

DEVELOPMENT OF RESPIRATORY CENTERS IN THE BULLFROG TADPOLE  
BRAINSTEM

By

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## Abstract

Among vertebrates, rhythmic motor behaviors such as breathing, swallowing, and sucking are controlled by rhythm generators or neural oscillators located at various sites in the medulla of the brainstem. That all vertebrates exhibit these behaviors, leads investigators to hypothesize common ancestry for the cellular networks responsible for homeostatic rhythm generation in the brainstem. While the locations and functions of rhythm generating sites controlling some of these behaviors have been well investigated, details regarding the development of these sites remain largely unknown. Recent work has suggested that neural oscillators in the rostral and caudal medulla, which contribute to ventilation in amphibians, may be homologous with those controlling breathing in mammals. I first investigated the developmental contributions of these regions to CO<sub>2</sub> sensitivity and rhythm generation in bullfrog tadpoles at different stages of metamorphosis. I then characterized the function and structure of a neural oscillator essential for lung rhythmogenesis in the tadpoles and compared it to similar oscillators in mammals. To investigate functional aspects of brainstem, I used a combination of single-unit and whole-nerve electrophysiology in the presence of pharmacological agents (neuronal receptor agonists and antagonists) or following removal of portions of the isolated brainstem of bullfrog tadpoles at different stages of metamorphosis. Structural studies were accomplished using immunohistochemistry, staining for phenotypic markers common to mammalian rhythmogenic sites, and assessing the difference between early and late metamorphic bullfrog tadpoles. Taken together, my results suggest that amphibians may indeed have a rhythmogenic site in the rostral medulla that is homologous to a mammalian rhythmogenic site; it is both structurally and functionally similar to the mammalian parafacial respiratory group/retrotrapezoid nucleus complex. This region undergoes structural and functional changes as tadpoles develop through metamorphosis. Understanding the development of respiration in amphibians may provide clues into the evolution and development of breathing in mammals.



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# Chapter 1

## Introduction

### 1.1 Introduction

A multitude of behaviors in mammals, such as breathing, chewing, swallowing, and sucking are driven by oscillators located in the brainstem (Marder and Bucher 2001). As abnormalities that manifest in these locations during neural development cause morbidity and mortality, the maturation of these neural systems are of interest. Investigations in mammals have led to new insights into the locations of putative oscillators controlling the behaviors described above (Dick et al. 1993; Smith et al. 2000; Broussard and Altschuler 2000; Sawczuk and Mosier 2001; Jean 2001; Janczewski and Feldman 2006; Smith et al. 2007; Matsuo and Palmer 2009; Bolser et al. 2013; Moore et al. 2014; Sugiyama et al. 2015; Huckstepp et al. 2016; Samson et al. 2017). However, questions remain regarding the development and evolution of these neural networks. The use of fish, amphibians, and reptiles to address phylogenetic differences in the aforementioned behaviors has revealed a number of shared characteristics. In particular, the control of key homeostatic behaviors such as breathing and cardiovascular interactions shows similarities (Brainerd and Owerkowicz 2006; Milsom and Burlinson 2007; Kinkead 2009; Wilson et al. 2009; Milsom 2010a; Milsom 2010b; Taylor et al. 2014). Amphibians transition from aquatic organisms in early metamorphosis to a terrestrial organism near the end of metamorphosis, offering investigators a link between fish and reptiles. During metamorphosis, amphibians undergo rapid development but are freely swimming, providing a chance to examine neurodevelopment with relative ease.

Breathing requires coordination between brainstem sites responsible for sensing internal changes in pH/CO<sub>2</sub> and rhythmogenic sites that create and shape respiratory output. While these respiratory sites are well studied in mammals, central chemoreception and rhythmogenesis have been only partially elucidated in amphibians. Questions still remain regarding the contribution of individual brainstem areas to respiratory motor patterns, and how these areas and functions relate to respiratory oscillators and chemoreception in mammals. Understanding the development of respiration in amphibians may provide clues into the evolution and development of breathing in mammals.

## 1.2 Buccal and pharyngeal pumping in gill ventilation

Amphibian respiration primarily relies on two forms of coordinated rhythmic behavior: gill and lung ventilation. It should also be noted that amphibians use a passive mechanism of cutaneous gas exchange through their skin, but this source of respiration is beyond the scope of this work, as it does not involve brainstem rhythmogenic circuitry. Gill and lung ventilation, however, occurs in a highly coordinated manner. Bullfrog (*Lithobates catesbeianus*) gill ventilation occurs in early metamorphosis during which the tadpole is completely aquatic. Like fish, tadpoles exchange respiratory gasses with their aquatic medium using gills that are ventilated by a unidirectional flow of water across the gill surfaces. Gills are present when tadpoles emerge from eggs, and are resorbed at the peak of metamorphosis when the animal transitions to terrestrial life. Gill ventilation in tadpoles is a two-phase repeating rhythmic behavior in which water is drawn into the buccal cavity and then expelled outward across the gills (Fig. 1.1). To accomplish this water movement, tadpoles begin by opening their mouths and nares, and depressing the buccal floor, which expands the buccal cavity. This act creates negative pressure that aspirates water into the buccal cavity and simultaneously moves the velum into a closed position, partitioning the buccal cavity from the pharyngeal cavity. At the same time, the pharyngeal cavity is compressed, forcing water over the gills and aiding in closure of the velum. Following buccal floor depression and pharyngeal compression, the mouth and nares close, and the buccal floor is raised compressing the buccal cavity and creating positive pressure inside. Simultaneously, the pharyngeal cavity floor depresses, creating slight negative pressure that aids in refilling the pharyngeal cavity. Buccal cavity compression/depression and pharyngeal compression/depression operate opposite each other, and together form the rhythmic basis for gill, or “buccal” ventilation in early metamorphic tadpoles (Gradwell 1972a; Gradwell 1972b; Wassersug and Hoff 1979).

## 1.3 Buccal pumping in lung breathing

Larval and adult amphibians also use lung inflation to exchange respiratory gasses, and this exchange is increasingly relied upon as tadpoles progress toward a terrestrial environment (Burggren and West 1982). The process of lung inflation is similar in some ways to buccal ventilation. Because amphibians do not contain innervated musculature to create negative

aspirating pressure in a thoracic cavity (largely accomplished in mammals by the diaphragm), amphibians use the buccal pump to force air into the lungs (Fig. 1.2). In practice, a lung breath consists of 4 phases: (1) buccal depression, (2) pulmonary expiration, (3) buccal compression resulting in pulmonary inspiration, and (4) glottal closure (Kogo et al. 1994). The buccal depression-compression sequence observed during lung inflation is similar to buccal depression-compression sequence observed during gill ventilation. During aerial ventilation as the mouth and nares open and air is drawn into the buccal cavity, nearly simultaneously, the glottis opens and air is drawn out of the lungs into the buccal cavity and out through the nares. Following this pulmonary expiration, the buccal cavity compresses and air is forced into the lungs, finishing with the closure of the glottis and a subsequent breath hold until the next lung breath is initiated (Kogo et al. 1994). The buccal cavity of amphibians is much smaller in volume than the capacity of their lungs, and as such, lung inflation often requires a series of inflation breaths called lung episodes. These episodes achieve two main outcomes, they allow for deflation (expiration) and inflation (inspiration) of the lungs. Expiration and inspiration are two phases of a single breath, with some breaths participating more for inspiration, and others more for expiration (Vitalis and Shelton 1990).

#### **1.4 Neural control of breathing in amphibians**

Respiratory musculature involved in buccal compression and expansion is largely innervated by the trigeminal (V), facial (VII), and hypoglossal (XII) cranial nerves, while innervation of the glottis is via the vagus (X) nerve (Kogo et al. 1994). The neural control of the coordination of muscle activation leading to buccal and lung rhythmic behaviors is located in the medulla. While afferent inputs into the brain can affect the coordination of buccal/lung rhythmicity, the generation of both these rhythms is driven centrally by neural oscillators (Sakakibara 1978; Sakakibara 1984; Kogo et al. 1994; Gdovin et al. 1998; Milsom et al. 1999; Sanders and Milsom 2001; Wilson et al. 2002; Gargaglioni and Milsom 2007). Neural oscillators are groups of neurons with coordinated activity responsible for driving rhythmic motor behaviors (Marder and Bucher 2001).

Investigations using isolated brainstem preparations have indicated locations in the medulla responsible for driving both of these rhythms (McLean et al. 1995; Gdovin et al. 1998;



Reid and Milsom 1998; Milsom et al. 1999). The neural oscillator responsible for driving buccal rhythmogenesis resides at the level of the vagus (X) nerve, and the lung oscillator at the level of the abducens nerve (VI). Addition of an AMPA/kainate glutamate receptor antagonist (CNQX) abolishes lung activity, but does not abolish gill ventilation, indicating that the lung oscillator might use glutamatergic neurotransmission (Wilson et al. 2002). Conversely, bath application of bicuculline or strychnine on the isolated frog brainstem abolishes buccal breathing, demonstrating that buccal rhythms are likely mediated by inhibitory neurotransmission (Galante et al. 1996; Straus et al. 2000b; Straus et al. 2000a; Broch et al. 2002).

### **1.5 Neural control of vertebrate respiration**

Among vertebrates, respiration, whether through gill and/or lung ventilation, is controlled by regions in the brainstem. Isolated brainstem preparations of fish, amphibians, reptiles, and mammals have all been shown to exhibit fictive respiration (given the term ‘fictive’ because the brainstem is isolated and respiration is determined by electrical activity from cranial nerves innervating respiratory musculature) that is modulated by central CO<sub>2</sub> chemosensitivity (for review see Milsom 2010a and Milsom 2010b). Despite this homology in central respiratory control, there are significant differences in the mechanics of ventilation among different groups of vertebrates.

In contrast to amphibians, mammals accomplish lung inflation using an aspiration pump that draws air into the lungs. This is largely accomplished by the contraction of the diaphragm via activation of the phrenic nerve. As the diaphragm contracts, space increases in the thoracic cavity and air is drawn into the lungs. Other muscles, such as intercostal and abdominal muscles are also involved in respiration, especially under conditions of high respiratory drive. Muscles innervated by the trigeminal (V), facial (VII), vagus (X), and hypoglossal (XII) nerves also innervate airway musculature that maintains airway patency during respiration (Sawczuk and Mosier 2001; Gestreau et al. 2005; Brainerd and Owerkowicz 2006; Shiba et al. 2007; Abdala et al. 2009; Fregosi and Ludlow 2014). Exhalation in mammals is largely passive and is mediated by elastic recoil of the lungs and thoracic cavity; however, active expiration via contraction of intercostal and abdominal muscles can be observed under conditions of high respiratory drive (Abdala et al. 2009; West 2012).

The site of respiratory rhythmogenesis in mammals is located in the medulla and consists of interaction between multiple oscillators. The primary oscillator controlling inspiration is the Pre-Bötzinger complex (PreBötC), located near the roots of the vagus and hypoglossal nerves (Smith et al. 1991; Feldman and Del Negro 2006; Solomon 2003). This group of cells is capable of producing pacemaker-like activity and is critical for eupneic breathing to occur. Furthermore, perturbations to this region result in apnea and are often lethal (Smith et al. 1991; Solomon et al. 2000; Janczewski and Feldman 2006; Feldman and Del Negro 2006; Tan et al. 2008). Other oscillators near the PreBötC include the rostral ventral respiratory group (rVRG), Bötzing complex (BötC), postinspiratory complex (PiCo), and parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN). These regions are generally more associated with expiration, with the pFRG/RTN playing an additional role in chemoreception (Fortuna et al. 2008; Abdala et al. 2009; Tomori et al. 2010; Smith et al. 2013; Molkov et al. 2014; Huckstepp et al. 2016; Anderson et al. 2016).

Interestingly, neural coordination of the aspiration pump in embryonic and neonatal mammals consists of two coupled oscillators: pFRG/RTN and the PreBötC (Thoby-Brisson et al. 2005; Fortin and Thoby-Brisson 2009; Onimaru et al. 2009; Onimaru et al. 2014). The pFRG/RTN is a group of glutamatergic neurons that reside ventral to the facial (VII) nucleus and produce pre-inspiratory oscillations in embryonic and neonatal mammals (Mulkey et al. 2004; Stornetta et al. 2006; Abbott et al. 2011). Rhythms generated in this region emerge earlier than those of the PreBötC in embryonic mammalian brainstems (Thoby-Brisson et al. 2005; Fortin and Thoby-Brisson 2009; Mellen and Thoby-Brisson 2012). While involved in pre-inspiratory activity in embryonic and neonatal animals, this region plays a more prominent role in expiration and chemosensitivity as development progresses (Dubreuil et al. 2009; Smith et al. 2009; Guyenet and Mulkey 2010; Marina et al. 2010; Pagliardini et al. 2011; Wang et al. 2013). The similarities shared between the mammalian PreBötC and RTN/pFRG and the amphibian buccal and lung oscillators have led investigators to propose homology between these sites; however, further work is needed to confirm this homology (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006; Milsom 2010a; Baghdadwala et al. 2015a).

## **1.6 Central chemoreception in amphibians and mammals**

Although amphibians are able to exchange CO<sub>2</sub> through their skin, hypercapnia has a potent effect on the drive to breathe (Feder and Burggren 1985; Gdovin et al. 1999; Wang et al. 1999). Central CO<sub>2</sub> sensors in amphibians are located in the brainstem, in three main locations: the locus coeruleus (LC), the rostral chemosensory area (located at the level of the trigeminal nerve root), and the caudal chemosensory area (located at the level of the vagus nerve root) (Kinkead et al. 1997; Torgerson et al. 1997; Taylor et al. 2003; Noronha-de-Souza et al. 2006; Santin and Hartzler 2013). Focal administration of CO<sub>2</sub> to any one of these locations stimulates fictive lung breathing in isolated brainstem preparations (Torgerson et al. 1997; Torgerson et al. 2001; Taylor et al. 2003; Santin and Hartzler 2013). While some studies are in dissent, it is generally accepted that chemosensitivity exists throughout metamorphosis of tadpoles and the central chemoreceptors found in amphibians are likely homologous with those of mammals (Torgerson et al. 1997; Torgerson et al. 2001; Taylor et al. 2003; Milsom 2010b).

In mammals, CO<sub>2</sub> sensitivity originating within the central nervous system is largely the result of activity in the raphe and the pFRG/RTN. Both locations contain cells that are intrinsically sensitive to increases in CO<sub>2</sub> (Wang et al. 2002; Mulkey et al. 2004; Guyenet et al. 2009; Hodges and Richerson 2010; Depuy et al. 2011; Wang et al. 2013; Iceman et al. 2013; Iceman et al. 2014). However, chemosensitive neurons have been identified in other regions of the brainstem including the nucleus of the solitary tract, the locus coeruleus, and astrocytes surrounding the pFRG/RTN (Nattie and Li 1996; Solomon et al. 2000; Biancardi et al. 2008; Nattie and Li 2009; Guyenet and Mulkey 2010; Gargaglioni et al. 2010; Mulkey and Wenker 2011; Huckstepp and Dale 2011; Barnett et al. 2016). While few locations have been identified in amphibians to date, it would be unsurprising to find a widespread network of CO<sub>2</sub> sensitivity similar to mammals.

## **1.7 Approach taken in this work**

The goal of this work is to investigate developmental changes involved in respiratory chemoreception and rhythmogenesis in the amphibian brainstem. Specifically, I investigate the potential homology between the amphibian lung oscillator and mammalian pFRG/RTN, and characterize function and anatomy of the amphibian lung oscillator. For this investigation, I used

the bullfrog isolated brainstem preparation from early and late metamorphic tadpoles. By recording from cranial nerves that innervate muscles that perform respiratory actions, measurements of respiratory activity under conditions of high drive (hypercapnia = high CO<sub>2</sub>) were made following removal of essential components of the respiratory neural network (Chapter 2). In other studies I isolated the lung oscillator and tested its function using pharmacological antagonists similar to work previously completed in mammals (Chapter 3). Finally, I used immunohistochemistry to investigate the presence of neurons in the amphibian lung oscillator that are histochemically similar to the pFRG/RTN neurons in mammals (Chapter 4).

## 1.8 Specific aims

*Chapter 2: Influence of ascending and descending projections on central CO<sub>2</sub> sensitivity and rhythm generation across metamorphosis in *Lithobates catesbeianus* tadpoles.*

The development of respiration in amphibians can provide insight into the evolution and development of breathing in mammals. Recent work has suggested that oscillators in the rostral and caudal medulla contributing to respiration in amphibians may be homologous with rhythmogenic sites controlling ventilation in mammals. These oscillators have also been proposed to be sensitive to increases in CO<sub>2</sub>, similar to those of mammals. While questions about the role both oscillators and chemosensitive regions take during metamorphosis have been investigated, conflicting reports have made comparisons to their potential mammalian counterparts challenging. In the current investigation, I assess the developmental influences of amphibian CO<sub>2</sub> sensitivity and rhythm generation. **I test the hypothesis that central CO<sub>2</sub> sensitivity is contained in the rostral medulla and present across metamorphosis.** To test this hypothesis, whole-nerve recordings from the facial (VII) and hypoglossal (XII) cranial nerves were made from intact and transected brainstems in early and late metamorphic tadpoles. Brainstems were either transected at the level of the trigeminal (V) nerve, removing the pons and locus coeruleus (LC), or at the level of the glossopharyngeal (IX) nerve, removing the putative buccal oscillator and caudal medulla. Results suggest that a developmental shift toward lung breathing is accompanied by a stark increase in CO<sub>2</sub> sensitivity. Further, buccal rhythmogenesis was sensitive to CO<sub>2</sub> following removal of the pons/LC. These results help resolve current

conflicts involving the change of CO<sub>2</sub> sensitivity through metamorphosis and the role specific regions of the amphibian brainstem play in generating and shaping rhythm.

*Chapter 3: Rhythmogenesis in the isolated rostral medulla of early metamorphic *Lithobates catesbeianus* tadpoles*

Respiratory behavior in terrestrial animals is primarily controlled via neural circuits in the medulla. A site in the amphibian rostral medulla at the level of the abducens nerve root (VI) is believed to be homologous to the PreBötC in mammals. The  $\mu$ -opioid receptor agonist DAMGO has a suppressive effect, and the GABA<sub>A</sub> receptor antagonist bicuculline has an excitatory effect on lung activity in mammals and amphibians. However, the specific locations of action in amphibians are unknown. Therefore, I examined the effect of DAMGO and bicuculline on isolated rostral brainstem tissue. **I test the hypothesis that amphibians have a rhythmogenic area in the rostral medulla that is functionally similar to the mammalian Pre-Bötzinger complex.** To test this hypothesis, isolated brainstems, from early metamorphic bullfrog tadpoles (*Lithobates catesbeianus*) were transected leaving a region of brainstem from the trigeminal nerve (V) to the glossopharyngeal nerve (IX) intact, isolating the lung oscillator and corresponding motor nerves. Whole-nerve recordings were made from the trigeminal (V) and facial (VII) nerves, while I tested the lung oscillator for sensitivity to opioids (DAMGO), GABAergic (bicuculline) and glycinergic (strychnine) inhibition, and glutamatergic transmission (CNQX). The transection alone resulted in a loss of discernable respiratory rhythm. However, bicuculline application caused a lung-like episodic rhythm to reappear. Contrary to the suppressive effect DAMGO has on lung activity in other preparations (including intact isolated amphibian brainstems), lung activity was not affected by the addition of DAMGO in my transected preparation. Results do not support the hypothesis that there is functional similarity between the lung oscillator in bullfrogs and the pre-Bötzinger complex in mammals. However, our results do support a homology with the pFRG/RTN, an anatomically similar rhythmogenic site in mammals.

#### *Chapter 4: Development of phenotypic markers for pFRG/RTN in *Lithobates catesbeianus**

Investigations of the neural substrates that control respiration can provide insight into the development and function of these sites. The pFRG/RTN plays an important role in both respiratory chemoreception and rhythmogenesis in prenatal and early postnatal mammals, and is essential for the development of respiration. In amphibians, the lung oscillator has been proposed to be functionally similar to the pFRG/RTN of mammals. While functional similarities have been investigated, the parallels of cellular phenotype have not been identified. **I test the hypothesis that the rostral medulla in amphibians is structurally and developmentally similar to pFRG/RTN in mammals.** To test this hypothesis, I stained for neuronal markers used to identify the pFRG/RTN region in mammals: paired-like homeobox gene 2b (Phox2b) transcription factor, tyrosine hydroxylase (TH), and choline acetyltransferase (ChAT), in both early and late metamorphic tadpoles. I then made extracellular recordings in the proposed lung oscillator to assess CO<sub>2</sub> sensitivity and association with respiration. A group of Phox2b immunoreactive cells were located ventral to the facial (VII) nuclei in both early and late metamorphic animals. These cells were not positive for ChAT or TH, and the number of cells observed in this region increased across metamorphosis. Extracellular recordings of neurons in the proposed area revealed CO<sub>2</sub> sensitive cells that fired coincidentally with lung bursts. Together, evidence supports the hypothesis that amphibians have a structurally homologous region to the mammalian pFRG/RTN. Furthermore, neurons in this population increase in number across metamorphosis, which could potentially contribute to increased CO<sub>2</sub> sensitivity in late metamorphic tadpoles.

#### **1.9 Major findings**

Taken together, my results suggest amphibians share a common respiratory oscillator with mammals; the amphibian lung oscillator and the mammalian pFRG/RTN are similar in function, anatomical location, and histology. This work supports a current shift in opinion regarding the homology of the amphibian lung and buccal oscillators and the mammalian pFRG/RTN and PreBötC (Baghdadwala et al. 2015a; Baghdadwala et al. 2015b). Additionally, my work provides clarification for inconsistencies found in amphibian literature regarding rhythm generation in the lung oscillator region and CO<sub>2</sub> sensitivity. This work contributes to the understanding of the

origins of air breathing in mammals and offers insight into the development of the neural circuits involved in the control of breathing.

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## 1.11 Figures

### Inspiration

“Buccal Expansion”  
buccal expansion  
mouth and nares open  
velum closed



### Inspiration / Expiration

“Buccal Expansion / Pharyngeal Compression”  
buccal expansion  
mouth and nares open  
velum closed  
pharyngeal compression



### Expiration

“Buccal Compression / Pharyngeal Expansion”  
buccal compression  
mouth and nares closed  
velum open  
pharyngeal expansion



Figure 1.1: **Buccal ventilation requires coordination between the buccal and pharyngeal force pumps.** Buccal ventilation is used in early metamorphic tadpoles to draw water into the buccal cavity and push it across gills and out the gill slits. (A) Ventilation begins with the buccal cavity expanding, drawing water in through the nares and mouth. (B) Nearly simultaneously, the pharyngeal cavity compresses, pushing water across the gills. (C) The buccal cavity then compresses during pharyngeal relaxation pushing water from the buccal cavity into the pharyngeal cavity, restarting the cycle. (This figure is from Gargaglioni & Milsom 2007)



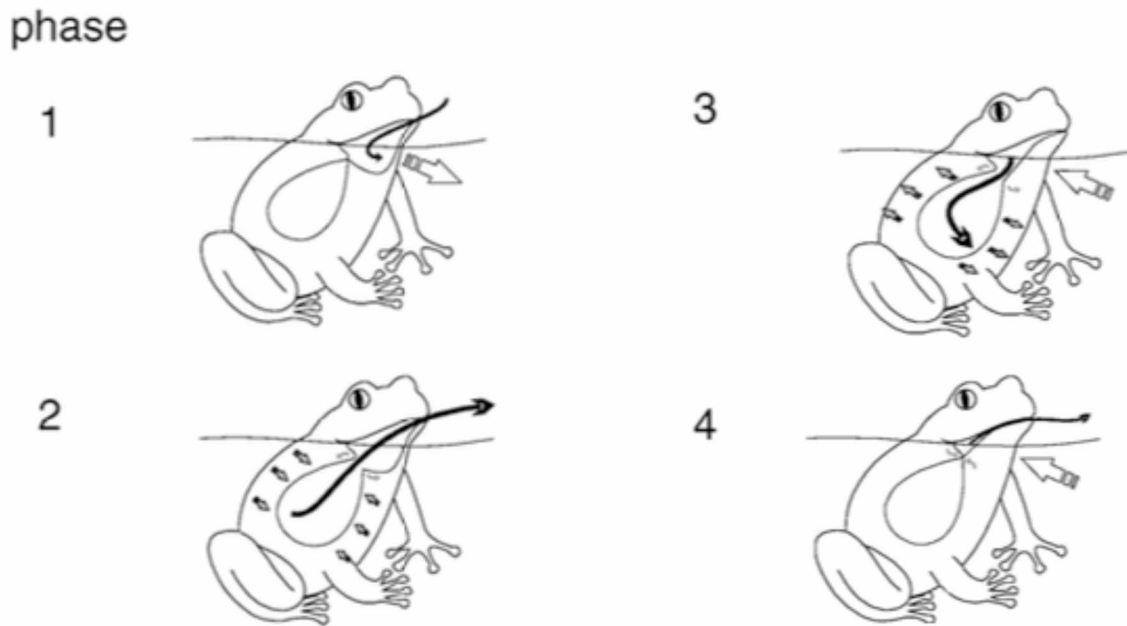


Figure 1.2: **Lung ventilation in amphibians consists of 4 phases.** (1) The beginning of lung ventilation is marked with a depression of the buccal cavity and the nares opening, drawing air into the buccal cavity. (2) Nearly simultaneously, the glottis opens and air from the lungs is drawn into the buccal cavity during from the depression in *phase 1*. (3) The nares then close and the buccal cavity compresses, forcing air into the lungs. (4) Following buccal compression, the glottis closes and a breath hold then proceeds. Figure has been adapted from Gargaglioni and Milsom 2007.

## Chapter 2

### Influence of ascending and descending projections on central CO<sub>2</sub> sensitivity and rhythm generation across metamorphosis in *Lithobates catesbeianus* tadpoles<sup>1</sup>

#### 2.1 Abstract

The development of respiration in amphibians can provide insight into the evolution and development of breathing in mammals. Recent work has suggested that neural oscillators in the rostral and caudal medulla of the brainstem, which drive ventilation in amphibians, may be homologous with those controlling breathing in mammals. The amphibian oscillators have also been proposed to be sensitive to increases in CO<sub>2</sub>, similar to mammals. While questions about the role both oscillators and chemosensitive regions take during metamorphosis have been investigated, conflicting reports have made comparisons to their potential mammalian counterparts challenging. In the current investigation, we assessed the influence of development on amphibian central CO<sub>2</sub> sensitivity and respiratory rhythm generation. Whole-nerve recordings from the facial (VII) and hypoglossal (XII) cranial nerves were made from intact and transected brainstems in bullfrog (*Lithobates catesbeianus*) tadpoles during early and late metamorphosis. Brainstems were either transected at the level of the trigeminal (V) nerve, removing the pons and locus coeruleus, or at the level of the glossopharyngeal (IX) nerve, removing the putative buccal oscillator and caudal medulla. Results suggest that a shift toward lung breathing during metamorphosis is accompanied by a stark increase in CO<sub>2</sub> sensitivity. Further, buccal rhythmogenesis was sensitive to CO<sub>2</sub> following removal of the pons and locus coeruleus. These results help resolve current controversies involving the change of CO<sub>2</sub> sensitivity during metamorphosis and the contributions made by specific regions of the amphibian brainstem to respiratory burst shape and rhythmogenesis.

#### 2.2 Introduction

Examination of bullfrog (*Lithobates catesbeianus*) brainstems *in vitro* has provided insight into the development of the respiratory control network (Torgerson et al. 1997a; Gdovin

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<sup>1</sup> Reed M, Iceman K, Harris M, Taylor B. Influence of ascending and descending projections on central CO<sub>2</sub> sensitivity and rhythm generation across metamorphosis in *Lithobates catesbeianus* tadpoles. *Comp Biochem Physiol* (In preparation)

et al. 1999a; Torgerson et al. 2001b; Taylor et al. 2003a; Winmill et al. 2005; Hedrick 2005; Chen and Hedrick 2008; Klingler and Hedrick 2013). The respiratory control network has been most studied in mammals; however, all vertebrates appear to have a similarly located respiratory control network comprised of rhythmogenic loci and CO<sub>2</sub>/pH-sensing loci (for review see (Milsom 2010b; Milsom 2010a)). This neural network in amphibians comprises several brainstem loci and is influenced by afferent stimuli and central changes in pH/CO<sub>2</sub> (Gargaglioni and Milsom 2007; Milsom 2010a). The comparison of brainstem respiratory regions between amphibians and mammals is of particular interest, but attempts to investigate the locations responsible for amphibian respiratory rhythm and chemoreception have produced conflicting results (Torgerson et al. 2001b; Wilson et al. 2002; Taylor et al. 2003a; Davies et al. 2009; Klingler and Hedrick 2013).

