.3 mm beyond the end of the guide cannula. The flow through the cannula was 0.12 ml/h. Push-pull cannulas resemble microdialysis in that the amount of tissue exposed to the agent is restricted, and the concentration of the test agent remains constant at the tissue adjacent to the probe throughout the perfusion period.

**Ventilatory responses to carbon dioxide.** Body weight and barometric pressure were recorded before each experiment. Each animal was placed in the plethysmograph and allowed to accommodate to the chamber for 10 min after the stabilization of CO₂ levels within the plethysmograph chamber. The chamber had a volume of 1 l, and the rate of airflow through the box was 1.25 l/min. Stable CO₂ values within the chamber were achieved in 2–3 min. The chamber carbon dioxide concentration (Applied Electrochemistry, CD3A) and pressure (Grass Instruments, PT5) were measured continuously, and the signals were low-pass filtered at 10 Hz, digitized at 20 Hz, smoothed using a moving-average transformation, and stored on a personal computer (World Precision Instruments, Biopac).

Both the chamber and rat body temperature were measured at the end of each step change in CO₂ concentration. Body temperature was measured using an infrared sensor (Linear Laboratories, C1600). For tidal volume calculations, the measured body temperature was corrected to rectal temperature based on previous experiments in which both body temperature determined by infrared emissivity and rectal temperature were measured simultaneously. The average pressure change of each breath, which reflects the magnitude of the tidal volume, was determined from 100 breaths during the last 2 min of each test period (i.e., room air or CO₂). The frequency of breathing was measured during the last minute of each period. Tidal volume was calculated using the method described by Pappenheimer (45).

**Histology.** After completing all experimental conditions, fast green was added to the aCSF and perfused into the RTN for ~1.5 h (a time similar to the duration of a typical experiment) to evaluate the location of each cannula and the distribution of perfusate within the medulla. After the dye was perfused, the rat was deeply anesthetized and perfused transcardially through the left ventricle with a tissue fixative (Streck S.T.F. pH 7.4, Streck Laboratories). After fixation, the brain was removed and placed in 30% sucrose for 1–3 days. The brain was blocked, mounted, flash frozen (Fisher Scientific, Histofreeze), and sectioned (40 μm thickness) with a cryostat maintained at −20°C (Reichert, Histostat). Tissue sections were mounted on poly-l-lysine-coated glass slides, stained with neutral red or cresyl violet, and viewed under brightfield. The images of the serial sections were photographed using a digital camera (Photometrics, Sensys 1400). The images were compared with photographic plates in a stereotaxic atlas to identify the location of the cannulas in each section (48). Each cannula made a small hole in the tissue at the end of the cannula track, and the placement of the cannula was determined by identifying the section with the largest lucent cross-sectional area. The tissue stained by fast green was located directly adjacent to the tip of the guide cannula (~600 μm in diameter) as in previous studies in the RTN (25).

**Experimental design and statistical analysis.** We performed four sets of experiments: two sets of experiments examining CO₂ chemosensitivity and two sets of control studies. In the first set of experiments, we focally perfused each cannula with either 10 μM AZ or aCSF equilibrated with 50% CO₂ (pH 6.5) to determine whether acidification of the RTN increased ventilation in intact animals (31, 39). We perfused aCSF alone (pH 7.48) into the RTN in control experiments. In the second set of experiments, we determined whether carbenoxolone (300 μM) would alter the ventilatory response to CO₂ when focally and unilaterally perfused in the RTN. These first two sets of experiments were done in three groups of animals: young animals aged 7–10 wk, a middle group of animals aged 11–12 wk, and an older group of animals aged 13–19 wk. The ventilatory responses were segregated by age on an a priori basis in light of our previous study (46) in which we found that carbenoxolone, focally perfused within the NTS, reduced the ventilatory response to CO₂ in younger animals (age range 7–10 wk; mean age 8.2 ± 0.5 wk), but not older animals (age range 11–15 wk; mean age 12.6 ± 0.7 wk). In some older animals, it seemed that carbenoxolone focally perfused in the NTS actually enhanced the ventilatory response to CO₂. To examine this possibility explicitly in the current study, we divided the animals >10 wk of age into two groups: a middle group (ages 11–12 wk) and an older group (ages 13–19 wk).

