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Abstract: The purpose of this study was to test the hypothesis that an intact cerebellar fastigial nucleus (CFN) is an important determinant of CO_2-H^+ sensitivity during wakefulness. Bilateral, stainless steel microtubules were implanted into the CFN (N = 9) for injection (0.5–10 μl) of the neurotoxin ibotenic acid. Two or more weeks after implantation of the microtubules, eupneic breathing and CO_2-H^+ sensitivity did not differ significantly ($P > 0.10$) from pre-implantation conditions. Injection of ibotenic acid (50 mM) did not significantly alter eupneic Pa_{CO2} ($P > 0.10$). The coefficient of variation of eupneic Pa_{CO2} was 4.0±0.6 and 3.7±0.4% over the 2 weeks before and after the lesion, respectively. CO_2-H^+ sensitivity expressed as inspired ventilation/Pa_{CO2} decreased from 2.15±0.17 pre-lesion to 1.58±0.26 l/(min mmHg) 3–6 days post-lesion ($P < 0.02$, −27%). There was no significant ($P > 0.10$) recovery of sensitivity between 7 and 10 days post-lesion. The lesion also increased ($P < 0.05$) the day-to-day variability of this index by nearly 100%. When CO_2 sensitivity was expressed as elevated inspired CO_2/room air $\dot{V}_I$, values at 7%, but not 3 and 5% inspired CO_2, were reduced and more variable ($P < 0.05$) after the ibotenic acid injections. We conclude that during wakefulness, the CFN contributes relatively more to overall ventilatory drive at high relative to low levels of hypercapnia.

Keywords: Control of breathing, Central chemosensitivity, Cerebellum, Hypercapnia, Awake

1. Introduction

There have been several previous studies on the role of cerebellar nuclei in the control of breathing. It was reported in the 1930s that electrical stimulation of the cortex of the anterior cerebellum inhibited carotid-sinus vasopressor and respiratory reflexes produced by carotid-sinus occlusion (Morruzzi, 1939). Subsequently, Stella demonstrated that electrical stimulation of the cerebellar cortex

altered heart rate and respiration, but the alterations were inconsistent (Stella, 1939). Years later, several groups found that electrical stimulation of the cerebellar fastigial nucleus (CFN) under anesthetized conditions, increased respiration, heart rate, and blood pressure in rats, cats, and dogs (Andrezik et al., 1984; Huang et al., 1989; Xu and Frazier, 1997; Xu and Frazier, 2000; Xu et al., 2001a; Asanome et al., 1998). Other investigators removed the cerebellum or lesioned the CFN in anesthetized animals and found attenuated ventilatory responses to systemic hypercapnia, systemic hypoxia, NaCN injections, respiratory load compensation, and to increases in blood pressure and heart rate caused by electrical stimulation of the CFN (Andrezik et al., 1984; Huang et al., 1989; Xu and Frazier, 1997; Asanome et al., 1998). It has also been reported that a focal acidosis in the CFN of anesthetized rats stimulates breathing (Xu et al., 2001b). Accordingly, in anesthetized mammals, it is clear that the CFN is part of the neural network that regulates breathing.

However, the role of the CFN in the control of breathing during the awake state has not been established; thus, we have undertaken a series of studies to gain insight into this topic. To date, we have reported (Martino et al., 2006a) that in awake goats, focal acidosis in the rostra1 CFN generated by micro-dialyzing mock cerebral spinal fluid with 50 and 80% CO_2 significantly increased ($P < 0.05$) inspired ventilation ($\dot{V}_I$) by 16 and 12%, respectively, and significantly increased ($P < 0.05$) heart rate (f_H) by 13 and 9%, respectively. In contrast, focal acidosis in the caudal CFN with 25 and 50% CO_2 significantly decreased ($P < 0.05$) $\dot{V}_I$ by 7 and 10%, respectively. At this CFN site, oxygen consumption was decreased during dialysis with 80% CO_2. These findings indicate that there is a heterogenous population of CO_2-H^+ chemoreceptor neurons in the CFN that affect multiple physiologic functions, including the control of breathing. In goats, we also found that ibotenic acid-induced lesions in the CFN attenuated the hyperpnea required to meet the gas-exchange needs of submaximal exercise (Martino et al., 2006b). These findings indicate that the CFN is part of the neural network regulating breathing during exercise.

Since focal acidosis in the CFN alters breathing, and since lesioning the CFN attenuates the exercise hyperpnea, we hypothesized that lesioning the CFN would also attenuate CO_2-H^+ ventilatory

sensitivity. The purpose of the present study was to test this hypothesis.

2. Methods

Physiologic data were obtained on 1 male and 10 female adult goats weighing 47.7±5.0 kg. The goats were housed and studied in an environmental chamber with a fixed ambient temperature and photoperiod. All goats were allowed free access to hay and water, except for periods of study. The goats were trained to stand comfortably in a stanchion during periods of study. Five other female goats were sacrificed immediately after arriving at the laboratory. The cerebellums of these unoperated goats were histologically analyzed to establish control neuronal numbers for goats. All aspects of the study were reviewed and approved by the Medical College of Wisconsin Animal Care Committee before the studies were initiated.

2.1. Experimental design

As described previously (Martino et al., 2006a), goats underwent an initial surgery to elevate and subcutaneously place the carotid arteries for eventual catheterization. Three weeks after recovery from this surgery, control data were obtained for eupneic breathing and CO_2-H^+ sensitivity (also for the exercise hyperpnea reported previously; Martino et al., 2006b). Then, stainless steel microtubules were bilaterally chronically implanted into the CFN (n = 9) or the cerebellar cortex (n = 2). Two to three weeks were required for recovery from this surgery. Once in excellent health (normal appetite, posture, ambulation, cardiorespiratory function), the goats were studied under control conditions listed above. Thereafter, reverse microdialysis of hypercapnic and acidic mock cerebral spinal fluid was utilized to create focal acidosis in the CFN (data previously reported; Martino et al., 2006a). After again establishing control conditions, the neurotoxin ibotenic acid (50 mM, irreversible glutamate receptor agonist) was injected through the micro-tubules into the cerebellum. Because of the uncertainty regarding the injection volume required to induce physiologic changes, the initial injection volume was 0.5 μl. This volume had respiratory effects in only one goat; thus, in the other goats, either 1 μl (n = 4) or 10 μl (n = 6) injections were made 3–5

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days after the first injection. Eupneic breathing and CO_2-H^+ sensitivity were assessed nearly daily after each injection, and the next volume of ibotenic acid was not injected until it was established that there was either no effect or a steady-state had been achieved. The goal was to create as large a lesion as possible without unduly compromising the health of the goats. The goats were sacrificed 1 day to 2 weeks after the final ibotenic acid injections. The cerebellums were intracranially perfused and fixed, and harvested for subsequent histological analysis described later.

