The Journal of Experimental Biology 210, 2046-2056
Published by The Company of Biologists 2007
doi:10.1242/jeb.003202

Effects of medullary Raphé stimulation on fictive lung ventilation during development in *Rana catesbeiana*

Olivier Belzile[1], Roumiana Gulemetova[2] and Richard Kinkead[2,*]

[1]*Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9039, USA and* [2]*Department of Pediatrics, Laval University, Centre de Recherche Hôpital St-François d'Assise, Québec City, Québec, G1L 3L5, Canada*

*Author for correspondence (e-mail: richard.kinkead@crsfa.ulaval.ca)

Accepted 13 March 2007

Summary

To better understand serotonergic modulation of air breathing during bullfrog development, we measured changes in fictive lung ventilation frequency associated with focal stimulation of the rostral region of the medullary Raphé neurons. Electrical (3 to 33 Hz) and chemical (glutamate microinjections; 0.5 mol l^{-1}, 0.3–10 nl) activation of Raphé neurons was performed in brainstem preparations from three developmental stages (pre- and metamorphic tadpoles and adult frogs). Fictive lung ventilation was recorded extracelluarly from the Vth and Xth cranial nerves. Electrical stimulation of Raphé neurons caused a frequency-dependent increase in lung burst frequency in pre-metamorphic tadpoles only. In metamorphic tadpoles, an increase in fictive lung ventilation was observed at 20 Hz only. Electrical stimulation had no effect in preparations from adult frogs. Glutamate microinjections elicited similar responses as a lung burst frequency increase was observed in the pre-metamorphic group only. Regardless of the stimulation technique used, the increase in fictive lung ventilation was attenuated by the selective 5-HT_3 antagonist tropisetron (5–20 μmol l^{-1}). Results from immunohistochemical analysis of the Raphé region stimulated do not correlate with functional data as the number of 5-HT immunoreactive neurons within this region increases during development. We conclude that, in this preparation, stimulation of lung ventilation by the medullary Raphé is restricted to early (pre-metamorphic) stages.

Key words: brainstem, bullfrog, control of breathing, development.

Introduction

The motor command driving lung ventilation appears early during development, even at stages when the lungs are not entirely functional. In mammals, for instance, foetal breathing movements can be recorded as early as the 11th week of gestation. At this stage, breathing movements are irregular and episodic, but their incidence progressively increases during gestation to become more regular and rhythmic at birth (Jansen and Chernick, 1991). A similar developmental pattern has been observed during the transition from aquatic to aerial breathing in bullfrog tadpoles (*Rana catesbeiana*). In this species, lung ventilation occurs infrequently during pre-metamorphic stages as their contribution towards gas exchange is negligible (Burggren and West, 1982). Lung ventilation frequency increases significantly during metamorphosis, and breathing episodes of more than one breath are observed more regularly (Burggren and Infantino, 1994). In adult bullfrogs, breathing is episodic also, but a continuous, high-frequency, breathing pattern can be observed when respiratory drive is elevated (Kinkead and Milsom, 1996). Although these data suggest that the ontogeny of the rhythmic motor activity generating lung ventilation is well preserved amongst vertebrates, the neural mechanism(s) at the basis of respiratory control maturation are not well understood.

Results of investigations on the ontogeny of other rhythmic motor behaviours such as locomotion have shown that, in developing tadpoles, the onset of serotonergic innervation of spinal motoneurons increases the intensity and duration of motor bursts necessary to the production of a more mature swimming pattern (for a review, see McLean et al., 2000). These results, combined with the fact that the number of brainstem (Raphé) serotonergic neurons increases steadily during tadpole development (van Mier et al., 1986), brought us to propose that ontogenic changes in serotonergic modulation facilitates the production of the motor output associated with lung ventilation during tadpole development.

This hypothesis was first addressed using bath application of physiologically relevant concentrations of 5-HT onto isolated brainstem preparations from bullfrog tadpoles of various developmental stages (Belzile et al., 2002; Kinkead et al., 2002). Results from these experiments showed that application of a low 5-HT concentration (0.5 μmol l^{-1}) to brainstem

preparations from pre-metamorphic tadpoles has little effect on fictive lung ventilation. Application of the same 5-HT concentration to brainstems from metamorphic tadpoles increases fictive lung ventilation (Kinkead et al., 2002). These results are consistent with the increase in the number 5-HT immunoreactive neurons in the hypothalamus and brainstem reported in *Xenopus laevis* (van Mier et al., 1986) and suggest that, as for locomotion, the progressive increase in the capacity for 5-HT release near respiratory neurons facilitates the emergence of lung ventilation over the course of development. However, the fact that bath application of 5-HT active agents can activate 5-HT receptors anywhere in the preparation (including pre-synaptic autoreceptors altering endogenous 5-HT release) combined with potential species-specific differences in the developmental pattern of the 5-HT system made it difficult to come to a clear conclusion.

To better understand serotonergic modulation of respiratory motor output associated with lung ventilation during development in *Rana catesbieana*, the present study used a more precise approach (electrical and chemical stimulation) and tested the hypothesis that rostral medullary Raphé stimulation increases lung burst frequency in a stage-dependent fashion. This brainstem area was chosen because it is easily accessible and corresponds to the Raphé magnus (Adli et al., 1999), a group of 5-HT neurons that, in mammals, send direct projections onto respiratory neurons (Manaker and Tischler, 1993; McCrimmon et al., 1995).

The 5-HT receptor system is highly complex with several receptor types (5-HT_1 up to 5-HT_7) and subtypes, many of which can modulate respiratory activity. Based on previous results suggesting that receptor subtypes other than 5-HT_{1A} and 5-$HT_{2A/C}$ are involved in 5-HT modulation of fictive lung ventilation (Belzile et al., 2002; Kinkead et al., 2002), and the fact that 5-HT_3 receptors are ligand-gated ion channels which, in turtles, exert important modulatory effects of ficitive breathing *in vitro* (Johnson et al., 2001), we combined medullary Raphé stimulation with bath application of a selective 5-HT_3 receptor antagonist to determine whether this receptor subtype contributes to the increase in fictive lung ventilation. For each stage group, the brainstem region stimulated was analysed by immunohistochemistry to, (1) demonstrate that 5-HT neurons are present within the region stimulated, and (2) determine whether the number of Raphé neurons observed within this brainstem region changes over the course of development in this species. Parts of this work have been reported in abstract form (Belzile et al., 2003).

Materials and methods

Experiments were performed on 51 tadpoles and 24 adult bullfrogs (*Rana catesbeiana* Shaw) obtained from a commercial supplier (Charles D. Sullivan, Nashville, TN, USA). Animals were housed in aquaria supplied with flowing, filtered, and dechlorinated tap water maintained between 19°C and 21°C (photoperiod: 12 h:12 h, light:dark). Tadpoles were fed a mixed diet of spinach and Nutrafin pellets for turtles and amphibians. Adult specimens were also fed live crickets. All experiments complied with the guidelines of the Canadian Council on Animal Care. The institutional animal care committee approved the specific protocols used in this study.