In the first set of control experiments, we perfused the “inactive” analog of carbenoxolone, glycerrhizic acid (GZA; 300 μM) dissolved in aCSF, to control for nonspecific effects of carbenoxolone. GZA is so insoluble in water at body temperature that it can be hard to test equivocal concentrations of carbenoxolone and GZA. Furthermore, carbenoxolone binds to glucocorticoid and mineralocorticoid receptors (67) and, at very low concentrations, inhibits enzymes [e.g., 11-hydroxysteroid dehydrogenase (11-HSD)] that may alter the mineralocorticoid-glucocorticoid balance. To address this issue, we performed a second set of control experiments in which we focally blocked mineralocorticoid receptors and 11-HSD by perfusing the RTN with spironolactone (10 μM) and reexamined the effect of perfusion of carbenoxolone on the ventilatory response to CO₂ in the presence of spironolactone. All compounds were added to aCSF before equilibration with 95% O₂ and 5% CO₂. Within each set of experiments, animals were perfused with aCSF alone or aCSF plus the treatment. No animal was exposed to more than one perfusate per day, and the order in which the perfusates were delivered was randomized within each set of experiments.

Within each study, the effects of animal age, the type of perfusate delivered (i.e., aCSF, aCSF plus test drug), and the FICO₂ (0–8%) were analyzed using an ANOVA. Animal age was a between-subjects factor, and drug treatment and CO₂ levels were repeated within-subjects factors. When the ANOVA indicated that significant differences existed among treatments, specific preplanned comparisons were made using P values adjusted by the Bonferroni method. Comparisons between aCSF and AZ or hypercapnic aCSF at inspired CO₂ levels of 0 and 8% were done using paired t-tests. Values reported are means ± SE. P values ≤0.05 were considered statistically significant.
RESULTS

Ventilatory effects of unilateral acidification of RTN with AZ or hypercapnic aCSF. We analyzed the ventilatory responses to focal unilateral acidification after perfusion of either AZ (10 μM) or hypercapnic aCSF (aCSF equilibrated with 50% CO2, pH ≈6.9) of the RTN in 19 animals. The average minute ventilation, tidal volume, and respiratory frequency during exposure to FICO2 equal 0 and 8% before and after unilateral acidification of the RTN are summarized for each age group in Fig. 1. In addition, the locations of the tip of each cannula within the RTN are shown in a series of cross-sections of the brain stem on Fig. 1, right. The ANOVA performed on the ventilatory responses before and after focal acidification of the RTN did not reveal any dependence of the ventilatory response to CO2 on the age of the animals. In addition, the ventilatory response to AZ at pH 7.48 was indistinguishable from the response to focal hypercapnic perfusion. Therefore, the data obtained during perfusion of AZ and hypercapnic aCSF were combined from all age groups for specific statistical comparisons (Fig. 1, right). As the inspired CO2 increased from 0 to 8%, minute ventilation, tidal volume, and respiratory frequency increased significantly (P < 0.001 for all 3 variables). Focal unilateral acidification of the RTN (AZ or focal hypercapnic perfusion) significantly increased minute ventilation, tidal volume, and respiratory frequency under room air conditions compared with aCSF alone (P < 0.002 for all 3 variables). There was, however, no significant change on any of the respiratory variables when focal acidification of the RTN was compared with focal perfusion of aCSF alone, whereas each animal inhaled 8% CO2. Minute ventilation, tidal volume, and frequency were decreased in the young animals, and minute ventilation and tidal volume were increased in the older animals, but these differences failed to reach statistical significance (0.05 < P < 0.10). The locations of all the cannulas in these animals fall within or closely adjacent to the RTN, which extends rostrocaudally from the rostral end of the facial nucleus to the rostral end of the nucleus ambiguus and mediolaterally between the pyramidal tract and the spinotrigeminal tract and is deep to the ventral surface 100–300 μm (49). These results indicate that the RTN is a CO2-chemosensitive area of the brain stem and are consistent with similar studies in adult rats (2, 18, 32, 41).