2.2. Surgical procedure

For all surgical procedures, the goats were initially anesthetized with a combination of ketamine and xylazine, intubated, and mechanically ventilated with 1–1.5% halothane in oxygen. Under sterile conditions, the carotid arteries were isolated from the vagi, elevated superficial to the muscle, and the skin sutured. After surgery, the goats received the antibiotic ceftifur sodium (2 mg/kg) daily as an antibiotic for 1 week.

In the second surgery, microtubules were chronically implanted bilaterally into the CFN (n = 9) or into the cerebellar cortex as a control (n = 2). Mean arterial blood pressure, f_H, and rectal temperature were continuously monitored throughout the duration of surgery. An occipital craniotomy was created, and the dura mater was excised to expose the dorsal cerebellum and dorsal aspect of the medulla for visualization of obex. The dorsal surface of the medulla, obex, and the midline were all used as reference points for stereotaxic coordinates in the dorso-ventral, rostro-caudal, and medio-lateral planes, respectively. The micro-tubules were 18-gauge stainless steel tubes 70 mm in length. They were attached to a micromanipulator, and then starting from obex, they were rotated to 45 degrees from the horizontal plane, elevated 18–21 mm above obex, moved 1–2 mm lateral to the midline, and then advanced until the tip made contact with the dorsal surface of the cerebellum. An electrical simulating electrode was inserted through the microtubule to 2 mm beyond the tip of the microtubule. All but the last 1 mm of the stimulating electrode was insulated. The electrode was grounded directly to the microtubule or at a site on the neck of the goat. A Grass SD9 square

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pulse stimulator was used to deliver 80 Hz, 5 V, 1 ms pulse trains for 30 s (Andrezik et al., 1984; Stella, 1939; Xu and Frazier, 1997). The microtubule was advanced 10 mm from the dorsal surface of the cerebellum. At this site, and subsequently after advancing the microtubule in 1–2 mm increments, the tissue was electrically stimulated. At the site that electrical stimulation elicited an increase in blood pressure and heart rate, the microtubules were secured with screws and dental acrylic to the bone. Other studies have shown an increase in blood pressure and heart rate when the rostra1 CFN is electrically stimulated (Andrezik et al., 1984; Morruzzi, 1939).

To maximize the potential for recovery from surgery, laboratory personnel monitored the goats continuously for a minimum of 24 h after the implantation surgery. Posture support, restraint, food, water, and pain medication were provided as needed, and vital signs were noted at 30 min intervals. Brain edema was minimized with dexamethasone injections (0.4 mg/(kg day), I.V. for 3 days, then decreasing by 0.05 mg/(kg day), T.I.D.) for 1 week. Infection was minimized with chloramphenicol injections (20 mg/kg, I.V., T.I.D.) for 3 days, and daily injections thereafter of ceftifur sodium (2 mg/kg, I.M., S.I.D.), and gentamycin (3 mg/kg, I.M., S.I.D.). Buprenorphine was administered 3–12 h after implantation as an analgesic.

2.3. Physiologic measurements

For all studies, $\dot{V}_I$ was measured with a pneumotach by attaching a breathing valve to a custom fitted mask secured firmly to the goat's snout. A chronically placed catheter in an elevated carotid artery was used to monitor blood pressure and f_H, and for arterial blood sampling to obtain pH, PO_2, and PCO_2 (model 278, Ciba-Corning). Rectal temperature of the animal was measured at regular intervals.

2.4. Assessment of CO_2-H^+ sensitivity

Inspired ventilation ($\dot{V}_I$), mean arterial blood pressure, and f_H were measured for 30 min while inhaling room air and during exposure to three levels (5 min each) of elevated inspired CO_2 (3.0, 5.0, and 7.0% CO_2 in room air). Arterial blood samples (2 cc) were drawn in

duplicate during the control period, and during the fourth and fifth minute of each CO_2 exposure level. The $\dot{V}_I$ and Pa_{CO2} at corresponding levels of inspired CO_2 were used to determine the slope of the relationship between $\dot{V}_I$ and Pa_{CO2} and used as one index of CO_2-H^+ sensitivity. Because (see Section 3) CFN lesioning greatly increased the day-to-day variation in this conventional index of CO_2-H^+ sensitivity, a second index of sensitivity was computed as the $\dot{V}_I$ divided by the arterial $[H^+]$. Finally, $\dot{V}_I$ for each minute of elevated CO_2 was expressed as a percent of the room air $\dot{V}_I$.

2.5. Assessment of the acute ibotenic acid effect

Respiratory and cardiovascular variables were measured for a 30 min control period and continuously for 5 h after injection of ibotenic acid. On most days, an injection was made into both microtubules an hour apart. However, because of the severe posture effects in some goats after bilateral injections of 10 μl (see Section 3), in three goats these injections were made a week apart. Arterial blood (2 cc) was drawn in duplicate during the control period and at 30 min intervals after each of the injections. Rectal temperature was continually monitored throughout the studies.

2.6. Histologic studies

After completion of these protocols, and also in the unoperated control goats, the animals were euthanized (Beuthanasia, pentobarbital and phenytoin) and the brain was intracranially perfused with PBS solution (pH = 7.35–7.4) and 4% paraformaldehyde fixative in PBS. The cerebellum was then removed, post-fixed in 4% paraformaldehyde solution for 24 h, dehydrated in a 30% sucrose solution, and cryoprotected. The cerebellum was then frozen and serially sectioned (25 μm) in a horizontal or transverse plane, and the sections adhered to chrom alum-coated slides. The tissue was then stained with hematoxylin and eosin (H&E), cover slipped, and examined microscopically. The microtubule implantation site was identifiable by visualization of an area of absent or disrupted tissue, approximately 0.9–1 mm along the rostral–caudal axis. In a previous study (Wenninger et al., 2001), microtubules were implanted into the medulla but no neurotoxin was injected. Tissue was destroyed 0.5 mm

along the lateral edges and for 0.5 mm beyond the tip of the microtubule. It was assumed that a similar volume of tissue destruction was caused by the implanted micro-tubule in the present study. Tissue destruction beyond this value caused by the microtubule was assumed due to injection of the neurotoxin. In both directions from the CFN midline, living and dead neurons were counted at 0.5 mm intervals and the values from the two sides were averaged for each goat.

2.7. Data and statistical analyses

In addition to V_I, breathing frequency (f_R), tidal volume (V_T), inspiratory (T_I) and expiratory (T_E) time, mean arterial blood pressure, and f_H were computed. The mean data were statistically analyzed using one-way ANOVA for repeated measures to compare pre-implant, post-implant, and post-ibotenic acid data for all of the physiological variables. A two-way ANOVA was also used to compare the differences in living and dead neurons between control unoperated goats and the goats that completed the protocols. If statistical significance was found, then a Bonferroni post-hoc analysis was used to isolate the specific differences. The threshold for significance was set at $P < 0.05$.

