Ontogeny of central CO₂ chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs

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Taylor, Barbara E., Michael B. Harris, J. C. Leiter, and Matthew J. Gdovin. Ontogeny of central CO₂ chemoreception: chemosensitivity in the ventral medulla of developing bullfrogs. Am J Physiol Regul Integr Comp Physiol 285: R1461–R1472, 2003; 10.1152/ajpregu.00256.2003.—Sites of central CO₂ chemosensitivity were investigated in isolated brain stems from Rana catesbeiana tadpoles and frogs. Respiratory neurograms were made from cranial nerve (CN) 7 and spinal nerve 2. Superfusion of the brain stem with hypercapnic artificial cerebrospinal fluid elicited increased fictive lung ventilation. The effect of focal perfusion of hypercapnic artificial cerebrospinal fluid on discrete areas of the ventral medulla was assessed. Sites of chemosensitivity, which are active continuously throughout development, were identified adjacent to CN 5 and CN 10 on the ventral surface of the medulla. In early- and middle-stage tadpoles and frogs, unilateral stimulation within either site was sufficient to elicit the hypercapnic response, but simultaneous stimulation within both sites was required in late-stage tadpoles. The chemosensitive sites were individually disrupted by unilateral application of 1 mg/ml protease, and the sensitivity to bath application or focal perfusion of hypercapnia was reassessed. Protease lesions at CN 10 abolished the entire hypercapnic response, but lesions at CN 5 affected only the hypercapnic response originating from the CN 5 site. Neurons within the chemosensitive sites were also destroyed by unilateral application of 1 mM kainic acid, and the sensitivity to bath or focal application of hypercapnia was reassessed. Kainic acid lesions within either site abolished the hypercapnic response. Using a vital dye, we determined that kainic acid destroyed neurons by only within 100 μm of the ventral medullary surface. Thus, regardless of developmental stage, neurons necessary for CO₂ sensitivity are located in the ventral medulla adjacent to CN 5 and 10.

CENTRAL CO₂ CHEMOSENSITIVITY exists in a wide variety of air-breathing animals (5–7, 19, 26, 34, 36, 44). Central chemoreceptors were first located in mammals superficially in rostral (28), intermediate (35), and caudal (23) areas of the ventral medulla. Additional chemosensory sites have been identified subsequently throughout the brain stem, including the fastigial nucleus of the cerebellum, the locus ceruleus, the medullary raphe, the nucleus of the solitary tract, the pre-Bötzinger complex, the retrotrapezoid nucleus, and the rostral aspect of the ventral respiratory group (29). To assess the individual contribution of these distributed chemosensitive sites to the overall CO₂ response, focal stimulation or focal disruption of discrete sites has been used. Stimulation of a single central chemosensitive site by focal acidification increases ventilation, but the increase is typically less than half of that seen with an equivalent systemic increase in PCO₂ (3, 9, 14, 31, 37). Disruption of a single central chemosensitive site by cooling (15), neurotoxic lesions (1, 2, 12), or neuronal inhibition (11, 24) reduces, but does not eliminate, the ventilatory response to CO₂ (14). Thus all of the identified sites of mammalian central chemosensitivity seem to contribute to the hypercapnic response, and it can be said of any site that its stimulation is necessary, but not sufficient, to evoke the full ventilatory response to CO₂. The distribution of the central CO₂ chemosensitive sites has been investigated less extensively in non-mammalian species, and rarely has the ontogeny of central CO₂ chemosensitivity been investigated in any species. Such an investigation is virtually impossible in mammals since most of ontogeny is experimentally inaccessible during early fetal life. The bullfrog is, therefore, particularly appropriate for investigations of the distribution and ontogeny of central CO₂ chemosensitivity because isolated brain stem preparations from the bullfrog are reliably robust and chemosensitive, and because bullfrog developmental stages are all free living and easily accessible. Torgerson et al. (43) conducted a series of transection studies in which the medulla of the tadpole was divided along the rostro-caudal axis. As a result of these studies, they concluded that the CO₂ chemosensory sites migrated from a caudal site in early-stage tadpoles to a more rostral site in late-stage tadpoles and frogs. However, the location of chemosensory sites within the transactions was not further defined. A subsequent study using hypercapnic microinjections to identify chemosensory sites placed the central chemosensors in the ventral two-thirds of the medulla but did not define the rostrocaudal loca-
tion of the chemosensory sites with any greater accuracy (40).

We hypothesized that caudal and rostral sites of CO₂ chemosensitivity would exist on the ventral surface of the medulla of the tadpole at sites indicated by the transection studies. We were intrigued by the possibility that the rostral and caudal sites corresponded to similar superficial rostral and caudal sites in the ventral medulla of mammals (33), a finding that would imply a phylogenetically ancient function for these ventral chemosensory sites. We used focal perfusion of CO₂ at discrete areas of the ventral medulla in this investigation to locate sites of chemosensitivity in the brain stem of bullfrogs over the entire range of developmental stages. Whereas focal CO₂ perfusion assessed discrete sites in terms of their sufficiency in evoking the hypercapnic response, we used focal disruption to assess the necessity of each site. We expected to find similarities to the mammalian system; stimulation of a discrete site would be sufficient to evoke a partial ventilatory response to CO₂, but not sufficient to evoke the full ventilatory response, and that each site’s functionality would be necessary for the expression of ventilatory responses from the system at large.

METHODS

Studies were performed on *Rana catesbeiana* tadpoles and frogs of either sex purchased from a commercial supplier (Sullivan, Nashville, TN). Tadpoles were assigned to one of four groups: early-stage tadpoles (forelimbs absent, hindlimbs paddle-like without joints or separated toes), middle-stage tadpoles (forelimbs absent, hindlimbs developing joints and toes), late-stage tadpoles (forelimbs developing, tail being resorbed), and juvenile frogs. For those familiar with the classification scheme of Taylor and Kollros (38), early corresponds with stages 1–10, middle to stages 11–17, and late to stages 18–25. Our decision to divide metamorphosis between middle- and late-stage tadpoles was based on the physiological changes in gas-exchange mode and blood PCO₂ and HCO₃⁻ buffering that occur between these stages: pulmonary gas exchange increases (8), and the PCO₂ and HCO₃⁻ concentrations rise dramatically at about stage 18 between the middle- and late-stage groups (21). All animals were maintained at 22°C in aquaria containing dechlorinated water and were fed tropical fish food (tadpoles) or crickets (frogs). The institutional animal resource committee approved the research and animal use protocols, and the experimental protocols conformed to local and national standards of ethics.