In vitro brainstem preparations

Tadpoles were anesthetized by immersion in a solution of tricaine methane sulfonate (1:10 000) buffered to pH 7.8 with $NaHCO_3$. Once unresponsive to tail pinch, tadpoles were decerebrated by a transection just rostral to the eyes. Animals were then placed under the dissection microscope for determination of the developmental stage, based on the criteria of Taylor and Kollros (Taylor and Kollros, 1946), and assigned to one of two groups: pre-metamorphic (stages VI to XIV; N=27) or metamorphic tadpoles (stages XVI to XXV; N=14). Adult bullfrogs were anesthetized by immersion in a solution of tricaine methane sulfonate (1:1 000) for 15 min then they were placed in a mixture of ice and oxygenated water for 30 min to slow metabolism and maintain anesthesia for subsequent dissection (Winmill and Hedrick, 2003). A hole was drilled in the cranium to allow decerebration. In all animals, the cranium was opened to expose the brainstem and rostral spinal cord and to allow dissection of the cranial nerves. To reduce axonal conductance throughout the dissection procedure, the brain was irrigated with ice-cold (0–5°C) artificial cerebrospinal fluid (aCSF). The composition of the aCSF was identical the one used for tadpoles and adult frogs in our previous studies (Belzile et al., 2002; Fournier and Kinkead, 2006; Kinkead et al., 2002) and consisted of (in mmol l^{-1}): 104 NaCl; 4 KCl; 1.4 $MgCl_2$; 10 D-glucose; 25 $NaHCO_3$; 2.4 $CaCl_2$ (tadpoles) or 75 NaCl; 4.5 KCl; 2.5 $CaCl_2$; 1.0 $MgCl_2$; 1.0 NaH_2PO_4; 7.5 D-glucose; 40 $NaHCO_3$ (adult frogs). The superfusate was equilibrated with a ~98% O_2/~2% CO_2 gas mixture to ensure adequate oxygenation of the preparation and to maintain the pH at physiological levels (7.8 or 7.9, for tadpoles and adults, respectively) (Kinkead et al., 1994; Torgerson et al., 1997). The brainstem was transected between the optic tectum and the forebrain and then caudal to the hypoglossal nerve before being transferred to a small Petri dish coated with Sylgard (Dow Corning, Midland, MI, USA) where it was immobilized with insect pins. The dura matter and parts of the arachnoid (where possible) were carefully removed, and the brain was moved to the recording chamber where it was placed dorsal side up.

Electrophysiological recordings

Bursts of respiratory-related motor activity were recorded simultaneously from the rootlets of cranial nerves V (trigeminal) and X (vagus) using suction electrodes (model 573000; A-M Systems, Everett, WA, USA). The pipettes were constructed from borosilicate glass (0.84 mm i.d.) pulled to a fine tip with a vertical microelectrode puller (Stoelting Instrument, Wood Dale, IL, USA). The tip was broken and bevelled to achieve appropriate tip diameter. Neural activity signals recorded from the suction electrodes were amplified (gain = 10 000) and filtered (low cut-off, 10 Hz; high cut-off,

1 kHz) using a differential AC amplifier (model 1700; A-M Systems, Everett, WA, USA). Vagal and trigeminal signals were then full-wave rectified and integrated (time constant: 100 ms) using a moving averager (model MA-821; CWE, Ardmore, PA, USA). The raw and integrated nerve signals were viewed on an oscilloscope and digitized for recording with a data acquisition system (model DI-720; Dataq Instruments, Akron, OH, USA). The sampling rate of the analog to digital conversion for the raw signal was 2.5 kHz.

Experimental protocols

Once the recording electrodes were in place, the brainstem preparation was superfused with control (drug free) aCSF at room temperature (20–22°C) equilibrated to pH 7.8 or 7.9 for tadpoles or adults, respectively, delivered at a rate ranging between 4–6 ml min^{-1}. The preparation was allowed to return to ambient temperature and stabilize for 45–60 min, until stable rhythmic neural activity was recorded from both nerves. After the stabilizing period, the experiments began with a recording of baseline activity for 10 min.

Series I: electrical stimulation

In a first series of experiments, a tungsten electrode (electrode diameter: 125 μmol l^{-1}, tapered tip size: 8°, AC resistance: 5 MΩ, model 575300; A-M Systems, Everett, WA, USA) was placed in the rostral portion of the medullary Raphé, according to the description of Tan and Miletic (Tan and Miletic, 1990) for adult *Rana pipiens*. These neurons are located on the floor of the 4th ventricle in the midline at the level of rootlets of the cranial nerves VII (facial). The presence of 5-HT immunoreactive neurons in this brain region of *Rana catesbeiana* tadpoles was confirmed by immunohistochemistry (Fig. 6). The tip of the electrode was positioned roughly 25 μm below the surface of the floor of the ventricle. The tungsten electrode was connected to an isolated pulse stimulator (model 2100, A-M Systems, Everett, WA, USA). Stimulation parameters were similar to those used in other studies (Fuller et al., 2002; Hilaire et al., 1989; Ling et al., 2001), and preliminary experiments conducted on tadpole brainstems confirmed their effectiveness in evoking a lung ventilation response (pulse duration, 0.2 ms; burst width, 1 s; frequency, 20 Hz). These parameters were then used for each preparation (pre-metamorphic, N=8; metamorphic, N=6; adults, N=11) to determine the minimum current necessary to evoke a response (typically between 1–2 μA). Once the current threshold was determined, the stimulator was set to deliver a current 10% greater than threshold, and the preparation was left undisturbed for another 10 min before the experiment started. Electrical stimulation (1 s) was then performed every 5 min, and the current frequency was increased progressively (3, 5, 10, 20 and 33 Hz). In preliminary experiments (N=1), this procedure was performed before and during bath application of a broad spectrum 5-HT receptor antagonist (methiothepin or methysergide) to confirm that the increase in lung burst frequency increase observed during electrical stimulation of the Raphé requires 5-HT receptor activation (Fig. 1).

To address the role of the 5-HT_3 receptor subtype in the response to electrical stimulation, the same protocol was used except that the selective 5-HT_3 antagonist tropisetron was added to the superfusate to try to block the frequency increase caused by the stimulation. The antagonist was added to another aCSF reservoir, the preparation was superfused by this solution at least 10 min before the second series of electrical stimuli began and until the end of the experiment. The tropisetron concentration used for the pre-metamorphic group was 20 μmol l^{-1}, in metamorphic animals, a 5 μmol l^{-1} concentration was found to be sufficient to attenuate the increase in lung burst frequency induced by the stimuli. In adults, Raphé stimulation had no significant effect on lung burst frequency so no attempt was made to try to block a frequency increase with this drug. To ensure that this effect of tropisetron was not due to a carry-over effect from the previous stimulation protocol, a series of control experiments was performed in which the second sequence of stimuli was performed while the preparation was superfused with drug-free aCSF (N=5). The second series of experiments elicited the same response as the first one (no drug-1 *vs* no drug-2: P=0.50, data not shown). All drugs were obtained from Sigma/RBI Aldrich (St Louis, MO, USA).

Series II: chemical stimulation

Electrical stimulation of Raphé neurons can elicit non-specific effects since it is difficult to control current spread and avoid stimulating axons of passage (Lipski et al., 1988). Consequently, the results obtained with the electrical stimulation protocol were validated by performing another series of experiments in which Raphé neurons were activated using glutamate microinjections. These experiments were performed on the same three developmental groups (pre-metamorphic, N=9; metamorphic, N=8; adults, N=6). In this series, a glass pipette filled with a glutamate solution (0.5 mol l^{-1} dissolved in aCSF) was placed in the medullary Raphé according to the coordinates described previously. The glutamate concentration and volume were based on those used by Lipski et al. (Lipski et al., 1988). The pipettes were constructed from borosilicate glass (0.58 mm i.d.) pulled to the finest possible tip (average tip diameter of 10–15 μm) with a vertical microelectrode puller (Stoelting Instrument, Wood Dale, IL, USA). This pipette was connected to a pneumatic picopump (model PV800, WPI Inc., Sarasota, FL, USA) set at roughly 137 kPa. Before each experiment, the pipette was placed under a microscope to determine the injection time required to inject specific volumes (0.3 to 10 nl). Although there was some variation between the experiments, the air pressure was kept constant for all the injections performed on the same preparation. The ejected volume was estimated by measuring the diameter of the drop. Preliminary experiments comparing the volume estimated with the drop diameter with the volume injected in the tissue calculated from the measured radius of the pipette and the change in meniscus height, confirmed that this method was adequate for the volumes used.

The protocol consisted of two glutamate microinjections of

increasing volumes (see below), separated by a 5-min recovery period. Following these injections, the 5-HT_3 antagonist tropisetron was added to the superfusate and the series of microinjections was repeated. Preliminary experiments showed that, although injections were performed in the same brainstem region, preparations from younger animals responded more vigorously than preparations from older animals. To avoid over-stimulation and a ceiling effect in the response, the glutamate volumes used varied according to the developmental stage of the preparation (pre-metamorphic, 0.3 and 1.0 nl; metamorphic, 1.0 and 2.5 nl; and adults, 5.0 and 10.0 nl). Control injections of glutamate 350 μm lateral to the regular injection site were performed. Control injections of different volumes of drug-free aCSF within the medullary Raphé were performed also. As for electrical stimulation, experiments were performed on the same three developmental groups (pre-metamorphic, *N*=9; metamorphic, *N*=8; adults, *N*=6).