3. Results

3.1. Histology

Histological studies verified that in nine goats, the microtubules were implanted in the CFN, whereas in two goats the microtubules were implanted in the cerebellar cortex (Fig. 1B). Typically, microtubules were bilaterally implanted in such a way that one was more rostral and the other was more caudal in the CFN, and therefore the ibotenic acid lesions similarly were primarily rostral or caudal (Fig. 1A). The average calculated volume of the cerebellum in goats is 13,718±488 mm^3, and of this volume, 71.4±8.3 mm^3 (0.5%) of the cerebellum was damaged by the combination of microtubule implantation and ibotenic acid injections. The total calculated bilateral volume of the CFN in goats is 116±1.1 mm^3, and the average volume destroyed (area absent of neurons) by the microtubules and ibotenic acid was 17±0.26 mm^3 (14.7%). Of this total, it is estimated that 8

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mm^3 (7%) of this destruction was due to the microtubules and 9 mm^3 (7.7%) was due to ibotenic acid.

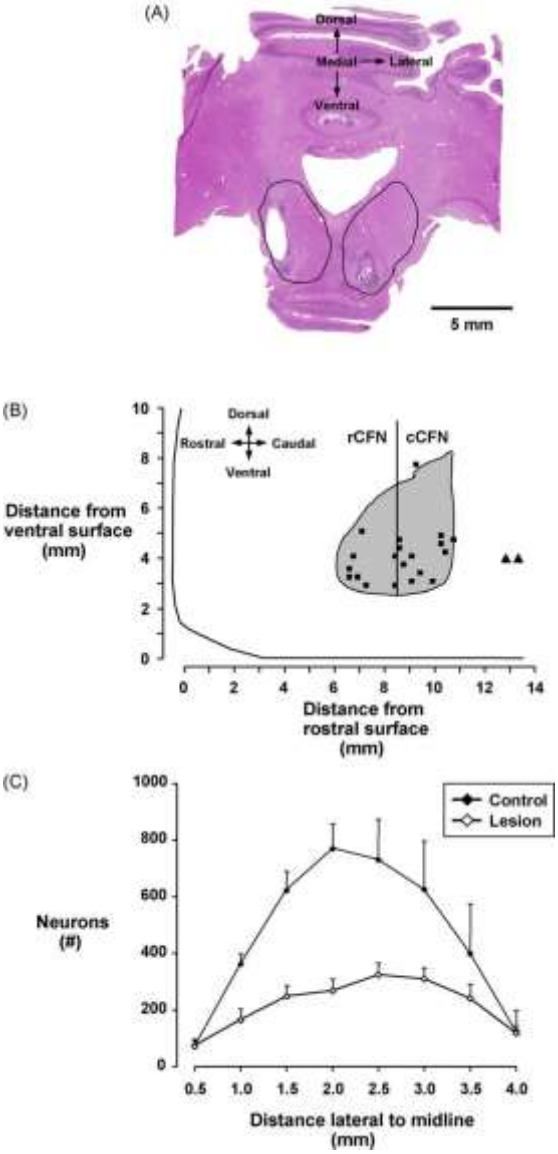

Fig. 1 Location in the cerebellum of the lesion sites in one goat (A), the site of microtubule placement in all goats (B), and the distribution of living neurons in the cerebellar fastigial nucleus (CFN) of unoperated control and lesioned goats. The image in panel A is a transversely sectioned cerebellum depicting a bilaterally lesioned CFN. Shown in panel B is a mid-sagittal sketch of the cerebellum including the CFN. The black squares indicate the location of the tips of the microtubules (two each in nine goats) that were implanted inside the CFN. The black triangles indicate the location of the tips of the microtubules (two each in two goats) that were implanted in the cerebellar cortex. The data in part C represent the average number of neurons counted on both halves of the CFN for all individual goats in both groups.

The H&E stained neurons that were amorphous in shape, dark purple in color, and had a visible nucleus were counted as living (Fig. 2A). The H&E stained neurons that were round in shape, light pink in color, and did not have visible nucleus were counted as dead (Fig. 2B). As shown in Fig. 1C, there was a significant 55% decrease ($P < 0.001$, $N = 9$) in living neurons in the CFNs of goats that had microtubules implanted compared to the CFNs of goats that either had microtubules implanted outside the CFN or had no microtubules implanted (1670±192 versus 3720±553), respectively.

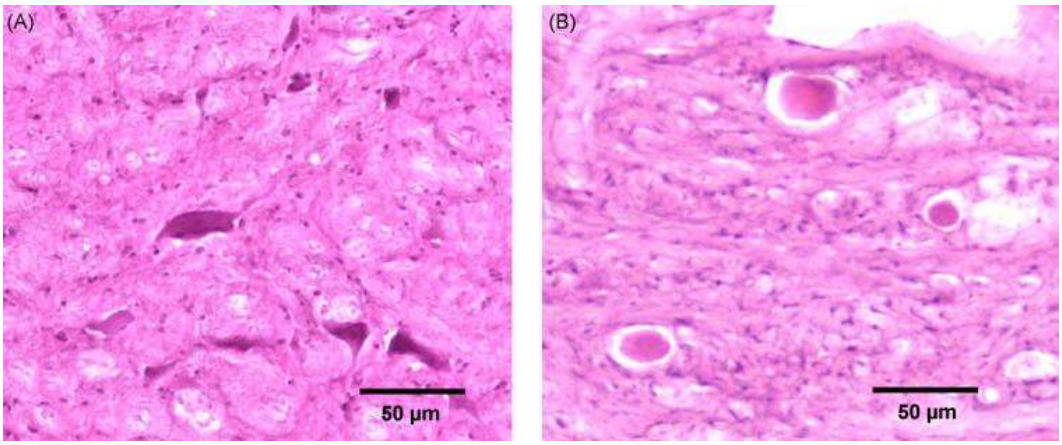

Fig. 2 Living and dead neurons can be distinguished by H&E staining. Presented in panel A is an image of living neurons that are characterized by their irregular shape, purple color, and a visible nucleus. Panel B is an image of dead neurons that are characterized by a round shape, light pink color, and no visible nucleus.

3.2. Postural and locomotor effects

Microtubule implantation into the CFN or into the cerebellar cortex created severe postural and locomotor problems. Goats could not maintain sternal recumbent posture, stand, or walk for several hours after implantation. In most goats, complete recovery required 2–3 weeks after implantation. There was a dose-dependent effect on posture and locomotion of the ibotenic acid injection with 0.5 μl injections lasting only a few hours, 1 μl injection effects lasting slightly longer and 10 μl injections effects still apparent the next day. One goat died about 14 h after 10 μl of ibotenic acid injected into the CFN. Since this goat was unable to maintain the sternal posture, laboratory personnel monitored her recovery at least every 30 min. There was no evidence of distress before or upon finding her deceased. Two other

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goats were sacrificed 24 h after the 10 μl injections because they had reached the endpoint criteria of failure to demonstrate improvement toward maintaining the sternal recumbent posture.

