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CO2/pH Signaling of Respiratory Control Neurons in the Bullfrog, Lithobates catesbeianus: Development of a Comparative Model

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CO_2/pH Signaling of Respiratory Control Neurons in the Bullfrog, *Lithobates catesbeianus*: Development of a Comparative Model

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Joseph M. Santin ENTITLED CO_2/pH Signaling in Respiratory Control Neurons in the Bullfrog, *Lithobates catesbeianus*: Development of a Comparative Model BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Santin, Joseph Santin. M.S., Department of Biological Sciences, Wright State University, 2013. CO_2/pH Signaling of Respiratory Control Neurons in the Bullfrog, *Lithobates catesbeianus*: Development of a Comparative Model

The locus coeruleus (LC) in the brainstem senses alterations in CO_2/pH and influences ventilatory adjustments that restore blood gas values to starting levels in bullfrogs (*Lithobates catesbeianus*). We hypothesized that neurons of the bullfrog LC are sensitive to changes in CO_2/pH and that chemosensitive responses are intrinsic to individual neurons. In addition, we hypothesized putative respiratory control neurons of the bullfrog LC would be stimulated by hypercapnic acidosis within physiological ranges of PCO2/pH. 84% of LC neurons depolarized and increased firing rates during exposure to hypercapnic acidosis (HA). A pH dose response curve shows LC neurons from bullfrogs increase firing rates during physiologically relevant CO_2/pH changes. With chemical synapses blocked, half of chemosensitive neurons lost sensitivity to HA; however, gap junction blockade did not alter chemosensitive responses. Intrinsically chemosensitive neurons increased input resistance during HA. These data demonstrate that the majority of neurons within the bullfrog LC elicit robust firing responses during physiological CO_2/pH, likely enabling adjustment of acid–base balance through breathing.

TABLE OF CONTENTS

List of Figures

Page

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Chapter 1: Review of the Literature

The Influence of Temperature on Respiratory Control in Anuran Amphibians

Introduction:

Temperature influences the activity of nearly all physiological functions. Unlike homeothermic animals, poikilotherms have variable body temperatures (T_b) which subjects their physiological systems to the stresses of both acute and chronic temperature fluctuation. The consequences of temperature on physiological processes have a profound impact on the integration of these regulatory systems in poikilothermic organisms (Lenz *et al.,* 2005; Roshbash, 2009; Tang *et al.,* 2010). An interesting problem, therefore presents itself: if temperature exerts such a prominent impact on biological processes how can physiological systems and behavioral functions remain exquisitely regulated over a broad temperature range. For example, bullfrogs are poikilothermic vertebrates that inhabit ponds from northern Canada to Mexico (Lannoo, 2005), and experience a wide-range of temperatures. Bullfrogs function normally (*e.g.,* forage and escape predators) during relatively warm summer months (>30°C). Additionally, bullfrogs overwinter by remaining submerged in water, although activity has been recorded during air temperatures as low as -8°C and water temperatures just exceeding 0°C (Stinner, 1994). These temperature changes will impose challenges on a variety of physiological processes. From the level of the cell, to physiological systems, bullfrogs, like many other poikilothermic organisms, have the ability to cope with large and rapid changes in temperature, while integrating multiple temperature-sensitive physiological systems to maintain homeostasis. Mechanisms poikilothermic vertebrates use to integrate temperature-dependent physiological systems to meet homeostatic demands at a range of physiological temperatures are largely unexplored.

Regulation of blood gas homeostasis through respiratory control involves various physiological systems that are influenced by temperature in anuran amphibians (Reeves, 1977; Burton, 2002); however, the influence of temperature on regulation of blood gasses, specifically through the integration of the control system has received less attention. Regulation of blood gases occurs through a respiratory negative feedback loop. Changes in the levels of blood gases (O_2/CO_2/pH) are detected by either central or peripheral chemoreceptors. Altered activity of these receptors either stimulates or dampens the pattern expressed by a central rhythm generator in the medulla (precise location unknown in amphibians) which then increases or decreases breathing through effector motor nuclei (*i.e.,* cranial nerves innervating buccal elevator/depressor muscles and the glottis). Given that temperature impacts all aspects of this feedback loop, maintenance of blood gases in amphibians presents an excellent model to study the integration of temperature-sensitive physiological systems at a multiple levels of organization (cell, organ system, and organism) during temperature change. The following sections will review the current understanding of the influence of temperature on the respiratory control system of anuran amphibians, focusing generation and modulation of a conditional respiratory rhythm. Further, I will discuss relevancy of respiratory chemosensing in the anuran model as a comparative approach to address enduring questions generated by chemosensing in mammals.

1. Impact of Temperature on Blood Gas Homeostasis in Anuran Amphibians

1.1 Temperature Influences on the Variables Regulated by the Respiratory System

Variable T_B in poikilothermic vertebrates imposes a challenge on the regulation of arterial blood gases (O_2/CO_2/pH) through breathing. In general, the rates of metabolic processes parallel T_B; rising T_B increases cellular metabolic rates, while lowering T_B decreases metabolic rates (Hochochka & Somero, 1984). Therefore, physiological temperature fluctuations modulate O_2 demands required to support cellular metabolism and varies the necessity to breathe depending on the temperature. If breathing is not tightly controlled to regulate temperature specific O_2 requirements, oxidative stress at low temperatures could result in the production of reactive oxygen species which are generally considered detrimental to cell function (Thannickal & Fanburg, 2000), while reduced consumption of O_2 relative to metabolic demands at elevated temperatures would not sustain cellular ATP requirements.

Despite the parallel relationship between oxygen demand and T_B in poikilothermic vertebrates, effects of temperature on acid-base balance are complex. An exhaustive review on the current understanding of acid-base balance during temperature change in poikilothermic vertebrates can be found in Burton (2002). As in homeothermic vertebrates, elevated rates of metabolism enhance, while decreased metabolic rates reduce the requirements of CO_2 excretion. For example, in toads at constant T_B, both digestion and activity cause acid-base disturbances requiring compensation through adjustments in Pco_2 (Andersen & Wang, 2003). Moreover, regulation of acid-base balance in poikilotherms is complicated by an inverse relationship of arterial pH with temperature; as temperature increases, arterial pH becomes relatively acidified, and *vice versa* during cooling (Robin, 1962; Howell *et al.*, 1970). Given that an arterial pH change of ~ -0.015pH U/°C exists in some poikilothermic vertebrates, Howell et al.

(1970) originally proposed the regulation of a constant arterial relative alkalinity (OH^-/H^+) compared to neutral water a various temperatures. Since arterial pH shows temperature-dependence in most, (Howell *et al.,* 1970; Reeves, 1972; Robin, 1962; Jackson *et al.*, 1974), but not all (Wood *et al.*, 1977) poikilothermic vertebrates, regulation of the fractional dissociation of protons from imidazole-histidine on intracellular/ cell surface proteins (Reeves, 1972) and net protein charge (Cameron, 1989) have been proposed, although no consensus has been reached as to which variable(s) are regulated to define pH-temperature relationships (Burton, 2002). Whatever the regulated parameter may be during changes in T_b, a temperature-specific arterial pH appears to maintain acid-base balance. For example, bullfrogs regulate an arterial pH of ~7.9 at ~20°C and conform to a new pH set-point of ~7.75 at ~30°C (Howell *et al.,* 1970; Reeves, 1972; Mackenzie & Jackson, 1978; Rocha & Branco, 1998). These values, however, show species-specific differences in poikilotherms (*e.g.,* temperature-specific pH for bullfrogs will not be the same temperature-specific pH for tegu lizards). In anuran amphibians and other poikilothermic vertebrates, it is therefore reasonable to posit that arterial acid-base imbalance occurring at different temperatures is compensated and returned to an arterial pH optimized for temperature and species-specific acid-base homeostasis. These data suggest that maintenance of cellular and organismal homeostasis necessitates that arterial blood gases O_2, CO_2, and pH be regulated at temperature- specific set-points.

1.2 Respiratory Rhythm During Temperature Change

As poikilothermic vertebrates, anuran amphibians cannot escape the regulatory challenges of maintaining respiratory gasses dictated by variable T_b. Adult anuran amphibians primarily use lung ventilation to obtain O_2 to support metabolism and compensate acid-base disturbances (Boutilier & Heisler, 1988; Branco *et al.*, 1992). Although developing anurans (*i.e*, tadpoles) are subjected to similar temperature challenges as adults, only adult anurans with a fully-developed pulmonary gas exchange system will be discussed here. At elevated temperatures and metabolic rates, amphibians require increases in lung ventilation to obtain O_2 and to excrete CO_2 in order to support accelerated rates of cellular metabolism and maintain acid-base balance, respectively. Conversely, preservation of acid-base homeostasis at decreased rates of metabolism caused by cooler temperatures may not require pulmonary ventilation to obtain O_2, allowing amphibians to exploit the skin as the primary mode for CO_2 elimination (Gottlieb & Jackson, 1976; Mackenzie & Jackson, 1978). Therefore, the necessity to breathe is dependent on T_b.

Anuran amphibians breathe episodically (Gargaglioni & Milsom, 2007). This respiratory pattern is characterized by breathing episodes with non-ventilatory periods, *i.e.*, apneas, of varying durations. Episodic breathing was originally thought to be driven by inherent oscillations in blood gases during breath-holds. Early dogma in the field suggested that accumulation of CO_2 and H^+ would increase respiratory drive throughout the non-ventilatory period which would stimulate a breathing episode once these variables exceeded a specific threshold (West *et al.*, 1987). This breathing style seemed perfectly adapted for amphibians (and other poikilothermic vertebrates); if breathing is not necessary, do not breathe and the accumulation of respiratory stimulators during a

non-ventilatory period will initiate the next episode. Kinkead & Milsom(1994) challenged this notion and found that by controlling arterial blood gases through unidirectional ventilation, thus removing inherent oscillations in blood gases, episodic respiratory patterns persisted in bullfrogs. Additionally, their results suggest that blood gas oscillations during the breath-holds were likely too small to stimulate the next breathing episode. These data demonstrated that episodic breathing is likely produced by an episodic rhythm generator within the brainstem.

The nucleus isthmus (NI) in the midbrain was initially thought to function as an on/off switch for episodic breathing (Kinkead & Milsom, 1997). Ablation of the NI increased ventilatory responses to hypoxia and hypercarbia, but did not eliminate breathing episodes (Gargaglioni *et al.,* 2002; Gargalioni & Branco, 2004). Therefore, the NI appears to acts as a "brake" when respiratory drive is high, while it could also be functioning as a relay site for additional afferent sensory information. Although the NI fails to serve as an on/off switch for breathing episodes, afferent input from disparate brainstem regions converge on the rhythm generating circuit and appear to influence the expression of an episodic respiratory rhythm. For example, prior to transection of the midbrain/pons from the medulla in bullfrogs, respiratory-related neural output from the brainstem is episodic, but after transection, the pattern transitions to one of low-frequency, evenly spaced breaths (Gargaglioni *et al.,* 2007). Yet, the continuous burst pattern converts back to being episodic over time either with (Gargaglioni *et al.,* 2007) or without (Milsom, 2009) tonic drive from receptors transducing the status of lung volume. Thus, it appears that descending modulation from the midbrain may influence episodic rhythm generated by the medulla. Although previous data suggested that the respiratory

rhythm of anuran amphibians is intrinsically episodic (*i.e.*, rhythm generating circuits produce an inherently irregular respiratory pattern) with the peripheral and central modulatory sources influencing the pattern generated, current data do not preclude the role of modulatory input as the sole driver of an episodic respiratory pattern (Milsom, 2009).

As previously discussed, breathing varies with temperature in anurans; therefore, as T_b changes, the necessity to generate a breathing rhythm should generally, but not necessarily (Hedrick *et al.*, 2007), be viewed as a function of O_2 demands and acid-base regulation requirements. At low temperatures the respiratory rhythm is not expressed, while at greater temperatures, the rhythm is continuous. The problem of manipulating a breathing rhythm within the brainstem to support regulatory requirements dictated by T_b is most parsimoniously addressed by modulatory control of a central rhythm generator that produces a regular rhythm over a range of temperatures. This contrasts the view that activity of rhythm generating circuits will cease at temperatures that do not require breathing. Data demonstrating elimination of the respiratory rhythm at cool temperatures has been interpreted to suggest that the rhythm generating circuitry has the inherent capacity to convert from being quiescent at low temperatures, to active and continuous at elevated temperatures (Mellen *et al.*, 2002; Morales & Hedrick, 2002). Although this interpretation may be sufficient to explain inhibition of a homeothermic mammalian respiratory network by cool temperatures (Mellen *et al.*, 2002), the amphibian respiratory network must be able maintain *control* of breathing at not just one, but at a variety of different of temperatures.

