

Nitric oxide changes its role as a modulator of respiratory motor activity during development in the bullfrog (*Rana catesbeiana*)[☆]

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Abstract

Nitric oxide (NO) is a unique chemical messenger that has been shown to play a role in the modulation of breathing in amphibians and other vertebrates. In the post-metamorphic tadpole and adult amphibian brainstem, NO, acting via the neuronal isoform of nitric oxide synthase (nNOS), is excitatory to the generation of lung burst activity. In this study, we examine the modulation of breathing by NO during development of the amphibian brainstem. Isolated brainstem preparations from pre-metamorphic and late-stage post-metamorphic tadpoles (*Rana catesbeiana*) were used to determine the role of NO in modulating central respiratory neural activity. Respiratory neural activity was monitored with suction electrodes recording extracellular activity of cranial nerve rootlets that innervate respiratory musculature. Brainstems were superfused with an artificial cerebrospinal fluid (aCSF) at 20–22 °C containing L-nitroarginine (L-NA; 1–10 mM), a non-selective NOS inhibitor. In pre-metamorphic tadpoles, L-NA increased fictive gill ventilation frequency and amplitude, and increased lung burst frequency. By contrast, L-NA applied to the post-metamorphic tadpole brainstem had little effect on fictive buccal activity, but significantly decreased lung burst frequency and the frequency of lung burst episodes. These data indicate that early in development, NO provides a tonic inhibitory input to gill and lung burst activity, but as development progresses, NO provides an excitatory input to lung ventilation. This changing role for NO coincides with the shift in importance in the different respiratory modes during development in amphibians; that is, pre-metamorphic tadpoles rely predominantly on gill ventilation whereas post-metamorphic tadpoles have lost the gills and are obligate air-breathers primarily using lungs for gas exchange. We hypothesize that NO provides a tonic input to the respiratory CPG during development and this changing role reflects the modulatory influence of NO on inhibitory or excitatory modulators or neurotransmitters involved in the generation of respiratory rhythm.

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1. Introduction

The mechanisms for breathing in amphibians change dramatically during development. During early larval (tadpole) stages, oxygen is acquired in a trimodal fashion with involvement of gills, skin and lungs (Burggren and West, 1982; Burggren and Doyle, 1986; Burggren and Infantino,

1994). Gill ventilation is the primary mechanism for oxygen acquisition in early stages, but as development proceeds, lung ventilation becomes more important until, at metamorphic climax, the gills involute and the animal becomes an obligate air-breathing post-metamorphic tadpole (Burggren and Pinder, 1991; Crowder et al., 1998). Throughout this remarkable morphological rearrangement of peripheral respiratory structures (gills and lungs), breathing occurs continuously with ventilatory muscles driven by motor output from neuronal circuits located in the ventral brainstem (Gradwell, 1972; Gdovin et al., 1998). Although the morphological changes in the peripheral structures that subserve ventilation are well known, the changes in the neuronal circuitry underlying respiratory rhythm that

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accompany development of the respiratory system are largely unknown.

Respiratory rhythm and pattern formation in amphibians and other vertebrates is generated by neuronal circuits, commonly referred to as central pattern generators (CPGs) (Delcomyn, 1980), localized to the brainstem. How CPGs for breathing and other motor behaviors are modified and modulated during the course of development is poorly understood. Central pattern generators exhibit a significant degree of plasticity throughout development and much of this plasticity arises from changes in developmental neuromodulation (Fenelon et al., 2004). Indeed, the adaptive plasticity of neural networks lends support to the hypothesis that neural networks underlying many motor behaviors are conserved during the course of evolution (Smith, 1994; Tierney, 1996; Katz and Harris-Warrick, 1999; Hedrick et al., 2001).

Recent evidence from North American bullfrogs suggests that gill and lung ventilation are modulated differently during development (Hedrick, in press). For example, in tadpoles, lung ventilation may be driven by a pacemaker-like rhythm (Galante et al., 1996; Broch et al., 2002), is voltage-dependent (Winmill and Hedrick, 2003a) and is dependent upon gap junctions (Winmill and Hedrick, 2003b). Gill ventilation in tadpoles also appears to rely on synaptic inhibition (Broch et al., 2002). In the adult bullfrog brainstem, lung ventilation appears to depend upon network synaptic inhibition (Broch et al., 2002), is not voltage-dependent (Winmill and Hedrick, 2003a), and has a different response to gap junction blockade compared with pre-metamorphic tadpoles (Winmill and Hedrick, 2003b). Taken together, these data indicate that modulation of gill and lung ventilation change over the course of development. Differential regulation of gill and lung ventilation in developing amphibians appears to have an anatomical basis since microinjections of inhibitory and excitatory neurotransmitters have effects on lung and gill bursts in different regions of the ventral brainstem (McLean et al., 1995; Wilson et al., 2002).

Nitric oxide (NO) is a unique chemical messenger that has shown to have modulatory effects on motor systems including respiration (Ling et al., 1992; Hedrick et al., 1998; Hedrick and Morales, 1999; Pierrefiche et al., 2002; Gargaglioni and Branco, 2001; Harris et al., 2002) and locomotion (McLean and Sillar, 2002, 2004). In amphibians, NO-producing neurons are localized to brainstem areas during early stages of development (McLean and Sillar, 2000; Lopez and Gonzalez, 2002) indicating that NO is present and may be capable of modulating brainstem neural circuits throughout development. These NO-containing regions are near putative respiratory-generating areas of the brainstem (Torgerson et al., 2001; Wilson et al., 2002), thus making it likely that NO contributes to the modulation of respiratory rhythm in amphibian development. Previous work has shown that NO modulates lung ventilation and buccal ventilation in the post-metamorphic tadpole (Harris et al., 2002) and lung ventilation in the adult (Hedrick et al.,

1998) bullfrog brainstem in vitro. Nitric oxide also provides an inhibitory signal for increased ventilatory drive during hypoxia or hypercapnia in the toad (Gargaglioni and Branco, 2001). In the post-metamorphic tadpole brainstem, NO appears to differentially regulate buccal and lung ventilation (Harris et al., 2002), supporting the hypothesis that there are distinct oscillators for these respiratory events and that NO is an important neuromodulator of central respiratory rhythms in the amphibian brainstem.

The present study further examines the role of NO on respiratory rhythm generation, but focuses on the effects of NO on respiratory rhythm generation at different stages of development in the North American bullfrog (*Rana catesbeiana*). Our results suggest that NO has a significant modulatory role on the neural circuits that drive respiratory rhythm, and the modulation of respiratory rhythm by NO changes during the course of development.

