

IS THE VERTEBRATE RESPIRATORY CENTRAL PATTERN GENERATOR CONSERVED?

Insights from in vitro and in vivo amphibian models

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1. INTRODUCTION

There have been considerable advances in our understanding of the anatomical substrates, neural connections and modulation of the mammalian respiratory central pattern generator (CPG). Despite these advances, however, relatively little is known about the regulation of CPGs for breathing in other vertebrates. It has been proposed that CPGs supporting a variety of motor behaviors such as breathing, locomotion, scratching and chewing, have been conserved in the course of vertebrate evolution.^{14, 15} At present, there is little experimental support for this hypothesis.

At first glance it would appear that breathing in 'lower' vertebrates, such as amphibians, and mammals have little in common save for the use of lungs for gas exchange. Amphibians use a positive-pressure, buccal force pump to inflate the lungs in contrast to the negative-pressure, aspiratory lung inflation mechanism present in mammals and other amniotes (birds and reptiles). In amphibians, the primary respiratory muscles responsible for generating airflow during breathing are innervated by cranial nerves, whereas in mammals the cranial nerves innervate muscles associated primarily with control of the upper airway. During the course of evolution the diaphragm, intercostals and abdominal muscles, which are innervated by spinal nerves, have assumed the role of the 'power plant' for generating airflow during breathing.

Despite these obvious differences in the mechanical aspects breathing, it is generally agreed that the peripheral structures associated with breathing are far less constrained by evolutionary change than the neural structures that support and control the peripheral elements.¹⁵ In other words, CPGs, in general, appear to be much more conserved than the structures with which they control. Direct evidence for conserved features of the respiratory CPG is sparse in vertebrates, but there are numerous examples of invertebrate

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CPGs that support the hypothesis of conserved neural substrates that control specific motor behaviors.⁶

In this paper, we outline some experiments using *in vitro* and *in vivo* preparations from amphibians that are aimed at testing the hypothesis of a conserved respiratory CPG in vertebrates. In the absence of any complete identification of the neurons that comprise the respiratory CPG for any vertebrate, it is difficult to test directly any hypotheses about the evolution of the respiratory CPG. Nevertheless, a comparative approach, like the one taken here, can provide some important insights into the behavior and possible changes in the respiratory CPG that may have occurred over the course of vertebrate evolution. Although similarity alone is insufficient for adequately testing this hypothesis¹⁴, this is a necessary first step in addressing this challenging question.

2. METHODS

2.1. In Vitro Studies

Whole brainstem preparations from larval (tadpole) and adult North American bullfrogs (*Rana catesbeiana*) are used for *in vitro* studies. General methods for surgical preparation and neural recording for tadpoles and adults have been described in detail elsewhere.^{3, 5} Briefly, tadpoles and adults are anesthetized in MS-222 until breathing movements cease. The brainstem is removed and placed in a recording chamber and continuously perfused with an artificial cerebrospinal fluid (aCSF) at 22 °C. Standard extracellular nerve recording from whole cranial nerve rootlets is made with glass suction electrodes. In tadpoles, neural activity from cranial nerves (CN) V (trigeminal), VII (facial), X (vagus) and spinal nerve II (SNII = hypoglossal) are recorded and used as indices of fictive gill and lung ventilation.³ In adults, neural activity from CN V, X and XII are used as indices of fictive lung ventilation.⁵

2.2. In Vivo Studies

Decerebrate, paralyzed and artificially-ventilated adult cane toads (*Bufo marinus*) are used for *in vivo* studies.¹⁶ Animals are anesthetized in MS-222 anesthesia until breathing movements cease. The animals are decerebrated, and both lungs are cannulated with PE-260 tubing for artificial ventilation. The animals are paralyzed with rocuronium (1 mg/kg) injected into the lymph space. A humidified gas mixture of 2.5% CO₂/20% O₂ is introduced unidirectionally through the lungs at a rate of 50-100 ml/min. Cranial motor nerves innervating the buccal pump muscles are placed on hook electrodes for whole nerve recording. In adult amphibians, CN V and the main branch of CN XII (XII_m) innervate the primary buccal elevator muscles whereas the sternohyoid branch of CN XII (XII_{sh}) innervates the primary buccal depressor muscle, and each of these branches are used as indices of fictive ventilation. Neural activity is recorded extracellularly by conventional methods.