3.3. The effects of microtubule implantation on physiologic variables

During the first 2 weeks after microtubule implantation all goats hyperventilated and CO_2-H^+ sensitivity varied from day to day. This trend has also been shown in previous studies after microtubules were implanted into medullary nuclei (Hodges et al., 2004b; Wenninger et al., 2001). Beyond 2 weeks after microtubule implantation into the CFN or the cerebellar cortex, the baseline ventilatory status was unchanged from preimplantation status. Resting Pa_{CO2} was 41.5±0.6 mmHg prior to implantation and 42.8±0.7 mmHg over the week prior to the first injection of ibotenic acid (Table 1; $P > 0.10$, $N = 9$). The conventional CO_2-H^+ sensitivity index ($\dot{V}_I/Pa_{CO2}$) was 2.14±0.18 and 2.15±0.15 l/(min mmHg) before implantation and before injections, respectively (Fig. 3; $P > 0.05$, $N = 9$). Sensitivity expressed in terms of arterial [H^+] also was unchanged as it was 3.44±0.32 and 3.20±0.23 l/(min nmol) before and after implantation, respectively (Fig. 4). Moreover, sensitivity expressed as $\dot{V}_I$ for each level of elevated inspired CO_2 as a percent of room air $\dot{V}_I$ was also unaffected ($P > 0.10$) by implantation (Fig. 4). Additionally, during room air conditions, $\dot{V}_I$, V_T, f_R, f_H, mean arterial blood pressure, Pa_{O2}, and arterial pH were not significantly ($P > 0.05$) altered by the microtubule implantation (Table 1).

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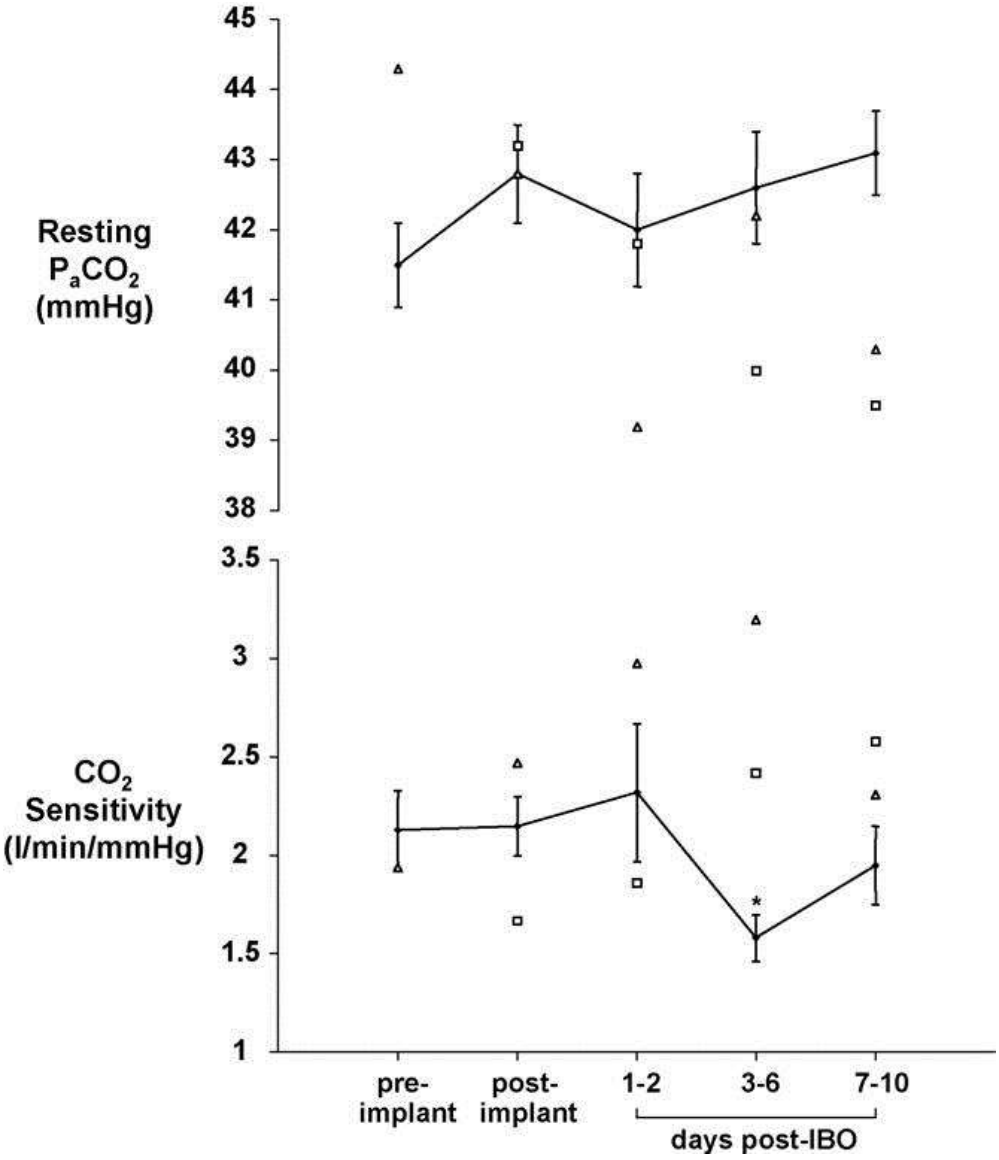

Fig. 3 Injection of ibotenic acid (ibo) into the CFN did not alter eupneic Pa_{CO2} , but CO_2-H^+ chemosensitivity was attenuated 3–6 days after the injection. Data joined by the line are mean (±S.E.M.) for the same nine goats for all periods before and after implantation or injections into the CFN except there were only six goats 7–10 days after the injection. The data represented by squares and triangles are from the two goats with microtubules implanted and ibotenic acid injections into the cerebellar cortex. The asterisk indicates significant differences ($P < 0.05$) between 3 and 6 days post-ibo and pre- and post-implantation and 1–2 days post-injection.

