

Development of respiratory rhythm generation in ectothermic vertebrates

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Abstract

Compared with birds and mammals, very little is known about the development and regulation of respiratory rhythm generation in ectothermic vertebrates. The development and regulation of respiratory rhythm generation in ectothermic vertebrates (fish, amphibians and reptiles) should provide insight into the evolution of these mechanisms. One useful model for examining the development of respiratory rhythm generation in ectothermic vertebrates has emerged from studies with the North American bullfrog (*Rana catesbeiana*). A major advantage of bullfrogs as a comparative model for respiratory rhythm generation is that respiratory output may be measured at all stages of development, both in vivo and in vitro. An emerging view of recent studies in developing bullfrogs is that many of the mechanisms of respiratory rhythm generation are very similar to those seen in birds and mammals. The overall conclusion from these studies is that respiratory rhythm generation during development may be highly conserved during evolution. The development of respiratory rhythm generation in mammals may, therefore, reflect the antecedent mechanisms seen in ectothermic vertebrates. The main focus of this brief review is to discuss recent data on the development of respiratory rhythm generation in ectothermic vertebrates, with particular emphasis on the North American bullfrog (*R. catesbeiana*) as a model.

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1. Introduction

It is well-established that respiratory rhythm in vertebrates is generated by neuronal circuits within the central nervous system that do not require sensory feedback. Although much progress has been made

in identifying important anatomical locations, cellular and synaptic mechanisms devoted to rhythm generation, many questions remain unanswered. Most of our knowledge regarding the development of respiratory rhythm generation has come from studies with mice, rats and chicks, but there has been some debate about which is the most appropriate model for studying rhythm generation (see Richter and Spyer, 2001, for review).

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Within the past 10–15 years, there has been a substantial increase in the number of in vitro ‘comparative’ models for investigating respiratory rhythm generation. These models include ectothermic species such as lamprey (Rovainen, 1983), bullfrogs (McLean et al., 1995a,b) and turtles (Johnson et al., 1998). Among these models, only the North American bullfrog (*Rana catesbeiana*) has provided any comprehensive data on the development of respiratory rhythm generation (Belzile et al., 2002; Kinkead et al., 2002; Winmill and Hedrick, 2003a,b). The bullfrog brainstem in vitro has proven to be an excellent model for studying the development of respiratory rhythm generation, primarily because of the ease of recording respiratory-related neural activity at all stages of development, thus providing direct developmental comparisons that are not possible with other vertebrate models. An emerging consensus from studies with comparative models, and particularly the bullfrog brainstem model, is that many underlying mechanisms for motor behaviors, including respiratory rhythm generation, are phylogenetically conserved (Tierney, 1996; Hedrick et al., 2001). The main purpose of this review is to highlight data from a number of ‘comparative’ models, with particular emphasis on recent advances that have been made in identifying mechanisms in the development of respiratory rhythm generation in *R. catesbeiana*.

2. Anuran amphibian development

The ontogenetic transition from an aquatic to a terrestrial habitat in amphibians is accompanied by major maturational changes of the respiratory system, including a shift from gill to lung ventilation (Burggren and Doyle, 1986), morphological changes associated with aquatic to aerial respiration (Torgerson et al., 1998), the development of central CO_2/H^+ receptors (Taylor et al., 2003) and a decreased tolerance to severe hypoxia (Stewart et al., 2004). Despite the profound physiological and morphological changes associated with the transition from aquatic to aerial ventilation, very little is known concerning the developmental neurobiology associated with the central regulation of breathing in amphibians and other ectothermic vertebrates.

A number of morphological changes occur during development in anuran (frogs and toads) amphibians, and these changes have been used to construct schemes

used to ‘stage’ animals to a particular time in development (e.g. Taylor and Köllros (T–K), 1946). This is a more useful definition for comparing developing bullfrogs since the larval (tadpole) period in bullfrogs is quite long (1–3 years) and animals of similar stages are not necessarily the same age. Post-embryonic bullfrogs are defined as those that are capable of independent feeding (Taylor and Köllros, 1946). The morphological terminology used by Taylor and Köllros (1946) is as follows: pre-metamorphosis (T–K stages I–XI), emergence of hind-limb buds and growth of trunk and tail; pro-metamorphosis (T–K stages XI–XX), rapid growth of limbs; metamorphosis (T–K stages XX–XXV), eruption of front limbs to completion of metamorphic climax. With respect to ventilation, tadpoles use gills to extract oxygen from the water from T–K stages I to about XX. During metamorphosis (stages XXI–XXV), the gills are resorbed and obligate air-breathing is assumed; the gape is increased and the tongue is innervated by the hypoglossal nerve (Matesz et al., 1999) to accommodate the prey-catching function typical of adult anurans. Overall, development in the bullfrog is a remarkable morphological transformation that supports the transition from an entirely aquatic lifestyle to a semi-aquatic, air-breathing adult.