2.3. Experimental Protocols

With the *in vitro* brainstem preparation from tadpole and adult bullfrogs, one hypothesis we wished to test was whether there is a developmental ‘switch’ in the mechanism used to generate respiratory rhythm. In mammals, there is evidence that respiratory rhythm is generated by a pacemaker-like mechanism in the neonate whereas in adults conventional synaptic inhibition is the primary mechanism for respiratory rhythmogenesis.¹² If the respiratory CPG is conserved, we would expect similar results during ontogenetic development in the amphibian larval-adult transition as with the mammalian neonate-adult transition. Our approach was to perfuse the tadpole and adult brainstem preparations with a Cl-free aCSF to determine if either preparation was dependent on conventional synaptic inhibition using Cl ions.

A second experimental approach using the *in vitro* preparation was to perfuse the brainstem with increasing concentrations of glycinergic and GABAergic agonists and antagonists. Our working hypothesis is that if the respiratory CPG is conserved, we should observe similar effects of GABA/glycine, and the antagonists, bicuculline and strychnine, on respiratory motor output during development that have been noted in developing mammals.⁹

Experiments with decerebrate toads involve the use of phase-resetting protocols that have been described in detail for mammals.^{7, 10} The recurrent laryngeal nerve (RLN) is stimulated in brief pulses to perturb the respiratory rhythm, and the aftereffects are recorded. Amphibians characteristically breathe in an episodic manner with periods of apnea interspersed with episodes of lung ventilation that contain from 3-10 lung breaths. The RLN is stimulated during a breathing episode and at various times during the respiratory cycle as defined by the phase of the XIIsh burst. As defined in mammalian studies¹⁷, old phase is the time of the onset of the XIIsh burst to the onset of the RLN stimulus. Cophase is the time from the offset of the stimulus to onset of the next breath. Type 0 (strong) resetting is defined as cophase having a net change of zero as old phase is varied through one complete cycle. Type 1 (weak) resetting occurs when cophase has a net change of one cycle per cycle of old phase. A phase singularity is defined as a dysrhythmic response to a discrete stimulus. Our hypothesis is that if the vertebrate respiratory CPG is conserved, we should observe similar responses to perturbation of the CPG by a brief stimulus that resets the respiratory rhythm.

3. RESULTS

Brainstem preparations from tadpoles (N=7) and adults (N=7) responded very differently to Cl-free aCSF (Fig. 1). In tadpoles, fictive lung ventilation frequency was not affected by Cl-free aCSF. By contrast, fictive lung ventilation in adult preparations was either abolished or significantly reduced by Cl-free aCSF. Addition of strychnine (2.5-25 μ M) in tadpole brainstem preparations caused a significant increase in fictive breathing frequency, whereas in adult preparations fictive breathing frequency was significantly reduced (Fig. 2). Addition of GABA or glycine caused significant reductions in fictive breathing frequency, however, adult preparations were inhibited at approximately ten-fold lower concentrations of the agonists (data not shown). We interpret this as an indication of a greater sensitivity of adults to receptor agonists compared with tadpoles.

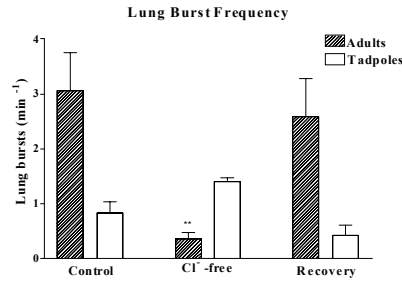


Figure 1. Effects of Cl-free aCSF on fictive breathing in tadpole and adult brainstems *in*

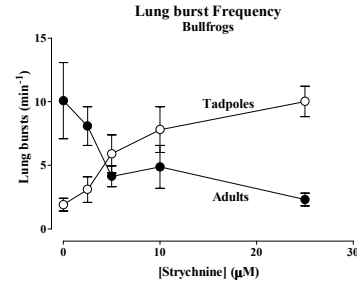


Figure 2. Effects of strychnine on fictive breathing in tadpole and adult brainstems

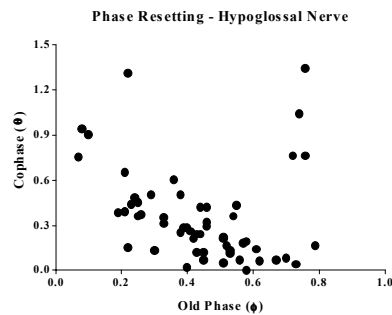


Figure 3. Effects of RLN stimulation showing Type 1 (weak) resetting with a discontinuity at