Application of high CO<sub>2</sub>/low pH (hypercapnia) is a potent stimulus for breathing in mammals and also excites respiration in amphibians (Torgerson et al. 1997a; Reid and Milsom 1998; Torgerson et al. 2001b; Taylor et al. 2003a). Effects of hypercapnia vary by investigator, preparation, and metamorphic stage of experimental animal. When Torgerson et al. (2001) bath applied hypercapnia to tadpole brainstems, both buccal and lung frequency increased. However, this result differed by metamorphic stage of the animals; in late metamorphosis only lung frequency increased, and in early/middle metamorphosis, only buccal frequency increased (Torgerson et al. 1997a). In a subsequent investigation, bath application of CO<sub>2</sub> increased buccal burst frequency in early metamorphosis only – not as metamorphosis progressed (Remmers et al. 2001; Torgerson et al. 2001b). In contrast, Taylor et al. (2003a) reported that both focally and bath applied hypercapnia increased lung frequency throughout development and had no effect on buccal frequency at any period of metamorphosis (Taylor et al. 2003a).

Although there are likely additional central chemosensory locations, three primary areas of the amphibian brainstem have received the most attention: the locus coeruleus (LC), a “rostral chemosensory area” located at the level of the trigeminal nerves, and a “caudal chemosensory area” located at the level of the vagus nerves (see Fig. 2.1a for a brainstem illustration) (Kinkead et al. 1997; Torgerson et al. 2001b; Taylor et al. 2003a; Noronha de Souza et al. 2006; Wilson et al. 2009; Santin and Hartzler 2013). Torgerson et al. (2001) reported that dominant chemoreceptive locations shift from a caudal region in early metamorphosis to a more rostral region in late

metamorphosis and adulthood (Torgerson et al. 2001b). However, Taylor et al. (2003) demonstrated that focal application of CO<sub>2</sub> to either rostral or caudal sites increased lung ventilation throughout metamorphosis (Taylor et al. 2003a). While studies of medullary chemosensitive sites in amphibians have produced conflicting results, results of studies of the LC consistently support its role as a chemosensitive region. In patch clamp recordings of adult bullfrog acute slices, LC neurons demonstrate intrinsic chemoreception (Santin and Hartzler 2013). In adult toads (*Bufo schneideri*), hypercapnia induces expression of the transcription factor and marker of increased cellular activity, cFos, in the LC, and LC lesions attenuate the increases in lung ventilation associated with hypercapnia (Noronha-de-Souza et al. 2006). While the LC is an important chemosensor in adult amphibians, its role during early metamorphosis has not been assessed. The amphibian LC is not essential for lung rhythmogenesis, but it does play a critical role in formation of episodic lung rhythms (Kinkead et al. 1997; Milsom et al. 1997; Reid et al. 2000).

Anatomically, the sites responsible for respiratory rhythm generation appear to be apposed to chemosensitive areas in the amphibian brainstem. Studies employing transections of (Torgerson et al. 2001a) and neurotransmitter injections into (Wilson et al. 2002) the bullfrog tadpole brainstem revealed a bilateral “lung oscillator” critical for lung rhythmogenesis, located between the facial (VII) and glossopharyngeal (IX) cranial nerves, near the rostral chemosensory area (Torgerson et al. 2001a; Wilson et al. 2002). A bilateral “buccal oscillator” was determined to be necessary for buccal ventilation, and located caudal to the lung oscillator at the level of the vagus nerves (X), near the caudal chemosensory area (Wilson et al. 2002). Two different studies examined the consequence of isolating the “lung oscillator” from the caudal portion of the brainstem containing the putative “buccal oscillator” in early metamorphic tadpoles. This isolated “lung oscillator” region was reported to be either capable (Klingler and Hedrick 2013) or incapable (Torgerson et al. 2001a) of producing lung bursts. These conflicting results may be due to differences in experimental preparations between the two studies. Nonetheless, the nature and putative necessity of interaction between the two rhythmogenic loci remain to be determined.

In the current investigation, we assessed developmental influences on amphibian CO<sub>2</sub> sensitivity and rhythm generation. We tested the hypothesis that central CO<sub>2</sub> sensitivity was present across development, and primarily contained in the rostral medulla. To resolve

conflicting results observed in previous studies, we performed similar experiments, wherein we recorded buccal and lung activity in an isolated brainstem preparation and made transections between putative chemosensitive and rhythmogenic regions. Results suggest that a developmental shift toward lung breathing is accompanied by a stark increase in CO<sub>2</sub> sensitivity.

## 2.3 Methods

### *Experimental Animals*

Studies were performed on 36 bullfrog tadpoles during early and late metamorphosis as defined by external anatomy. One group was selected during early metamorphosis characterized by the absence of forelimbs, and the presence of paddle-like hind limbs lacking joints or separated toes (n=17). The second group was selected during late metamorphosis, characterized by the presence of both forelimbs and hind limbs, and a partially resorbed tail (n=19). These groups corresponded to developmental stages 7–12 and 20–25, respectively, in the classification scheme of tadpole development originally proposed by Taylor and Kollros (Taylor and Kollros 1946). Tadpoles were purchased from a commercial supplier (Pond Megastore, [www.pondmegastore.com](http://www.pondmegastore.com)), and maintained at room temperature housed in aquaria with dechlorinated water, and were fed goldfish food daily. All care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines.

### *Surgical preparation*

Each tadpole was anesthetized by immersion for 1–2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222; Sigma, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) in dechlorinated water buffered to pH 7.8 with NaHCO<sub>3</sub>. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostrally to the optic tectum and caudal to the brachial nerves. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 MgCl<sub>2</sub>, 10 d-glucose, 25 NaHCO<sub>3</sub> and 2.4 CaCl<sub>2</sub> equilibrated with 100% O<sub>2</sub>. The aCSF

HCO<sub>3</sub> concentration is similar to that of plasma from late metamorphic tadpoles and frogs but higher than that in plasma from early metamorphic tadpoles (Just et al. 1973). This HCO<sub>3</sub> concentration has been used in previous tadpole studies (Taylor et al. 2003a; Taylor et al. 2008) and was selected here to ensure comparability with previous studies and between experiments on animals of different stages of metamorphosis.

The isolated brainstem was transferred *en bloc* to a 2.5-ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O<sub>2</sub> –CO<sub>2</sub> mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. The pH of the aCSF was maintained at pH 7.8 (1.5 % CO<sub>2</sub> : 98.5 % O<sub>2</sub>; normocapnia) by adjusting the fractional concentrations of O<sub>2</sub> and CO<sub>2</sub> in the equilibration gas, and the latter was monitored with a CO<sub>2</sub> analyzer (AMETEK CD-3A CO<sub>2</sub> analyzer, [www.ametek.com](http://www.ametek.com)). After isolation the brainstem was allowed to stabilize for 1 hour while superfused with aCSF at 23 °C and pH 7.8 (~9 Torr PCO<sub>2</sub>).

### *Nerve recordings*

Roots of the trigeminal (V), facial (VII), or hypoglossal (XII) cranial nerves were drawn into glass suction electrodes pulled from 1-mm diameter capillary glass to tip diameters that fit the nerve roots. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, [www.wpiinc.com](http://www.wpiinc.com); X1000 by a model 1700 amplifier, A-M Systems, [www.a-msystems.com](http://www.a-msystems.com)) and filtered (100Hz high pass to 1kHz low pass). The amplified and filtered nerve output was sent to a data acquisition system (Power 1401, Cambridge Electronic Design, [ced.co.uk](http://ced.co.uk)), which sampled at 25 kHz. Data were archived as neurograms of whole-nerve discharge, and duplicate integrated (root mean squared and averaged over 200 ms). Neurograms were acquired simultaneously from two nerves prior to transections. Such recordings were made during the initial 1-h post-isolation stabilization period and recorded continuously throughout the duration of each treatment protocol.

### *Transections and experimental protocol*

Whole-nerve recordings were made from intact, rostral transected, or caudal transected brainstems. Experiments began by treating isolated intact brainstems with 15 min of normocapnia (pH 7.8), and 30 min of hypercapnic (pH 7.4; 5.0 % CO<sub>2</sub> :95.0 % O<sub>2</sub>) gas treatment for 30 min, followed by a recovery period of 1 h at normocapnia. After recovery, brainstems were either: 1) left intact as controls (Fig. 2.1a); 2) transected at the glossopharyngeal nerve (caudal transection), which removed the buccal oscillator and the caudal chemosensory area (Fig. 2.1b); or 3) transected at the level of the trigeminal nerves (rostral transection), which removed the chemosensory influence of the LC (Fig. 2.1c). Brainstems were allowed to recover for 1 h following transection, and then given the same gas treatment administered prior to transection. For each gas treatment the most representative 5-min sample within the last 10 min was used for analysis.

### *Data and statistical analyses*

Patterns of activity within recorded neurograms were designated as either putative “buccal,” or putative “lung” bursts on the basis of the amplitude of the integrated nerve activity and the presence or absence of coincident firing in both the facial and hypoglossal nerves of the intact brainstem preparation, as previously described (Kogo and Remmers 1994; Torgerson et al. 1997b; Milsom et al. 1999; Gdovin et al. 1999b; Gdovin et al. 1999a; Taylor et al. 2003a). Lower amplitude activity bursts from the facial nerve with little or no coincident activity from the hypoglossal nerve were designated as “buccal bursts.” Higher amplitude activity bursts from the facial nerve with coincident activity from the hypoglossal nerve were designated “lung bursts.”

Lung and buccal bursts from each treatment group were analyzed for burst frequency (bursts/min), instantaneous frequency (Hz), burst duration (sec), time to peak duration (sec), and time to trough duration (sec). The normally distributed measures were compared using one-way repeated-measures analysis of variance (RMANOVA; SigmaStat, [www.systat.com](http://www.systat.com)). When comparing effects of hypercapnia between early and late metamorphic animals or between transections, a factorial two-way RMANOVA (one-factor repetition) was used. In both cases, when RMANOVA indicated that significant differences existed, a *post hoc* Holm-Sidak multiple

comparison test was used to identify the significantly different data groups. Values are reported as means  $\pm$  standard error.

## 2.4 Results

*Rhythmic respiratory nerve discharge was recorded in early and late metamorphic animals regardless of transection.*

To investigate developmental changes of ascending and descending inputs to lung rhythms, we transected brainstems immediately rostral to the trigeminal cranial nerves (V) or immediately rostral to the glossopharyngeal cranial nerves (IX). Whole-nerve recordings were made from intact, rostrally transected (transected at V), or caudally transected (transected at IX). Recordings from intact brainstems of early and late metamorphic tadpoles produced lung and buccal activity bursts (Fig. 2.2a). Lung and buccal bursts were also detected in early and late metamorphic, rostrally transected brainstems (Fig. 2.2c). Lung bursts were recorded from caudally transected brainstems of early and late metamorphic tadpoles, and anecdotally, small amplitude bursts similar to buccal bursts were also detected (Fig. 2.2b).

*Hypercapnia stimulates lung breathing in late but not early metamorphic tadpoles.*

We then tested the difference in CO<sub>2</sub> sensitivity between intact brainstems from early and late metamorphic tadpoles. Whole-nerve discharge was recorded from the facial (VII) and hypoglossal (XII) cranial nerves in normocapnic (pH 7.8) and hypercapnic (pH 7.4) conditions from intact brainstems of early (n = 6) and late (n = 4) metamorphic tadpoles. Both buccal and lung bursts were observed in early and late metamorphic tadpoles in both normocapnic and hypercapnic conditions (Fig. 2.3a & b). Lung bursts were observed less frequently in early metamorphic animals (3.5 bursts/min) compared to late metamorphic (23.2 bursts/min), regardless of pH (Fig. 2.3c, factorial two-way RMANOVA; metamorphic stage factor  $F = 64.58$ ,  $p < 0.001$ ). Hypercapnia produced a significant increase in lung burst frequency in late metamorphic animals (30.7 bursts/min; one-way RMANOVA;  $F = 10.06$ ,  $p = 0.012$ ), but not early metamorphic animals (Fig. 2.3d, 3.4 bursts/min; one-way RMANOVA;  $F = 0.87$ ,  $p = 0.450$ ). The duration of putative lung bursts differed between early (1.54 sec/burst) and late metamorphic (0.69 sec/burst) animals (factorial two-way RMANOVA; metamorphic stage factor



$F = 33.49$ ,  $p < 0.001$ ), but increases in burst frequency in response to hypercapnia were not associated with changes in lung burst duration in early or late metamorphic tadpoles (factorial two-way RMANOVA; gas treatment factor  $F = 2.77$ ,  $p = 0.093$ ). Figure 2.3d indicates waveform averages of lung bursts (gray lines =  $\pm 1$  standard error of the mean) differed between early and late metamorphic animals, and did not change with hypercapnia.

*Rostral transection of the brainstem affects lung burst frequency, but not hypercapnic response.*

Whole-nerve activity was recorded from the facial (VII) and hypoglossal (XII) nerves during normocapnic (pH 7.8) and hypercapnic (pH 7.4) conditions in rostrally transected brainstems of early and late metamorphic tadpoles. Lung and buccal bursts were observed following transection in both metamorphic tadpole groups (Fig. 2.4a & b). Figure 2.4c shows that hypercapnia stimulated lung burst frequency in late metamorphic (pH 7.8 = 5.3 bursts/min; pH 7.4 = 17.8 bursts/min) animals (one-way RMANOVA;  $F = 13.34$ ,  $p = 0.002$ ), but not early metamorphic animals (pH 7.8 = 1.16 bursts/min; pH 7.4 = 0.9 bursts/min, one-way RMANOVA;  $F = 0.53$ ,  $p = 0.606$ ). Lung burst frequency and effects of hypercapnia in early and late metamorphic animals were significantly different (factorial two-way RMANOVA; metamorphic stage factor  $F = 49.41$ ,  $p < 0.001$ ; gas treatment factor  $F = 13.59$ ,  $p < 0.001$ ). Lung burst durations were similar between early (1.16 sec/lung burst) and late (1.57 sec/lung burst) metamorphic animals (factorial two-way RMANOVA; metamorphic stage factor  $F = 3.21$ ,  $p = 0.103$ , Fig. 2.4d), and were not significantly affected by hypercapnia (factorial two-way RMANOVA; gas treatment factor  $F = 1.88$ ,  $p = 0.180$ , Fig. 2.4d). Waveform averages of lung bursts (gray lines =  $\pm 1$  standard error of the mean) differed between early and late metamorphic animals, and displayed increased variability in early metamorphic animals (Fig. 2.4e).

*Hypercapnic responses were observed in both early and late metamorphic animals following caudal transection.*

Whole-nerve discharge was recorded from the trigeminal (CNV) and facial (CNVII) cranial nerves in normocapnic (pH 7.8) and hypercapnic (pH 7.4) conditions from caudally transected brainstems of early and late metamorphic tadpoles. Lung bursts were observed following transection in both early and late metamorphic animals (Fig. 2.5a & b). Figure 2.5c

demonstrates lung bursts in early metamorphic animals (10.4 bursts/min) were more frequent compared to late metamorphic animals (5.2 bursts/min), but both metamorphic groups were equally sensitive to increased CO<sub>2</sub> in regards to lung bursting (factorial two-way RMANOVA; gas treatment factor  $F = 14.20$ ,  $p < 0.001$ ). Durations of lung bursts, shown in Figure 2.5d, were not significantly different between early (1.50 sec/burst) and late metamorphic animals (1.99 sec/burst, factorial two-way RMANOVA; metamorphic stage factor  $F = 0.19$ ,  $p = 0.674$ ), or during hypercapnia (factorial two-way RMANOVA; gas treatment factor  $F = 0.55$ ,  $p = 0.585$ ). Lung burst waveform averages (Fig. 2.5e) in early and late metamorphic animals were largely similar and did not change in response to hypercapnia.

*Rostral transection affects lung burst duration while caudal transection affects lung burst frequency in early metamorphic animals.*

Recordings made following transections were compared to recordings from intact early metamorphic brainstems with respect to burst frequency (bursts/min), burst duration (sec), bursts time to peak (sec), and burst time to trough (sec). Lung burst frequency was significantly affected by caudal transection of the brainstem (Fig. 2.6a, factorial two-way RMANOVA; transection factor  $F = 30.58$ ,  $p = <0.001$ ). As reported above, caudal transection revealed a CO<sub>2</sub> sensitive network, a unique observation in early metamorphic tadpoles (Fig. 2.6a). Rostral transection produced a significant reduction in lung burst duration compared to control brainstems (Fig. 2.6b, factorial two-way RMANOVA; transection factor  $F = 4.95$ ,  $p = 0.024$ ). The reduction in lung burst duration observed after removal of the rostral area was primarily due to a significant reduction in the time to peak (0.46 sec compared to 0.80 sec) seen in control recordings (Fig. 2.6c; factorial two-way RMANOVA, transection factor  $F = 12.08$ ,  $p < 0.001$ ). The time to trough for control (0.73 sec), rostral transection (0.69 sec), and caudal transection (0.714 sec) were not significantly different (Fig. 2.6d; factorial two-way RMANOVA, transection factor  $F = 0.51$ ,  $p = 0.610$ ).

*Rostral or caudal transection each affected both lung burst frequency and duration, but not sensitivity to CO<sub>2</sub>.*

Recordings made following transections were compared to intact recordings from late metamorphic brainstems with respect to burst frequency (bursts/min), burst duration (sec), bursts time to peak (sec), and burst time to trough (sec). Either rostral or caudal transections reduced lung burst frequency (Fig. 2.7a; factorial two-way RMANOVA; transection factor  $F = 19.50$ ,  $p < 0.001$ ). While lung burst frequency differed depending on transection, the hypercapnic response was preserved (Fig. 2.7a; factorial two-way RMANOVA; gas treatment factor  $F = 25.98$ ,  $p < 0.001$ ). Lung burst duration was significantly increased following caudal but not rostral transection (Fig. 2.7b; factorial two-way RMANOVA; transection factor  $F = 6.64$ ,  $p = 0.010$ ). The increase in duration varied based on the type of transection. Caudal transection produced significantly larger time to peak durations (1.07 sec) compared to intact control (0.34 sec) brainstems (Fig. 2.7c; factorial two-way RMANOVA; transection factor  $F = 4.90$ ,  $p = 0.026$ ). Rostral and caudal transections both produced significant changes in time to trough durations (control = 0.35 sec, rostral transection = 0.97 sec, and caudal transection = 0.92 sec) in lung bursts (Fig. 2.7d; factorial two-way RMANOVA; transection factor  $F = 6.18$ ,  $p = 0.013$ ).

*Hypercapnia affects the shape of buccal bursts in rostrally transected brainstems from early and late metamorphic animals.*

To test the effect transections have on buccal bursts, recordings from control brainstems were compared to recordings made after transections with respect to instantaneous frequency (Hz), burst duration (sec), burst time to peak (sec), and burst time to trough (sec) in preparations derived from early and late metamorphic animals. Waveform averages of buccal burst shapes from intact control and rostrally transected brainstems exposed to hypercapnia were created for both early and late metamorphic animals (Fig. 2.8). Average burst shapes demonstrate that hypercapnia decreased burst duration in early and late metamorphic brainstems with the rostral area removed (Fig. 2.8b).

Instantaneous buccal burst frequency and burst duration of buccal bursts recorded from intact or rostrally transected early and late metamorphic brainstems also differed. Late metamorphic brainstems had significantly higher buccal burst instantaneous frequencies (1.10

Hz) compared to intact early metamorphic brainstems (0.65 Hz; Fig. 2.8a; factorial two-way RMANOVA; metamorphic stage factor  $F = 34.93$ ,  $p < 0.001$ ). Additionally, buccal burst durations in intact brainstems were significantly longer in early metamorphic (1.26 sec) compared to late metamorphic (0.66 sec) tadpoles (Fig. 2.8b; factorial two-way RMANOVA; metamorphic stage factor  $F = 40.03$ ,  $p < 0.001$ ). Rostral transection of early and late metamorphic brainstems revealed a CO<sub>2</sub>-sensitive buccal rhythm. Instantaneous frequency of rostrally transected early metamorphic brainstems changed from 0.64 Hz under normocapnic conditions to 0.79 Hz during hypercapnia (Fig. 2.8c; factorial two-way RMANOVA; gas treatment factor  $F = 7.246$ ,  $p = 0.010$ ). Buccal bursts from rostrally transected late metamorphic brainstems were also sensitive to CO<sub>2</sub> (normocapnia = 0.52 Hz, hypercapnia = 0.77 Hz; factorial two-way RMANOVA; gas treatment factor  $F = 7.25$ ,  $p = 0.010$ ). Buccal burst durations decreased under increased CO<sub>2</sub> in rostrally transected early (normocapnia = 1.24 seconds/burst, hypercapnia = 1.03 seconds/burst) and late (normocapnia = 1.58 seconds/burst, hypercapnia = 1.02 seconds/burst) metamorphic brainstems (Fig. 2.8d; factorial two-way RMANOVA; gas treatment factor  $F = 9.363$ ,  $p = 0.004$ ).

## 2.5 Discussion

### *Central CO<sub>2</sub> sensitivity is present throughout metamorphosis*

Central CO<sub>2</sub> sensitivity drives lung burst generation and has been demonstrated in early metamorphosis of bullfrog tadpoles, becoming more robust as tadpoles transition to a more terrestrial lifestyle (Torgerson et al. 1997a; Gdovin et al. 1999a; Torgerson et al. 2001b; Taylor et al. 2003a; Milsom 2010a). Similar to the classical Miller and Loeschcke chemosensitive areas (Loeschcke 1982), Taylor et al. (2003a) reported rostral (near V) and caudal (near X) chemosensory areas on the ventromedullary surface of brainstems from tadpoles at different stages of metamorphic development (Taylor et al. 2003a). Additionally, the bullfrog locus coeruleus contributes to CO<sub>2</sub> sensitivity, and is located near the rostral chemosensory area (Taylor et al. 2003a; Noronha-de-Souza et al. 2006; Gargaglioni et al. 2010; Santin and Hartzler 2013). To assess the regional influences on respiratory bursts following administration of hypercapnia across bullfrog metamorphosis, we isolated these areas using transections. We observed that CO<sub>2</sub> sensitivity increases from early to late metamorphosis of bullfrogs (Fig. 2.3).

We report lack of central chemosensitivity in early metamorphic tadpoles, except when the brainstem is transected caudal to the glossopharyngeal nerves (Fig. 2.6a). The caudal portion of the brainstem has been proposed to contain buccal rhythm generating elements that have a considerable inhibitory effect on lung bursts (Wilson et al. 2002; Vasilakos et al. 2005; Baghdadwala et al. 2015b). Thus, removing the caudal section of the brainstem may remove inhibition of the lung oscillator and reveal CO<sub>2</sub> sensitivity via this disinhibition. Figure 2.7 shows that lung bursts in late metamorphic tadpoles are robustly chemosensitive, no matter the level of transection. However, this is only observed in early metamorphic tadpole brainstems following caudal transection (Fig. 2.6a). We propose that the primary location for CO<sub>2</sub> sensitivity in the amphibian brainstem is near the previously described lung oscillators, and that these rostral loci contribute to CO<sub>2</sub>-modulated respiratory drive in early metamorphic tadpoles.

Intriguingly, the anatomical position of the amphibian lung oscillator is similar to that of the mammalian parafacial respiratory group and retrotrapezoid nucleus (pFRG/RTN), near the facial nucleus (Wilson et al. 2002; Mulkey et al. 2004; Stornetta et al. 2006). In mammals, this region contributes to CO<sub>2</sub> sensitivity and active expiration, and is essential for lung rhythmogenesis in pre-natal and early post-natal animals (Nattie and Li 1994; Mulkey et al. 2004; Thoby-Brisson et al. 2005; Onimaru et al. 2009; Fortin and Thoby-Brisson 2009; Abbott et al. 2011; Onimaru et al. 2014). Investigators have variously proposed that the pFRG/RTN is homologous to either the lung or buccal oscillators in amphibians (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006; Milsom 2010a; Baghdadwala et al. 2015b). Focal administration of CO<sub>2</sub> into either the rostral chemosensory area, located near the lung oscillators, or the caudal chemosensory area, located near the buccal oscillators, increases fictive lung ventilation (Torgerson et al. 2001b; Taylor et al. 2003a). We removed the buccal oscillator located at the level of the vagus nerves and subsequently recorded lung bursts while exposing the brainstem to varying levels of CO<sub>2</sub>. The results, depicted in Figure 2.7a, suggest that the removed region does not contribute to CO<sub>2</sub> sensitivity. This idea is also supported by the results of Leclère et al. (2012), who found that CO<sub>2</sub> sensitivity persisted after the buccal oscillator were silenced by bathing the isolated brainstem in chloride-free aCSF (Leclère et al. 2012). Thus, we propose that the amphibian buccal oscillator and the mammalian pFRG/RTN are not homologous.

Multiple central CO<sub>2</sub>-sensitive locations have been reported in amphibians and mammals (Loeschcke 1982; Branco et al. 1992; Torgerson et al. 1997a; Torgerson et al. 2001b; Wilson et al. 2002; Taylor et al. 2003a; Noronha-de-Souza et al. 2006; Biancardi et al. 2008; Nattie and Li 2009; Huckstepp and Dale 2011). The locations include the pons and locus coeruleus, pFRG/RTN, and raphe, all of which show some functional homology between amphibians and mammals (Torgerson et al. 1997a; Kinkead et al. 2002; Taylor et al. 2003a; Nattie and Li 2009). While in minor conflict with investigations showing CO<sub>2</sub> sensitivity residing near the buccal oscillators, the current data are consistent with such a diffuse network of brainstem chemosensitivity being present throughout metamorphosis (Torgerson et al. 1997a; Torgerson et al. 2001b; Taylor et al. 2003a).

#### *Rostral pontine influences on the generation of lung and buccal bursts in the amphibian brainstem*

That the pontine areas of the rostral brainstem of mammals contributes to respiration is well documented (see Deutschmann and Dick 2012 for recent review (Deutschmann and Dick 2012)). Kolliker-Fuse (KF) neurons in the mammalian rostral pons are involved in phase shifting from inspiration to expiration (Cohen and Feldman 1977; Rybak et al. 2004; Segers et al. 2008; Smith et al. 2009; Smith et al. 2013). KF neurons are also involved in the Hering-Breuer Reflex, a sensory feedback mechanism from pulmonary stretch receptors that synapse in the nucleus tract solitarius (NTS) (Breuer 1868; Kubin et al. 2006; Deutschmann and Herbert 2006). These second-order NTS neurons relay information to KF neurons, which then inhibit inspiration. These interactions are reciprocal, as both the NTS and pons send and receive projections from each other (Stella 1938; Deutschmann et al. 2000; Alheid et al. 2004; Kubin et al. 2006; Deutschmann and Herbert 2006). In amphibians, structures in the pons – specifically the nucleus isthmi and locus coeruleus (NI and LC, respectively) – contribute to episodic lung burst rhythms (Kinkead et al. 1997; Milsom et al. 1997; Reid et al. 2000). Amphibians typically breathe via episodic lung breaths, consisting of a short expiratory breath and a series of inflation breaths (Vitalis and Shelton 1990; Kogo et al. 1994). Figure 2.2a & c show that episodic lung bursts are lost and single bursts resume following transection of the KF/LC/NI region *in vitro* in frogs, confirming the results of Kinkead et al. (1997), Milsom et al. (1997), and Reid et al. (2000). In late metamorphic tadpoles, post-transection burst shapes show increased time to trough durations (Fig. 2.7d). It is

possible the rostral pons in amphibians contributes to timing and phase shifting of lung bursts, similar to its function in mammals. However, in mammals, lesion of KF neurons increases tidal volume via inhibition of post-inspiratory activity, while removing this area in amphibians reduces episodic breathing and seemingly reduces overall tidal volume (Berger et al. 1978; Alheid et al. 2004; Dutschmann and Herbert 2006; Smith et al. 2009).

Apart from influencing the episodicity of lung bursts in amphibians, the rostral pontine neurons in amphibians seem to play a role in timing and frequency of buccal bursts. Following transection of the rostral pons, buccal bursts were sensitive to changes in CO<sub>2</sub>, and increased in duration both in “time to peak” and “time to trough” (Fig. 2.9). These changes were more apparent in late metamorphic animals. In early metamorphic animals, buccal bursts were detected in 4/6 preparations following transection, but burst shapes were more consistent under hypercapnic conditions. The effects of hypercapnia on buccal burst shape appear to be similar in both early and late metamorphic animals, reducing burst duration and increasing the frequency of events. Taken together these results suggest that the rostral pons likely sends excitatory descending projections to both buccal and lung rhythm generating sites in the medulla and are involved in burst termination for buccal and lung bursts in amphibians.

