You might find this additional information useful...

This article cites 28 articles, 7 of which you can access free at:
http://ajpregu.physiology.org/cgi/content/full/293/1/R447#BIBL

Updated information and services including high-resolution figures, can be found at:
http://ajpregu.physiology.org/cgi/content/full/293/1/R447

Additional material and information about *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* can be found at:
http://www.the-aps.org/publications/ajpregu

This information is current as of November 8, 2007 .

Role of midbrain in the control of breathing in anuran amphibians

Luciane H. Gargaglioni,[1] Janice T. Meier,[2] Luiz G. S. Branco,[3] and William K. Milsom[2]

[1]*Department of Animal Morphology and Physiology, UNESP-Sao Paulo State University, FCAV at Jaboticabal, Sao Paulo, Brazil;*
[2]*Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada; and* [3]*Department of
Morphology, Stomatology and Physiology, Dental School of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil*

Gargaglioni LH, Meier JT, Branco LG, Milsom WK. Role of
midbrain in the control of breathing in anuran amphibians. *Am J
Physiol Regul Integr Comp Physiol* 293: R447–R457, 2007. First
published April 9, 2007; doi:10.1152/ajpregu.00793.2006.—The
present study was designed to explore systematically the midbrain of
unanesthetized, decerebrate anuran amphibians (bullfrogs), using
chemical and electrical stimulation and midbrain transections to
identify sites capable of exciting and inhibiting breathing. Ventilation
was measured as fictive motor output from the mandibular branch of
the trigeminal nerve and the laryngeal branch of the vagus nerve. The
results of our transection studies suggest that, under resting condi-
tions, the net effect of inputs from sites within the rostral half of the
midbrain is to increase fictive breathing frequency, whereas inputs
from sites within the caudal half of the midbrain have no net effect on
fictive breathing frequency but appear to act on the medullary central
rhythm generator to produce episodic breathing. The results of our
stimulation experiments indicate that the principal sites in the mid-
brain that are capable of exciting or inhibiting the fictive frequency of
lung ventilation, and potentially clustering breaths into episodes,
appear to be those primarily involved in visual and auditory integra-
tion, motor functions, and attentional state.

control of ventilation; central nervous system; mesencephalon; frog;
Rana catesbeiana

IN ALL VERTEBRATES, THE MIDBRAIN links the sensory, motor, and
integrative components of the pons and medulla with those of
the forebrain. The midbrain contains three major regions: the
tectum, the tegmentum, and the isthmus. These regions contain
nuclei intrinsic to each, as well as ascending and descending
fiber tracts that pass through them (2). Several of these nuclei
control the synthesis of important neurotransmitters (notably
the catecholamines and serotonin) and their distribution to
other brain regions (2). In general, the midbrain plays a
fundamental role in integrating the behaviors of multiple organ
systems to produce coordinated responses. Many of these
behaviors modulate respiration, and thus stimulation of mid-
brain regions would be expected to affect descending inputs
that exert a modulatory influence on the respiratory rhythm-
generating networks located in the caudal brain stem (medulla
and pons) (see Ref. 13).

In mammals, electrical stimulation of the midbrain produces
increases in both respiratory frequency and depth (3), and
midbrain lesions can prevent the respiratory changes associated
with changes in EEG state (4). Moreover, some recording
experiments have shown that neurons in midbrain areas, in-
cluding the superior colliculus, red nucleus, and the central

tegmental field, have firing patterns temporally related to the
respiratory cycle, implying that they receive respiratory-related
inputs (29), although others have failed to substantially confirm
this (24). The sum of these data suggest that midbrain sites may
receive information from, and modulate, brain stem respiratory
centers, although these data do not suggest that they play a
significant role in the production of the normal respiratory
rhythm or pattern.

Recent studies of the role of midbrain structures on the
regulation of breathing pattern in amphibians suggest that the
midbrain may play a more important respiratory role in am-
phibians than in mammals. The breathing pattern of these
animals can range from random single breaths, to regular
single breaths, to breathing in episodes, and finally to contin-
uous breathing, depending on several factors, including the
level of respiratory drive (see Ref. 22). In in vitro, brain
stem-spinal cord preparations of the American bullfrog, *Rana
catesbeiana*, a transection in front of the midbrain at a level
slightly caudal to the optic nerve roots transformed the fictive,
episodic breathing pattern into one of relatively evenly spaced
breaths in which the overall breathing frequency increased but
breath amplitude remained constant (21, 26). The effects of this
transection in decerebrate, paralyzed in situ preparations, how-
ever, was somewhat different. Although this level of transec-
tion produced a pattern of evenly distributed breaths, it also
dramatically reduced breathing frequency and increased breath
amplitude. Furthermore, this pattern could be converted back
to a pattern of smaller amplitude breaths occurring in episodes
again by lung inflation (22). In these preparations, episodic
breathing was only completely eliminated by transection be-
hind the optic lobes (i.e., behind the midbrain) just in front of
the cerebellum (23). These data suggested that structures
within the midbrain of anurans, besides providing tonic drive
for breathing, may be important for clustering breaths into
episodes and in producing prolonged periods of apnea (26). It
was suggested that the nucleus isthmi, a mesencephalic struc-
ture located in the roof of the amphibian midbrain below the
bridge to the cerebellum, might be such a structure (15), but the
episodic breathing pattern was not completely eliminated either
in decerebrate frogs (*Rana catesbeiana*) after pharmacological
lesion of this area (15) or in unanesthetized toads (*Chaunus
schneideri*) after electrolytic lesion of this area (7).

The variety of breathing patterns observed in in vitro prep-
arations with midbrain attached, the differences seen after
midbrain transections in different preparations, and the differ-
ences seen after midbrain lesions in different preparations
suggest that the balance of inputs arising from the midbrain

Address for reprint requests and other correspondence: L. H. Gargaglioni,
Via de acesso Paulo Donato Castellane s/n, 14870-000, Departamento de
Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinár-
ias, Universidade Estadual Paulista Júlio de Mesquita Filho, Jaboticabal, SP,
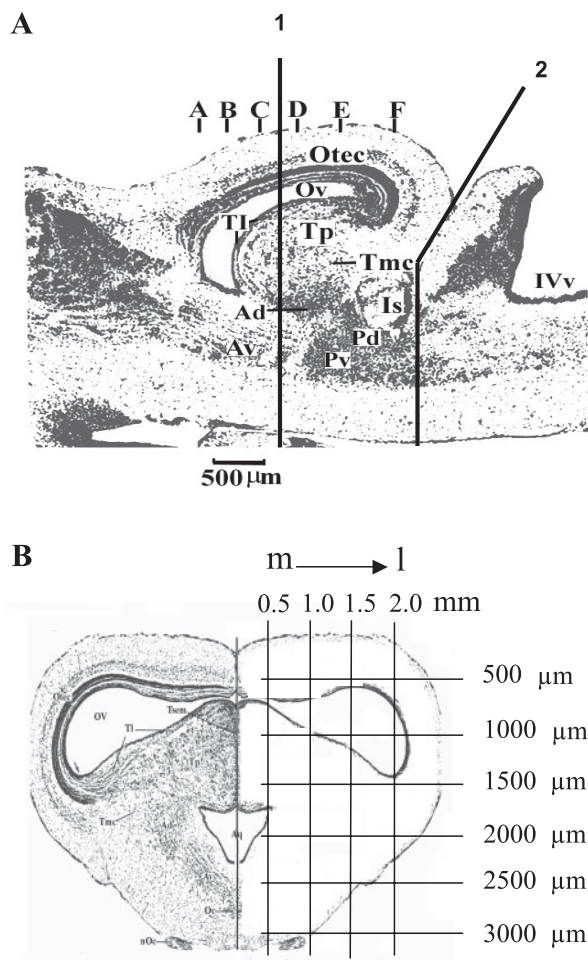Brasil (e-mail: lucihel@fcav.unesp.br.).