Fig. 1. Ventilatory effects of focal, unilateral acidification of the retrotrapezoid nucleus (RTN) with either acetazolamide (●) or hypercapnic artificial cerebrospinal fluid (aCSF; ■) in conscious rats are shown above as a function of animal age (n = 19). Pattern of ventilatory responses to CO2 and focal acidification of the RTN were similar across ages, and pooled average responses from all ages (“combined”) are shown at right. In the control period during which the RTN was perfused with aCSF, ventilation, tidal volume, and respiratory frequency all increased significantly as the FICO2 was elevated from 0 to 8% (**main effect of FICO2; P < 0.001 for all 3 variables). Focal unilateral perfusion of the RTN with either acetazolamide (AZ) or hypercapnic aCSF significantly increased minute ventilation, tidal volume, and frequency during normocapnia (**P < 0.01). However, focal acidification of the RTN during hypercapnia did not alter minute ventilation, tidal volume, or frequency compared with focal perfusion of normocapnic aCSF alone during hypercapnic stimulation. Sites of focal perfusion of AZ or hypercapnic aCSF within the RTN are shown on a series of schematic drawings of cross-sections of the brain stem. ■, Young animals; ♀, middle-age group; ♂, older group. Volume of tissue affected by the perfusion was larger than the size of the symbols. Symbols mark only the location of the tip of the perfusion probe.
Effects of carbenoxolone on CO2 responses. The response to focal perfusion of carbenoxolone differed significantly as a function of age among the animals tested. There were eight animals in the young group (average age = 9.3 ± 0.3 wk), and the ventilatory responses to CO2 with and without focal carbenoxolone perfusion in the RTN are shown in Fig. 2, left. The locations of the cannulas within the RTN in these animals are shown in Fig. 2, right. Increasing the $F_{CO2}$ caused a significant increase in minute ventilation, tidal volume, and respiratory frequency (all $P < 0.001$) when aCSF alone or aCSF plus carbenoxolone was perfused in the RTN. There was a significant interaction between CO2 and carbenoxolone treatment within this age group, and specific comparisons between focal perfusion of aCSF and carbenoxolone at each $F_{CO2}$ level revealed a significant reduction in minute ventilation at 8% $F_{CO2}$ ($P < 0.05$). Tidal volumes were significantly depressed at 4 and 8% $F_{CO2}$ (both $P < 0.05$). There was no effect of focal perfusion of carbenoxolone in the RTN on respiratory frequency.

We evaluated the effect of focal perfusion of carbenoxolone in the RTN in the middle-age group of animals ($n = 8$; mean age 11.1 ± 0.1 wk). In this group, hypercapnia remained a potent respiratory stimulus during all of the drug treatments; minute ventilation, tidal volume, and frequency all increased significantly as the $F_{CO2}$ increased ($P < 0.001$ for all 3 variables as shown in Fig. 3, left). However, there was no effect of focal perfusion of carbenoxolone on minute ventilation, tidal volume, or respiratory frequency at any level of $F_{CO2}$. The locations of the cannulas in these eight animals are shown in Fig. 3, right. The locations of the cannulas were not different from the locations in younger animals.

In the older group, which consisted of eight animals with a mean age of 14.5 ± 0.4 wk, minute ventilation and tidal volume were actually increased during focal perfusion of carbenoxolone compared with perfusion with aCSF alone at inspired CO2 values of 4, 6, and 8% ($P < 0.05$ for all comparisons). The respiratory frequency was not altered by focal perfusion of carbenoxolone into the RTN. As was true in all age groups, increasing the inspired CO2 incrementally from 0 to 8% significantly increased minute ventilation, tidal volume, and respiratory frequency in both treatment conditions ($P < 0.001$ for all variables; Fig. 4).