Series III: quantification of 5-HT immunoreactive neurons in the rostral medullary Raphé

Immunohistochemistry was used to quantify the number of serotonergic neurons present in the brainstem region stimulated in the previous two series of experiments. Tadpoles and bullfrogs were deeply anesthetised according to the protocols described in the previous series, and brains were harvested from animals representing all three developmental groups (pre-metamorphic, *N*=7; metamorphic, *N*=3; adults, *N*=7). The tissue was fixed in 4% paraformaldehyde (PFA) in 0.1 mol l^{-1} phosphate buffer solution (PB, pH 7.4 at 4°C) overnight. The next day, brains were placed in 30% sucrose–4% PFA/PB solution until they sank (~48 h at 4°C). Brains were then imbedded in Histoprep (Fisher Scientific, Ottawa, ON, Canada) and frozen on dry ice. The tissue block was mounted in a cryostat (Leica CM 1900), and cross sections (20 μm) of the medullary region of the Raphé at the level of nerve VII were cut in series. On average, five to six sections were obtained from each brain. Sections were first rinsed in 0.1 mol l^{-1} PB (pH 7.4) and then incubated in 0.3% H_2O_2/PB for 10 min. Sections were rinsed in PB and permeabilized in a solution of 0.2% Triton X-100/0.1 mol l^{-1} PB for 10 min at room temperature. This was followed by incubation in a solution of 0.1% bovine serum albumin (BSA fraction V; Sigma, St Louis, MO, USA) to reduce non-

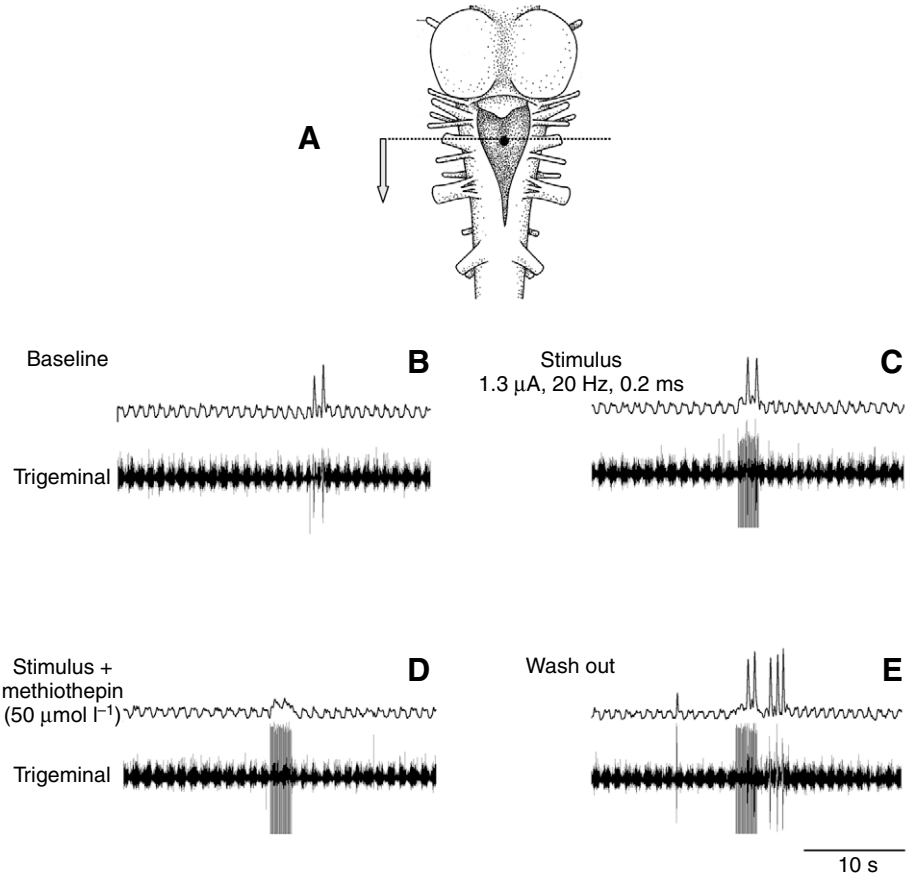

Fig. 1. (A) Schematic representation of bullfrog brainstem showing the Raphé stimulation site. (B–D) Integrated (top trace) and raw (bottom trace) trigeminal neurograms from a pre-metamorphic tadpole, (B) under baseline condition, (C) during and after electrical stimulation of the medullary Raphé with drug-free artificial cerebrospinal fluid (aCSF) and (D) in the presence of methiothepin in aCSF (50 μmol l^{-1}). (E) The changes in respiratory-related motor output after a 20-min 'wash out' period with drug-free aCSF.

specific activity before being incubated with the primary antibody, rabbit anti-5-HT (Sigma) at a dilution of 1:4 000 in a solution of 0.1% BSA/PB at 4°C overnight. Tissue sections were washed in 0.1 mol l^{-1} PB for 10 min and then incubated in biotinylated goat anti-rabbit secondary antiserum (Vector laboratories, Burlington, ON, Canada), diluted 1:200 for 1 h at room temperature. Sections were rinsed and biotin amplification was performed with the avidin–biotin peroxydase reaction (Vectastain Elite ABC kit, Vector Laboratories, Burlington, ON, Canada) for 1 h at room temperature. Sections were rinsed in PB and the reaction complex was stained with the 3-amino-9-ethylcarbazole reaction (AEC substrate kit for peroxydase; Vector Laboratories, Burlington, ON, Canada) for 10 min at room temperature. The reaction was stopped by rinsing the sections in PB. Sections were then mounted onto slides before being dried and sealed under a coverslip with mounting medium. Control experiments were performed by omitting the primary antibody, resulting in negative staining.

Data analyses

Frequency values for lung-related activity (large amplitude bursts) were obtained by analyzing the neurogram for 30 s following electrical or chemical stimulation of medullary Raphé neurons. Cranial nerve burst amplitude from a single electroneurogram is not always sufficient to adequately identify fictive gill and lung bursts (Sanders and Milsom, 2001). Based upon the criterion proposed by Torgerson and colleagues (Torgerson et al., 1998), both nerve signals were analyzed simultaneously, and vagal nerve activity was used as a sensitive marker of fictive lung activity to distinguish between gill- and lung-related signals (Kogo et al., 1994; Kogo and Remmers, 1994). Occasionally, Raphé stimulation altered the burst pattern. Under those circumstances, only bursts that were similar (in terms of amplitude and duration) to those recorded prior to Raphé stimulation were counted as respiratory related. Fig. 4 shows neurograms in which glutamate microinjection elicited burst activity that differs from respiratory-related activity (e.g. prolonged burst duration). Such bursts were not included in the analysis. Lung burst frequencies were obtained by counting the number of lung-related burst events for the 30 sec period analyzed, and calculated for a 1 min period. To facilitate comparisons between stage groups, frequency data were also normalised in two ways: (1) as a percentage change from baseline, and (2) as a percentage change from baseline expressed as the percentage of the maximum lung burst frequency produced by the preparation. The latter form of normalisation obviates concerns about expressing date in terms of the percentage increase above a low baseline value (e.g. pre-metamorphic).

On average, five to six brain sections were obtained from each animal. The number of 5-HT immunoreactive neurons present in the Raphé area was counted for each section, and a mean value was calculated for each animal. These values were then pooled for each developmental group to produce a group-specific value.

All measurements are reported as the mean ± 1 s.e.m. The results were analyzed statistically using a two-way analysis of variance (ANOVA) for repeated measures when appropriate. Expressing data as a percentage change can bias data distribution away from normality, which violates a key assumption underlying ANOVA. This issue was addressed by performing the ANOVAs both on normalised data and normalised data following arcsine transformation. The conclusions emerging from both analyses were identical. Results from the immunohistochemistry experiments were analysed using a one-way ANOVA (Statview version 5.01; SAS Institute, Cary, NC, USA). These analyses were followed by Fisher's protected least significant difference (PLSD) test when appropriate ($P<0.05$).