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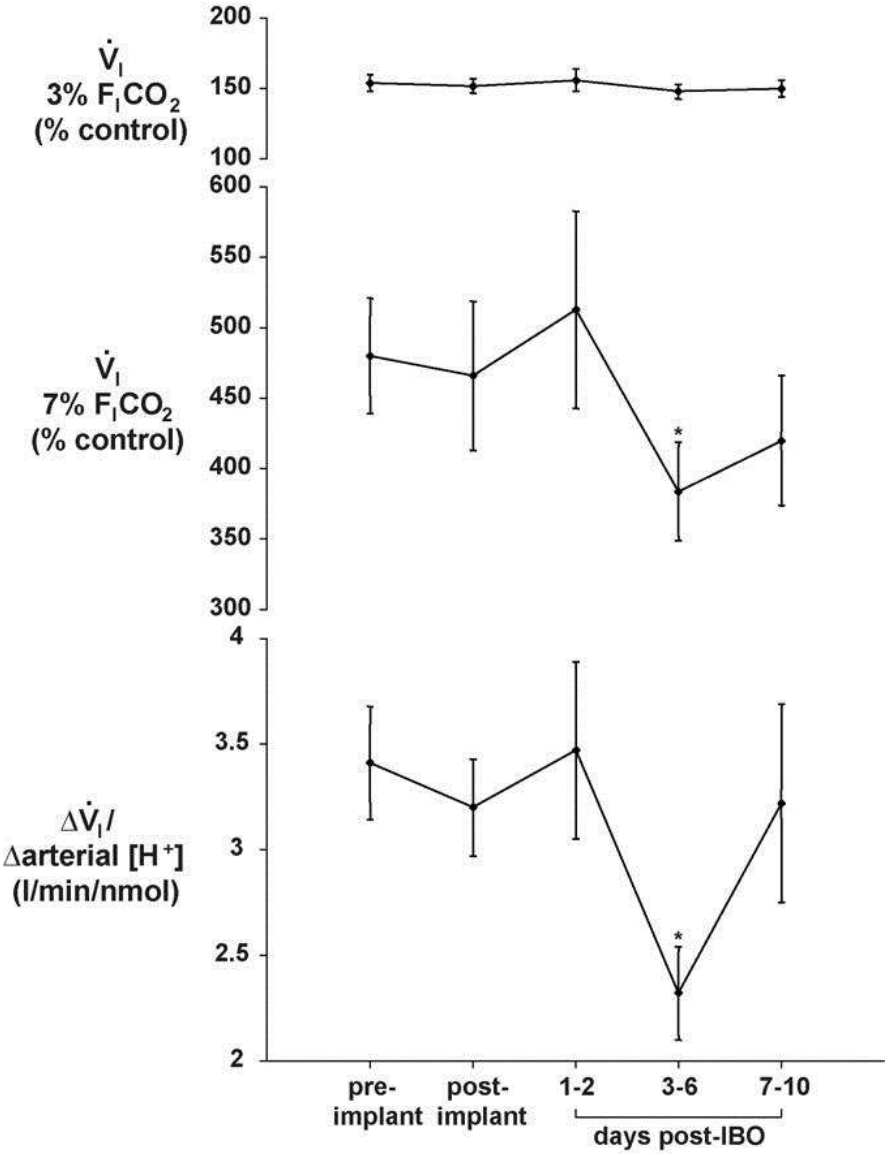

Fig. 4

CO_2-H^+ ventilatory chemosensitivity before and after implantation of microtubules into the CFN and after injection of ibotenic acid (ibo) into the CFN (N = 9). CO_2-H^+ sensitivity is expressed as the change in inspired ventilation (V_I) for each nmol change in arterial [H^+] (V_I/arterial H^+). In addition, V_I at 3% (2 min) and 7% (5 min) inspired CO_2 is also expressed as a percent of the control V_I and 1–2 days post-injection. The asterisks indicate significant ($P < 0.05$) differences between pre- and post-lesioning. Note that CFN lesioning did not affect the V_I response to 3% inspired CO_2.

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Table 1
Absolute values of physiologic variables of awake goats while inhaling room air before and at least 2 weeks after implantation of microtubules into the cerebellar fastigial nucleus(CFN), and again 7–10 days after injection of ibotenic acid into the CFN

	Pre-implant	Post-implant	Post-ibotenic acid
$\dot{V}_I$ (l/min)	8.2 ± 0.9	7.7 ± 1.2	7.8 ± 1.7
f_R (breaths/min)	18.2 ± 1.7	18.1 ± 2.1	18.2 ± 3.3
V_T (l/breath)	0.45 ± 0.06	0.43 ± 0.08	0.43 ± 0.09
T_I (s)	1.12 ± 0.10	1.11 ± 0.07	1.00 ± 0.09
T_E (s)	2.17 ± 0.17	2.20 ± 0.12	2.19 ± 0.20
MABP (mmHg)	70.9 ± 7.1	71.7 ± 4.4	77.1 ± 8.1
f_H (beats/min)	93.0 ± 9.5	90.8 ± 6.3	88.8 ± 3.8
Pa_{CO_2} (mmHg)	41.5 ± 0.67	42.8 ± 0.72	43.1 ± 0.67
Pa_{O_2} (mmHg)	103.3 ± 1.3	100.3 ± 6.3	108.8 ± 6.3
pH	7.43 ± 0.01	7.44 ± 0.01	7.45 ± 0.01

Values are means for inspired ventilation ($\dot{V}_I$), breathing frequency (f_R), tidal volume (V_T), inspiratory (T_I) and expiratory (T_E) times, mean arterial blood pressure (MABP), heart rate (f_H), arterial PCO_2 (Pa_{CO_2}), arterial PO_2 (Pa_{O_2}), and pH ± the standard error of the mean. The number of animals in each group were pre-implant, $N = 9$; post-implant, $N = 9$; post-ibotenic acid, $N = 6$.

3.4. Acute physiologic effects of ibotenic acid injections into the CFN

V_I was significantly ($P < 0.05$) but transiently elevated by 35±6% 30 min after the injections of 10 μl of ibotenic acid into the rostral CFN (Fig. 5). The effect of this volume of ibotenic acid contrasts to the lack of a significant ($P < 0.05$) effect on V_I when either 0.5 or 1 μl ibotenic acid was injected in the rostral CFN or caudal CFN, or when 10 μl was injected into the caudal CFN (Fig. 5). No other physiologic variables were significantly altered by any volume of ibotenic acid injected into the CFN.

