Hollerman, Julianne, Melissa Babbie, and Joseph S. Erlichman. Ventilatory effects of impaired glial function in a brain stem chemoreceptor region in the conscious rat. J Appl Physiol 90: 1539–1547, 2001.—Glia are thought to regulate ion homeostasis, including extracellular pH; however, their role in modulating central CO2 chemosensitivity is unclear. Using a push-pull cannula in chronically instrumented and conscious rats, we administered a glial toxin, fluorocitrate (FC; 1 mM) into the retrotrapezoid nucleus (RTN), a putative chemosensitive site, during normocapnia and hypercapnia. FC exposure significantly increased expired minute ventilation (Ve) to a value 38% above the control level during normocapnia. During hypercapnia, FC also significantly increased both breathing frequency and expired Ve. During FC administration, maximal ventilation was achieved at ~4% CO2 compared with 8–10% CO2 during control hypercapnic trials. RTN perfusion of control solutions had little effect on any ventilatory measures (Ve, tidal volume, or breathing frequency) during normocapnic or hypercapnic conditions. We conclude that unilateral impairment of glial function in the RTN of the conscious rat results in stimulation of respiratory output.

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level during fluorocitrate perfusion. Our findings support our hypothesis that glia in the region of the RTN play an important role in respiratory control in the conscious rat.

**METHODS**

The care of the animals and the experimental protocol used in this study were approved by the institution’s Animal Review Committee. Adult Sprague-Dawley rats of either sex, weighing between 350 and 450 g, were used for this study. Animals were kept on a 12:12-h light/dark cycle and provided food and water ad libitum.

Rats were initially anesthetized with an intraperitoneal injection of a mixture of ketamine-HCl (70 mg/kg) and xylazine (6 mg/kg). Before surgery, the depth of anesthesia was determined by the lack of a pinch reflex in the hind paw. In the event that an animal needed additional anesthesia during the course of the surgery, one-fourth to one-third of the original dose of ketamine-xylazine was administered.

The scalp was shaved, and the animal was secured in a stereotaxic apparatus (Kopf Instruments). A midline incision was made, and a hole was drilled in the skull 2 mm caudal to lambda and 1.5 mm lateral to midline. Three additional holes were drilled into the skull for placement of anchor screws. A push-pull guide cannula (0.29 mm ID, 0.56 mm OD; Plastics One, Roanoke, VA) was placed with the tip dorsal to the RTN level during fluorocitrate perfusion. Our findings support our hypothesis that glia in the region of the RTN play an important role in respiratory control in the conscious rat.

The guide cannula and the anchor screws were attached to the skull with cranioplast (Plastics One), and care was taken to ensure that the exposed end of the guide cannula was free of the acrylic cement. Rough spots in the cement were leveled, and the scalp was sutured up to the border of the cement cap. A dummy cannula was screwed into the guide cannula, the return cannula to maintain patency of the lumen during the recovery period and between experiments. The dummy cannula extended 0.3 mm beyond the end of the guide cannula.

After surgery, animals were examined for signs of infection, such as redness, swelling, and discharge. In one instance, an animal developed signs of infection and was given oral antibiotics. This animal and two others who pulled out their cannulas during the recovery period were excluded from the study. Approximately 3–4 wk after surgery, ventilatory measurements were made on the remaining six animals.

**Solutions.** A 1 mM fluorocitrate solution was prepared as previously described (11). Briefly, the barium salt of fluorocitric acid was dissolved in 0.1 M HCl, and the barium was precipitated by the addition of Na2SO4 (0.1 M) to the solution. This solution was then buffered with NaHCO3 (0.1 M) and centrifuged at 800 g for 10 min. The supernatant was removed and added to artificial cerebral spinal fluid (aCSF).

aCSF was composed of the following salts (in mM): 124 NaCl, 5 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. For citrate control experiments, citrate was dissolved directly into aCSF at a final concentration of 1 mM.

**Solution delivery.** Solutions were delivered to the RTN by 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; 0.1 mm ID, 0.2 mm OD). Before perfusate delivery, the line and inner cannula were flushed with either aCSF, aCSF + citrate, or aCSF + fluorocitrate. The inner cannula was then screwed into the guide cannula, the return vaco line was connected (Fig. 1B), and the animal was placed in a whole body plethysmograph. 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 0.06 ml/h. Test solutions were continuously perfused throughout the experiment.