The mechanisms for breathing in amphibians change dramatically during development. During early tadpole stages, oxygen is acquired in a trimodal fashion with involvement of gills, skin and lungs (Burggren and Doyle, 1986). 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 metamorphic tadpole (Crowder et al., 1998). More recently, however, it has been shown that bullfrog tadpoles use lung ventilation throughout development, with early stages breathing more frequently than later stages (Crowder et al., 1998). During the early stages of development lung ventilation is not required for oxygen acquisition, suggesting that lung ventilation serves to promote lung development (Crowder et al., 1998). Another possibility, not yet tested experimentally, is that continued activation of central neural circuits for lung ventilation may strengthen relevant synaptic connections during development. Intermittent hypoxia in developing tadpoles has been shown to increase ventilatory responses to hypercarbia, thus illustrating the

plasticity of central respiratory circuits during bullfrog development (Simard et al., 2003).

In tadpoles, water flow over the gills occurs unidirectionally by activation of oropharyngeal musculature innervated by trigeminal, facial and hypoglossal nerves (Gradwell, 1972). During air-breathing events, airflow through the glottis is regulated by the vagus nerve. Gill ventilation occurs almost continuously and is occasionally interrupted by lung ventilation events. In the decerebrate, spontaneously breathing tadpole and the *in vitro* brainstem from tadpoles, recordings from trigeminal, facial, vagus and hypoglossal nerves exhibit both gill and lung motor patterns that closely resemble the pattern of breathing that occurs in tadpoles *in vivo* (Gdovin et al., 1998).

In late stage metamorphic (T–K stages XXIII–XXV) and adult bullfrogs, ventilation consists of two basic motor behaviors: buccal oscillations and lung ventilations (DeJongh and Gans, 1969). Buccal oscillations are rhythmic elevations and depressions of the buccal cavity that result in tidal airflow between the atmosphere and buccal cavity through the open nares. Although it is assumed that buccal ventilation in adults is the remnant of gill ventilation in tadpoles, this has not been experimentally demonstrated. Lung inflation occurs by positive pressure, with air from the buccal cavity forced into the lungs through an open glottis. Lung ventilation events can occur singly, but often occur in episodes in which the lungs are inflated by two or more breaths in succession. Episodic breathing occurs with *in situ* and *in vitro* preparations from amphibians (Kinkead and Milsom, 1994; Hedrick and Winmill, 2003; Winmill and Hedrick, 2003a,b) and is often more pronounced under hypoxia or hypercapnia in intact animals (Wang et al., 1999) and with *in vitro* brainstem preparations (Winmill et al., 2005). This indicates that the production of episodes does not require higher brain structures or peripheral sensory feedback. The adult turtle brainstem *in vitro* also produces phasic expiratory and inspiratory activity similar to that seen in intact animals (Johnson and Mitchell, 1998). Expiration in amphibians is generally considered to be a passive event, generated by elastic recoil of the lungs and body wall with the glottis open, but muscle activity during the expiratory phase can occur under certain conditions such as vocalization (Girgenrath and Marsh, 1997), indicating that expiration can be an active event under specific conditions. It remains to be demonstrated that

active expiration is involved in normal, quiet breathing in anurans.

Episodic breathing is also produced during development in chicks (Fortin et al., 1994) and mice (Abadie et al., 2000) during the segmentation period when the hindbrain develops distinct rhombomeres. Bursts of motor activity occur simultaneously in hindbrain cranial nerves indicating that in this early stage of development the neuronal respiratory network is organized with distinct reticular and motor neurons (Borday et al., 2003). Activities from trigeminal, facial and glossopharyngeal motor nerves indicate that respiratory rhythm is intersegmentally coordinated through multisynaptic connections (Fortin et al., 1995). However, it has been shown that individual rhombomeres have the ability to produce a respiratory motor output from cranial nerves originating in each segment, indicating that each rhombomere has the capacity to generate a rhythmic motor output (Champagnat and Fortin, 1997). Although the development of rhombomeres is a transient event in birds and mammals, rhombomeric organization is retained throughout development in anuran amphibians thus providing a unique model for mapping physiologically identified respiratory neurons in the adult frog within persistent developmental rhombomeres (Straka et al., 2002).