The respiratory rhythm of amphibians is modulated by various peripheral and central afferent inputs to the central rhythm generator (Reid, 2006). It is therefore possible that during increases in T_b, output from the rhythm generating circuit may increase breathing because of afferent input from respiratory modulating brainstem/midbrain areas which may detect changes in temperature, CO_2, or pH, as opposed to Q_{10} effects on intrinsic ionic conductances of rhythm generating neurons, *i.e.*, as implied by Mellen et al. (2002). In bullfrogs, experimental evidence demonstrates that respiratory frequency *in vivo* and respiratory rhythm of the brainstem *in vitro* ceases at cool temperatures (Bícego-Nahas & Branco, 1999; Morales & Hedrick, 2002). This inhibition of breathing due to cool T_b, however, can be overridden by modulatory inputs during stress and handling (personal observation). Despite evidence that breathing is not required to support metabolism and regulate acid-base balance at low temperatures, *e.g.*, 10°C, (McKenzie & Jackson, 1976) and that no respiratory rhythm persists *in vitro*; breathing, in fact, *can* occur at low temperatures. Breathing-related nerve output in hypothermic hamsters supports the idea of a network-modulated control of rhythm generating respiratory circuits during changes in T_b (Zimmer & Milsom, 2004). For example, at hypothermic temperatures (≤22°) no respiratory rhythm was expressed in the hamster brainstem preparation. Removal of the pons, however, caused spontaneous respiratory activity at low temperatures, suggesting that descending inhibitory drive from nuclei originating in the pons mediates the respiratory inhibition during cooling. Given the diverse regulatory requirements determined by T_b in amphibians, inherent inhibition or hyperactivation of the rhythm generating circuits by Q_{10} effects during cooling and

warming, respectively, may provide too little control over the breathing process required to maintain temperature-specific blood gas homeostasis.

A model of temperature compensation to preserve neuronal function over a range of temperatures has been proposed in order to explain behavioral phenomena which are precisely regulated over a range of temperatures (Robertson & Money, 2012). Temperature compensation of neurons and neuronal circuits is achieved mechanistically through the expression of opposing ionic conductances with similar Q_{10}s. For example, during an increase in temperature, ionic conductances stimulated by the increase in temperature must be balanced by an equal number ionic conductances decreased by increased temperature in order for a neuron to preserve stable firing frequency or a neuronal circuit to maintain constant phase frequency. From the level of individual neurons to the level of neuronal circuits, temperature compensation within the central nervous system allows for automatic regulation of crucial biological processes. This model has been applied to the rhythmic chewing behavior in crabs (Tang *et al.,* 2010), escape response in copepods (Lenz *et al.*, 2005), and circadian rhythms in *Drosophila* (Mehra *et al.,* 2009). Each example is an integrative biological function that must maintain function over a broad range of temperatures.

Temperature compensation would provide a mechanism to maintain constant function of the amphibian respiratory rhythm generating circuits over a range of temperatures. If the respiratory rhythm generating circuit of the amphibian brainstem was temperature compensated to maintain constant function over a range of physiological temperatures, modulatory input from any number of sources would ensure tuned control of the rhythm underlying breathing. Specifically, temperature compensation of rhythm

generating neurons at cool T_b would be advantageous so that a modulatory input could suppress expression of rhythm generating neurons; however, if breathing became obligatory, *i.e.*, during stress, escaping prey, or foraging, another modulatory site could disinhibit the respiratory rhythm. Complete inhibition of the respiratory rhythm generator during exposure to cold temperatures would not allow for this level of breathing control. In fact, Milsom (2009) suggests that regardless of expression of a respiratory rhythm, there is likely some endogenous rhythmic activity within the primary-rhythm generating circuits even during periods of low respiratory drive. Conversely, at warm temperatures, when arterial pH acidifies and metabolism increases, brainstem area(s) sensitive to CO_2/pH (Taylor *et al.,* 2003; Noronha de Souza *et al.,* 2006) could facilitate expression of a regular respiratory rhythm (Milsom, 2009), while the intrinsic properties of the rhythm generating circuit would remain temperature compensated. The involvement of temperature compensation of the respiratory rhythm generator, nevertheless, would require that activity of neurons and circuits from modulatory sources remain sensitive to stimuli varied with changes in T_b, *i.e.*, warm/cold temperatures, changes in CO_2/pH, membrane fluidity, *etc*. Maintaining temperature-sensitivity in modulatory regions, instead of temperature compensation, would ensure that these areas could control the respiratory rhythm under a variety of conditions.

Studies investigating temperature compensation of physiological functions have mainly been performed in a simple neuronal circuit responsible for chewing in the crab (Tang *et al.,* 2010). In this circuit, temperature compensation of neuronal phase firing resulted in preservation of circuit function over a range of physiological temperatures. If the respiratory rhythm generating circuits were to behave in this manner while being

influenced by modulators sensitive to stimuli varying with temperature, the net result would be precise temperature compensation of breathing relative to homeostatic regulatory demands over a wide-range of temperatures. Collectively, it seems as though a "modulator" model of a temperature compensated respiratory rhythm generator would provide fine-tuned control of ventilation regardless of the temperature in order to best meet energetic demands of poikilothermic vertebrates in a variety of conditions. In addition, the neuronal circuitry controlling breathing in anuran amphibians provides an excellent, albeit highly complex, opportunity to explore temperature compensation of a vital function in a vertebrate and test the model developed in the invertebrate CNS.

1.3 Modulation of the Respiratory Rhythm By Chemosensitive Regions During Temperature Change

Modulatory areas within the brain provide excitatory and inhibitory input to central rhythm generating circuits. When elevated CO_2 and acidified pH increase respiratory drive, chemosensitive regions stimulate breathing in order to maintain blood gas homeostasis through activation of chemosensitive neurons (Feldman *et al.*, 2003). The ability to detect changes in arterial CO_2/pH and elicit ventilatory compensation during acid-base disturbance is common among terrestrial tetrapods and these regions are primarily localized to distinct areas within the brainstem (Coates *et al.*, 1993). In mammals, several areas have been identified and implicated in respiratory control (for a recent review; see Huckstepp & Dale, 2011). Experimental evidence indicates the potential for wide-spread chemosensitivity in the brain of anuran amphibians, but this has not been directly assessed. In amphibians, focal acidification of both the rostral and

caudal ventral lateral medulla (VLM) increase respiratory-related motor output *in vitro* (Taylor *et al.,* 2003). Additionally, focal acidification of the locus coeruleus (LC) increases minute ventilation (V_E) *in vivo* (Noronha de Souza *et al.,* 2006). The role of central chemoreception during T_b changes in amphibians has received little attention. It is conceivable that chemosensitive regions that detect changes in CO_2/pH in amphibians could provide excitatory drive to the respiratory rhythm generator during both acidifying acid-base disturbances, as well as increasing temperatures (see section 1.1). Conversely, during T_b cooling, chemosensitive regions may slow firing of accelerator neurons through alkalization and therefore decrease excitatory respiratory drive. Mechanisms underlying the source of temperature-dependent respiratory drive in amphibians have not been investigated. Although amphibians utilize a derived, positive pressure mechanism to ventilate the lung (Gans *et al.,* 1969), central control of breathing appears well conserved in vertebrates (Wilson *et al.,* 2002). The benefits from studying the function of central chemoreceptive brainstem regions in amphibians will be two-fold. First, investigating the function of multiple chemosensitive areas in the brainstem of amphibians will establish understanding as to its adaptive role in amphibian ventilatory control. Secondly, given the similarities among central control of breathing in vertebrates, exploring wide-spread chemosensitivity throughout the brain of amphibians may provide insight into its role in vertebrate respiratory control (discussed further in section 2).

In addition to the possibility that chemosensitive regions mediate changes in respiratory drive during changes in temperature, ventilatory sensitivity to chemosensitive stimuli must be altered in order to regulate blood gasses at temperature-specific set-points. Given that amphibians experience broad changes in T_B which vary the necessity

to breathe, their CO_2/pH sensory system must be able to influence ventilatory adjustments to satisfy the greatest of metabolic and acid-base regulation demands at high T_B, while concurrently, have the propensity to become relatively insensitive to changes in CO_2/pH during circumstances of low T_B. The respiratory frequency of bullfrogs is highly sensitive to hypercarbia at 20˚C and even more so at 30 ˚C, while at 10 ˚C, respiratory frequency is completely hypercarbia-insensitive (Bícego-Nahas & Branco, 1999). Likewise, breathing-related nerve activity from the bullfrog brainstem is CO_2/pH sensitive at 20˚C and 25˚C, but exposure of the brainstem to 15˚C reduces sensitivity to CO_2/pH changes (Morales & Hedrick, 2002). Despite extraordinarily high-levels of respiratory drive under these circumstances, cooling eliminates central chemoreceptive drive to breathe in bullfrogs, while warming enhances this drive. Furthermore, these examples provide evidence that CO_2/pH sensitive neurons within brainstem may be equipped with mechanisms to up or downregulate cellular chemosensitivity during changes in temperature depending on the necessity for breathing to meet homeostatic requirements. Investigating the influence of temperature on neuronal sensors of CO_2/pH provides an opportunity to assess temperature-dependent tuning of CO_2/pH sensitivity of the amphibian respiratory control system at the cellular level.

2. Implication of the Anuran Model

2.1 Comparative Approach to Understanding the Complexity of the Chemosensory System

Many cellular mechanisms within the brainstem have been implicated in the transduction of changes of CO_2/pH into breathing responses (Reviewed in Putnam *et al.,* 2004; Jiang *et al.,* 2005; Huckstepp & Dale, 2011). The purpose of this section is not to provide an exhaustive review of the current understanding of chemosensory mechanisms influencing ventilatory control, but rather to demonstrate how anuran amphibians can serve as an alternative model to address inherent issues encountered by respiratory chemosensing in mammals.

Widely varied sensory mechanisms involved in transducing changes in cerebral spinal fluid or interstitial fluid CO_2/pH continue to intrigue respiratory physiologists. These varied mechanisms are located within neurons of a single nucleus or distributed across anatomically distinct chemosensory regions within the brainstem (Jiang *et al.,* 2005; Nattie & Li, 2005). Furthermore, molecular sensors of pH on chemosensitive neurons have a wide-range of sensitivities (Jiang *et al.,* 2005). An increase in Pco_2 by 1 torr can elevate breathing by 20-30% (Ren and Robbins, 1999), yet studies in mammals cannot find any single sensing molecule, or combination of sensing molecules expressed in chemosensitive neurons able to detect this miniscule change.

Su et al. (2007) propose that a combination of serial and parallel processing amplifies small CO_2/pH signals to achieve large breathing responses. Parallel processing indicates that common chemosensory transduction molecules are expressed at distinct chemoreceptive nuclei. Many chemosensory mechanisms discussed in Putnam et al. (2004) and Jiang (2005) coincide in discrete regions throughout the brain (Su *et al.,* 2007). Expression of a variety of channels capable of sensing various degrees of CO_2/pH changes throughout the brainstem would allow for detection of a diverse range of

chemosensory signals. In addition, serial processing involves expressing CO_2/pH receptors at both pre- and postsynaptic neurons and glia in chemosensory pathways. By expressing transduction molecules at both pre- (*i.e.,* respiratory modulators) and postsynaptic (*i.e.,* respiratory rhythm generator) sites, stimulation of individual chemosensitive neurons and chemosensitive nuclei arranged in series would amplify the chemosensitive response within the respiratory network; therefore, facilitating larger changes in breathing compared to individual chemoreceptive sites influencing the respiratory rhythm separately. Although experimental evidence is sparse, the combined expression of both parallel and serial processing throughout the respiratory network provides a reasonable explanation as to how the mammalian respiratory network can be so exquisitely sensitive to minute changes in CO_2/pH.

The proposition that diverse sensing molecules in disparate brainstem areas provide a mechanism for exquisite respiratory sensitivity to changes in CO_2/pH is adequate considering most mammalian *in vitro* models of central chemosensitivity do respond to physiological CO_2/pH changes (Fukuda, 1983; Wang *et al.,* 1998; Song *et al.,* 2012). Parallel and serial processing of chemosensory information assumes that the primary function of multiple sensing molecules with broad sensitivities is to generate high chemoreceptor sensitivity used to evoke large breathing responses; therefore, maintaining regulated gasses within a narrow range during the slightest perturbation. Although this model explains the issue inherent to mammalian respiratory control, poikilothermic vertebrates regulate a variety of temperature-specific pH set points (see section 1.1). A chemosensory system with the propensity to be sensitive over a range of

pH changes seems perfectly adapted for anuran, or more generally, poikilothermic vertebrate respiratory control.