A

B

Fig. 1. *A*: parasagittal section of the bullfrog brain ~0.63 mm from the midline. *Left*: rostral. *Right*: caudal. Electrical stimulations were administered at 3 or 4 medial-to-lateral sites at each of 6 rostral-to-caudal levels of the midbrain (*levels A–F*), and transections were made at 2 rostral-to-caudal levels of the midbrain (*1* and *2*). *B*: transverse section of the bullfrog brain to illustrate the 4 medial-to-lateral sites used for electrode penetration and the 6 dorsal-to-ventral sites at which stimuli were applied. Ad, anterodorsal tegmentum; Aq, aqueduct of Sylvius; Av, anteroventral tegmentum; Is, nucleus isthmi; nOc, oculomotor nerve; Oc, nucleus of oculomotor nerve, Otec, optic tectum; Ov, optic ventricle; Pd, posterodorsal tegmentum; Pv, posteroventral tegmentum; Tl, laminar nucleus of torus semicircularis; Tmc, magnocellular nucleus of torus semicircularis; Tp, principal nucleus of torus semicircularis; Tsem, subpendymal midline nucleus. [From Gargaglioni and Milsom (10).]

bodies. The transection experiments were designed to determine which half of the midbrain contains the sites that are instrumental in producing the episodic breathing pattern.

METHODS

Animals

Frogs (*Rana catesbeiana*; 220 ± 13.2 g) of undetermined sex were obtained from a commercial supplier. The animals were maintained
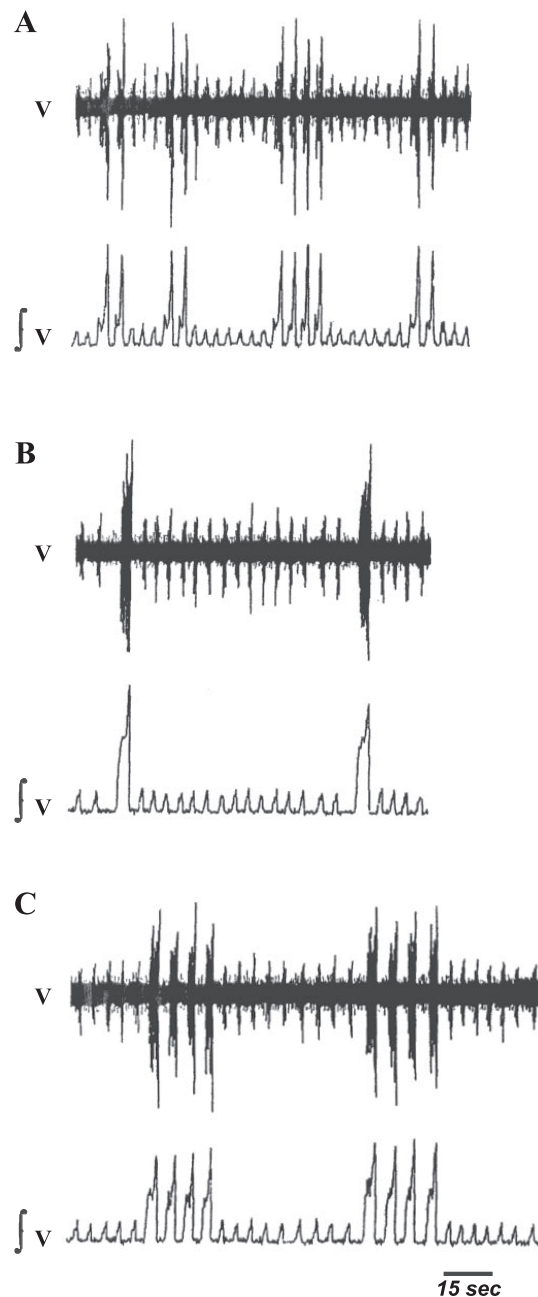
A

B

C

Fig. 2. *A*: episodic pattern of fictive lung breaths exhibited by a decerebrate bullfrog ventilated with 2.5% CO_2 before any transections. Note the buccal oscillations (small bursts of activity) between lung breaths (large bursts of activity). *B*: pattern of slow, single breaths exhibited by the same bullfrog after a transection at the level of the midoptic tectum. *C*: episodic breathing pattern exhibited by the same bullfrog after a subsequent increase in lung inflation. Each pair of traces illustrate the raw (*top*) and integrated (*bottom*) discharges recorded from the fifth cranial nerve (V).

must vary from preparation to preparation. That notwithstanding, the overall results of these studies also highlight the importance of neurons in the region between the optic chiasma and the rostral border of the medulla in influencing breathing pattern. In view of this, the present study was designed to explore the anuran midbrain systematically to begin to define sites that modulate breathing pattern. This was done in frogs (*Rana catesbeiana*) using chemical (glutamate) and electrical stimulation and transection protocols. The chemical and electrical stimulation experiments were designed to determine generalized midbrain sites capable of exciting or inhibiting breathing. The electrical stimulations were performed to stimulate both axons of passage and cell bodies, and glutamate injections were performed to more specifically excite only cell

indoors in fiberglass tanks with running water and free access to heating lamps. The animals were fed locusts and crickets once per week and maintained on a daily photoperiod of 12 h of light and 12 h of dark. Food was withheld for 2 days before surgery. Animal holding and experimental procedures followed Canadian Council on Animal Care Guidelines and were approved by University of British Columbia Animal Care Committee (certificate no. 980294).

Surgical Procedures

All experiments were performed on decerebrate animals. The reduced preparation was similar to the one developed by Kogo et al. (16). Animals were anesthetized by immersion in an aqueous solution of ethyl-*m*-aminobenzoate (0.15% MS-222; Sigma, St. Louis, MO) buffered to pH 7.8 with $NaHCO_3$ until the toe pinch reflex was abolished. An incision was made along the saggital plane on top of the head to cut the skin. With the use of a small drill, a hole was then drilled into the skull. The brain was transected between the optic

tectum and the rostral forebrain by electrocautery, and the entire forebrain was removed by aspiration with a suction device. Additionally, a small opening was made in the skull to expose the midbrain region. Small cotton pellets soaked with physiological saline were placed over the area to prevent desiccation. The hole in the cranium was then sealed with bone wax, a small piece of dental dam was affixed to the top of the skull with cyanoacrylate glue, and the skin was subsequently closed with sutures. The animals were allowed to fully recover from the anesthesia, and the experiments were performed at least 24 h after the decerebration.

Experimental Setup

The following day, all animals had recovered from decerebration and were spontaneously active. They were restrained, and the apex of each lung was cannulated with polyethylene tubing (PE-240) to establish unidirectional ventilation of the lungs with humidified air containing 2.5% CO_2 The exit cannula for the unidirectional ventila-
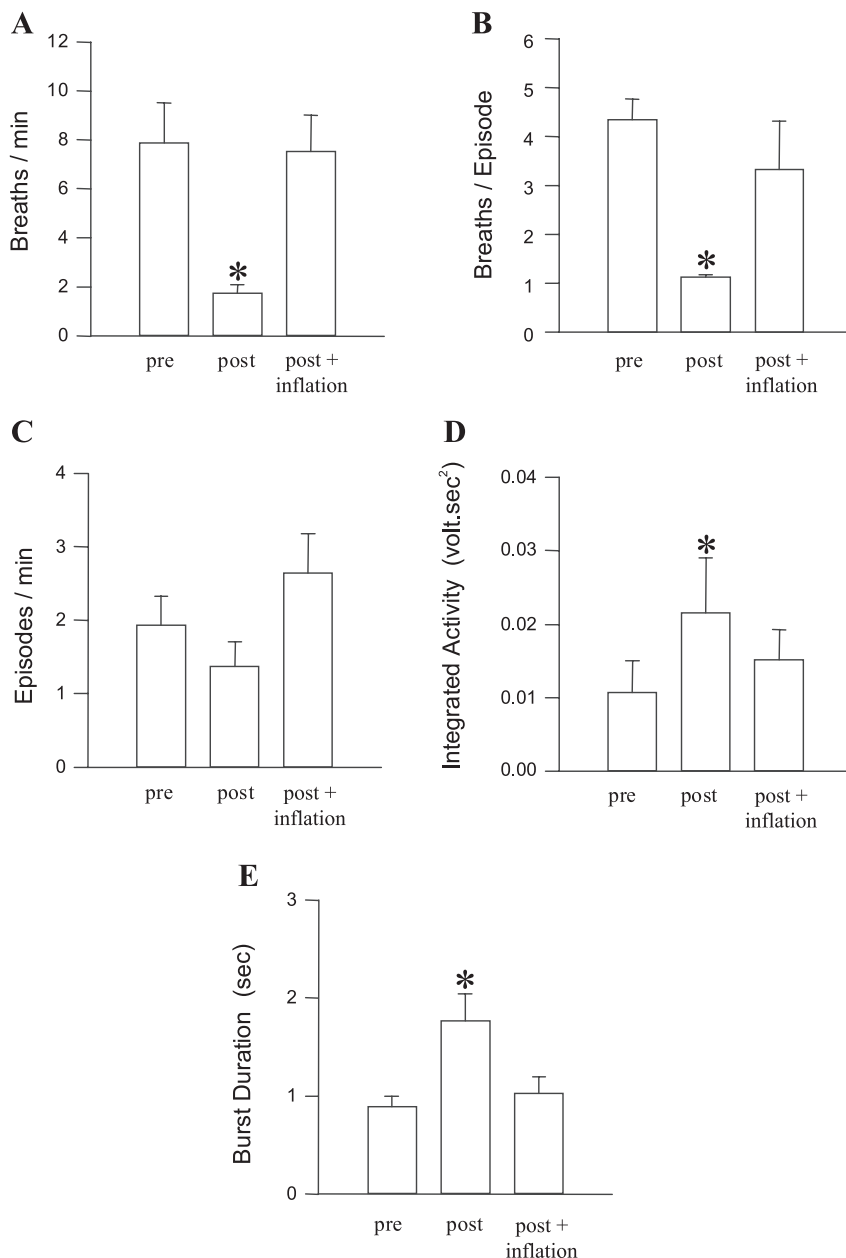