Results

Rostral medullary Raphé activation and modulation of respiratory motor output during bullfrog development

I: electrical stimulation

Trigeminal neurograms were obtained from a pre-metamorphic tadpole prior to electrical stimulation of the medullary Raphé (Fig. 2A,B). These neurograms illustrate respiratory-related activity produced by brainstems from pre-metamorphic tadpoles under baseline conditions and show that bath application of the selective 5-HT_3 receptor antagonist tropisetron has little effect on fictive breathing. Electrical stimulation of the medullary Raphé increased fictive lung ventilation frequency in a frequency-dependent manner and this effect was attenuated by tropisetron (Fig. 2C-F, lower panels). Compilation of the mean data indicate that lung burst frequency changes with development (stage effect: $P=0.002$) and that electrical stimulation of the medullary Raphé increased lung burst frequency in a frequency-dependent fashion with a plateau at 20 Hz (stimulation effect: $P=0.004$; Fig. 2A,C,E and Fig. 3A). However, subsequent analysis showed that this effect was statistically significant in preparation from pre-metamorphic tadpoles only. Bath application of the selective 5-HT_3 antagonist tropisetron onto preparations from pre- and metamorphic tadpoles had no effect on baseline lung burst frequency. However, this drug eliminated the increase in fictive lung ventilation frequency (drug effect: $P=0.01$) that occurred in no-drug preparations following Raphé stimulation (Fig. 3A,B).

In preparations from metamorphic tadpoles, the overall effect of electrical activation of medullary Raphé on lung burst frequency was not statistically significant (stimulation effect: $P=0.39$). It is noteworthy, however, that at 20 Hz, five out of six preparations increased their fictive lung ventilation (Fig. 3B). Stimulation during bath application of 5 μmol l^{-1} tropisetron did not affect fictive lung activity at any stimulation frequency (stimulation effect: $P=0.8$).

Electrical stimulation of the medullary Raphé performed on preparations from adult bullfrogs elicited mixed responses: fictive lung ventilation increased in 45% (5/11) of the preparations, whereas a decrease was observed in 55% (6/11)

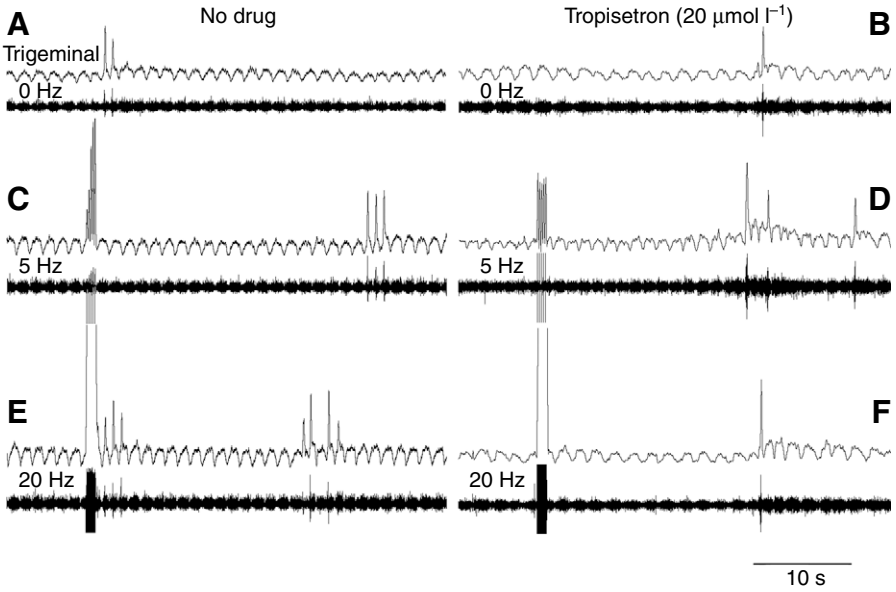

Fig. 2. Trigeminal neurograms comparing responsiveness to electrical stimulation of medullary Raphé neurons before (A,C,E) and during (B,D,F) bath application of the selective 5-HT_3 receptor antagonist tropisetron. Recordings were obtained from brainstem preparations from pre-metamorphic tadpoles.

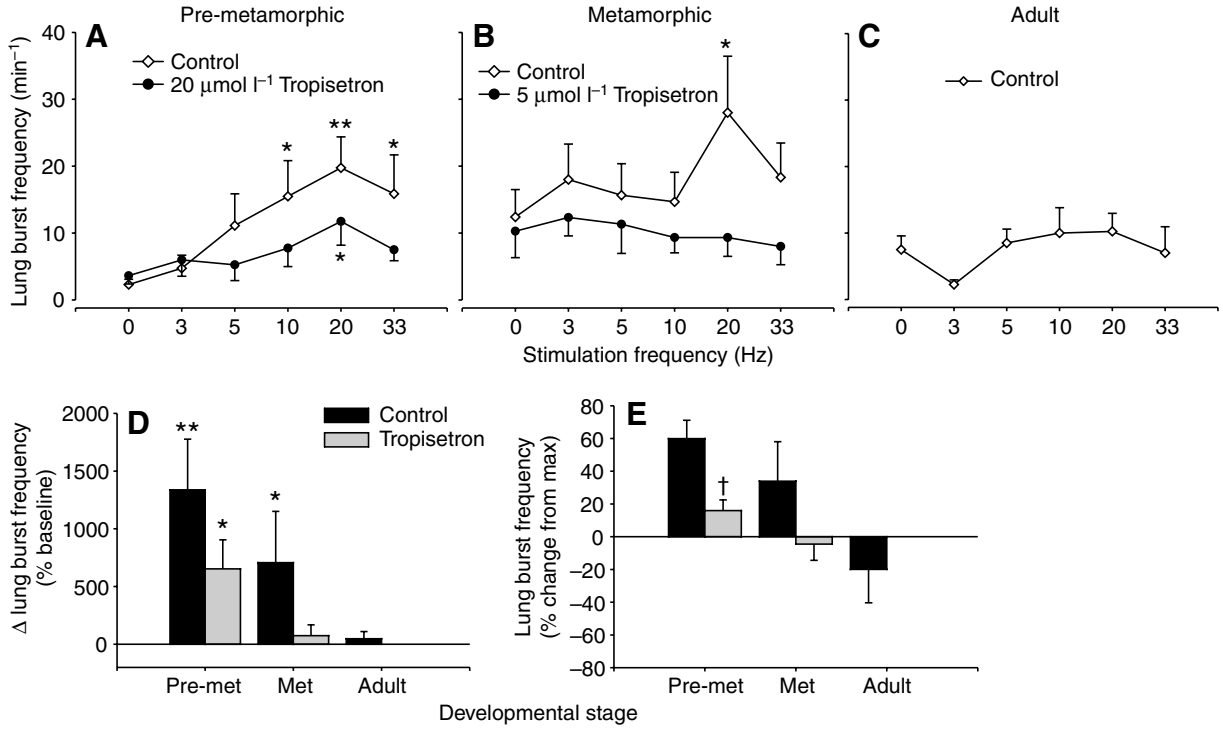

Fig. 3. Effects of electrical stimulation of medullary Raphé neurons on fictive lung ventilation frequency. (A,C) Responses were measured in brainstem preparations from (A) pre-metamorphic tadpoles, (B) metamorphic tadpoles, and (C) adult bullfrogs under control conditions [artificial cerebrospinal fluid (aCSF) only; open diamonds] and in the presence of a 5-HT_3 receptor antagonist (tropisetron; closed circles). Note that the tropisetron concentration required to affect the frequency response (when present) decreases during development. (D,E) Stage-dependent lung burst frequency responsiveness to medullary Raphé stimulation expressed as the percentage change from baseline of the frequency response following 20 Hz stimulation (D), and as percentage change from baseline expressed as the percentage of the maximum lung burst frequency produced by each preparation (E). The black bars represent the control response (aCSF) and the grey bars represent the response in the presence of the 5-HT_3 antagonist (when necessary). Values are expressed as means ± s.e.m. Asterisks indicate values statistically different from baseline values (no stimulation) at $*P<0.05$ and $*P<0.01$; † indicates a value significantly different from control at $P<0.01$.

of the preparations. The increase seen in five adult preparations was smaller than the increases seen in the two other groups. Consequently, the overall (mean) data show no significant effect of electrical stimulation on fictive lung ventilation in this group (Fig. 3C; P=0.69).