**Surgical preparation.** Each animal was anesthetized by immersion in a cold 0.2 mM solution of tricaine methanesulfonate (MS222; Sigma, St. Louis, MO) in dechlorinated water buffered with NaHCO₃ to pH 7.4. Under a dissecting microscope, the dorsal cranium was removed, the forebrain rostral to the optic lobes was resected, and the fourth ventricle was exposed by removing the choroid plexus. The brain stem and spinal cord were removed en bloc from the cranium and spinal canal, the dura mater was stripped away, and the brain was transacted rostral to the optic tectum and caudal to the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebrospinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 MgCl₂, 10 d-glucose, 25 NaHCO₃, and 2.4 CaCl₂ equilibrated with 100% O₂. The aCSF HCO₃⁻ concentration is similar to that of late-stage tadpole and frog plasma, but higher than the HCO₃⁻ concentration in early-stage tadpole plasma. This concentration has been used in our previous tadpole studies, and to ensure comparability between experiments on animals of different stages, the aCSF composition was kept consistent in all experiments.

The isolated brain stem was transferred to a low volume (0.5 ml) flow-through recording chamber (41, 42). Each isolated brain stem was supported ventral side up between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal regions at a rate of 5 ml/min. A supply of aCSF, equilibrated with O₂–CO₂ mixtures to produce the desired pH, flowed through glass tubing to the chamber. The pH of the aCSF was maintained at either pH 7.8 (normocapnia) or pH 7.4 (hypercapnia) by adjusting the fractional concentrations of O₂ and CO₂ in the equilibration gas. CO₂ was monitored with a CO₂ analyzer (Capstar-100, CWE, Ardmore, PA). After dissection, the brain stem was allowed to stabilize while superfused with aCSF at 22°C, pH 7.8, and ~9 Torr PCO₂ for 1 h.

**Nerve recording.** Roots of cranial nerve (CN) 7 and spinal nerve (SN) 2 were drawn into glass suction electrodes pulled from 1-mm-diameter capillary glass to-tip diameters of 30–60 μm. Whole nerve discharge was amplified (×100 by DAM-50 amplifier, World Precision Instruments, Sarasota, FL; ×1,000 by model 1700 A-M Systems, Carlsborg, WA), filtered (1 Hz low pass to 500 Hz high pass) and integrated (full-wave rectified and averaged over 50 ms; MA-821/RSP, CWE, Ardmore, PA). Both amplified raw and integrated signals were digitized at 200 Hz/channel, and these neurograms were archived as computer files (Datapac, RUN Technologies, Mission Viejo, CA) for subsequent analysis.

**Focal perfusion.** A stainless steel Babcock spinal needle with a 40-μm-diameter tip (Popper & Sons, New York) was used to construct the focal-perfusion pipette (13). A 20-ml glass syringe, held in a syringe pump (Orion Research, Boston, MA), was connected to the spinal needle by polyethylene tubing. The sleeve of the spinal needle was connected to the laboratory vacuum line. The syringe pump was set to dispense 0.25 ml/min. The dispersed volume plus some aCSF from the recording chamber was immediately removed by vacuum up the sleeve. Thus only a small area on the brain stem was exposed to the perfusion solution. To determine the area perfused by the pipette, aCSF containing trypan blue (1 mg/ml; Sigma) was applied with the pipette, and immediately after this perfusion, the brain stems were viewed with a dissecting microscope, and the stained areas were measured by using a graduated reticule. These measurements indicated that focal perfusion affects an area 500 ± 35 μm in diameter; this represents 25–35% of the width of the isolated brain stem, depending on the age and size of the animal.

Before focal perfusion in each isolated brain stem was studied, a baseline response to normocapnia was first established by superfusing with aCSF at pH 7.8 (equilibrated with 1.3% CO₂–98.7% O₂) for 20 min. We increased the CO₂ of the aCSF superfuse to a hypercapnic level (equilibrated with 4.7% CO₂–95.3% O₂; pH 7.4) for 20 min to confirm that the preparation was able to generate a hypercapnic response. After the baseline fictive lung frequency was reestablished by superfusing with normocapnic aCSF for 20–40 min, focal perfusions were carried out. Brain stems from 46 tadpoles and frogs received focal perfusions of hypercapnic aCSF (the same as used for bath application of normocapnia) applied for 20 min to the ventral medullary surface adjacent to CN 5, CN 8, CN 10, or SN 2. Using brain stems from four late-stage tadpoles, we focally perfused normocapnic aCSF (the same as used for bath application of normocapnia) for 20 min to the
ventral medullary surface adjacent to CN 5 and CN 10. This focal normocapnic perfusion was carried out while the remainder of the brain stem was bathed by hypercapnic aCSF. In all unilateral focal perfusion studies, we varied the side of brain stem (left vs. right) that was perfused.

In addition to applying different levels of CO$_2$, the focal perfusion pipette was used to create lesions within identified chemosensitive sites in the ventral medulla. Lesions were created by either a 13-min focal perfusion of aCSF containing 1 mg/ml protease (Sigma) or a 10-min focal perfusion of aCSF containing 1 mM kainic acid (Sigma). After a lesion was created, the baseline fictive lung frequency was reestablished by superfusing with normocapnic aCSF for 40–60 min, and the capacity for a hypercapnic response was tested. First, hypercapnic aCSF was focally perfused at the lesion site for 20 min to confirm that chemosensitivity at that site had been eliminated. Then we applied hypercapnic aCSF in the bath for 20 min to test the effect of the lesion on the brain stem’s overall hypercapnic response. Isolated brain stems from 16 bullfrogs of all developmental groups received a protease lesion adjacent to CN 5; 18 received a protease lesion adjacent to CN 7; 17 received kainic acid lesion adjacent to CN 5; and 17 received kainic acid lesion adjacent to CN 10.