2. Materials and methods

2.1. Animals

Experiments were performed on 7 pre-metamorphic (body mass 5.9–14.8 g) and 6 post-metamorphic (body mass 6.3–7.8 g) North American bullfrogs (*R. catesbeiana*). Tadpoles were classified according to the staging criteria of Taylor and Kollros (T–K: 1946). Pre-metamorphic tadpoles from foot paddle stages (VI–X) with undifferentiated limbs and foot stage tadpoles (XI–XVII) were used. The pre-metamorphic tadpoles used in this study ranged from T–K stages IX to XIV. Late-stage post-metamorphic tadpoles that had involuted gills and were obligate air-breathers (T–K stages XXIV–XXV) were used. Animals were purchased from a commercial supplier (Charles D. Sullivan Co., Inc.; Nashville, TN, USA). Tadpoles were kept in fiberglass aquaria with aerated, dechlorinated tapwater and fed boiled spinach several times per week. All animals were maintained at room temperature (20–22°C). All experimental procedures were approved by the CSUEB Institutional Animal Care and Use Committee.

2.2. In vitro brainstem preparation

Prior to surgery, tadpoles were anesthetized by submersion in an aqueous solution of ethyl-*m*-aminobenzoate (MS-222, Sigma-Aldrich Chemical Co., St. Louis, MO, USA; 0.5g/L) buffered to pH 7.8 with sodium bicarbonate. When breathing movements ceased and the withdrawal and corneal reflexes were abolished (2–5 min), animals were removed from anesthesia. Tadpoles were placed in ice water for 20 min to slow metabolism and maintain anesthesia for subsequent dissection.

A small opening was then made in the cranium with iris scissors, allowing for the transection and removal of the forebrain rostral to the optic lobes. During decerebration and

subsequent dissection, the brainstem was constantly perfused with cold (5–10 °C) artificial CSF (aCSF) with the following composition: (in mM) NaCl, 104.0; KCl, 4.0; MgCl₂, 1.4; NaHCO₃, 25.0, CaCl₂, 2.4; glucose, 10.0 (adapted from Torgerson et al., 2001), and equilibrated with 98% O₂/2% CO₂. The spinal cord was transected caudal to the brachial nerves and cranial nerve roots were severed at their exit from the cranium. The entire dissection required approximately 15–20 min to complete.

The isolated brainstem was pinned ventral side up in a sylgard-lined (Dow Corning) recording chamber (7 mL) and the dura and arachnoid were removed. Throughout this process, and during all subsequent experiments, the recording chamber was continuously perfused with oxygenated aCSF (pH 7.8, 20–22 °C) from a gravity-fed reservoir (350 mL) at a flow rate of 5–10 mL/min.

Suction electrodes, fabricated from thin-walled capillary glass and held in micromanipulators (Narashige), were attached to three of the following four cranial nerve (CN) rootlets to record respiratory-related neural activity: CN V (trigeminal), CN VII (facial), CN X (vagus) and CN XII (hypoglossal). These nerves innervate buccal elevator and depressor muscles in the oropharyngeal region of anurans and are responsible for generating water flow and airflow, and controlling glottal airflow, associated with small amplitude, non-ventilatory gill/buccal oscillations and larger amplitude, positive-pressure lung ventilatory events. Previous studies with tadpole and adult amphibian preparations have verified that neural activity from CN V, VII, X and XII is correlated with breathing movements in intact animals (Sakakibara, 1984; Gdovin et al., 1998). Nerve activity was amplified 10,000 times with a differential AC amplifier (A-M systems model 1700; Everett, WA, USA), filtered (100 Hz–5 kHz) and moving time averaged (CWE model MA-821-4). Raw and processed signals were simultaneously recorded on a computer (Dell, Pentium 4) that interfaced with a data acquisition system sampling at 2 kHz (Powerlab 8/SP; AD Instruments, Milford, MA, USA).

2.3. Experimental protocol

L-nitroarginine (L-NA; Sigma) was used as a non-selective inhibitor of nitric oxide synthase (NOS) in the amphibian brainstem preparation. L-NA was dissolved in aCSF to achieve a final concentration of 1–10 mM. The brainstem preparation was superfused with aCSF for 1 h, or until the signal was stable, before a 20 min control recording was obtained. After the initial control recording was taken, each brainstem was switched to a superfusate of aCSF containing L-NA. L-NA was superfused in increasing concentrations from 1.0 mM, 5.0 mM and 10.0 mM before returning to the control aCSF (washout). Each concentration of L-NA was superfused for 25 min before increasing the concentration. Respiratory related motor output was recorded throughout the L-NA superfusion and the last 10 min of data collected during the L-NA exposure was used

for analysis. Fictive respiratory-related neural discharges were classified based on previously described criteria, obtained from comparison of the *in vitro* motor output to fictive breathing in the less reduced decerebrate, paralyzed, unidirectionally-ventilated *in situ* preparation (Gdovin et al., 1998). Fictive gill activity in the tadpole and buccal activity in the post-metamorphic tadpole are characterized as low amplitude, cyclic neural burst discharge. Fictive lung ventilation was characterized as neural bursts occurring singly or in clusters and having amplitudes higher than that of fictive gill or buccal bursts. Fictive lung bursts also occur simultaneously in CN V, CN VII and CN XII in pre-metamorphic and post-metamorphic tadpoles and have burst durations of about 1 s (Gdovin et al., 1998; Torgerson et al., 2001). Discharges of motor output not meeting these criteria were assumed to be associated with events other than normal fictive breathing and were excluded from analysis (Hedrick and Winmill, 2003). Absolute burst frequency is defined as neural bursts per minute, regardless of the pattern of lung burst events (i.e. single breaths or episodes). Burst duration was measured from the onset of deviation from the baseline to the return to baseline in the integrated neural trace. Burst amplitude was measured in arbitrary units and analyzed as a percentage of control from the integrated neural trace. Lung bursts in amphibians often occur in clusters or episodes (Kinkead and Milsom, 1994). Episodes were defined as two or more bursts occurring in succession separated by a pause not longer than the average duration of two ventilatory cycles (Kinkead and Milsom, 1994). Lung burst episodes were also quantified by measuring the instantaneous frequency of lung bursts within an episode; that is, measuring the period between successive lung bursts in an episode and converting this value to bursts min⁻¹ (Kinkead and Milsom, 1994).

2.4. Statistical analysis

A one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test (Zar, 1974) was used for evaluation of statistical significance between fictive breaths during drug administration compared with the control. Data expressed as percentages were first transformed to their arcsine values prior to statistical testing (Zar, 1974). Statistical significance was assumed if $P < 0.05$. All statistical analyses were carried out using a commercial software package (Graphpad Prism, v. 4.0 (PC version), San Diego, CA, USA).