Fig. 3D compares the lung burst frequency responses to electrical stimulation (expressed as a percentage change from baseline) recorded at 20 Hz, which elicited the maximum response in all three developmental stages. Expressed this way, the data show that the response to medullary Raphé stimulation was stage dependent (stage effect: P=0.016) and that the increase, when present, tended to be attenuated by tropisetron (drug effect: P=0.07). Expressing these data as a percentage change from maximum response revealed similar effects (Fig. 3E).

II: chemical stimulation

Results obtained in this series of experiments were specific to Raphé neuron activation because control glutamate injection 350 μm lateral to the injection site did not affect lung-related neural activity (Fig. 4C). Whenever observed, trigeminal bursts were longer than those recorded under any other experimental condition, and vagal activity was often seizure-like. Thus the activity recorded could not be interpreted as respiratory related. Furthermore, control injections of different volumes of drug-free aCSF in the medullary Raphé had no effect (Fig. 4D).

Overall, data obtained in this series of experiments mainly concur with those reported for electrical stimulation. Only preparations from pre-metamorphic tadpoles showed a lung burst frequency increase following the glutamate microinjections (Fig. 5A; P=0.01). Tropisetron bath application attenuated this response (glutamate effect, P=0.15; drug effect, P=0.02). In metamorphic tadpoles and adult frogs, glutamate microinjections had no significant effect (P>0.05), and in adults an inhibition of fictive lung ventilation was observed in four out of six preparations at 5 and 10 nl (Fig. 5C).

Expressing these results as a percentage change from baseline indicate that the response to medullary Raphé stimulation was stage dependent (stage effect, P<0.0001) and that the increase, when present, was attenuated by tropisetron (drug effect, P<0.0001). Expressing these data as percent change from maximum response revealed similar effects (Fig. 5E).

Stage-dependent changes in 5-HT immunoreactive neurons in the dorsal medullary Raphé

5-HT immunoreactive neurons are present in the rostral portion of the medullary Raphé at the level of the VIIth cranial nerve where electrical and chemical stimulation was performed in all stage groups (Fig. 6). Micrographs and mean data show that the number of 5-HT immunoreactive neurons present in this region increased during development with a peak during metamorphic stages (stage effect, P=0.0006; Fig. 6B).

Discussion

Our study shows that, in *Rana catesbeiana*, medullary Raphé neuron activation increases fictive lung ventilation in brainstem preparations from pre-metamorphic tadpoles only, and that 5-HT_3 receptor activation is necessary for the full expression of this response. The attenuation of 5-HT-mediated facilitation of fictive lung ventilation that takes place during development occurs despite an overall increase in the number of 5-HT immunoreactive neurons observed in the rostral medullary Raphé. Whereas the present data do not discount the possibility that other groups of 5-HT neurons (e.g. isthmic region) modulate respiratory activity in a different way, they nonetheless indicate that the neural pathways involved in

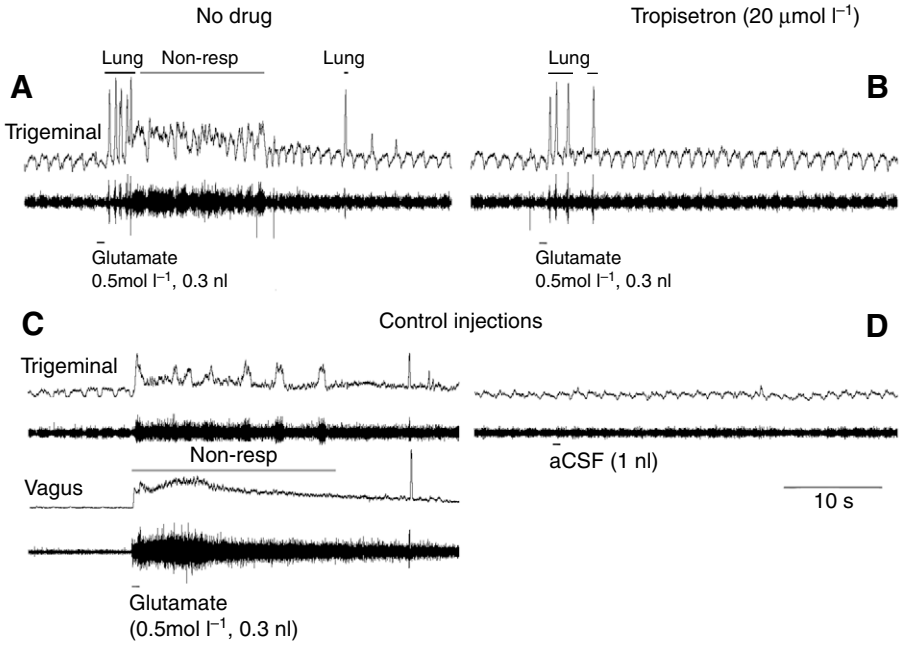

Fig. 4. Changes in respiratory motor output during microinjection in the medullary Raphé. (A,B) Trigeminal neurograms showing the effects of glutamate microinjections in the medullary Raphé on fictive breathing (A) in the presence of artificial cerebrospinal fluid (aCSF) only and (B) in the presence of the selective 5-HT_3 receptor antagonist tropisetron. (C,D) Control experiments consisted of measuring changes in trigeminal activity following (C) glutamate microinjection (1 nl, 0.5 mol l^{-1}) 350 μm lateral to the medullary Raphé or (D) aCSF injection into the medullary Raphé. This figure also shows various types of burst activity observed following glutamate microinjection. Note that only bursts with a duration and amplitude similar to those observed prior to injections were counted as respiratory related (black bars). Bursts of a seizure-like activity or that were too long were not counted (grey bars).