3. The amphibian brainstem as a model for the development of rhythm generation

There are several advantages to using the *in vitro* amphibian brainstem as a model system for studying the development of central neural processes (see Luksch et al., 1996), including respiratory rhythm generation. First, and perhaps most importantly, the amphibian brainstem *in vitro* provides for direct developmental comparisons since this preparation is capable of producing a quantifiable, spontaneous respiratory motor output at all stages of development (Fig. 1). This feature of the amphibian brainstem is unique among vertebrates and eliminates the problems of interpretation when comparing whole brainstems or slice preparations from animals of different ages that are common to mammalian models (Richter and Spyer, 2001). Second, this preparation produces fictive gill/lung bursts in tadpoles and fictive buccal/lung bursts in adults that have been shown to closely represent *in vivo*

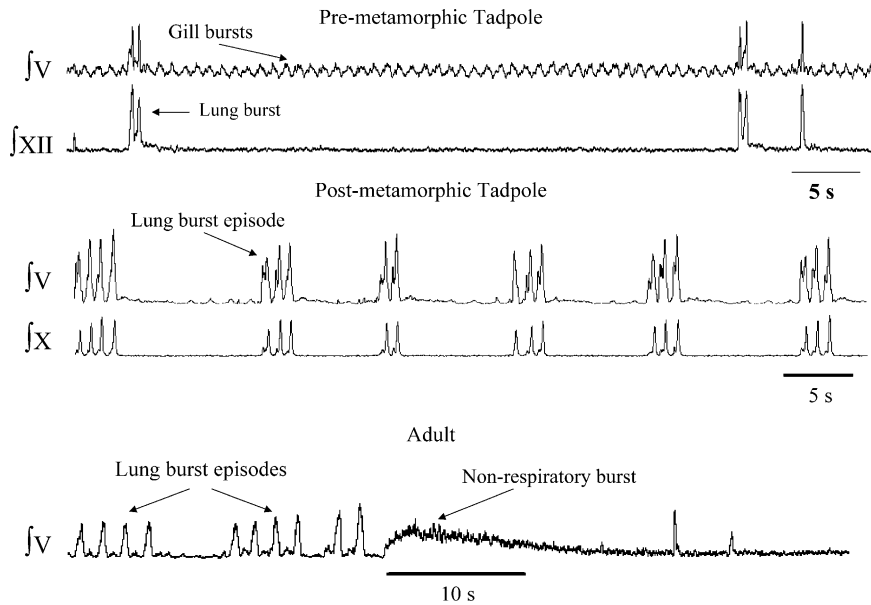


Fig. 1. Integrated cranial nerve (CN) activity from a pre-metamorphic tadpole (CN V and CN XII), a post-metamorphic tadpole (CN V and CN XII) and an adult (CN V) brainstem in vitro. In the pre-metamorphic tadpole brainstem, high frequency, low amplitude gill bursts and low frequency, high amplitude lung bursts are apparent; in the post-metamorphic tadpole brainstem, lung bursts are episodic; in the adult brainstem, episodic lung bursts and a single 'non-respiratory' burst is evident (see Section 5).

ventilatory behaviors (Gdovin et al., 1998; Sakakibara, 1984). Third, breathing frequencies in vitro are closely matched to normal, in vivo breathing frequencies. In mammals, respiratory frequencies with in vitro preparations are at least an order of magnitude lower than in vivo breathing rates. Fourth, the amphibian brainstem preparation can be studied at physiologically relevant temperatures, rather than having to reduce temperature to maintain tissue viability, as is often the case with mammalian brainstems in vitro. Fifth, amphibian brainstems preparations can be studied at physiologically relevant extracellular K^+ ($[K^+]_o$) concentrations and are viable over a broad range of $[K^+]_o$ (Winmill and Hedrick, 2003a). Very often, rhythmically active slices from mammalian preparations are not active until $[K^+]_o$ is raised to higher than normal levels (Rybak et al., 2003); however, in some cases slices are active at normal $[K^+]_o$ depending on the level of synaptic inhibition (Tryba et al., 2003). Finally, lower tissue metabolism of larval amphibian brain tissue allows higher PO_2 values under in vitro conditions (Torgerson et al., 1997). However, this assumption may be invalid since we have found that the adult bullfrog brainstem has an

anoxic core even when superfused with 98% O_2 (see below).

4. Organization of the amphibian central respiratory network

Two key questions regarding respiratory rhythm generation in vertebrates are: (1) where are the relevant respiratory rhythm generating sites in the medulla? (2) What are the mechanisms that produce rhythm generation? In mammals, there is considerable evidence to suggest that the pre-Bötzinger Complex (pBC) is a critical region for respiratory rhythm generation; data from in vitro and in vivo models support this hypothesis (Smith et al., 1991; Ramirez et al., 1998; Gray et al., 2001; Wenninger et al., 2004). Recent data also suggest that the pBC and the more rostral para-facial respiratory group (pFRG; Onimaru and Homma, 2003) represent synaptically coupled networks that interact to produce a rhythmic respiratory motor output (Janczewski et al., 2002). This dual network hypothesis for respiratory rhythm generation is reminiscent of – and perhaps

homologous to – the dual lung and gill/buccal oscillators of the amphibian brainstem (Wilson et al., 2002), but this remains unresolved.