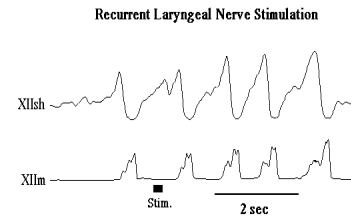


Figure 4. Effects of RLN stimulation on XIIsh and XIIIm. Note phase advance in XIIsh and

Both Type 1 and Type 0 phase resetting responses have been observed with RLN stimulation during episodic lung bursts. The effects of RLN stimulation on phase resetting for one toad is shown in Fig. 3. Type 1 (weak) resetting occurs over most of the respiratory cycle with a discontinuity evident at a cophase of approximately 0.8. This indicates that both phase advances (Fig. 4) and phase delays occur at about 80% of the duty cycle of the XIIsh burst and occur with unpredictable results.

4. DISCUSSION

The results presented here suggest that the lung CPG of tadpoles uses a pacemaker-like mechanism, similar to that hypothesized for neonate mammals.² In tadpoles and neonate mammals, fictive lung ventilation persists in the absence of extracellular Cl ions.

Because conventional synaptic inhibition requires Cl ions, this is evidence for a pacemaker-driven respiratory CPG. Adult amphibians (Fig. 1) and mammals⁴ appear to use conventional inhibitory synaptic inhibition because lung ventilation is abolished with Cl-free aCSF. Thus, there appears to be a developmental 'switch' in the mechanism used to generate respiratory rhythm. If the respiratory CPG has indeed been conserved, it is most parsimonious to hypothesize that common developmental mechanisms should be preserved during the course of evolution. Moreover, results from the adult lamprey, a primitive vertebrate, indicate that the respiratory CPG relies on a pacemaker-like mechanism.¹³ Thus, ontogeny of the respiratory CPG may reflect the phylogeny of the respiratory CPG.

There are also similarities in the results from inhibitory amino acids in developing amphibians and mammals. If there is a developmental 'switch' from a pacemaker to a network-driven CPG, increased receptor density and sensitivity to GABA and/or glycine receptor agonists and antagonists should be a component of this process. The tadpole to adult transition in amphibians is accompanied by increasing sensitivity to GABA and glycine¹ and differential responses to the glycinergic antagonist strychnine (Fig. 2). These results are similar to those found for developing mammals⁹ suggesting that common inhibitory mechanisms are found in disparate groups of vertebrates.

Phase-resetting experiments have proven useful for examining the behavior of oscillatory processes, such as breathing, to brief perturbations of the rhythmic activity.¹⁷ These experiments are useful because the oscillator may be treated as a 'black box' without explicit knowledge of the structure of the oscillator. In mammals, phase-resetting experiments predict that precisely-timed stimuli can result in dysrhythmic breathing.¹¹ In toads, brief perturbations of the respiratory oscillator by RLN stimulation cause phase advances, phase delays and discontinuities in respiratory rhythm (Fig. 3,4). These preliminary results suggest that perturbations of the amphibian respiratory oscillator produce similar effects as in mammals, which argues for conservation of the respiratory CPG. Our long-term goal is to model the breathing patterns of amphibians to examine whether episodic breathing in amphibians has features in common with pathologic breathing disorders in mammals.

Direct evidence for evolutionary conservation of CPGs is generally lacking in vertebrates, but there is substantial support for this hypothesis in invertebrates where 'simple' CPGs have been described in detail. The primary reason that CPGs are highly conserved is that neural networks tend to be 'generalists' rather than 'specialists'.⁶ Individual CPGs are capable of producing a variety of motor outputs that depend on neuromodulatory inputs or sensory feedback. Thus, novel CPGs need not evolve to produce new motor behaviors. A recent example of this was found for the mammalian respiratory CPG with a medullary rhythmic 'slice' containing the pre-Botzinger complex that produced a variety of motor outputs including eupnea, sighs and gasps.⁸

So, what, if anything, has changed with respect to the vertebrate respiratory CPG? It could be hypothesized that the evolution of the vertebrate respiratory CPG is associated with increased 'complexity' which may involve increased modulatory inputs, greater flexibility in the respiratory neural circuitry and more 'degrees of freedom' for respiratory motor outputs. Perhaps a greater flexibility of the respiratory CPG in mammals was associated with the evolutionary change in spinal, rather than cranial, motoneuronal control of the primary respiratory pump muscles.

5. ACKNOWLEDGMENTS

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