**Distribution of cells affected protease or kainic acid lesion.** The fluorescent, nucleic acid stain DEAD red (Molecular Probes, Eugene, OR) was used to determine the location of cells whose function was impaired by the focal perfusion of either protease or kainic acid. DEAD red is a cell-impermeant dye that can only diffuse across leaky or otherwise compromised cell membranes (18). Brain stems were completely immersed in ~300 µl of aCSF containing DEAD red (1:50 dilution) for 15 min, and the brain stems were frozen in OCT compound (Fischer, Pittsburgh, PA) and sliced in a cryostat at 20-µm-thick coronal sections. Tissue sections were mounted on glass slides and photographed by using an epifluorescence microscope (BH2 Olympus, Melville, NY) equipped with a 480- to 550-nm excitation and 590-nm emission filter set and a charge-coupled device camera (Optronics, Goleta, CA). Images were saved on a personal computer and analyzed by using ImagePro software (Media Cybernetics, Silver Springs, MD).

**Data analyses and statistics.** Burst activity patterns in the neurograms were designated as either buccal or lung on the basis of the amplitude of the integrated nerve activity and the presence or absence of coincident firing in both CN 7 and SN 2, as previously described (17, 42). Buccal bursts had lower amplitude in CN 7 than lung bursts and no coincident firing in SN 2, whereas lung bursts were of greater amplitude in CN 7 with coincident firing in SN 2. Our laboratory previously demonstrated (39) that changes in the frequency of fictive lung ventilation are the primary manifestation of the CO$_2$ response. The amplitude of fictive lung ventilation and the pattern of gill ventilation are unresponsive to hypercapnia. Thus only the frequency of fictive lung ventilation was quantified by counting the number of lung bursts in 2 consecutive minutes of each treatment condition.

Frequencies of fictive lung ventilation were analyzed by using a repeated-measures ANOVA in which metamorphic group (4 levels) was a continuous between-subject factor and CO$_2$ level (room air vs. hypercapnia) was a within-subject factor (GLM procedure, Systat 9.0, Chicago, IL). When ventilatory responses to focal perfusion of CO$_2$ were analyzed within a developmental group, site of CO$_2$ application was a between-subject factor and CO$_2$ level (normocapnia vs. hypercapnia) was a within-subject factor. When ventilatory responses to lesions were compared, CO$_2$ was analyzed within a chemosensitive site, lesion type (protease vs. kainic acid) and developmental group were between-subject factors, and CO$_2$ level (normocapnia vs. hypercapnia) was a within-subject factor. When an ANOVA indicated that significant differences existed between the treatment groups, multiple preplanned comparisons were made by using t-values adjusted by the Bonferroni method. Values reported in the text represent means ± SD.

**RESULTS**

The neuroventilatory responses to bath-applied normocapnia and hypercapnia and to focal perfusions of hypercapnic aCSF at four sites on the ventral surface of the medulla are illustrated by the representative neurograms in Fig. 1, which were recorded from the brain stems of four different juvenile frogs. Neural recordings were made from CN 7 and SN 2. Measurements of fictive ventilatory frequency were made from CN 7 neurograms, where both buccal and lung bursts were evident. SN 2 neurograms demonstrated lung bursts coincident with those on CN 7. Both recorded nerves innervate the buccal musculature, which provides the main ventilatory pump for both buccal and lung ventilation. As seen in the figure, lung bursts were easily distinguished from buccal bursts by virtue of their greater amplitude. Lung burst frequencies are the only measures of fictive ventilation presented here because our previous investigation (39) indicated that hypercapnia affects only fictive lung ventilation, not fictive buccal ventilation.

**Lung frequency responses to bath-applied CO$_2$.** Mean frequencies of fictive lung ventilation are presented in Fig. 2 as a function of developmental group and level of CO$_2$ (normocapnia or hypercapnia). During normocapnia, there is a linear increase in lung burst frequency with increased development ($P < 0.001$, $r^2 = 0.50$; higher order curve fits were not significant). Under normocapnic conditions, lung burst frequency increased an average of 4.22 bursts/min between successive developmental groups from 0.58 ± 0.13 burst/min in early tadpoles to 11.66 ± 1.86 bursts/min in frogs. Under hypercapnic conditions, lung burst frequency increased an average of 6.77 bursts/min between successive developmental groups from 3.27 ± 0.42 burst/min in early tadpoles to 20.47 ± 3.29 bursts/min in frogs, and again the increase in lung burst frequency was significant ($P < 0.001$, $r^2 = 0.43$). Moreover, the changes in lung burst frequency between normocapnic and hypercapnic treatments within each metamorphic group were also significant ($P < 0.001$ for all groups), but the increase in lung burst frequency was significantly greater in the older metamorphic groups (the slope of the hypercapnic line was significantly greater than the slope of the normocapnic response; $P < 0.001$). Thus development and CO$_2$ each caused an increase in lung burst frequency, and bullfrogs at each metamorphic stage exhibited a hypercapnic response. Although the absolute size of the hypercapnic response increased as tadpoles aged, the relative change in frequency was remarkably similar across stages; at each stage, hypercapnia caused a 2- to 2.5-fold increase in lung burst frequency.
Responses to focal perfusion of CO2 at CN 8 or SN 2. Mean frequencies of fictive lung ventilation are presented in Fig. 3 as a function of developmental group, CO2 level, and CO2 focal perfusion site. Values for bath normocapnia and hypercapnia are taken from Fig. 2 and repeated in this figure for the sake of comparison. For each developmental stage, lung burst frequency during focal perfusion of CO2 adjacent to either CN 8 or SN 2 was not different from the lung burst frequency measured during normocapnia (P = 0.10, 0.62, 0.09, and 0.71 for early-, middle-, and late-stage tadpoles, and frogs, respectively). Thus the ventral medullary sites adjacent to CN 8 and SN 2 are not chemosensitive in the bullfrog brain stem.