**Experimental design.** Baseline, presurgical ventilatory measurements were made in each rat before cannulation during normocapnic conditions. Animals were placed in the plethysmograph and allowed to accommodate to the chamber for 30 min before the chamber CO2 was elevated. In cannulated animals, perfusion was initiated at the beginning of the 30-min accommodation period in the plethysmograph and was terminated after the animal was exposed to incremental hypercapnia. Cannulated animals were perfused with either aCSF alone, aCSF + citrate, or aCSF + fluorocitrate, or no perfusate was delivered. All rats were exposed to each experimental treatment. No animal was exposed to more than one perfusate per day, and the order that the perfusates were delivered was randomized. Body weight and barometric pressure were determined before each experiment. After the completion of all experimental conditions, fast green was added to aCSF, and this solution was perfused into the RTN for a similar amount of time as the experimental trials (~1.5 h) to evaluate the distribution of perfusate within the medulla.

**Ventilatory responses to CO2.** The rats were exposed to increasing levels of inspired CO2 in the plethysmograph, beginning with room air and progressing in 2% increments to a final concentration of ~10% CO2. Before delivery into the chamber, the compressed air and CO2 were mixed and humidified by bubbling of the air through a water column. Animals were maintained at each level of CO2 for ~10 min after the stabilization of CO2 levels within the plethysmograph chamber. The chamber had an approximate volume of 5 liters, and the rate of air flow through the box was 1.5–2 l/min. Stable CO2 values within the chamber were achieved in 3–4 min.

![Fig. 1.](http://jap.physiology.org/) Inner delivery cannula (A) and outer guide cannula (B). The inner delivery cannula has an inner diameter of 0.1 mm and an outer diameter of 0.2 mm. The outer guide cannula has an inner diameter of 0.29 mm and an outer diameter of 0.56 mm. Solutions were delivered through the cannula using a push-pull syringe pump.
Changes in chamber CO₂ (CD3A, Applied Electrochemistry) and pressure (PT5, Grass Instruments) were measured continuously, digitized, and stored on a personal computer (Biopac, World Precision Instruments). Both the chamber and rat body temperature were measured at the end of each step change in CO₂ concentration. Body temperature was measured by using an infrared sensor (C1600, Linear Laboratories). For tidal volume (VT) calculations, this value was measured by using an infrared sensor (C1600, Linear Labo-

mograph chamber temperature (K), and T₂, is body tem-

perature (K). Typical pressure tracings using this method are

shown in Fig. 2.

Histology. Examination of the histological sections revealed that the placement of the terminal portion of the cannula was either in the RTN or in the superficial

margin of neighboring nuclei (Fig. 3). Tissue staining with Fast Green outside the site of the lesion was minimal and present only on the lateral margins of the

lesion, suggesting that the mobility of the perfusate was restricted to the tissue adjacent to the cannula.

Effects of surgery on ventilation. The presurgical values for VT per kilogram, Vₖ per kilogram, and breathing frequency were not significantly different among the no perfusion, αCSF, or αCSF + citrate conditions during normocapnia (P ≈ 0.24). The mean CO₂ concentration in the plethysmograph, while room air was delivered to the chamber at a rate of 1.5–2 l/min, was 0.58 ± 0.08% and was likely the result of incomplete washout of expired air from the chamber. VT per kilogram, Vₖ per kilogram, and breathing frequency were not different during unilateral perfusion in the RTN among the no perfusion, αCSF perfusion, or αCSF + citrate conditions across CO₂ levels (main effect of perfusion, P ≈ 0.10; CO₂, P ≈ 0.02; interaction of flow and CO₂, P ≈ 0.38). For control conditions (i.e., no flow, αCSF alone, αCSF + citrate), the increase in maximal Vₖ during hypercapnia was the result of an ~50% increase in frequency and a 50% increase in Vₖ.

Effects of perfusate type on ventilation. Although hypercapnia increased Vₖ in all experimental conditions, Vₖ was significantly greater during αCSF + fluorocitrate treatment compared with other conditions (P = 0.007; Fig. 4). In addition, there was no statistical difference in

2% CO₂

A

B

6% CO₂

A

B

8% CO₂

A

B

Fig. 2. Typical pressure measurements collected using the whole body plethysmograph. A: plots utilizing raw pressure values. B: plots of the same data at a higher gain that have been smoothed using a moving average transformation. Bar = 5 s.
Post hoc analysis revealed that expired V˙E during the fluorocitrate condition was significantly higher compared with control conditions at all CO2 levels tested (P ≤ 0.025), and the control conditions were not significantly different from each other (P ≥ 0.51). During the fluorocitrate trial, all animals were capable of tolerating elevations in CO2 up to ~4% (Fig. 5). In three of six rats, the experiment was terminated at the end of the 4% CO2 condition (Fig. 5, C, E, and F) due to apparent respiratory distress experienced by the animal. This discomfort was manifest as one of two behaviors: either the rat showed labored breathing while on its side and was incapable of righting itself (2 of 3 rats) or the rat attempted to chew through the rubber stoppers used as sampling ports in the side of the plethysmograph (1 of 3 rats). All three of these animals recovered when the level of hypercapnia was reduced. None of these behaviors was observed during any of the other trials (i.e., no flow, aCSF, or aCSF + citrate), implicating fluorocitrate as the probable cause.