#### *Buccal rhythms appear to be chemosensitive after rostral transection*

The pons, lung oscillator, and buccal oscillator form a potential circuit whose interactions can explain buccal respiratory sensitivity to CO<sub>2</sub>. Studies of the effects of CO<sub>2</sub> on buccal bursts in amphibians have produced conflicting reports (Torgerson et al. 1997b; Gdovin et al. 1999b; Remmers et al. 2001; Torgerson et al. 2001b; Taylor et al. 2003b; Quenet et al. 2014). Torgerson et al (1998) reported that buccal bursts are centrally sensitive to pH changes in the middle stages of metamorphosis, while Taylor et al (2003) reported that CO<sub>2</sub> does not influence buccal ventilation (Torgerson et al. 1997b; Torgerson et al. 2001b; Taylor et al. 2003b). Focal acidification of CO<sub>2</sub> sensitive sites near the buccal oscillator did not stimulate buccal bursts in bullfrogs (Taylor et al. 2003b; Taylor et al. 2003a), and there is no evidence of CO<sub>2</sub> influencing buccal bursts in isolated fish brainstems (Sundin et al. 2007; Côté et al. 2014). Our data show buccal bursts are sensitive to changes in pH/CO<sub>2</sub>, but only after the rostral brainstem has been removed. A possible explanation for the discrepancy in findings among these studies is the methodology of isolated brainstem

preparations differed between Torgerson and Taylor (Torgerson et al. 1997b; Torgerson et al. 2001b; Taylor et al. 2003b). Torgerson severed the brainstem immediately rostral to the trigeminal nerves (V), while Taylor severed the brainstem rostral to the optic tectum (Torgerson et al. 1997b; Torgerson et al. 2001b; Taylor et al. 2003b). Our data support the results of both methods, in that isolated brainstems that include the optic tectum do not display buccal CO<sub>2</sub> sensitivity, but those transected at the level of the trigeminal nerves do (Fig. 2.9). While this yields insight into discrepant results, it does not reveal the mechanism by which transecting the rostral pons would produce or unmask a CO<sub>2</sub>-sensitive buccal rhythm.

The buccal oscillator drives the rhythmic movements of the buccal cavity that are responsible for gill breathing in fish and larval amphibians. This ventilatory behavior is influenced by peripheral chemoreceptors primarily found on gill arches (Jia and Burggren 1997a; Jia and Burggren 1997b; Smatresk and Smits 1991; Reyes et al. 2014). However, there is some evidence that central chemoreception exists near the buccal oscillators, but these sites only promoted changes in lung bursts in late metamorphic bullfrog tadpoles (Torgerson et al. 2001b; Taylor et al. 2003b). While it is possible the buccal oscillator are intrinsically CO<sub>2</sub> sensitive but under tonic inhibition from the pons, it is unclear why a central CO<sub>2</sub>-sensitive mechanism directly influencing the buccal oscillator would modulate gill ventilation.

Alternatively, and perhaps more likely, the lung oscillator may provide mild CO<sub>2</sub>-sensitive stimulation to the buccal oscillators. The lung oscillator in amphibians has been proposed to be coupled with, and have a stimulatory effect on, the buccal oscillator (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006). While buccal bursts are at most mildly chemosensitive in isolated bullfrog brainstems, CO<sub>2</sub> has a vigorous effect on lung bursts in late metamorphic brainstems (Fig. 2.7). In mammals, the coupled pFRG/RTN and Pre-Bötzinger Complex (PreBötC) oscillators demonstrate a similar response to CO<sub>2</sub> (Thoby-Brisson and Greer 2008; Fortin and Thoby-Brisson 2009; Thoby-Brisson et al. 2009; Dubreuil et al. 2009; Guyenet and Mulkey 2010; Marina et al. 2010; Mellen and Thoby-Brisson 2012; Wang et al. 2013). Focal administration of CO<sub>2</sub> on the pFRG/RTN increases neuron activity and efferent output to the lungs (Mulkey et al. 2004; Dubreuil et al. 2009; Marina et al. 2010; Guyenet and Mulkey 2010; Wang et al. 2013), but when applied to the PreBötC, focal CO<sub>2</sub> induces at most mild changes in activity (Solomon 2003). Baghdawala et al. (2015) proposed amphibian lung and buccal



oscillators may be homologous to the mammalian pFRG/RTN and PreBötC, respectively (Baghdadwala et al. 2015a). In this case, hypercapnia would be expected to increase buccal bursts.

As described above, the pons is thought to influence respiratory burst shape, in part by stimulating inhibitory neurons, which effectively terminate the burst. The pons (directly) and the lung oscillator (indirectly/directly) are sensitive to CO<sub>2</sub> (Kinkead et al. 1997; Torgerson et al. 1997b; Torgerson et al. 2001b; Taylor et al. 2003b; Noronha-de-Souza et al. 2006; Santin and Hartzler 2013). We propose these areas have connections to the buccal oscillators. According to this model, buccal oscillations would be stimulated by CO<sub>2</sub> due to influence from the lung oscillators. However, this would not be observable in the intact brainstem because this mild stimulation of buccal rhythm by the lung oscillator would be counteracted by inhibitory influences from the pons. The pons is CO<sub>2</sub> sensitive and hypercapnia would increase pontine stimulation of inhibitory neurons to inhibit buccal bursts. Therefore, pontine transection would result in increases of buccal burst duration, by removing inhibitory drive from the buccal oscillators. This would allow the CO<sub>2</sub>-sensitive lung oscillator to stimulate buccal oscillator directly, absent the counteracting inhibitory influence of the pons. In this model, removing the pons would “reveal” mild buccal sensitivity to CO<sub>2</sub>, as observed in this investigation and that of Torgerson and colleagues (Torgerson et al. 1997b; Torgerson et al. 2001b).

*Do inhibitory cells surround and provide tonic inhibition to the lung oscillators?*

Inhibition plays a critical role in rhythmogenesis (Smith et al. 2000; Dutschmann and Paton 2002; Smith et al. 2009; Janczewski et al. 2013; Koizumi et al. 2013). In mammals, rhythmogenic circuits have strong surrounding inhibitory inputs, as seen in the Böttinger Complex and the PreBötC (Smith et al. 2000; Dutschmann and Paton 2002; Smith et al. 2009; Morgado-Valle et al. 2010; Janczewski et al. 2013; Koizumi et al. 2013). Further, the pFRG/RTN appears to be under tonic inhibition, and can be disinhibited via antagonists to GABA<sub>A</sub> receptors (Nattie et al. 2001; Pagliardini et al. 2011). It is unknown if a similar excitatory network embedded within a large inhibitory network exists in amphibians. An intriguing observation gleaned from Klingler et al. (2013), Torgerson et al. (1998 and 2001b), and the current results may provide insight to this question. In the Torgerson et al. (2001b) transection investigation, the rostral portion of the medulla, between the trigeminal and glossopharyngeal nerves, was

isolated resulting in an absence of lung activity. However, a brainstem preparation including the portion rostral to the trigeminal nerves (V) and transected at the glossopharyngeal nerves (IX) produces fictive lung breaths as seen in Klingler et al. (2013) and this work (Fig. 2.5). If the reverse experiment is conducted by transecting at the trigeminal nerves and leaving the rest of the brainstem intact, lung rhythms persist (Fig. 2.4). The lung oscillator appears to be sufficient to exhibit rhythmic lung bursts (Wilson et al. 2002). As we demonstrate here, neither the area rostral to the trigeminal nerves, nor caudal to the glossopharyngeal nerves appear to be critical for lung rhythmogenesis to persist. However, when both areas are removed and this small region between the trigeminal and glossopharyngeal nerves is isolated, lung bursts are absent.

The amphibian lung oscillators, specifically their generation of lung bursts, may be under control from either the rostral or caudal portions of the brainstem. While this is possible, based on studies to date, both the mammalian pFRG/RTN and PreBötC exhibit endogenous burst activity in isolation and when coupled together (Thoby-Brisson et al. 2005; Fortin and Thoby-Brisson 2009; Onimaru et al. 2009; Onimaru et al. 2014). Alternatively, inhibitory cells providing either tonic or phasic inhibition may surround the amphibian lung oscillator. If inhibitory cells surround an excitatory lung oscillator, transecting the pons and caudal brainstem may create a state of low excitation and high inhibition in regards to the lung oscillator activity.

## **2.6 Conclusion**

The amphibian brainstem generates lung bursts following removal of ascending or descending inputs to the lung oscillator across metamorphosis. Sensitivity of lung bursts to CO<sub>2</sub> increases during metamorphosis, but is present even in early metamorphic tadpoles. The current results help clarify previous contradictory findings, and provide insight into pontine influences on both the frequency and shape of buccal and lung bursts. We propose that the buccal oscillator is not homologous to the pFRG/RTN of mammals. We also propose the amphibian lung oscillator is tonically inhibited by surrounding inhibitory neurons. If this is the case, then application of GABA and glycine receptor antagonists would potentially reveal intrinsic burst activity. Identifying functional characteristics of the isolated lung oscillator may provide useful insight into the evolution and potential homologies of brainstem respiratory activity among vertebrates.

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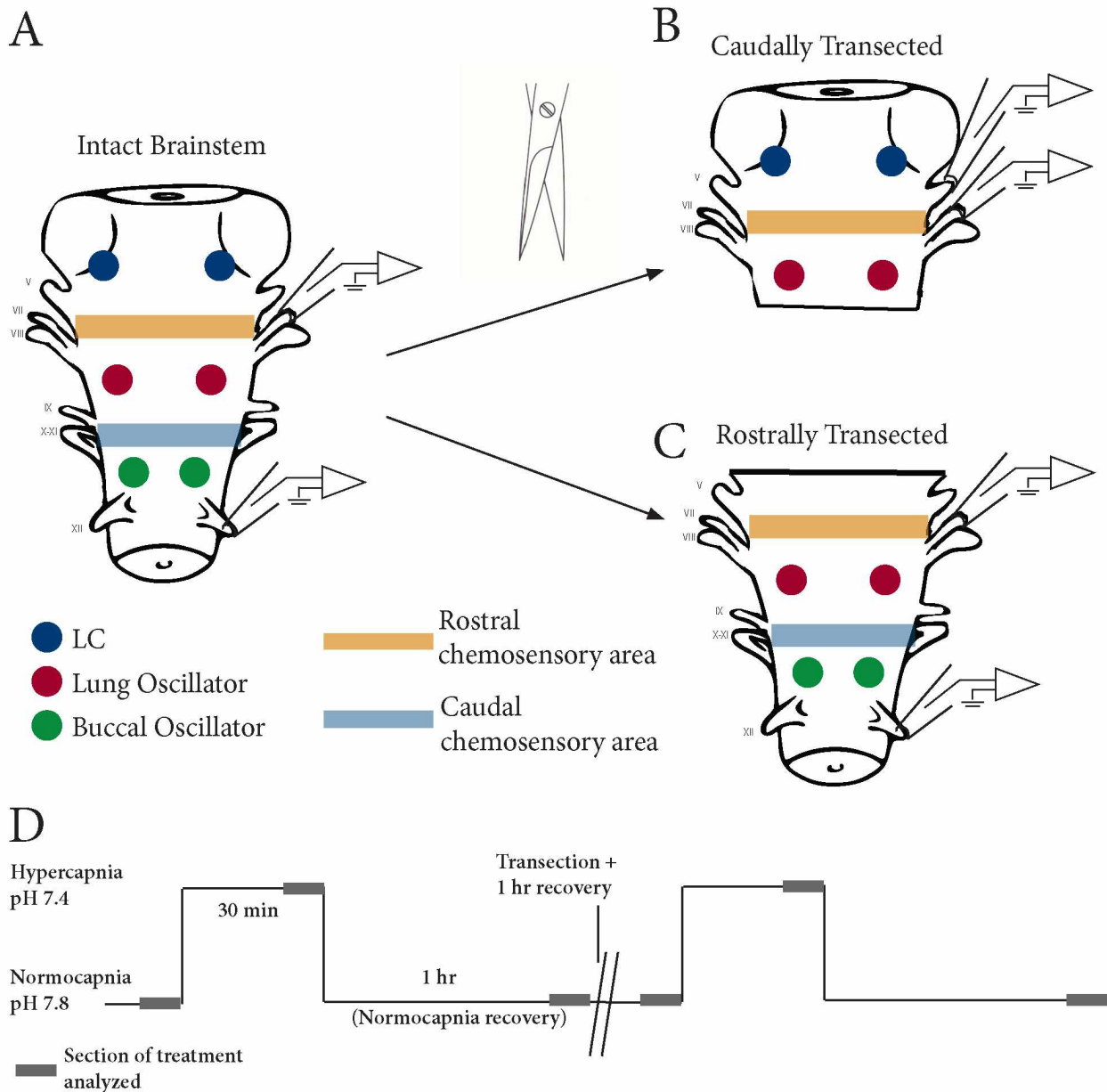
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## 2.8 Figures



**Figure 2.1: Recordings were made from intact, rostrally transected, and caudally transected early and late metamorphic tadpole brainstems.** (A) Intact brainstems were transected (B) between the lung and buccal oscillator at the level of the glossopharyngeal nerve (IX, Caudally Transected), or (C) between the LC and lung oscillator at the level of the trigeminal nerve (V, Rostrally Transected). (A-C) Burst discharges were measured from the trigeminal (V), facial (VII), and hypoglossal (XII) nerves in each brainstem (intact, rostrally transected, or caudally transected). (D) A hypercapnic treatment was administered both before and after transection, and the last 5 minutes of each gas treatment was measured.

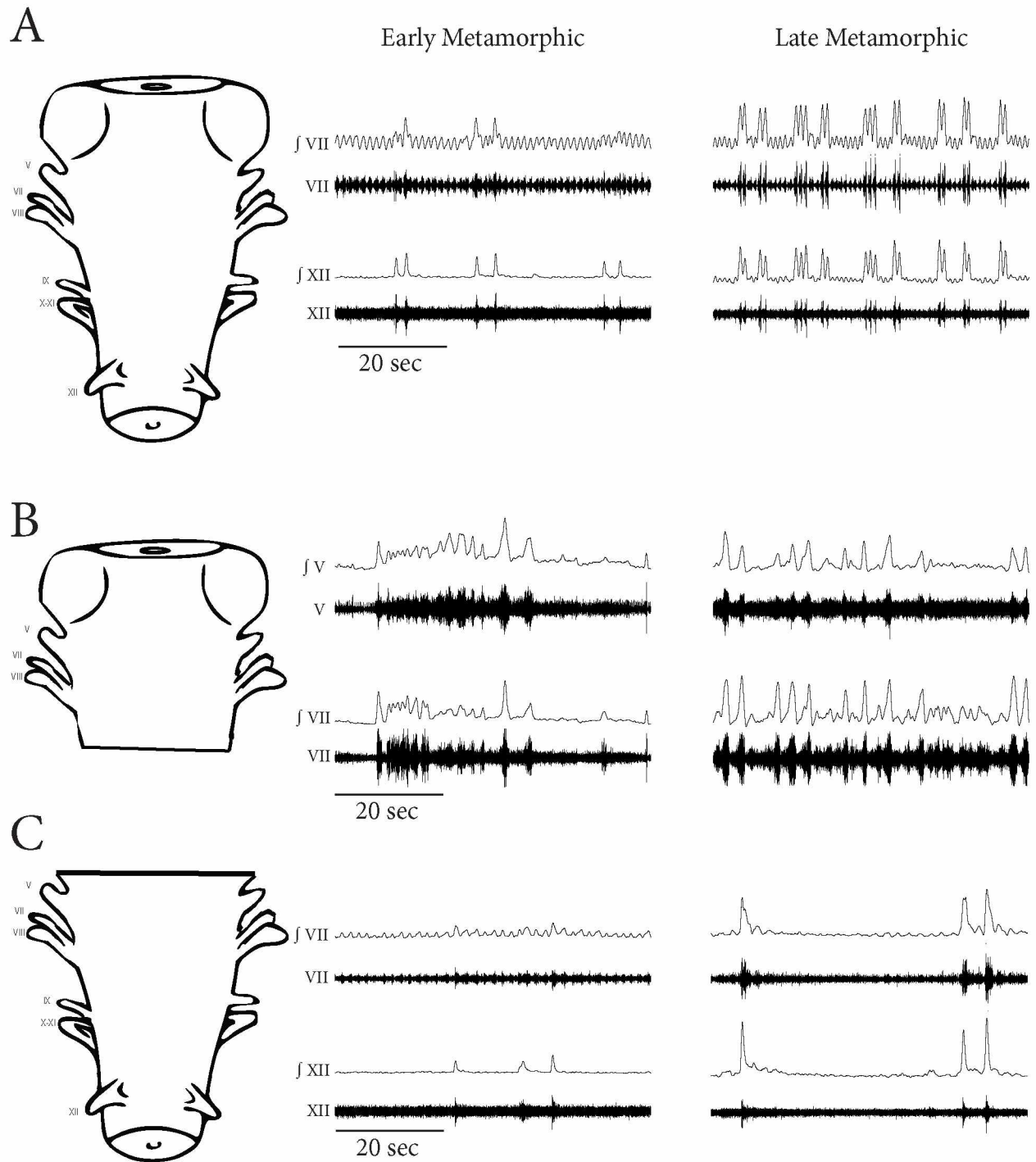
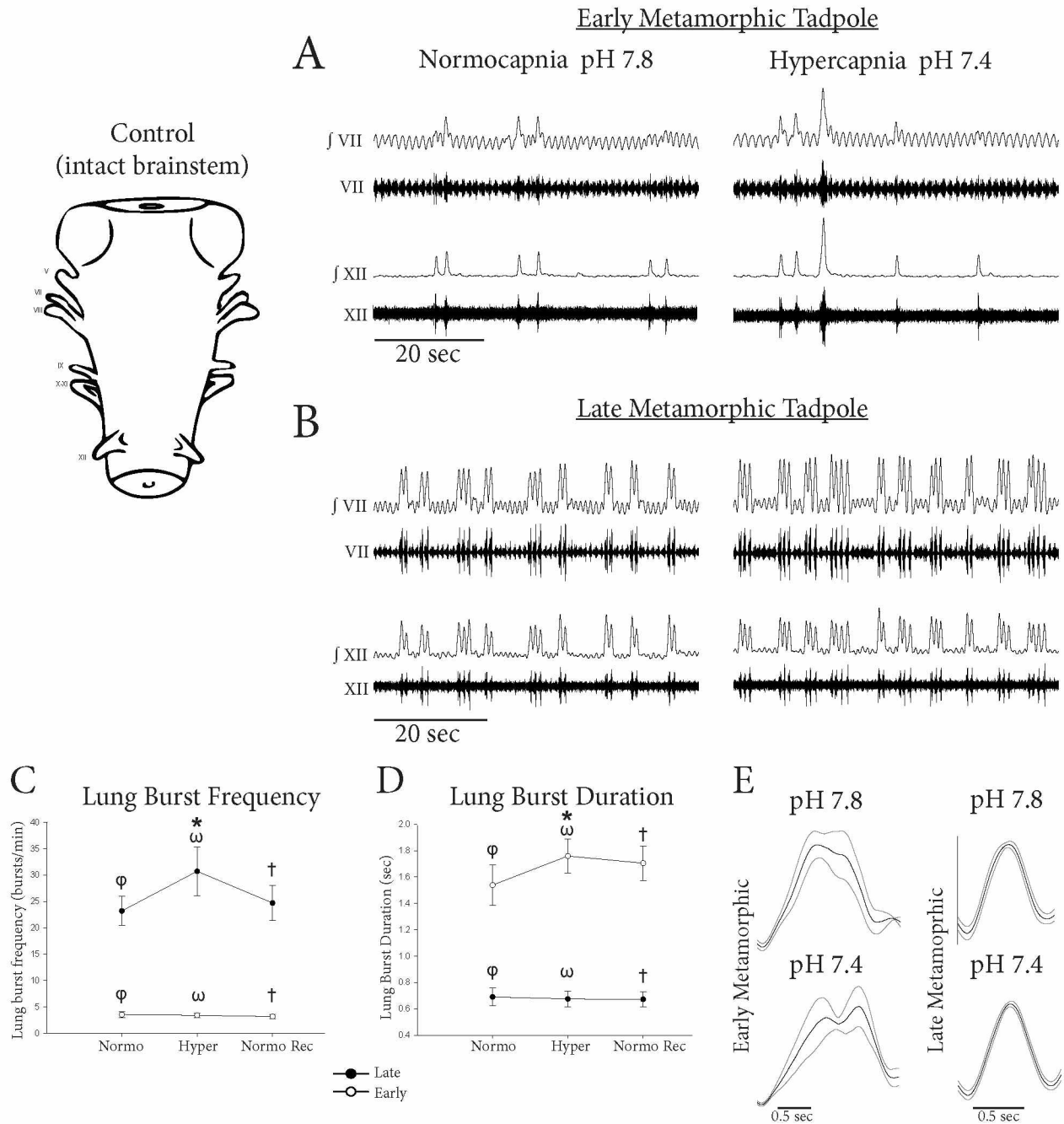


Figure 2.2: Rhythmic respiratory bursts were observed in early and late metamorphic animals regardless of transection. Whole-nerve recordings (raw and integrated “f”) were made from intact, rostrally transected (transected at the level of the trigeminal nerves), and caudally transected (transected at the glossopharyngeal nerves) brainstems. (A) Recordings from intact brainstems from early and late metamorphic tadpoles produced both buccal and lung bursts. (B) Lung and small amplitude bursts were detected in caudally transected brainstem recordings from

Figure 2.2 cont.

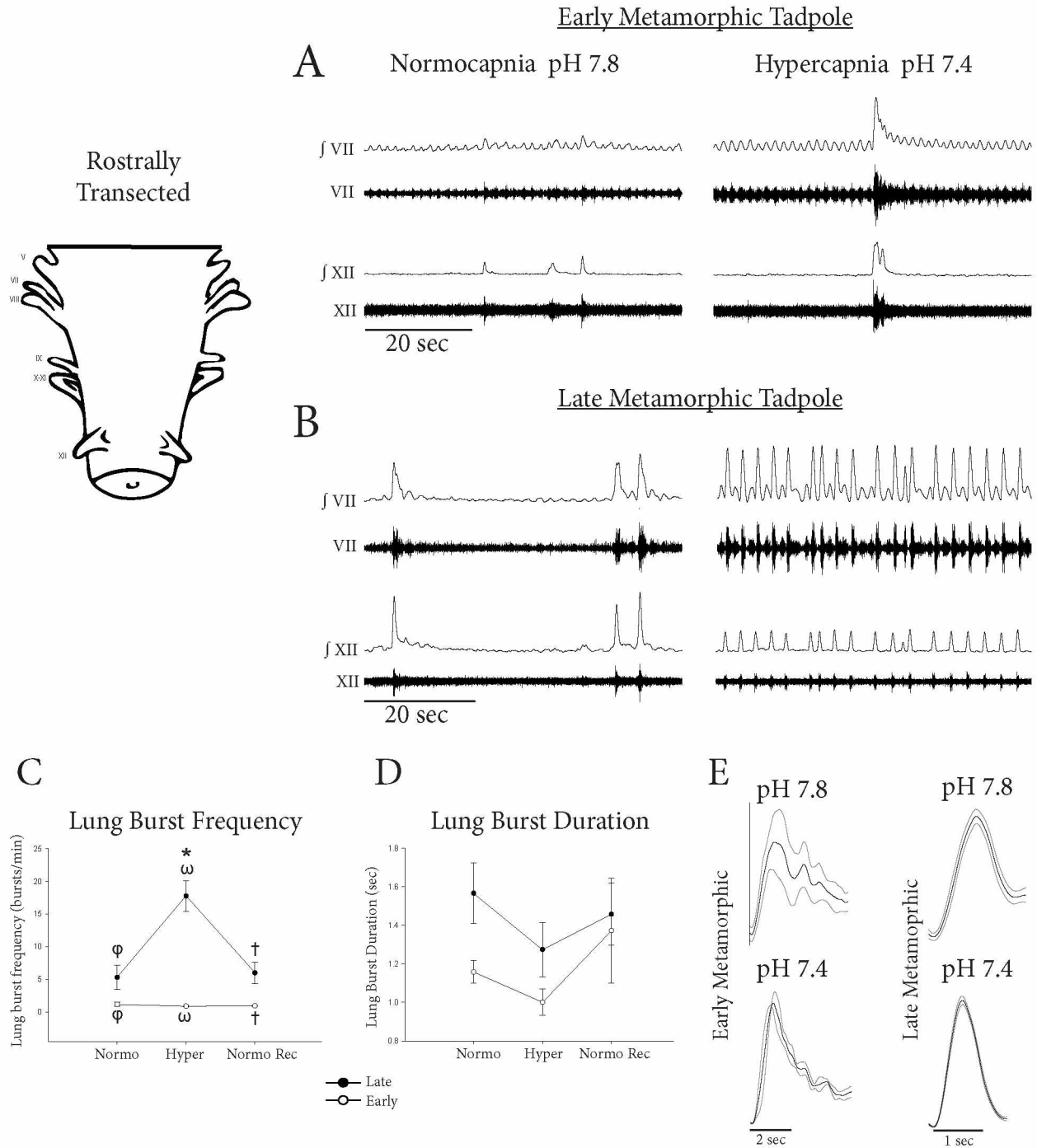
early and late metamorphic brainstems. (C) Lung and buccal bursts were also detected in early and late metamorphic rostrally transected brainstems.



**Figure 2.3: Hypercapnia stimulates lung bursts in late, but not early, metamorphic tadpoles.** Whole-nerve discharge (raw and integrated “f”) was recorded from the facial (VII) and hypoglossal (XII) cranial nerves in normocapnic conditions (Normo, pH 7.8) and hypercapnic (Hyper, pH 7.4) conditions from intact brainstems of early and late metamorphic tadpoles. (A and B) Both buccal and lung bursts were observed in early and late metamorphic tadpoles in both normocapnic and hypercapnic conditions. (C) Lung bursts were observed less frequently in early stage animals (open circles) compared to late stage (filled circles), regardless of pH. Hypercapnia

Figure 2.3 cont.

produces a significant increase in lung frequency (bursts/min) in late, but not early, metamorphic tadpoles. (D) Increases in burst frequency were not associated with changes in lung burst duration in early or late metamorphic tadpoles. (E) Waveform averages of lung bursts (gray lines =  $\pm 1$  SEM) differed between early and late metamorphic animals, but did not change with hypercapnia. Significant differences ( $p < 0.05$ ) between gas treatments are denoted by (\*). Symbols denote  $p < 0.05$  between means (e.g. means labeled “ $\phi$ ” differ from each other, means labeled “ $\omega$ ” differ from each other, etc.).



**Figure 2.4: Rostral transection affects lung burst frequency, but not hypercapnic response.**

Whole-nerve discharge (raw and integrated “f”) was recorded from the facial (VII) and hypoglossal (XII) cranial nerves in normocapnic conditions (Normo, pH 7.8) and hypercapnic (Hyper, pH 7.4) conditions from rostrally transected brainstems of early and late metamorphic tadpoles. (A and B) Lung and buccal bursts were observed following transection in both early and late metamorphic animals. (C) Hypercapnia stimulated lung burst frequency (bursts/min) in late

Figure 2.4 cont.

metamorphic animals (filled circles), but not early (open circles). (D) Lung burst durations were similar between early and late metamorphic animals, and were not significantly affected by hypercapnia. (E) Waveform averages of lung bursts (gray lines =  $\pm 1$  SEM) differed between early and late metamorphosis, and demonstrated increased variability in early metamorphic animals. Significant differences ( $p < 0.05$ ) between gas treatments are denoted by (\*). Symbols denote  $p < 0.05$  between means (e.g. means labeled “ $\varphi$ ” differ from each other, means labeled “ $\omega$ ” differ from each other, etc.).