Responses to focal perfusion of CO2 at CN 5 or CN 10. Mean frequencies of fictive lung ventilation are presented in Fig. 4 as a function of developmental group, CO2 level, and CO2 focal perfusion site. Values for bath normocapnia and hypercapnia are the same as in Fig. 2 and are repeated in this figure for the sake of comparison. Applying CO2 to the ventral medullary surface adjacent to either CN 5 or CN 10 elicited a hypercapnic response in all but the late-stage tadpoles. In early- and middle-stage tadpoles and frogs, lung burst frequency increased above the normocapnia values (frequency increased from 0.84 ± 0.33 to 5.30 ± 1.98 bursts/min for early-stage tadpoles, P < 0.001; from 0.76 ± 0.33 to 4.57 ± 2.47 bursts/min for middle-stage tadpoles, P < 0.001; from 8.58 ± 2.46 to 19.75 ± 6.83 bursts/min for frogs, P < 0.001). Similarly, when CO2 was perfused adjacent to CN 5 (frequency increased from 0.84 ± 0.33 to 5.30 ± 1.98 bursts/min for early-stage tadpoles, P < 0.001; from 0.76 ± 0.33 to 4.57 ± 2.47 bursts/min for middle-stage tadpoles, P < 0.001; from 8.58 ± 2.46 to 19.75 ± 6.83 bursts/min for frogs, P < 0.001). During hypercapnic stimulation of the ventral medulla adjacent to CN 5 and CN 10, brain stems from late-stage tadpoles exhibited lung burst frequencies of 6.90 ± 3.23 and 8.40 ± 3.68, respectively. These frequencies...
were not different from those measured during normocapnia ($P = 0.067$). Thus the ventral medullary surface adjacent to CN 5 and CN 10 contains central chemosensitive sites in bullfrogs of most developmental stages, the exception being late-stage tadpoles, and each CO$_2$-sensitive site is capable, after only unilateral stimulation, of recapitulating the entire hypercapnic response to equivalent bath-applied stimulation.

Responses to focal normocapnia at CN 5 and CN 10 during bath application of CO$_2$. Mean frequencies of fictive lung ventilation are presented in Fig. 5 as a function of CO$_2$ level and application of a normocapnic stimulus to the chemosensitive sites adjacent to CN 5 and CN 10. These experiments were performed only on late-stage tadpoles because they were meant to determine why brain stems from this developmental group exhibited a hypercapnic response to bath-applied CO$_2$ but not to focal perfusion of CO$_2$ at any one site on the brain stem. Lung burst frequency during bath application of CO$_2$ (28.50 $\pm$ 4.60 bursts/min) was higher ($P = 0.002$) than that during normocapnia (14.33 $\pm$ 4.83 bursts/min) or during coincident bath application of CO$_2$ and focal perfusion of normocapnic aCSF to either the CN 5 chemosensitive site (14.67 $\pm$ 5.81 bursts/min) or the CN 10 chemosensitive site (14.50 $\pm$ 5.07 bursts/min). Thus the sites adjacent to CN 5 and CN 10 are chemosensitive, but the hypercapnic response of isolated brain stems from late-stage tadpoles requires simultaneous bilateral stimulation of both of these chemosensitive sites.

**Fig. 3.** Neuroventilatory responses to normocapnia, hypercapnia, and hypercapnic focal perfusions to the ventral medulla adjacent to CN 8 and SN 2 generated by the isolated brain stem of bullfrogs. Early- (A), middle- (B), and late-stage tadpoles (C), and frogs (D) respond to bath application of hypercapnia but not to focal perfusion of hypercapnia at CN 8 or SN 2. Each symbol represents mean lung burst frequency $\pm$ SD for 4–6 tadpoles or frogs when the brain stem was treated with normocapnia ($\circ$) or hypercapnic ($\bullet$) aCSF at the specified location. *Significant difference between lung burst frequency during normocapnic and hypercapnic treatment.

**Fig. 4.** Neuroventilatory responses to normocapnia, hypercapnia, and hypercapnic focal perfusions to the ventral medulla adjacent to CN 5 and CN 10 generated by the isolated brain stem of bullfrogs. Early- (A) and middle-stage tadpoles (B) and frogs (D) respond to bath application of hypercapnia and to focal perfusion of hypercapnia at CN 5 or CN 10. Late-stage tadpoles (C) respond to bath application of hypercapnia but not to focal perfusion at either site alone. Each symbol represents mean lung burst frequency $\pm$ SD for 4–6 tadpoles or frogs when the brain stem was treated with normocapnia ($\circ$) or hypercapnic ($\bullet$) aCSF at the specified location. *Significant difference between lung burst frequency during normocapnic and hypercapnic treatment.
Fig. 5. Neuroventilatory responses, as generated by the isolated brain stem of late-stage tadpoles to bath-applied normocapnic or hypercapnic aCSF or to normocapnic focal perfusion to sites in the ventral medulla during bath application of hypercapnia. Hypercapnic response of late-stage tadpoles is not maintained unless hypercapnia is simultaneously applied to the ventral medulla adjacent to CN 5 and CN 10. Each symbol represents mean lung burst frequency ± SD for 4 late-stage tadpoles. *Significant difference between lung burst frequency during normocapnic and bath hypercapnic-only treatment.

Responses to CO2 after unilateral rostral lesions (CN 5). Mean frequencies of fictive lung ventilation are presented in Fig. 6 as a function of developmental group, CO2 level, and unilateral damage caused by either protease (Fig. 6A) or kainic acid (Fig. 6B) applied within the chemosensitive site adjacent to CN 5 in the ventral medulla. There was a developmental increase in lung burst frequency regardless of CO2 level or lesion treatment (P < 0.001), and this increase was consistent with the previously demonstrated developmental increase in lung frequency (see Fig. 2). The ability of the isolated brain stem to generate a hypercapnic response was influenced by the type of lesion created (there was a significant interaction between the effect of CO2 and lesion type; P = 0.003). Figure 6A shows the frequency of lung bursts under normocapnic control conditions and hypercapnic conditions after the protease lesion was created. Irrespective of the developmental group, bath application of hypercapnia elicited an increase in lung burst frequency despite the protease lesion in the rostral chemosensitive site. Although protease lesions in the rostral site did not affect the overall hypercapnic response of the brain stem, they did eliminate the chemosensitivity of that site. Focal application of hypercapnic aCSF to the rostral chemosensitive site after a protease lesion had been made elicited no increase in the frequency of fictive lung ventilation in any animal (data not shown). Figure 6B shows the frequency of lung bursts under normocapnic control conditions and hypercapnic conditions after a lesion was created with kainic acid applied to the ventral medullary surface adjacent to CN 5. Here, both focal and bath application of hypercapnia failed to elicit a change in lung burst frequency after lesion placement at the CN 5 site. Thus, although the ability of the isolated brain stem to respond to hypercapnia was not compromised by the protease lesion in the rostral chemosensitive site, the hypercapnic response could no longer be elicited when a lesion was made at the same site with kainic acid.