Fig. 3. Effects of transection at the level of the midoptic tectum of bullfrogs on overall frequency of fictive breathing (*A*), number of breaths per episode (*B*), number of episodes per minute (*C*), total integrated activity associated with each fictive burst of motor output (*D*), and duration of each fictive breath (*E*). "Pre" represents values for preparations before transection, and "post" represents values for preparations after transection. *Significantly different from the pre condition, $P < 0.05$.

tion was open to air, minimizing outflow resistance and maintaining the lungs in a partially inflated state. This level of CO_2 was used to produce normal blood-gas levels of CO_2 and pH (15). The level of lung inflation ranged from 1 to 2 cmH_2O. These levels remained constant throughout all protocols except the transection study. The glottis was occluded with tissue cement to prevent the escape of gas through the mouth. The brain was reexposed, and the animal was placed in a stereotaxic apparatus. Animals were kept moist with damp towels at all times to prevent desiccation. No paralyzing agents were used, and animals remained calm and motionless once the setup was complete.

Measurements of Fictive Ventilation

Ventilation was assessed from the mandibular branch of the trigeminal nerve (fifth cranial nerve) and the laryngeal branch of the vagus nerve (tenth cranial nerve). The skin covering the mandible was cut and retracted, and the fifth nerve was located and isolated. A piece of 5-0-gauge silk was placed around the nerve before it was cut distally. The vagus nerve was located on one side from a dorsal approach. An incision was made above the scapula, the muscles were cut, and the scapula was retracted to isolate the laryngeal branch of the tenth nerve. The branches of the fifth and tenth nerves were then each positioned on bipolar platinum hook electrodes and covered with a 1:1 mixture of Vaseline and mineral oil. Nerve activity recorded from the bipolar electrodes was amplified and filtered (10 Hz to 2 kHz), full wave rectified, and integrated in 67-ms intervals. The signal was monitored visually with an oscilloscope and acoustically with an audio monitor (Grass AM8). The electroneurogram was recorded on computer (Windaq v1.32 data acquisition system, DI-720; DATAQ Instruments) sampling at a frequency of 2,500 Hz per channel.

Brain Transections

Transection experiments were conducted only on the bullfrogs. In these experiments, episodic breathing was first produced by altering respiratory drive by increasing or decreasing the level of CO_2 in the ventilation gas. The level of respiratory drive required to produce episodic breathing in anurans varies from individual to individual; however, by altering the level of CO_2 at the starting level of gas flow used for unidirectional ventilation, episodic breathing could always be produced. Once steady-state fictive ventilation had been recorded in the decerebrate preparation ($n = 20$) (usually for at least 1 h), the brain was further transected at either the level of the midoptic ($n = 10$) or caudal optic ($n = 10$) tectum (1 and 2 in Fig. 1). Transections were performed with either a fine microblade fitted to a holder mounted in a micromanipulator or with a pair of extra-fine iris scissors.

In all cases, nerve discharge (not necessarily fictive breathing) was stimulated by the initial transection, but the spurious discharge disappeared and fictive breathing returned and then stabilized at some new level, usually within 15 min. We then waited at least 30 min before beginning to record and then recorded for a minimum of 20 min. Throughout this time, the fictive breathing pattern was stable and persistent. After transections at both levels, the episodic breathing was eliminated, and, after this recording period, attempts were made to restore episodic breathing by manipulating respiratory inputs (lung volume, alteration of the outflow resistance of the exit cannula used for unidirectional ventilation, and/or the level of CO_2).

Only one transection was performed in each animal. At the end of each experiment, the brains were removed and fixed in 10% neutral buffered formalin for at least 24 h. The tissue was subsequently embedded in paraffin, sectioned, and then stained with eosin-hematoxylin to determine the exact level of each transection.

Midbrain Stimulations

Electrical stimulations. In this series of experiments, once the animals ($n = 4$) were placed in the stereotaxic apparatus, a tungsten

electrode (0.4 μm tip diameter; A-M Systems, Everett, WA) was placed into an electrode holder and positioned at the rostral end of the midbrain. Electrical stimulations were then administered at three or four medial to lateral sites (see Fig. 1B) at each of six rostral-to-caudal levels of the midbrain according to the fields described by Potter (25) (*levels A–F* in Fig. 1A). At each site, stimulations were made at sequential 500-μm steps up to a total depth of 3,000 μm from the dorsal surface (Fig. 1B). The midbrain was stimulated electrically with rectangular pulses 6 ms in duration, at 10–150 μA using each of two protocols. The first protocol consisted of a train of five stimuli applied every 10 s for 60 s in total, and the second protocol consisted of a single stimulus applied every 2 s for 30 s in total. These parameters were selected based on the results of pilot experiments.

Chemical stimulations. In this series of experiments, we used only frogs ($n = 6$). Once the animals were placed in the stereotaxic apparatus, the glass micropipettes were positioned at the rostral end of the midbrain. We stimulated the same sites used in the electrical
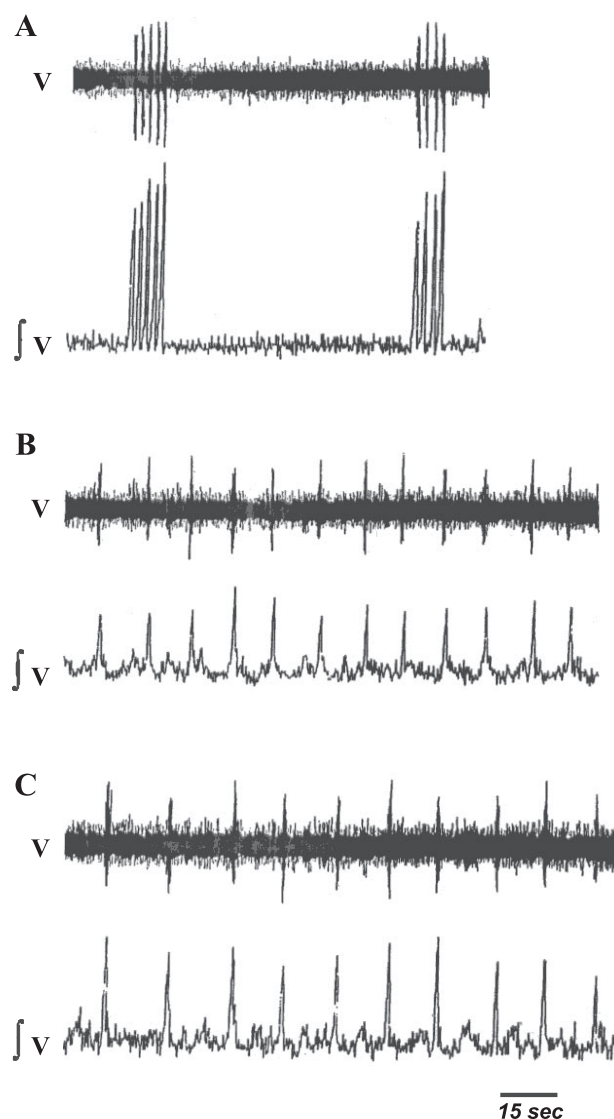