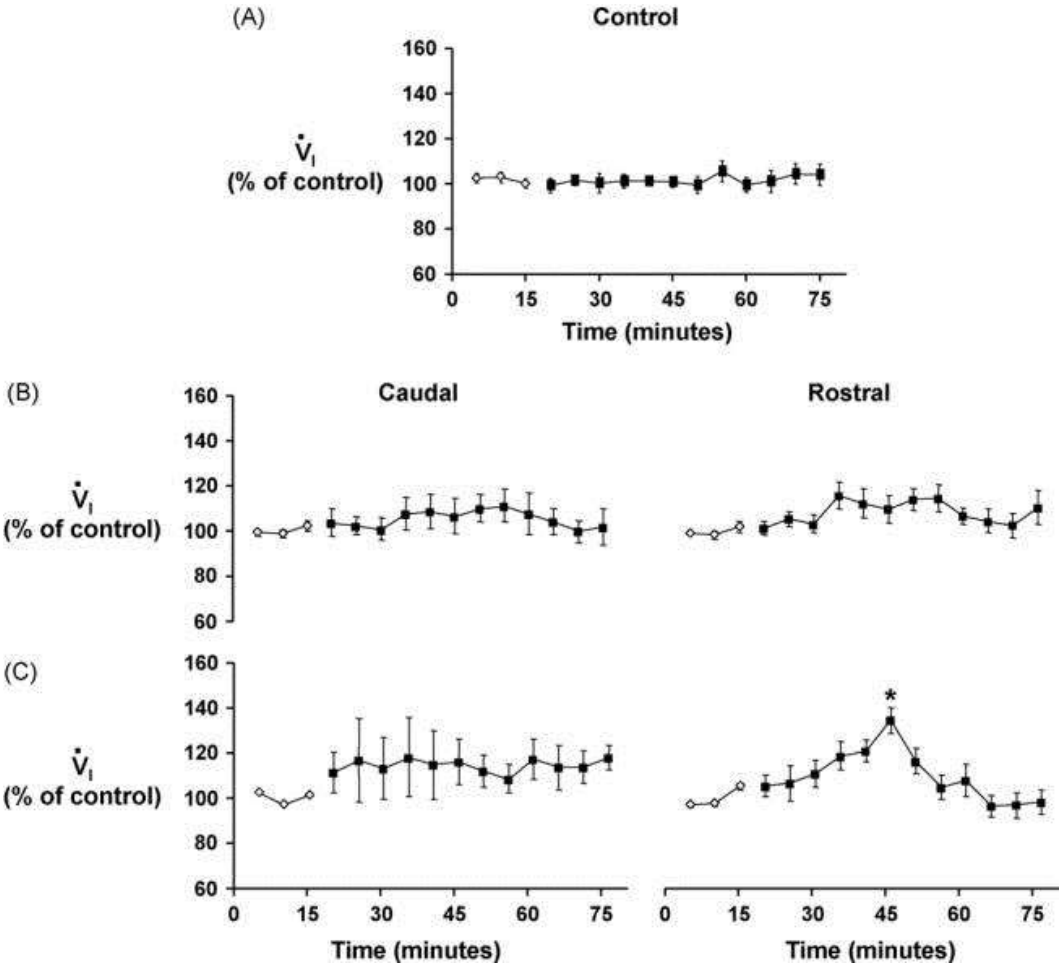

Fig. 5 Injections of 0.5 and 10 μl ibotenic acid into the rostral CFN, but not the caudal CFN, acutely increased inspired ventilation ($\dot{V}_I$). All values are 5 min averages of $\dot{V}_I$ normalized as a percent of control±the standard error of the mean. The first 3 points in each graph represent a 15 min control period prior to injections or the beginning of the 60 min control study. Panel A presents data from a control study (N = 6), Panel B demonstrates the effect of injecting 0.5 μl, and Panel C demonstrates the effect of injecting 10 μl of ibotenic acid (N = 6) into the caudal and rostral CFN.

3.5. Chronic physiologic effects of ibotenic acid injections into the CFN

Injection of ibotenic acid into the CFN did not significantly alter eupneic Pa_{CO2} throughout the 3–10 days after any injection (Fig. 3). Moreover, there was little day-to-day variation in Pa_{CO2} as the coefficients of variations were 4.0±0.6 and 3.7±0.4 for the pre- and post-lesion periods, respectively (Table 2). Compared to pre-injection, there were no significant (P < 0.1) changes after the lesions in V_I, V_T,

f_R, f_H, mean arterial blood pressure, Pa_{O2}, and arterial pH (Table 1). Finally, the ibotenic acid lesions did not result in any consistent abnormal respiratory rhythms and patterns.

Table 2
Neurotoxic lesions in the cerebral fastigial nucleus do not destabilize eupneic Pa_{CO_2} nor breathing at low levels of inspired, but the lesions destabilize CO_2-H^+ sensitivity at high levels of inspired CO_2

	Pre-lesion	Post-lesion
Eupneic Pa_{CO_2}	4.0 ± 0.6	3.7 ± 0.4
3% $F_{I_{CO_2}}$	14.8 ± 1.5	14.6 ± 0.7
$\Delta \dot{V}_I/Pa_{CO_2}$	14.1 ± 3.1	26.6 ± 4.0*
$\Delta \dot{V}_I$/arterial $[H^+]$	20.8 ± 1.6	28.7 ± 2.6*
7% $F_{I_{CO_2}}$	17.1 ± 1.4	24.9 ± 2.4*

Values are coefficient of variation ((S.D./mean) × 100).
* $P < 0.05$ compared to pre-lesion.

Between 3 and 6 days after injection of ibotenic acid into the CFN, the conventional CO_2-H^+ sensitivity index was reduced from the control of 2.15±0.17−1.58±0.26 l/(min mmHg) (−28%, $P < 0.05$; Fig. 3). In one goat, 0.5 μl of ibotenic acid elicited this change, but in four goats, 1.0 μl was required and in the other four goats, 10 μl volume injection was required. There was a slight but insignificant recovery of sensitivity between 7 and 10 days after the injections. Since changes in this index of sensitivity could be due to changes in arterial H^+ buffering, sensitivity was also computed relative to changes in arterial $[H^+]$. This index of sensitivity also decreased between 3 and 6 days after the injection from 3.20±0.23 to 2.32±0.22 l/(min nmol) after the injection (−28%, $P < 0.05$; Fig. 4). Because both the CO_2 and H^+ indexes were changed, V_I during CO_2 inhalation was also expressed as a percent of room air V_I. Values at 7.0% inspired CO_2 were decreased by 18.2±3.4% ($P < 0.05$; Fig. 4). However, values at 3.0 (Fig. 4) and 5.0% (not shown) inspired CO_2 did not significantly ($P > 0.01$) differ before and after the injections.

The day-to-day variability in CO_2-H^+ sensitivity was increased after the injections of ibotenic acid (Fig. 6 and Table 2). The average coefficient of variation ((S.D./mean) × 100) in the conventional CO_2-H^+ sensitivity index prior to injection of ibotenic acid was 14.1±3.1%,

but after the injections, the average coefficient of variation was 26.6±4.0% ($P < 0.001$). Similarly, the variation in the H^+ index increased from 20.8±1.6 to 28.7±2.6% after the injection. Finally, the variation in $\dot{V}_I$ during CO_2 inhalation expressed as a percent of the room air $\dot{V}_I$ increased ($P < 0.05$) for values at 7.0% inspired CO_2 (17–24%, pre- and post-injection, respectively) but not for values at 3.0 and 5.0% inspired CO_2 (Table 2).