3. Results

3.1. Respiratory-related effects of L-NA in tadpole brainstems

L-NA applied to pre-metamorphic brainstems significantly increased gill burst frequency (Fig. 1A). A consistent

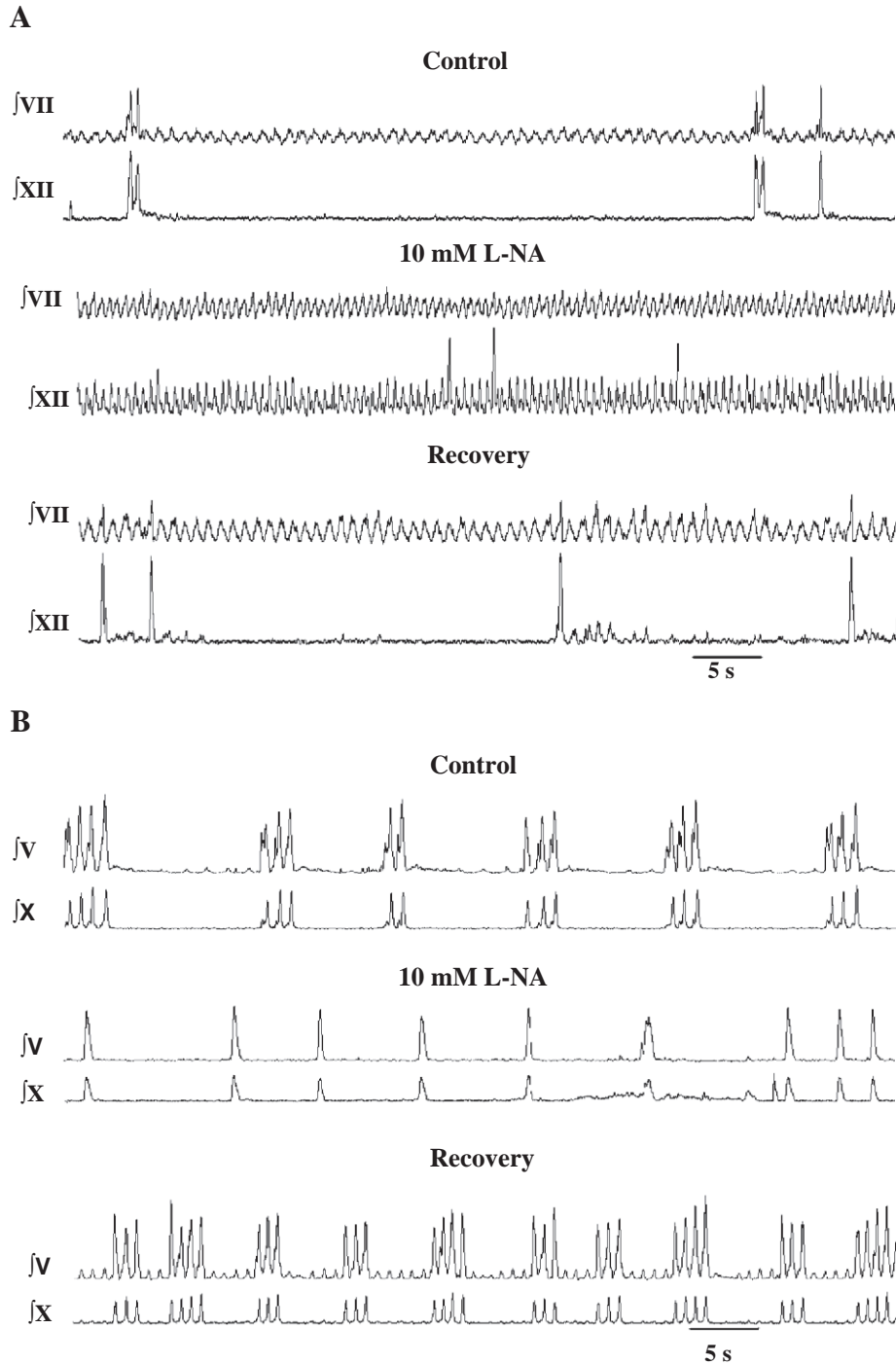


Fig. 1. Integrated respiratory motor activity recorded from one (A) pre-metamorphic and one (B) post-metamorphic brainstem during superfusion with aCSF (control), L-NA (10 mM) and washout (recovery) with normal aCSF. In the pre-metamorphic preparation, integrated neural activity from the facial nerve (∫VII) and hypoglossal nerve (∫XII) is shown. In the post-metamorphic preparation, integrated neural activity from the trigeminal nerve (∫V) and vagus nerve (∫X) is shown. Note the appearance of respiratory activity in CN XII after application of L-NA in the pre-metamorphic tadpole and the inhibition of episodic breathing in the post-metamorphic tadpole.

observation from the application of 10 mM L-NA to pre-metamorphic brainstems is that neural burst activity in CN XII, which normally exhibits only lung burst activity in pre-metamorphic brainstems (Torgerson et al., 1998), exhibits neural activity that resembles gill bursts after L-NA superfusion (Fig. 1A). This was readily reversible upon super-

fusion with control aCSF (Fig. 1A). Application of L-NA to post-metamorphic brainstems reduced the overall lung burst frequency and the frequency of lung burst episodes (Fig. 1B). Application of L-NA to post-metamorphic brainstems also elicited bursts of activity that were not present under control conditions (Fig. 1B).

Gill burst frequency in pre-metamorphic animals was 61 ± 5.8 bursts min^{-1} with control aCSF superfusion. Inhibition of NOS in pre-metamorphic brainstems significantly increased gill burst frequency to 96 ± 5.7 bursts min^{-1} with 5 mM L-NA ($q'_{4,30}=5.3$; $P<0.01$) and to 115 ± 7.9 bursts min^{-1} with 10 mM L-NA ($q'_{4,30}=8.1$; $P<0.01$) (Fig. 2A). The increased gill burst frequency was accompanied by significant reductions in gill burst duration (Fig. 2B). Burst duration decreased from 0.75 ± 0.07 s (control) to 0.59 ± 0.02 s ($q'_{4,30}=2.8$; $P<0.05$) with 5 mM L-NA and 0.47 ± 0.02 s ($q'_{4,30}=5.0$; $P<0.01$) with 10 mM L-NA. Gill burst amplitude (Fig. 2C) also increased significantly upon exposure to higher concentrations of L-NA, increasing to $180 \pm 15\%$ ($q'_{4,30}=3.8$; $P<0.01$) and $164 \pm 26\%$