Unlike mammals, bullfrogs regulate arterial pH from values as alkaline as 8.2 at < 10˚C to as acidic as 7.5 at 35˚C (Howell *et al.,* 1970; Reeves, 1972). These changes in T_b can happen slowly (hours) or quickly (minutes) during normal life history (Reeves, 1977; Stinner *et al.,* 1994). At temperatures ≤ 10 ˚C, however, it is plausible that cutaneous elimination of CO_2 may be sufficient to maintain temperature-specific acid-base balance negating the necessity for pulmonary ventilation (Tattersall & Boutilier, 1999). Given the spectrum of physiological T_b's experienced and pH values regulated by anuran amphibians, a sensory system with a large bandwidth may allow these animals to detect deviations from and maintain a wide range of temperature-specific pH set-points and influence various levels of basal respiratory drive (see section 1.3). As demonstrated by *in vitro* and *in vivo* data (see section 1.3), central chemosensitivity is altered by acute changes in temperature. These findings likely reflect the necessity of the amphibian respiratory control system to blunt or enhance breathing responses to chemosensitive stimuli depending on the temperature to best meet homeostatic demands. Whether these changes in respiratory chemosensitivity are the result of temperature altering the sensitivity of a single receptor/ receptor group or influencing the activity of different "pH specific" receptors with different sensitivities is not known. Multiple chemotransducing molecules with various pH sensitivities offers a mechanistic explanation for the ability to differentially regulate breathing during changes in T_b. Although speculative, diversity in cellular mechanisms of CO_2/pH sensing throughout the brainstem of mammals may be the ancestral condition of a flexible respiratory control system necessary to control

breathing in a vertebrates with variable body temperatures. An exquisitely sensitive regulatory system capable of maintaining pH within a narrow range is possibly an exaptation to endothermy.

Although cellular chemotransduction has not been investigated in amphibians, evidence suggests that the mechanisms used by neurons to enable respiratory chemosensing may be common. Several mechanisms of CO_2/pH sensing in respiratory control overlap between mammals (Putnam *et al.*, 2004), and terrestrial invertebrates (Denton *et al.*, 2007). *Helix aspersa*, the terrestrial pulomonate snail, has been used to study CO_2/pH sensing in respiratory control because direct stimulation of chemosensitive neurons increases opening of the pneumostome leading to ventilation of the mantle (Erlichman & Leiter, 1994). Similarly to many chemosensitive regions in mammals, Denton et al. (2007) found that delayed-rectifier (K_{DR}), A-type (K_A), and calcium-activated (K_{Ca}^{2+}) potassium currents mediated the chemosensitive response of dorsal subesophageal ganglia neurons in *Helix aspersa*. Each of these pH-sensitive K^+ currents are fundamental components of excitable cells. Tetraethylamine (TEA) - sensitive K_{DR} currents are present in green algae (Findlay & Coleman, 1983) and plants (Schroeder & Hedrich, 1989). In fact, K_{DR} and K_{Ca}^{2+} currents play a role in the avoidance response of *paramecium* (Eckert & Brehm, 1979). In contrast to other sensory processes including sight, taste, and olfaction, these receptors involved in CO_2/pH transduction are not necessarily specialized for respiratory control. Given the commonalties between chemotransduction in two different phyla with completely distinct mechanisms of gas exchange, these ubiquitous K^+ channels expressed in neurons controlling breathing are plausible candidates to mediate central chemosensitivity in amphibians; however, the

possibility remains that mechanisms uniquely adapted for amphibian respiratory control may exist.

2.2 Phylogeny of Central Chemoreception: A Reassessment

The phylogeny of central CO_2/pH sensing is not well elucidated among vertebrates, but it seems to have arisen at multiple sites in multiple lineages. Confusion regarding phylogenetic trends in central chemoreception arises from the presence of hypercapnic ventilatory responses in some, but not all air-breathing fish (Milsom, 2002; Milsom, 2010). Despite some evidence supporting phylogenetic relationships of central sensitivity to CO_2/pH among air-breathing vertebrates, it appears that the "bare essentials" for neurons to become chemosensitive are incredibly ordinary across phyla, as demonstrated by the similarities between *Helix aspersa* and mammals. Expression of these basic components in respiratory modulating cells/networks of organisms exhibiting chemosensitive responses may be the result an adaptation to obligate or even facultative air-breathing. Therefore, absence of central chemosensitivity in water-breathing and some air-breathing fish must result from either not expressing CO_2/pH sensing machinery in neuromodulator centers that project to respiratory rhythm generating centers, a lack of connectivity between neurons expressing CO_2/pH sensing mechanisms and the central rhythm generator, or a deficiency in both chemotransduction mechanisms as well as anatomical connections between modulatory regions to the rhythm generating circuit.

The current view with respect to the evolution of central chemoreception suggests that multiple chemosensitive areas developed throughout vertebrate evolution as the requirements for a more complex control system arose (Feldman *et al.*, 2003; Milsom, 2010). Feldman et al. (2003) propose, "Presumably, new sites [chemosensitive regions]

developed in parallel with or as part of major steps in vertebrate evolution, *e.g.*, air breathing, homeothermy, and sleep." Given the highly conserved organization of the vertebrate hindbrain (Murakami *et al.*, 2004), it is improbable that novel chemosensitive areas developed both CO_2/pH sensing machinery, as well as nervous projections to rhythm generating centers as a result of air-breathing. Because the minimum requirements for neurons to function as a respiratory chemoreceptor are so basic, it is interesting to speculate that evolution drove the selection of developmental processes controlling projection of key neuromodulatory nuclei (*e.g.*, locus coeruleus, medullary raphe, and retrotrapezoid nucleus) to ancient rhythm generating circuits in the hindbrain (Bass & Baker, 1997). When regulatory requirements necessitated increasingly stringent control of ventilation, *e.g.*, during the transition to air-breathing, network connections would already be established and further selective pressures may have favored expression of rather generic pH sensing ion channels in a variety of brain areas to mediate chemosensitivity in the respiratory network. If this is the case, then I would expect water breathing and some air-breathing fish to increase respiratory output during electrical stimulation of neuromodulatory regions, but not during focal acid injection. Utilizing focal stimulation of putative chemosensory regions (*i.e.*, establishing connection of modulatory sites to the effector) as well as acid injection (*i.e.*, determining presence of chemosensing machinery within modulatory sites) could provide powerful insight into determining evolutionary trends in central chemoreception. Phylogenetic patterns will become more apparent using a "multi-factor" method compared to the current "presence-absence" approach to central respiratory chemosensitivity. Discerning phylogenetic patterns of central chemoreception in vertebrates will attribute greatly benefit from

knowing which animals contain centrally originating ventilatory response to elevated CO_2, in addition to chemosensitive or non-chemosensitive neuromodualtory regions that project to respiratory rhythm generating centers.

The amphibian model shows promise in answering unresolved questions in the field of CO_2/pH sensing in respiratory control. First, amphibians provide a unique opportunity to study activity of chemosensitive neurons and chemotransduction mechanisms in a facultative air-breather. Amphibians exhibit a robust hypercapnic ventilatory response at room temperature (Noronha de Souza *et al.*, 2006); however, when cooled, ventilatory sensitivity to hypercarbia and respiratory-nerve activity ceases (Bícego-Nahas & Branco, 1999; Morales & Hedrick, 2002). Exploring how acute and chronic changes in temperature influence activity of respiratory chemoreceptors in amphibians will provide insight into their role in regulation of breathing in air-breathers. Although these studies will not provide direct insight into the phylogeny of central chemoreception, inferences can be made regarding the function of central chemoreceptors and neuromodulatory connections in animals that do not require pulmonary ventilation for gas exchange. For example, I hypothesize that acute decreases in temperature would suppress CO_2/pH sensitivity in chemosensitive neurons, thus reflecting their role in decreasing sensitivity of the respiratory system during cooling. I expect that these changes in sensitivity will be immediately reversible, demonstrating the inherent flexibility present in the respiratory control system of poikilotherms. In contrast, I presume that chronic acclimation to temperatures negating pulmonary ventilation for gas exchange ($\leq$10°C) will eliminate sensitivity to CO_2/pH in chemosensitive neurons influencing lung breathing; however, electrical stimulation of the chemosensitive area at

≤10°C would likely lead to increases in respiratory output. These results would provide strong evidence that central chemosensitivity may be an adaptation to air-breathing which can be turned off by decreasing expression of CO_2/pH sensitive molecules within/on these cells when air-breathing is no longer required to regulate blood gas homeostasis.

There is clearly a potential role for the diverse range in pH sensing molecules in poikilothermic vertebrates. Since amphibians regulate temperature-specific pH to maintain acid-base balance, distinct ion channels with different pH sensitivities expressed either in similar or disparate brainstem regions may influence ventilatory adjustments to maintain these set points at various temperatures. In addition, ventilatory adjustments made in response to temperature changes (*i.e.*, increase ventilatory drive during transition from 20°C to 30°C or decrease ventilatory drive during transition from 20°C to 10°C) may be mechanistically achieved through altered activity of chemosensory neurons expressing pH sensitive mechanisms with varied sensitivities.

3. Bullfrog Locus Coeruleus Neurons as a Model for Amphibian Chemosensitivity During Changes in T_b

3.1 Involvement of the LC in Amphibian Respiratory Control

Of the chemosensitive regions identified in the brain of amphibians, the locus coeruleus (LC) is the only brainstem nucleus to date that has been implicated as an important chemosensory site in amphibian ventilatory control. Focal acidification of the LC *in vivo* increases minute volume, while ablation attenuates the hypercapnic ventilatory response (Noronha-de-Souza *et al.*, 2006). Due to its noradrenergic content

and projections to the telencephalon, the LC of amphibians and mammals appear to be homologous (Marin *et al.,* 1996). In both amphibians and mammals, ablation of the LC results in reductions of the increase in tidal volume during hypercarbic exposure (Biancardi *et al.,* 2008) suggesting that LC neurons have an analogous role in the regulation of respiratory pattern expression during hypercarbia. The role of the LC in respiratory control during changes in T_b has not been assessed. Given that amphibians cope with a wide range of temperatures, the repertoire of cellular mechanisms used by LC neurons to execute regulation of blood gas homeostasis and basal breathing level at a various T_bs are likely quite sensitive to and dependent on temperature.

A great deal of insight into the mechanisms of CO_2/pH chemotransduction has been gained from using mammalian preparations (Putnam *et al.*, 2004); however, no information exists for other vertebrate classes. The comparative approach described previously offers many advantages for delineating the issues that have plagued the field of respiratory chemosensing. Discrete chemosensory areas containing molecules with a wide range of sensitivities may be adaptive in amphibians, providing insight into the nature of chemosensing in air-breathing vertebrates. Before we can determine how LC neurons of amphibians function during changes in temperature, we must first establish the baseline physiology of these neurons during normo- and hypercapnia. For example, how many neurons are sensitive to CO_2/pH changes, how sensitive are these neurons, and how does CO_2/pH influence passive membrane properties? The following manuscript provides the first account of signaling properties of respiratory control neurons from a chemosensitive region in a non-mammal. In addition, this work establishes the background for further studies involving temperature changes in LC neurons, while

providing a context for the role of LC neurons in regulating breathing during both changes in temperature, as well acid-base disturbances at a variety of T_bs.

Chapter Two: Manuscript

Respiratory signaling of locus coeruleus neurons during hypercapnic acidosis in the bullfrog, *Lithobates catesbeianus*

Santin, J.M., Hartzler, L.K., 2013. Respiratory Physiology and Neurobiology 185, 553-561

1. Introduction

For amphibians, the transition from water to air-breathing during metamorphosis shifts respiratory control from being exclusively O_2 to primarily CO_2/pH driven (Gargaglioni and Milsom, 2007). Tadpoles increase gill ventilation during exposure to modest hypoxia whereas adult amphibians show blunted ventilatory responses to hypoxia, but marked increases in breathing during inhalation of hypercapnic gasses and acidified arterial pH (pHa; Macintyre and Toews, 1976; Branco et al., 1992, 1993). When unidirectionally ventilated, elevating arterial PCO2 stimulates ventilation, while large changes in PO_2 minimally alter breathing in bullfrogs (Kinkead and Milsom, 1994). Moreover, exposing the isolating bullfrog brainstem preparation to hypercapnic acidosis (HA) increases respiratory motor nerve output (Morales and Hedrick, 2002; Taylor et al., 2003a,b), demonstrating that the brainstem of bullfrogs intrinsically detects CO_2/pH and alters motor output independently of peripheral input during hypercapnia. Discrete regions in the amphibian brainstem including the caudal and rostral ventral lateral medulla (VLM) and locus coeruleus (LC) have been identified as CO_2/pH chemoreceptive sites because focal acidification of these areas increases ventilation and ablation attenuates the hypercapnic ventilatory response (Noronha-de-Souza et al., 2006; Taylor et al., 2003a,b). Collectively, these data show that maintenance of normal breathing in amphibians requires signaling from chemosensitive brainstem regions.

In both amphibians and mammals, CO_2/pH-sensitive brainstem areas involved in respiratory chemosensing have been located in regions surrounding the fourth ventricle (Coates et al., 1993; Torgerson et al., 2001). The relative contribution that each mammalian chemosensitive area makes towards respiratory control has been debated

(Nattie and Li, 2005; Guyenet et al., 2008). Difficulty in determining the contribution of each brainstem area to respiratory control emanates, in part, from the varied percentage of neurons responding to acid challenges, as well as diverse neuronal sensitivities among regions (Putnam et al., 2004). For example, neurons of the medullary raphé elicit robust responses to CO_2/pH changes, yet only ~20% of these neurons are stimulated upon challenge (Wang et al., 1998). In contrast, LC neurons exhibit modest responses, although >80% of these neurons increase firing rates during acidification (Filosa et al., 2002). Because of the abundance of intrinsically chemosensitive neurons relative to other chemosensitive areas (Nichols et al., 2008), the mammalian LC has been of particular interest for the study of chemosensory mechanisms in respiratory control. Intrinsically chemosensitive LC neurons increase firing rates during acidification with chemical synapses blocked and electrical synapses uncoupled, providing a convenient model to study cellular mechanisms of CO_2/pH-sensing. Conversely, network-driven chemosensitive neurons increase discharge rates during acidification with synaptic connections intact, but do not exhibit chemosensitive responses when synapses are blocked (Nichols et al., 2008).