Fig. 6. Neuroventilatory responses of lung burst frequency expressed as a function of developmental level during bath-applied normocapnic or bath-applied hypercapnic after a lesion in the rostral chemosensitive site (CN 5). Protease lesions in the ventral medulla adjacent to CN 5 (A) do not interfere with the hypercapnic response, but kainic acid lesions in the same site (B) block the hypercapnic response. Each symbol represents mean lung burst frequency ± SD for 4–6 tadpoles or frogs when the bath contained normocapnic (○) or hypercapnic (●) aCSF. *Significant difference between lung burst frequency during normocapnic and hypercapnic treatment.
tease or kainic acid lesions in the ventral medulla are
ment. The locations of cells destroyed by either pro-

The locations of cells destroyed by either pro-

average of 100 μm below the ventral surface of the medulla. Brain stems treated with focal perfusion of protease (Fig. 9B) showed an intense area of staining at the site of perfusion and extending an average of 50 ± 20 μm below the ventral surface of the medulla. Brain stems treated with focal perfusion of kainic acid (Fig. 9C) showed an even more intense staining at the site of perfusion, which extended an average of 100 ± 20 μm below the ventral surface of the medulla. Figure 9, E–L, illustrates the distribution throughout the me-
dula of cells affected by protease and kainic acid les-
ions within the chemosensitive sites. Kainic acid was
ocally perfused in the ventral medulla adjacent to CN 5 (Fig. 9D), and the resulting cell death is reflected by int
ense DEAD red staining in the slice containing that
ite. Intense staining is seen only at the site of perfu-
sion (Fig. 9F) and not on the contralateral side adjacent
to CN 5 (Fig. 9E). Lesions within one chemosensitive
ite did not destroy cells in the other chemosensitive
ite, as seen in the whole slice at the level of CN 10
(Fig. 9I) and the higher magnification of the contralat-
eral side of that slice (Fig. 9J). Nor did the lesions
destroy cells in any other area of the medulla, as seen
in the whole slices at the level of CN 8 (Fig. 9G) and

bursts under normocapnic control conditions and hy-
percappnic conditions after a lesion was created with
kainic acid applied to the ventral medullary surface
adjacent to CN 10. Here again, hypercapnia failed to
elicit an increase in lung burst frequency under these
conditions in any of the developmental groups. Thus
either type of lesion in the caudal chemosensitive site
eliminated the hypercapnic response, suggesting that
the caudal site is necessary for expression of the hy-
percapnic response.

Nortocapnic lung frequency before and after unilat-
eral lesion in a chemosensitive site. Mean frequencies of
factive lung ventilation during normocapnia are pre-
sented in Fig. 8 as a function of lesions that disrupted
overall hypercapnic sensitivity or disrupted hypercap-
nic sensitivity only at the lesion site. Focal perfusion of
kainic acid to either chemosensitive site or protease to
the caudal site had similar effects. These lesions, which
disrupted the brain stem’s overall chemosensitivity,
also caused a decrease in lung burst frequency during
normocapnia (P = 0.009). In contrast, focal perfusion of
protease to the rostral chemosensitive site disrupted
the chemosensitivity of that site when stimulated by
focal hypercapnic but did not disrupt the response to
bath-applied hypercapnic stimulation and had no ef-
fect on the frequency of lung bursts during normocap-
nia (P = 0.618)

Cell destruction after protease and kainic acid treat-
ment. The locations of cells destroyed by either pro-
tease or kainic acid lesions in the ventral medulla are
illustrated in Fig. 9 by photomicrographs of DEAD red
staining in representative slices from the brain stems
of three different late-stage tadpoles. Figure 9, A–C,
compares the penetration of protease and kainic acid
into the ventral medulla. Untreated brain stems (Fig.
9A) exhibited sporadic staining throughout, but the
densest staining was at the outermost edges of the
medulla. Brain stems treated with focal perfusion of
protease (Fig. 9B) showed an intense area of staining
at the site of perfusion and extending an average of
50 ± 20 μm below the ventral surface of the medulla.
Brain stems treated with focal perfusion of kainic acid
(Fig. 9C) showed an even more intense staining at the
site of perfusion, which extended an average of 100 ±
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9, D–L, illustrates the distribution throughout the me-
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5 (Fig. 9D), and the resulting cell death is reflected by
intense DEAD red staining in the slice containing that
site. Intense staining is seen only at the site of perfu-
sion (Fig. 9F) and not on the contralateral side adjacent
to CN 5 (Fig. 9E). Lesions within one chemosensitive
site did not destroy cells in the other chemosensitive
site, as seen in the whole slice at the level of CN 10
(Fig. 9I) and the higher magnification of the contralat-
eral side of that slice (Fig. 9J). Nor did the lesions
destroy cells in any other area of the medulla, as seen
in the whole slices at the level of CN 8 (Fig. 9G) and SN

Fig. 7. Neuroventilatory responses of lung burst frequency as a function of develop-
mental level during bath-applied normo-
capnia and bath-applied hypercapnia af-
after a lesion in the caudal chemosensitive
site (CN 10). Protease (A) and kainic acid
(B) lesions in the ventral medulla adjacent
to CN 10 both block the hypercapnic re-
response. Each symbol represents mean
lung burst frequency ± SD for 4–6 tadpoles or frogs when the brain stem was
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Fig. 8. Neuroventilatory responses of lung burst frequency as a function of develop-
mental level during bath-applied normo-
capnia before and after a lesion in a
chemosensitive site. The normocapnic data from Figs. 6
and 7 have been combined to demonstrate the effect of
kainic acid and protease lesions on the unstimulated
ventilatory output of each brain stem. Lesions that
blocked the hypercapnic response (A) also caused a
decrease in lung burst frequency during normocapnia,
whereas the lesion that did not affect the brain stem’s
overall chemosensitivity (B; protease placed rostrally)
also had no affect on the normocapnic fictive lung ven-
tilation frequency. Each symbol represents the mean
lung burst frequency ± SD for 4–6 tadpoles or frogs
before (○) and after (●) a lesion was made. See text for
details of statistical comparisons.