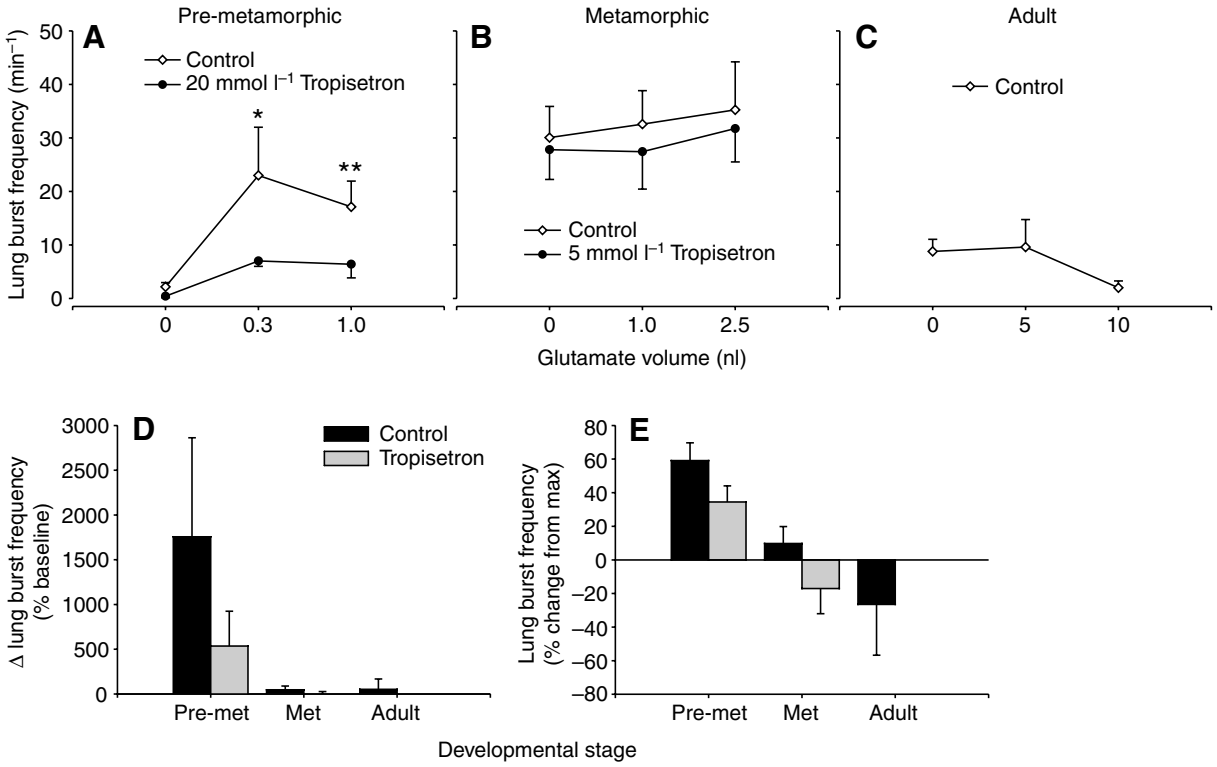

Fig. 5. Effects of glutamate microinjections in the medullary Raphé on fictive lung ventilation frequency. (A-C) Responses were measured in brainstem preparations from (A) pre-metamorphic tadpoles, (B) metamorphic tadpoles, and (C) adult bullfrogs under control conditions [artificial cerebrospinal fluid (aCSF) only; open diamonds)] and in the presence of a 5-HT_3 receptor antagonist (Tropisetron; closed circle). Note that the tropisetron concentration required to affect the frequency response (when present) decreased during development. (D,E) A between stage-group comparison was performed by expressing the frequency response following glutamate microinjection (1 nl pre- and metamorphic tadpoles; 5 nl adult frogs) as (D) the percentage change from baseline, and (E) the percentage change from baseline expressed as the percentage of the maximum lung burst frequency produced by each preparation. The black bars represent the control response (aCSF); the grey bars represent the response in the presence of the 5-HT_3 antagonist (when necessary). Values are expressed as means ± s.e.m. Asterisks indicate values statistically different from baseline values (no stimulation) at $*P<0.05$ and $**P<0.01$.

serotonergic modulation of lung ventilation undergo important reorganisation over the course of development. The net effect of these changes tends to minimise the impact of 5-HT modulation provided by the rostral medullary Raphé on this function and suggest that, in this preparation, change in serotonergic modulation from this structure does not contribute to the progressive increase in lung ventilation that occurs during development.

Critique of methods

Various approaches can be used to stimulate a group of neurons located within a specific CNS area; however, each technique has inherent limitations that must be considered in our interpretation of the results. For instance, electrical stimulation can elicit non-specific effects since it is difficult to control current spread and avoid stimulating axons of passage (Lipski et al., 1988). In the case of chemical stimulation, the same glutamate concentration may have excitotoxic effects that are not constant throughout development.

Another important limitation relates to the difficulty in determining precisely the area influenced by the stimulation (whether electric or chemical). For instance, developmental change in the number of glial cell could affect the spread of the electric stimulus and injection of excitatory amino acids with volume ranges similar to those we used can elicit effects in regions quite distant from the injection site, depending on the concentration used (Lipski et al., 1988; Nicholson, 1985). Based on the equations proposed by Nicholson (Nicholson, 1985) and assuming that the frog brain tissue has a volume fraction (α) and tortuosity factor (λ) similar to that of mammals (0.21 and 1.6, respectively) the glutamate volumes used could affect neurons as far as 300 μm away from the injection site.

To address these issues two different experimental approaches were used to stimulate medullary Raphé neurons: electrical and chemical stimulation, and in addition immunohistochemistry was used to evaluate the number of 5-HT-positive neurons located in the immediate vicinity of the stimulation site (~50 μm radius) for each stage group. Results obtained were essentially similar in both series of experiments as we showed that (1) brainstems from pre-metamorphic tadpoles were the most responsive to medullary Raphé neuron stimulation, regardless of the technique used, and (2) for all

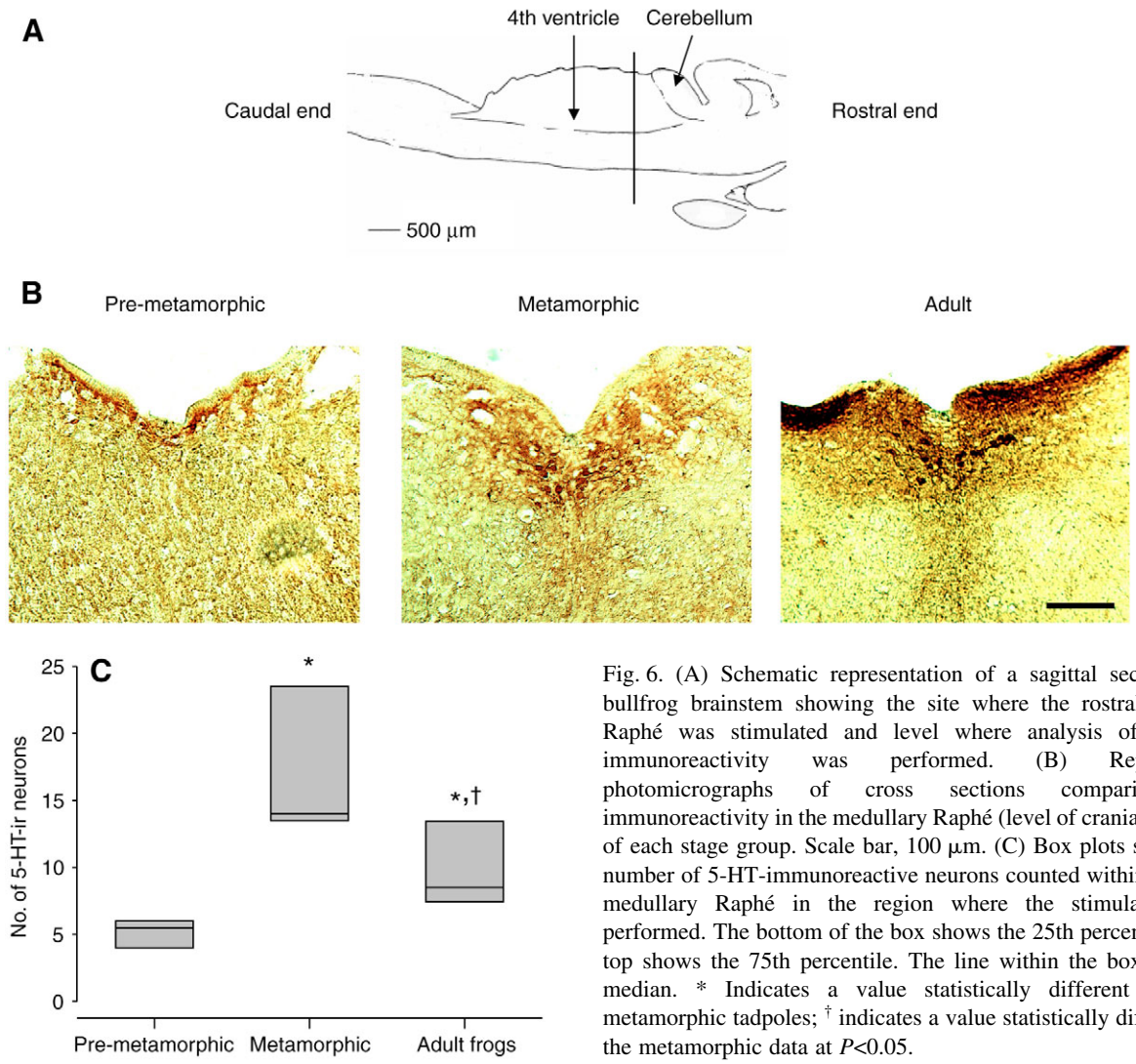

Fig. 6. (A) Schematic representation of a sagittal section of the bullfrog brainstem showing the site where the rostral medullary Raphé was stimulated and level where analysis of the 5-HT immunoreactivity was performed. (B) Representative photomicrographs of cross sections comparing 5-HT immunoreactivity in the medullary Raphé (level of cranial nerve VII) of each stage group. Scale bar, 100 μm. (C) Box plots showing the number of 5-HT-immunoreactive neurons counted within the rostral medullary Raphé in the region where the stimulations were performed. The bottom of the box shows the 25th percentile and the top shows the 75th percentile. The line within the box shows the median. * Indicates a value statistically different from pre-metamorphic tadpoles; † indicates a value statistically different from the metamorphic data at $P<0.05$.