In addition to the wide-spread distribution of chemosensitive regions, diversity of cell signaling mechanisms within individual regions introduces further complexity. For example, in mammalian LC neurons, acidification inhibits several different types of K^+ channels and activates TRP channels (Putnam et al., 2004; Jiang et al., 2005; Putnam, 2010; Gargaglioni et al., 2010; Cui et al., 2011), while bicarbonate activates L-type Ca^{2+} channels (Imber and Putnam, 2012) resulting in increased neuronal excitability.

Specifics regarding these signal transduction events in LC neurons and other CO_2/pH-sensitive respiratory control neurons do not exist for amphibians.

Noronha-de-Souza et al. (2006) established that breathing a hypercapnic gas mixture increased c-fos expression within the LC of toads, suggesting that hypercapnia stimulated these neurons. Following ablation of the LC in toads, the hypercapnic ventilatory response significantly decreased when breathing hypercapnic air, while focal acidification (pH $\leq$7.6) of the intact LC increased minute ventilation. These data provide direct evidence that the LC of amphibians plays a significant role in sensing CO_2/pH and influencing changes in pulmonary ventilation during acid–base perturbations. Due to its noradrenergic content and projections to the spinal cord and telencephalon, the LC of amphibians has been considered homologous to the LC of mammals (Marin et al., 1996). Although the LC regions of mammals and amphibians have analogous functions in respiratory control, it is unknown whether bullfrog LC neurons share similar signaling properties. Previous studies have identified output from respiratory motor nerves and whole-animal ventilatory responses as CO2/pH sensitive, but measurements directly assessing signaling properties of respiratory control neurons from any chemosensitive area in amphibians are lacking. We hypothesized that neurons of the bullfrog LC would be sensitive to changes in CO_2/pH and that chemosensitive responses would be intrinsic to individual neurons. In addition, we hypothesized putative respiratory control neurons of the bullfrog LC would be stimulated by hypercapnic acidosis within physiological ranges of Pco_2/pH. To test these hypotheses we used the whole-cell patch clamp technique to measure changes in action potential firing frequency of LC neurons during acidification by elevated CO_2 with chemical synapses and gap junctions intact and

blocked. In addition, we recorded changes in membrane resistance induced by acidification with CO_2.

2. Materials and methods

2.1. Brainstem slices

Adult bullfrogs (either sex; 98.04 ± 4.67 g; N = 43), *Lithobates catesbeianus*, were maintained at 22°C water with access to wet and dry areas, 12:12 light dark cycles, and fed crickets ad libitum. Animals were handled following Wright State University Institutional Animal Care and Use Committee guidelines. Following rapid decapitation, the head was placed in 4°C artificial cerebral spinal fluid (aCSF; for composition see Section 2.2) equilibrated with 97.5% O_2 and 2.5% CO_2. The frontoparietal bone was removed and the brainstem was dissected. 400 um-thick, transverse brainstem slices were cut using a Vibratome tissue slicer (Leica Microsystems Inc., Buffalo Grove, IL, USA). The locus coeruleus (LC) area has been identified through tyrosine hydroxylase immunoreactive staining in amphibians including *Xenopus laevis*, *Rana ridibunda* (González and Smeets, 1993; González et al., 1994), *Rana perezi*, *Pleurodeles waltl* (Sánchez-Camacho et al., 2003; Marin et al., 1996), *Bufo schneideri* (Noronha-de-Souza et al., 2006), and *Lithobates catesbeiana* (Fournier and Kinkead, 2008), and is illustrated here in a cartoon (Fig. 1).

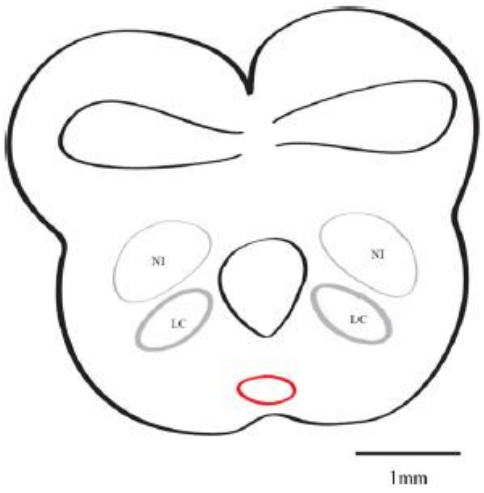


Fig. 1. Cartoon of the brain slice containing the region previously identified as the LC. Neurons were examined within the bold, gray oval (LC area). Neurons located on the ventral part of the slice, within the red oval (peri-LC neurons) served as negative controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Brainstem slices were equilibrated with control gas mixture (80% O_2, 1.3% CO_2, balanced N_2) at 22°C and given 1 h to recover from slicing prior to experimentation. Slices containing the LC were placed in a polyethylene recording chamber with a glass coverslip base and stabilized using a nylon grid. LC slices were superfused withaCSF at a rate of ~2.5 mL/min via gravity-fed, stainless-steel drip lines. Solutions were maintained at 22°C and bubbled with equilibrated gas mixtures (MFC-4 Mass Flow Controller, gas mixer, Sable Systems International, Las Vegas, NV, USA).

2.2. Solutions

aCSF was composed (in mM) of 104 NaCl, 4 KCl, 1.4 MgCl2, 7.5 glucose, 40 $NaHCO_3$, 2.5 $CaCl_2$, and 1 NaH_2PO_4 (Taylor et al., 2003a,b, 2008). Control aCSF was equilibrated with 80% O_2, 1.3% CO_2, balanced N_2 (pH 8). 1.3% CO_2 was used as the control because bullfrogs typically experience resting arterial PCO_2 near 10 Torr at 20°C (Howell et al., 1970; Reeves, 1972; Gottlieb and Jackson, 1976). Hypercapnic aCSF was identical to control aCSF except the CO2 was elevated (percent CO2 determined by experiment; see Section 2.4.1). Gases were mixed to the desired composition using

infrared gas mixers. Synaptic blockade media (SNB; pH 8) was produced by lowering the CaCl2 to 0.2 mM and raising MgCl2 to 11.4 mM with balanced NaCl to maintain osmolarity (Dean and Boulant, 1989; Nichols et al., 2008). Gap junctions were uncoupled using 100 uM carbenoxolone (CBX; Sigma–Aldrich Co., St. Louis, MO, USA) in aCSF (Davidson and Baumgarten, 1988; Masaru and Williams, 1996a,b; Alvarez-Maubecin et al., 2000; Winmill and Hedrick, 2003; Nichols et al., 2009).

2.3. Whole-cell patch clamp recordings

The whole-cell patch clamp technique was used to record membrane potential (V_m) in individual neurons (n = 85) located within the region of the locus coeruleus (LC neurons) of bullfrog brain-stem slices. Micropipettes were fabricated from borosilicate glass capillary tubes using a two-stage pipette puller (Model PC-10, Narishige, East Meadow, NY, U.S.A.) with resistances of 3–7 MΩ. Pipettes were back-filled with mock-intracellular solution (composition in mM: 110 K-gluconate, 2 $MgCl_2$, 10 HEPES, 1 Na_2-ATP, 0.1 Na_2-GTP, 2.5 EGTA; pH 7.2) (Martini et al., 2009) and placed over a $AgCl_2$-coated Ag wire connected to Axon instruments CV 203BU headstage (Molecular Devices, Sunnyvale, CA, USA). The LC area was visualized with a Nikon FN1 fixed stage microscope (Nikon, Elgin, IL, USA) using NIS Elements Imaging Software (Nikon, Elgin, IL, USA) at 4× magnification and individual neurons of interest were identified at 60×. A 10 mL syringe was connected to the headstage which was used to apply positive pressure through the pipette tip in order to clear the tip of debris. A micromanipulator

(Burleigh PCS 5000, Thorlabs, Newton, NJ, USA) was used to position the pipette adjacent to the neuron of interest. Once the pipette produced a slight indentation on the soma, positive pressure was removed and gentle suction applied until a ≥1 GΩ seal existed between the pipette tip and cell membrane. After formation of the GΩ seal, slight, rapid negative pressure was applied to rupture the seal and obtain whole-cell electrochemical access to the neuron. Neurons located on the ventral part of the slice, outside of the LC area (peri-LC neurons) served as negative controls. Access resistance ranged from 20 to 70 MΩ and signals were filtered at 2 kHz. Changes in Vm were recorded in 'current-clamp mode' (Axopatch 200 B integrating patch clamp amplifier, Molecular Devices, Sunny-vale, CA, USA) and collected using Molecular Devices P10 Clampex software (Molecular Devicies, Sunnyvale, CA, USA). Current-clamp recordings were analyzed for firing rates and V_m using pCLAMP software (Molecular Devices, Sunnyvale, CA, USA).

2.4. Experimental procedures

2.4.1. Determine LC chemosensitivity

LC neurons were exposed to hypercapnic aCSF to determine sensitivity to CO_2/pH. After gaining whole-cell access, V_m was recorded for 3–5 min under the influence of aCSF bubbled with control gas mixture (80% O_2, 1.3% CO_2, N_2 balanced) to establish baseline firing rate. Neurons were exposed to 10% CO_2 (n = 13) or 5% CO_2 (n = 21) for 3–5 min to elicit increases in firing rate in response to hypercapnic acidosis (HA) with a return to control aCSF. Only neurons returning to near-control firing rates

following HA were included in analysis. A dose response curve was established to determine the minimum amount of CO_2/pH change required to elicit firing rate changes in response to pH changes from 8 to 7.58, which occur within the reported physiological range in bullfrogs of ~8.2 to ~7.5 (Howell et al., 1970; Reeves, 1972). aCSF bubbled with control gas mixture was used to determine baseline firing rate and percent CO_2 composing the hypercapnic gas mixture was increased to either 1.7% (n = 4), 1.8% (n = 6), 1.9% (n = 6), 2% (n = 8), 2.1% (n = 6), 3% (n = 8), and 5% (n = 21), (ΔpH 0.07, 0.085, 0.10, 0.115, 0.165, 0.285, and 0.42, respectively). Neurons excited by HA as defined by chemosensitivity index (see Section 2.5) were included on the dose response curve. Time to firing rate increase in chemosensitive neurons was dependent upon individual neuron sensitivity and the depth of the neuron in the slice. Neurons unresponsive after 5 min of HA exposure in all described experiments were considered non-chemosensitive (n = 14).

2.4.2. Chemical synapse blockade and electrical synapse uncoupling

Although LC neuron sensitivity was determined by applying hypercapnic aCSF, neurons within the brainstem are connected through synapses; without assessing sensitivity to CO_2/pH in neurons devoid of synaptic communication with other neurons, we cannot assume the chemosensitive responses observed with synapses intact were intrinsic to the individual neuron. To determine whether firing rate changes in LC neurons due to HA were intrinsic or due to synaptic coupling, either 0.2 mM Ca^{2+}/11.4 mM Mg^{2+} (SNB) or 100uM carbenoxolone (CBX) in aCSF were used to block chemical synapses and gap junctions, respectively. In these experiments, a neuron was first exposed to control aCSF and then to HA (5% CO_2). Once the response to HA was

determined, the solution was switched from control aCSF to either SNB (n = 11) or CBX (n = 4) equilibrated with control gas mixture. HA was then initiated in either SNB or CBX. Effects of SNB were reversed upon returning to control aCSF; however, slices containing neurons exposed to CBX were discarded following CBX experiments because removal of CBX could not be determined. The protocol to determine intrinsic chemosensitivity typically lasted <40 min. Because we aimed to investigate firing properties of neurons intrinsically stimulated by HA, only neurons exhibiting increases in firing during HA in aCSF were assessed; however neurons unresponsive to HA during the aCSF exposure protocol were included in the percentage of neurons responding to HA.

2.4.3. Input resistance

According to the model of neuronal chemosensitivity in mammals, acidification induces inhibition of potassium channels which functions to depolarize the membrane and increase neuronal firing rate (Putnam et al., 2004). Changes of neuron input resistance indicate whether depolarization was caused by HA inhibiting or activating channels (i.e., neuron depolarization with a net resistance increase indicates inhibition of channels, and a net decrease in resistance indicates channel opening). Input resistance was used as an indicator of neuron resistance (Denton et al., 2007) in intrinsically chemosensitive and non-chemosensitive neurons. Resistance was measured in aCSF by injecting three −25 pA current pulses for 800 ms, which elicited a hyperpolarization of the membrane. Average ΔVm (Vm during plateau of the hyperpolarization − control Vm) from the three current injections was used in Ohm's law (ΔVm = IR) to calculate input

resistance of intrinsically chemosensitive (n = 5) and non-chemosensitive (n = 4) LC neurons in both *control and hypercapnic aCSF*.