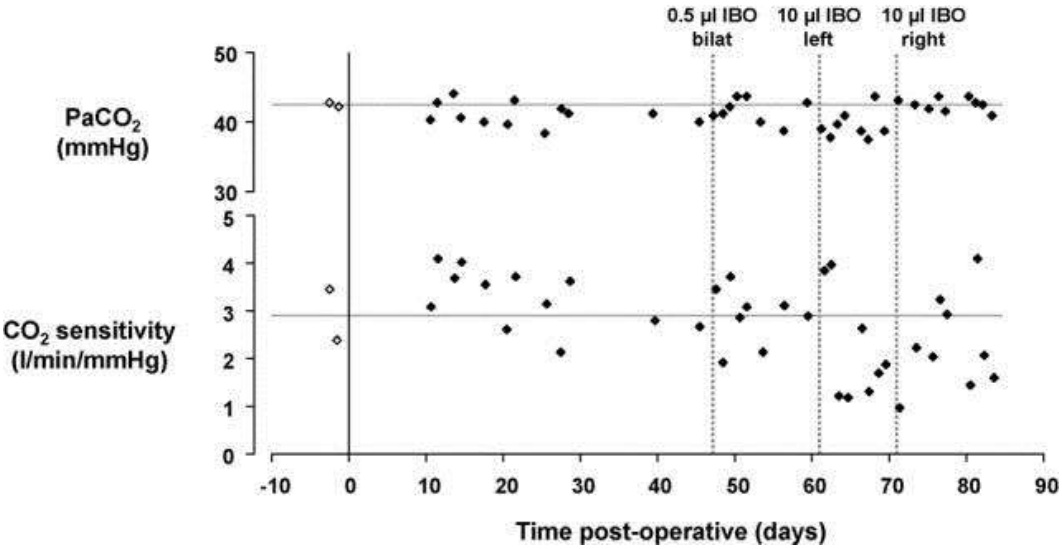

Fig. 6 The effects of microtubule implantation and ibotenic acid injections into the CFN on resting Pa_{CO2} and CO_2-H^+ sensitivity of one goat. Each value represents an individual value for resting Pa_{CO2} and CO_2-H^+ sensitivity prior to and following microtubule implantation and following several injections of ibotenic acid into the CFN. The horizontal line is the average of the pre-implantation responses. Zero represents the day that surgery was performed to bilaterally implant microtubules into the CFN. Note the overall reduction and destabilization of CO_2-H^+ sensitivity after the 10 µl injections while Pa_{CO2} was minimally affected.

In contrast to the decreased CO_2-H^+ sensitivity observed when ibotenic acid was injected into the CFN, CO_2-H^+ sensitivity changed differently in two goats in which microtubules were implanted and injections were made in the cerebellar cortex. In one of these goats, CO_2-H^+ sensitivity was 1.94, 2.94, and 3.39 l/(min mmHg) before implantation, after implantation, and after ibotenic acid injection, respectively. In the second goat, CO_2-H^+ sensitivity likewise increased from 0.97 to 1.37 and finally, after the injection to 1.60 l/(min mmHg). These listed values are averages over several days for each period. In both goats, eupneic Pa_{CO2} did not change over these periods.

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4. Discussion

The physical destruction caused by implanting the micro-tubules and the destruction caused by the neurotoxin resulted in 14.7% of the CFN totally devoid of neurons, and there was a 55% reduction in living neurons in the CFN. This deficit in neurons relative to the small volume totally devoid of neurons is consistent with the finding that there is a heterogeneous distribution of neurons within the CFN (Fig. 1C). Specifically, the highest density of neurons is near the center of the CFN, which is the site where the lesion was created. Since one goat died and two had to be sacrificed after the 10 μl injection, there was no attempt to increase the size of the (partial) lesion by injecting a larger volume of the neurotoxin. The finding that there was a dose-dependent effect of the neurotoxin on posture and locomotion is supportive evidence that the neurotoxin (with even the smallest injection) bound to glutamate receptor neurons. The posture and locomotor effects presently observed are consistent with the established role of the cerebellum in motor control.

4.1. Chronic effects on breathing of cerebellar lesions

Over the first 2 weeks after implanting the microtubules, the goats hyperventilated, had varied CO_2- H^+ sensitivities, and breathing was unstable. Similar effects have been previously observed after craniotomies and implantation of microtubules into the medulla (Hodges et al., 2004b; Wenninger et al., 2001). Thereafter, there were no overall significant chronic effects on Pa_{CO2} and CO_2-H^+ sensitivity of the physical lesion created by implanting the microtubules. The general effects of the craniotomy, the physical lesion of the CFN, and any plasticity cannot be separated, but it is clear that the lesions created by the implant did not create a critical, sustained deficit in the control of breathing.

Since the microtubules were not implanted at precisely the same CFN sites in each goat, there was not a uniform physical lesion across all goats. For this reason, and because there is a heterogeneous population of neuronal phenotype and neuronal density in the CFN, it is not unexpected that there was lack of uniformity among the goats in the injectate volume required to attenuate CO_2 sensitivity. However,

there was uniformity in the temporal pattern of the attenuated response, as in all goats it occurred between 3 and 6 days after the injection. The effects of lesioning were uniform even though the site and size of the lesions were not uniform, and even though the effects of focal acidosis previously reported (Martino et al., 2006a) were not uniform. The temporal pattern and magnitude of the attenuation is similar to that previously found after lesioning medullary raphe nuclei (Hodges et al., 2004b). Most impressive was that neurotoxic lesions in the cerebellar cortex had exactly opposite effects, indicating the unique effects of the CFN lesions. The effect of lesioning the cerebellar cortex is consistent with data of other studies which indicate that this portion of the cerebellum has primarily inhibitory effects on breathing (Xu et al., 2004).

