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Evolution of lung breathing from a lungless primitive vertebrate

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ABSTRACT

Air breathing was critical to the terrestrial radiation and evolution of tetrapods and arose in fish. The vertebrate lung originated from a progenitor structure present in primitive boney fish. The origin of the neural substrates, which are sensitive to metabolically produced CO_2 and which rhythmically activate respiratory muscles to match lung ventilation to metabolic demand, is enigmatic. We have found that a distinct periodic centrally generated rhythm, described as "cough" and occurring in lamprey in vivo and in vitro, is modulated by central sensitivity to CO_2. This suggests that elements critical for the evolution of breathing in tetrapods, were present in the most basal vertebrate ancestors prior to the evolution of the lung. We propose that the evolution of breathing in all vertebrates occurred through exaptations derived from these critical basal elements.

1. Introduction

Air breathing in tetrapods is achieved via lungs, which likely arose from gas-filled bladders functioning for gas exchange and/or buoyancy control in primitive air-breathing fish prior to the radiation of ray-finned (Actinopterygi) and lobe-finned (Sarcopterygi) fishes (Perry et al., 2001; Remmers et al., 2001; Wilson et al., 2000, 2009). Air breathing in tetrapods, however, requires more than just a lung. Also required are a "breath", output from a brainstem central rhythm generator (CRG) activating respiratory muscles to ventilate the lung, and populations of brainstem chemoreceptors sensitive to CO_2/pH that modulate CRG activity. Together with the lung this system produces breathing matched to metabolic demand. The CO_2/pH-modulated air-breathing CRG (CRG_{AB}) is anatomically and functionally distinct from the CRG producing gill ventilation, which is not modulated by CO_2/pH (Milsom, 2010; Wilson et al., 2002). The origin of the requisite CO_2/pH-modulated CRG_{AB} is unknown, but may have preceded the evolution of the lung (Perry et al., 2001).

Lamprey are cartilaginous and jawless fish reminiscent of the basal vertebrate lineage. The larval (ammocoete) stage is considered among the most "primitive" living vertebrates, resembling non-vertebrate chordates. Ammocoetes are microphagous suspension feeders that form burrows in soft sediment. Water flow is generated by continuous rhythmic ventilation of the pharyngeal pouch, which acquires nutrients but also satisfies metabolic gas exchange requirements. O_2 diffuses from water across the surface areas of exchange epithelia including the pharynx and gills (Hsia et al., 2013; Mallatt, 1981; Rovainen, 1996), and metabolically produced CO_2 easily diffuses across all body surfaces and dissipates into surrounding water. Brainstems isolated from lamprey exhibit rhythmic discharge on cranial nerves that innervate ventilatory muscles. This discharge results from a central rhythm generator for pharyngeal ventilation (CRG_P) (Cinelli et al., 2013; Gariépy et al., 2012; Martel et al., 2007; Rovainen, 1996). An additional pattern of periodic activity occurs, characterized as a "slow rhythm" or "cough" (Martel et al., 2007; Rovainen, 1996, 1977). These patterns of putative pharyngeal ventilation and "cough" result from anatomically distinct CRGs and resemble patterns of activity present in isolated brainstems of larval amphibians, which represent the products of distinct CRGs for gill ventilation and a functionally and anatomically distinct tetrapod CRG for air breathing (CRG_{AB}) (Martel et al., 2007; Missaghi et al., 2013; Wilson et al., 2002). In amphibians and all higher amniotes, the CRG_{AB} is responsive to CO_2/pH-sensitive central chemoreceptors that modulate ventilation to meet metabolic demand. The origin of the vertebrate CRG_{AB} is unknown and the presence of central CO_2/pH-sensitive chemoreceptors prior to the amphibians is controversial (Milsom,

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2010; Wilson et al., 2000, 2009). We propose that the lamprey "slow rhythm" or "cough" CRG is the progenitor to the tetrapod CRG_{AB} and test the hypothesis that this progenitor will be modulated by CO_2/pH.

We predict the existence, in the basal vertebrate, of a CO_2/pH-modulated CRG distinct from that producing gill ventilation. Subsequent evolution of a lung provided the substrates that, through exaptation (Gould and Vrba, 1982) or functional retasking, resulted in the complex combination of lung, chemoreceptor and CO_2/pH-modulated CRG_{AB}. This new system was then capable of producing and regulating air breathing to meet metabolic demand for ventilation critical for subsequent evolution of amphibians, the transition to terrestrial habitats and further evolution of reptiles, birds, and mammals. Here we show that a CO_2/pH-modulated CRG, distinct from that producing gill ventilation occurs in lamprey, a jawless, lungless and exclusively water-breathing "primitive" fish representative of the basal ancestor common to all vertebrates. We propose this CO_2/pH-modulated CRG represents a critical progenitor characteristic, present in basal vertebrates epochs prior to evolution of the lung.