Fig. 4. *A*: episodic pattern of fictive lung breaths exhibited by a decerebrate bullfrog ventilated with 2.5% CO_2 before any transections. *B*: pattern of slower, evenly spaced breaths exhibited by the same bullfrog after a transection at the caudal margin of the optic tectum. *C*: pattern of evenly spaced breaths remains after a subsequent increase in lung inflation and inspired CO_2. Each pair of traces illustrates the raw (*top*) and integrated (*bottom*) discharge recorded from the fifth cranial nerve.

stimulation protocol (*levels A–F* in Fig. 1*A*). Multibarrel glass micropipettes (35–40 μm outer tip diameter) were fabricated from five-barrel microfilament capillary glass (1.2 mm outer diameter, 0.6 mm inner diameter; AM Systems) using a Narashige pipette puller, which was broken back until the total tip diameter measured ∼10–15 μm. Barrels were two-thirds filled with either *1*) mock cerebrospinal fluid (in mM: 104 NaCl, 4 KCl, 1.4 $MgCl_2$, 10 D-glucose, 25 $NaHCO_3$, 2.4 $CaCl_2$) (1 barrel); *2*) glutamate solution (Sigma, St. Louis, MO) (10 mM in cerebrospinal fluid, 3 barrels); or *3*) Evans blue dye (1 barrel). Barrels were filled such that each meniscus was visible. A piece of polyethylene tubing (PE-10; Clay Adams) was secured into the top of each barrel with epoxy adhesive at one end and a manifold constructed of a series of five three-way stopcocks at the other. The manifold was connected to a pressure ejection system (Picospritzer II; General Valve) such that only one barrel of the multibarrel micropipette was exposed to any given pressure pulse. Both the duration and magnitude of the pressure pulse could be adjusted on the pressure ejector to regulate injection volume, and the injection volume was calculated from the radius of the micropipette barrel and the distance traveled by the meniscus during the injection as measured with a microscope equipped with a fine reticule. The injections were performed with a pressure of 80–100 psi applied for 300–1,000 ms, resulting in small injection volumes (∼10 nl).

Histology

For the chemical stimulation protocol, not all injection sites were marked. In each animal, microinjection sites located at least at two different rostrocaudal and one dorsoventral level were marked with microinjections of Evans blue dye (2%). Microinjections were made at least 5 min apart by pressure injection with the picospritzer. The volume of each injection was monitored by the movement of the meniscus of the drug against gradations on a calibrated scale. At the end of the experiments, the animals were deeply anesthetized with 0.3% MS-222 (Sigma), Ringer solution followed by 10% formalin was perfused through the heart, and the animals' heads were removed
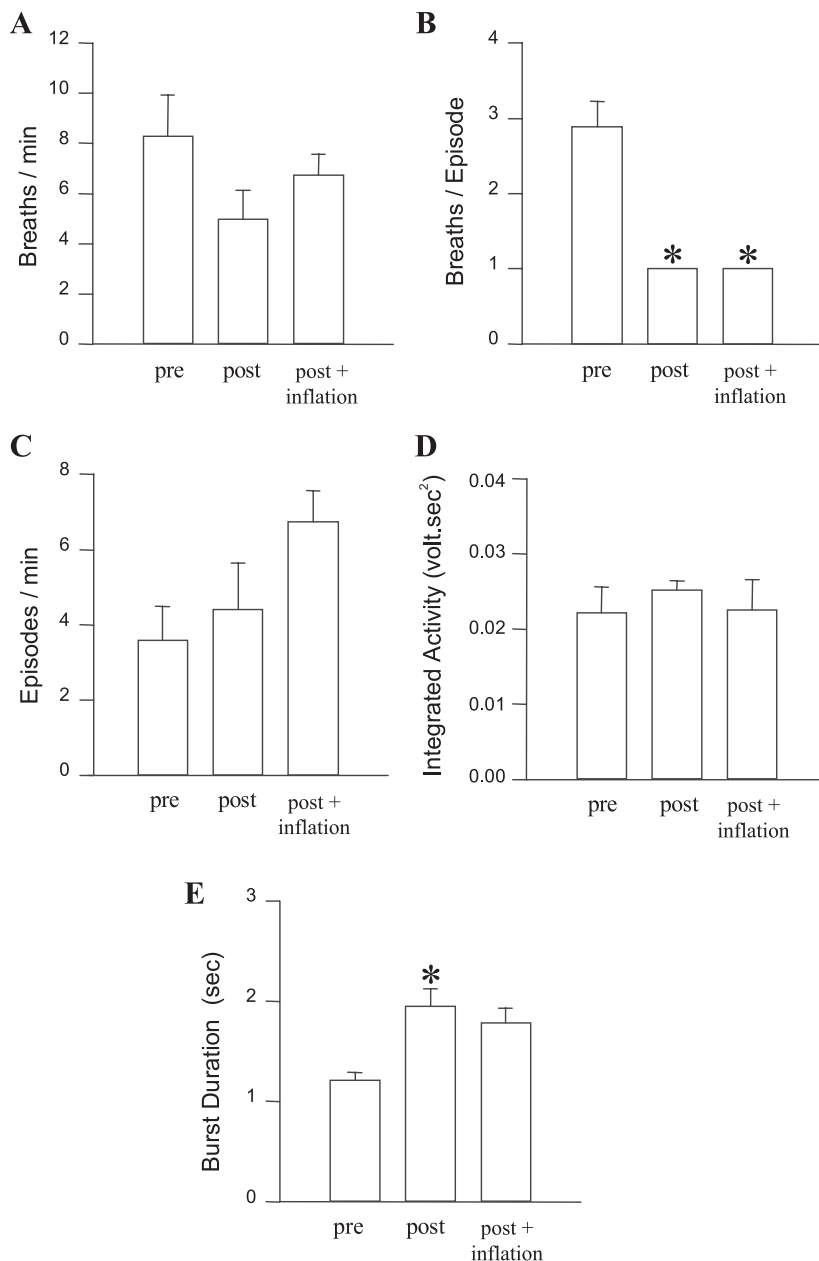

Fig. 5. Effects of transection at the level of the caudal margin of the optic tectum of bullfrogs on overall frequency of fictive breathing (*A*), number of breaths per episode (*B*), number of episodes per minute (*C*), total integrated activity associated with each fictive burst of motor output (*D*), and duration of each fictive breath (*E*). "Pre" represents values for preparations before transection, and "post" illustrates the values for preparations after transection. *Significantly different from the pre condition, $P < 0.05$.

Table 1. *Sites in anuran midbrain in the vicinity of which electrical stimulation altered fictive lung ventilation frequency*

Level of Midbrain Stimulation	Coordinates	Prestimulation Period	Stimulation
A	1.0 mm of midline, 0.5 mm deep	29.5±7.5	10.2±4.1*
B	1.5 mm of midline, 0.5 mm deep	18.6±7.7	6.6±3.5*
C	1.0 mm of midline, 2.0 mm deep	9.3±2.3	16.8±3.7*
C	1.5 mm of midline, 0.5 mm deep	18.6±5.1	4.0±3.0*
D	1.0 mm of midline, 2.0 mm deep	17.4±5.2	3.0±3.0*
D	1.5 mm of midline, 1.5 mm deep	17.0±4.7	10.0±5.0*
E	1.5 mm of midline, 0.5 mm deep	19.0±2.0	0.0±0.0*
F	0.5 mm of midline, 1.5 mm deep	9.2±4.1	17.5±3.0*
F	1.5 mm of midline, 0.5 mm deep	19.0±2.7	8.2±0.8*

Values are means ± SE in breaths/min. *Significant difference between the prestimulation and stimulation period, $P < 0.05$ (Student's t-test).

and fixed in 10% formalin. The next day, the brains were removed from the skull, immersed in paraffin, sectioned with a microtome, and stained by the cresyl-Nissl method for light microscopy determination of the injection sites.