Early Metamorphic Tadpole

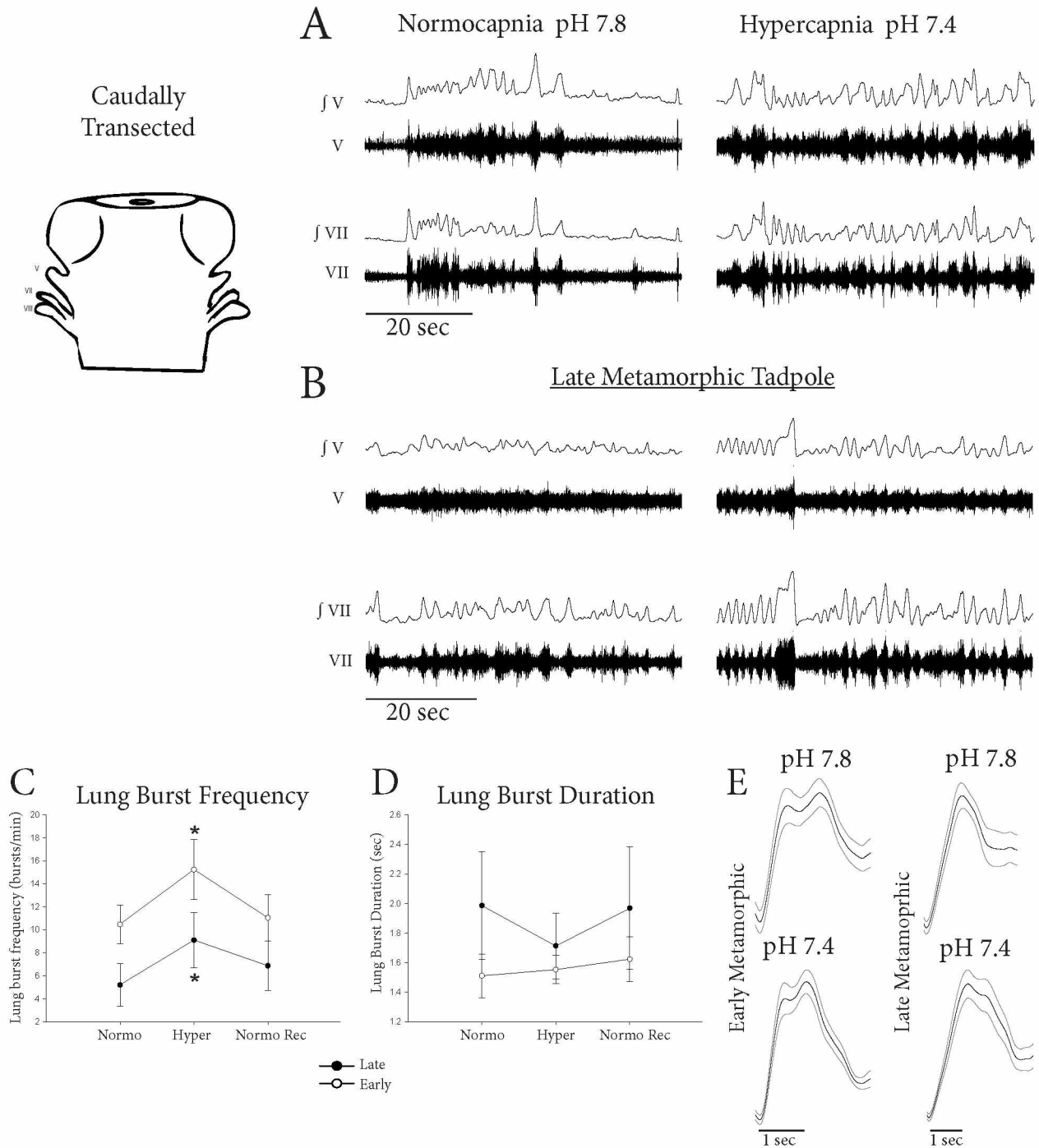
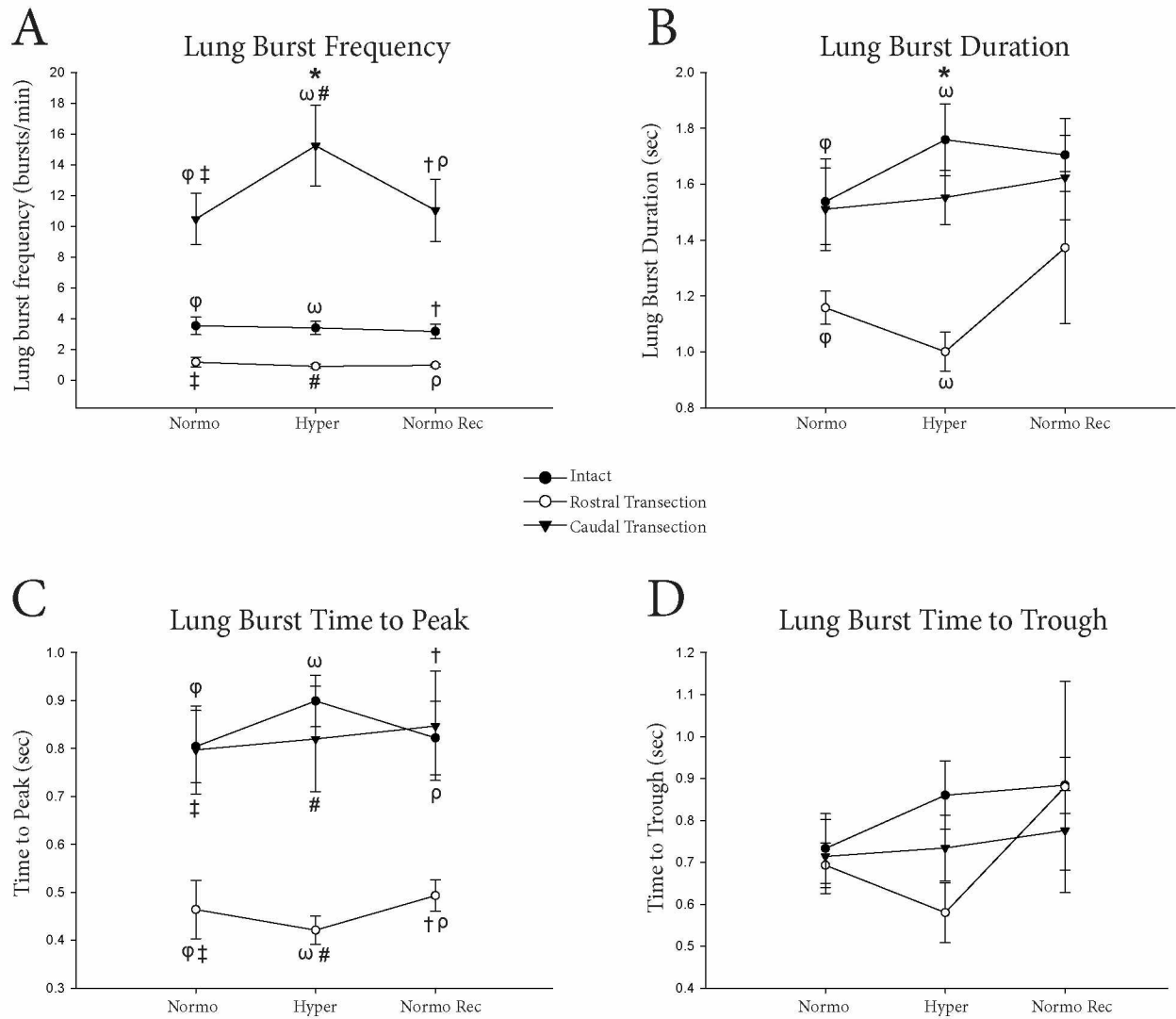


Figure 2.5: Hypercapnic responses were observed in both early and late metamorphic animals following caudal transection. Whole-nerve discharge (raw and integrated “f”) was recorded from the trigeminal (V) and facial (VII) nerves in normocapnic conditions (Normo, pH 7.8) and hypercapnic (Hyper, pH 7.4) conditions from caudally transected brainstems of early and late metamorphic tadpoles. (A and B) Lung and small amplitude bursts were observed following

Figure 2.5 cont.

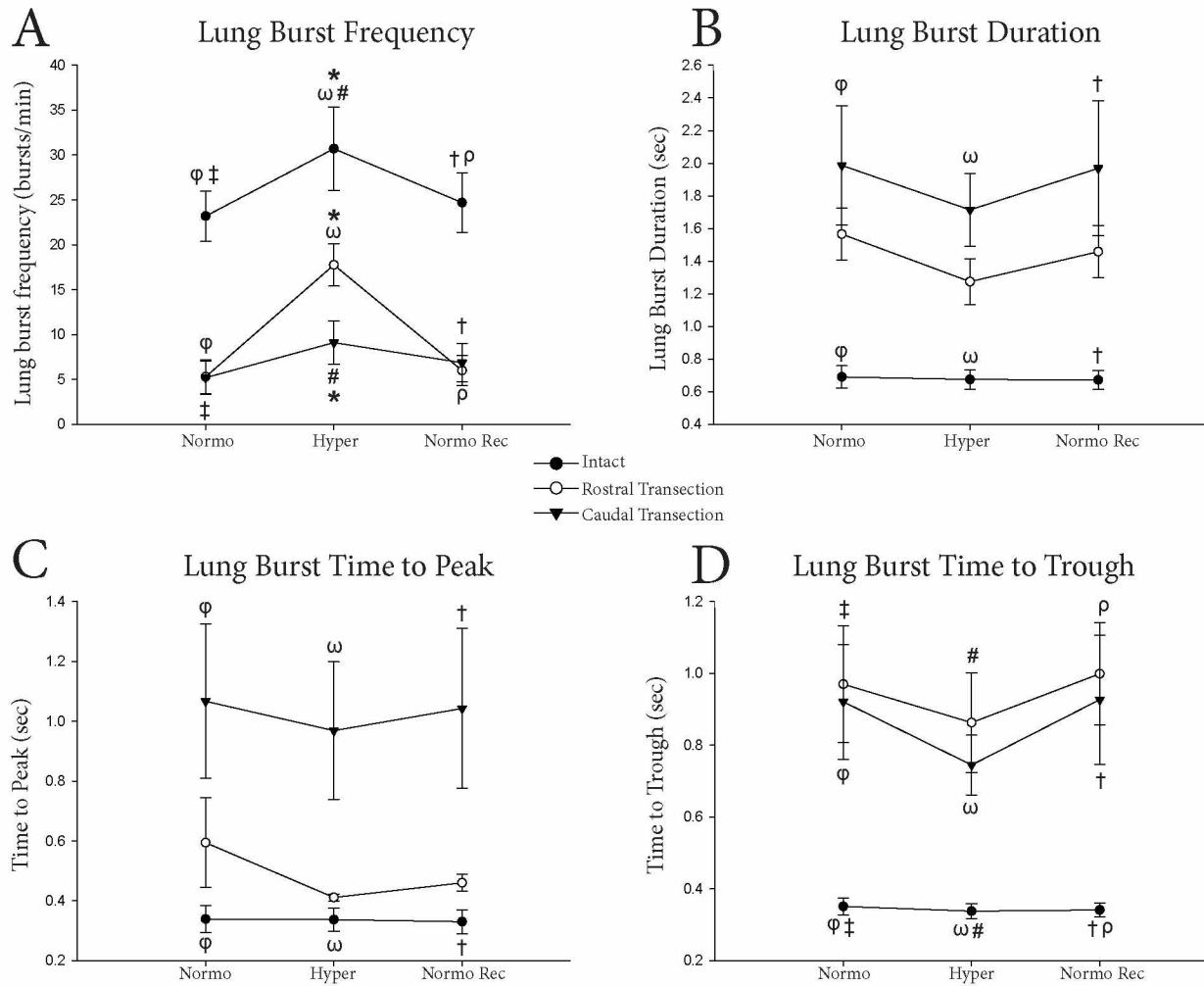
transection in both early and late metamorphic animals. (C) Lung burst frequency (bursts/min) in early metamorphic animals (open circles) was more frequent compared to late (filled circles), but both metamorphic groups were equally sensitive to increased CO<sub>2</sub>. (D) Durations of lung bursts were not significantly different between early and late metamorphic animals, or during hypercapnia. (E) Waveform averages for lung bursts (gray lines = ±1 SEM) in early and late metamorphic animals were largely similar and did not change in response to hypercapnia. Significant differences ( $p < 0.05$ ) between gas treatments are denoted by (\*).

## Early Metamorphic Lung Bursting

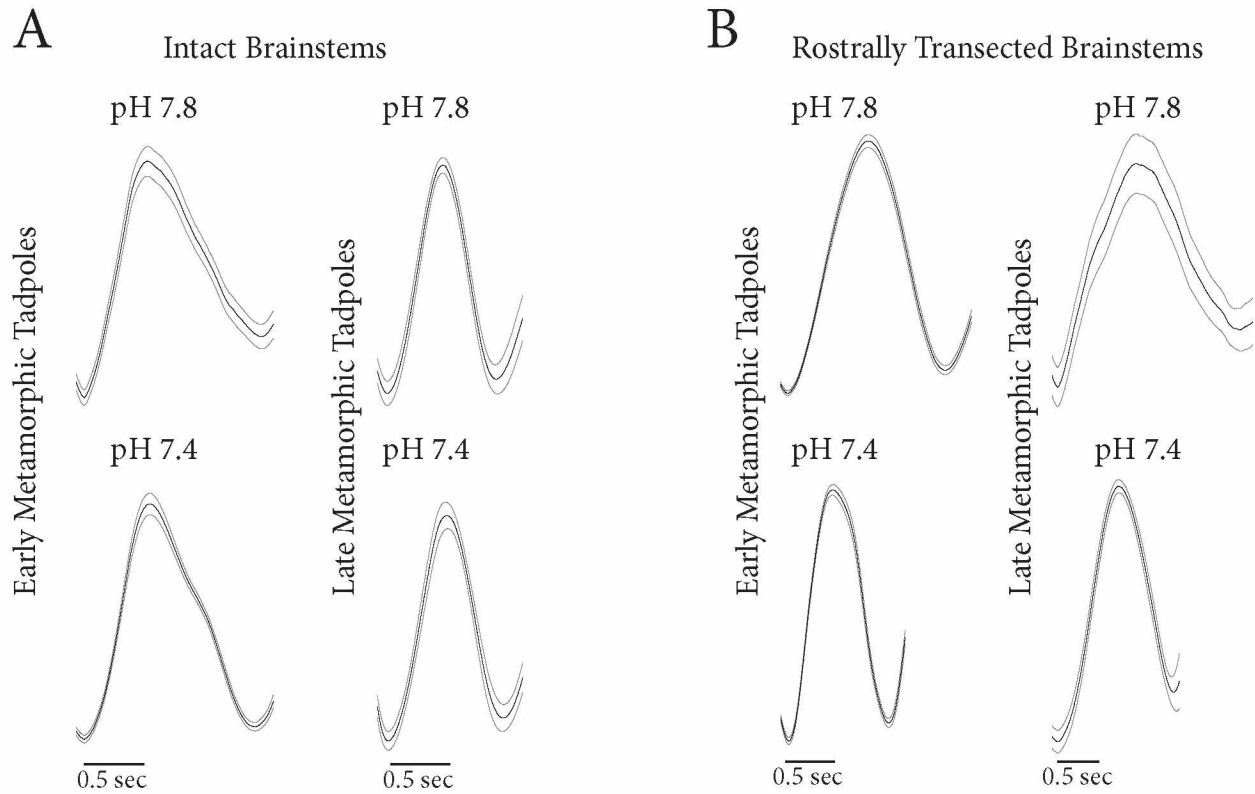


**Figure 2.6: Rostral transection affects lung burst duration while caudal transection affects lung burst frequency in early metamorphic animals.** The effects of transections (caudal transection = filled triangles, rostral transection = open circles) compared to intact (filled circles) early metamorphic brainstems were compared measuring burst frequency (bursts/min), burst duration (sec), time to peak (sec), and time to trough (sec). (A) Lung burst frequency and sensitivity to hypercapnia (Hyper), as compared to normocapnia (Normo), was significantly affected following caudal transection of the brainstem. (B) Rostral transections produced a significant reduction in lung burst duration compared to intact brainstems. (C & D) Reduction in duration by rostral transections manifested as a significant decrease in time to peak duration, while time to trough remained unchanged. Significant differences ( $p < 0.05$ ) between gas treatments are denoted by (\*). Symbols denote  $p < 0.05$  between means (e.g. means labeled “ $\phi$ ” differ from each other, means labeled “ $\omega$ ” differ from each other, etc.).

## Late Metamorphic Lung Bursting

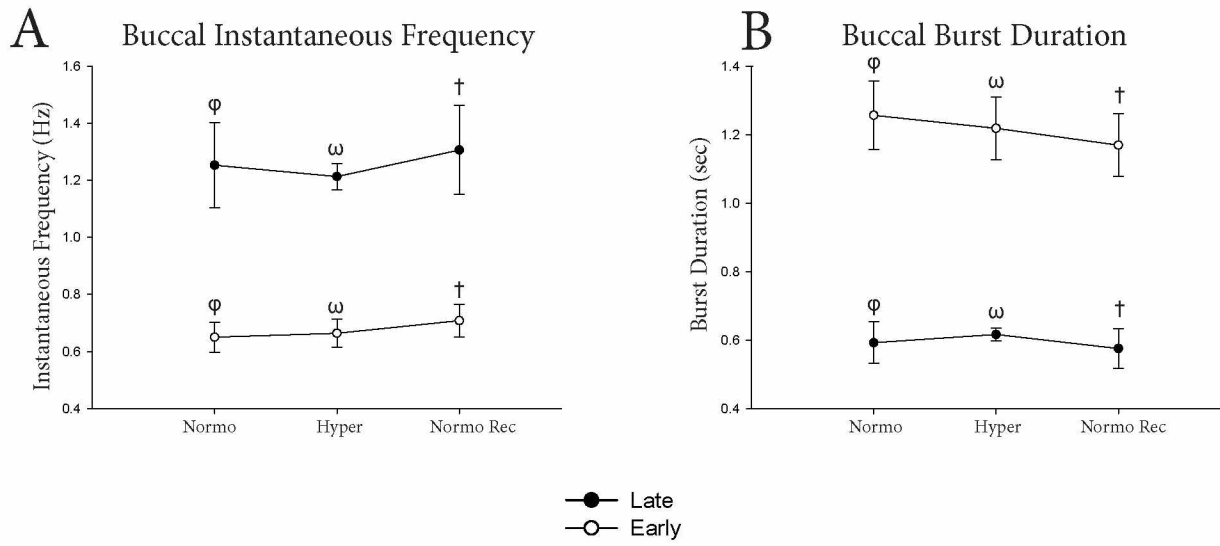


**Figure 2.7: Rostral or caudal transection each affected both lung burst frequency and duration, but not sensitivity to CO<sub>2</sub>.** The effects of transections (caudal transection = filled triangles, rostral transection = open circles) compared to intact (filled circles) late metamorphic brainstems were compared measuring burst frequency (bursts/min), duration (sec), time to peak (sec), and time to trough (sec). (A) Either rostral or caudal transections reduced lung burst frequency but did not affect CO<sub>2</sub> sensitivity (Normo vs Hyper) as compared to intact brainstems (filled circles). (B) Lung burst duration was significantly increased following caudal, but not rostral, transections of brainstems from late metamorphic animals. (C) Caudal transections also produced significantly larger time to peak durations compared to intact brainstems. (D) Rostral and caudal transections produced significant changes in time to trough durations for lung bursts as compared to intact brainstems. Significant differences ( $p < 0.05$ ) between gas treatments are denoted by (\*). Symbols denote  $p < 0.05$  between means (e.g. means labeled “ $\phi$ ” differ from each other, means labeled “ $\omega$ ” differ from each other, etc.).

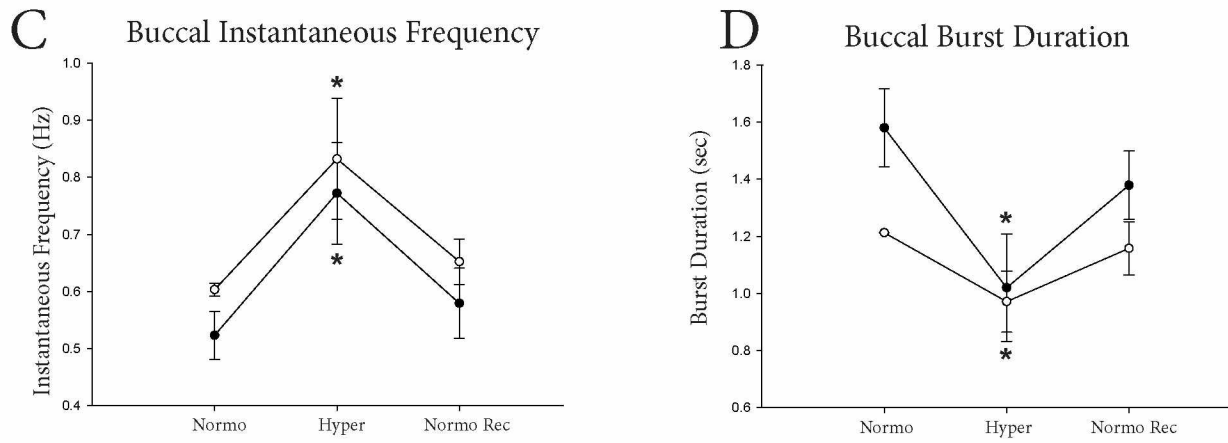


**Figure 2.8: Hypercapnia affects the shape of buccal bursts in rostrally transected brainstems.** Waveform averages for buccal bursts (gray lines =  $\pm 1$  SEM) were generated for control and rostral transections in early and late stage brainstems. (A) In intact brainstems, hypercapnia (pH 7.4) did not alter the average shape of buccal bursts regardless of developmental stage as compared to normocapnia (pH 7.8). (B) The shape of buccal bursts were altered by hypercapnia in brainstems that were rostrally transected.

### Intact Brainstem



### Rostrally Transected Brainstem



**Figure 2.9: Rostral transection produces buccal rhythms sensitive to hypercapnia in early and late metamorphic animals.** Instantaneous frequency (Hz) and buccal burst duration were compared from intact (filled circles) or rostrally (open circles) transected early and late metamorphic brainstems. (A) Late metamorphic (filled circles) brainstems had a significantly higher buccal burst frequency compared to early metamorphic (open circles) in intact brainstems. (B) Buccal burst durations measured from intact brainstems were significantly longer in early, as compared to late, metamorphic tadpoles. (C) Rostrally transecting early and late metamorphic brainstems produced a buccal burst rhythm that was sensitive to hypercapnia (Hyper) as compared to normocapnia (Normo). (D) Sensitivity was mediated by a decrease in burst duration in both early and late metamorphic animals. Significant differences ( $p < 0.05$ ) between gas treatments are denoted by (\*). Symbols denote  $p < 0.05$  between means (e.g. means labeled “ $\phi$ ” differ from each other, means labeled “ $\omega$ ” differ from each other, etc.).





## Chapter 3

### Respiratory rhythmogenesis in the isolated rostral medulla of the tadpole brainstem<sup>1</sup>

#### 3.1 Abstract

Respiratory behavior in terrestrial animals is primarily controlled via neural circuits in the medulla. A site in the amphibian rostral medulla at the level of the abducens cranial nerve (VI) is believed to be homologous to the Pre-Bötzinger Complex in mammals. The  $\mu$ -opioid receptor agonist DAMGO has a suppressive effect, and the GABA<sub>A</sub> receptor antagonist bicuculline has an excitatory effect on lung activity in mammals and amphibians. However, the locations of action in amphibians are unknown. In the current study we test the hypothesis that there is functional similarity between the lung oscillator in bullfrogs (*Lithobates catesbeianus*) and structures responsible for respiratory rhythmogenesis in mammals. We examined the effect of DAMGO and bicuculline on the isolated rostral medulla of the brainstem. Isolated brainstems from early metamorphic bullfrog tadpoles were transected leaving a region of brainstem from the trigeminal cranial nerve (V) to the glossopharyngeal cranial nerve (IX) intact, isolating the lung oscillator and corresponding motor nerves. Whole-nerve recordings were made from the trigeminal (V) and facial (VII) cranial nerves while we tested the lung oscillator for sensitivity to opioids (DAMGO), GABAergic (bicuculline) and glycinergic (strychnine) inhibition, and glutamatergic neurotransmission (CNQX). The transection alone resulted in a loss of discernable respiratory rhythm. However, bicuculline application caused a lung-like episodic rhythm to reappear. Contrary to the suppressive effect DAMGO has on lung activity in other preparations (including intact isolated amphibian brainstems), lung activity was not affected by the addition of DAMGO in our transected preparation. Results support the conclusion that there is functional similarity between the lung oscillator in bullfrogs and the pFRG/RTN in mammals.

#### 3.2 Introduction

Gas exchange is essential for life because it provides O<sub>2</sub> as an electron acceptor in metabolic reactions and removes CO<sub>2</sub>, a metabolic waste. Similarities in central respiratory

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<sup>1</sup> Reed MD, Iceman KE, Harris MB, Taylor BE. Respiratory rhythmogenesis in the isolated rostral medulla of the tadpole brainstem. *Am J Physiol Regul Integr Comp Physiol*. (In preparation)



control in amphibians, reptiles, and mammals have led many to conclude that components of the respiratory control network are evolutionarily conserved (Milsom et al. 1999; Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006; Milsom 2010; Baghdadwala et al. 2015). However, respiratory control networks differ between vertebrate groups, and these differences have not been fully elucidated in amphibians.

Amphibians breathe using a buccal force pump, which is responsible for both gill and lung rhythmic ventilation depending on the medium – water or air (Kogo et al. 1994; Milsom et al. 1999; Gargaglioni and Milsom 2007). The neural coordination of this pump appears to be generated from two sets of coupled oscillators located in the brainstem: the lung and buccal oscillators (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006). The buccal oscillator, located at the level of the vagus cranial nerve (X), generates the timing of buccal movements involved in gill ventilation (Wilson et al. 2002). Isolated brainstem preparations derived from bullfrog (*Lithobates catesbeianus*) tadpoles produce patterns of activity in cranial and spinal nerves corresponding to buccal and lung ventilation. Microinjections of GABA into the buccal oscillator region reduce buccal burst occurrence and increase lung burst occurrence, suggesting the buccal oscillator inhibits the lung oscillator (Wilson et al. 2002). This observation, and the fact that buccal rhythmicity is the baseline respiratory-related activity from cranial nerves, has led investigators to infer that the buccal oscillator is the dominant oscillator in the amphibian brainstem (Wilson et al. 2002; Vasilakos et al. 2005; Bose et al. 2005; Baghdadwala et al. 2015). Buccal bursts are dependent on chloride-mediated inhibition and are insensitive to opioids (Galante et al. 1996; Straus et al. 2000; Broch et al. 2002; Vasilakos et al. 2006; Leclère et al. 2012). Generation of lung bursts in amphibian brainstems is attributed to rhythmic activity of the lung oscillator located at the level of the abducens cranial nerve (VI), between the facial (VII) and glossopharyngeal (IX) cranial nerves (Wilson et al. 2002). Similar to investigations of the buccal oscillator, injections of GABA into this region inhibit lung bursts but do not disrupt buccal activity. Lung bursts are also abolished by application of opioids or the non-NMDA glutamate receptor antagonist CNQX (Vasilakos et al. 2005; Vasilakos et al. 2006; Chen and Hedrick 2008; Ranohavimparany et al. 2014).

Mammals breathe using an aspiration pump that draws air into the lungs. Neural coordination of this pump in embryonic and neonatal mammals involves two coupled oscillators: the parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN) and the Pre-Bötzinger complex (PreBötC) (Onimaru and Homma 2002; Thoby-Brisson and Greer 2008; Thoby-Brisson et al. 2009; Champagnat et al. 2009; Fortin and Thoby-Brisson 2009; Champagnat et al. 2011; Mellen and Thoby-Brisson 2012). The PreBötC has been called the “kernel” of respiration, and is crucial for inspiratory rhythm generation in mammals (Smith et al. 1991; Solomon 2003; Feldman and Del Negro 2006). Residing at the level of the hypoglossal nerve in mammals, the PreBötC contains a population of pacemaker-like neurons that appear to be mildly chemosensitive (Smith et al. 1991; Solomon et al. 2000; Janczewski and Feldman 2006; Feldman and Del Negro 2006; Tan et al. 2008). Similar to the lung oscillator in amphibians, application of the  $\mu$ -opioid receptor agonist DAMGO attenuates activity produced at this site (Janczewski et al. 2002; Mellen et al. 2003; Thoby-Brisson et al. 2005; Onimaru et al. 2006; Davies et al. 2009).

The pFRG/RTN resides ventral to the facial nucleus and produces pre-inspiratory oscillations in embryonic and neonatal mammals (Onimaru and Homma 2002; Thoby-Brisson et al. 2005; Thoby-Brisson and Greer 2008; Thoby-Brisson et al. 2009; Fortin and Thoby-Brisson 2009; Mellen and Thoby-Brisson 2012). Rhythmic activity generated in this region emerges prior to that of the PreBötC during development of embryonic mammalian brainstems (Thoby-Brisson et al. 2005; Fortin and Thoby-Brisson 2009). While involved in pre-inspiratory activity in embryonic and neonatal animals, this region takes a more prominent role in active expiration and chemosensitivity as development progresses (Smith et al. 2009; Dubreuil et al. 2009; Marina et al. 2010; Guyenet and Mulkey 2010; Pagliardini et al. 2011; Wang et al. 2013). The pFRG/RTN oscillator is not affected by opioid application and is excited by the GABA<sub>A</sub> receptor antagonist bicuculline (Nattie et al. 2001; Mellen et al. 2003; Thoby-Brisson et al. 2005; Onimaru et al. 2006).