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Fig. 9. Photomicrographs of the brain stem of late tadpoles stained with DEAD red after no treatment, protease lesion, or kainic acid lesion to the rostral chemosensitive site. The untreated brain stems (A) exhibited sporadic staining throughout but densest staining at the outermost edges of the medulla. After focal perfusion of protease (B), there was an intense area of staining at the site of perfusion, which extended an average of 50 ± 20 μm below the ventral surface of the medulla. After focal perfusion of kainic acid (C), there was an even more intense staining at the site of perfusion and extending an average of 100 ± 20 μm below the ventral surface of the medulla. E–L: distribution throughout the medulla of cells affected by protease and kainic acid lesions within the chemosensitive sites. Kainic acid was focally perfused in the ventral medulla adjacent to CN 5 (D). Intense staining is seen only at the site of perfusion (F) and not on the contralateral side adjacent to CN 5 (E). Lesions within 1 chemosensitive site did not destroy cells in the other chemosensitive site, as seen in the whole slice at the level of CN 10 (I) and at the higher magnification on the contralateral side of that slice (J). Nor did the lesions destroy cells in any other area of the medulla, as seen in the whole slices at the level of CN 8 (G) and SN 2 (K) and in the higher magnification of the contralateral (H) and ipsolateral (L) sides of the two slices, respectively.

DISCUSSION

This study of central CO₂ chemosensitivity in developing bullfrogs yielded five novel findings. First, the hypercapnic response of the isolated bullfrog brain stem can be elicited by focal hypercapnic stimulation within two discrete sites (a rostral site adjacent to the CN 5 roots and a caudal site adjacent to the CN 10 roots) in the superficial layer of the ventral medulla at all stages of metamorphosis. We found no evidence that the chemosensory sites migrate (40), and these sites do not seem to colocalize with the gill and lung central pattern generators (45), contrary to previous studies (40). Second, in most developmental stages of bullfrogs, unilateral CO₂ stimulation within either of these sites is sufficient to evoke a hypercapnic response that is indistinguishable from the response to hypercapnic stimulation of the entire brain stem. Third, for a hypercapnic response to be elicited from any developmental stage, it is necessary that neurons within both sites be functional, but both sites need not be stimulated by hypercapnia simultaneously. This pattern of interactions among sites differs substantially from the interactions among multiple chemoreceptor sites in many mammalian preparations. Fourth, late tadpole stages are anomalous in that both chemosensitive sites must be stimulated simultaneously to elicit a hypercapnic response. Fifth, neurons that are essential to the hypercapnic response also contribute to baseline ventilatory drive because destruction of neurons in these sites leads to a decrease in the frequency of fictive lung ventilation during normocapnia.

Hypercapnic responses over the course of development. The ontogeny of the neuroventilatory response to hypercapnia, evident as an increase in fictive ventilation, has been investigated previously in bullfrogs, but conflicting results were found as to whether a hypercapnic response was evident as an increase in fictive lung ventilation in the earliest stages of metamorphosis (39) or evident only in the latter half of metamorphosis (42). Once again, we found that a hypercapnic response was present from the earliest stages of bullfrog development. Furthermore, the strength of this response steadily increases over the course of development, and a constant level of hypercapnic stimulation elicits a greater increase in the frequency of fictive lung ventilation, although there is a relatively consistent 2-
to 2.5-fold increase in lung frequency in each metamorphic group. There are two possible ways to interpret the interactions between chemosensors and the lung central pattern generator (CPG). As a first approximation, the interaction may be additive or multiplicative. If the hypercapnic stimulus adds to the ventilatory activity already present, then the increase in lung bursts during hypercapnia as metamorphosis progresses is evidence of a developmental increase in the neuroventilatory response to CO$_2$. On the other hand, if CO$_2$ multiplies the basic lung CPG output (and that is consistent with other aspects of the hypercapnic response in tadpoles [see below]), then there is no increase in responsiveness to CO$_2$ during metamorphosis since the relative change in lung frequency during hypercapnia remained constant over the course of metamorphosis. However, there was a metamorphic change in the activity of the lung CPG since the normocapnic lung frequency increased during metamorphosis. Regardless of the nature of the interaction between chemosensors and the lung CPG or the neuronal mechanism of this ontogenetic change, it is clear that central CO$_2$ chemoreceptors are present in the brain stem and influence the lung rhythm generator from the earliest stages of bullfrog metamorphosis.

**Ventral medullary sites of CO$_2$ chemosensitivity.** In mammals, the specific sites that contribute to central chemosensitivity have been investigated extensively. Among the possible “proofs” of chemosensitivity, the most unequivocal evidence that an area of the brain stem is involved in respiratory chemosensitivity is that discrete application of CO$_2$ and/or H$^+$ in the particular area leads to an increase in ventilation or neuroventilatory activity (3, 9, 31, 37). Reported here is similar evidence that there are two sites of CO$_2$ chemosensitivity on the ventral surface of the bullfrog medulla, a rostral one adjacent to the roots of CN 5 and a caudal one adjacent to the roots of CN 10. Chemosensitivity is not a general property of the ventral medullary surface in bullfrogs, as shown by the failure of similar hypercapnic stimulation at other ventral medullary sites adjacent to CN 8 and SN 2 to stimulate ventilatory activity (Fig. 3), but is a property specific to these two locations. Thus, like mammals, the bullfrog ventral medulla has identifiable sites of chemosensitivity, but the locations of these sites do not correspond to the rostral (28), intermediate (35), and caudal (23) areas of the ventral medulla identified in mammals.