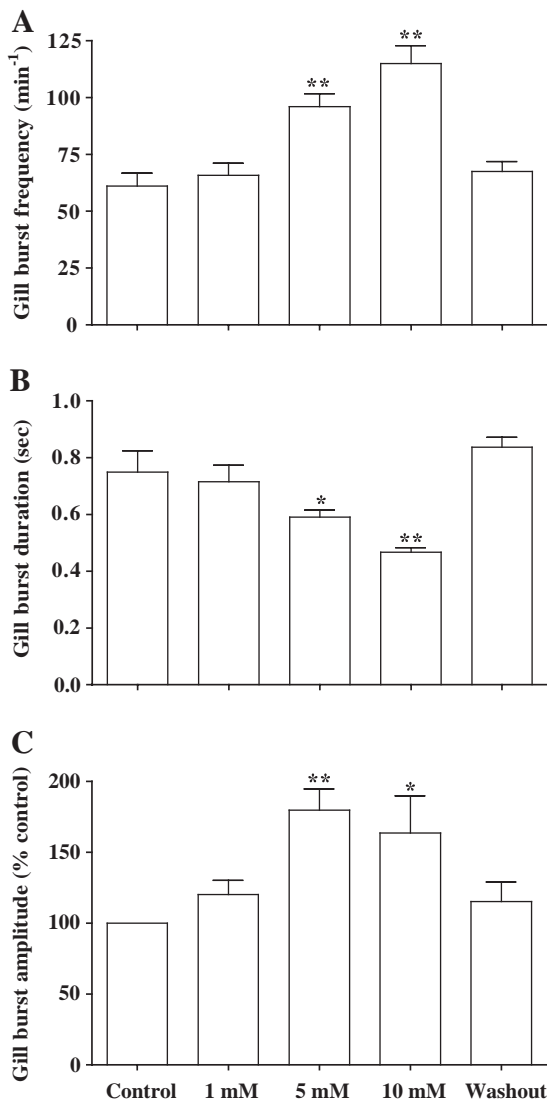


Fig. 2. Effects of L-NA on gill burst activity in pre-metamorphic tadpole brainstems ($N=7$). Data from control, L-NA (1 mM, 5 mM and 10 mM) and washout (recovery) are shown for (A) gill burst frequency (min^{-1}), (B) gill burst duration (s) and (C) gill burst amplitude (% control). * $P<0.05$, ** $P<0.01$ from control.

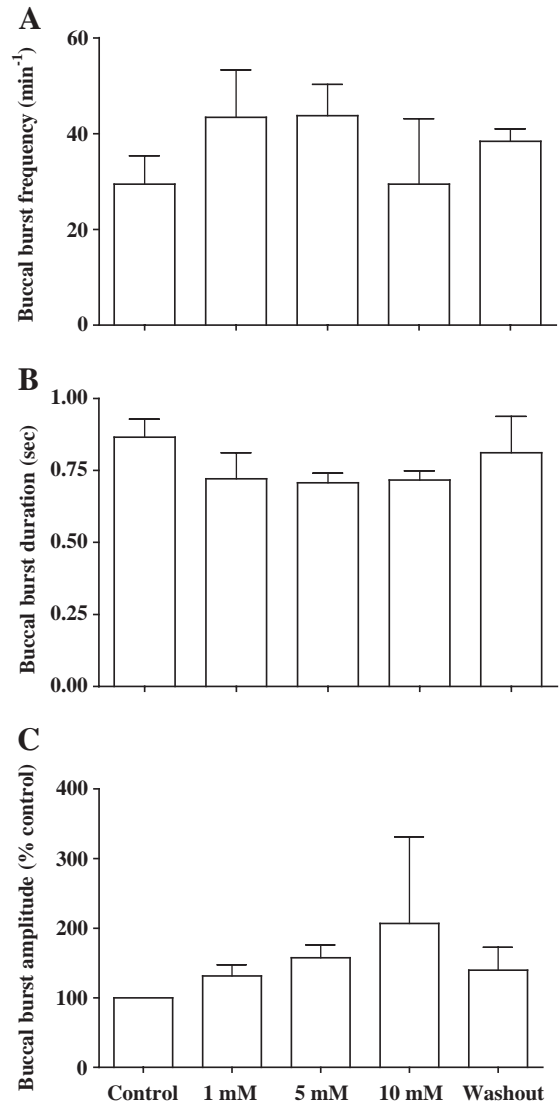


Fig. 3. Effects of L-NA on buccal burst activity in post-metamorphic tadpole brainstems ($N=6$). Control, L-NA and washout are shown for (A) buccal burst frequency (min^{-1}), (B) buccal burst duration (s) and buccal burst amplitude (% control). There were no significant effects of L-NA on buccal burst activity.

($q'_{4,30}=3.3$; $P<0.05$) of control at 5 and 10 mM L-NA, respectively.

Buccal burst frequency was present in 5 of 6 post-metamorphic tadpole brainstems. Buccal frequency was 29 ± 6 bursts min^{-1} with control aCSF and exhibited considerable variability in response to L-NA, including the abolition of buccal activity in 2 of 5 preparations with 10 mM L-NA. Overall, there was no effect of L-NA on buccal burst frequency in post-metamorphic brainstems. L-NA also did not affect buccal burst duration or amplitude (Fig. 3).

Lung burst frequency in pre-metamorphic tadpoles was 4.8 ± 0.8 bursts min^{-1} in control conditions and remained constant with 1.0 mM and 5.0 mM L-NA, but superfusion with 10 mM L-NA approximately doubled lung burst frequency to 9.9 ± 1.6 burst min^{-1} ($q'_{4,30}=3.6$; $P<0.01$;

Fig. 4A). Lung burst duration in control aCSF was 0.54 ± 0.04 s and was not affected by application of L-NA; similarly, lung burst amplitude was also unaffected by application of L-NA (data not shown).

Lung burst frequency was 21.6 ± 3.7 bursts min^{-1} in post-metamorphic brainstems with control superfusion and significantly decreased to 5.9 ± 1.3 bursts min^{-1} ($q'_{4,24} = 3.8$; $P < 0.01$) with 10 mM L-NA (Fig. 4B), but application of L-NA had no significant effects on lung burst duration or amplitude (data not shown).