Because the buccal oscillator and pFRG/RTN are both involved in pre-inspiratory activity and are insensitive to opioids, investigators have proposed homology between these oscillators (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006). Conversely, the lung oscillator and PreBötC are sensitive to opioid application and are involved in the inspiration phase of breathing (Smith et al. 1991; Mellen et al. 2003; Solomon 2003; Thoby-Brisson et al. 2005; Feldman and Del Negro 2006; Onimaru et al. 2006). While support for a functional homology of these oscillators

exists, it is also important to note the anatomical orientation of these regions appear to be transposed in amphibians and mammals (Vasilakos et al. 2005; Wilson et al. 2006). Further complicating interpretation is the fact that, in frogs, opioids have only been applied in bath fashion to isolated brainstem preparations (Vasilakos et al. 2005; Davies et al. 2009). The pontine locus coeruleus (LC) is a source of tonic drive to respiratory control centers in both mammals and amphibians, and it is extremely sensitive to opioid administration in mammals (Arvidsson et al. 1995; Noronha-de-Souza et al. 2006; Gray et al. 2006; Biancardi et al. 2008; Gargaglioni et al. 2010; Santin and Hartzler 2013). Thus, we hypothesized that opioid sensitivity in amphibians would not remain following removal of this portion of the brainstem. Another result contrary to homology of buccal oscillator and pFRG/RTN is the response to bicuculline. In amphibians, bicuculline abolishes buccal bursting; however, it increases pFRG/RTN activity in mammals (Galante et al. 1996; Nattie et al. 2001; Broch et al. 2002). The aim of the current study was to observe lung burst activity in bullfrog tadpole brainstems when the lung oscillator was isolated from input from both the LC and the buccal oscillator. While previous studies have transected the amphibian brainstem in a similar way, an investigation of the effects of bicuculline, strychnine, CNQX, and DAMGO on the transected rostral medulla has not been previously attempted.

### **3.3 Methods**

#### *Animals*

Studies were performed on 12 bullfrog tadpoles during early stages of metamorphosis, defined by external anatomy. Early metamorphic animals were selected based on the absence of forelimbs and the presence paddle-like hind limbs lacking joints or separated toes. This group corresponded to developmental stages 7–12 in the classification scheme of tadpole development originally proposed by Taylor and Kollros (Taylor and Kollros 1946). Tadpoles were purchased from a commercial supplier (Pond Megastore, [www.pondmegastore.com](http://www.pondmegastore.com)) and maintained at room temperature housed in aquaria with dechlorinated water, and fed goldfish food daily. All care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines.

### *Surgical preparation*

Each tadpole was anesthetized by immersion for 1–2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222; Sigma, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) in dechlorinated water buffered to pH 7.8 with NaHCO<sub>3</sub>. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostrally at the optic tectum and caudally at the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM): 104 NaCl, 4 KCl, 1.4 MgCl<sub>2</sub>, 10 d-glucose, 25 NaHCO<sub>3</sub> and 2.4 CaCl<sub>2</sub> equilibrated with 100% O<sub>2</sub>. The aCSF HCO<sub>3</sub> concentration is similar to that of plasma from late metamorphic tadpoles and frogs but higher than that in plasma from early metamorphic tadpoles (Just et al. 1973). This HCO<sub>3</sub> concentration has been used in previous tadpole studies (Taylor et al. 2003a; Taylor et al. 2003b; Taylor et al. 2008) and was selected here to ensure comparability with previous studies.

The isolated brainstem was transferred *en bloc* to a 2.5-ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O<sub>2</sub> –CO<sub>2</sub> mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. The pH of the aCSF was maintained at either pH 7.8 (1.5 % CO<sub>2</sub>:98.5 % O<sub>2</sub>; normocapnia) or pH 7.4 (5.0 % CO<sub>2</sub>:95.0 % O<sub>2</sub>; hypercapnia) by adjusting the fractional concentrations of O<sub>2</sub> and CO<sub>2</sub> in the equilibration gas, and CO<sub>2</sub> was monitored with a CO<sub>2</sub> analyzer (AMETEK CD-3A CO<sub>2</sub> analyzer and AMETEK P-61B CO<sub>2</sub> sensor). After isolation, the brainstem was allowed to stabilize for 1 h while superfused with aCSF at 23 °C and pH 7.8 (~9 Torr PCO<sub>2</sub>).

### *Nerve recording*

Roots of the facial (VII) and hypoglossal (XII) nerves were drawn into glass suction electrodes pulled from 1-mm diameter capillary glass to tip diameters that fit the nerve roots. Whole-nerve discharge was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, [www.wpiinc.com](http://www.wpiinc.com); X1000 by a model 1700 amplifier, A-M Systems, [63](http://www.a-</a></p></div><div data-bbox=)

msystems.com) and filtered (100Hz high pass to 1kHz low pass). The amplified and filtered nerve output was sent to a data acquisition system (Power1401, Cambridge Electronic Design, ced.co.uk), which sampled at 25 kHz. Data were archived as whole-nerve discharge, and duplicate integrated (root mean squared and averaged over 200 ms) neurograms were acquired simultaneously. Such recordings were made during the initial 1-h, post-isolation stabilization period and recorded continuously throughout the duration of each treatment protocol following transection of rostral medulla.

#### *Transection and bath application of antagonists*

Following recovery, brainstems were transected immediately rostral to the trigeminal (V) nerves, and at the level of the glossopharyngeal nerves (IX), isolating the rostral medulla (Fig. 3.1). Activity from the trigeminal (V) and facial (VII) nerves were recorded following isolation of the rostral medulla for 1 h under control conditions (1.5 % CO<sub>2</sub> 98.5 % O<sub>2</sub>). A cocktail containing either [D-Ala<sub>2</sub>, N-MePhe<sub>4</sub>, Gly-ol]-enkephalin (DAMGO; 120 nM;  $\mu$ -opioid receptor agonist) and bicuculline methochloride (5  $\mu$ M; GABA<sub>A</sub> receptor antagonist), strychnine (10  $\mu$ M; Glycine receptor antagonist) and bicuculline methochloride, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 1  $\mu$ M;  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor antagonist) and bicuculline methochloride, or bicuculline methochloride alone was subsequently bath applied for 30 min.

#### *Data and statistical analyses*

Recordings during each treatment condition were made for 30 min, and a representative 10-min segment in the last 15 min of each treatment was selected for analysis. Whole-nerve recordings were made from the facial nerve (VII), and were analyzed with respect to the frequency of episodes, frequency of lung and small amplitude bursts within episodes, and amplitude of lung and small amplitude bursts. Nerve activity was deemed a lung burst if the integrated burst amplitude was more than double the baseline activity and if bursts were less than 2 sec in duration, and were designated as a “large amplitude burst” if greater than 2 sec in duration. Activity was designated a “small amplitude burst” if more than 3 bursts were observed, less than 2 sec in duration per burst with half the amplitude of lung bursts. Small amplitude

bursts resembled buccal bursts observed in previous studies with intact brainstems and were included in analysis in the current investigation. However, large amplitude bursts resembled bursts deemed non-respiratory in Milsom et al. (1999) and were not included in analysis (Milsom et al. 1999). Activity was designated an episode if it contained at least 2 lung bursts and had a clear start and end compared to background nerve activity. Differences in lung bursts, burst episodes, and small amplitude bursts during treatments were compared using paired t-tests using SigmaPlot12 (systatsoftware.com).

### 3.4 Results

#### *Bicuculline induces rhythmic activity and lung bursts in the isolated rostral medulla*

We propose that the lung oscillator is functionally homologous to either the mammalian PreBötC or pFRG/RTN. As such, we isolated the lung oscillator, between the glossopharyngeal and vagus nerves (IX and X, respectively), in early metamorphic tadpoles and recorded whole-nerve discharge from the facial nerve (VII) (Fig. 3.1). Recordings from the isolated rostral brainstem during normocapnia in control aCSF did not show discernable bursts (Fig. 3.2a). Application of the GABA<sub>A</sub> receptor antagonist bicuculline produced rhythmic discharges that contained putative lung bursts, large amplitude bursts, and small amplitude bursts (Fig. 3.2b). Large amplitude bursts were deemed non-respiratory, and were not analyzed further; however, small amplitude bursts were similar to buccal bursts and were included in subsequent analysis.

#### *DAMGO attenuates small amplitude burst activity during bicuculline-induced episodes*

To assess sensitivity of the bicuculline-induced rhythms to the  $\mu$ -opioid agonist DAMGO (recall the mammalian PreBötC rhythm is sensitive to DAMGO) we exposed isolated rostral brainstem to aCSF containing both 120 nM DAMGO and bicuculline. DAMGO treatment did not abolish episodes or lung bursting but did disrupt small amplitude bursts (Fig. 3.3a and b). Episodes induced by bicuculline (5.2 episodes/10 min, n = 4) were not significantly disrupted when combined with DAMGO treatment (4.7 episodes/10 min, n = 4; paired t-test, t = 1.000, p = 0.391; Fig. 3.3c). Lung bursts observed in episodes during bath application of bicuculline (5.3 bursts/episode) were not significantly different when DAMGO was subsequently applied (4.9 bursts/episode, paired t-test, t = 0.39, p = 0.725; Fig. 3.3d). The frequency of small amplitude

bursts within episodes (9.6 bursts/episode) was attenuated following addition of DAMGO (3.9 bursts/episode, paired t-test,  $t=3.240$ ,  $p = 0.040$ ; Fig. 3.3d).

#### *CNQX abolishes episodic activity induced by bicuculline*

We assessed the role of glutamatergic neurotransmission on bicuculline-induced episodic rhythms of isolated rostral medulla with application of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist CNQX combined with bicuculline. These rhythms were sensitive to bath application of 1  $\mu$ M CNQX ( $n = 3$ ), which abolished the episodic rhythms induced by bicuculline ( $n = 3$ ; Fig. 3.4a and b). Both the episodes and the bursts within episodes (lung and small amplitude) were eliminated following administration of CNQX (Fig. 3.4a and b).

#### *Strychnine increases burst amplitude, but does not affect burst frequency in episodes induced by bicuculline.*

We assessed the role of glycinergic inhibition in bicuculline-induced episodic rhythms in the isolated rostral medulla by application of strychnine (a glycine receptor antagonist; Fig. 3.5a and b). Strychnine (10  $\mu$ M) did not significantly affect the frequency of episodes or individual lung bursts when combined with bicuculline ( $n = 3$ , 3.24 bursts/episode, 9.6 episodes/10 min) as compared to bicuculline alone ( $n = 3$ , 3.44 bursts/episode, 12.6 episodes/10 min; paired t-test,  $t = 0.792$   $p = 0.511$ ; Fig. 3.5c and d). The addition of strychnine did produce significant changes to lung burst amplitude (1.05 mV) compared to bicuculline alone (0.72 mV, paired t-test,  $t = 6.130$ ,  $p = 0.026$ , Fig. 3.5e).

### **3.5 Discussion**

#### *The current framework of amphibian respiration*

In mammals, application of DAMGO to acute slices containing the PreBötC and pFRG/RTN attenuates PreBötC rhythm generation but does not affect pFRG/RTN rhythms (Takeda et al. 2001; Mellen et al. 2003; Onimaru et al. 2006; Thoby-Brisson et al. 2009; Fortin and Thoby-Brisson 2009). In amphibians, bath application of DAMGO to intact brainstems attenuates lung rhythms but does not affect buccal rhythm generation, leading investigators to

conclude that the lung oscillator is homologous to the PreBötC and the buccal oscillator is homologous to the pFRG/RTN (Wilson et al. 2002; Vasilakos et al. 2005; Davies et al. 2009; Ranohavimparany et al. 2014). While this is one explanation for DAMGO inhibition of lung bursts in amphibians, it is complicated by the comparisons of intact respiratory networks of amphibians to acute slices in mammals. Amphibians share a similar structure to mammals in the locus coeruleus (LC), an area containing CO<sub>2</sub>-sensitive neurons and a number of cells expressing u-opioid receptors (Arvidsson et al. 1995; Gray et al. 2006; Noronha-de-Souza et al. 2006; Biancardi et al. 2008; Gargaglioni et al. 2010; Santin and Hartzler 2013). In both amphibians and mammals, this region provides drive to respiratory centers (Milsom et al. 1997; Kinkead et al. 1997; Reid et al. 2000; Noronha-de-Souza et al. 2006; Biancardi et al. 2008; Fournier and Kinkead 2008; Gargaglioni et al. 2010). As such, the attenuation of lung bursts by bath-applied DAMGO in amphibians may be due to a disruption of LC neurons.

The pFRG/RTN plays a role in active expiration and chemoreception in adult mammals (Dubreuil et al. 2009; Smith et al. 2009; Marina et al. 2010; Guyenet and Mulkey 2010; Pagliardini et al. 2011; Wang et al. 2013; Huckstepp et al. 2015). Mammalian active expiration involves contraction of abdominal muscles in conditions of high drive of the respiratory network (Pagliardini et al. 2011; Molkov et al. 2014; Huckstepp et al. 2015). Amphibians breathe by gulping air and subsequently forcing it into their lungs in a series of “inflation” breaths (Burggren and West 1982; Vitalis and Shelton 1990; Kogo et al. 1994). Instead of passively expiring air, some amphibians actively expire air from their lungs via contraction of abdominal muscles prior to inflation breaths (Brainerd 1994; Brainerd and Owerkowicz 2006). Much of the work in amphibians has used the isolated brainstem preparation, and it can be difficult to distinguish between inspiratory or expiratory breaths in these preparations. While amphibians do show central sensitivity to CO<sub>2</sub>, it is unknown if the resulting enhanced ventilation is reflected in inspiration and expiration equally. Due to the mechanics of amphibian ventilation, it is enticing to draw connections between active expiration in mammals and active expiration in amphibians; however, it is unknown if CO<sub>2</sub> can stimulate active expiration in amphibians.

We speculate that the lung oscillator in amphibians may be homologous with the pFRG/RTN in mammals. While a homology between buccal oscillator and PreBötC is an inviting proposition, an investigation using an acute slice containing the buccal oscillator has not been



conducted. Caution should be used when declaring homologies, as the buccal oscillator could be homologous to any number of other rhythmically controlled behaviors such as swallowing, suckling, or airway protection (Gestreau et al. 2005; Barlow and Estep 2006; Milsom 2010; Moore et al. 2014; Samson et al. 2017).

*Evidence for functional homology between amphibian “lung oscillator” and mammalian pFRG/RTN*

In prenatal animals, the pFRG/RTN serves as the dominant oscillator critical for providing rhythm generation for respiratory activity (Thoby-Brisson et al. 2005; Fortin and Thoby-Brisson 2009; Onimaru et al. 2009; Onimaru et al. 2014). As development progresses, the PreBötC becomes the dominant oscillator for inspiratory activity, while the pFRG/RTN takes a larger role in chemoreception and active expiration (Smith et al. 2009; Dubreuil et al. 2009; Marina et al. 2010; Guyenet and Mulkey 2010; Pagliardini et al. 2011; Wang et al. 2013; Huckstepp et al. 2015). Bicuculline has been reported to increase pFRG/RTN activity when applied to this region via micro-dialysis in awake rats (Nattie et al. 2001) or focally injected in adult rats (Pagliardini et al. 2011). Conversely, focal injections of bicuculline into the PreBötC region decrease breathing in anesthetized cats (Pierrefiche et al. 1998). However, systemic administration of bicuculline in mammals produces a general increase in frequency and amplitude of phrenic nerve output, output that activates the diaphragm (Hayashi and Lipski 1992; Sica et al. 1993).

As observed in this study, the rostral medulla of amphibians contains a rhythmogenic site that is stimulated by bicuculline, i.e., activated by GABA<sub>A</sub> inhibition and insensitive to DAMGO, i.e.,  $\mu$ -opioid stimulation. Our data suggest two similarities between the pFRG/RTN and lung oscillator. (1) Lung bursts and episodes could be stimulated by application of bicuculline within the rostral medulla, similar to pFRG/RTN in mammals (Nattie et al. 2001; Pagliardini et al. 2011). (2) Lung activity stimulated by bicuculline was not sensitive to DAMGO, similar to the pFRG/RTN in mammals. The rhythms induced by bicuculline were extremely sensitive to CNQX, which blocks glutamate at AMPA/kainate receptors. Rhythms produced by the mammalian embryonic parafacial region (ePF, later differentiated as pFRG/RTN) are insensitive to CNQX (Fortin and Thoby-Brisson 2009). While CNQX drastically reduces lung oscillator

activity in the current study, it is important to note that we recorded whole-nerve output from the brainstem, whereas Fortin et al (2009) made cell recordings in the ePF region. Although we cannot make the presumption that the rhythm observed in this study is driven entirely by glutamatergic input, the reduction in activity from CNQX suggests that it is mediated by glutamate at some level.

*Functional homology of the lung oscillator and pFRG/RTN regions of amphibians and mammals, respectively*

Results of this study suggest that the isolated lung oscillator of amphibians functions in a similar manner to that of the pFRG/RTN of mammals. It is possible the portion of medulla isolated in this study contains components from other respiratory centers, but the evidence still supports shared respiratory characteristics between the rostral medulla in amphibians and the pons/rostral medulla in mammals. More evidence is needed, however, to definitively support the proposed homology. Future studies could include individually recorded neurons in the lung oscillator region in the presence of DAMGO and labeling of these neurons for the characteristic glutamatergic/Phox2b staining of the pFRG/RTN. A model showing loss of CO<sub>2</sub> sensitivity following chronic exposure to nicotine and ethanol in bullfrog tadpoles has been established (Brundage et al. 2010; Brundage and Taylor 2010; Taylor and Brundage 2013; Taylor et al. 2013). These two toxins are associated with increased rates of the Sudden Infant Death Syndrome (SIDS; (Sullivan and Barlow 2001; Bajanowski et al. 2007; Lavezzi et al. 2010; Phillips et al. 2011). Alterations and abnormalities of cell morphology and population have also been identified in the pFRG/RTN in babies that have died from SIDS (Lavezzi et al. 2012). Observing an amphibian homologue of the pFRG/RTN during developmental exposure to nicotine or ethanol could provide information that would further the understanding of respiratory pathologies such as SIDS.

### 3.6 Bibliography

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### 3.7 Figures

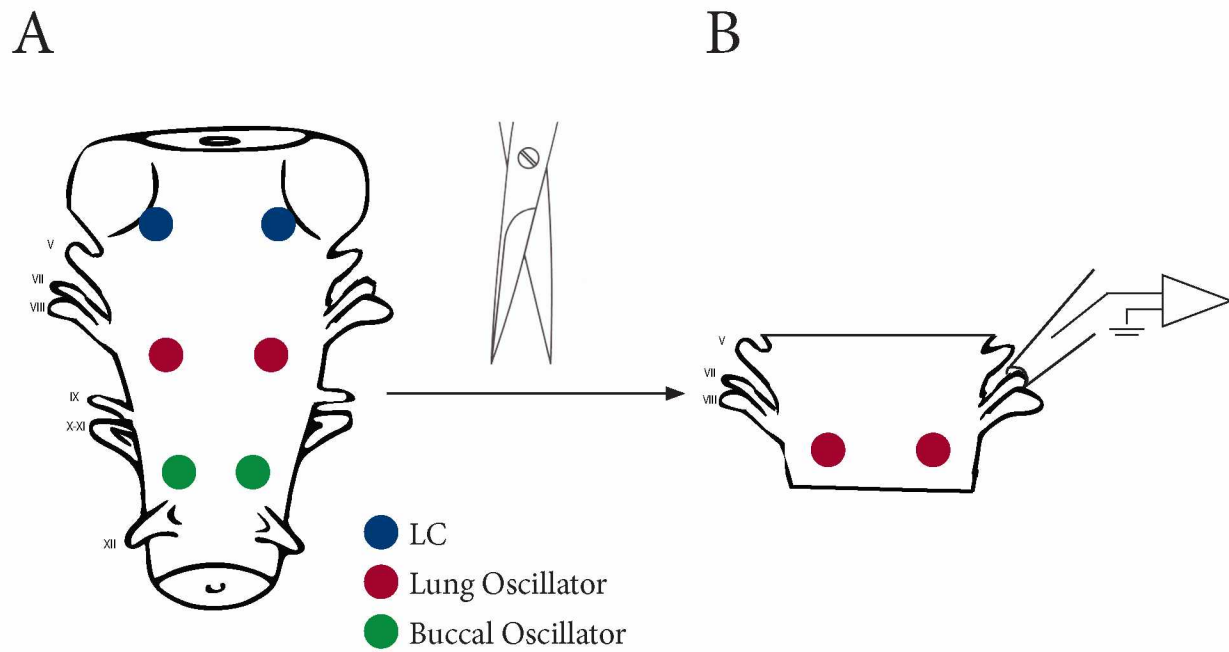


Figure 3.1: **Whole-nerve recordings from the isolated rostral medulla.** (A) Intact brainstems were removed from early metamorphic tadpoles and transected, leaving the a block of tissue from the trigeminal (V) to the glossopharyngeal nerves (IX) remaining (A and B). Transections isolated the lung oscillator from the locus coeruleus (LC) and the buccal oscillator, which were removed. (B) Whole-nerve recordings were made from the facial (VII) nerve in the isolated rostral medulla.

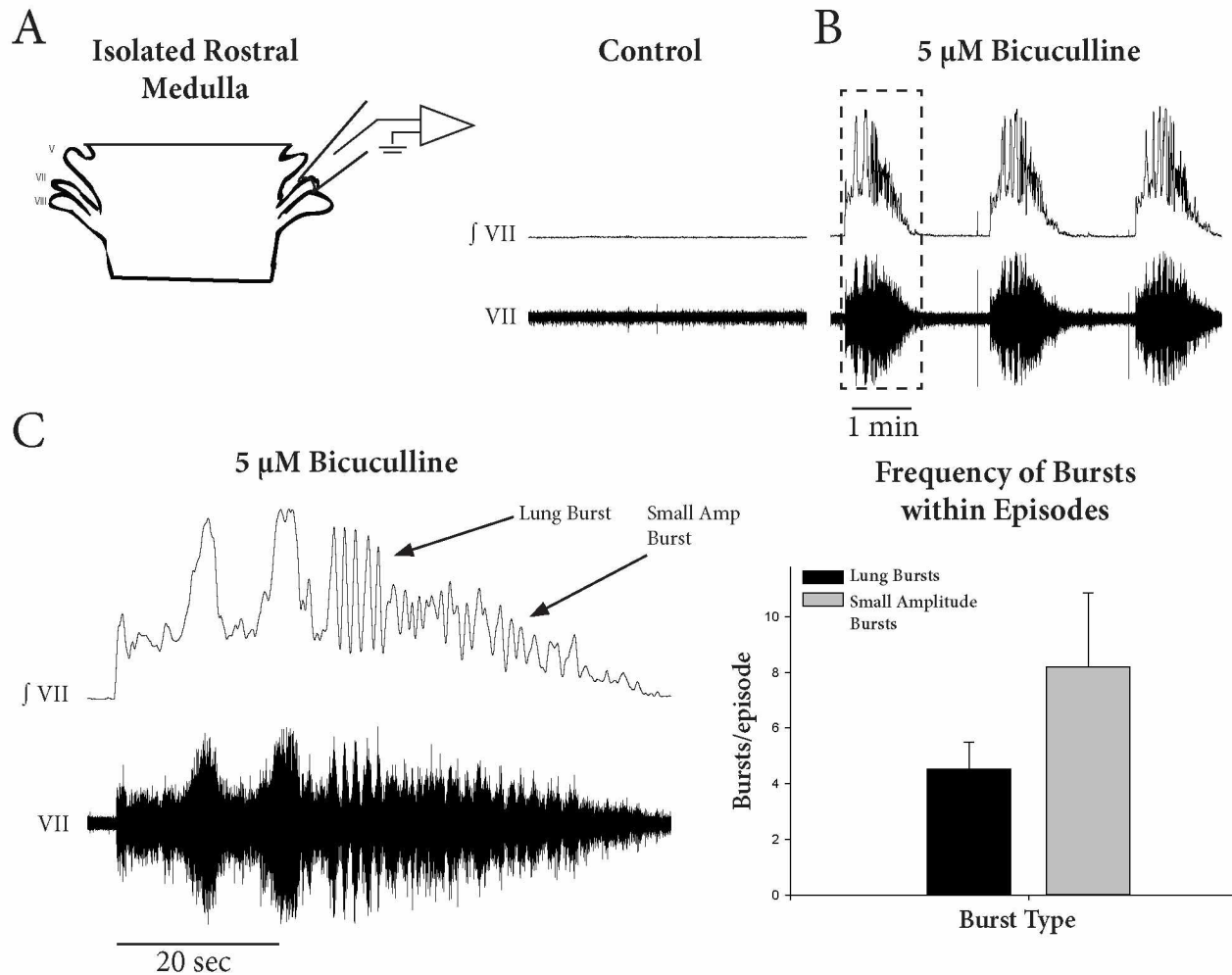
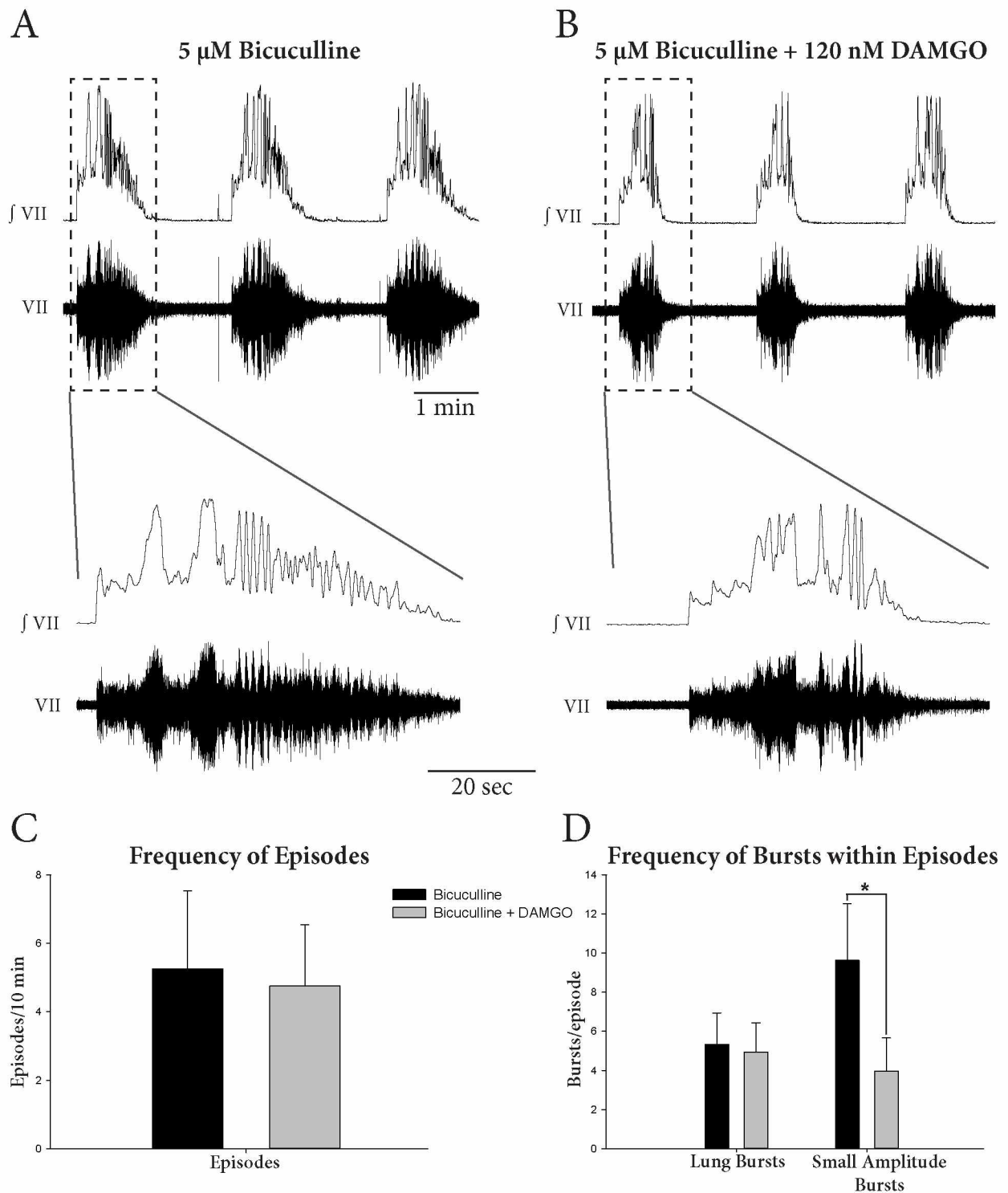


Figure 3.2: **Bicuculline induces rhythmic bursts in the isolated rostral medulla of early metamorphic tadpoles.** Whole-nerve discharges (raw and integrated “f”) from the facial (VII) nerve in the isolated rostral medulla of early metamorphic tadpole brainstems were recorded. (A) In the presence of control aCSF, brainstems produced no rhythmic bursts. (B) Bath application of 5 $\mu$ M bicuculline produced episodic rhythmic bursts. (C) Each episode contained two bursts that were measured: lung bursts and small amplitude bursts.