4.1. Localization of the amphibian respiratory rhythm generator during development

The precise locations of brainstem regions directly involved with respiratory rhythm generation in amphibians and other ectotherms are largely unknown; even less is known about the organization of respiratory brainstem circuits of ectotherms during development. However, recent experiments have made some progress in localizing the rhythm generating areas in tadpole and adult brainstems. Despite this, there has been no discovery of a ‘*noed vital*’ (cf. Rekling and Feldman, 1998) in amphibians or other ectotherms, equivalent to the pBC in mammals.

Utilizing a variety of techniques including brainstem transection, microinjection of neurotransmitters and intracellular/extracellular recording of respiratory-related neurons, there appear to be at least two broadly circumscribed regions responsible for respiratory rhythm generation (Kogo and Remmers, 1994; McLean et al., 1995a; McLean and Remmers, 1997; Torgerson et al., 2001; Wilson et al., 2002). Based upon small-volume microinjections of glutamate and the non-NMDA receptor agonist, AMPA, and the inhibitory neurotransmitter, GABA, in post-metamorphic tadpoles and adult frogs, a rostral medullary region, lying between the acoustic and vagus nerves, appears to be directly involved with generating lung burst activity (McLean et al., 1995a; Wilson et al., 2002). Transection between the rostral lung region and the caudal buccal region indicates that both regions are independently capable of producing respiratory-related bursts, suggesting that there are at least two distinct oscillators responsible for generating respiratory rhythm in the post-metamorphic frog brainstem (Wilson et al., 2002). This may represent the retention of rhombomeric organization in the adult frog and the capability of individual segments to retain the ability to produce independent, but coupled, respiratory rhythms.

The specific regions that generate respiratory rhythm in early, pre-metamorphic tadpoles are less clear. There have been no comparable microinjection studies in pre-metamorphic tadpole preparations to further localize the important brainstem regions

that generate respiratory activity. Transection experiments early stage tadpole brainstems suggest that lung burst activity is located more caudally than in the post-metamorphic or adult brainstem (Torgerson et al., 2001). Owing to the differences in the general location of brainstem regions that generate lung burst activity, Torgerson et al. (2001) have suggested that there is a caudal to rostral ‘translocation’ of the lung burst rhythmogenic region during development. It is possible that neural circuits in specific rhombomeric segments generate or drive the overall brainstem rhythm at different stages of development, which would account for the transection and microinjection studies (Torgerson et al., 2001; Wilson et al., 2002). In support of this, transection of embryonic rhombomeres in the chick hindbrain into discrete segments containing a specific cranial nerve retains the ability to generate rhythmic bursts (Champagnat and Fortin, 1997).

4.2. Respiratory-related neurons in the amphibian brainstem

There are very few studies that have examined the firing properties or burst characteristics of respiratory-related neurons or motoneurons in the amphibian brainstem. Recordings of respiratory modulated neurons have been recorded from facial cranial motoneurons in pre-metamorphic tadpoles (Liao et al., 1996) reveal the majority of neurons were modulated by both gill and lung activity, or by lung activity alone; no solely gill modulated neurons were recorded (Liao et al., 1996). These neurons had variable membrane potentials and exhibited phasic, Cl⁻-mediated synaptic inhibition. In adults, a wider variety of respiratory modulated neurons have been recorded in the rostral and caudal medullary region including lung/non-lung modulated bursting and phase-spanning neurons (Kogo and Remmers, 1994; McLean and Remmers, 1997). Lung modulated neurons did not exhibit any voltage-dependence or endogenous bursting characteristic of pacemaker neurons (McLean and Remmers, 1997), which are present in the pFRG (Onimaru et al., 1995) and the PBC (Smith et al., 1991; Tryba et al., 2003) in the neonatal mammalian medulla. Embryonic respiratory-related neurons of mammals undergo several developmental changes including increased burst duration, increased input resistance, decreased cell capacitance and increased action potential amplitude (Onimaru and

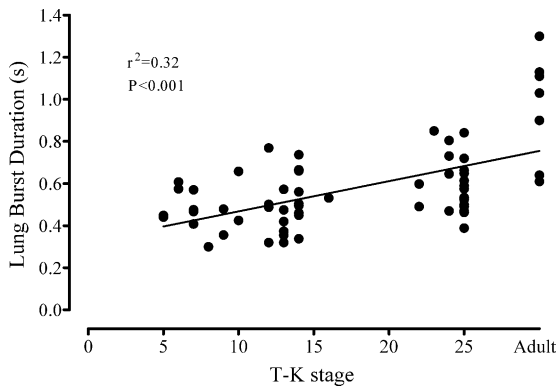


Fig. 2. Lung burst duration (s) measured as cranial nerve motor output, increases significantly during development from T–K stage V to adult (Hedrick, Winmill, Chen and Jessop, unpublished results).