Lung burst episodes were present in both pre-metamorphic and post-metamorphic brainstems (Fig. 5), but not every brainstem exhibited episodic lung bursts. Four of 7 pre-metamorphic brainstems exhibited episodic lung bursts and 4 of 6 post-metamorphic brainstems exhibited episodic lung bursts. Frequency of lung burst episodes for pre-metamorphic tadpoles was 1.6 ± 0.3 episodes min^{-1} with control aCSF and decreased significantly to 0.3 ± 0.2 episodes min^{-1} with 10 mM L-NA ($q'_{4,25} = 3.3$; $P < 0.05$). The effect of L-NA on episodic breathing in pre-metamorphic tadpoles was irreversible since episodes during washout was significantly different from control ($q'_{4,25} = 3.4$; $P < 0.05$; Fig. 5A). In post-metamorphic tadpoles, L-NA

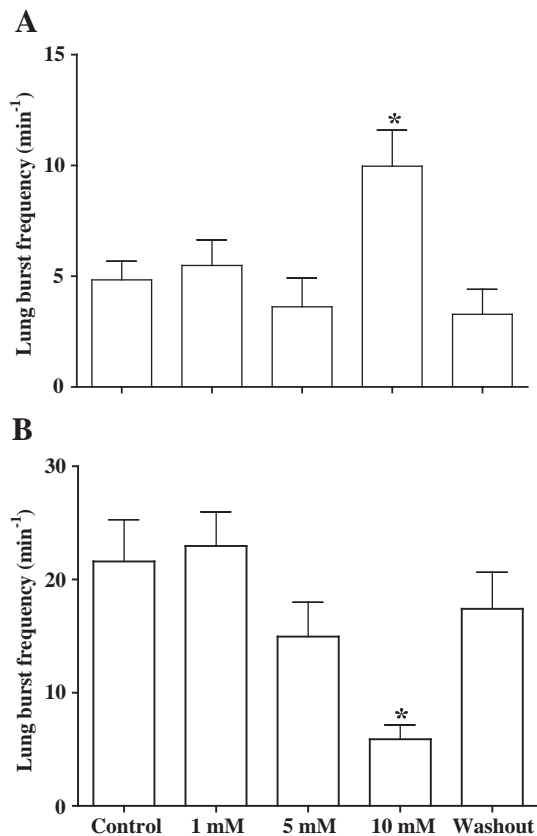


Fig. 4. Effects of L-NA on lung burst frequency (min^{-1}) for (A) pre-metamorphic and (B) post-metamorphic tadpole brainstems. Superfusion with 10 mM L-NA significantly increased lung burst frequency in pre-metamorphic tadpoles and significantly inhibited lung burst frequency in post-metamorphic tadpoles. * $P < 0.05$ from control.

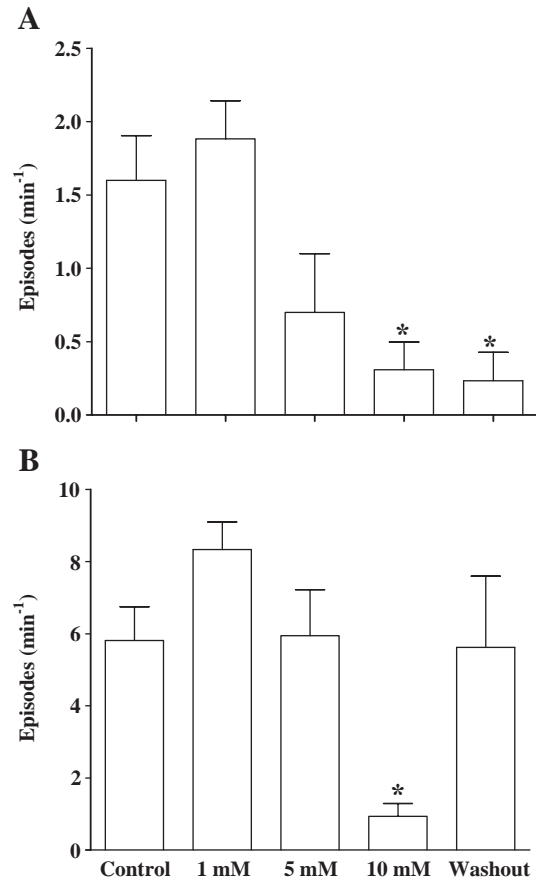


Fig. 5. Effects of L-NA on lung burst episodes (min^{-1}) in (A) pre-metamorphic and (B) post-metamorphic tadpole brainstems. Superfusion with 10 mM L-NA significantly reduced episode frequency in both pre-metamorphic and post-metamorphic brainstems, but was reversible only in the post-metamorphic brainstems. * $P < 0.05$ from control.

significantly inhibited episodic breathing at 10 mM (Fig. 5B) and this effect was completely reversible (Fig. 5B). Although the effects of L-NA were to significantly reduce episode frequency in pre-metamorphic and post-metamorphic tadpoles, there was no significant change in the number of lung bursts per episode in either group before, during or following L-NA exposure. Under control conditions, pre-metamorphic brainstems averaged 2.7 ± 0.2 bursts episode⁻¹ and post-metamorphic brainstems averaged 3.3 ± 0.6 bursts episode⁻¹ (data not shown).

4. Discussion

This study has shown that NO changes its role as a modulator of respiratory activity during development in the bullfrog. In early stage (pre-metamorphic) tadpoles, NO acts primarily as an inhibitory modulator of respiratory activity since application of L-NA significantly increased gill and lung burst activity. Following metamorphosis, L-NA inhibits lung burst activity, indicating that NO is an excitatory modulator of respiratory activity.

This ‘switch’ in the role of NO during development appears to coincide with the loss of gills and the initiation of obligate air-breathing in post-metamorphic tadpoles. Because NO is an important modulator of neural circuits, it is likely that the changing role for NO throughout development reflects its modulation of other neurotransmitters and neuromodulators that have a direct impact on respiratory rhythm generation.

4.1. Nitric oxide as neuromodulator of respiratory rhythm generation

Based upon the results from NOS inhibition, the most surprising aspect of this study is that NO initially inhibits respiratory motor activity (gill and lung) early in development, but later in development excites fictive breathing (lung only). The inhibitory actions of NO on lung ventilation is a novel finding since previous work has generally found that NO is an excitatory modulator of lung burst activity in the post-metamorphic tadpole and adult amphibian brainstem (Hedrick et al., 1998; Hedrick and Morales, 1999; Harris et al., 2002). However, NO has been shown to inhibit buccal burst activity in the post-metamorphic brainstem (Harris et al., 2002) as it does with gill ventilation in pre-metamorphic tadpoles. In awake toads, inhibition of NO synthesis in the nucleus isthmi increases tidal volume during hypoxia or hypercapnia, indicating that NO normally inhibits ventilation when respiratory drive is high (Gargaglioni and Branco, 2001). The excitatory actions of NO appear to coincide with the metamorphic transition associated with the loss of gills and the obligate air-breathing in the post-metamorphic animal. However, the precise timing or the mechanisms underlying the switch from an inhibitory to an excitatory neuromodulator have not been examined.