Data Analysis

All data recorded for respiratory variables are reported as means ± SE. For the transection experiments, measurements were made of the overall fictive breathing frequency, the mean duration of each burst of motor output, the mean integrated area of each fictive burst, the number of breaths per episode, and the number of fictive episodes per minute both pre- and posttransection. For the purposes of this part of the study, breathing episodes were defined as sequences of breaths with no pause in between (see Figs. 2 and 4). By this definition, continuous slow breathing, in which single breaths were separated by periods of apnea, computes as a pattern of episodes of one breath each (and hence breaths/episode × episodes/min = breaths/min). Data were analyzed from the last 20 min of recording both before and after the transection. The differences between pretransection and posttransection and postinflation periods were accessed by one-way repeated measures ANOVA followed by Tukey's test.

For the stimulation experiments, measurements were made of the mean frequency, amplitude, and duration of the fictive lung breaths. We did not analyze the results of the stimulation experiments for effects on breathing episodes because the stimulus protocol interfered with the normal production of breathing episodes. The differences between prestimulation and stimulation period (electrical stimulation) and between vehicle and glutamate group were accessed by paired t-tests.

RESULTS

Transections at the Level of the Midoptic Tectum

Transections at the level of the midoptic tectum decreased the overall frequency of fictive breathing ($P = 0.007$; Fig. 3A) and also converted the episodic breathing pattern into a pattern of uniform (evenly spaced) breaths (Figs. 2 and 3, B and C). Both the average integrated activity ($P = 0.022$; Fig. 3D) and the average burst duration ($P = 0.009$; Fig. 3E) increased. If the lungs were subsequently inflated, the fictive breathing pattern returned to an episodic pattern that did not appear different from that seen before the transection (Fig. 2C).

Transections at the Level of the Caudal Optic Tectum

Transections at the level of the caudal optic tectum did not alter the overall frequency of fictive breathing (see Fig. 5A) but did convert the episodic breathing pattern into one of relatively slow, continuousbreathing (Figs. 4 and 5, B and C). After this

transection, alteration of neither lung volume (Figs. 4C and 5B) nor the level of CO_2 in the ventilation gas (not shown) could restore episodic breathing. Although the average burst duration increased significantly ($P = 0.001$; Fig. 5E) after the transection, the average integrated activity was not significantly altered (Fig. 5D).

Electrical and Chemical Stimulations in the Rostral Half of the Midbrain

Electrical and chemical stimulation at specific midbrain locations evoked reproducible changes in the frequency and amplitude of the respiratory discharge recorded from the fifth or tenth cranial nerve roots. The two different stimuli produced similar results.

Effects on fictive lung breathing. At *level A*, a decrease was observed with stimulation in the area of the pretectal gray (Tables 1 and 2 and Fig. 6). At *levels B* and *C*, electrical stimulation of the area of the optic tectum decreased fictive lung ventilation frequency. Chemical stimulation at *level B*, at a site roughly corresponding to the laminar nucleus of the torus semicircularis, induced a decrease in fictive lung ventilation frequency, whereas an increase was observed with stimulation in areas corresponding to the magnocellular nucleus of the torus semicircularis. At *level C*, chemical stimulation in the area of the anteroventral tegmentum decreased in fictive lung ventilation frequency, whereas only electrical stimulation of the anterodorsal tegmentum increased fictive lung ventilation frequency (Fig. 6, *left*). At *level D*, a decrease in the frequency of fictive lung breaths was observed with both stimulations in

Table 2. *Sites in anuran midbrain in the vicinity of which glutamate stimulation altered lung oscillation frequency*

Level of Midbrain Stimulation	Coordinates	Vehicle	Glutamate
A	1.0 mm of midline, 1.0 mm deep	30.0±3.5	3.3±0.6*
B	1.0 mm of midline, 2.0 mm deep	15.0±1.0	36.0±1.5*
B	1.5 mm of midline, 1.5 mm deep	16.0±8.3	8.3±3.1*
C	1.0 mm of midline, 2.5 mm deep	15.1±2.4	8.4±2.4*
C	1.5 mm of midline, 2.5 mm deep	17.8±3.9	8.6±3.4*
D	1.5 mm of midline, 1.5 mm deep	19.6±3.4	8.5±5.5*
E	1.0 mm of midline, 0.5 mm deep	22.0±0.5	17.0±4.0*
F	0.5 mm of midline, 2.5 mm deep	10.0±2.7	18.0±1.9*
F	1.0 mm of midline, 2.5 mm deep	8.8±2.9	16.7±2.2*

Values are means ± SE in breaths/min. *Significant difference between the saline and glutamate results, $P < 0.05$ (Student's t-test).
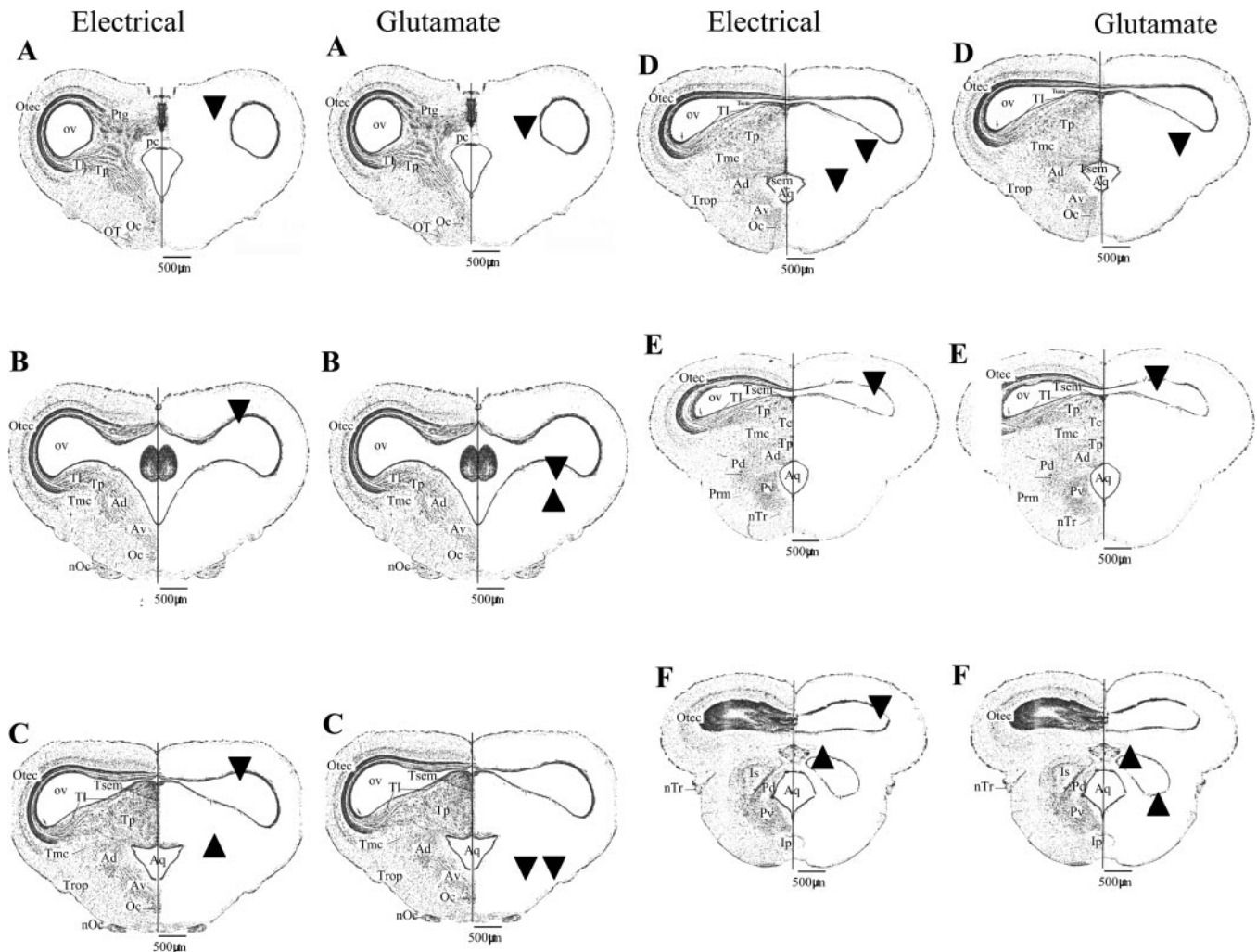
Fig. 6. *Left 2 columns*: representative sections of midbrain from rostral to caudal (*levels A* to *C*; see Fig. 1 for details) showing the general sites where electrical and chemical stimulations produced significant increases (▲) and decreases (▼) in fictive lung oscillation frequency. *Right 2 columns*: representative sections of midbrain from rostral to caudal (*levels D* to *F*; see Fig. 1 for details) showing the general sites where electrical and chemical stimulations produced significant increases (▲) and decreases (▼) in fictive lung oscillation frequency. IP, interpeduncular nucleus; nTr, trochlear nerve nucleus; OT, optic tegmental nucleus; pc, posterior commissure; Prm, nucleus profundus mesencephali; Ptg, pre tectal gray; Tbsp, tectobulbospinal tract; Tc, commissural nucleus of torus semicircularis; Trop, ventrolateral optic tract; Tsem, subpendymal midline nucleus.