developmental stages, the effects of Raphé neuron stimulation (when they occurred) could be blocked by a 5-HT antagonist. These results differ from those obtained using bath application of low 5-HT concentrations (0.5 μmol l^{-1}), which enhanced fictive lung ventilation in preparations from metamorphic but not pre-metamorphic tadpoles (Kinkead et al., 2002). An important distinction between the two techniques is that during bath application, 5-HT activates both post- and pre-synaptic (auto) receptors located on Raphé neurons to reduce endogenous 5-HT release. In mammals, 5-HT_{1A} receptors located on cell bodies regulate 5-HT release. In pre-metamorphic tadpoles, 5-HT bath application in the presence of the selective 5-HT_{1A} antagonist NAN-190 increased fictive lung ventilation frequency (Kinkead et al., 2002). These data are consistent with those obtained with focal Raphé stimulation and suggest that the result obtained with 5-HT bath application reflects a compromise between two opposing effects.

Although 5-HT immunoreactive neurons are predominant in the medullary Raphé nucleus, this structure is relatively heterogeneous in comparison with other nuclei. Thus, focal stimulation within this region may elicit release of neurotransmitters other than 5-HT. However, the fact that the response observed could be blocked by a low concentration of a selective 5-HT antagonist indicates that 5-HT release was the predominant effect elicited in our experiments.

The role of the 5-HT_3 receptor in modulation of lung burst frequency during development

Since application of pharmacological agents inactivating 5-HT_{1A} or 5-$HT_{2A/C}$ receptor subtypes had little effect on the fictive lung ventilation response to 5-HT application (Belzile et al., 2002; Kinkead et al., 2002), we proposed that 5-HT_3 receptors are involved in serotonergic modulation of lung burst frequency. Our results showing that, in pre-metamorphic tadpoles, these receptors participate in the strong excitation of fictive lung activity that follows Raphé neuron stimulation support this interpretation. These results concur with those of Johnson et al. (Johnson et al., 2001) who reported a 5-HT_3-mediated enhancement of fictive lung ventilation for *in vitro* preparations from adult turtles, but do not exclude the

possibility that other receptor types are involved also. In that regard, 5-HT_{4A} receptors may contribute to these effects since their activation stimulates respiratory activity both *in vitro* and *in vivo* in rats (Manzke et al., 2003).

Results from metamorphic animals show that the decline in the excitatory influence of medullary Raphé neurons on fictive lung ventilation becomes apparent with the onset of metamorphosis, thereby suggesting that the endocrine changes that take place over that period influence modulation of respiratory activity by 5-HT. It is important to note that, unlike adult frogs, the modest effect of Raphé neuron stimulation observed in the metamorphic group was consistent amongst preparations. Experiments on preparations from adult frogs yielded mixed results even though the animals used for this study were purchased together and were used in a short period of time to exclude seasonal changes as a potential confounding factor; however, frogs' gender was not determined prior to experiments. Knowing that serotonergic modulation of respiratory activity shows substantial sexual dimorphism in rats (Behan et al., 2003), the different responses may reflect the influence of sexual steroids that becomes apparent only in fully mature adults.

The sum of these data leads us to conclude that the developmental period during which serotonergic modulation facilitates fictive lung ventilation is restricted to the early developmental stages. These results are similar to those observed with noradrenaline bath application (Fournier and Kinkead, 2006) but are difficult to reconcile with the overall increase in 5-HT immunoreactive neurons reported over the course of development in the brainstem of anurans, *Rana* (present study) and *Xenopus* (van Mier et al., 1986). Thus, unless the number of serotonergic boutons near respiratory neurons decreases substantially, the capacity for 5-HT synthesis (and release) by medullary Raphé neurons likely increases during bullfrog development. Results from the present experiments do not allow us to provide a clear description of the mechanisms responsible for this sudden change in the effect of 5-HT modulation of fictive lung ventilation; however, our data showing the important role that 5-HT_3 receptors play in this function provides valuable clues in that regard.

The 5-HT_3 receptor is a ligand-gated ion channel which, in mammals, modulates fast synaptic neurotransmission and is located mainly on GABAergic interneurons (van Hooft and Vijverberg, 2000). GABA is a neurotransmitter that is excitatory during early development but inhibitory in the mature nervous system (Ben-Ari, 2002). This stage-dependent response to GABA reflects the low expression of chloride transporters and the inefficiency of neurons in maintaining chloride gradients across the membrane. Consequently, $GABA_A$ receptor activation results in neuron depolarisation during early life but causes a hyperpolarization in more mature stages (Ben-Ari, 2002). In *Rana catesbeiana*, *in vitro* bath application of GABA supports this interpretation because it enhances fictive lung ventilation in preparations from tadpoles but inhibits this activity in preparations from adult frogs (Broch et al., 2002). We therefore propose that modulation of fictive lung ventilation by medullary Raphé neurons acts mainly *via* 5-HT_3 receptors located on GABA interneurons. According to this hypothesis, the developmental change could reflect the appearance of ion transporters necessary to establish and maintain chloride gradients in these GABAergic neurons.

Perspectives

In adult mammals, stimulation of the Raphé magnus inhibits, whereas stimulation of the Raphé pallidus facilitates respiratory activity (Cao et al., 2006; Lalley, 1986). Conversely, electrical stimulation of the Raphé obscurus induces both types of respiratory responses, depending on the stimulation sites (Cao et al., 2006). However, these effects seem more consistent *in vitro* where Raphé obscurus stimulation in brainstems preparations from newborn rat augments fictive breathing frequency (Al-Zubaidy et al., 1996; Peever et al., 2001). While comparing our results with those obtained in mammals must be done cautiously, our data (especially those obtained from more mature preparations) is nonetheless consistent with our initial assumption that the medullary Raphé region stimulated corresponds to the Raphé magnus.

Bath application of selective 5-HT antagonists such as tropisetron (5-HT_3; present study) or NAN-190 ($5HT_{1A}$) (Kinkead et al., 2002) have little effect on 'resting' lung burst frequency, indicating that tonic 5-HT modulation of fictive lung ventilation is minimal in this preparation. Specific stimuli such as hypoxia may therefore be necessary to trigger 5-HT release from Raphé neurons. Oxygen-sensitive peripheral chemoreceptors like those located in the first gill arch of tadpoles (Straus et al., 2001) relay their sensory signal to the CNS to increase both gill and lung ventilation during hypoxia. Since peripheral chemoreceptor stimulation activates Raphé neurons in mammals (Erickson and Millhorn, 1994), it is likely that hypoxia elicits such response in amphibians also. This, in turn, would facilitate air breathing owing to (at least in part) 5-HT_3 receptor activation. Since lungs of pre-metamorphic tadpoles contribute little to gas exchange, an increase in air breathing could promote lung development.

The authors would like to thank Dr Michael Hedrick for providing CNS tissue samples from pre-metamorphic tadpoles to complete parts of the neuroanatomical study. This research was supported by an operating and equipment grant from the National Science and Engineering Research Council of Canada (NSERC) to R.K. O.B. was the recipient of studentships from NSERC and Centre de recherche de l'Hôpital St-François d'Assise. R.K. is the holder of the Canada Research Chair in Respiratory Neurobiology. We would like to thank Norbert Balon, Pierre De Grandpré, and Edith Simard for excellent comments and technical assistance.

References

Adli, D. S., Stuesse, S. L. and Cruce, W. L. (1999). Immunohistochemistry and spinal projections of the reticular formation in the northern leopard frog, *Rana pipiens*. *J. Comp. Neurol.* **404**, 387-407.

Al-Zubaidy, Z. A., Erickson, R. L. and Greer, J. J. (1996). Serotonergic and

noradrenergic effects on respiratory neural discharge in the medullary slice preparation of neonatal rats. *Pflugers Arch.* **431**, 942-949.