The ANOVA also indicated that the ventilatory and tidal volume responses to carbenoxolone differed significantly among age groups independent of the level of inspired CO2; there were significant interactions between age group and carbenoxolone treatment with respect to minute ventilation and tidal volume ($P = 0.007$ and $P = 0.020$, respectively). Furthermore, the magnitude of the carbenoxolone effect varied as the level of ventilation varied (there were larger absolute changes after carbenoxolone treatment at higher levels of ventilation). Therefore, the consistent, but small, changes in minute ventilation and tidal volume, present when the $F_{CO2}$ was low, were not well represented in the previous analysis. To assess the drug response without the interfering effect of CO2, we expressed the ventilatory responses to carbenoxolone as a function of the CO2 concentration (Fig. 2).
percent of the ventilatory response to focal perfusion of aCSF alone in each animal at each level of FiCO₂. This has the further advantage that the magnitude of the drug effect can be examined across conditions in which the changing levels of ventilation have been factored out. The results of this analysis are shown in Fig. 5. It is apparent that carbenoxolone generally changed minute ventilation and tidal volume by similar relative amounts across all FiCO₂ levels. Focal perfusion of carbenoxolone into the RTN decreased minute ventilation and tidal volume by ∼17% in the young group of animals across all CO₂ levels (P < 0.001 for both variables), but did not alter the respiratory frequency. In contrast, unilateral perfusion of carbenoxolone in the RTN in the older group of animals increased minute ventilation by ∼42% (P < 0.001), increased tidal volume by ∼28% (P < 0.001), and increased the respiratory frequency by ∼8% (P = 0.02) when analyzed across all FiCO₂ values. Minute ventilation, tidal volume, and frequency were not affected by carbenoxolone treatment in the middle group of animals.

Control studies: an analysis of “misses.” In our previous study of the effect of focal perfusion of carbenoxolone in the NTS, the perfusion cannulas frequently missed the NTS, and these misses constituted an anatomic control group (46). The RTN is an easier target to hit, and, as a consequence, we have many fewer misses, only three. The ages of these animals were 9, 9, and 18 wk. Across all inspired CO₂ values, carbenoxolone (300 μM) perfused focally outside the RTN changed minute ventilation <1% in these animals (data not shown). Tidal volume and respiratory frequency were similarly unaffected by carbenoxolone treatment. Thus carbenoxolone treatment had no effect on the minute ventilation, tidal volume, or respiratory frequency response to CO₂ in these three animals.

Control studies: the effect of unilateral perfusion of GZA in the RTN. GZA is a congener of carbenoxolone, and it has been used as a control substance to test for nonspecific effects of carbenoxolone (46, 63). We studied the effect of unilateral perfusion of GZA in the RTN on ventilatory responses to CO₂ in five animals (mean age 10.2 ± 0.9 wk). There was no effect of GZA on minute ventilation, tidal volume, or respiratory frequency (Fig. 6). However, incremental elevation of the FiCO₂ remained an effective stimulus, and minute ventilation, tidal volume, and the respiratory frequency rose significantly during focal perfusion with either aCSF or aCSF plus GZA (P < 0.001 for all 3 variables).

We conducted additional control studies to ensure that the effect of carbenoxolone was not mediated through mineralocorticoid effects in the RTN. We compared the response to increasing inspired CO₂ during unilateral perfusion of the RTN among three conditions: aCSF alone, aCSF plus spironolactone (10 μM), and spironolactone plus carbenoxolone in aCSF. Carbenoxolone alone had the largest effect in older animals in the preceding studies. Therefore, we examined the effect of
spironolactone in a subset of the older animals because any confounding effect of spironolactone should have been most apparent in this group. When we did study spironolactone and carbenoxolone in three younger animals, we saw no evidence that spironolactone blocked the effect of carbenoxolone in these animals (data not shown). The results of the studies in six older animals (average age 14.2 ± 0.4 wk) are summarized in Fig. 7. The stimulatory effect of increasing FICO2 was apparent in all the respiratory variables (P < 0.001 in all cases). Unilateral perfusion of the RTN with spironolactone alone did not alter the ventilatory response to CO2 compared with aCSF alone. Minute ventilation and tidal volume were significantly increased during perfusion with both spironolactone and carbenoxolone. Specific comparisons indicated that both tidal volume and minute ventilation were increased at an FICO2 of 6% (P < 0.05 for both variables). We also expressed the ventilatory responses as a percent of the control response during aCSF perfusion of the RTN. The results of this analysis are also shown in Fig. 7, right. Spironolactone alone did not alter any of the respiratory variables. Carbenoxolone, even when combined with spironolactone, increased minute ventilation by ~48% (P < 0.001) and increased tidal volume by ~36% (P < 0.001). The respiratory frequency was not altered by combined carbenoxolone and spironolactone treatment in this group of animals. This is the same pattern and magnitude of responses seen in the older animals when treated with carbenoxolone alone. Therefore, there is no evidence that the carbenoxolone effects were mediated by mineralocorticoid receptor activation.