2. Methods

2.1. Animals

Animal use was done in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee. Animal collection was approved by the State of Alaska Department of Fish and Game. Larval (ammocoete, 7–15 cm, 1–5 g) lamprey (*Lampetra camtschatica* or *L. alaskense*; Tilesius) were collected through sediment sifting or electroshock, from natural populations occurring in shallow, slow-moving fresh-water streams in interior Alaska. Captive animals were housed at 12 °C in 20-L aquaria containing 3.125 g salt (Instant Ocean) per L deionized water. Ammocoetes were fed dry yeast three times weekly. Filters were used to maintain water quality and remove excess food. Aquaria were continuously aerated.

2.2. Isolated-brainstem preparation

Procedures to isolate the brainstem and spinal cord en bloc from ammocoetes, and record from whole nerves associated with ventilation, were slightly modified from those we use to isolate and record from similar tissues derived from larval amphibians (Davies et al., 2009; Taylor and Brundage, 2013; Taylor et al., 2003). Ammocoetes were anesthetized using tricaine methanesulfonate (MS222, Sigma-Aldrich; 0.3 g/L in deionized water buffered with 2.4 g/L $NaHCO_3$) until unresponsive to a tail pinch. Anesthetized animals were transected caudal to the branchial pores and the ventral half of the head was removed so the cranium rested on a dissection tray. Subsequent dissection occurred with tissues constantly irrigated with an artificial cerebrospinal fluid (aCSF) equilibrated with 1% CO_2, balance O_2 (Davies et al., 2009; Martel et al., 2007). The aCSF comprised (in mM) NaCl (130), KCl (2.1), $CaCl_2$ (2.6), $MgCl_2$ (1.8), HEPES (4), D-glucose (4), and $NaHCO_3$ (1) buffered to pH 7.4 with NaOH. The dorsal cranium was removed to expose the brain and spinal cord. Each brain was transected at the optic lobes, the spinal cord was cut approximately 5 mm caudal to obex, and meninges and choroid plexus were removed from regions of the 3rd and 4th ventricles. Remaining cranial and spinal nerves were cut and the decerebrated brainstem was removed en bloc and transferred to an acrylic superfusion recording chamber supplied with aCSF equilibrated with 1.5% CO_2 balance O_2. Cranial nerves V and X (CN V/X) were drawn into glass suction electrodes. Whole-nerve signals were amplified (bipolar recording of whole nerve relative to recording chamber) and filtered (first stage 100x, 10 Hz low, 1 kHz high, DAM 50, World Precision Instruments (Sarasota, FL, USA); 2nd stage 1000x, 100 Hz low, 1 kHz high, Model 1700 DAC Amplifier, A-M Systems, Carlsborg, WA, USA), and recorded using a computer analogue-to-digital data acquisition system (Powerlab, AD Instruments, Colorado Springs, CO, USA). After dissection, tissues were allowed at least 40 min to recover. During the experiment, cranial nerve discharge was recorded for 30 min during superfusion with normocapnic aCSF (equilibrated with 1.5% CO_2, balance O_2), followed by 10 min hypercapnia (5% CO_2, balance O_2), and at least 30 min of subsequent normocapnia. All experiments were conducted at constant temperatures approximating animal chamber temperature 10–15 °C.

2.3. Control experiments

Each experiment was concluded with tissues exposed to normocapnia subsequent to hypercapnia. Hypercapnic "cough" frequency was reduced during subsequent normocapnia, and was no difference from that during initial normocapnia. Separate trials were conducted during which brainstems were maintained under constant normocapnia. In no case was "cough" frequency observed to increase with time independent of hypercapnic exposure.

2.4. Data analysis

Periods for analysis (10 min), for each experiment, were extracted from original data records representing the final period of initial normocapnia, the period of hypercapnia and normocapnia subsequent to hypercapnia. Neurograms were scored for rhythmic patterns of burst activity previously characterized as fictive ventilation of the pharynx (a "fast rhythm") and a distinct periodic burst pattern characterized as a "slow rhythm" or "cough" (Martel et al., 2007; Rovainen, 1996, 1977). The total number of "coughs" occurring during each period was determined and treatment means were statistically assessed.

2.5. Statistical analysis

Data were analyzed using repeated measures analysis of variance (RM-ANOVA) comparing "cough" frequency in each preparation during normocapnia and hypercapnia. Where significance was found, RM-ANOVAs were followed by post-hoc multiple comparison using the Holm–Sidak method.

2.6. Data exclusion

The described dataset is derived from 5 in vitro preparations treated with identical protocols. Additional individual replicates had been done but were excluded. One replicate was excluded as it was inadvertently exposed to 2% CO_2 during "normocapnia" prior to the 5% hypercapnia (rather than 1.5%); three replicates were excluded because tissues received a different treatment followed by a prolonged recovery prior to the hypercapnic exposure trial; one replicate was excluded because experiments were conducted in a different recording apparatus using recirculated solutions (Wilson et al., 1999). None of these differences would be expected to confound the experiment and, were these trials included, we would still find a statistically significant increase in "cough" frequency with hypercapnia ($N = 10$; $P = 0.007$; NC vs. HC $P = 0.011$, $t = 3.371$; HC vs. RNC, $P = 0.018$, $t = 2.943$, *1-way RMANOVA*). Fictive pharyngeal ventilation was relatively regular and appeared consistent within each preparation. However, the amplitude was variable and at times individual bursts were difficult to distinguish