the areas of the magnocellular nucleus of the torus semicircularis. Electrical stimulation of the anterodorsal tegmentum also decreased fictive lung breaths (Fig. 6, *right*). At *level E*, a decrease in fictive lung ventilation frequency was observed with chemical and electrical stimulation in the area of the optic tectum (Fig. 6, *right*). At *level F*, a decrease in fictive lung ventilation frequency was observed with electrical stimulation in the area of the optic tectum, whereas an increase was observed with chemical and electrical stimulation of the nucleus isthmi and posteroventral tegmentum (Fig. 6, *right*).

Stimulation in the rostral half of the midbrain had no effect on the amplitude of the fictive lung bursts, whereas stimulation in the area of the optic tectum increased the duration of the fictive lung burst in one animal. Stimulation also caused an increase in the amplitude of the fictive lung bursts at *level E* in the areas of the posterodorsal tegmentum and the magnocellular nucleus of the torus semicircularis, as well as at *level F* in the vicinity of the nucleus isthmi. Stimulation caused an increase in the duration of the fictive lung burst at *level E* in the

areas of the posterodorsal tegmentum and the nucleus profundus mesencephali and at *level F* in the areas of the nucleus isthmi and the nucleus of the trochlear nerve (data not shown).

Figure 7 shows coronal midbrain sections depicting microinjection sites at *levels B* and *F* in parallel with their effects on lung ventilation.

DISCUSSION

In the present study, we identified sites within the midbrain of anuran amphibians that can influence the frequency of fictive lung breaths and present data to suggest that sites within the caudal half of the midbrain provide the inputs essential for the production of episodic breathing.

We acknowledge that care must be taken when interpreting the results of the different protocols used in this study. *1*) The transection experiments reveal information about the net balance of tonic inputs arising from each half of the midbrain, whereas the stimulation experiments reveal information about
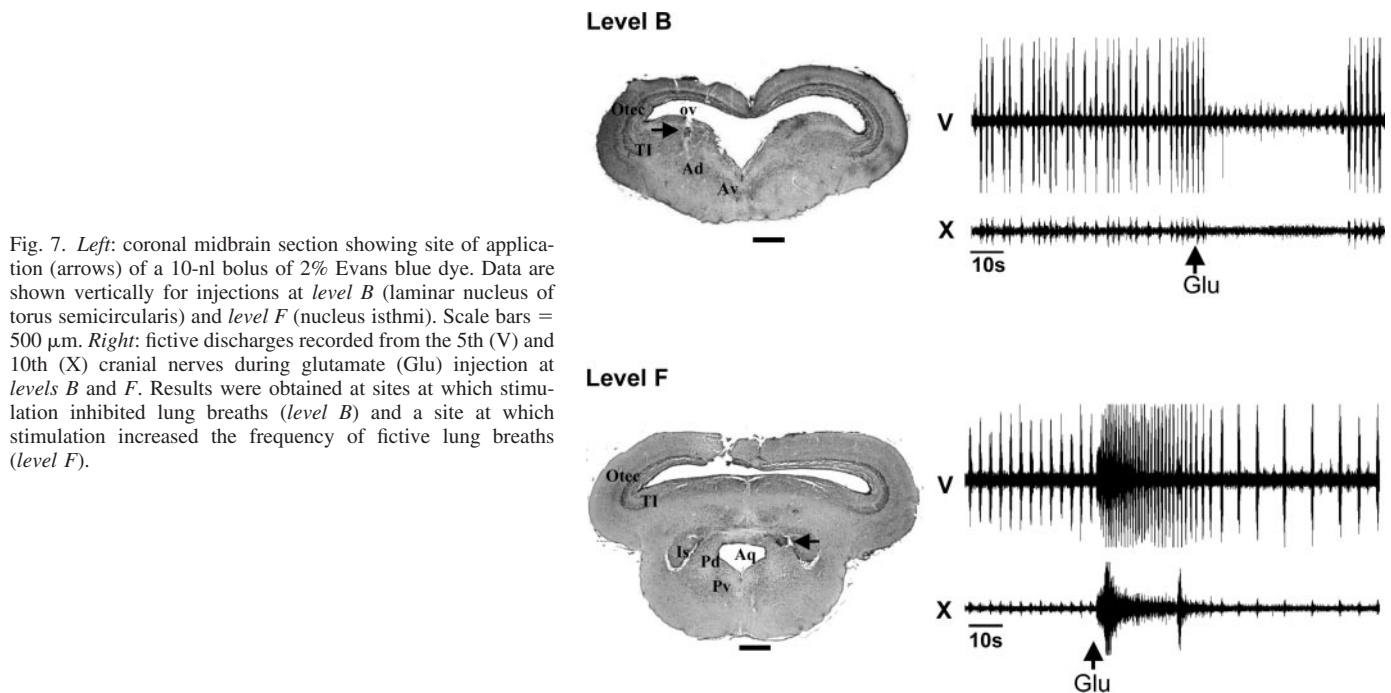
Fig. 7. *Left*: coronal midbrain section showing site of application (arrows) of a 10-nl bolus of 2% Evans blue dye. Data are shown vertically for injections at *level B* (laminar nucleus of torus semicircularis) and *level F* (nucleus isthmi). Scale bars = 500 μm. *Right*: fictive discharges recorded from the 5th (V) and 10th (X) cranial nerves during glutamate (Glu) injection at *levels B* and *F*. Results were obtained at sites at which stimulation inhibited lung breaths (*level B*) and a site at which stimulation increased the frequency of fictive lung breaths (*level F*).

the ability of specific areas to modulate the output of the medullary centers but do not indicate whether descending inputs were coming from that area under resting conditions. *2*) The electrical stimulations could elicit effects by acting on cell bodies or axons of passage, whereas the glutamate injections act only on cell bodies containing appropriate receptors. *3*) The increase in frequency could represent an increase in the frequency of inflation, deflation, or balanced breaths (10, 28). With the measurements made in this study, we know nothing of the timing between glottal opening and buccal contraction, nor of the force of that contraction. In all cases, however, an increase in frequency must reflect increased excitation of the rhythm-generating circuits and a decrease in the respiratory cycle time.

Transections Through the Midbrain

There are numerous studies in amphibian tadpoles and adults that suggest that the endogenous respiratory rhythm is generated by the medulla (16, 20, 26, 31). There is also strong evidence indicating that separate areas within the medulla are involved in generating the buccal and lung oscillations (6, 31) and to suggest that separate areas within the medulla influence the frequency and amplitude of the motor output (20, 31). The episodic breathing pattern that is a hallmark of breathing in most anuran amphibians, however, has been suggested to arise as a result of influences descending from higher brain structures acting on the medullary centers, and it has been suggested that such influences could arise from the midbrain (22, 26). The results of our transection experiments are consistent with this. It should be noted that episodic breathing is part of a continuum of breathing patterns that span from continuous breathing to episodic breathing to a pattern of single breaths (either randomly or evenly spaced) to apnea. As such, episodic breathing can be produced or eliminated by changing drive. At an appropriate level of drive, however, episodic breathing appears to be produced by inputs that turn the central rhythm generator

for breathing on (to produce an episode of continuous breathing) and off (to produce the apnea).