2.5. Data analysis

All values are reported as mean ± SEM. A repeated measures analysis of variance (RM-ANOVA) was used to determine differences among control, 5% or 10% CO_2, and washout neuronal firing rates. The magnitude of the response to HA was quantified as chemosensitivity index (CI) as well as the proportion of neurons with an excitatory response to CO_2/pH. CI expresses the percent firing rate change due to HA normalized for a pH change of 0.2 pH units. Integrated firing rate was measured 2 min prior to the application of HA and 2 min before returning to normocapnia. CI ≥120% was used to categorize percentage of neurons excited by HA (Wang et al., 1998). A CI <120% signifies no CO_2/pH response. All statistics were computed using GraphPad Prism (GraphPad Software, San Diego CA, USA). The percentage of LC and peri-LC neurons responding to HA were assessed using Fisher's exact test. Firing rate changes due to SNB were established by comparing 1 min of integrated firing rate before application of SNB to firing for 1 min after 1 min of SNB exposure. Comparisons between two means were per-formed using Student's t tests or paired t tests. One-way ANOVA with Tukey's post hoc test were used to determine differences in data sets with greater than two means. A one-site, specific-binding curve was fitted to the pH dose response. One-sample t-tests were used to determine fire rate differences from zero for ΔpH values in dose response experiments and effects of CBX on basal membrane characteristics. Significance was accepted with $P < 0.05$; indicated by *.

3. Results

3.1. Chemosensitivity in bullfrog LC neurons

Integrated firing rates and representative traces of V_m for LC neurons excited by and unresponsive to HA are presented in Fig. 2.

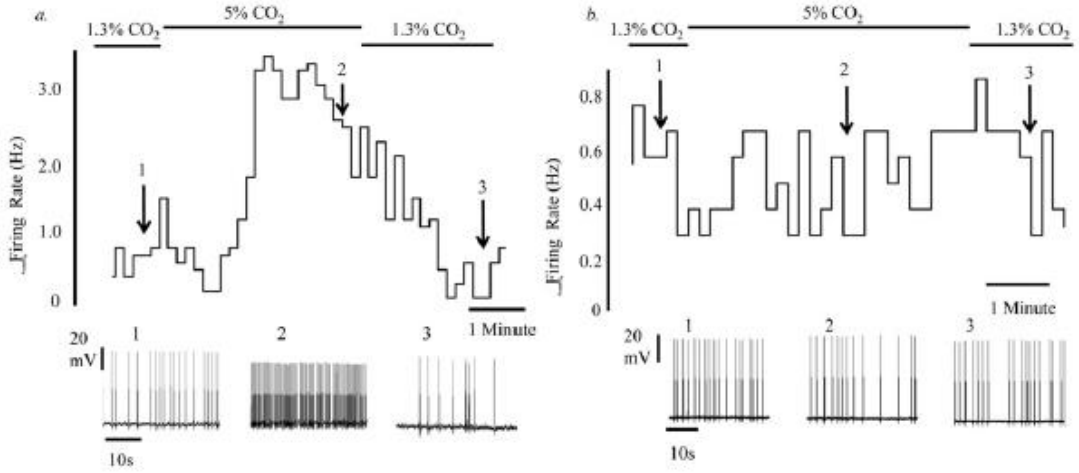
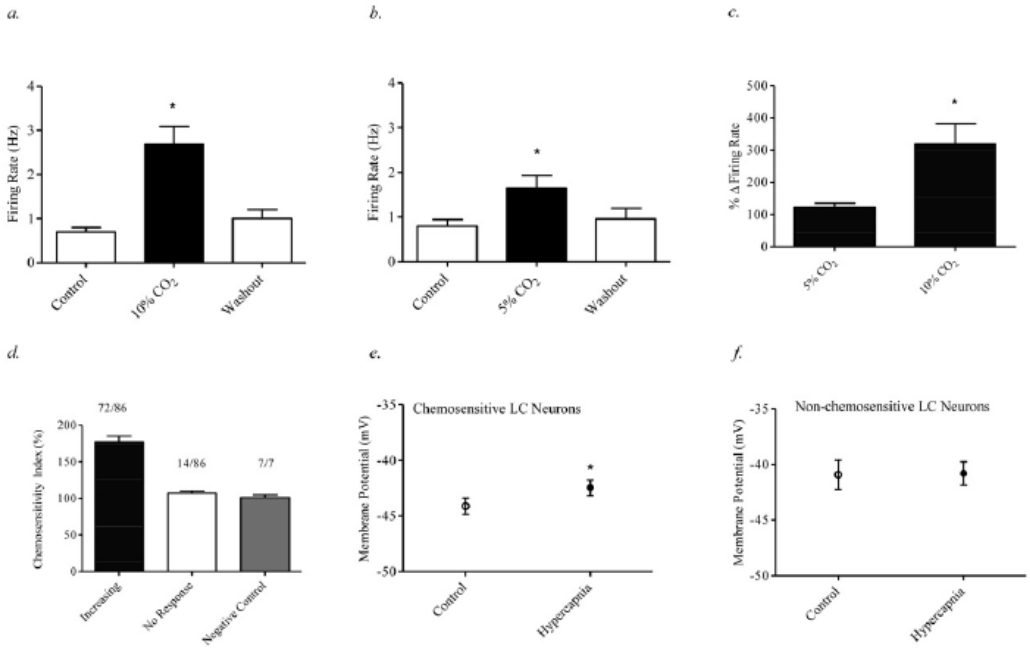


Fig. 2. Integrated firing rates (top) and representative traces (bottom) from LC neurons stimulated by (a) and unresponsive to (b) hypercapnic acidosis (HA). Hypercapnic exposure of this chemosensitive LC neuron causes firing rate increased from ~0.5 Hz to 3.0 Hz within 1 min, whereas changes in CO2/pH did not alter control firing rate in unresponsive LC neurons or negative control cells. Recovery time of chemosensitive LC neurons ranged from 1 and 5 min. 30-s segments of individual action potentials are shown in the bottom trace. The segments are expanded from times indicated by the corresponding numbered arrows in the integrated firing rate trace.

Bullfrog LC neurons had control firing rates of 0.70 ± 0.10 Hz and hypercapnic (10% CO_2; n = 13) firing rates of 2.69 ± 0.40 Hz with washout (return to control) firing rates of 1.00 ± 0.19 Hz (RM-ANOVA; $P < 0.05$; Fig. 3a). Neurons exposed to 5% CO_2 (n = 21) had firing rates of 0.79 ± 0.15 Hz and 1.65 ± 0.27 Hz, respectively, with washout firing

rates of 0.96 ± 0.23 Hz (RM-ANOVA; $P < 0.05$; Fig. 3b). 10% CO_2 elicited a larger change in firing rate compared to 5% CO_2 (two-tailed t test; $P < 0.05$; Fig. 3c). To determine percentage of neurons responding to HA (1.7–10% CO_2), we used normalized firing rate changes, expressed as CI, to account for differences in control firing rates and varied percentages of CO_2 used. Neurons excited (72/86; 84%) by HA had a mean CI of 177.6 ± 7.5%, while 14/86 neurons (16%) were not stimulated by HA. Peri-LC neurons located on the ventral part of the slice, below the fourth ventricle (Fig. 1; region indicated by red circle) were used as negative controls, and were not stimulated by HA (Fig. 3d). Responses of non-chemosensitive LC neurons and peri-LC neurons to HA were not different (two tailed t-test; $P > 0.05$). HA had an overall stimulatory effect on firing rate (two-tailed Fisher's exact test; $P < 0.0001$). V_m was depolarized by exposure to 5% CO_2 in excited LC neurons (Fig. 3e; n = 21; paired t-test; $P < 0.05$) whereas HA did not alter control V_m in non-chemosenstive LC neurons (Fig. 3f; n = 4; paired t-test; $P > 0.05$).

Fig. 3. Firing rate of LC neurons at control and 10% (a; n = 13) or 5% (b; n = 21) CO2



exposure. 10% and 5% CO_2 both induced increases in firing rate (RM-ANOVA; $P < 0.05$). (c) 10% CO_2 causes a greater change in firing rate compared with 5% CO_2 (two-tailed t test $P < 0.05$). 10% CO_2 was initially used to identify chemosensitive neurons within the area of the LC, thus serving as a positive control. (d) Chemosensitivity indices (CI) for neurons stimulated by and unresponsive to HA. 84% (72/86) of neurons were excited in HA with an average CI of 177%. A CI of 100 ± 19.9% indicates no change in firing rate. CIs of unresponsive (16% of neurons; 14/86) and peri-LC (negative control) neurons were not different (two-tailed t test; $P > 0.05$). (e) Change in control Vm induced by HA (5% CO2; n = 21). HA caused a ~2 mV depolarization the cell membrane of chemosensitive LC neurons (paired t test; $P < 0.05$), while non-chemosensitive neurons (f; n = 4) did not change Vm during exposure to 5% CO2 (paired t test; $P > 0.05$).

We fitted a sigmoidal curve to data relating CO_2/pH to firing rate and obtained a Hill coefficient (h) of 2.1. Based on that curve, we determined the pH change required to elicit 50% maximal firing (FR50) was Δ0.12 pH U (Fig. 4). All changes in firing rate at each ΔpH were significantly different from zero (one-sample t-test: $P < 0.05$).

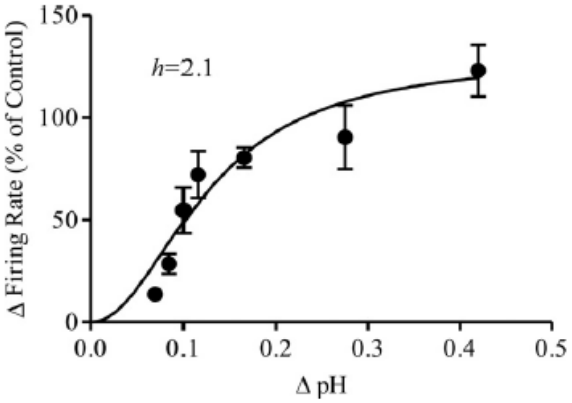


Fig. 4. Firing rate dose response curve during extracellular acidification was achieved by increasing the percentage of CO2 from control (1.3%; ΔpH 0) to HA (1.7%, n = 4; 1.8%, n = 6; 1.9%, n = 6; 2%, n = 8; 2.1%, n = 6; 3%, n = 8; 5%, n = 21; ΔpH 0.07, 0.085, 0.10, 0.115, 0.165, 0.285, and 0.42, respectively). Hypercapnia induced significant firing rate increases across the range of _pH (one-sample t test; $P < 0.05$). Small changes in extracellular pH caused half maximal firing at Δ0.12 pH units; h = 2.1. All pH changes used are within physiological ranges normally experienced by bullfrogs.

3.2. Putative intrinsic chemosensitivity in LC neurons

To establish putative intrinsic responses of LC neurons to HA, either low Ca^{2+}/high Mg^{2+} synaptic blockade (SNB; n = 11) or carbenoxolone (CBX; n = 4) were used to block chemical and uncouple electrical synapses, respectively. 6/11 excited LC neurons increased firing rates upon exposure to HA with chemical synapses intact and blocked. 5/11 excited LC neurons increased firing rates only with synapses intact suggesting that chemosensitive responses of these neurons were network driven (Fig. 5a and b). In putative intrinsically chemosensitive LC neurons, (i.e. neurons that maintained excitatory responses to HA during chemical synapses blackade), the magnitude of the chemosensitive response with synapses blocked and intact were not different (Fig. 5b.; two-tailed t test; $P > 0.05$). Without input from chemical synapses, intrinsically chemosensitive neurons maintained resting V_m (paired t test; $P = 0.7$; Fig. 6a and b), while the V_m of network-driven chemosensitive neurons tended to hyperpolarize (paired t test; $P = 0.06$; Fig. 6c and b). Firing rates of both intrinsically chemosensitive and network-driven chemosensitive neurons decreased during blockade of chemical synaptic

input; however intrinsically chemosensitive neurons decreased to a lesser extent compared to network-driven chemosensitive neurons (two-tailed t test; $P < 0.05$; Fig. 6d).