DISCUSSION

There are two essential findings in this study. First, the portion of the RTN in which we placed perfusion probes is sensitive to CO2. Second, carbenoxolone, a pharmacological inhibitor of gap junctions, blunted the ventilatory response to CO2 when perfused unilaterally into the RTN in awake animals <11 wk of age, but increased the ventilatory response to CO2 in animals >12 wk of age. The effect of blocking gap junctions was specific to carbenoxolone; GZA, an “inactive” congener of carbenoxolone, did not similarly affect the ventilatory response to CO2 in older animals, and the response was not prevented by blocking mineralocorticoid receptors with spironolactone.

Stimulation of the RTN increases ventilation in the conscious rat. The RTN was identified as a respiratory-related site in the brain stem as a result of retrograde labeling studies in which wheat germ agglutinin-horseradish peroxidase was injected into the dorsal and ventral respiratory groups in cats (61). Subsequently, lesion studies in anesthetized animals revealed that the RTN contributed to CO2 chemosensitivity in rats (40).
The effects of unilateral lesions are more modest in intact animals studied during wakefulness than in anesthetized animals, but lesions in the RTN still reduced the ventilatory response to 7% FICO2 in intact animals (2). Furthermore, unilateral focal stimulation of the RTN by microdialysis of hypercapnic saline (25% CO2) into the RTN increased ventilation during wakefulness, but not during sleep (32). Our results are consistent with the foregoing studies. Focal perfusion in the RTN with hypercapnic aCSF (equilibrated with 50% CO2) or aCSF containing AZ increased minute ventilation, tidal volume, and respiratory frequency in conscious animals. Acetazolamide treatment and hypercapnic aCSF both generate an extracellular acidosis and probably an intracellular acidosis (9). In our sample, the response to both treatments was similar. Unlike the results of Li et al. (32), minute ventilation increased as a result of both increased tidal volume and increased respiratory frequency.

The combination of an FICO2 of 8% and focal dialysis of AZ or hypercapnic aCSF did not further increase minute ventilation, tidal volume, or respiratory frequency above values achieved in the control condition when the FICO2 was increased to 8%, but the RTN was perfused with aCSF. Failure of focal AZ or hypercapnic aCSF to stimulate ventilation when the inspired CO2 was elevated may indicate that the focal pH changes associated with the perfusion treatments in the region of the microperfusion probe were not much different from the pH change associated with an FICO2 of 8% alone or the neuronal response was already maximal at the FICO2 of 8% (31). With the use of AZ alone, unilateral microperfusion of the NTS also increased minute ventilation during room air breathing, but had a more modest effect when the FICO2 was increased to 8% (46). In summary, there are differences among studies in respect to the combination of tidal volume and frequency responses elicited by focal acidic stimulation of the RTN, but all of the studies cited support the thesis that the RTN contributes to the ventilatory response to CO2 in awake animals.

Fig. 5. Effect of carbenoxolone on minute ventilation, tidal volume, and frequency has been expressed as a percent of control measurements at each FICO2 level in each age group. This analysis demonstrates that across all FICO2 levels, carbenoxolone decreased minute ventilation and tidal volume in the young animals compared with the control condition in these animals (*main effect of carbenoxolone treatment: P < 0.05), had no effect on breathing in the middle age animal group, and increased minute ventilation, tidal volume, and respiratory frequency in the older animals compared with the control condition in these animals (*main effect of carbenoxolone treatment: P < 0.05). Arrows indicate the magnitude and direction of the average effect of carbenoxolone treatment across all CO2 levels in young (down arrows) and older animals (up arrows).

Fig. 6. Ventilatory effects of focal, unilateral perfusion of glycyrrhizic acid (GZA) in the RTN of conscious, rats (aged 8–13 wk) are shown. Carbenoxolone treatment had no effect on minute ventilation, tidal volume, or respiratory frequency at any level of FICO2. Elevating the FICO2 from 0 to 8% increased minute ventilation, tidal volume, and respiratory frequency whether GZA was present in the perfusate or not (***P < 0.001).
Effect of carbenoxolone on the ventilatory response to CO₂.