Statistical comparisons of V˙E per kilogram during fluorocitrate exposure at 4 vs. ~10% CO2 during control conditions (i.e., aCSF and aCSF + citrate) revealed that V˙E was not statistically different between these conditions, suggesting that animals had reached their maximal ventilation (P ≥ 0.84). Although three of six rats tolerated higher levels of CO2, none of the animals could achieve the same maximal level of CO2 used during perfusions with aCSF or aCSF + citrate, and little change in ventilation was evident at CO2 concentrations >4% (Fig. 5, A, B, and D).

Because V˙E is a product of both frequency of breathing and VT, both factors could account for the increase in V˙E per kilogram during fluorocitrate perfusion. STATISTICAL ANALYSIS showed that the increase in V˙E was due principally to an increase in breathing frequency evident at all CO2 concentrations during fluorocitrate
perfusion ($P = 0.01$; Fig. 6). There was no significant change in frequency across CO$_2$ levels in any of the groups ($P = 0.87$). The breathing frequencies associated with control conditions were significantly lower than those of the fluorocitrate trials ($P \leq 0.04$). The aCSF + citrate resulted in higher breathing frequencies that approached significance compared with trials with no perfusate or aCSF alone ($P = 0.08$). Although fluorocitrate resulted in an increase in both $V_E$ per kilogram and breathing frequency, it had less of an effect on VT per kilogram. Hypercapnia resulted in an increased VT per kilogram; however, this change was not statistically significant and there was no statistical effect of the type of perfusate or the interaction between these terms ($P \geq 0.10$; Fig. 7). Body temperature decreased slightly during hypercapnia in all groups; however, this decrease was not statistically significant ($P = 0.24$) nor were there statistical differences among treatments ($P = 0.13$). The effect of hypercapnia on body temperature for all animals and conditions is shown in Fig. 8.

**DISCUSSION**

**Role of glia in central chemoreception.** The ventilatory effects that we observed with fluorocitrate perfusion into the RTN were marked. At all levels of CO$_2$, $V_E$ and breathing frequency were statistically higher compared with other conditions. Our observation that maximal $V_E$ was not different between the fluorocitrate trials during 4% CO$_2$ exposure compared with control trials (aCSF, aCSF + citrate) utilizing 8–10% CO$_2$ indicates that the rats attained the same maximal ventilation albeit at a lower CO$_2$ during fluorocitrate perfusion. Although one-half of the animals in this study could tolerate higher levels of CO$_2$ >4%, the
small, additional increase in \( \dot{V}\text{E} \) was not significant, indicating that maximal ventilation had been reached.

Similar to previous reports, maximal ventilation in all trials except fluorocitrate was observed between 8 and 10% \( \text{CO}_2 \) (2, 4, 16). Our data showed a significant increase in \( \dot{V}\text{E} \) per kilogram during hypercapnia for all groups that was due principally to an increase in breathing frequency. \( V_T \) also increased during hypercapnia; however, this was most evident at \( \text{CO}_2 \) levels >4%. The change in \( V_T \) was incremental and more variable during mild hypercapnia (2 and 4% \( \text{CO}_2 \)), and the change in \( V_T \) was not statistically significant, even though resting and maximal \( V_T \) values were different from each other and similar to those reported by others (2, 16, 28).

The precise mechanism underlying the selective nature of fluorocitrate impairing glial function is not known. Previous studies in vitro have shown that fluorocitrate rapidly decreases ATP and glutamine levels by inhibiting the tricarboxylic acid cycle enzyme aconitase (13). In both the hippocampus and the medulla, changes in neuronal morphology and function during fluorocitrate perfusion in situ are minimal when tissue is exposed to this agent for <4 h at 1 mM concentrations (11, 17). Here we have shown that the ventilatory effects of this compound are reversible, suggesting that the ability of fluorocitrate to impair glial function is likely restricted to the time during exposure. This is supported by our findings that the functional effects of fluorocitrate impairment are not carried over from one trial to another.