The data presented here for pre-metamorphic tadpoles are consistent with an inhibitory role for NO in the locomotor system of developing amphibians (McLean and Sillar, 2000, 2002, 2004; Sillar et al., 2002). The inhibitory actions of NO in the spinal locomotor system of amphibians occur by the facilitation of glycinergic and GABAergic inhibition and the direct actions of NO on spinal motoneurons, without any apparent involvement of excitatory neurotransmitters such as glutamate (McLean and Sillar, 2002). Thus, NO acts as a ‘brake’ on swimming motor behavior in *Xenopus* tadpoles (Sillar et al., 2002). The direct actions of NO are to depolarize motoneurons while decreasing whole cell conductance, which is likely caused by a decrease in K^+ conductance (McLean and Sillar, 2002). Because we did not make intracellular recordings, we cannot be certain if NO depolarizes motoneurons in the tadpole brainstem preparation. The appearance in activity within CN XII following L-NA application (Fig. 1A) could result from the direct actions of L-NA on hypoglossal motoneurons, disinhibition of NO on motoneurons or on pre-motor neurons upstream of

motoneuronal output. There is no evidence for the presence of NOS in the cranial motoneurons of amphibians (Munoz et al., 1996), suggesting that the appearance of gill-like bursting activity in the hypoglossal nerve results from the inhibition of NO at the level of the respiratory CPG.

Nitric oxide has been shown to act as a modulator of other neuromodulators such as noradrenaline, thus serving as a ‘meta’-modulator of the amphibian locomotor network (McLean and Sillar, 2004). Because neurotransmitters such as glycine, GABA and serotonin (5-HT) inhibit respiratory rhythm generation in the developing amphibian brainstem, the interaction of NO with these neuromodulators has important implications for the current study. Superfusion of GABA inhibits gill and lung burst activity in pre-metamorphic tadpoles (Galante et al., 1996; Broch et al., 2002) and in adult brainstems in vitro (Broch et al., 2002). Application of baclofen, a GABA_B receptor agonist, also inhibits lung burst activity, but also inhibits episodic breathing in post-metamorphic tadpole brainstems (Straus et al., 2000). The role of 5-HT also changes during the course of development (Kinkead et al., 2002). Gill ventilation is inhibited by 5-HT in pre- and post-metamorphic tadpole brainstems, but has a differential effect on lung ventilation during development (Kinkead et al., 2002). Given that NO-synthesizing neurons are located in the caudal raphe’ group along with serotonergic neurons (Lopez and Gonzalez, 2002; McCrimmon et al., 1995), and both systems exhibit developmentally-dependent modulation of breathing in amphibians, an interaction between NO and 5-HT in the amphibian brainstem is very likely. The interactions of NO and glycinergic, GABAergic and serotonergic pathways in the amphibian respiratory system have not been examined, but it is also likely that NO may be modulating these and other pathways to affect breathing, as previously demonstrated in the locomotor system.

Neurons that express NOS or NADPH-diaphorase are found in the hindbrain reticular formation of early larval stages of *Xenopus*, and are present throughout development (McLean and Sillar, 2001; Lopez and Gonzalez, 2002). These brainstem nitrenergic neurons are located in regions associated with respiratory rhythm generation in vertebrates (McLean et al., 1995; Wilson et al., 2002; Milsom et al., 2004). In adult amphibians, NADPH-diaphorase staining is found in the medullary reticular formation (Munoz et al., 1996), thus making it more likely that NO is exerting its effects directly or indirectly on the respiratory CPG rather than modulating motoneurons.

Nitric oxide is synthesized in neurons by post-synaptic stimulation of the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor (Brenman and Bredt, 1997). Calcium influx through the NMDA channel activates nNOS producing NO during the conversion of L-arginine to L-citrulline. Nitric oxide acts intracellularly on the heme group of soluble guanylate cyclase (GC) to raise the

intracellular concentration of cyclic GMP (cGMP). Owing to its ability to readily cross cell membranes, NO also increases cGMP in nearby cells thus acting as an intercellular signaling molecule. Given its unique properties as a gas, NO has been implicated as having a role as a ‘synchronizing’ modulator of neural networks (Anbar, 1995) associated with olfaction (Gelperin, 1994), thalamo-cortical oscillations (Pape and Mager, 1992), feeding (Elphick et al., 1995) and neural network plasticity (Scholz et al., 2001). Recent work implicates NO as an important molecule during amphibian metamorphosis for regulating limb development (Cristino et al., 2004) and controlling cell proliferation and differentiation in brain development (Peunova et al., 2001). An intriguing suggestion is that NO is important for the morphogenesis of the amphibian nervous system, coordinating the switch from cell proliferation to differentiation and functional connectivity between cell groups (Cristino et al., 2004). Applied to the respiratory system, this may suggest that NO is important as a neuromodulator of respiratory-related neurons during the transition from facultative to obligate air-breathing during metamorphosis.

Previous work has shown that glutamatergic pathways facilitate lung ventilation in the post-metamorphic and adult amphibian brainstem, but this pathway has not been examined in pre-metamorphic tadpoles. For example, microinjection of glutamate into rostral areas of the medulla near the facial motor nucleus increases respiratory burst frequency, whereas microinjections at more caudal locations inhibits neural output or has no effect (McLean et al., 1995). Microinjections of the non-NMDA receptor agonist, AMPA, also stimulate lung bursts when injected in rostral brainstem locations between CN VIII and CN X (Wilson et al., 2002). We are not aware of any studies that have examined the role of glutamatergic NMDA receptors on fictive breathing in amphibians. At present, a direct link between glutamate receptor activation and NO production in the amphibian brain has not been established, but the results from this study and previous studies (Hedrick and Morales, 1999; Hedrick et al., 1998; Harris et al., 2002) are consistent with the excitatory effect of glutamate on respiratory activity in the post-metamorphic and adult amphibian brainstem.