Behan, M., Zabka, A. G., Thomas, C. F. and Mitchell, G. S. (2003). Sex steroid hormones and the neural control of breathing. *Respir. Physiol. Neurobiol.* **136**, 249-263.

Belzile, O., Gulemetova, R. and Kinkead, R. (2002). Role of 5-$HT_{2A/C}$ receptors in serotonergic modulation of respiratory motor output during tadpole development. *Respir. Physiol. Neurobiol.* **133**, 277-282.

Belzile, O., Gulemetova, R. and Kinkead, R. (2003). Stage-dependent effect of raphe neuron stimulation on fictive lung ventilation in Rana catesbeiana: role of 5-HT_3 receptors. *FASEB J.* **17**, A422.

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. *Nat. Rev. Neurosci.* **3**, 728-739.

Broch, L., Morales, R. D., Sandoval, A. V. and Hedrick, M. S. (2002). Regulation of the respiratory central pattern generator by chloride-dependent inhibition during development in the bullfrog (*Rana catesbeiana*). *J. Exp. Biol.* **205**, 1161-1169.

Burggren, W. W. and Infantino, R. L. (1994). The respiratory transition from water to air breathing during amphibian metamorphosis. *Am. Zool.* **34**, 238-246.

Burggren, W. W. and West, N. H. (1982). Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog Rana catesbeiana. *Respir. Physiol.* **47**, 151-164.

Cao, Y., Fujito, Y., Matsuyama, K. and Aoki, M. (2006). Effects of electrical stimulation of the medullary raphe nuclei on respiratory movement in rats. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **192**, 497-505.

Erickson, J. T. and Millhorn, D. E. (1994). Hypoxia and electrical stimulation of the carotid sinus nerve induce Fos-like immunoreactivity within catecholaminergic and serotoninergic neurons of the rat brainstem. *J. Comp. Neurol.* **348**, 161-182.

Fournier, S. and Kinkead, R. (2006). Noradrenergic modulation of respiratory motor output during tadpole development: role of α-adrenoceptors. *J. Exp. Biol.* **209**, 3685-3694.

Fuller, D., Bavis, R. W., Vidruk, E. H., Wang, Z. Y., Olson, E. B., Jr, Bisgard, G. E. and Mitchell, G. S. (2002). Life-long impairment of hypoxic phrenic responses in rats following 1 month of developmental hyperoxia. *J. Physiol.* **538**, 947-955.

Hilaire, G., Monteau, R. and Errchidi, S. (1989). Possible modulation of the medullary respiratory rhythm generator by the noradrenergic A5 area: an in vitro study in the newborn rat. *Brain Res.* **485**, 325-332.

Jansen, A. H. and Chernick, V. (1991). Fetal breathing and development of control of breathing. *J. Appl. Physiol.* **70**, 1431-1446.

Johnson, S. M., Wilkerson, J. E., Henderson, D. R., Wenninger, M. R. and Mitchell, G. S. (2001). Serotonin elicits long-lasting enhancement of rhythmic respiratory activity in turtle brain stems in vitro. *J. Appl. Physiol.* **91**, 2703-2712.

Kinkead, R. and Milsom, W. (1996). CO_2-sensitive olfactory and pulmonary receptor modulation of episodic breathing in bullfrogs. *Am. J. Physiol.* **270**, R134-R144.

Kinkead, R., Filmyer, W. G., Mitchell, G. S. and Milsom, W. K. (1994). Vagal input enhances responsiveness of respiratory discharge to central changes in pH/CO_2 in bullfrogs. *J. Appl. Physiol.* **77**, 2048-2051.

Kinkead, R., Belzile, O. and Gulemetova, R. (2002). Serotonergic modulation of respiratory motor output during tadpole development. *J. Appl. Physiol.* **93**, 936-946.

Kogo, N. and Remmers, J. E. (1994). Neural organization of the ventilatory activity in the frog, *Rana catesbeiana*. II. *J. Neurobiol.* **25**, 1080-1094.

Kogo, N., Perry, S. F. and Remmers, J. E. (1994). Neural organization of the ventilatory activity in the frog, *Rana catesbeiana*. I. *J. Neurobiol.* **25**, 1067-1079.

Lalley, P. M. (1986). Responses of phrenic motoneurones of the cat to stimulation of medullary raphe nuclei. *J. Physiol. Lond.* **380**, 349-371.

Ling, L., Fuller, D. D., Bach, K. B., Kinkead, R., Olson, E. B., Jr and Mitchell, G. S. (2001). Chronic intermittent hypoxia elicits serotonin-dependent plasticity in the central neural control of breathing. *J. Neurosci.* **21**, 5381-5388.

Lipski, J., Bellingham, M. C., West, M. J. and Pilowsky, P. (1988). Limitations of the technique of pressure microinjection of excitatory amino acids for evoking responses from localized regions of the CNS. *J. Neurosci. Methods* **26**, 169-179.

Manaker, S. and Tischler, L. J. (1993). Origin of serotoninergic afferents to the hypoglossal nucleus in the rat. *J. Comp. Neurol.* **334**, 466-476.

Manzke, T., Guenther, U., Ponimaskin, E. G., Haller, M., Dutschmann, M., Schwarzacher, S. and Richter, D. W. (2003). 5-HT4(a) receptors avert opioid-induced breathing depression without loss of analgesia. *Science* **301**, 226-229.

McCrimmon, D. R., Mitchell, G. S. and Dekin, M. (1995). Glutamate, GABA, and serotonin in ventilatory control. In *Lung Biology in Health and Disease; 79 Regulation of Breathing* (ed. J. A. Dempsey and A. I. Pack), pp. 151-218. New York: M. Dekker.

McLean, D. L., Merrywest, S. D. and Sillar, K. T. (2000). The development of neuromodulatory systems and the maturation of motor patterns in amphibian tadpoles. *Brain Res. Bull.* **53**, 595-603.

Nicholson, C. (1985). Diffusion from an injected volume of a substance in brain tissue with arbitrary volume fraction and tortuosity. *Brain Res.* **333**, 325-329.

Peever, J. H., Necakov, A. and Duffin, J. (2001). Nucleus raphe obscurus modulates hypoglossal output of neonatal rat in vitro transverse brain stem slices. *J. Appl. Physiol.* **90**, 269-279.

Sanders, C. E. and Milsom, W. K. (2001). The effects of tonic lung inflation on ventilation in the American bullfrog *Rana catesbeiana* Shaw. *J. Exp. Biol.* **204**, 2647-2656.

Straus, C., Wilson, R. J. and Remmers, J. E. (2001). Oxygen sensitive chemoreceptors in the first gill arch of the tadpole, *Rana catesbeiana*. *Can. J. Physiol. Pharmacol.* **79**, 959-962.

Tan, H. J. and Miletic, V. (1990). Bulbospinal serotoninergic pathways in the frog *Rana pipiens. J. Comp. Neurol.* **292**, 291-302.

Taylor, A. C. and Kollros, J. J. (1946). Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* **94**, 7-24.

Torgerson, C., Gdovin, M. and Remmers, J. (1997). Ontogeny of central chemoreception during fictive gill and lung ventilation in an in vitro brainstem preparation of *Rana catesbeiana*. *J. Exp. Biol.* **200**, 2063-2072.

Torgerson, C. S., Gdovin, M. J. and Remmers, J. E. (1998). Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. *J. Neurophysiol.* **80**, 2015-2022.

van Hooft, J. A. and Vijverberg, H. P. (2000). 5-HT(3) receptors and neurotransmitter release in the CNS: a nerve ending story? *Trends Neurosci.* **23**, 605-610.

van Mier, P., Joosten, H. W., van Rheden, R. and ten Donkelaar, H. J. (1986). The development of serotonergic raphe spinal projections in *Xenopus laevis*. *Int. J. Dev. Neurosci.* **4**, 465-475.

Winmill, R. E. and Hedrick, M. S. (2003). Developmental changes in the modulation of respiratory rhythm generation by extracellular K^+ in the isolated bullfrog brainstem. *J. Neurobiol.* **55**, 278-287.