There is increasing evidence suggesting that glia can influence neuronal function by at least three mechanisms. First, neuronal excitability can be altered by glial uptake of glutamate from the synaptic cleft (3). Second, the bidirectional, \( \text{Ca}^{2+} \)-dependent communication reported between glia and neurons appears to play a role in synaptic plasticity (39). Third, glia appear to regulate the extracellular ionic milieu surrounding neurons (11, 17, 18, 27). In the case of central chemoreceptors, changes in accumulation of neurotransmitters, \( \text{K}^+ \), or protons could all potentially alter chemoreceptor function, thereby altering ventilatory control. Regarding the role of \( \text{pH} \), we showed previously that transient impairment of glial function in the RTN in the anesthetized rat led to a rapid and reversible acidification of the extracellular space that correlated well with increased phrenic nerve activity (11). Although the extent to which changes in \( \text{pH}_i \) or \( \text{pH}_a \) mediate central chemoreceptor activity is unknown, it has been shown that the application of other acidifying stimuli in the region of the RTN increases ventilation during normocapnia (6, 22). Hence, it is intriguing to consider the role glia may play in \( \text{pH}_a \) regulation. Previously, Deitmer (8) showed that \( \text{pH}_a \) buffering in the leech neuropile was dependent on the availability of bicar-
bonate and linked to the Na\(^+\)-HCO\(_3\) cotransporter present in the glial membrane. Na\(^+\)-HCO\(_3\) cotransport has been described in many vertebrate glial cell types; however, this transporter has not been identified in neurons (35). An essential feature of this proton transporter is that it is electrogenic, having a stoichiometry of 1 (Na\(^+\)):2 or 3 (HCO\(_3\)), depending on the cell type. The reversal potential for this transporter is near the resting membrane potential of most glia. Therefore, membrane hyperpolarization in RTN glia may result in intracellular acidification, whereas membrane depolarization may lead to intracellular alkalinization. Thus the linkage of glial membrane potential and pH\(_i\) may enable the glia to influence pH\(_e\) by transporting acid equivalents between the extracellular and intracellular compartments (9, 32). Recently, Largo et al. (18) showed that glial cells depolarized in the hippocampus in the presence of fluorocitrate in a time-dependent manner. In light of this finding and our previous observations, this suggests that the metabolic inhibition generated by fluorocitrate exposure may lead to a decrease in membrane potential in glia in the RTN, resulting in the movement of HCO\(_3\)\(^-\) from the extracellular space into glia, thus leading to extracellular acidification via Na\(^+\)-HCO\(_3\) cotransport. Using medullary brain slices pretreated with kainic acid to generate slices devoid of neurons (24), we are presently examining the proton transport mechanisms present in the RTN glia in vitro and have preliminary data supporting the presence of Na\(^+\)-HCO\(_3\) cotransport. Determining the role of glia in brain stem pH regulation is crucial to our understanding of ventilatory control, given the important role of pH\(_e\) and pH\(_i\) in chemoreceptor function (10, 11, 22, 34).

The findings in this study are consistent with an increase in the gain of the respiratory system rather than an increase in CO\(_2\) sensitivity. Such a change would be expected if there were an acid shift in the extracellular space, consequent to fluorocitrate exposure, that was additive of the acidification associated with CO\(_2\). Previously, our laboratory (11) showed, in the anesthetized rat in situ, that unilateral perfusion of fluorocitrate resulted in an acidification of the extracellular fluid and an increase in phrenic nerve activity of ~43%. Consistent with these findings, we observed a 38% increase in \(\dot{V}E\) during normocapnia with the perfusion of the fluorocitrate into the RTN in the conscious rat in this study. Using a microdialysis probe, Li and Nattie (19) found that focal acidification with CO\(_2\) in the RTN resulted in a 24% increase in \(\dot{V}E\) above baseline in the anesthetized rat. Using electrodes to measure pH\(_{wa}\), they showed that this method resulted in acidification that was largely restricted to the RTN. Here we report a slightly greater increase in resting ventilation with fluorocitrate perfusion. Assuming that the increase in \(\dot{V}E\) is associated with acidification of the extracellular space, resulting from glial impairment, we can speculate either that fluorocitrate results in greater acidification of the RTN or more tissue is acidified, thus affecting additional chemosensitive sites compared with focal hypercapnia. The extent of acidification that our laboratory measured in our previous study (11) using fluorocitrate in the anesthetized rat in situ is similar to the values reported by Li and Nattie (19), suggesting that the effects of fluorocitrate may have extended beyond the RTN rather than result in greater acidification. Although the Fast Green we added to the perfusate was restricted to the tissue directly adjacent to the perfusion pipette, it is unlikely that this stain was reflective of the true mobility of fluorocitrate. Once taken up by glia, fluorocitrate could diffuse to distant sites via intracellular coupling of glia by gap junctions. Labeling of up to 100 astrocytes has been reported after injection of the fluorescent dye Lucifer Yellow into a single glial cell (31). Alternatively, the clearance of fluorocitrate by blood flow may have transported this compound to other areas of the brain stem. Thus it is likely that we have underestimated the region of tissue affected by fluorocitrate in this study.