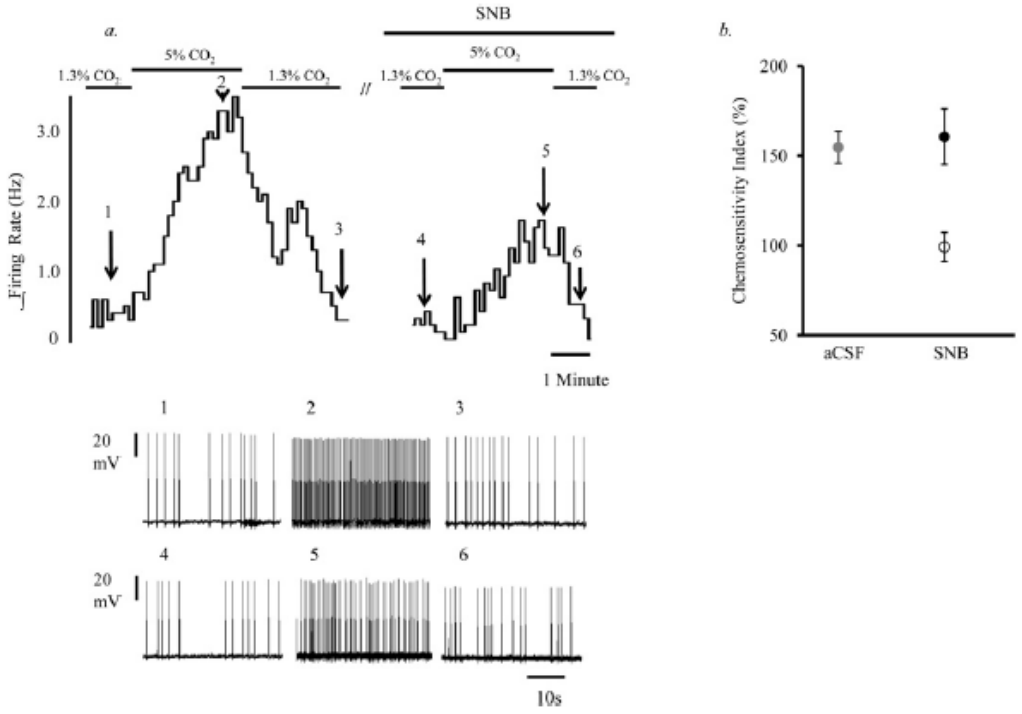
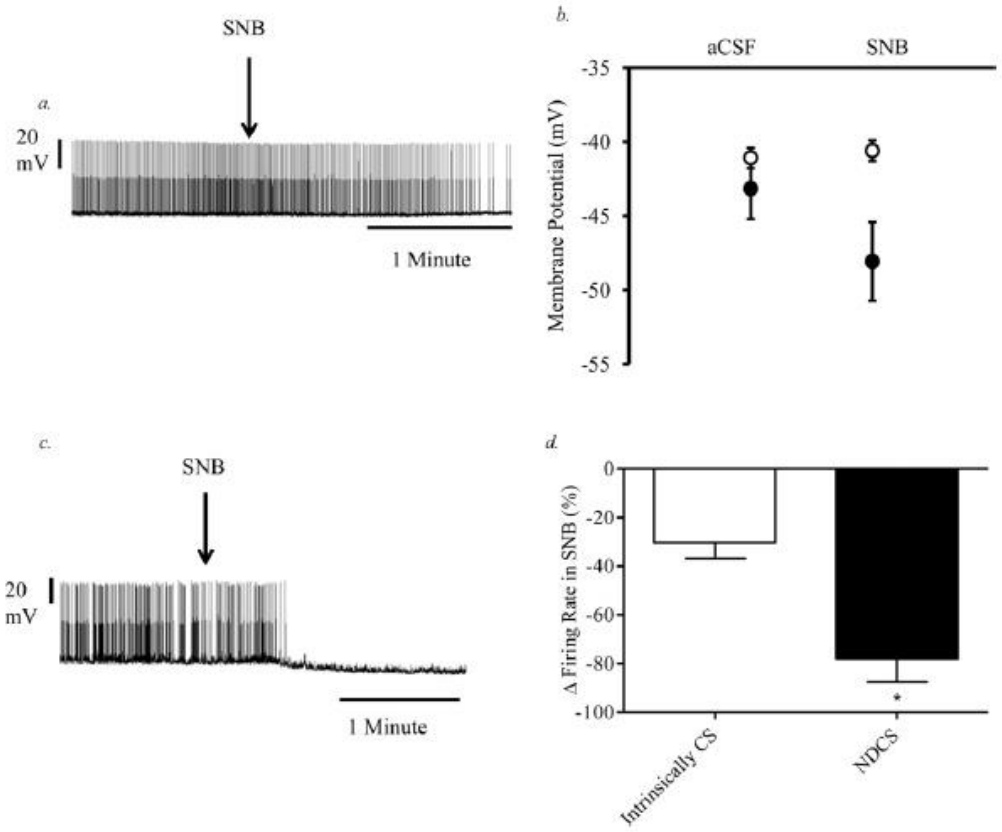


Fig. 5. (a) Integrated firing rate and representative trace of a LC neuron increasing firing rate during HA with and without synaptic input. This particular neuron had a larger chemosensitive response with synapses intact; however, mean responses of putative intrinsically chemosensitive neurons in synaptic blockade media (SNB) had similar firing responses of LC neurons with chemical synapses intact. 30-s segments of individual action potentials are shown in the bottom trace. The segments are expanded from times indicated by the corresponding numbered arrows in the integrated firing rate trace. (b) CIs for LC neurons excited by HA with chemical synapses intact in aCSF (gray circle; 11/11). During SNB, 6/11 LC neurons retained chemosensitive responses (black circle); the other 5 LC neurons were no longer chemosensitive (open circle). The excitatory responses of these five LC neurons in aCSF were likely network-driven rather than

intrinsic. Responses of neurons excited by HA in aCSF (gray circle) and SNB (black circle) were not different (two-tailed t test; $P > 0.05$).

Fig. 6. Sample trace of a putative intrinsically chemosensitive neuron during the



transition from aCSF to SNB (a). Blocking chemical synapses of putative intrinsically chemosensitive neurons (n = 6) did not change resting V_m (b; paired t test; $P = 0.7$; open circles). In contrast, a sample trace representing firing rate of a network-driven chemosensitive (NDC) neuron shows hyperpolarization upon exposure to SNB (c). Average Vm of network-driven chemosensitive neurons (b; closed circles; n = 5) shows a trend towards hyperpolarization (paired t test; $P = 0.06$). The removal of synaptic input slowed firing rates of all LC neurons, but to a significantly greater degree in network-driven chemosensitive neurons (d; two-tailed t test; $P < 0.05$).

Excited LC neurons in aCSF exposed to CBX maintained chemosensitive responses to HA (4/4; two-tailed Fisher exact test; $P = 1$). CBX did not significantly alter mean CI (139.93 ± 7.52% in aCSF vs. 133.15 ± 6.64% in CBX; two-tailed t test), control firing rate (10.27 ± 15.68% of control aCSF), and resting V_m ($\Delta V_{mCBX} - \Delta V_{mACSF} = 0.47 \pm 0.15$ mV from control aCSF (one sample t test; $P > 0.05$ for all characteristics).

3.3. Membrane resistance

To investigate the mechanism by which CO_2/pH increases firing rates and V_m in intrinsically chemosensitive neurons, input resistance was measured during normocapnia and hypercapnia. Input resistances of intrinsically chemosensitive LC neurons ($n = 5$) increased from 1004 ± 107 MΩ to 1104 ± 123 MΩ during hypercapnia (paired t test; $P < 0.05$), while input resistances of non-chemosensitive neurons ($n = 4$) did not change (584 ± 158 MΩ to 577 ± 164 MΩ; paired t test; $P > 0.05$). HA increased average input resistance by 9.65 ± 2.93% in intrinsically chemosensitive neurons and decreased input resistance by 2.31 ± 1.25% in non-chemosensitive neurons (two-tailed t test; $P < 0.05$).

4. Discussion

4.1. Chemosensitivity in LC neurons of bullfrogs

The bullfrog brainstem slice preparation is the first in which it has been possible to measure changes in firing rates of chemosensitive neurons in response to physiological changes in pH. In mammals, altered ventilation compensates for small pH_a changes (Feldman et al., 2003), but it has been difficult to study these minute changes in chemosensory neurons within brain slice preparations. Mammalian chemosensory neurons in vitro typically require pH changes from 0.2 U to as large as 0.65 U to elicit maximal firing responses (Fukuda, 1983; Wang et al., 1998; Song et al., 2012). These rather large pH changes are outside of normal physiological ranges and even the smaller pH changes (ΔpH 0.1–0.2 U) are only likely to occur in vivo during extreme situations like severe hemorrhage (~7.35–7.1), heavy exercise (7.46–7.29), voluntary hypoventilation (7.4–7.26), or chronic hypoxia (7.41–7.31) (Alfaro et al., 1999; González et al., 1991; Jonville et al., 2002; Powell et al., 2000). In contrast, bullfrog LC neurons used in our current study increased firing rates in response to pH changes that bullfrogs likely experience naturally during ambient temperature fluctuation. 1.3% CO_2 (10 Torr), the value we used as our normocapnic control, is typical for bullfrog CSF at 20°C (Howell et al., 1970; Reeves, 1972). pH values under physiological conditions range from >8 to <7.6 and change ~0.015pH units/◦C during temperature change in bullfrog (Howell et al., 1970; Reeves, 1972; Gottlieb and Jackson, 1976; Mackenzie and Jackson, 1978; Rocha and Branco, 1998). Thus, our dose response demonstrates that bullfrog LC neurons in vitro increase firing rates during physiologically relevant Pco_2/pH changes (Fig. 4). A pH change of 0.12 units required to give half-maximal firing rate increases in

LC neurons suggests that pH changes associated with ambient temperature fluctuation could alter LC output. Because bullfrog LC neurons are highly sensitive to a narrow range of pH changes at 22°C, the temperature-dependence of pHa that bullfrogs experience could impact both chemosensitivity and the contribution of LC neurons to breathing control at temperatures other than 22°C. Widely varied sensory mechanisms involved in transducing changes in cerebral spinal fluid CO2/pH continue to intrigue respiratory physiologists. These varied mechanisms could be within neurons of a single nucleus or distributed across anatomically distinct chemosensory regions within the brainstem (Jiang et al., 2005; Nattie and Li, 2005; Huckstepp and Dale, 2011; Mellen, 2010). The rostral and caudal VLM (Taylor et al., 2003a,b) and the LC (Noronha-de-Souza et al., 2006) have been implicated in CO2/pH sensing mechanisms of amphibians, but the broad distribution of chemosensitive brainstem regions reported in rats has yet to be examined. Further, the diversity of cellular CO2/pH mechanisms documented in mammals remains completely unexplored in amphibians.

4.2. Chemical and electrical coupling effects on CO2/pH chemosensitivity

Our work is the first to demonstrate signaling and network characteristics of chemosensitive respiratory control neurons in an amphibian. We observed dye-coupling within LC neurons in brainstem slices from *L. catesbeianus* (unpublished observation); in addition, dye-coupling in the brainstem and spinal cord of Rana esculenta is inhibited by the gap junction blocker glycyrrhetinic acid (Bácskai and Matesz, 2002). These two examples demonstrate the presence of gap junctions in the central nervous system of anuran amphibians. In the isolated bullfrog brainstem preparation, bath application of 100 µM CBX has been shown to increase fictive lung bursting after 10 min of exposure,

but measurements at times after 10 min showed no differences compared to control lung bursting. Both lung and gill burst frequencies in tadpoles were decreased by 100µM CBX which demonstrates a role for gap junction coupling in breathing development from tadpoles to bullfrogs (Winmill and Hedrick, 2003). Application of CBX did not change V_m, control firing rate, nor chemosensitive responses in LC neurons; therefore, our data do not support a role for gap junction involvement in the chemosensitive response within the LC of adult bullfrogs. It would be interesting to investigate the influence of gap junction coupling on chemoresponsiveness starting from the earliest of larval stages in tadpole LC neurons and observe (1) if gap junctions play a role in maintenance of chemosensitive circuitry in immature amphibians and (2) if so, when chemosensitivity shifts to the chemically controlled network that we observed (Fig. 5). We show that coupling through chemical synapses plays an important role in bullfrog LC neuron chemosensitivity (Fig. 5). These data, in spite of CBX results, suggest that LC neurons responding to HA when devoid of chemical synaptic input are intrinsically chemosensitive. With synaptic networks intact, >80% of LC neurons increased firing rates during HA exposure. In contrast, blocking chemical synapses with SNB reduced the number of chemosensitive neurons by half; neurons that do not exhibit chemosensitive response during synaptic blockade are network-driven chemosensitive neurons. When devoid of synaptic input, putative intrinsically chemosensitive neurons maintained resting V_m and decreased firing rate by ~30%, while network-driven chemosensitive neurons decreased both V_m by ~10% and firing rate by ~80% (Fig. 6). These data suggest that network-driven chemosensitive neurons receive more tonic excitatory input compared with putative intrinsically chemosensitive neurons. Moreover,

decreased firing rates during chemical synaptic blockade in both network-driven chemosensitive and intrinsically chemosensitive LC neurons suggest that all neurons we studied within the LC receive tonic excitatory input from either an internal network composed of LC neurons or networks from neighboring nuclei. Observed excitatory input in the reduced slice preparation could be coming from any number of distinct nuclei within the slice; however, the nucleus isthmi (NI; see Section 4.3) likely provides tonic inhibitory, rather than excitatory, input to its targets (Gargaglioni and Branco, 2004). Future work should investigate whether the excitatory drive originates within the LC or a nearby nucleus.