We recently reported (Martino et al., 2006b) that lesioning the CFN attenuated the hyperventilation of these same goats during light and moderate treadmill exercise by 1.3 and 2.8 mmHg, respectively. These effects were consistent and not characterized by day-to-day variation as presently shown for CO_2-H^+ sensitivity. Moreover, the exercise effect was relatively small compared to the 28% reduction in CO_2-H^+ sensitivity (Fig. 3) between 3 and 6 days after ibotenic acid lesions. Since focal acidosis in the CFN alters breathing (Martino et al., 2006a), it seems feasible to consider that lesioning in the CFN had a specific effect on CO_2-H^+ chemoreceptors. However, the effects of focal acidosis differed between the rostra1 and caudal CFN, whereas the lesion effects were consistent over the entire CFN; thus, it appears the lesion effects are probably not solely due to the destruction of chemoreceptor neurons. Another possibility is that the CFN also has a more general non-chemoreceptor modulatory role in ventilatory control, explaining why there was attenuated breathing during exercise and during CO_2 inhalation after the lesions. Previous findings have suggested a chemoreceptor and a non-chemoreceptor effect on breathing of neurons in the medullary raphe nucleus (Hodges et al., 2004b) and neurons near the ventrolateral medularlly surface (VLM) (Forster et al., 1995). The present data do not clearly distinguish between these two possible effects for the CFN, but it is clear that there was dissociation between effects on CO_2-H^+ chemosensitivity and effects on eupneic breathing. This dissociation was evident in that CFN lesioning decreased and destabilized CO_2-H^+ sensitivity but had no

effect on eupneic breathing. This dissociation may suggest that the CFN has a specific role in the ventilatory response to CO_2-H^+.

One possible role of the CFN is as an integrator for the neural control of breathing such as it serves for posture and locomotion. Conceivably, the role of the CFN is to integrate respiratory related afferents irrespective of the system it is modulating. Since there is a widespread distribution of chemoreceptors in the brain (Coates et al., 1993; Dean et al., 1990; Hodges et al., 2004a; Nattie, 2000; Severson et al., 2003), it seems intuitive that at some site or sites there would be integration of all or some chemoreceptor activity. Indeed, it has been postulated that a VLM site served this integrator function (Schlaefke et al., 1970), and some (Takakura et al., 2006) but not all (Forster et al., 1995) more recent findings support this concept. Disruption in integrator function of the CFN may underlie the high degree of variability specific to CO_2-H^+ sensitivity after CFN lesioning.

Previous studies have found that acute and chronic neuronal dysfunction within known intracranial chemoreceptor areas results in a dissociation between effects on eupneic breathing and CO_2-H^+ ventilatory chemosensitivity (Forster et al., 1995; Hodges et al., 2004b; Nattie and Li, 2006). For example, in rats during wakefulness and NREM sleep, selective neurotoxic destruction of a majority of Neurokinin 1 receptor expressing neurons in ventral medullary nuclei reduced CO_2-H^+ sensitivity by about 60%, but only reduced eupneic breathing by about 10% (Nattie and Li, 2006). Similarly, in awake goats acute or chronic neuronal dysfunction in medullary nuclei reduced CO_2-H^+ sensitivity by 20–50% with no or minimal hypercapnia during eupnea (Forster et al., 1998; Hodges et al., 2004b). Other examples of this dissociation are the clinical condition of chronic congenital alveolar hypoventilation (Shea et al., 1993) and during the neonatal periods of both rats and piglets (Serra et al., 2002; Davis et al., 2006).

Data from the present study may suggest one potential contributing factor to the dissociation between eupneic Pa_{CO2} and CO_2-H^+ sensitivity. Specifically, lesioning the CFN decreased the absolute values and increased the variation of the $\dot{V}_I/Pa_{CO2}$, the $\dot{V}_I/[H^+]$, and $\dot{V}_I$ at 7.0% inspired CO_2 expressed as a percent of the room air $\dot{V}_I$. However, lesioning the CFN did not alter either the absolute value or

variation in $\dot{V}_I$ at 3 and 5% inspired CO_2 expressed as a percent of the room air. The unaltered values at low levels of inspired CO_2 contrast to findings in awake goats in whom carotid body denervation (CBD) significantly ($P < 0.05$) decreased all values after the first minutes at 3.0% inspired CO_2 (Hodges et al., 2004b and unpublished data). Conceivably, tonic carotid chemoreceptor activity and the rapid response of carotid chemoreceptors to changes in Pa_{CO2} (Smith et al., 2006) maintain a near normal and stable Pa_{CO2} in spite of changes in intracranial CO_2-H^+ chemosensitivity.

It is of interest that a small change in CFN pH with focal acidosis stimulated breathing (Martino et al., 2006a), but a lesion at the same site in the same goats did not alter the ventilatory response to 3 and 5% inspired CO_2. We have no definitive explanation for this apparent inconsistency. We speculate that redundancy in CO_2-H^+ chemoreception is sufficient to maintain a normal response to low levels inspired CO_2 when the chemoreceptors at one site are lesioned.

Thus, there seems to be a difference between the carotid and the intracranial chemoreceptors in regulation of eupneic breathing. CBD clearly induces severe hypoventilation during eupnea (Hodges et al., 2005), but partial lesioning of intracranial chemoreceptor sites has minimal effect on eupneic breathing (Forster et al., 1998; Hodges et al., 2004b; Nattie and Li, 2006). It is unclear whether this difference could be due to the fact that CBD eliminates the entire carotid complex, but the lesions in the brain only eliminate a small portion of the central chemoreceptors. Conceivably, the intracranial chemoreceptors (including those within the CFN) provide, relative to other inputs including carotid afferents, a relatively low level of input to downstream respiratory neurons. Attenuation of this input may thus have minimal effect on eupneic breathing. Another possibility is that compensatory mechanisms for CO_2-H^+ sensitivity might differ from those for eupneic Pa_{CO2} with the former being more limited than the latter.

4.2. Clinical relevance

Damage has been documented in the cerebellar deep nuclei and cerebellar cortex in clinical conditions of congenital central hypoventilation syndrome (CCHS), heart failure, and sleep disordered

breathing (Kumar et al., 2005; Woo et al., 2003). These conditions have impaired regulation of arterial blood pressure, impaired ventilatory responses to CO_2 and hypoxia, and impaired regulation of ventilation during sleep (Hla et al., 1994; Suzuki et al., 1996; Woo et al., 2005; Shea et al., 1993). The present findings indicate the deficits in CO_2 sensitivity in CCHS patients could in part be due to cerebellar damage. We did not formally examine breathing during sleep in the CFN-lesioned goats, and thus can make no definitive statements regarding sleep disordered breathing. However, the lesioned goats were often observed in sternal recumbency with their eyes closed and thus presumably asleep. During these conditions, the respiratory rhythm appeared to be regular. The possibility remains that even though we found no hypoventilation in these goats while awake, they may have been hypoventilating during sleep. Finally, injection of ibotenic acid into the CFN had no measurable acute or chronic effect on regulation of arterial blood pressure; thus, the observed effects on posture were not secondary to arterial hypotension. Also our data do not provide support for the concept that cerebellar damage contributes to deficits in blood pressure regulation during certain clinical conditions (Rector et al., 2006).

5. Conclusions

The attenuated CO_2-H^+ sensitivity and increased coefficient of variation of the CO_2-H^+ sensitivity after neurotoxic lesions provide strong evidence that the CFN is an important determinant of ventilatory CO_2-H^+ chemosensitivity in the awake state.

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