Homma, 2002). We have observed increased burst duration of cranial nerve motor output during development in bullfrogs (Fig. 2) similar to that seen in developing mammals (Onimaru and Homma, 2002; Viemari et al., 2003), although the mechanisms underlying this change in burst properties in amphibians are unknown. In the African clawed frog (*Xenopus laevis*), maturation of spinal nerve firing properties are associated with a down-regulation of delayed rectifier K^+ currents and an increase in Ca^{2+} -dependent K^+ currents (Sun and Dale, 1998).

4.3. Mechanisms of respiratory rhythm generation

How respiratory rhythm is generated in vertebrates remains an elusive yet fundamentally important question. In general, rhythmic motor patterns can be generated by two basic mechanisms that are not mutually exclusive: neural network interactions that require synaptic inhibition and pacemaker neurons that generate an endogenous rhythm independent of synaptic inhibition (Richter and Spyer, 2001). In the context of respiratory rhythm generation, both pacemaker, network and ‘hybrid’ pacemaker–network models have been hypothesized to be responsible for rhythm generation (Richter and Spyer, 2001; Smith et al., 1991). Another view hypothesizes that pacemakers may be embedded within a respiratory network, but not obligatory for rhythm generation (Rekling and Feldman, 1998). In this ‘group pacemaker’ hypothesis, pacemaker neurons contribute to rhythmogenesis by pro-

moting excitability, but are not essential for generating respiratory rhythm (Del Negro et al., 2005). Although no models have been developed to explain respiratory rhythm generation in ectothermic vertebrates, there is indirect support for a maturational hypothesis for the development of respiratory rhythm generation in amphibians (cf. Richter and Spyer, 2001). We hypothesize that pacemaker neurons are important for generating respiratory rhythm early in development, but network interactions (possibly including pacemaker neurons) are more important for rhythm generation following metamorphosis. At present, there is no direct evidence for pacemaker neurons in any ectothermic brainstem.

The lamprey (*Ichthyomyzon unicuspis*) brainstem in vitro exhibits very weak synaptic inhibition and maintains respiratory neural activity when superfused with a Cl^- -free solution (Rovainen, 1983). These experiments suggest that in the lamprey, a primitive jawless vertebrate, respiratory activity may be generated by pacemaker-like activity (Rovainen, 1983). Superfusion of the tadpole brainstem with Cl^- -free artificial cerebrospinal fluid (aCSF) abolishes fictive gill bursts, but not lung bursts (Galante et al., 1996; Broch et al., 2002), suggesting that Cl^- -mediated synaptic inhibition is required for gill burst activity but not lung burst activity. Superfusion with Cl^- -free aCSF abolishes lung bursts in the adult brainstem (Broch et al., 2002). These data suggest that there may be a ‘pacemaker to network’ switch in the mechanism for lung burst activity in the bullfrog brainstem. Synaptic inhibition blockade with bicuculline and strychnine, however, reveal the persistence of lung bursts in both tadpole and adult brainstem preparations (Hedrick and Chen, unpublished results), suggesting that pacemaker activity may also be present in the adult. This is consistent with a recent experiment with the in vitro turtle brainstem, which maintains rhythmic neural activity despite blockade of $GABA_A$ and glycine receptors (Johnson et al., 2002). These recent experiments with adult turtle and adult bullfrog brainstems suggest that pacemaker activity may be present in the mature brainstem, which has yet to be clearly demonstrated in the adult mammalian brainstem.

Gill and lung burst frequencies in the pre-metamorphic tadpole brainstem are significantly dependent upon extracellular K^+ concentration, suggesting that respiratory activity is voltage-dependent (Fig. 3). Because endogenously bursting neurons are

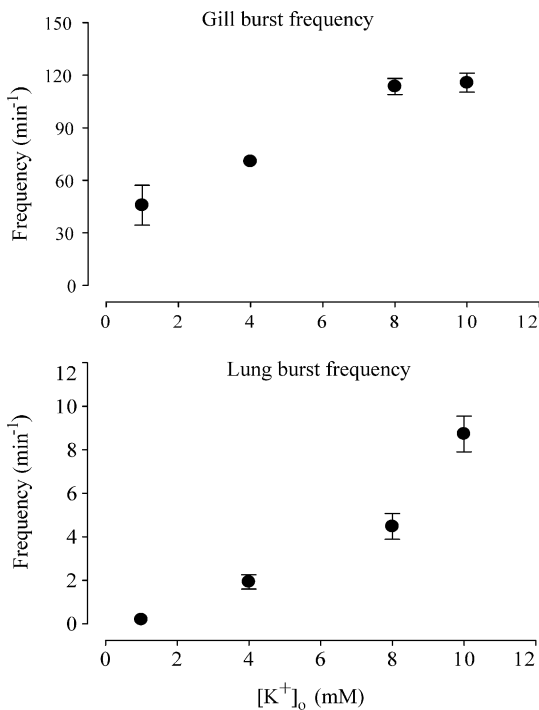


Fig. 3. Gill burst frequency and lung burst frequency are significantly dependent upon extracellular K⁺ ([K⁺]_o) in the pre-metamorphic tadpole brainstem (modified from Winmill and Hedrick, 2003a).