The ventilatory response to unilateral perfusion of carbenoxolone (300 μM) was biphasic: carbenoxolone reduced the ventilatory response to CO₂ in younger animals aged 7–10 wk, had no effect during a brief 2-wk age range (11–12 wk), and actually stimulated ventilation in older animals aged 13–19 wk. In striking contrast, the RTN was consistently stimulated by focal acidification across all ages tested. This finding raises at least two issues: how is carbenoxolone altering the ventilatory response to CO₂, and why should this effect vary as a function of animal age? Carbenoxolone disrupts gap junctions. Therefore, any explanation for the biphasic response to carbenoxolone must involve a change in the function of gap junctions within the RTN over the age range of the animals that we studied.

Development of chemical and electrical synapses. The mechanisms of synaptic communication change during development. Electrical synapses are ubiquitous early in development, but diminish in number (but do not disappear) as animals grow older (26, 62, 64). Chemical synapses seem to be less important early in development, but come to dominate interneuronal communication as animals mature.

Gap junctions are common in the central nervous system in the early postnatal period, and electrical coupling and dye-coupling are extensive. As many as 60–80 neurons may be connected by gap junctions (10, 68). Over the first weeks of life in rodents, the fraction of neurons that are electrically coupled diminishes and the extent of coupling decreases so that it is unusual to find more than two neurons in any electrically coupled aggregate in juvenile animals (8, 10, 36). Although the number of gap junctions and the extent of coupling declines as animals mature, morphological and electrophysiologi-

and adult brain (8, 10, 26, 62, 64). For example, expression of connexin 32 (Cx32) mRNA, which appeared in brain stem nuclei by postnatal day 3, continued to increase until postnatal day 30 when the level of message reached a plateau and remained stable during adulthood (15). Expression of astrocyte-specific connexins, Cx30 and Cx43, also increased progressively over the first 2–3 wk of life and remained elevated throughout adulthood similar to expression of Cx32 (1, 54). Within the brain stem, Cx36 shows the most dramatic developmental regulation: it is abundant in the early postnatal period but declines as animals mature (26). Nonetheless, all of the connexins studied to date have some detectable expression levels in adults. The function of gap junctions in developing animals may be to amplify inputs and enhance the formation of local circuits that will ultimately depend on chemical synaptic transmission (68). The low resistance electrical pathway provided by gap junctions may also synchronize activity among cells and synchronization of multiple cells may coordinate and amplify responses to particular stimuli (7, 12, 14, 42).

Although electrical coupling between neurons diminishes as animals develop (33), chemical synaptic activity increases. Synaptophysin is a major membrane protein of synaptic vesicles that has been used as an immunohistochemical marker for presynaptic terminals associated with chemical synaptic transmis-

sion (5). Rao et al. (52) calculated the change in synaptophysin density as a function of NTS volume during development and found that synaptophysin staining increased threefold between birth and postnatal day 9 and twofold between postnatal days 9 and 70. Furthermore, Miller et al. (37) showed a 1.3-fold increase in presynaptic boutons/unit volume between postnatal day 30 and adulthood in the NTS. The foregoing summary of the development and purpose of electrical and chemical synapses establishes three things: neurons are more
extensively coupled by gap junctions in young animals, gap junctions persist well into adulthood, and chemical synapses, which are less common in very young animals, mature relatively slowly over a time course that extends up to at least 10 wk of age in the rat.

The developmental time courses of maturation of electrical and chemical synapses are consistent with the hypothesis that early in development, electrical coupling plays an important role in CO₂ chemosensory function, but this function may diminish or change as chemical synapses mature. Although we studied “older” animals, these animals fell within an age range during which the function and distribution of chemical and electrical synapses were still changing. We believe that gap junctions synchronize activity among multiple chemosensory cells and amplify the ventilatory response to CO₂ in younger animals. This is consistent with the reduced ventilatory response to CO₂ that we observed after blocking gap junctions with carbenoxolone. Furthermore, gap junctions between CO₂ sensitive neurons are known to exist in other chemosensory sites. Using brain slices prepared from neonatal rats at postnatal days 0 to 21, Huang et al. (27) found electrotonic and anatomical coupling between CO₂-chemosensitive neurons in the NTS. There are no similar electrophysiological studies of gap junctions in the RTN in neonatal animals. Nonetheless, immunohistochemical studies indicate that gap junctions are ubiquitous in the NTS and RTN in neonates (62, 64).