4.3. Role of the LC in bullfrog respiratory chemosensing

Studies demonstrating functions performed by specific brain-stem nuclei in amphibian ventilatory control are limited. The most investigated area to date is the nucleus isthmi (NI), a structure located in the rostral hindbrain, near the optic tectum (Fig. 1). The NI was originally thought to generate episodic breathing pat-terns in amphibians (Kinkead et al., 1997); however, when the NI is ablated with ibotenic acid, ventilatory responses to inhaled hypercapnic gas mixture are increased indicating that the NI limits some aspect of the hypercapnic ventilatory response in bullfrogs (Gargaglioni et al., 2002; Gargaglioni and Branco, 2004). NI and LC neurons are located adjacent (Fig. 1) to each other in the rostral hindbrain (Marin et al., 1996). The close proximity of NI and LC neurons suggests that the LC would be a strong candidate for the NI to carry out its inhibitory effects on the hypercapnic ventilatory response; however, our data do not support that notion. If bullfrog LC neurons were under inhibitory influence of the NI during hypercapnia, chemical synaptic blockade experiments should have caused

elevated sensitivity to CO_2/pH. Chemosensitive responses were nearly identical with chemical synapses intact or blocked and gap junctions intact or uncoupled (Fig. 6b), suggesting that the NI may not inhibit chemosensitive responses of LC neurons. The location of the NI projections and how they act to dampen the hypercapnic ventilatory response are currently unknown; therefore future experiments should directly explore a functional relationship, or lack thereof, between the LC and NI regions. Many regions have been implicated in central CO2/pH chemoreception in the brainstem of mammals including, but not limited to, the retrotrapezoid nucleus, solitary tract nucleus, LC, medullary raphé, ventral medulla (Mulkey et al., 2007; Nichols et al., 2009; Filosa et al., 2002; Wang et al., 1998; Fukuda, 1983, respectively). While the LC in neonatal rats appears to have a relatively low CI (121%), in bullfrogs the LC has greater sensitivity (177%), comparable to the rat NTS (177%; Fig. 7). We propose that the LC may be one of the vital CO2-sensing elements in bullfrogs, whose signals are integrated in structures elsewhere (i.e., the central pattern gener ator or another nucleus in the chemosensing pathway) which may in turn be inhibited by the NI. Further studies should investigate functional relationships between the LC and other chemosensitive regions in order to delineate the processing of chemosensory information in bullfrog respiratory control.

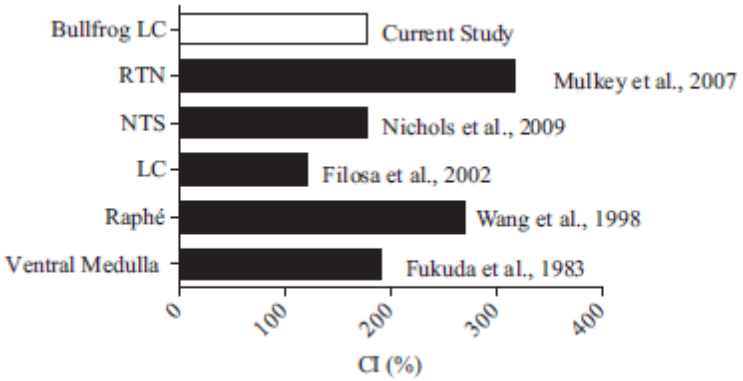


Fig. 7. Chemosensitivity indices comparing neurons from respiratory control nuclei in bullfrogs (white bar; current study) and rats (black bars, literature values). CI of LC neurons of bullfrogs is greater than CI of LC neurons of rats and is comparable to CI of NTS neurons of neonatal rats.

5. Conclusions

This is the first study to establish putative intrinsically chemosensitive responses to CO2/pH and signaling properties of individual neurons from the brainstem of amphibians through whole-cell patch clamp electrophysiology. In bullfrogs, >80% of LC neurons increased firing rates during HA ranging from 1.7 to 10% CO_2 (ΔpH = 0.07–0.89 U), which includes chemosensitivity within physiologically relevant pH changes. Since input resistance of putative intrinsically chemosensitive neurons increases during HA, there is a possible role for pH-sensitive K^+ currents in chemosensory mechanisms of bullfrog LC neurons. Since chemosensitive responses can be evoked from bullfrogs LC neurons within physiological ranges, brainstem slices from bullfrogs are excellent alternative model for investigating cellular chemosensory mechanisms. Furthermore, given that the brainstem of tadpoles responds strongly to acidification (Taylor et al., 2003a,b), bullfrogs are useful model for studying the development of CO_2/pH sensing. Because bullfrogs experience daily and seasonal alterations in T_b and pH their central chemosensors likely have the ability to sense and regulate breathing over a wide-range of pH changes. Investigations into the underlying function of widespread chemosensing and potentially diverse chemosensory mechanisms in bullfrogs offers a comparative approach to understanding the observed diversity of chemosensitive regions and chemosensory mechanisms in mammals.

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Chapter 3: Future Directions

Taken from the National Science Foundation Graduate Research Fellowship Proposal (NSF GRFP) submitted by Joseph M. Santin on November 19, 2012

Bullfrogs inhabit all parts of North America which requires seasonal acclimatization; consequently, temperature changes accompanying seasonal acclimatization impose challenges on ventilatory control. Warm ambient temperatures increase metabolic rate and raise ventilatory drive. At these elevated temperatures, bullfrogs require lung ventilation to obtain O_2 and excrete CO_2 to match cellular metabolic rates. Conversely, decreases in ambient temperatures lower metabolic rate and diminish respiratory drive, allowing cutaneous elimination of CO_2 to be sufficient for maintenance of acid-base homeostasis (Gottlieb & Jackson, 1976). Activities, including hunting and foraging, across a variety of ambient temperatures experienced by bullfrogs impose further metabolic and acid-base stresses which likely are compensated primarily by altered ventilation (Andersen & Wang, 2003). Despite well-studied breathing responses as a result of changing temperatures at rest, a fundamental understanding of ventilatory control (*e.g.,* ventilatory compensation during acid-base disturbances) over a range of temperatures remains lacking.

The ability to detect changes in arterial CO_2/pH and elicit a ventilatory response during acid-base disturbance is common among terrestrial tetrapods. In tadpoles, O_2 provides the chemical drive which stimulates gill ventilation, but following metamorphosis, CO_2/pH assumes primary control of lung ventilation. A majority of this CO_2/pH drive emanates from chemosensitive areas in the brainstem (Milsom, 2010). Given that bullfrogs experience broad ranges in body temperatures, their CO_2/pH sensory system must be able to sense a wide range of CO_2/ pH changes and influence ventilatory adjustments to satisfy variable metabolic demands at different body temperatures. Neural

breathing activity from the bullfrog brainstem has been identified as CO_2/pH sensitive at 20˚C and 25˚C, but exposure of the brainstem to 15˚C reduced CO_2/pH sensitivity (Morales & Hedrick, 2002). These data reflect that CO_2/pH-driven breathing activity of the brainstem changes to meet temperature-specific metabolic and acid-base regulation requirements; however, neuronal mechanisms underpinning CO_2/pH sensory transduction which influence altered ventilatory drive at different temperatures have not been addressed.

Central CO_2/pH sensitivity has been identified in distinct regions throughout the brainstem of amphibians, but the effect of temperature on chemosensitivity in neurons of these areas has not been investigated. The locus coeruleus (LC) has been implicated as an important chemosensory site in ventilatory control because focal acidification of the LC increases breathing, while ablation attenuates the hypercapnic ventilatory response (Noronha-de-Souza et al., 2006). LC neurons of bullfrogs exhibit a high degree of sensitivity within physiological pH ranges (8-7.56) experienced by bullfrogs (Santin & Hartzler, 2013), demonstrating the ability of LC neurons to transduce changes in cerebral spinal fluid CO_2/pH into elevated firing responses (and presumably altered ventilation). Preliminary data indicate that chemosensitive responses of LC neurons observed at 22˚C are completely eliminated at 15˚C. These data provide evidence that respiratory control of bullfrogs involves LC chemotransduction at 22˚C; however, altered sensitivity of LC neurons and neurons of other chemosensitive regions of the brainstem during temperature change may present a mechanism for the observed modulation of CO_2/pH sensitivity at the level of the brainstem.

Regions including the rostral and caudal ventral lateral medulla (VLM) of the bullfrog brainstem have also been identified as CO_2/pH sensitive (Taylor et al., 2003). Given the inhibitory effect of cool temperatures on chemosensitivity observed in LC neurons and the whole-brainstem, neurons of other chemosensory regions, such as the VLM, likely decrease responsiveness to CO_2/pH during exposure to cool temperatures. Although chemosensitivity in response to warm temperatures has not been measured in the LC or the VLM, the stimulatory effect of warm temperatures on whole brainstem chemosensitivity suggest that warm temperatures will increase individual neuronal sensitivity to CO_2/pH. Additionally, the influence of temperature on breathing control during acid-base disturbances of intact bullfrogs has not been examined. We expect that inducing hypercapnic acidosis will proportionally stimulate lung ventilation relative to ambient temperature. For example, higher temperatures will elicit greater ventilatory sensitivity to hypercapnic challenges and vice versa.

Hypotheses:

H_1: Inhaling 5% CO_2 in air will cause increases in minute ventilation (V_E; tidal volume/breathing frequency) at 15˚C and induce relatively greater increases in V_E at 22˚C and 29˚C.

H_2: Individual LC neurons will exhibit decreased chemosensitivity at 15˚C compared to 22˚C, while at 29˚C, chemosensitivity will be increased compared to 22˚C.

H_3: VML neurons will exhibit decreased chemosensitivity at 15˚C compared to 22˚C, while at 29˚C chemosensitivity will be increased compared to 22˚C.

Given that breathing-related motor output from the brainstem of bullfrogs decreases sensitivity to CO_2/pH during exposure to cool temperatures and increases

chemosensitivity at elevated temperatures (Morales & Hedrick, 2002); modulation of LC and VLM chemosensitivity during temperature change presents a mechanistic substrate for altered ventilatory drive at different temperatures. Understanding the influence of temperature on CO_2/pH sensitivity of neurons within chemosensitive brainstem regions will provide mechanistic insight into how bullfrogs control breathing at different temperatures to cope with metabolic and acid-base balance challenges experienced as a result of activity in different climates. Additionally, investigating respiratory control in bullfrogs at multiple levels of organization (i.e., whole-animal and cellular level) during temperature changes will provide insight into how the respiratory controller of amphibians evolved to cope with the metabolic and acid-base challenges associated with variable body temperature.

References:

Alfaro, V., Pesquero, J., Palacios, L., 1999. Acid–base disturbance during hemorrhage in rats: significant role of strong inorganic ions. Journal of Applied Physiology 85, 1617–1625.

Alvarez-Maubecin, V., García-Hernández, F., Williams, J.T., Van Bockstaele, E.J., 2000. Functional coupling betweens neurons and glia. Journal of Neuroscience 20, 4091–4096.

Andersen, J.B., Wang, T., 2003. Cardiorespiratory effects of forced activity and digestion in toads. Physiological and Biochemical Zoology 76, 459-470.

Bácskai, T., Matesz, C., 2002. Primary afferent fibers establish dye-coupled connections in the frog central nervous system. Brain Research Bulletin 57, 317–319.

Bass, A.H., Baker, R., 1997. Phenotypic specification of hindbrain rhombomeres and the origins of rhythmic circuits in vertebrates. Brain Behavior and Evolution 50, 3-16.

Biancardi, V., Bícego, K.C., Almeida, M.C., Gargaglioni, L.H., 2008. Locus coeruleus noradrenergic neurons and CO_2 drive to breathing. Pflugers Archives- European Journal of Physiology 455, 119-1128.

Bícego-Nahas, K., C., Branco, L.G.S., 1999. Seasonal changes in the cardiorespiratory responses to hypercarbia and temperature in the bullfrog, *Rana catesbeiana*. Comparative Biochemistry and Physiology Part A 124, 221-229.

Boutilier, R.G., Heisler, N., 1987. Acid-base regulation and blood gases in the anuran amphibians, *Bufo marinus*, during environmental hypercapnia. The Journal of Experimental Biology 134, 79-98.

Branco, L.G.S., Glass, M.L., Hoffmann, A., 1992. Central chemoreceptor drive to breathing in unanesthetized toads, Bufo paracnemis. Respiration Physiology 87, 195–204.

Branco, L.G.S., Glass, M.L., Wang, T., Hoffman, A., 1993. Temperature and central chemoreceptor drive to ventilation in toad (*Bufo paracnemis*). Respiratory Physiology 93, 337–346.

Burton, R.F., 2002. Temperature and acid-base balance in ectothermic vertebrates: the imidazole alphastat hypothesis and beyond. The Journal of Experimental Biology 205, 3587-3600.

Cameron, J.N., 1989. Acid-base homeostasis: past and present perspectives. Physiological Zoology 62, 845-865.