generally voltage-dependent, these data support the hypothesis that both gill and lung burst activity in tadpoles is driven by pacemaker activity. However, previous results (Galante et al., 1996; Broch et al., 2002) suggest that gill ventilation is driven by synaptic inhibition. By contrast, lung bursts in the adult bullfrog brainstem are not dependent upon K⁺ concentration (Winmill and Hedrick, 2003a). The apparent lack of voltage-dependence of lung bursts in the adult does not rule out the possibility of pacemaker activity driving these bursts given that some pacemaker neurons in the PBC of mammals have stable membrane potentials that are insensitive to voltage modulation induced by changes in extracellular K⁺ (Tryba et al., 2003).

A recent study from our laboratory suggests that gap junctions are important for respiratory rhythm generation in the tadpole brainstem, and perhaps for expression of chemoreception in the adult bullfrog brainstem (Winmill and Hedrick, 2003b). Gap junction blockade with carbenoxolone (CBX) inhibited gill and lung burst activity in pre-metamorphic tadpole brainstems, and

reversibly abolished neural activity at higher concentrations of CBX. These results suggest that in larval amphibians, electrical coupling between respiratory-related neurons may be important for respiratory rhythmogenesis, and is consistent with the hypothesis that gap junctions are important for maturation of neural networks (Kandler and Katz, 1995). By contrast, CBX applied to adult bullfrog brainstems initially increased lung burst activity before inhibiting neural activity at high concentrations and, in addition, blocked the facilitation of breathing by hypercapnia (Winmill and Hedrick, 2003b). What is particularly interesting is that the responses of larval and adult amphibian brainstems to CBX are very similar to respiratory inhibitory responses of neonatal medullary slice preparations (Bou-Flores and Berger, 2001) and excitatory responses of arterially perfused adult rats (Solomon et al., 2003) exposed to CBX. Taken together, respiratory responses to CBX in amphibians and mammals strongly suggests a common developmental role for gap junctions in the regulation of respiratory rhythmogenesis, thus providing additional evidence that developmental mechanisms of rhythm generation are conserved.

4.4. Neurotransmitters and neuromodulators involved in respiratory rhythm generation

Several recent studies with amphibians have demonstrated that modulation of respiratory rhythm generation by specific neurotransmitters and/or neuromodulators changes during development. Both excitatory and inhibitory modulation of respiratory rhythm has been demonstrated in the bullfrog brainstem at different stages of development. Inhibitory neurotransmission via GABA_A and glycine receptors plays a major role in shaping respiratory motor activity in developing bullfrogs. GABA or glycine application to the bullfrog brainstem produces different responses depending on stage of development (Galante et al., 1996; Broch et al., 2002). For example, GABAergic and glycinergic inhibition of respiratory activity occurs at a 10-fold lower concentration in adult compared with tadpole brainstem preparations (Broch et al., 2002). In larval amphibians, GABA_A receptor blockade with bicuculline (Galante et al., 1996; Broch et al., 2002), GABA_B receptor blockade with 2-hydroxy-saclofen (Straus et al., 2000a) or glycine receptor blockade with strychnine (Galante et al., 1996; Broch et al., 2002)

increase lung burst activity, suggesting there is a general suppression of lung burst activity by GABAergic and glycinergic mechanisms that is disinhibited during development (Straus et al., 2000a).

Recent studies also indicate that serotonin (5-HT) plays a significant neuromodulatory role in the development of respiratory rhythmogenesis in the amphibian brainstem. For example, 5-HT significantly depresses gill burst activity in both pre- and post-metamorphic brainstems *in vitro*, but only post-metamorphic brainstems exhibit an increase in lung burst activity in response to low (<0.5 μM) 5-HT (Kinkead et al., 2002). Depression of gill burst activity is mediated partially by 5-HT_{1A} receptors, but there appears to be no involvement of 5-HT_{2A/C} receptors in mediating the effects of 5-HT in pre- or post-metamorphic tadpoles (Belzile et al., 2002).

Previous work has established that nitric oxide (NO) is an excitatory neuromodulator of fictive breathing in the adult bullfrog brainstem (Hedrick et al., 1998; Hedrick and Morales, 1999). We have recently re-examined the role of NO on respiratory-related gill and lung activity during development in the bullfrog brainstem (Hedrick et al., *in press*). Application of the general NO synthase (NOS) inhibitor L-nitroarginine (L-NA) to pre- and post-metamorphic bullfrog brainstems produces contrasting results. In the pre-metamorphic brainstem, L-NA increases gill burst frequency and amplitude, and increases lung burst frequency. By contrast, L-NA or 7-nitroindazole (7-NI), a specific inhibitor of neuronal NOS, applied to the post-metamorphic brainstem depresses lung burst frequency and virtually eliminates episodic lung bursts (Harris et al., 2002; Hedrick et al., *in press*). These data indicate that NO acts as an inhibitory modulator of respiratory activity early in development, but becomes an excitatory modulator of frequency and pattern (episodic breathing) late in development. The 'switch' from inhibition to excitation appears to coincide with the loss of gills and emergence of obligate air-breathing in bullfrogs. Although it is unclear how NO initially acts as an inhibitory molecule and later as an excitatory molecule, this changing role of NO is similar to the role of NO in modulating the spinal rhythm generator for swimming in the amphibian spinal cord (McLean and Sillar, 2004). In this model, the inhibitory–excitatory modulatory role of NO appears to be related to the changing interaction of NO with the inhibitory neurotransmitters