4.2. NO as a modulator of episodic breathing

Episodic breathing is normally a characteristic feature of the breathing pattern of late-stage post-metamorphic and adult amphibians (Milsom, 1991). Although it is clear that episodic breathing is generated from brainstem circuits that produce a respiratory motor output, episodic breathing is also modified by afferent feedback from peripheral chemo- and mechanoreceptors (Kinkead and Milsom, 1994, 1997). However, the underlying mechanisms and anatomical locations that generate or modulate episodic breathing are unclear (see Milsom et al., 2004). The present study has

shown that inhibiting endogenous NO production with L-NA significantly reduces the production of lung burst episodes in tadpoles, suggesting that NO is important for the production of episodic breathing in the amphibian brainstem. These results are consistent with a previous study showing that inhibiting nNOS with 7-nitroindazole inhibits the production of lung burst episodes in the post-metamorphic tadpole brainstem (Harris et al., 2002). Application of baclofen, a GABA_B agonist, also inhibits the production of episodes in the post-metamorphic brainstem in vitro (Straus et al., 2000), suggesting that multiple pathways or interactions between nitrergic and GABAergic systems modulate episodic breathing in the amphibian brainstem. We have recently found that severe brainstem hypoxia is capable of increasing the frequency of lung burst episodes in post-metamorphic and adult brainstems in vitro (Winmill et al., 2005). Although it is not clear how hypoxia exerts this effect, one possibility is that the release of glutamate during hypoxia could activate NMDA channels and facilitate the production of NO. In support of this, glutamate microinjection into the mid-rostral region of the medulla in the adult bullfrog in vitro can produce episodic-like lung bursts (McLean et al., 1995). The increased production of NO during severe hypoxia (see Gozal and Torres, 2001) through activation of the NMDA receptor pathway could lead to increased episodic breathing, but the link between glutamate, NO and episodic breathing remains to be tested.

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References

- Anbar, M., 1995. Nitric oxide: a synchronizing chemical messenger. *Experientia* 51, 545–550.
- Brenman, J.E., Bredt, D.S., 1997. Synaptic signaling by nitric oxide. *Curr. Opin. Neurobiol.* 7, 374–378.
- Broch, L., Morales, R.D., Sandoval, A.V., 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., Doyle, M., 1986. Ontogeny of regulation of gill and lung ventilation in the bullfrog, *Rana catesbeiana*. *Respir. Physiol.* 66, 279–291.
- Burggren, W.W., Infantino, R.L., 1994. The respiratory transition from water to air breathing during amphibian metamorphosis. *Am. Zool.* 34, 238–246.