In the present study, transections of the midbrain at the level of the midoptic tectum eliminated episodic breathing by slowing breathing dramatically to a pattern of large, widely spaced single breaths. It would appear that this transection removed a site(s) providing a tonic excitatory drive to breathing frequency, since both the number of breaths per episodes and the overall breathing frequency were significantly reduced after the transection. However, regarding the burst pattern, transection at the level of the midoptic tectum led to slower (increased burst duration) and larger breaths (delayed inspiratory off switch). In all cases, increasing lung volume restored the episodic breathing pattern, decreased burst duration, and integrated activity and increased fictive breathing frequency overall. This effect could be eliminated by vagotomy (data not shown).

When the transection was made at the caudal border of the midbrain (the border between the optic lobes and the medulla), once again the breathing pattern was converted to a continuous one; however, this time there was no change in the total level of ventilation, and neither changes in the level of CO_2 in the ventilation gas nor changes in lung volume could restore the episodic breathing pattern. These data are consistent with the suggestion that there is a site (or sites) within the caudal midbrain that is responsible for clustering the breaths into episodes. The data also suggest that caudal midbrain transection disrupts the normal integration of pulmonary stretch receptor information in the medulla or that vagal inputs alter breathing pattern via projections to a site or sites within the caudal midbrain.

The midoptic tectum transection had effects that were different from those for caudal optic tectum transection. The differences indicate that that, under resting conditions, the net effect of inputs from sites within the rostral half of the

midbrain is to increase the breathing frequency through a projection to the medullary rhythm generator, whereas inputs from sites within the caudal half of the midbrain have no net effect on fictive breathing frequency per se but appear to act on the medullary central rhythm generator to produce episodic breathing. Given that lung inflation was capable of reversing all effects caused by midoptic tectum transection but not caudal midbrain transection, this suggests that pulmonary stretch receptor input can act to replace the excitatory drive arising from the rostral midbrain.

Electrical and Chemical Stimulation in the Midbrain

Electrical stimulation activates both neurons in the region of stimulation and fibers of passage and was used as a preliminary approach to provide the basis for more refined localization. The present study also used excitatory amino acid microinjections to identify whether the excitation of neurons in the midbrain or fibers of passage accounts for the respiratory responses.

In the present study, we did not find any sites where stimulation consistently produced significant changes in the amplitude or duration of the fictive buccal oscillations or lung breaths. All effects were primarily on the frequency of these events. This would suggest that midbrain structures primarily modulate rhythm generation rather than pattern formation.

The midbrain tectum contains the optic tectum and torus semicircularis, which are involved in visual and auditory inte-gration, respectively (19). According to Sánchez-Camacho et al. (27), the main differences between amphibians and others anamniotes concern the presence of extensive pretectospinal projections. These projections are absent in amniotes. In amphibians, they play an important role in visuomotor behavior. Our study suggests that the output from these general areas (tectum and torus semicircularis) may act to decrease the occurrence of lung breaths. This would suggest that certain visual and auditory stimuli may act to produce apnea or breath holding.

Studies in which GABA and DL-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) have been microinjected into the medulla of bullfrogs have located two specific rhythmogenic sites within the ventral medullary reticular formation that appear to be involved in the generation of endogenous respiratory activity: a lung oscillator just caudal and lateral to the root of the sixth cranial nerve and a buccal oscillator at the level of the posterior root of the tenth cranial nerve (20, 31). Wilson et al. (31) demonstrated that, when the buccal rhythm was accelerated by injecting AMPA into the buccal area, lung bursts were abolished. On the basis of these data, the authors suggested that inhibitory inputs from the buccal oscillator onto the lung oscillator coordinate lung bursts with the buccal rhythm. Thus, in the present study, although the reciprocal effects of stimulation on buccal (data not shown) and lung oscillations (when one increased the other decreased)
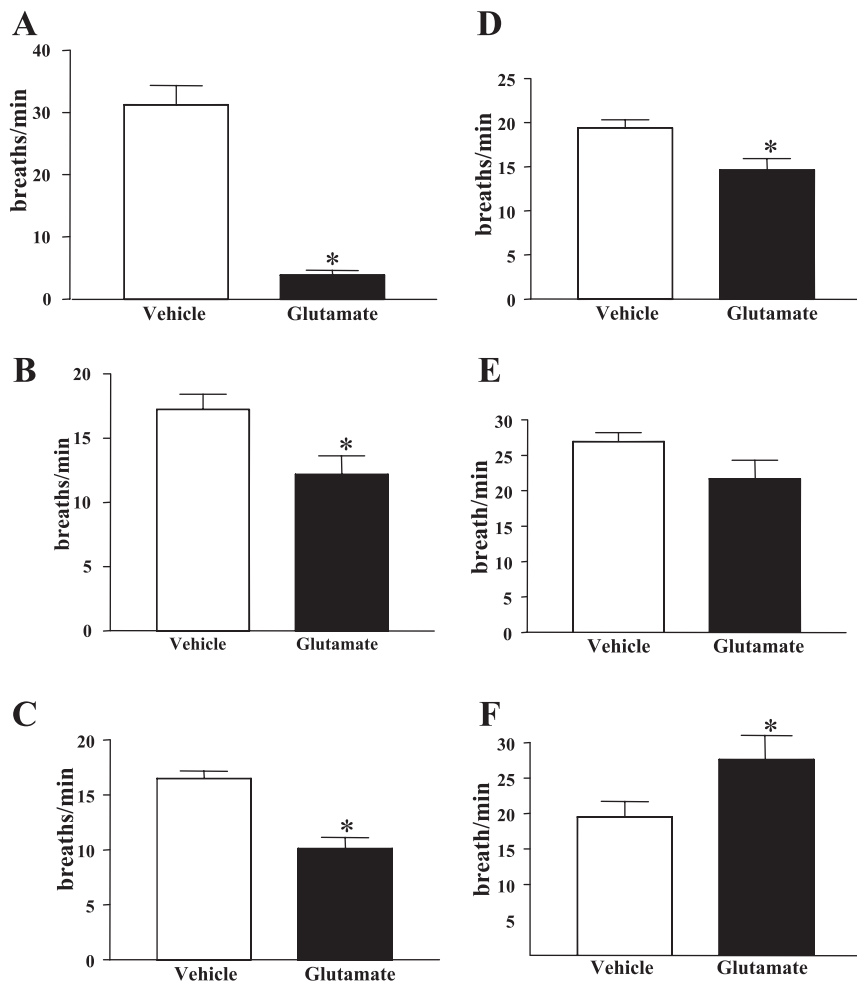

Fig. 8. Mean effects of vehicle and glutamate microinjections in the frequency of fictive lung ventilations at sites ranging from 0.5 to 2.0 mm lateral to the midline at depths ranging from 500 to 3,000 μm below the brain surface at *levels A*, *B*, *C*, *D*, *E*, and *F* (*A–F*) in Fig. 1. *Statistical difference from vehicle values, $P < 0.05$ (*t*-test). [Adapted from Gargaglioni and Milsom (10).]

in the tectum and torus semicircularis may indicate that these areas project to each of the two oscillators, it may also simply reflect the fact it is not possible to have more of one without having fewer of the other.

The tegmentum lies rostral to the isthmus and ventral to the tectum (12) and contains structures that are involved in the production and control of various motor functions (1), including defensive behaviors (9). Our present results suggest that excitation in the rough vicinity of these areas, especially the posteroventral and posterodosal tegmentum (*level F*), can stimulate lung breaths.

Finally, the isthmus region lies at the rostral end of the hindbrain. Among the isthmic structures are the locus coeruleus and nucleus isthmi. The axons of the locus coeruleus in both amphibians and mammals are distributed widely throughout the brain but mainly project to the cerebral and cerebellar cortices (18) and play a role in modulating the activity of sensory neurons and attentional state (30). In the present study, only electrical stimulation believed to be in the vicinity of the locus coeruleus caused an increase in lung frequency, but the chemical stimulations had no effect. These differences may be because electrical stimulation excites both fibers of passage and cell bodies, whereas chemical stimulation activates only cell bodies. Another possibility is that electrical stimulation activates a larger region vs. more discrete chemical stimulations (10 nl/site).