An important finding of this study was our observation that unilateral impairment of glial function in the RTN of the conscious rat is sufficient to stimulate ventilation. The depressant effects of anesthesia and the state-dependent nature of ventilatory control make it difficult to extend many of the previous findings to the conscious animal (19, 26). In anesthetized and decerebrate animals, destruction of the RTN markedly reduces ventilatory responses to hypercapnia. In contrast, bilateral cooling of rostral regions of the ventral lateral medulla surface in goats results in a sustained apnea under anesthesia but has only modest effects on breathing in the unanesthetized animal. Similarly, unilateral lesions in the RTN attenuate the ventilatory responses to CO\(_2\) much less compared with anesthetized animals (2). Recently, Li and Nattie (19) showed that focal CO\(_2\) stimulation increases ventilation in anesthetized, decerebrate, and conscious, but not sleeping, animals.

**Technical considerations.** The whole body plethysmography method used in this study permits noninvasive and unrestricted ventilatory measurements to be made in the conscious rat. Our presurgical values for \(VT\) per kilogram of 5.78 ml/kg during room-air breathing are similar to those reported previously by Pappenheimer (28) of 5.66 ml/kg in awake rats using a similar technique. In addition, our frequency data of 102 breaths/min are similar to the values reported by both Akilesh et al. (2) and Lai et al. (16). Our observation that maximal \(\dot{V}E\) during hypercapnia was the result of an approximately equal increase in both breathing frequency and \(VT\) is also consistent with previous studies in vivo in rats (2, 19). The decrease in body temperature that we observed in the present study was not statistically significant; however, the magnitude of the decrease was similar to values reported in other studies (19, 21). Although most studies in the rat report a hypothermic response during CO\(_2\) exposure, Saiki and Mortola (36) showed in the rat that decreases in body temperature during hypercapnia are dependent on ambient temperature, suggesting that some of the variability among studies may be attributed to this factor.
In their study, maintaining an ambient temperature of 25°C eliminated decreases in body temperature during hypercapnia. In the present study, the ambient temperature range was 25–29°C.

The effects of cannulation alone on the ventilatory measurements in this study were minimal. Cannulation resulted in a slight decrease in both VT (5.78 vs. 5.63 ml/kg) and frequency (103 vs. 101 breaths/min) in baseline conditions; however, neither of these decreases was statistically significant. In addition, the ventilatory effects of perfusion of the vehicle alone into the RTN, compared with the no perfusion or presurgical values, were not significant. Hence both the vehicle (aCSF) and inactive analog citrate had minimal effects on ventilation during normocapnia. The small increase in breathing frequency that we observed during citrate perfusion may have been due to increased neuronal excitability of cells exposed to this compound. Previous studies have shown that citrate is selectively sequestered, produced, and released by astrocytes (39, 41). Citrate can chelate extracellular zinc and other normally occurring divalent ions, resulting in enhanced neuronal firing (40, 42). Thus the addition of exogenous citrate may have resulted in increased chemoreceptor activity, leading to a slight increase in ventilation. In contrast, the application of fluorocitrate has little effect on biophysical properties of neurons and no known effect on glutamate receptor activity (5, 15, 17, 18).

Summary. Our data suggest that glia in the RTN can dramatically affect the ventilatory responses arising from this chemosensitive site. The increase in ventilation was likely due to a decrease in pHo associated with impaired glial function; an observation that our laboratory has previously reported (11). We hypothesize that glia may regulate pHo possibly through the activation of Na⁺–HCO₃⁻ cotransport, to maintain a stable milieu surrounding central chemoreceptors. Our data support the findings of others (2, 19) that the RTN is an important site in ventilatory control during wakefulness and anesthesia. Together, these findings raise the possibility that the contribution that a given chemoreceptor site may have on shaping central ventilatory control may critically depend on arousal state.

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