- Burggren, W.W., Pinder, A.W., 1991. Ontogeny of cardiovascular and respiratory physiology in lower vertebrates. *Annu. Rev. Physiol.* 53, 107–135.
- Burggren, W.W., West, N.H., 1982. Changing respiratory importance of gills, lungs and skin during metamorphosis in the bullfrog, *Rana catesbeiana*. *Respir. Physiol.* 47, 151–164.
- Cristino, L., Florenzano, F., Bentivoglio, M., Guglielmotti, V., 2004. Nitric oxide synthase expression in dorsal root ganglia and spinal dorsal horn of developing and adult *Rana esculenta* indicate a role of nitric oxide in limb metamorphosis. *J. Comp. Neurol.* 472, 423–436.
- Crowder, W.C., Nie, M., Ultsch, G.R., 1998. Oxygen uptake in bullfrog tadpoles (*Rana catesbeiana*). *J. Exp. Zool.* 280, 121–134.
- Delcomyn, F., 1980. Neural basis of rhythmic behavior in animals. *Science* 210, 492–498.
- Elphick, M.R., Kemenes, G., Staras, K., O'Shea, M., 1995. Behavioral role for nitric oxide in chemosensory activation of feeding in a mollusk. *J. Neurosci.* 15, 7653–7664.
- Fenelon, V.S., Le Feuvre, Y., Meyrand, P., 2004. Phylogenetic, ontogenetic and adult adaptive plasticity of rhythmic neural networks: a common neuromodulatory mechanism? *J. Comp. Physiol. A* 190, 691–705.
- Galante, R.J., Kubin, L., Fishman, A.P., Pack, A.I., 1996. Role of chloride-mediated inhibition in respiratory rhythmogenesis in an in vitro brainstem of tadpole *Rana catesbeiana*. *J. Physiol. Lond.* 492, 545–558.
- Gargaglioni, L.H., Branco, L.G.S., 2001. Effect of nitric oxide in the nucleus isthmi on the hypoxic and hypercarbic drive to breathing of toads. *Am. J. Physiol.* 281, R338–R345.
- Gdovin, M.J., Torgerson, C.S., Remmers, J.E., 1998. Neurorespiratory pattern of gill and lung ventilation in the decerebrate spontaneously breathing tadpole. *Respir. Physiol.* 113, 135–146.
- Gelperin, A., 1994. Nitric oxide mediates network oscillations of olfactory interneurons in a terrestrial mollusk. *Nature* 369, 61–63.
- Gozal, D., Torres, J.E., 2001. Brainstem nitric oxide tissue levels correlate with anoxia-induced gasping activity in the developing rat. *Biol. Neonate* 79, 122–130.
- Gradwell, N., 1972. Gill irrigation in *Rana catesbeiana*: Part II. On the musculoskeletal mechanism. *Can. J. Zool.* 50, 501–521.
- Harris, M.B., Wilson, R.J.A., Vasilakos, K., Taylor, B.E., Remmers, J.E., 2002. Central respiratory activity of the tadpole in vitro brain stem is modulated diversely by nitric oxide. *Am. J. Physiol.* 283, R417–R428.
- Hedrick, M.S., in press. Development of respiratory rhythm generation in ectothermic vertebrates. *Respir. Physiol. Neurobiol.*
- Hedrick, M.S., Morales, R.D., 1999. Nitric oxide as a modulator of central respiratory rhythm in the isolated brainstem of the bullfrog (*Rana catesbeiana*). *Comp. Biochem. Physiol. A* 124, 243–251.
- Hedrick, M.S., Winmill, R.E., 2003. Excitatory and inhibitory effects of tricaine (MS-222) on fictive breathing in the bullfrog brainstem. *Am. J. Physiol.* 284, R405–R412.
- Hedrick, M.S., Morales, R.D., Parker, J.M., Pacheco, J.L.H., 1998. Nitric oxide modulates respiratory-related neural activity in the isolated brainstem of the bullfrog. *Neurosci. Lett.* 251, 81–84.
- Hedrick, M.S., Broch, L., Martinez, M., Powell, J.L., Wade, R.E., 2001. Is the vertebrate respiratory central pattern generator conserved? Insights from in vitro and in vivo amphibian models. In: Poon, C.S., Kazemi, M. (Eds.), *Frontiers in Modeling and Control of Breathing: Integration at Molecular, Cellular and Systems Levels*, Advances in Experimental Medicine and Biology Series, vol. 499. Kluwer Academic/Plenum Publishers, Inc., New York, pp. 127–132.
- Katz, P.S., Harris-Warrick, R.M., 1999. The evolution of neuronal circuits underlying species-specific behavior. *Curr. Opin. Neurobiol.* 9, 628–633.
- Kinkead, R., Milsom, W.K., 1994. Chemoreceptors and control of episodic breathing in the bullfrog (*Rana catesbeiana*). *Respir. Physiol.* 95, 81–98.
- Kinkead, R., Milsom, W.K., 1997. Role of pulmonary stretch receptor feedback control of episodic breathing in the bullfrog. *Am. J. Physiol.* 270, R497–R508.
- Kinkead, R., Belzile, O., Gulemetova, R., 2002. Serotonergic modulation of respiratory motor output during tadpole development. *J. Appl. Physiol.* 93, 936–946.
- Ling, L., Karius, D.R., Fiscus, R.R., Speck, D.F., 1992. Endogenous nitric oxide required for an integrative respiratory function in the cat brain. *J. Neurophysiol.* 68, 1910–1912.
- Lopez, J.M., Gonzalez, A., 2002. Ontogeny of NADPH diaphorase/nitric oxide synthase reactivity in the brain of *Xenopus laevis*. *J. Comp. Neurol.* 445, 59–77.
- McCrimmon, D.R., Mitchell, G.S., Dekin, M., 1995. Glutamate, GABA, and serotonin in ventilatory control. In: Dempsey, J.A., Pack, A.I. (Eds.), *Regulation of Breathing*, 2nd ed. vol. 79. Dekker, New York, pp. 151–218.
- McLean, D.L., Sillar, K.T., 2000. The distribution of NADPH-diaphorase labeled interneurons and the role of nitric oxide in the swimming system of *Xenopus laevis* larvae. *J. Exp. Biol.* 203, 705–713.
- McLean, D.L., Sillar, K.T., 2001. Spatiotemporal pattern of nicotinamide adenine dinucleotide phosphate–diaphorase reactivity in the developing central nervous system of premetamorphic *Xenopus laevis* tadpoles. *J. Comp. Neurol.* 437, 350–362.
- McLean, D.L., Sillar, K.T., 2002. Nitric oxide selectively tunes inhibitory synapses during vertebrate locomotion. *J. Neurosci.* 22, 4175–4184.
- McLean, D.L., Sillar, K.T., 2004. Metamodulation of a spinal locomotor network by nitric oxide. *J. Neurosci.* 24, 9561–9571.
- McLean, H.A., Perry, S.F., Remmers, J.E., 1995. Two regions in the isolated brainstem of the frog that modulate respiratory-related activity. *J. Comp. Physiol. A* 177, 135–144.
- Milsom, W.K., 1991. Intermittent breathing in vertebrates. *Annu. Rev. Physiol.* 53, 87–105.
- Milsom, W.K., Chatburn, J., Zimmer, M.B., 2004. Pontine influences on respiratory control in ectothermic and heterothermic vertebrates. *Respir. Physiol. Neurobiol.* 143, 263–280.
- Munoz, M., Munoz, A., Marin, O., Alonso, J.R., Arevalo, R., Porteros, A., Gonzales, A., 1996. Topographical distribution of NADPH-diaphorase activity in the central nervous system of the frog, *Rana perezi*. *J. Comp. Neurol.* 367, 54–69.
- Pape, H.C., Mager, R., 1992. Nitric oxide controls oscillatory activity in thalamocortical neurons. *Neuron* 9, 441–448.
- Peunova, N., Scheinker, V., Cline, H., Enikolopov, G., 2001. Nitric oxide is an essential negative regulator of cell proliferation in *Xenopus* brain. *J. Neurosci.* 21, 8809–8818.
- Pierrefiche, O., Maniak, F., Lamicol, N., 2002. Rhythmic activity from transverse brainstem slice of neonatal rat is modulated by nitric oxide. *Neuropharmacology* 43, 85–94.
- Sakakibara, Y., 1984. The pattern of respiratory nerve activity in the bullfrog. *Jpn. J. Physiol.* 34, 269–282.
- Scholz, N.L., de Vente, J., Truman, J.W., Graubard, K., 2001. Neural network partitioning by NO and cGMP. *J. Neurosci.* 21, 1610–1618.
- Sillar, K.T., McLean, D.L., Fischer, H., Merrywest, S.D., 2002. Fast inhibitory synapses: targets for neuromodulation and development of vertebrate motor behaviour. *Brains Res. Rev.* 40, 130–140.
- Smith, K.K., 1994. Are neuromotor systems conserved in evolution? *Brain Behav. Evol.* 43, 293–305.
- Straus, C., Wilson, R.J.A., Tezenas du Montcel, S., Remmers, J.E., 2000. Baclofen eliminates cluster lung breathing of the tadpole brainstem, in vitro. *Neurosci. Lett.* 292, 13–16.
- Taylor, A.C., Kollros, J.J., 1946. Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* 94, 7–24.
- Tierney, A.J., 1996. Evolutionary implications of neural circuit structure and function. *Behav. Processes* 35, 171–182.
- Torgerson, C.S., Gdovin, M.J., Remmers, J.E., 1998. Fictive gill and lung ventilation in the pre- and postmetamorphic tadpole brain stem. *J. Neurophysiol.* 80, 2015–2022.
- Torgerson, C.S., Gdovin, M.J., Remmers, J.E., 2001. Sites of respiratory rhythmogenesis during development in the tadpole. *Am. J. Physiol.* 280, R913–R920.

- Wilson, R.J.A., Vasilakos, K., Harris, M.B., Straus, C., Remmers, J.E., 2002. Evidence that ventilatory rhythmogenesis in the frog involves two distinct neuronal oscillators. *J. Physiol.* 540, 557–570.
- Winmill, R.E., Hedrick, M.S., 2003a. Developmental changes in the modulation of respiratory rhythm generation by extracellular K^+ in the isolated bullfrog brainstem. *J. Neurobiol.* 55, 278–287.
- Winmill, R.E., Hedrick, M.S., 2003b. Gap junction blockade with carboxolone differentially affects fictive breathing in larval and adult bullfrogs. *Respir. Physiol. Neurobiol.* 138, 239–251.
- Winmill, R.E., Chen, A.K., Hedrick, M.S., 2005. Development of the respiratory response to hypoxia in the isolated brainstem of the bullfrog, *Rana catesbeiana*. *J. Exp. Biol.* 208, 213–222.
- Zar, J.H., 1974. *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, NJ.