In older animals, it seems more likely that carbenoxolone is disrupting the function of inhibitory processes. Gap junctions seem to play a particularly prominent role in inhibitory GABAergic networks in adult animals in the cortex (21) and brain stem (34). For example, connexins 32, 36, and 43 are associated with parvalbumin-positive neurons in the cortex (51); parvalbumin is a marker for GABAergic neurons. Within the RTN, GABAergic mechanisms seem to inhibit minute ventilation in adult rats (38). Moreover, the GABAergic input seems to limit tidal volume rather than respiratory frequency, just as we found when carbenoxolone blocked gap junctions in the older age group. Thus gap junctions are associated with GABAergic inhibitory networks; GABAergic mechanisms within the RTN seem to inhibit ventilation and the ventilatory response to CO₂; Cx26, Cx32, and Cx36 are present in the RTN in adult rats (62, 64); and in our own studies with carbenoxolone, disruption of gap junctions dis inhibited the ventilatory response to CO₂ in older animals. Finally, hypercapnia seemed to activate a set of neurons in the ventrolateral medulla (c-Fos-positive cells) in neonatal piglets that were also positive for parvalbumin (69). Similar dual-labeled cells were not found in the NTS. Thus it is possible that hypercapnia activates GABAergic neurons in the ventrolateral medulla, but not in the NTS, and this may be the reason that we found no disinhibition of the ventilatory response to CO₂ in older animals when carbenoxolone was microperfused into the NTS (46). Fortunately, the RTN was just outside the region studied by dual labeling of c-Fos and parvalbumin after hypercapnic stimulation (69). Therefore, we lack complete anatomic information in the ventrolateral medulla about the colocalization of hypercapnia-activated cells and parvalbumin-positive cells.

In summary, it is our hypothesis that carbenoxolone disrupts electrical synapses among chemosensory neurons in younger animals. We believe that electrical synapses supplement and enhance the emerging activity of chemical synapses, but the importance of electrical synapses wanes among CO₂ chemosensory neurons as the animals mature. As a consequence, the inhibitory effect of carbenoxolone diminishes as the animals mature. We believe that gap junctions are more specifically associated with inhibitory GABAergic networks in older animals, but these are not fully developed before 12 wk of age. The GABAergic inhibitory network modulates the ventilatory response to CO₂, and the efficacy of GABAergic networks is enhanced by synchronization of network activity. To the extent carbenoxolone disrupts synchronization, the inhibitory potency of GABAergic networks within the brain stem will be reduced, and the ventilatory response to CO₂ diminished. If our hypothesis is correct, gap junctions actually enhance inhibitory chemical synaptic efficiency by synchronizing activity among neurons in the GABAergic network in older animals.

Limitations of the methods. There are significant limitations in our study and our conception of how carbenoxolone may effect changes in the ventilatory response to CO₂. First, gap junctions are not uniquely interneuronal: they exist between astrocytes and between oligodendrocytes (53) and possibly between neurons and astrocytes (3, 54). Carbenoxolone has no specificity for particular connexins or particular classes of intercellular gap junctions. We believe that astrocytes play an important role in central CO₂ chemosensitivity, particularly in the RTN (17, 25). Astrocytes may amplify the ventilatory responses to CO₂ by modifying the extracellular pH in the region of chemosensory neurons. If carbenoxolone interfered with astrocytic spatial buffering of pH, the ventilatory response to a given CO₂ level might be altered or the pH modulation of neuronal gap junctions might be changed (65). Thus the ventilatory changes after carbenoxolone treatment that we observed might arise from disruption of connexins between astrocytes rather than interneuronal gap junctions. However, astrocytic gap junctions are well represented in the brain stem in juvenile and adult animals, and we are not aware of any developmental changes in the function of astrocytic gap junctions over the age range we studied. Therefore, we would have expected similar carbenoxolone effects in younger and older animals were carbenoxolone modifying the ventilatory response to CO₂ by an astrocytic mechanism.

In any drug study, one must be concerned that the putative drug effect is nonspecific, and carbenoxolone may modify neuronal function in specific nuclei or specific reduced systems through processes that do not involve gap junctions (56, 58). However, other authors studying gap junctions in more intact systems, found that carbenoxolone blocked gap junction reversibly without affecting membrane potential, input resistance, or excitability in neurons in the NTS, locus ceruleus, and type II cells in the pre-Bötzingher complex (11, 14, 50, 66). Furthermore, two aspects of our study argue against a nonspecific effect of carbenoxolone. First, focal perfusion of GZA did not alter the ventilatory or tidal volume response to CO₂, whereas carbenoxolone did, and carbenoxolone disrupts gap junctions, but GZA does not. Also, spiironolactone did not block the effect of carbenoxolone. Second, we would have expected any nonspecific effect of carbenoxolone to be consistent across all age groups, which is contrary to what we actually found.