**Figure 3.3: DAMGO inhibits small amplitude bursts but not lung bursts or episodes.** Whole-nerve discharge (raw and integrated “f”) from the facial nerve (VII) were recorded in the presence of 5 $\mu\text{M}$  bicuculline and 5 $\mu\text{M}$  bicuculline + 120 nM DAMGO. (A and B) Episodic rhythmic bursts were observed in the presence of both bicuculline and bicuculline + DAMGO. (C) The number of episodes observed in the presence of bicuculline alone was not effect by the

Figure 3.3 cont.

addition of DAMGO. (D) Small amplitude bursts, but not lung bursts, were inhibited by the addition of DAMGO.

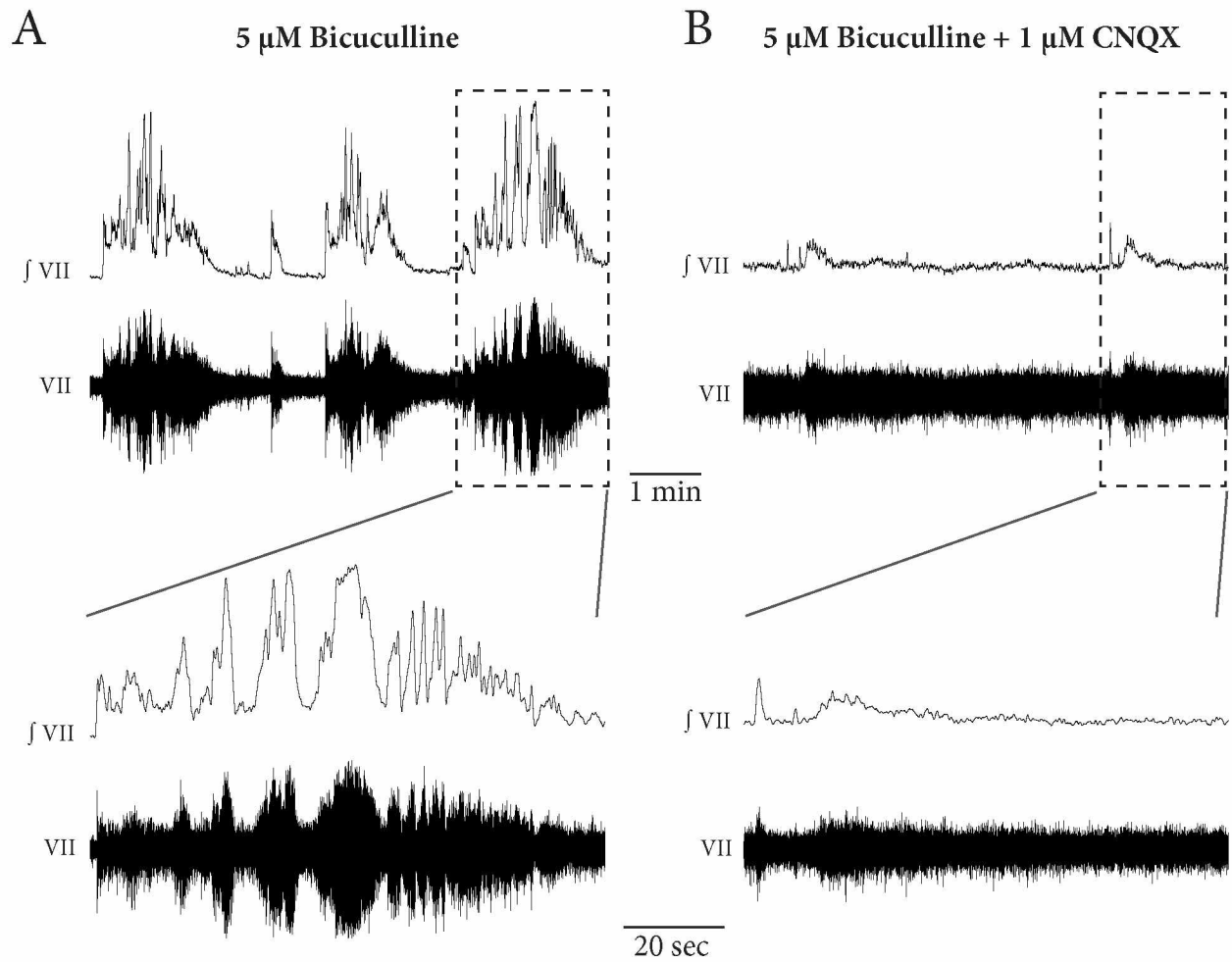
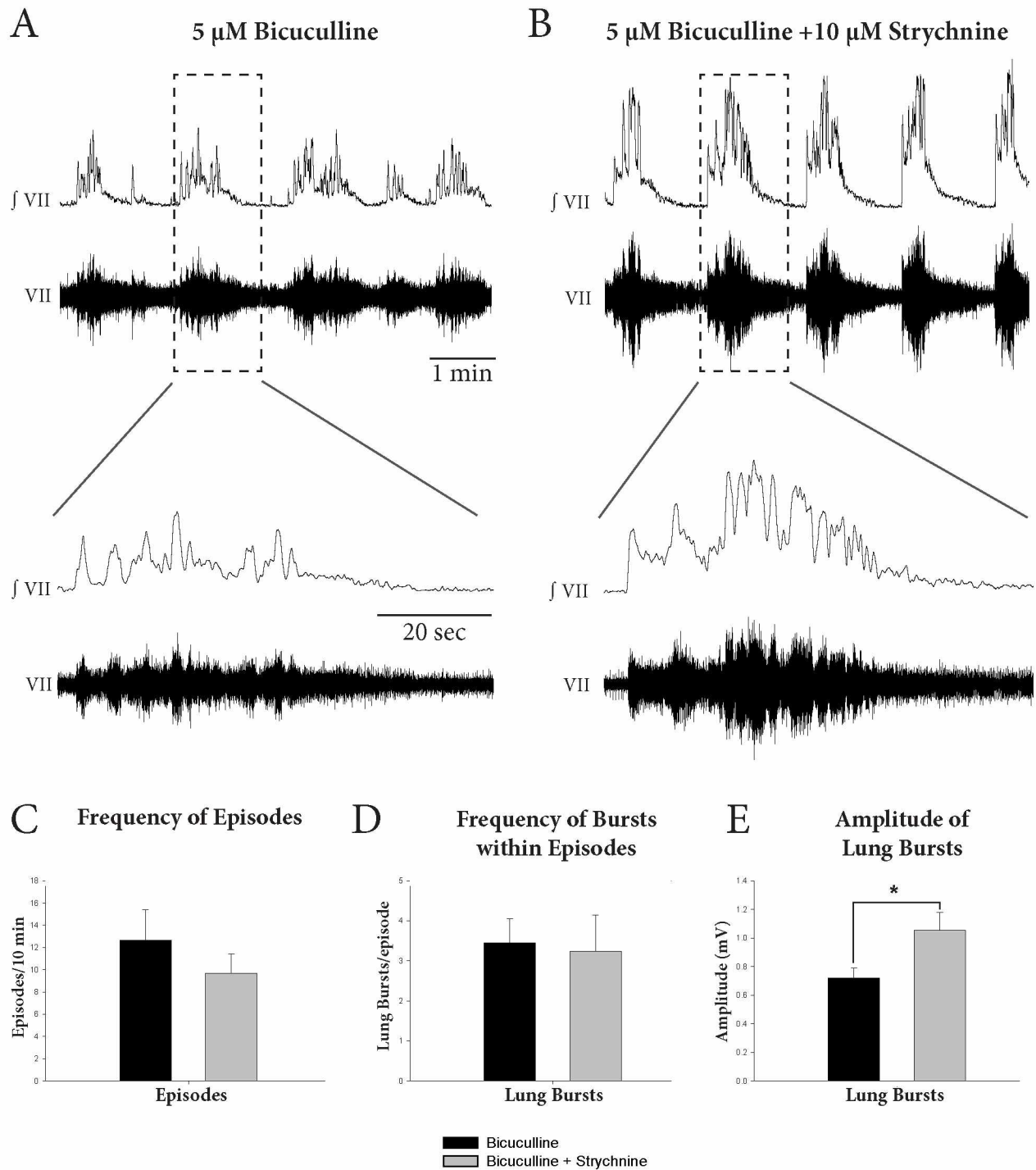


Figure 3.4: **CNQX abolishes episodic bursts induced by bicuculline.** Whole-nerve activity (raw and integrated “f”) from the facial (VII) nerve was measured in the presence 1  $\mu$ M CNQX combined with 5 $\mu$ M bicuculline. (A and B) Episodic bursts induced by bicuculline were virtually abolished upon application of CNQX. (B) Small amplitude activity was observed following CNQX, but did not contain identifiable bursts.



**Figure 3.5: Strychnine increases burst amplitude but does not affect burst frequency in episodes induced by bicuculline.** Whole-nerve activity (raw and integrated “f”) from the facial nerve (VII) were recorded in the presence of 5  $\mu\text{M}$  bicuculline and bicuculline + 10  $\mu\text{M}$  strychnine. (A and B) Strychnine in the presence of bicuculline induced activity similar to that of bicuculline alone. (C) Addition of strychnine did not affect the number of episodic bursts or (D)

Figure 3.5 cont.

the number of lung bursts per episode. (E) However, strychnine in the presence of bicuculline did increase the amplitude of lung bursts within episodes.





## Chapter 4

### Development of phenotypic markers for pFRG/RTN in *Lithobates catesbeianus*<sup>1</sup>

#### 4.1 Abstract

Investigations of the neural substrates that control respiration can provide insight into the development and function of these sites. The pFRG/RTN plays an important role in both chemoreception and rhythmogenesis in prenatal and early postnatal mammals, and it is essential for the development of respiratory behavior. In amphibians, the lung oscillator has been proposed to be functionally similar to the pFRG/RTN of mammals. While functional similarities have been investigated, parallels of cellular phenotype have not been identified. We tested the hypothesis that the rostral medulla in amphibians is structurally and developmentally similar to pFRG/RTN in mammals. To test this hypothesis, we stained for neuronal markers used to identify the pFRG/RTN region in mammals: paired-like homeobox 2b (Phox2b) transcription factor and the enzymes tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), in both early and late metamorphic bullfrog (*Lithobates catesbeianus*) tadpoles. We then made extracellular recordings in the proposed lung oscillator to assess CO<sub>2</sub> sensitivity and the coincidence of neuronal activity with fictive respiration. A group of Phox2b immunoreactive cells were located ventral to the facial nucleus in both early and late metamorphic animals. These cells were not positive for ChAT or TH, and the number of cells observed in this region increased during metamorphosis. Extracellular recordings of neurons in the proposed lung oscillator revealed CO<sub>2</sub>-sensitive cells that fired coincidentally with lung bursts. Together, the results support the hypothesis that amphibians have a structurally homologous region to the mammalian pFRG/RTN. Furthermore, these neurons increase in number during metamorphosis, which could potentially contribute to increased CO<sub>2</sub> sensitivity in late metamorphic tadpoles.

#### 4.2 Introduction

Investigations into the neural respiratory control network of amphibians have provided an interesting comparative perspective on the origins and development of breathing in mammals

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<sup>1</sup> Reed MD, Iceman KE, Harris MB, Taylor BE. Development of phenotypic markers for pFRG/RTN in *Lithobates catesbeianus*. *Am J Physiol Regul Integr Comp Physiol*. (In preparation)

(For reviews see: Wilson et al. 2006; Gargaglioni and Milsom 2007; Milsom 2010a; Milsom 2010b). Whole-nerve recordings from amphibian tadpole brainstems *in vitro* reveal a network capable of producing fictive lung and gill ventilation (McLean et al. 1995; Liao et al. 1996; Reid and Milsom 1998). Lung activity recorded in tadpoles increases as tadpoles progress through metamorphosis (Torgerson et al. 1997; Gdovin et al. 1999; Torgerson et al. 2001b; Hedrick 2005). Fictive lung breathing can be observed in newly hatched tadpoles as periodic single bursts, and by late metamorphosis it occurs as episodic bursts (Reid and Milsom 1998; Milsom et al. 1999; Fong et al. 2009). Stimulation of peripheral chemoreceptors by hypercapnia, acidosis, or hypoxia stimulates lung burst activity of the respiratory control network (Ishii and Kusakabe 1985a; Ishii and Kusakabe 1985b; Van Vliet and West 1992; West and Van Vliet 1992; Jia and Burggren 1997; Milsom and Burleson 2007; Reyes et al. 2014). Additionally, bath application of high CO<sub>2</sub>/low pH (hypercapnia) to isolated brainstems induces increases in the frequency of fictive lung breathing, indicating that amphibians have both peripheral and central chemoreception (Feder and Burggren 1985; Gdovin et al. 1999; Wang et al. 1999). Central chemoreception also develops through metamorphosis; as tadpoles enter late metamorphic development, they become more sensitive to CO<sub>2</sub>/low pH (Torgerson et al. 1997; Torgerson et al. 2001a; Taylor et al. 2003a). Three discrete locations of central chemoreception in amphibians are proposed: locus coeruleus (LC, in the pons), rostral chemosensory area (RCA, in the rostral medulla), and caudal chemosensory area (CCA, in the caudal medulla) (Kinkead et al. 1997; Torgerson et al. 1997; Taylor et al. 2003a; Noronha-de-Souza et al. 2006; Santin and Hartzler 2013). While there are numerous functional studies of the areas responsible for central chemosensitivity in bullfrogs (*Lithobates catesbeianus*), far fewer investigations of the structure of chemosensitive regions have been attempted.

Seemingly conserved, the traditional Loeschcke central chemoreceptive sites on the ventromedullary surface of mammalian brainstems also exist in similar anatomical locations in amphibians (Loeschcke 1982; Torgerson et al. 1997; Torgerson et al. 2001a; Taylor et al. 2003a; Santin and Hartzler 2013). While the LC provides CO<sub>2</sub> sensitivity in both mammals and amphibians, chemoreception does not appear to be critically reliant on this area (Chapter 2, (Noronha-de-Souza et al. 2006; Biancardi et al. 2008)). In contrast, the parafacial respiratory group and retrotrapezoid nucleus (pFRG/RTN) region in mammals appears to be a critical site

for chemoreception and drive to respiratory centers (Mulkey et al. 2004; Stornetta et al. 2006; Guyenet et al. 2009; Abbott et al. 2011). This region is intrinsically sensitive to changes in CO<sub>2</sub>/low pH, and disruption of this area in mammals produces respiratory abnormalities and loss of CO<sub>2</sub> sensitivity (Mulkey et al. 2004; Takakura et al. 2008; Dubreuil et al. 2009; Amiel et al. 2009; Marina et al. 2010; Wang et al. 2013).

Anatomically, the pFRG/RTN in mammals is similar to the RCA in amphibians. We have previously demonstrated potential functional homology between these two regions (Chapter 3), leading to the current investigation of structural homologies. The pFRG/RTN region in mammals has been well studied and a number of neuronal markers identifying its location in the medulla have been established (Cream et al. 2002; Mulkey et al. 2004; Stornetta et al. 2006; Onimaru et al. 2008; Guyenet et al. 2009). All pFRG/RTN cells are glutamatergic cells, express the paired-like homeobox 2b (Phox2b) transcription factor and not tyrosine hydroxylase (TH) (Cream et al. 2002; Stornetta et al. 2006; Onimaru et al. 2008; Guyenet et al. 2009). The pFRG/RTN region is ventral to the facial nucleus (VII), which also expresses Phox2b (Stornetta et al. 2006; Onimaru et al. 2008).

The importance of the pFRG/RTN's role in both chemoreception and rhythm generation during early mammalian development led investigators to suggest a potential homology with the amphibian lung oscillator/RCA (Milsom 2010a; Jenkin and Milsom 2014; Baghdadwala et al. 2015b). The added evidence of metamorphic changes in CO<sub>2</sub> sensitivity in amphibians provokes further speculation about the development and homology of this area. In the present study, we tested the hypothesis that the rostral medulla in amphibians is structurally and developmentally similar to pFRG/RTN in mammals.

### **4.3 Methods**

#### *Animals*

Studies were performed on bullfrog tadpoles (n = 15) purchased from a commercial supplier (Pond Megastore, [www.pondmegastore.com](http://www.pondmegastore.com)). Tadpoles were maintained at room temperature housed in aquaria with dechlorinated water, and they were fed goldfish food daily. At the time of dissection each tadpole was designated as either early metamorphic (n = 6; forelimbs absent, hind limbs paddle-like without joints or separated toes) or late metamorphic (n

= 9; forelimbs and hind limbs present, tail being resorbed), which correspond to developmental stages 7–12 or 20–25, respectively, in the classification scheme of Taylor and Kollros (Taylor and Kollros 1946). All care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alaska Fairbanks and complied with all state and federal ethical guidelines.

### *Surgical preparation*

Each tadpole was anesthetized by immersion for 1–2 min in cold (4 °C) 0.2 mM tricaine methanesulfonate (MS222; Sigma, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)) in dechlorinated water buffered to pH 7.8 with NaHCO<sub>3</sub>. The front of the head rostral to the nares and the back of the body (hind limbs and tail, if present) were removed. The dorsal cranium and forebrain rostral to the diencephalon were resected and the fourth ventricle opened by removing the choroid plexus. The remaining brainstem and spinal cord were removed *en bloc* and further trimmed rostral to the optic tectum and caudal to the brachial nerve. During dissection, exposed tissues were superfused with cold artificial cerebral spinal fluid (aCSF) composed of (in mM) 104 NaCl, 4 KCl, 1.4 MgCl<sub>2</sub>, 10 d-glucose, 25 NaHCO<sub>3</sub> and 2.4 CaCl<sub>2</sub> equilibrated with 100 % O<sub>2</sub>. The aCSF HCO<sub>3</sub><sup>-</sup> concentration is similar to that of plasma from late metamorphic tadpoles and frogs but higher than that in plasma from early metamorphic tadpoles (Just et al. 1973). This HCO<sub>3</sub><sup>-</sup> concentration has been used in previous tadpole studies (Taylor et al. 2003a; Taylor et al. 2008) and was selected here to ensure comparability between experiments and with previous studies on animals of different metamorphic stages.

The isolated brainstem was transferred *en bloc* to a 2.5-ml, Plexiglas, flow-through recording chamber and was supported, ventral side up, between coarse nylon mesh such that all surfaces were bathed with aCSF flowing from rostral to caudal at a rate of 5 ml/min. A supply of aCSF, equilibrated with O<sub>2</sub> –CO<sub>2</sub> mixtures that produced the desired pH, flowed through plastic tubing to the recording chamber and bathed the isolated brainstem. The pH of the aCSF was maintained at either pH 7.8 (1.5 % CO<sub>2</sub>:98.5 % O<sub>2</sub>; normocapnia) or pH 7.4 (5.0 % CO<sub>2</sub>:95.0 % O<sub>2</sub>; hypercapnia) by adjusting the fractional concentrations of O<sub>2</sub> and CO<sub>2</sub> in the equilibration gas. CO<sub>2</sub> was monitored with a CO<sub>2</sub> analyzer (CD-3A, Applied Electrochemistry, [ametec.com](http://ametec.com)).

After isolation the brainstem was allowed to stabilize for 1 h while superfused at 23 °C, with aCSF of pH 7.8 (~9 Torr PCO<sub>2</sub>).

### *Electrophysiology*

Roots of the facial and hypoglossal nerves were drawn into glass suction electrodes pulled from 1-mm diameter capillary glass to tip diameters that fit the nerve roots. Whole-nerve activity was amplified (X100 by DAM 50 amplifiers, World Precision Instruments, [www.wpiinc.com](http://www.wpiinc.com); and X1000 by a model 1700 amplifier, A-M Systems, [www.a-msystems.com](http://www.a-msystems.com)) and filtered (100 Hz high pass to 1k Hz low pass). The amplified and filtered nerve activity was recorded by a data acquisition system (Power1401, Cambridge Electronic Design, [ced.co.uk](http://ced.co.uk)), which sampled at 25 kHz. Data were archived as whole-nerve activity, and duplicate integrated (root mean squared and averaged over 200 ms). Neurograms from two nerve roots were acquired simultaneously. Such recordings were made during the initial 1-h, post-isolation, stabilization period and continuously throughout the duration of each treatment protocol.

Extracellular recordings of medullary neurons were made using pulled glass capillary electrodes (15-40 MΩ), filled with biotinamide hydrobromide (Life Technologies, Carlsbad, CA, USA) dissolved at 5 % in 0.5 M sodium acetate. We targeted regions of the medulla between the glossopharyngeal (IX) and facial (VII) nerves, the area of the lung oscillator. Electrodes were placed above the target area and driven into the tissue using a fine stepping motor (1 μm steps; Burleigh Inchworm, Victor, NY, USA). Baseline firing was recorded for each neuron during normocapnia, followed by a 20-min hypercapnic challenge, and then a 40-min normocapnic recovery period. Electrodes were connected to an Axon Multiclamp 700B intracellular amplifier (Molecular Devices, Sunnyvale, CA, USA) with high-pass filter at 300 Hz and low-pass filter at 1 kHz Bessel via an Axon CV7B high impedance headstage (Molecular Devices). Signals were digitized using Spike 2 (CED, Cambridge, UK) or LabChart (AD Instruments, Colorado Springs, CO), sampled (>10 kHz), and stored as computer data files for subsequent analysis.

### *Histology*

Brainstems were fixed in 4 % paraformaldehyde overnight, and subsequently placed in a 30 % sucrose 1X PBS solution. Tissue was flash frozen in optimal cutting temperature compound

(Tissue-Tek OCT compound, sakura.eu) that was immersed in hexanes, cooled by a dry-ice/ethanol slurry, and sectioned into 20- $\mu\text{m}$  slices using a cryostat microtome. Slides with brainstem slices were dried for 3 h prior to processing. Following 1X PBS washes to remove OCT, slides were incubated in blocking buffer (5 % normal donkey serum, 0.3 % Triton 100X, 1X PBS) for 1-2 h, and subsequently incubated in primary antibody overnight (blocking buffer plus a combination of antibodies; Table 4.1). Following primary incubation, slides were washed in 1X PBS and then incubated in a biotinylated or fluorescently tagged secondary antibody for 1-2 h (Table 4.1). Tissues processed enzymatically were incubated in ABC solution (VectorLabs ABC elite kit, vectorlabs.com) for 30 min and Ni-DAB for 8 min, followed by an abrupt 1-min  $\text{H}_2\text{O}_2$  reaction. Slides were cleared using reverse osmosis  $\text{H}_2\text{O}$ , allowed to dry fully, and cover-slipped with Vecta Shield mounting media (VectorLabs, vectorlabs.com). Tissue slices were visualized using either an inverted fluorescent microscope (Olympus FSX-100) or confocal inverted microscope (Zeiss LSM 710).

#### *Analysis of histology*

Images were made of slices every 80  $\mu\text{m}$  along the brainstem, and results for Phox2b, TH, and ChAT immunostaining were plotted to brainstem diagrams. Phox2b immunoreactive cells ventral to the facial motor nucleus were counted in a blind analysis using Image J software (<https://imagej.nih.gov/ij/>). Mean fluorescence values of Phox2b-immunoreactive (Phox2b-ir) neurons ventral to the facial motor nuclei were compared between early and late metamorphic tadpole brainstems (student t-test; Systat software, systatsoftware.com).

## **4.4 Results**

### *Phox2b immunoreactivity is found throughout the brainstem in early and late metamorphic tadpoles*

We hypothesized amphibians would have a brainstem structure histochemically similar to the pFRG/RTN in mammals. As such, we used immunohistochemistry to identify the homeodomain transcription factor, paired-like homeobox 2b (Phox2b) and the acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) in early and late metamorphic tadpole brainstems. Figures 4.1 and 4.2 show locations of Phox2b-ir cells in early and late metamorphic

tadpole brainstems. Regardless of metamorphosis, Phox2b-ir neurons were present bilaterally in each medullary section (Fig. 4.1a-h & 4.2a-h). Phox2b-ir and ChAT immunoreactivity (ChAT-ir) colocalized in each region previously identified as a motor nucleus (Fig. 4.1a-g & 4.2a-g), and not in the hypoglossal nuclei (Fig. 4.1h & 4.2h).

*Catecholaminergic neurons are absent between the glossopharyngeal and trigeminal nerves*

We then stained for the presence of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) to test for overlap with Phox2b-ir neurons in early and late metamorphic tadpole brainstems. TH immunoreactive cells (TH-ir) were found bilaterally at the level of the cerebellum (Fig. 4.1A & 2A) and caudal to the glossopharyngeal (IX) nerves at the level of the vagus (X) nerves (Fig. 4.1f-h, 4.2f-h, & Fig. 4.3g-f). TH-ir neurons were not present between the trigeminal (V) and glossopharyngeal (IX) nerves (Fig. 4.3c-e).

*Phox2b-ir neurons are found ventral to the facial motor nuclei and are more numerous in the brainstems of late metamorphic tadpoles*

We assessed the distribution of Phox2b-ir neurons in relation to ChAT+Phox2b-ir motor neurons. Phox2b-ir neurons were found lateral and dorsal, but not ventral, to ChAT+Phox2b-ir neurons between the glossopharyngeal (IX) and hypoglossal (XII) nerves in early and late metamorphic brainstems (Fig. 4.4a-f). Between the glossopharyngeal and trigeminal (V) nerves, Phox2b-ir neurons were found ventral to ChAT+Phox2b-ir motor nuclei in early and late metamorphic brainstems (Fig. 4.5 a-f). We then analyzed the number of Phox2b-ir neurons ventral to motor nuclei between the glossopharyngeal and trigeminal nerves in both early and late metamorphic brainstems. A greater number of Phox2b-ir neurons were found ventral to motor neurons in late metamorphic (n = 3) compared to early metamorphic (n = 3) brainstems, 31.1 vs 20.3 respectively (Fig. 4.6, paired t-test,  $t = 4.81$ ,  $p = 0.008$ ).

*Neurons recorded between the facial and glossopharyngeal nerves are chemosensitive and associated with lung bursts*

Following identification of Phox2b-ir neurons ventral to the facial motor nuclei, extracellular neuron recordings were made at the level of the facial nuclei (Fig. 4.7a). Neurons (n



= 4) recorded in late metamorphic tadpoles from this area were CO<sub>2</sub> sensitive and fired coincidentally with lung bursts (Fig. 4.7b). Attempts to record neurons in this area from early metamorphic animals were unsuccessful.

#### 4.5 Discussion

##### *Developmental similarities among amphibians and mammals of Phox2b expression near the facial nucleus*

As in mammals, the Phox2b-ir cells of amphibians colocalize with many of the ChAT-ir motor nuclei in the brainstem, with the exception of the hypoglossal nucleus (Albersheim-Carter et al. 2015; Fig. 4.3 & 4.4). As reported in previous literature, TH-ir neurons exist in pontine regions and caudal to the vagus nerve in the amphibian brainstem (Fig. 4.1 & 4.2, González et al. 1994). The development of the central nervous system is a tightly controlled process that relies on a variety of cues for proper neural differentiation and positioning. Phox2b is a transcription factor that aids in the migration of neurons involved in visceral control, which includes control of the lungs (Coppola et al. 2010; Gray 2013). In mammals, this transcription factor is expressed in the brainstem and parts of the spinal cord, and it is important for proper development of the pFRG/RTN (Gray 2008; Dubreuil et al. 2009; Coppola et al. 2010; Gray 2013). Near the facial nucleus specifically, Phox2b-ir cells fated to become the pFRG/RTN emerge dorsally and progress radially around the facial nucleus, ultimately localizing to the ventral surface of the medulla (Champagnat et al. 2011; Gray 2013).

Results of the present study show a similar transition between early and late metamorphic tadpoles. Cellular migration from dorsal to ventral regions around the facial nucleus may reflect maturation of the respiratory control network and is consistent with observations of Phox2b neurons in a similar anatomical region in mammals. Phox2b pFRG/RTN fated cells originate in rhombomere 3 and migrate to rhombomere 5 during embryonic development, eventually residing in the parafacial region (Champagnat et al. 2009). A shift in Phox2b-ir between these rhombomeres was not indicated in this investigation; however, it is likely that the Phox2b-ir neurons in question have already traveled rostro-caudally, arriving in rhombomere 5 and merely moved ventrally during the period we observed. The rhombomeric organization of the

amphibian brainstem has been identified, and like mammals, the amphibian facial nucleus resides in a region associated with rhombomere 5 (Straka et al. 2002; Straka et al. 2006).