GABA and glycine. Thus, the role of NO in modulating respiratory rhythm generation in the developing bullfrog brainstem likely stems from an indirect interaction with inhibitory, and perhaps excitatory (e.g. glutamate), neurotransmitters.

Nitric oxide has been implicated as having a role as a 'synchronizing' modulator of neural networks (Anbar, 1995). 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). One possibility 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). This may be important during the development of the anuran respiratory system during the transition from aquatic to terrestrial environments.

5. Brain metabolism and effects of hypoxia on respiratory rhythm generation

Brain metabolism in endotherms is approximately 10-fold higher than brain tissue from ectotherms, when size is taken into account. The high metabolism of mammalian brain tissue results in whole brainstems being acidotic and hypoxic *in vitro* (Okada et al., 1993), which has necessitated the use of perfused preparations that maintain adequate oxygenation (Wilson et al., 2001) or rhythmic slices that maintain oxygenation through diffusion (Ramirez et al., 1996). Each of these preparations has advantages and disadvantages (Richter and Spyer, 2001). Because of the lower metabolism and generally smaller size of ectothermic brain tissue, it has been assumed that brainstem preparations from ectotherms are well-oxygenated *in vitro*. One study has confirmed that the bullfrog tadpole brainstem is well-oxygenated throughout the tissue when superfused with oxygenated (98% O₂) aCSF (Torgerson et al., 1997). We have confirmed these findings in pre-metamorphic tadpoles (Hedrick, Winmill and Chen, unpublished results), but have found that the adult bullfrog brainstem superfused with 98% O₂ is severely hypoxic at depths ranging from about 300–700 μm (Fig. 4). This indicates that diffusion alone is inadequate to support the metabolism of the adult bullfrog

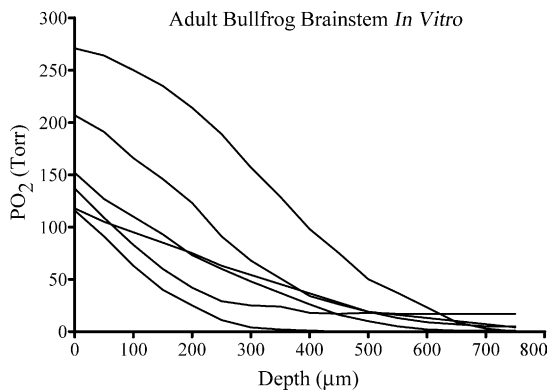


Fig. 4. Brain tissue PO₂ (Torr) decreases with depth in the brain tissue of adult *Rana catesbeiana*. Tissue PO₂ was measured at various depths using a polarographic microelectrode (50 µm tip; Diamond General Corp., Ann Arbor, MI) in six adult brainstems superfused with artificial cerebrospinal fluid bubbled with 98% O₂/2% CO₂. PO₂ drops to near zero in five of six brainstems at depths ranging from about 300 to 700 µm (Hedrick, Winnill and Chen, unpublished results).

brainstem in vitro. Nevertheless, the adult bullfrog brainstem generates a spontaneous respiratory output for several hours, and microinjection studies suggest that the relevant respiratory areas are at depths of about 100–300 µm (McLean et al., 1995a) which would be well-oxygenated (Fig. 3). However, these data do raise concerns about the microenvironment of ectothermic brainstems and the assumption that all ectothermic brainstems are well-oxygenated owing to their lower metabolism should be questioned.

Severe hypoxia or anoxia has profound effects on respiratory rhythm that change with development (Ballanyi et al., 1999). Brainstem preparations or rhythmically active medullary slices from the neonatal mouse do not show any significant change in respiratory rhythm for 30 min or more; however, preparations from more mature animals exhibit an initial augmentation of respiratory rhythm followed by respiratory depression and apnea occurring within a few minutes (Ballanyi et al., 1999). A recent study using medullary preparations from mice indicates that at E 16 severe hypoxia has no effect on fictive breathing frequency, but at E 18 to PO-P2, hypoxia significantly depresses respiratory rhythm (Viemari et al., 2003). The neonate is relatively insensitive to anoxia due to maintenance of extracellular ion concentrations of K⁺ and Ca²⁺, and a reliance on anaerobic metabolism (Hansen, 1985),

compared with adults that have limited abilities to tolerate hypoxia. Neuronal death during anoxia is generally associated with increased cell calcium entering primarily through the *N*-methyl-D-aspartate (NMDA) channel, and significant increases in cell calcium produce a cascade of cellular events leading to neuronal death (Kristian and Siesjo, 1998).