Our findings do not establish any lower age limit on the excitation actions of gap junctions in the ventilatory response
to CO₂. Animals <7 wk of age were too small to be tested with our perfusion probes. Given the patterns of electrical and chemical synaptic development, we certainly expect carbeneoxolone to decrease the ventilatory response to CO₂ in animals <7 wk, but we did not perform those experiments.

**Limitations of the hypothesis.** We have no information about the specific mechanistic details whereby gap junctions might achieve the effects we observed in intact animals. In younger animals, we do not know for certain that CO₂ chemosensory cells are synchronized within the RTN and, even if they are synchronized, synchronization of electrical activity may not alter CO₂ chemosensitivities. In the locus ceruleus, the activity of CO₂ chemosensory cells appears to be synchronized by gap junctions, but carbeneoxolone treatment, which disrupted electrical synchronization in these neurons, did not alter the capacity of individual neurons to respond to CO₂ (4). In light of the current findings, this may indicate that synchronization among neurons amplifies CO₂ chemosensory responses, but the presence or absence of gap junctions may not alter the intrinsic CO₂ sensitivity of individual neurons.

In older animals, we have no evidence that gap junctions are actually associated with GABAergic inhibitory networks in the RTN, and we know of no studies demonstrating that synchronization of GABAergic neurons within the RTN occurs. A GABAergic hypothesis is attractive because the primary effect of carbeneoxolone was disinhibitory in older animals. However, GABAergic activation need not be exclusively inhibitory (28). Furthermore, synchronization of inhibitory neurons may also depend on glutamatergic inputs (29, 35), and nothing in our data points exclusively to one neurotransmitter or another.

Finally, we do not know for certain that the gap junction effect is really specific to CO₂ sensitivity. The blunted response to CO₂ might result from an effect of gap junctions on nonchemosensory neurons that provide a tonic drive to either CO₂ chemosensory cells or other elements in the respiratory control system. Many studies indicate that neurons within the ventral medulla, including the RTN, may provide such a tonic drive to ventilation (20, 38, 43). In summary, we do not have any evidence that carbeneoxolone disrupted the actual cellular process of CO₂ chemotransduction within the RTN.

**Perspectives**

Gap junctions may synchronize or coordinate CO₂ chemosensory responses to sustain the ventilatory response to CO₂ when chemical synaptic connectivity is not fully established. Once chemical synaptic function is fully established, the role of gap junctions in CO₂ chemosensitivities seems to wane. We recently reported that carbeneoxolone focally perfused into the NTS also inhibited the ventilatory response to CO₂ in animals <10 wk of age. Thus gap junctions seem to enhance or amplify the ventilatory response to CO₂ in younger animals in both the RTN and NTS. Gap junctions are present in all CO₂ chemosensory sites identified to date, and gap junctions may enhance chemosensory responsiveness in all CO₂ chemosensory sites in younger animals.

In older animals, blockade of gap junctions in the RTN disinhibited the ventilatory response to CO₂, but we did not find a similar disinhibition of the ventilatory response to CO₂ when we focally perfused carbeneoxolone into the NTS in animals of similar age. Thus the responses to carbeneoxolone within the RTN and NTS, although both sites are sensitive to CO₂, diverge in older animals. The heterogeneous nature of the response to carbeneoxolone in older animals suggests that there ought to be differences between the RTN and NTS with respect to the distribution of gap junctions, the connexin types expressed, patterns of cellular expression, or association of connexin types with particular neurotransmitters. Moreover, the divergent ventilatory responses to carbeneoxolone between the RTN and NTS are further evidence that the nature of the chemosensory process, the particular value of the chemosensory stimulus, the role of individual chemosensory sites in different states of wakefulness and sleep, and the processing of chemosensory information differ among chemosensory sites despite, at least nominally, a similar function (17, 19). Why multiple sites in the brain stem should have a similar function but achieve this function by diverse mechanisms is unresolved at this time.

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