*Phox2b-ir neurons ventral to the facial nucleus may be part of the amphibian lung oscillator*

The lung oscillator in amphibians has been proposed to reside between the glossopharyngeal (IX) and facial (VII) nerves (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006). Interestingly, key chemosensitive regions have been reported to exist in this region (Torgerson et al. 2001a; Taylor et al. 2003b; Taylor et al. 2003a). In mammals, the pFRG/RTN acts as both an oscillator for expiration and critical CO<sub>2</sub> sensor (Smith et al. 2009; Dubreuil et al. 2009; Marina et al. 2010; Guyenet and Mulkey 2010; Pagliardini et al. 2011; Wang et al. 2013). Developmentally, the pFRG/RTN is demarcated by Phox2b-ir in the vicinity of the facial nucleus (Cream et al. 2002; Stornetta et al. 2006; Onimaru et al. 2008; Guyenet et al. 2009). Neurons expressing Phox2b originate dorsally and migrate ventrally during development (Champagnat et al. 2011; Gray 2013).

*Increase in Phox2b-ir neurons ventral to the facial nuclei corresponds with an increase in CO<sub>2</sub> sensitivity*

Central chemosensitivity is a homeostatic mechanism present in all tetrapod vertebrates, including larval amphibians, and is present in some fish as well (Torgerson et al. 1997; Milsom 2010b; Guyenet and Mulkey 2010; Huckstepp and Dale 2011; Mulkey and Wenker 2011; Côté et al. 2014; Hoffman et al. 2016). In amphibians, isolated brainstem preparations from early metamorphic tadpoles display central CO<sub>2</sub> sensitivity; however, in late metamorphic tadpoles and frogs, it is a more developed and robust feature (Chapter 2; (Torgerson et al. 1997; Torgerson et al. 2001a; Taylor et al. 2003b; Taylor et al. 2003a)). Depending on the investigator, increased CO<sub>2</sub> has been reported to either stimulate or have no effect on fictive lung respiration in intact brainstem preparations (Torgerson et al. 1997; Taylor et al. 2003b). However, in late metamorphic tadpoles and frogs, increased CO<sub>2</sub> produces vigorous increases in lung burst frequency (Chapter 2; (Torgerson et al. 1997; Torgerson et al. 2001a; Taylor et al. 2003b; Taylor et al. 2003a)). While several locations in the medulla of amphibians have been proposed to contain central chemoreceptors, a key area congruent in all literature centers near the proposed lung oscillator (Torgerson et al. 1997; Torgerson et al. 2001a; Taylor et al. 2003b; Taylor et al.

2003a). The lung oscillator, located near the abducens (VI) nerves, between the facial (VII) and glossopharyngeal (IX) nerves, is in the precise region Phox2b-ir neurons appear more ventral when comparing early and late metamorphic brainstems.

An interesting observation in relation to the increase in Phox2b neurons ventral to the facial nucleus is the rather large distance from the ventral surface (Fig. 4.4). Previous work has indicated CO<sub>2</sub> sensitivity in this region depends on cells located near the ventral surface, as indicated by DEAD RED staining following kainic acid application (Taylor et al. 2003b). However, few neurons have been reported to reside near the ventral surface in amphibians near this region (Kemali and Braitenberg 1969). Further, investigations using cFos staining for CO<sub>2</sub>-activated cells did not reveal any CO<sub>2</sub>-activated cFos-positive neurons near the ventral medullary surface (Reed, unpublished). An alternative interpretation may reconcile these conflicting observations. In mammals, Phox2b-ir pFRG/RTN cells are closely juxtaposed to neighboring blood vessels, putatively sensing changes in pH/CO<sub>2</sub> near blood vessels (Onimaru et al. 2012). The blood brain barrier is made of both astrocytes and endothelial cells, forming tight junctions, and astrocytes forming this barrier express ionotropic and AMPA/kainate glutamate receptors, the receptors for kainic acid (Brand-Schieber et al. 2004; Abbott et al. 2006). It is possible that the Phox2b-ir neurons ventral to the facial nucleus in amphibians are in close proximity to blood vessels and kainic acid applications used by Taylor (2003) destroyed the integrity of these vessels near the ventral surface (Taylor et al. 2003b). Furthermore, pFRG/RTN neurons in mammals send projections to the ventral medullary surface, possibly to monitor changes in pH/CO<sub>2</sub> (Mulkey et al. 2004; Guyenet et al. 2009). If these processes exist in the Phox2b-ir neurons in amphibians, they also may have been disrupted when kainic acid was applied (Taylor et al. 2003b). A staining investigation of cell proximity to blood vessels or processes extending to the ventral surface in amphibians has not been attempted, but might resolve these conflicting findings.

#### *Is there homology between the amphibian lung oscillator and the mammalian pFRG/RTN?*

We explored potential homology of function in the amphibian lung oscillator and the mammalian pFRG/RTN in Chapter 2. As the pFRG/RTN is most commonly identified using positive immunoreactivity of Phox2b and type 2 vesicular glutamate transporter (VGlut2) and

negative immunoreactivity for TH (Cream et al. 2002; Stornetta et al. 2006; Onimaru et al. 2008; Guyenet et al. 2009), we propose that we have demonstrated a similar area in amphibians. No single study can prove homology, and other alternatives must be considered.

First, an equivalent functional site need not reside in anatomical homology. One would expect the drastic difference in the size and complexity of the mammalian brainstem compared to the amphibian brainstem to result in different anatomical regions responsible for similar functions in at least some cases. Two examples of this are the optic tectum (also called superior colliculus in mammals) and the cerebellum. Amphibians have a large optic tectum and small cerebellum compared to the smaller superior colliculus and larger cerebellum found in mammals (Northcutt 2002). Further, the majority of motor nuclei in the amphibian brainstem are found relatively more dorsal, when compared to the proximity of mammalian motor nuclei to the ventral surface.

Second, without staining for VGlut2, it is not known if the Phox2b neurons observed ventral to the facial nucleus are glutamatergic. However, lung oscillator rhythms can be abolished by application of the AMPA/kainate receptor antagonist CNQX, suggesting a population of rhythmic glutamatergic neurons exist in this location (Chapter 2). Additional staining for glutamatergic markers may reveal the specific phenotype of Phox2b neurons surrounding the facial nucleus. Staining for the mRNA of VGlut2 would provide conclusive evidence for a glutamatergic population of cells.

Third, it is possible that the increase in Phox2b neurons observed here may be more related to a priming oscillator in amphibians than the lung oscillator. Recent work from Baghdawala et al. has suggested a third oscillator, termed the priming oscillator, located between the facial (VII) and glossopharyngeal nerves (IX), is responsible for producing priming bursts observed immediately prior to lung bursts (Baghdadwala et al. 2015a). Neurons recorded from this area seem to fire at both the priming phase immediately before lung bursts and immediately after lung bursts, similar to the recorded neurons we describe here (Fig. 4.7). Because the described priming oscillator spans a region encompassing that of the ventral shift in Phox2b neurons observed in this study, the most parsimonious explanation may be that Phox2b neurons are related more to the diffuse priming oscillator than the much more discrete lung oscillator.

#### **4.6 Conclusion**

The identification of Phox2b as a marker for the pFRG/RTN respiratory group in mammals has been useful for understanding the development of a crucial respiratory circuit in mammals. Here we show a conspicuous ventral migration of Phox2b neurons surrounding the facial nucleus that occurs during metamorphosis. That both in mammals and amphibians a population of Phox2b-ir neurons migrates ventrally, in an anatomically and functionally similar region, leads us to speculate that this population of neurons may be homologous either to the lung oscillator or the priming oscillator.

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#### 4.8 Figures

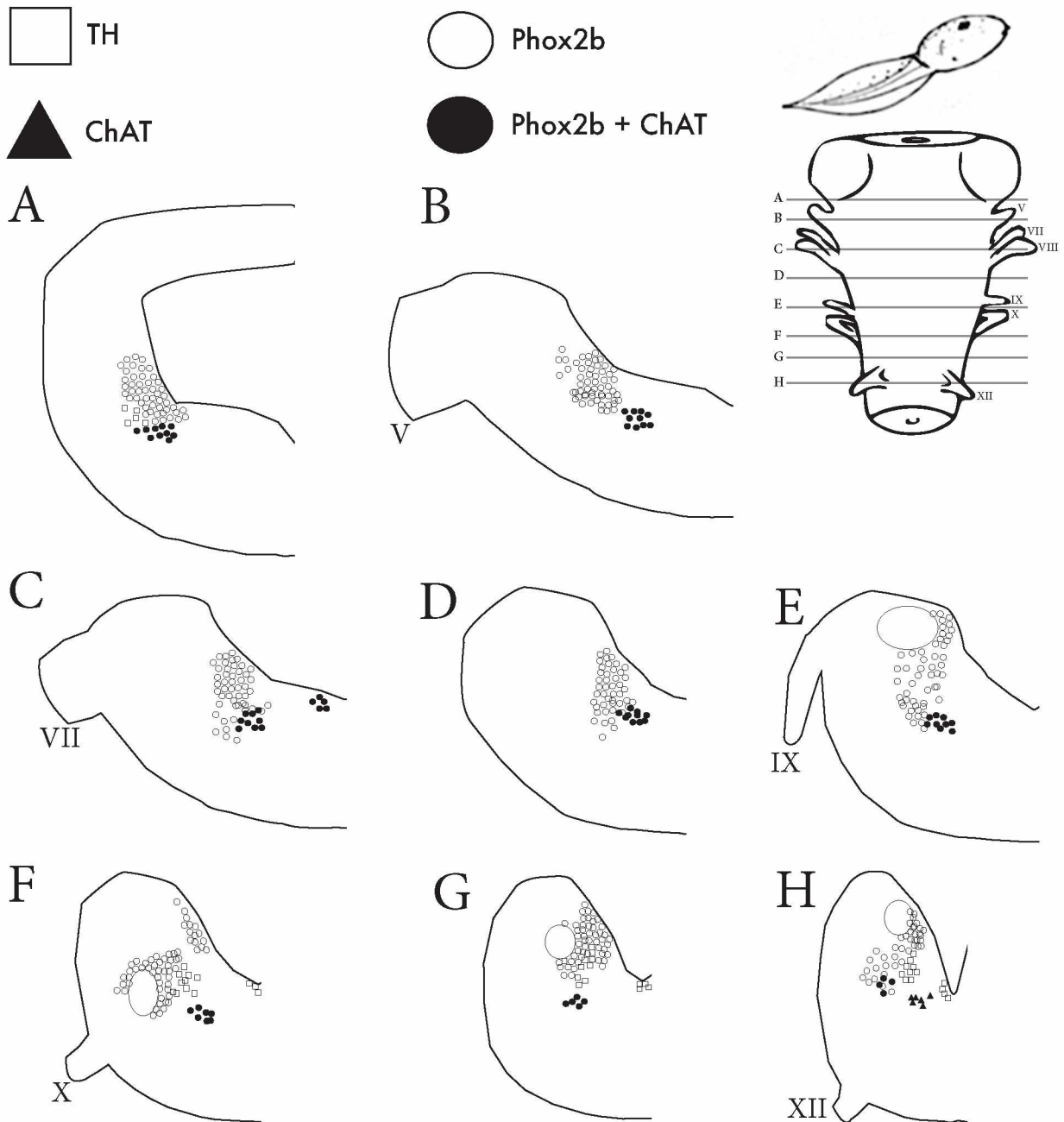


Figure 4.1: **Phox2b is present throughout the brainstem and closely associates with catecholaminergic neurons of the motor nuclei in early metamorphic tadpoles.** Early metamorphic tadpole brainstems were stained for presence of paired-like homeobox 2b (Phox2b) transcription factor, choline acetyltransferase (ChAT), and tyrosine hydroxylase (TH) from the level of the cerebellum (A) to the hypoglossal nerve (H). (A-H) Phox2b-immunoreactive (Phox2b-ir) neurons (open circles) were observed at each level of the brainstem. ChAT-ir neurons co-localized with Phox2b (filled circles) throughout the brainstem, with the exception of the

Figure 4.1 cont.

hypoglossal (XII) motor nuclei (filled triangles, H). TH-ir (open squares) neurons were found at the level of the cerebellum (A) and caudal to the glossopharyngeal (IX) nerve (F-H), but were not found between the trigeminal (V) and glossopharyngeal nerves (B-E).

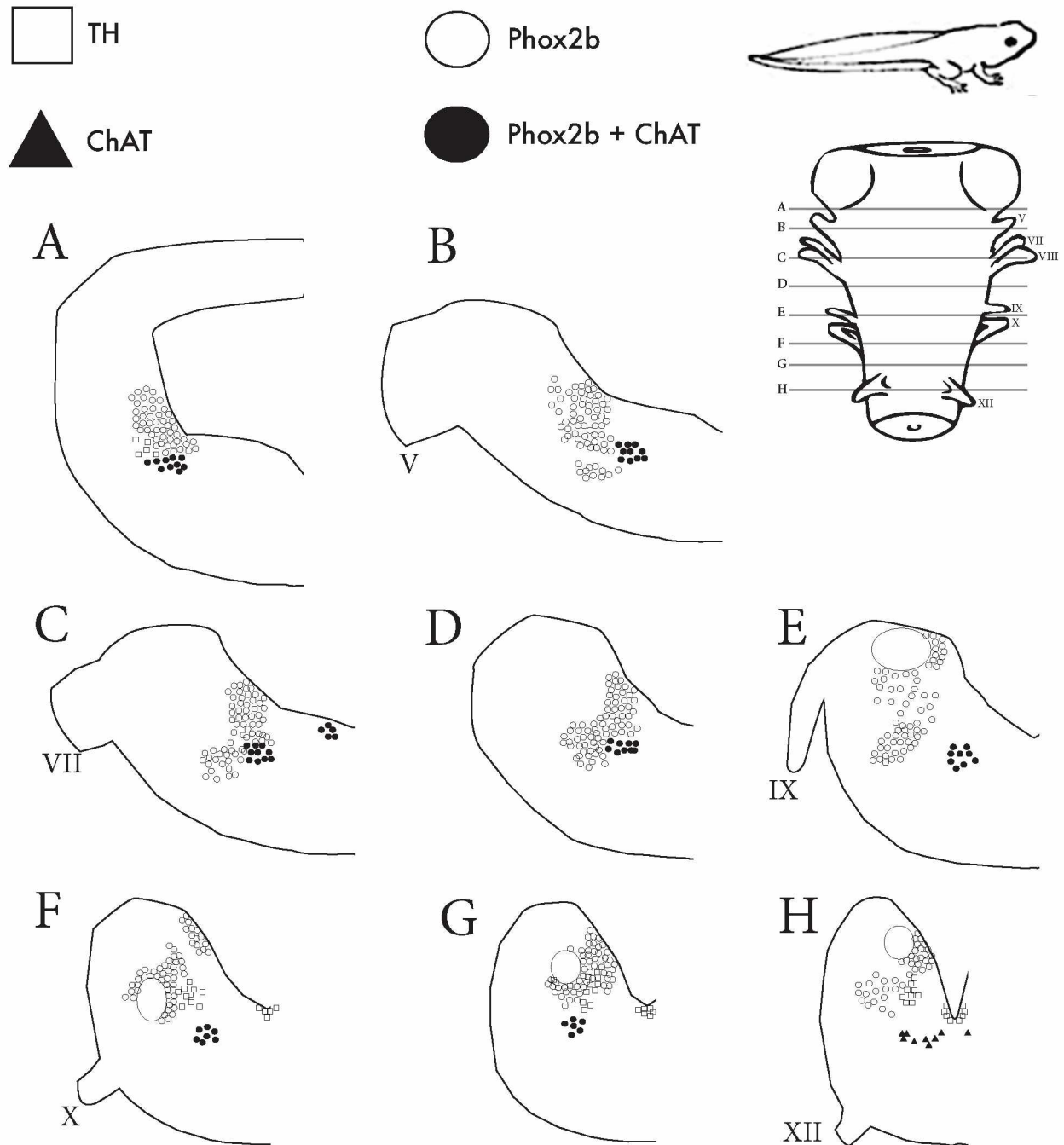


Figure 4.2: **Phox2b immunoreactivity persists throughout the brainstem in late metamorphic animals similar to the distribution observed in early metamorphic animals.** Late metamorphic brainstems were stained for presence of paired-like homeobox 2b (Phox2b) transcription factor, choline acetyltransferase (ChAT), and tyrosine hydroxylase (TH) from the level of the cerebellum (A) to the hypoglossal nerve (H). (A-H) Similar to early stage distribution, Phox2b-immunoreactive (Phox2b-ir) neurons (open circles) were observed at each level of the brainstem. ChAT-ir neurons co-localized with Phox2b (filled circles) throughout the brainstem,

Figure 4.2 cont.

with exception to the hypoglossal (XII) motor nuclei (filled triangles, H). TH-ir (open squares) neurons were found at the level of the cerebellum (A) and caudal to the glossopharyngeal (IX) nerve (F-H), but were not found between the trigeminal (V) and glossopharyngeal nerves (IX; B-E).

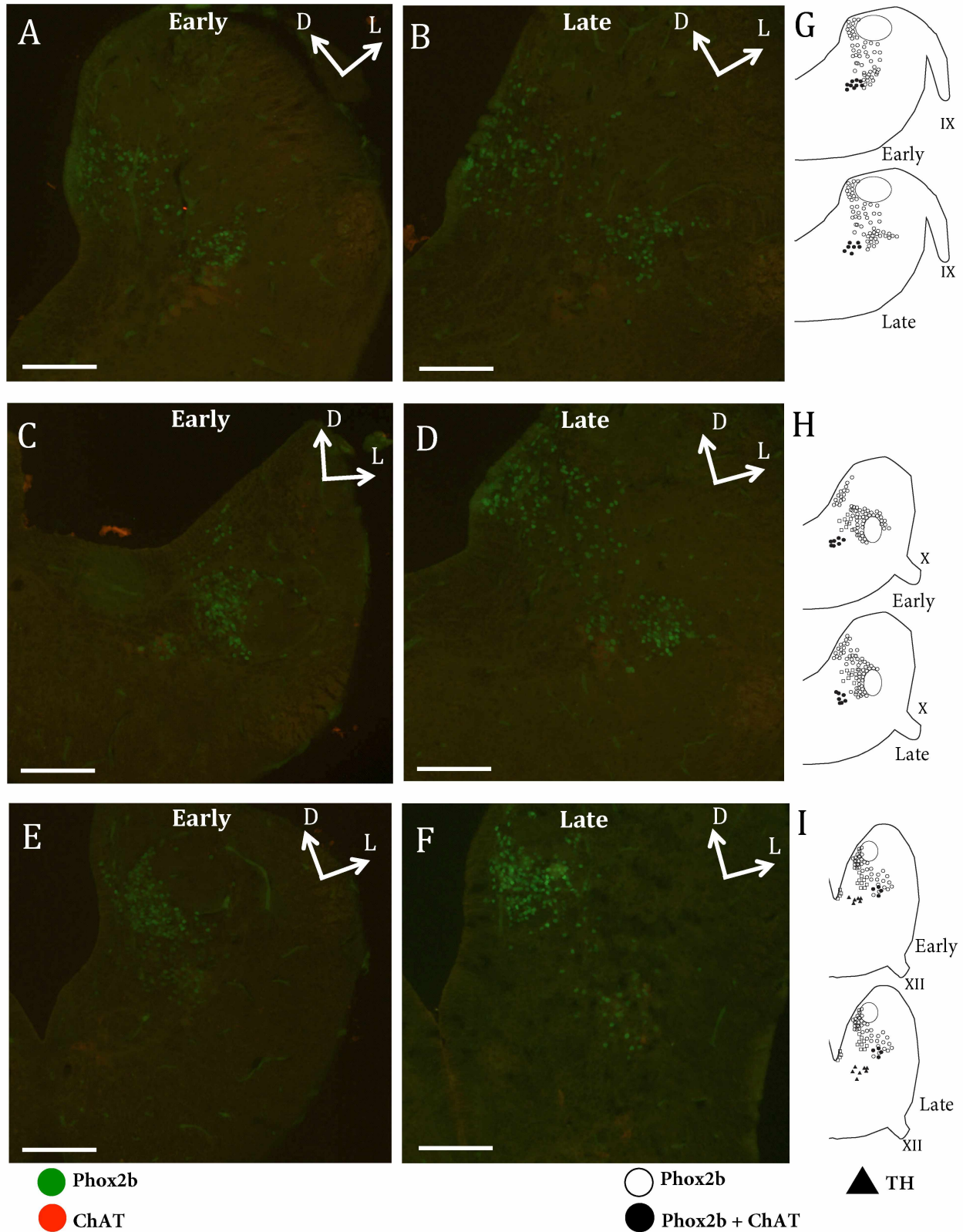


Figure 4.3: **Phox2b immunoreactive neurons between the glossopharyngeal and hypoglossal nerves co-localized with and were found lateral and dorsal to ChAT immunoreactive motor neurons.**



Figure 4.3 cont.

Early and late metamorphic tadpole brainstems (Early and Late respectively) were stained for the presence of paired-like homeobox 2b (Phox2b) transcription factor and choline acetyltransferase (ChAT). (A-I) In both early and late metamorphic animals, Phox2b-immunoreactive (Phox2b-ir) neurons (green in panels A-F; open circles in panels G-I) co-localized with ChAT (red in panels A-F; filled circles in panels G-I) except in the hypoglossal motor nucleus (red, filled triangle in panels E, F, and I). (A-I) Phox2b-ir neurons were found dorsal and lateral (arrows orienting dorsal “D” and lateral “L”), but not ventral to Phox2b+ChAT-ir motor neurons between the glossopharyngeal (IX) and hypoglossal (XII) nerves (scale bars = 200  $\mu\text{m}$ ).

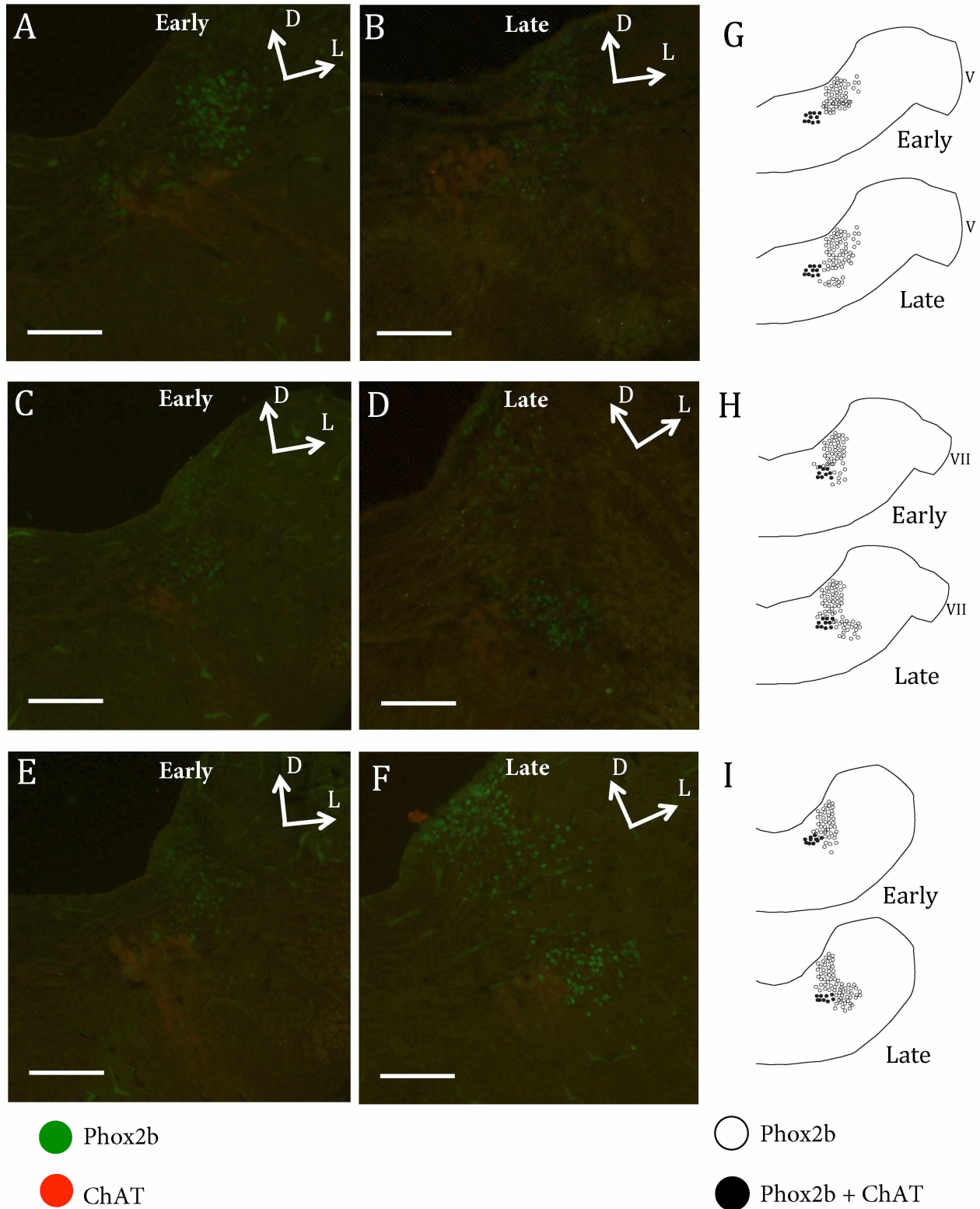
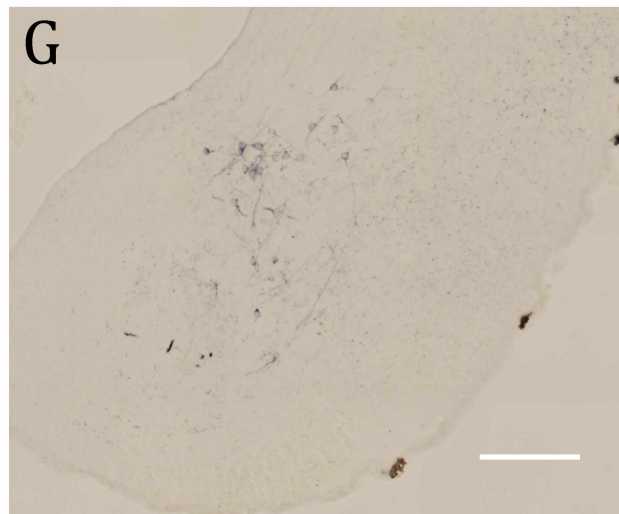
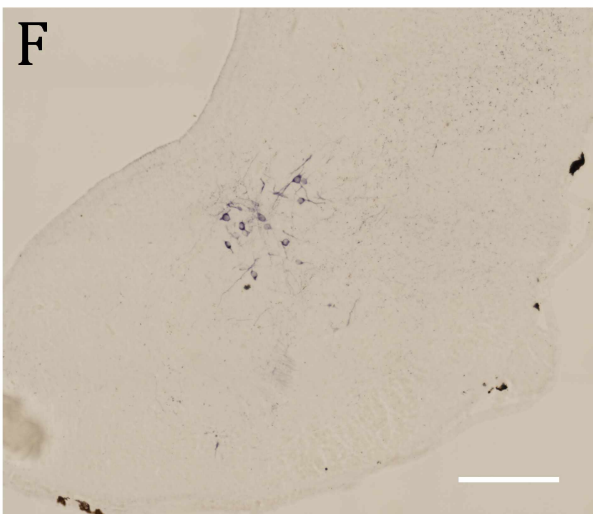
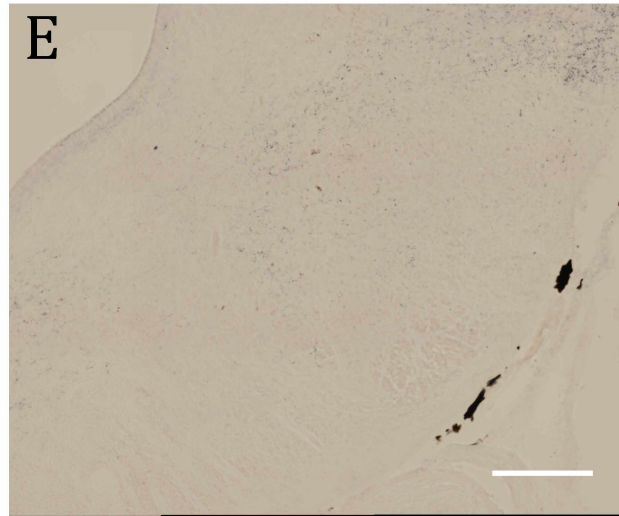
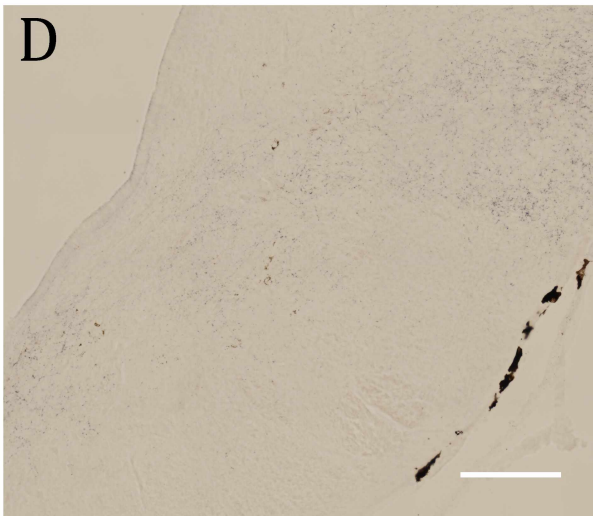
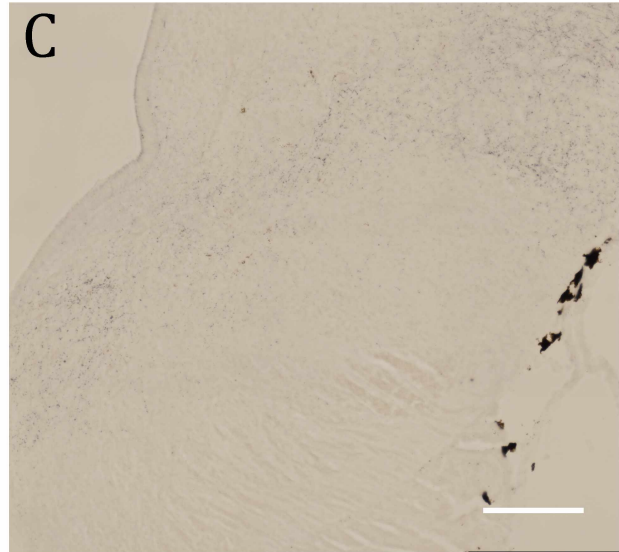
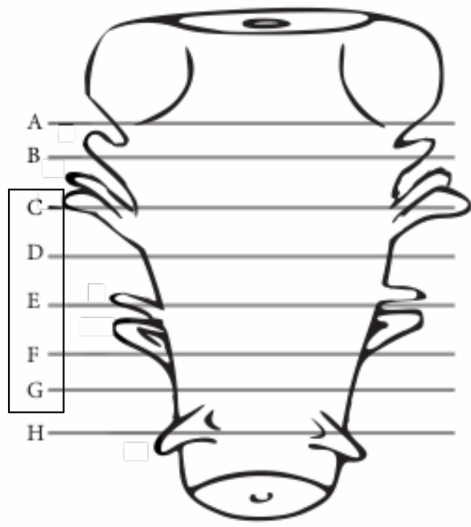


Figure 4.4: **Phox2b** immunoreactive neurons between the trigeminal and glossopharyngeal nerves co-localized with and were found lateral, dorsal, and ventral to ChAT immunoreactive motor neurons.