Focal stimulation within either site of central chemosensitivity in the bullfrog elicits a hypercapnic response that is statistically indistinguishable from the response to equivalent CO$_2$ stimulation of the entire isolated brain stem. In mammals, discrete stimulation at any one of the central chemosensitive sites elicits a significant increase in ventilation that is 15–40% of the increase seen when the body’s entire complement of chemosensitive areas (central and peripheral) are stimulated simultaneously by an increase in arterial PCO$_2$ in intact animals. Thus it appears that the various sites of mammalian central chemosensitivity are each capable of contributing partially to the hypercapnic response. The capacity to initiate only a portion of the total ventilatory response in mammals likely reflects three issues. There is a complicated algebraic relationship among the central CO$_2$ chemoreceptors in intact unanesthetized animals. The peripheral chemoreceptors contribute to the ventilatory response to CO$_2$ in intact animals, and other respiratory and nonrespiratory inputs provide tonic excitation to the respiratory centers and enhance the ventilatory response for any level of chemical drive. The details of interactions among central CO$_2$ chemoreceptors depend on the animal’s arousal state and the presence or absence of anesthesia, but in no case is the relationship mathematically straightforward (simple addition among sites or multiplication). In respect to peripheral chemoreceptors in mammals, the peripheral chemoreceptors contribute up to 50% of the ventilatory response to transitory changes in PCO$_2$ and/or pH (25). Thus peripheral chemoreceptor input may add to the stimulatory influences of central chemoreceptors and/or augment the sensitivity of these receptors. Because the present study used isolated brain stem preparations in the tadpole, the response of ventral medullary chemosensitive sites to focal stimulation was never compared with a whole body hypercapnic response, i.e., one measured when both peripheral and central chemoreceptors were stimulated. The response of the isolated brain stem to bath application of hypercapnic aCSF is likely to represent only part of the response of the intact whole brain hypercapnic response (27). Nonetheless, the central chemosensory sites in most bullfrog developmental stages have a clearer pattern of interdependence of function than in mammals since each ventral medullary site of chemosensitivity is capable of eliciting an equal hypercapnic response when it alone is stimulated, but no response can be elicited if the neurons in either region are destroyed.

**Location of chemosensory sites within the ventral medulla.** In addition to confirming that neuronal output from the two ventral medullary chemosensitive sites is necessary for both the hypercapnic ventilatory response and baseline ventilation, the kainic acid lesion studies provide information about the location of the chemosensory neurons associated with these sites. The kainic acid lesions indicate that the chemosensory neurons associated with each site all lay within 100 μm of the surface where the kainic acid was applied. Staining of neurons destroyed by kainic acid was limited to this area, which precludes the possibility that chemosensitivity of the sites was conferred by projections to these areas from neurons located elsewhere in the brain stem. The protease lesions provide further information about the location of the chemosensory neurons associated with these sites, as well as the CO$_2$ sensors associated with these neurons. Focal perfusion of protease destroyed cells within 50 μm of the surface where it was applied. Because protease lesions at the caudal chemosensitive site eliminated the overall hypercapnic ventilatory response, the chemosensory neurons associated with this site are likely located within 50 μm of the ventral medullary surface adjacent to the roots of...
CN 10. At the rostral site, focal perfusion of protease destroyed the chemosensitivity of that site alone without affecting the overall hypercapnic ventilatory response or baseline ventilation. Protease treatment seemed to destroy the chemosensitivity of neurons at this site without destroying the neurons themselves. A possible explanation is that the neurons lie 50 to 100 μm below the ventral surface, but they possess CO2-sensing elements that project toward the surface and lie within 50 μm of it. Chemosensitive neurons with such projections have been identified in the neonatal rat brain stem (22). According to our studies, these CO2-sensing components consist of protein moieties for their CO2 sensitivity because protease destroyed that sensitivity. However, destruction of these protein moieties does not breach the integrity of the cell membrane because protease treatment did not allow the DEAD red stain to penetrate the cell. Thus, at both sites, CO2 sensors lie within 50 μm of the ventral surface. In the caudal site, these sensors are located close to the cell body, whereas in the case of the rostral site these protein-bearing sensors appear to be located on projections up to 100 μm away from the cell body. A similar superficial ventral location has been implicated in mammals (33).

Taken together, the kainic acid and protease studies indicate that neurons whose individual output is essential to both the hypercapnic ventilatory response and baseline ventilation are located on both sides of the ventral medulla in the outermost 100 μm near the roots of CN 5 and 10. The fact that unilateral lesions had such a large effect on chemosensitivity was somewhat surprising. We surmise that output from chemosensory neurons on both sides of the site is necessary for the hypercapnic response, although stimulation of one side is sufficient to elicit the response if the contralateral side is intact but not stimulated. To account for the unilateral effectiveness of lesions, the chemosensory neurons must have decussating projections between the two sides of the brain stem within a given site. Furthermore, the loss of chemosensory function when only one of two bilaterally symmetrical sites was inhibited suggests that there is a strong interdependence between the sites. Similar difficulties exist in mammalian studies, but the circumstances in which unilateral ablation has a large effect in mammals may help understand our results in tadpoles. In anesthetized rats and cats, unilateral lesions of small CO2-sensitive areas often wipe out all ventilatory activity for an extended time (30–90 min) and dramatically reduce the CO2 responsiveness when respiratory activity does resume (32). Some of the animals remain apneic once the lesion is made and regular ventilatory activity is never restored. In contrast, lesions in unanesthetized animals in exactly the same location have remarkably modest effects on ventilation and CO2 responsiveness (1). We did not study intact tadpoles, and the deafferented state of the isolated tadpole brain stem may resemble the anesthetic state in which tonic nonchemosensory excitatory inputs to the lung CPG were considerably reduced, and therefore, the importance of central chemosensory drive to the lung CPG was enhanced. The dramatic reduction in responsiveness when only one of a bilaterally paired structure was inhibited or ablated suggests that the CO2 sensors or neurons within the affected area have the effect of amplifying (multiplying) the respiratory output. Thus, if the amplifier multiplies by zero after ablation, the output of the system is zero. The system does not seem to be additive in respect to CO2 chemosensory responses, in which case ablation of a single site would simply reduce ventilation by a fixed amount. The system is plastic as well. If one waits long enough in mammals, the ventilatory output recovers (although chemosensory responses remain low; Ref. 32). Small, unilateral electrolytic lesions in one of the chemosensory sites often completely suppress lung bursts in tadpoles. About half the time, the lung rhythm is restored after 30–60 min (B. E. Taylor, unpublished observations). Thus the isolated tadpole brain stem demonstrates similar plasticity. Anesthesia and isolation of the brain stem seem to reduce the number of excitatory inputs to the respiratory system and reveal the multiplicative interaction between the lung CPG and chemosensory sites. In more intact systems, the chemosensors represent a much smaller fraction of the total drive to breath acting on the lung CPG, and consequently ablation of any single site has a much smaller effect in intact, unanesthetized animals. However, no studies have yet investigated the electrophysiological interactions or anatomic connections between adjacent sites within a chemosensory region in tadpoles to explore the cellular mechanisms whereby this apparent multiplicative interaction might originate.