The nucleus isthmi has been described in all vertebrates except cyclostomes and mammals (12). According to Larsell (17) this nucleus in the frog appears from its connections and relationships to correspond to the medial geniculate body in mammals, which is involved in auditory processing. Our results suggest that activation near this site can stimulate lung breaths. Paradoxically, it has been reported that electrolytic lesions of the nucleus isthmi in unanesthetized toads produced no change in lung ventilation (7, 8), whereas pharmacological lesions (lidocaine and kainic acid) in decerebrate frogs reduced ventilation (15). The nucleus isthmi is a large structure containing several subregions, and these discrepancies may either arise from differences in the location of the stimulations or lesions or from differences in the preparations used in each of these studies.

Thus our results indicate that outputs from areas in the midbrain involved in visual and auditory integration and in motor functions, including defensive behaviors and attentional state, may modulate respiration directly. Similar results have been obtained in mammals (11). Perhaps best studied are the midbrain influences on breathing associated with speech (periaquaductal gray; Ref. 13) and locomotion (hypothalamus; Ref. 5) and the phase resetting of breathing elicited by a variety of central and peripheral stimuli (midbrain reticular formation and periaquaductal gray; Ref. 13). These data obtained previously from studies in mammals suggest that midbrain sites can modulate brain stem respiratory centers but do not suggest that they play a significant role in the production of the normal respiratory rhythm or pattern. Our data for frogs are consistent with such a view.

Conclusions and Perspectives

If we group all of the chemical stimulations points at each level [Fig. 8, adapted from Gargaglioni and Milsom (10)], it seems that there is a tendency for more rostral regions of the midbrain to inhibit lung ventilation and for the isthmus region (*level F*) to excite lung ventilation. Similar data exist for fish; carp also breathe episodically under normoxic or hyperoxic conditions, and it has been shown that there are neurons in the dorsal mesencephalic tegmentum, more specifically in a small area near the oculomotor nucleus, that discharge before the first breath in a breathing episode (14). Electrical stimulation at this site shortened the interbout interval and brought forward the onset of the next bout of breathing (14). These tegmental neurons receive cerebellar, vestibular, and reticular input and send efferent information to the cerebellum, the reticular formation, and the trigeminal motor system (14). The nucleus of the oculomotor nerve in the frog lies in the medioventral portion of the tegmentum, just rostral to the nucleus isthmi (Ref. 12, Fig. 1), and electrical stimulation in this general area in the present study stimulated fictive ventilation in roughly one-half of the preparations. This interesting similarity suggests that further investigation is needed on this area.

REFERENCES

1. **Abbie AA, Adey WR.** Motor mechanisms of the anuran brain. *J Comp Neurol* 92: 241–291, 1950.
2. **Butler AB, Hodos W.** *Comparative Vertebrate Neuroanatomy: Evolution and Adaptation.* New York: Wiley-Liss, 1996.
3. **Cohen MI, Hugelin A.** Suprapontine reticular control of intrinsic respiratory mechanisms. *Arch Ital Biol* 103: 317–334, 1965.
4. **Doty RW, Beck EC, Kooi KA.** Effect of brain stem lesions on conditional responses of cats. *Exp Neurol* 1: 360–385, 1959.
5. **Eldridge FL, Milhorn DE, Waldrop T.** Exercise hyperpnea and locomotion: parallel activation from the hypothalamus. *Science* 211: 844–846, 1981.
6. **Galante RJ, Kubin L, Fishman AP, Pack AI.** Role of chloride-mediated inhibition in respiratory rhythmogenesis in an in vitro brainstem of tadpole, *Rana catesbeiana. J Physiol* 492: 545–558, 1996.
7. **Gargaglioni LH, Branco LGS.** Role of nucleus isthmi in the ventilatory response to hypoxia of *Bufo paracnemis. Respir Physiol* 119: 31–39, 2000.
8. **Gargaglioni LH, Branco LG.** Effect of nitric oxide in the nucleus isthmi on the hypoxic- and hypercarbic-drive to breathing of toads. *Am J Physiol Regul Integr Comp Physiol* 281: R338–R345, 2001.
9. **Gargaglioni LH, Pereira AS, Hoffmann A.** Basal midbrain modulation of tonic immobility in the toad *Bufo paracnemis. Physiol Behav* 72: 297–303, 2001.
10. **Gargaglioni LH, Milsom WK.** Control of breathing in anuran amphibians. *Comp Biochem Physiol A Mol Integr Physiol.* In press.
11. **Gatán SP, Pásaro R, Coulon P, Bevengut M, Hilaire G.** Identification of central nervous system neurons innervating the respiratory muscles of the mouse: a transneuronal tracing study. *Brain Res Bull* 57: 335–339, 2002.
12. **Hoffmann A.** Stereotaxic atlas of the toad's brain. *Acta Anat (Basel)* 84: 416–451, 1973.
13. **Horn NH, Waldrop TG.** Suprapontine control of respiration. *Respir Physiol* 114: 201–211, 1998.
14. **Jüch PJW, Ballintijn CM.** Tegmental neurons controlling medullary respiratory centre activity in the carp. *Respir Physiol* 51: 95–107, 1983.
15. **Kinkead R, Harris MB, Milsom WK.** The role of the nucleus isthmi in respiratory pattern formation in bullfrogs. *J Exp Biol* 200: 1781–1793, 1997.
16. **Kogo N, Perry SF, Remmers JE.** Neural organization of the ventilatory activity in the frog, *Rana catesbeiana. J Neurobiol* 25: 1067–1079, 1994.
17. **Larsell O.** The nucleus isthmi of the frog. *J Comp Neurol* 36: 309–322, 1924.
18. **Marín O, Smeets WJAJ, González A.** Do amphibians have a true locus coeruleus? *Neuroreport* 7: 1447–1451, 1996.

19. **McCormick CA.** Evolution of auditory pathways in the amphibia. In: *The Evolution of the Amphibian Auditory System*, edited by Fritzsch B, Ryan MJ, Wilczynski W, Hetherington TE, Walkowiak W. New York: Wiley-Liss, 1988, p. 587–612.

20. **McLean HA, Kimura N, Kogo N, Perry SF, Remmers JE.** Fictive respiratory rhythm in the isolated brainstem of frogs. *J Comp Physiol [A]* 176: 703–713, 1995.

21. **Milsom WK, Harris MB, Reid SG.** Do descending influences alternate to produce episodic breathing? *Respir Physiol* 110: 307–317, 1997.

22. **Milsom WK, Reid SG, Meier JT, Kinkead R.** Central respiratory pattern generation in the bullfrog, *Rana catesbeiana*. *Comp Biochem Physiol A Mol Integr Physiol* 124: 253–264, 1999.

23. **Oka K.** The influence of the transection of the brain upon the respiratory movement of the frog. *J Physiol Soc Jpn* 20: 513–519, 1958.

24. **Orem J, Netick A.** Characteristics of midbrain respiratory neurons in sleep and wakefulness in the cat. *Brain Res* 244: 231–241, 1982.

25. **Potter HD.** Mesencephalic auditory region of the bullfrog. *J Neurophysiol* 28: 1132–1154, 1965.

26. **Reid SG, Meier JT, Milsom WK.** The influence of descending inputs on breathing pattern formation in the isolated bullfrog brainstem-spinal cord. *Respir Physiol* 120: 197–211, 2000.

27. **Sanchez-Camacho C, Marin O, Ten Donkelaar HJ, Gonzalez A.** Descending supraspinal pathways in amphibians. I. A dextran amine tracing study of their cells of origin. *J Comp Neurol* 434: 186–208, 2001.

28. **Sanders CE, Milsom WK.** The effects of tonic lung inflation on ventilation in the American bullfrog *Rana catesbeiana* Shaw. *J Exp Biol* 204: 2647–2656. 2001.

29. **Vibert JF, Bertrand F, Denavit-Saubié Hugelin A.** Three dimensional representation of bulbo-pontine respiratory networks architecture from unit density maps. *Brain Res* 114: 227–244, 1976.

30. **Waterhouse RD, Border B, Wahl L, Mihailoff GA.** Topographical organization of rat locus coeruleus and dorsal raphe nuclei: distribution of cells projecting to visual system structures. *J Comp Neurol* 336: 345–361, 1993.

31. **Wilson RJ, Vasilakos K, Harris MB, Straus C, Remmers JE.** Evidence that ventilatory rhythmogenesis in the frog involves two distinct neuronal oscillators. *J Physiol* 540: 557–570, 2002.