Figure 4.4 cont.

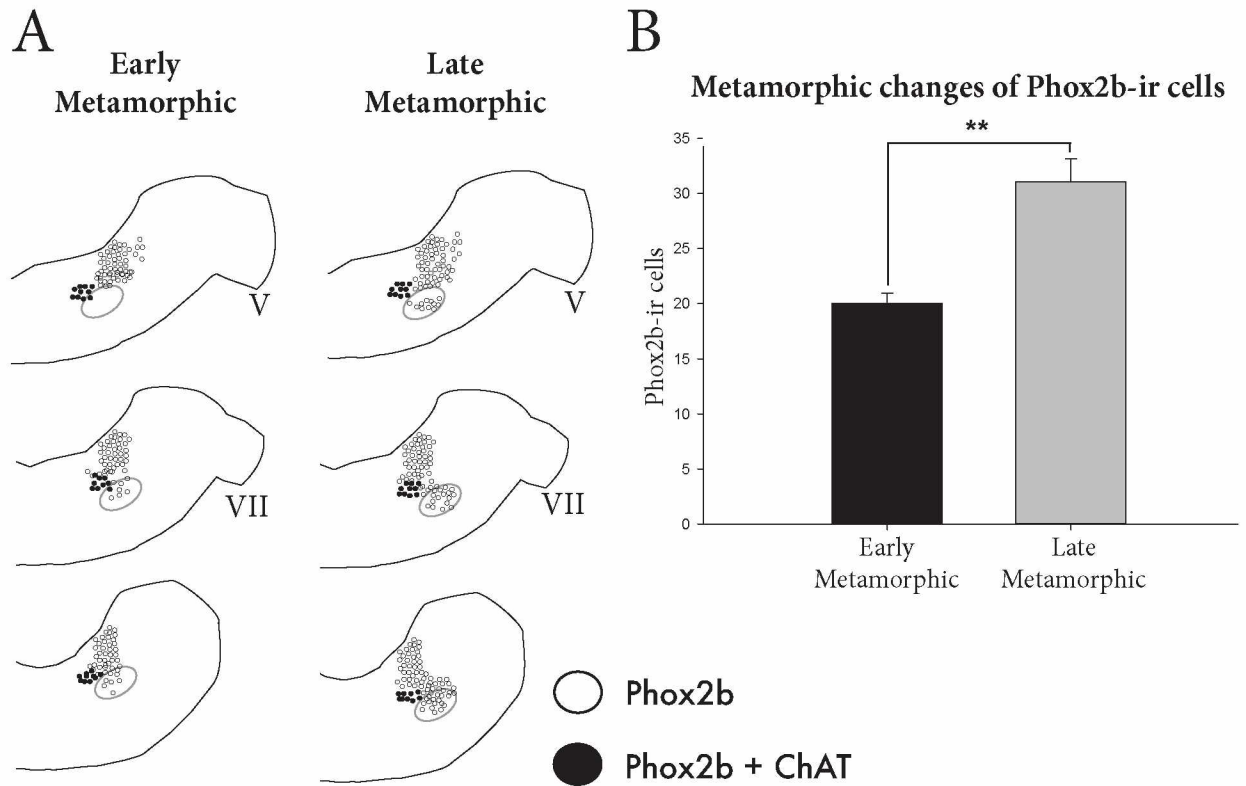
Early and late metamorphic tadpole brainstems were stained for the presence of paired-like homeobox 2b (Phox2b) transcription factor and choline acetyltransferase (ChAT). (A-I) In both early and late metamorphic animals, Phox2b-immunoreactive (Phox2b-ir) neurons (green in panels A-F; open circles in panels G-I) co-localized with ChAT (red in panels A-F; filled circles in panels G-I) motor neurons. (A-I) Phox2b-ir neurons were found dorsal, lateral, and ventral (arrows orienting dorsal “D” and lateral “L”) to the trigeminal (V) and facial (VII) ChAT-ir motor nuclei (scale bars = 200  $\mu\text{m}$ ).



**Figure 4.5: Tyrosine hydroxylase immunoreactive neurons are absent between the trigeminal and glossopharyngeal nerves.**

Figure 4.5 cont.

Brainstems of early and late metamorphic tadpoles were stained for the presence of tyrosine hydroxylase (TH). (C-G) TH-immunoreactivity (TH-ir) was not observed between the trigeminal (CN V) and glossopharyngeal (CN IX) nerves (C-E), but were found at the level of the vagus (CN X) nerve and caudal to it (F and G) All scale bars = 200  $\mu$ m.



**Figure 4.6: Phox2b immunoreactive neurons are more numerous ventral to the facial nucleus in late compared to early metamorphic tadpole brainstems.** Early and late metamorphic tadpole brainstems were stained for the presence of paired-like homeobox 2b (Phox2b) transcription factor and choline acetyltransferase (ChAT). (A) Phox2b-immunoreactive (Phox2b-ir) cells (open circles in panel A) were found both dorsally and ventrally relative to Phox2b + ChAT-ir (filled circles) motor neurons in early and late metamorphic brainstems. (B) Phox2b-ir cells (open circles in panel A) ventral to Phox2b + ChAT-ir motor neurons (filled circles in panel A) were, on average, more numerous in late metamorphic tadpoles compared to early metamorphic tadpoles. Significant differences ( $p < 0.01$ ) between early and late metamorphic Phox2b-ir cells ventral to Phox2b + ChAT-ir cells are denoted by (\*\*).

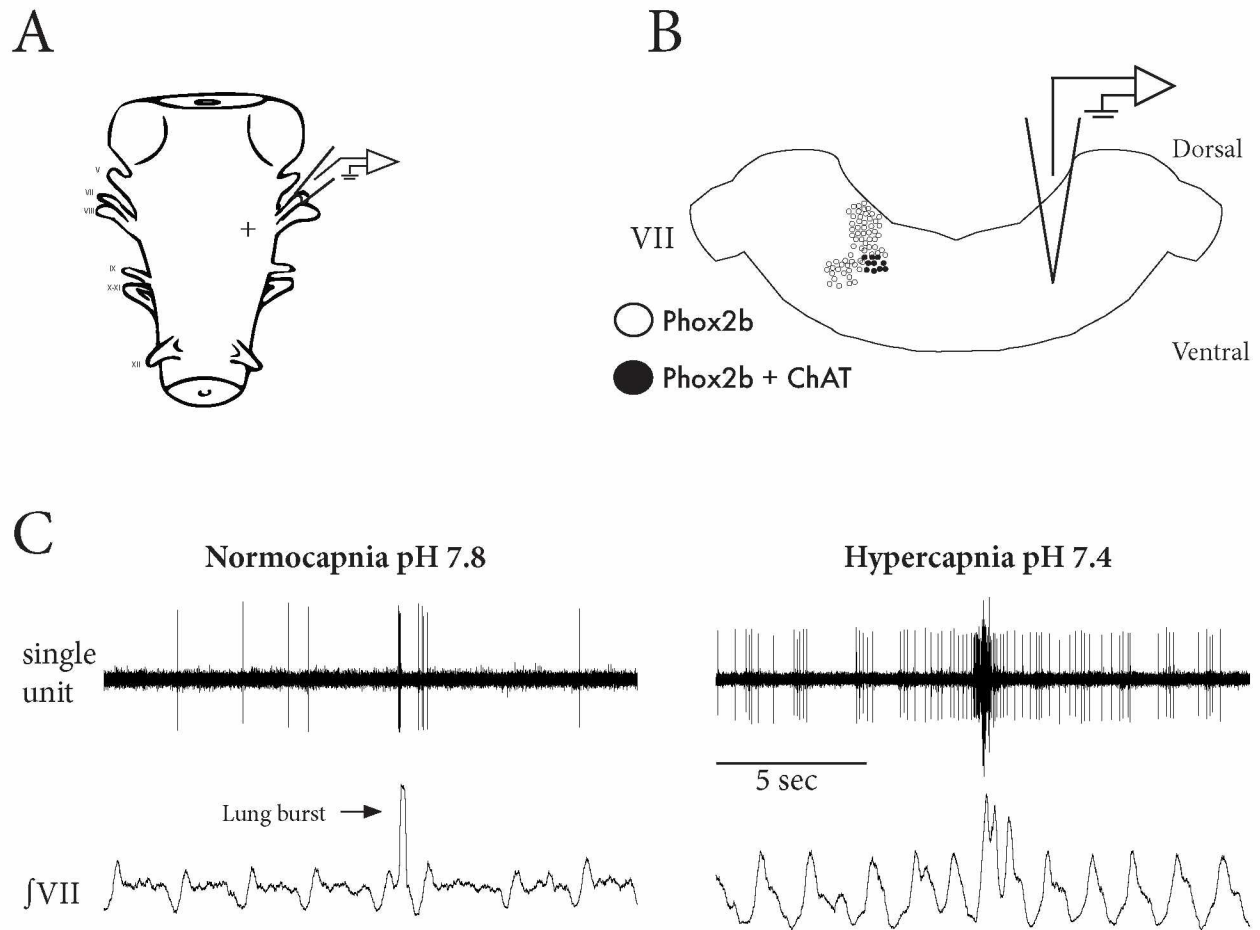


Figure 4.7: **Neurons recorded between the facial and glossopharyngeal nerves were chemosensitive and associated with lung bursts.** (A) Whole-nerve bursts (raw and integrated “f”) were recorded from the facial nerve (VII) and (B) single-unit recordings were made between the facial (VII) and glossopharyngeal nerves (IX), during normo- and hypercapnia to test for CO<sub>2</sub> sensitivity. Recordings were made approximately 400-600 μm from the dorsal surface, into areas of paired-like homeobox 2b (Phox2b)-immunoreactive (Phox2b-ir; open circles in panel B) neurons ventral to the Phox2b+ choline acetyltransferase (ChAT)-ir (closed circles in panel B) facial (VII) motor nuclei. (C) Recorded neurons fired during lung bursts and demonstrated increased firing frequency when exposed to hypercapnia (pH 7.4).



**Table 4.1**  
**List of primary and secondary antibodies used**

<i>Primary Antibody</i>	<i>Manufacturer + (ID)</i>	<i>Concentration</i>	<i>Host Species</i>
Paired-like homeobox 2b (Phox2b)	Santa Cruz Biotech (B-11)	1:100	Mouse
Choline acetyltransferase (ChAT)	EMD Millipore (AB144P)	1:100	Goat
Tyrosine Hydroxylase (TH)	Immunostar, INC (22941)	1:1000	Mouse
<i>Secondary Antibody</i>			
Alexa 488	Invitrogen	1:500	Donkey
Alexa 546	Invitrogen	1:500	Donkey
Biotinylated anti-Mouse	Jackson ImmunoResearch Laboratories	1:600	Donkey





## Chapter 5

### Conclusion

#### 5.1 Summary of results

My results confirm that amphibians have a respiratory control network in the brainstem that undergoes changes as metamorphosis advances and demonstrate that the amphibian lung oscillator shares functional and structural similarities with the mammalian parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN). I have provided evidence that a robust rhythmogenic site exists in the rostral medulla of developing bullfrog tadpoles and is capable of providing CO<sub>2</sub> drive to lung rhythmogenesis. My results show that this region, when isolated, can be disinhibited by the addition of the  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor antagonist bicuculline, producing episodic rhythmic activity containing lung bursts. The episodes and lung bursts induced by bicuculline could be abolished by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) antagonism of AMPA/kainate receptors and were insensitive to administration of the  $\mu$ -opioid receptor agonist [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin (DAMGO). Application of the glycine receptor antagonist strychnine increased burst amplitude but did not affect episodes or lung bursts. This rostral medullary region of the tadpole brainstem also contained a high number of neurons that are immunoreactive for the paired-like homeobox 2b (Phox2b-ir) transcription factor that were not immunoreactive for the tyrosine hydroxylase or choline acetyltransferase proteins (TH-ir and ChAT-ir, respectively) that migrated ventrally and laterally around the facial motor nucleus. Further, neurons recorded from this area were CO<sub>2</sub> sensitive and had activity patterns that synchronized with fictive lung breaths. Taken together, these results suggest a site located in the rostral medulla that is homologous to the pFRG/RTN in mammals and plays a large role in chemosensitivity and lung rhythmogenesis in the bullfrog tadpole.

#### 5.2 Buccal rhythmogenesis is sensitive to CO<sub>2</sub>

I have helped resolve conflicting reports regarding the effect CO<sub>2</sub> has on buccal rhythmogenesis in the isolated rostral medulla (Torgerson et al. 1997; Gdovin et al. 1999; Remmers et al. 2001; Torgerson et al. 2001; Taylor et al. 2003; Quenet et al. 2014). Torgerson reported that buccal rhythmogenesis can be stimulated by CO<sub>2</sub>, however, Taylor and others have

suggested buccal rhythms are not affected by hypercapnia (Torgerson et al. 1997; Torgerson et al. 2001; Taylor et al. 2003). These differences may be explained by the way in which the brainstem was isolated. The rostral transection of the isolated brainstem preparations made of Torgerson et al. (1997 and 2001) was made immediately rostral to the trigeminal (V) cranial nerve, whereas Taylor et al. (2003) made this transection immediately rostral to the optic tectum (Torgerson et al. 1997; Torgerson et al. 2001; Taylor et al. 2003). While these details of preparation were reported in the methods sections of both papers, it was likely overlooked because the isolated brainstem preparation is generally made at the borders described in Taylor et al. (2003). In Chapter 2, I show that rostrally transecting the brainstem immediately to the trigeminal (V) nerve reveals a buccal rhythm (normally insensitive to changes in CO<sub>2</sub> within an intact brainstem) that is stimulated by increases in CO<sub>2</sub>. This data supports the work of both investigators and raises interesting questions about the physiological significance of a CO<sub>2</sub> sensitive buccal rhythm.

### **5.3 Is the buccal oscillator in amphibians homologous to the Pre-Bötzinger Complex in mammals?**

In mammals, the anatomical region similar to the location of the buccal oscillator in amphibians contains the Pre-Bötzinger Complex (Smith et al. 1991; Solomon 2003; Feldman and Del Negro 2006). This area is excited by CO<sub>2</sub> and provides efferent stimuli to phrenic motor neurons causing contraction of the diaphragm (Smith et al. 1991; Solomon 2003; Feldman and Del Negro 2006; Janczewski and Feldman 2006). Homologies have been proposed between the lung and buccal oscillator in amphibians to the Pre-Bötzinger Complex in mammals (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006; Baghdadwala et al. 2015). Because lung ventilation in amphibians and lung ventilation in mammals are both sensitive to hypercapnia, researchers have suggested similarities between the lung oscillator and the Pre-Bötzinger Complex (Wilson et al. 2002; Vasilakos et al. 2005; Wilson et al. 2006). While this is not an anatomical similarity, the change in activity in the presence of CO<sub>2</sub> and role in lung generation is intriguing and suggests homology in function. However, the current demonstration of mildly chemosensitive buccal rhythmogenesis adds a new layer of complexity to the discussion of the evolutionary origins of respiratory oscillators in amphibians and mammals.

#### 5.4 Buccal oscillations without the buccal oscillator?

The location in the brainstem that generates rhythmic output necessary for buccal behavior in metamorphic amphibians, termed the “buccal oscillator”, has been identified in the caudal half of the medulla, caudal to the vagus (X) nerve (Wilson et al. 2002; Kottick et al. 2013; Baghdadwala et al. 2015). However, in the current study, nerve recordings following transections rostral to this site exhibited buccal-like, small amplitude bursts, albeit these bursts differed in duration and timing compared to intact brainstems (Fig. 2.5a-b). The most likely explanations for these disparate observations are:

1. The rostral border of the buccal oscillator is further rostral than previously reported.
2. The buccal rhythm generating network is more diffuse than previously reported.
3. The small amplitude bursts observed following transections are not buccal bursts.

1. The rostral border of the putative buccal oscillator has been reported to lie at the caudal level of the vagus nerve (X). In the current study, transections were made at the level of the glossopharyngeal nerve (IX), removing the putative buccal oscillator from that portion of the brainstem from which I recorded. Results from previous transection studies suggest buccal bursts are abolished following transection at the level of the glossopharyngeal nerve (Torgerson et al. 2001; Wilson et al. 2002; Klingler and Hedrick 2013), providing evidence for a discrete buccal oscillator residing at the level of the vagus nerve. While different than previously reported, the bursts observed in my experiments may be due to the inclusion of a portion of the buccal oscillator following transection, suggesting a larger buccal oscillator region than previously reported.

2. While inclusion of the previously described buccal oscillator is possible, it is also possible the network responsible for generating buccal bursts is more diffuse in amphibians than previously proposed. Buccal ventilation in amphibians is a remnant of gill ventilation in fish. Gill ventilation in both lamprey and goldfish is reported to be produced by a more diffuse network than the respiratory control network of mammals (Duchcherer et al. 2010; Cinelli et al. 2014). In lamprey specifically, two sites have been implicated in gill ventilation, a paratrigeminal respiratory group

(pTRG) and paravagal respiratory group (pVRG; (Cinelli et al. 2014)). In goldfish, presumed respiratory rhythmogenesis was observed in multiple locations, including a rostral portion of the medulla near the trigeminal nerve (Duchcherer et al. 2010). While more work will be needed for confirmation, it is likely that lamprey and fish possess a more diffuse network to produce a conserved rhythmic behavior as compared to amphibians.

3. It is possible that the bursts observed were not related to buccal ventilation. Lung rhythms observed in amphibians have been noted to contain a pre-inspiratory component, thought to prime the buccal cavity for a subsequent lung burst (Baghdadwala et al. 2015). Investigation into this component of lung burst rhythms has led to the identification of a potential new oscillator (termed the priming oscillator) surrounding the lung oscillator (Baghdadwala et al. 2015). While the small amplitude bursts may be a result of increased priming oscillator activity, Baghdadwala et al. (2015) were not able to uncouple the priming oscillator from lung oscillator as observed in the small amplitude bursts presented here. The brainstem also possesses neurons responsible for producing numerous rhythmic behaviors, such as breathing, feeding, swallowing, and suckling (Jean 2001; Milsom 2010; Moore et al. 2014; Samson et al. 2017). Additionally, each rhombomere (a transiently divided segment of the developing neural tube in a vertebrate embryo) within the brainstem has been proposed to contain an oscillator capable of producing rhythmic neural activity (Gust et al. 2003; Ren and Greer 2003; Milsom 2010). Removing any input to these oscillators could change their “common” rhythm to something undefinable. As such, it is possible the small amplitude rhythm observed may be related to swallowing, feeding, or other oropharyngeal rhythms.

### **5.5 Is the priming oscillator or lung oscillator in amphibians more likely to be homologous with the pFRG/RTN in mammals?**

As previously mentioned, the priming oscillator surrounds the lung oscillator in the rostral medulla of bullfrog brainstems (Baghdadwala et al. 2015). In my experiments, both these oscillators were kept intact during transections and pharmacological manipulations and, as such, were not able to be uncoupled. A curious observation is the distribution of Phox2b-ir neurons ventral to motor nuclei between the glossopharyngeal (IX) and trigeminal (V) nerves as reported

in Chapter 4. This distribution resembles the border of the priming oscillator nuclei drawn by Baghdawala and is not as discrete as the lung oscillator is reported to be. Further, extracellular recordings reported in Chapter 4 are similar to the priming neuron discharge reported by Baghdawala. As such, it is possible that Phox2b-ir cells in this region are both lung and priming oscillator neurons. In mammals, the pFRG/RTN is a heterogeneous group of glutamatergic, Phox2b-ir cells (Mulkey et al. 2004; Stornetta et al. 2006; Onimaru et al. 2008; Guyenet et al. 2009; Abbott et al. 2011). A portion of neurons in this region have a more phasic, burst-like firing pattern, while the others have a more steady tonic firing pattern (Onimaru and Homma 2003; Onimaru et al. 2009; Huckstepp et al. 2015). Both groups of cells seem to play a role in chemoreception and active expiration rhythmogenesis (Onimaru and Homma 2003; Mulkey et al. 2004; Guyenet et al. 2009; Onimaru et al. 2009; Huckstepp et al. 2015). Because both groups of pFRG/RTN neurons are phenotypically similar, separating these two populations in mammals has proven difficult. In amphibians, the lung oscillator is a discrete region at the level of the abducens (VI) nerve, where the priming oscillator is far more diffuse (Wilson et al. 2002; Baghdadwala et al. 2015). It is possible these cells reflect a primitive heterogeneous population of cells similar to the pFRG/RTN. One possible way to test this observation would be intracellular filling of cells with fluorescent dye to label lung and priming oscillator neurons, and followed by staining for Phox2b and other neuronal markers to determine neurotransmitter phenotype.

## 5.6 Critique of methods

While the work described in these chapters helps advance our understanding of the evolutionary origins of respiratory rhythmogenesis, it is not without limitations. First, the transections described in Chapter 2 were completed using micro-dissection scissors. It is possible that during transections more damage was caused to the remaining brainstem than intended. Also, the location of transection was determined by cranial nerve position. Because the freely floating isolated brainstem could not be secured during transections, it is possible transections were not as consistent as intended. However, the recordings produced from this set of experiments were consistent. In Chapter 3, the addition of 5  $\mu$ M bicuculline was chosen because of previous published work examining the role of chloride-mediated neurotransmission (Broch et al. 2002; Galante et al. 1996). Because the addition of higher concentrations of bicuculline can

cause seizure-like activity, it cannot be assumed that the rhythmic episodes observed during experiments described in Chapter 3 were not seizures (Fig. 4.2). In Chapter 4 experiments, immunohistochemistry targeting Phox2b transcription factor may produce non-specific binding of other proteins within the nuclei of neurons. Because so little immunohistochemistry has been completed in amphibians, some reservation about the specificity of the Phox2b antibody used in this study is reasonable. A study comparing conservation of transcription factors across phylogeny using *in situ* hybridization demonstrated Phox2b is present throughout the amphibian brainstem (Albersheim-Carter et al. 2015). Distributions of Phox2b presented by Albersheim-Carter et al. were similar to our findings. For example, Albersheim-Carter et al. report Phox2b-ir was found in motor neurons throughout the brainstem, except the hypoglossal motor nucleus (Albersheim-Carter et al. 2015). A similar observation is made in Chapter 4, suggesting Phox2b-ir presented in this work shows specificity for the targeted transcription factor (Fig. 4.3).

## 5.7 Summary of conclusion

The results presented here demonstrate some similarities in the neural control of breathing shared by amphibians and mammals. Similar to mammals, amphibian respiration can be stimulated by exposure to CO<sub>2</sub>. Chemosensitivity in amphibians develops as tadpoles develop toward the terrestrial lifestyle. The location providing a large component of CO<sub>2</sub> sensitivity is in the rostral medulla of amphibians, similar to mammals. Further, this location exhibits rhythmogenesis that is insensitive to DAMGO and can be abolished by the addition of CNQX, suggesting it is glutamatergic. The rostral medulla also exhibited a high degree of Phox2b positive neurons ventral to the facial motor nucleus, and increases in Phox2b-ir were observed during metamorphosis. Taken together, I propose that there is compelling evidence for a pFRG/RTN homologue in the rostral medulla in bullfrog tadpoles.

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## Appendix



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### **Institutional Animal Care and Use Committee**

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

April 27, 2012

To: Barbara Taylor, PhD  
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [155879-9] Development of a respiratory neural circuit: Ontogeny of respiratory drive

The IACUC has reviewed the Progress Report for 2011-12 by Full Committee Review and the Protocol has been approved for an additional year.

Received:	April 9, 2012
Initial Approval Date:	April 7, 2010
Effective Date:	April 27, 2012
Expiration Date:	April 7, 2013

This action is included on the April 24, 2012 IACUC Agenda.

*If you have any questions about how to submit the required information through IRBNet please contact the Office of Research Integrity for assistance (email [fyori@uaf.edu](mailto:fyori@uaf.edu) or call x7800/x7832).*



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## Institutional Animal Care and Use Committee

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

March 22, 2013

To: Barbara Taylor, PhD  
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [155879-13] Development of a respiratory neural circuit: Ontogeny of respiratory drive

The IACUC has reviewed the Progress Report by Full Committee Review and the Protocol has been approved for an additional year.

Received:	March 11, 2013
Initial Approval Date:	April 7, 2010
Effective Date:	March 21, 2013
Expiration Date:	April 7, 2014

This action is included on the March 21, 2013 IACUC Agenda.

### ***PI responsibilities:***

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures detailed in the form 005 "Reporting Concerns".*



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## Institutional Animal Care and Use Committee

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

March 14, 2014

To: Barbara Taylor, PhD  
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [155879-17] Development of a respiratory neural circuit: Ontogeny of respiratory drive

The IACUC has reviewed the Progress Report by Full Committee Review and the Protocol has been approved for an additional year.

Received:	March 5, 2014
Initial Approval Date:	April 7, 2010
Effective Date:	March 13, 2014
Expiration Date:	April 7, 2015

This action is included on the March 13, 2014 IACUC Agenda.

### ***PI responsibilities:***

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures detailed in the form 005 "Reporting Concerns".*



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## Institutional Animal Care and Use Committee

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

April 27, 2015

To: Barbara Taylor, PhD  
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [155879-20] Development of a respiratory neural circuit: Ontogeny of respiratory drive

The IACUC has reviewed the Progress Report by Designated Member Review and the Protocol has been approved for an additional year.

Received:	April 27, 2015
Initial Approval Date:	April 7, 2010
Effective Date:	April 27, 2015
Expiration Date:	April 7, 2016

This action is included on the May 7, 2015 IACUC Agenda.

### ***PI responsibilities:***

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures detailed in the form 005 "Reporting Concerns".*



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## Institutional Animal Care and Use Committee

909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

April 19, 2016

To: Barbara Taylor, PhD  
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [155879-24] Development of a respiratory neural circuit: Ontogeny of respiratory drive

The IACUC has reviewed the Progress Report by Administrative Review and the Protocol has been approved for an additional year.

Received:	April 18, 2016
Initial Approval Date:	April 7, 2010
Effective Date:	April 19, 2016
Expiration Date:	April 7, 2017

This action is included on the May 12, 2016 IACUC Agenda.

### ***PI responsibilities:***

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures detailed in the form 005 "Reporting Concerns".*