Anomalous chemosensory function in late-stage tadpoles. In late-stage tadpoles, focal stimulation of a single ventral medullary chemosensitive site is not sufficient to elicit a hypercapnic response. Rather, it is necessary that the two identified sites be simultaneously stimulated to produce a response. This necessity is illustrated by the disruption of an ongoing hypercapnic response by focal, unilateral application of normocapnic aCSF at either chemosensitive site. We believe that this ontogenetic change reflects a transient reduction in the robustness of chemosensitivity at both ventral medullary sites even though the overall chemosensory response to hypercapnia is not reduced. It appears that neither site loses its chemosensitivity (Fig. 4), but seemingly the individual signals of each site fall below the threshold of responsiveness (Fig. 5). If chemosensitivity is in fact less robust, one might look for a reduced cellular responsiveness (fewer cells and/or less-sensitive cells) or a reduced transmission from chemoreceptors to the lung rhythm generator. Because overall chemosensory responses consistently increase throughout bullfrog development, including the late tadpole stages, reduced cellular responsiveness seems unlikely. We think it is more likely that a neural reorganization, which is known to occur in late-stage tadpoles (4, 16), creates a temporary situation in which paired stimulation at both ventral medullary chemosensitive sites is necessary for a hypercapnic response.
response. In a previous study (39), our laboratory found that acetazolamide produced a paradoxical reduction in the hypercapnic ventilatory response in late-stage tadpoles, a finding we also attributed to a loss of robustness in chemosensory responses. Thus, in two sets of experiments, we found that chemosensory function in late-stage tadpoles was more susceptible to disruption. The transition from aquatic to terrestrial life occurs in the late stages of metamorphosis, and a variety of sensory systems undergo a transformation associated with reduced sensory function at this stage in metamorphosis. The auditory system and visual systems seem to be rewired for aerial acoustic and visual function. In the case of the acoustic system, this may actually be associated with transient deafness (4).

The dominant method of gas exchange changes at this stage of metamorphosis, and the PCO₂ of blood rises dramatically in late metamorphosis. Thus we feel that it is likely that central CO₂ chemosensory mechanisms are also undergoing a transformation that amplifies the interdependence of the rostral and caudal chemosensory sites as the system is prepared for aerial respiration.

Role of central chemoreceptors in respiratory rhythm generation. The lesion studies suggest that output from both the rostral and caudal chemosensitive sites is required for a hypercapnic response at any stage of development. In all but late-stage tadpoles, focal stimulation of a single ventral medullary chemosensitive site was sufficient to elicit a hypercapnic response, but only when the nonstimulated site was intact. Destroying cells with a kainic acid lesion at either site or a protease lesion at the caudal site disrupted the overall hypercapnic response of the brain stem. This suggests that, even when not stimulated, chemosensitive neurons at these sites have a tonic baseline output that contributes to the hypercapnic response. Nattie (30) and Cohen (10) have proposed that tonic baseline output from all chemoreceptor sites is required for full expression of the hypercapnic response in mammals. That the amphibian and mammalian brain stem share this characteristic suggests that integration of central chemosensory outputs from multiple sites is phylogenetically older than previously thought.

Central chemosensitivity sustains baseline, normocapnic ventilation as well as ventilatory responses to changes in CO₂/H⁺ in mammals (29). Baseline ventilation in bullfrogs also seems to require input from central chemoreceptors. Lesions within the ventral medullary chemosensitive sites that eliminated the hypercapnic response also decreased the frequency of fictive lung ventilation during normocapnia. It is significant that, although the lesions decreased the frequency of fictive lung ventilation, they did not abolish it. Nor did they abolish fictive buccal ventilation. Because the destruction of central chemoreceptors did not prevent the generation of the lung or buccal rhythm, the central chemoreceptors are not obligatory components of the central respiratory rhythm generators, and the neurons responsible for central rhythm generation are probably not intrinsically CO₂ chemosensitive.

Perspectives

During the late tadpole phase, the peak of metamorphosis, there is a transient change in the integration of chemosensation from the two ventral medullary sites such that stimulation of a single site is insufficient to elicit a hypercapnic response. The excitatory effects of acidosis are attenuated or less effectively communicated to the lung rhythm pattern generator, and the late-stage tadpole presents, therefore, an interesting conundrum regarding the ontogeny of central CO₂ chemosensitivity. At the peak of metamorphosis, the bullfrog tadpole exhibits significant changes in the perception and/or transmission of chemosensory information, but at the close of metamorphosis, the bullfrog emerges with a central CO₂ chemosensitivity that is remarkably similar to that which it possessed as a hatching tadpole. The developmental stages apparent in tadpoles are recapitulated to some extent in mammals, but the period of mammalian development comparable to late-stage metamorphosis occurs early in fetal life and is inaccessible to study. Our study has suggested a homology of amphibian and mammalian central chemoreception because it identified similarities in the location and interplay between central chemosensitive sites. As we assess the reasons for the lack of robustness of chemosensitivity in late-stage tadpoles, we may extrapolate a similar respiratory transformation in fetal mammals, one that is not currently accessible to study, but a transformation that may represent a period of developmental vulnerability with implications for condition such as the sudden infant death syndrome in which maternal risk factors (alcohol and smoking) seem to modify the subsequent development of the respiratory system in the infant (20).

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DISCLOSURES

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