The effects of hypoxia on respiratory rhythm generation in non-mammalian vertebrates are poorly understood, despite the large number of studies that have examined the cellular effects of anoxia in anoxia-tolerant species such as turtles and carp (see Lutz and Nilsson, 2004). The turtle brainstem in vitro is insensitive to anoxia over a 2 h exposure (Johnson et al., 1998), which may not be surprising given the extreme anoxia tolerance of freshwater turtles (Lutz and Nilsson, 2004).

We have recently examined the role of hypoxia on respiratory rhythm generation and pattern formation during development in the bullfrog brainstem (Winnill et al., 2005). Brainstem preparations from pre-metamorphic tadpoles are relatively insensitive to the effects of severe hypoxia for up to 3 h or more. Metamorphic tadpole and adult brainstems, on the other hand, exhibit a complete cessation of respiratory motor activity within 30–40 min exposure to hypoxia, which is reversible upon reoxygenation. Blocking anaerobic metabolism with iodoacetate (IAA) significantly reduces the ability of the tadpole brain to produce respiratory activity in hypoxia, indicating a large contribution from glycolysis to maintain ATP levels. Metamorphic/adult brainstems, however, showed no difference in the response to hypoxia with IAA. This suggests that following metamorphosis to the obligate air-breathing stages, hypoxia effectively shuts down neural activity until oxygen is available. This may be an adaptive, energy-saving response to hypoxia that develops upon metamorphosis during the transition from water to land. The reversible cessation of fictive breathing may also suggest that the post-metamorphic brainstem is capable of sensing ATP and/or oxygen levels and this may be linked to the respiratory cessation. The respiratory cessation in adults during anoxia may be an adaptive response to brief periods of hypoxic stress that occur during overwintering (Stewart et al., 2004).

Tadpoles are known to be more hypoxia tolerant than adult frogs (Crowder et al., 1998), and there are likely to be several neuronal mechanisms that account

for this differential tolerance to hypoxia. One possible mechanism that accounts for the increased tolerance of tadpole brains to hypoxia is the tolerance to high intracellular calcium levels that accompany hypoxic conditions. Tadpole forebrain cells exposed to anoxia develop significant increases in cell calcium, but this is not associated with increased cell death for at least 4–6 h exposure to anoxia (Hedrick et al., 2005). It is not clear how tadpole neurons tolerate such high levels of calcium during hypoxia, but one hypothesis is that increased calcium may act as a neuroprotectant during anoxia, consistent with recent studies from turtles and mammals (Bickler and Fahlman, 2004).

Severe hypoxia also produces an increase in episodic breathing in metamorphic/adult, but not in pre-metamorphic, brainstems (Winmill et al., 2005). Episodic breathing is clearly an endogenous feature of the mature bullfrog brainstem, but the factors that contribute to episodic breathing are poorly understood. Our data indicate that hypoxia, in addition to NO and/or stimulation of GABA_B receptors (Straus et al., 2000b), by some unknown mechanism, is capable of inducing episodic breathing in the mature bullfrog brainstem. During severe hypoxia, there are no measurable changes in gill or lung burst characteristics that would be indicative of gasping that is found in mammals exposed to severe hypoxia (St. John and Knuth, 1981).

Although gasping does not appear to be a feature of the amphibian brainstem, we have found that ‘non-respiratory bursts’ (see Fig. 1, adult trace) are modulated by hypoxia. Non-respiratory bursts are more resistant to the effects of anesthesia than lung bursts (Hedrick and Winmill, 2003) and show a reversible increase in frequency during hypoxia (Winmill et al., 2005). These bursts are similar to ‘arousal’ breathing in the lamprey brainstem (Thompson, 1985), and we hypothesize that they serve an analogous function with gasping in mammals; that is, a widespread increase in neural activity to restart respiratory activity when normal respiratory efforts fail.

6. Conclusions

Currently, there is little information on the mechanisms underlying the development of respiratory rhythm generation in ectothermic vertebrates, but progress is being made. The mechanisms that have

been described show strong similarities to studies with mammalian preparations suggesting that development of respiratory rhythm generation is a conserved feature of the vertebrate brainstem. Future studies that examine the development of respiratory rhythm generation in ectothermic vertebrates will necessitate further characterization of respiratory-related neurons, their location, synaptic connections and localization within the brainstem. These details are generally lacking in ectotherms, but will be necessary to achieve a greater understanding of the mechanisms underlying respiratory rhythm development in non-mammalian vertebrates.

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