Coates, E.L., Li, A., Nattie, E.E., 1993. Widespread sites of brain stem ventilatory chemoreceptors. Journal of Applied Physiology 75, 5–14.

Cui, N., Zhang, X., Tadepalli, J.S., Yu, L., Gai, H., Petit, J., Pamulapati, R.T., Jin, X., Jiang, C., 2011. Involvement of TRP channels in the CO_2 chemosensitivity of locus coeruleus neurons. Journal of Neurophysiology 105, 2791–2801.

Davidson, J.S., Baumgarten, I.M., 1988. Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure–activity relationships. Journal of Pharmacology and Experimental Therapeutics 245, 1104–1107.

Dean, J.B., Boulant, J.A., 1989. Effects of synaptic blockade on thermosensitive neurons in rat diencephalon in in vitro. American Journal of Physiology 257, R65–R73.

Denton, J.S., McCann, F.V., Leiter, J.C., 2007. CO2 chemosensitivity in Helix aspersa: three potassium currents mediate pH-sensitive neuronal spike timing. American Journal of Physiology – Cell Physiology 292, C292–C304.

Eckert, R., Brehm, P., 1979. Ionic mechanisms of excitation in *Paramecium*. Annual Review of Biophysics and Bioengineering 8, 353-383.

Erlichman, J.S., Leiter, J.C., 1993. CO_2 chemoreceptoin in the pulomonate snail, *Helix aspersa*. Respiration Physiology 93, 347-363.

Feldman, J.L., Mitchell, G.S., Nattie, E.E., 2003. Breathing: rhythmicity, plasticity, chemosensitivity. Annual Review of Neuroscience 26, 239–266.

Findlay, G.P., Coleman, H.A., Potassium channels in the membrane of *Hydrodictyon africanum*. Journal of Membrane Biology 75, 241-251.

Fukuda, Y., 1983. Difference between actions of high PCO2 and low [HCO3−] on neurons in the rat medullary chemosensitive areas in vitro. European Journal of Physiology 398, 324–330.

Filosa, J.A., Dean, J.B., Putnam, R.W., 2002. Role of intracellular and extracellular pH in the chemosensitive response of rat locus coeruleus neurones. Journal of Physiology 541, 493–509.

Fournier, S., Kinkead, R., 2008. Role of pontine neurons in central O_2 chemoreflex during development in bullfrogs (*Lithobates catesbeiana).* Neuroscience 155, 983–996.

Fukuda, Y., 1983. Difference between actions of high Pco_2 and low [HCO_3^-] on neurons in the rat medullary chemosensitive areas in vitro. European Journal of Physiology 398, 324–330.

Gans, C., De Jogh, H.J., Farber, J., 1969. Bullfrog (Rana catesbeiana) ventilation: how does the frog breathe? Science 163, 1223-1225.

Gargaglioni, L.H., Branco, L.G.S., 2004. Nucleus isthmi and control of breathing in amphibians. Respiratory Physiology and Neurobiology 143, 177–186.

Gargaglioni, L.H., Coimbra, N.C., Branco, L.G.S., 2002. Chemical lesions of the nucleus isthmi increase hypoxic and hypercarbic drive to breathing of toads. Respiratory Physiology and Neurobiology 132, 289–299.

Gargaglioni, L.H., Milsom, W.K., 2007. Control of breathing in anuran amphibians. Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology 147, 665–684.

Gargaglioni, L.H., Hartzler, L.K., Putnam, R.W., 2010. The locus coeruleus and central chemosensitivity. Respiratory Physiology and Neurobiology 173, 264–273.

González, A., Smeets, W.J.A.J., 1993. Noradrenaline in the brain of the South African clawed frog Xenopus laevis: a study with antibodies against noradrenaline and dopamine hydroxylase. The Journal of Comparative Neurobiology 331, 363–373.

Gottlieb, G., Jackson, D.C., 1976. Importance of pulmonary ventilation in respiratory control in the bullfrog. American Journal of Physiology 230, 608–613.

Guyenet, P.G., Stornetta, R.L., Bayliss, D.A., 2008. Retrotrapezoid nucleus and central chemoreception. Journal of Physiology 586, 2043–2048.

Hedrich, R., Schroeder, J.I., 1989. The physiology of ion channels and electrogenic pumps in higher plants. Annual Review of Plant Physiology 40, 539-569.

Hedrick, M.S., Drewes, R.C., Hillman, S.S., Withers, P.C., 2007. Lung ventilation contributes to vertical lymph movement in anurans. The Journal of Experimental Biology 210, 3940-3945.

Hochochka, P.W., Somero, G.N., 1984. Biochemical adaptation. Princeton, New Jersey, Princeton University Press 355-449.

Howell, B., Baumgardner, F., Bondi, K., Rahn, H., 1970. Acid–base balance in cold-blooded vertebrates as a function of body temperature. American Journal of Physiology 218, 600–606.

Huckstepp, R.T.R., Dale, N., 2011. Redefining the components of central CO_2 chemosensitivity—towards a better understanding of mechanism. Journal of Physiology 589.23, 5561–5579.

Imber, A.N., Putnam, R.W., 2012. Postnatal development and activation of L-type Ca^{2+} currents in locus coeruleus neurons: implications for a role for Ca^{2+} in central chemosensitivity. Journal of Applied Physiology 112, 1715–1726.

Jackon, D.C., Palmer, S.E., Meadow, W.L., 1974. The effects of temperature and carbon dioxide breathing on ventilation and acid-base status of turtles. Respiration Physiology 20, 131-146.

Jiang, C., Rojas, A., Wang, R., Wang, X., 2005. CO2 central chemosensitivity: why are there so many sensing molecules? Respiratory Physiology & Neurobiology 145, 115–126.

Jonville, S., Delcech, N., Denjean, A., 2002. Contribution of respiratory acidosis to diaphragmatic fatigue at exercise. European Respiratory Journal 19, 1079–1086.

Kinkead, R., 1997. Episodic breathing in frogs: converging hypotheses on neural control of respiration in air breathing vertebrates. American Zoology 37, 31-40.

Kinkead, R., Harris, M.B., Milsom, W.K., 1997. The role of the nucleus isthmi in respiratory pattern formation in bullfrogs. Journal of Experimental Biology 200, 1781–1793.

Kinkead, R., Milsom, W.K., 1994. Chemoreceptors and control of episodic breathing in the bullfrog (*Rana catesbeiana*). Respiratory Physiology 95, 81–98.

Lannoo, M. (ed.), 2005. Chapter title. In Amphibian Declines: The Conservation Status of United States Species. Berkeley, Los Angeles: University of California Press, 540-543.

Lenz, P.H, Hower, A.E., Hartline, D.K., 2005. Temperature Compensation in the Escape Response of a Marine Copepod, *Calanus finmarchicus* (Crustacea). Biology Bulletin 209, 75-85.

Macintyre, M., Toews, D., 1976. The mechanics of lung ventilation and the effects of hypercapnia on respiration in *Bufo marinus*. Canadian Journal of Zoology 54, 1364–1374.

Mackenzie, J.A., Jackson, D.C., 1978. The effect of temperature on cutaneous CO_2 loss and conductance in the bullfrog. Respiratory Physiology 32, 313–323.

Masaru, I., Williams, J.T., 1996a. Synchronous activity in locus coeruleus results from dendritic interactions in pericoerulear regions. The Journal of Comparative Neurology 346, 63–79.

Masaru, I., Williams, J.T., 1996b. Synchronous activity in locus coeruleus results from dendritic interactions in pericoerulear regions. The Journal of Neuroscience 16, 5196–5204.

Marin, O., Smeets, W.J.A.J., Gonzalez, A., 1996. Do amphibians have a true locus coeruleus? Neuroreport 7, 1447–1451.

Martini, M., Canella, R., Leparulo, A., Prigioni, I., Fesce, R., Rossi, M.L., 2009. Ionic currents in hair cells dissociated from frog semicircular canals after preconditioning under microgravity conditions. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 296, R1585–R1597.

Mehra A, Shi M, Baker CL, Colot HV, Loros JJ, et al. 2009. A role for casein kinase 2 in the mechanism underlying circadian temperature compensation. Cell 137, 749–760.

Mellen, N.M., Milsom, W.K., Feldman, J.L., 2002. Hypothermia and recovery from respiratory arrest in a neonatal rat in vitro brainstem preparation. American Journal of Physiology- Regulatory, Integrative, and Comparative Physiology 282, 484-491.

Milsom, W.K., 2002. Phylogeny of CO_2/pH chemoreception in vertebrates. Respiratory Physiology and Neurobiology 131, 29-41.

Milsom, W.K., 2009. Adaptive trends in respiratory control: a comparative perspective. American Journal of Physiology- Regulatory, Integrative, and Comparative Physiology 299, 1-10.

Milsom, W.K., 2010. Phylogeny of central chemoreception. Respiratory Physiology and Neurobiology. 173, 195-200.

Morales, R.D., Hedrick, M.S., 2002. Temperature and pH/CO2 modulate respiratory activity in the isolated brainstem of the bullfrog (*Rana catesbeiana*). Comparative Physiology and Biochemistry 132, 477–487.

Murakami, Y., Pasqualetti, M., Takio, Y., Hirano, S., Rijli, F.M., Kuratani, S., 2004. Segmental development of reticulospinal and branchiomotor neurons in lamprey: insights into the evolution of the vertebrate hindbrain. Development 131, 989-995.

Mulkey, D.K., Talley, E.M., Stornetta, R.L., Siegel, A.R., West, G.H., Chen, X., Sen, N., Mistry, A.M., Guyenet, P.G., Bayliss, D.A., 2007. TASK channels determine pH sensitivity in select respiratory neurons but do not contribute to central respiratory chemosensitivity. Journal of Neuroscience 27, 14049–14058.

Nattie, E., Li, A., 2005. Central chemoreception 2005: a brief review. Autonomic Neuroscience: Basic and Clinical 126-127, 332–338.

Nichols, N., Hartzler, L., Conrad, S., Dean, J., Putnam, R., 2008. Intrinsic chemosensitivity of individual nucleus tractus solitarius (NTS) and locus coeruleus (LC) neurons from neonatal rats. Advances in Experimental Medicine and Biology 605, 348–352.

Nichols, N.L., Mulkey, D.K., Wilkinson, K.A., Powell, F.L., Dean, J.B., Putnam, R.W., 2009. Characterization of the chemosensitive response of individual solitary complex

neurons from adult rats. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 296, R763–R773.

Noronha-de-Souza, C.R., Bícego, K.C., Michel, G., Glass, M.L., Branco, L.G.S., Gargaglioni, L.H., 2006. Locus coeruleus is a central chemoreceptive site in toads. American Journal of Physiology – Regulatory, Integrative and Comparative Phys-iology 291, R997–R1006.

Pineda, J., Aghajanian, G.K., 1997. Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton- and polyamine-sensitive inward rectifier potassium current. Neuroscience 77, 723–743.

Powell, F.L., Dwinell, M.R., Aarron, E.A., 2000. Measuring ventilatory acclimatization to hypoxia: comparative aspects. Respiratory Physiology 122, 271–284.

Putnam, R.W., Filosa, J.A., Ritucci, N.A., 2004. Cellular mechanisms involved in CO2 and acid signaling in chemosensitive neurons. American Journal of Physiology & Cell Physiology 287, C1493–C1526.

Reeves, R.B., 1972. An imidazole alphastat hypothesis for vertebrate acid–base regulation: Tissue carbon dioxide content and body temperature in bullfrogs. Respiration Physiology 14, 219–236.

Reeves, R.B., 1977. The interaction of body temperature and acid-base balance in ectothermic vertebrates. Annual Review of Physiology 39, 559-586.

Reid, S.G., 2006. Chemoreceptor and pulmonary stretch receptor interactions within amphibian respiratory control systems. Respiratory Physiology and Neurobiology 154, 153-164.

Ren, X., Robbins, P.A., 1999. Ventilatory responses to hypercapnia and hypoxia after 6 h passive hyperventilation in humans. Journal of Physiology 514.3, 885-894.

Robertson, R.M., Money, T.G.A., 2012. Temperature and neuronal circuit function: compensation, tuning, and tolerance. Current Opinion in Neurobiology 22, 724-734.

Robin, E.D., 1962. Relationship between temperature and plasma pH and carbon dioxide tension in the turtle. Nature 4838, 249-251.

Rocha, P.L., Branco, L.G.S., 1998. Seasonal changes in the cardiovascular, respira-tory, and metabolic responses to temperatura and hipoxia in the bullfrog, Rana catesbeiana. Journal of Experimental Biology 201, 761–768.

Rosbash, M., 2009. The implications of multiple circadian clock origins. PLoS Biology 7, e62.

Sánchez-Camacho, C., Peña, J.J., González, A., 2003. Catecholaminergic innervation of the septum in the frog: a combined immunohistochemical and tract-tracing study. Journal of Comparative Neurology 455, 310–323.

Santin, J.M., Hartzler, L.K., 2013. Respiratory signaling of locus coeruleus neurons during hypercapnic acidosis in the bullfrog, *Lithobates catesbeianus* Respiratory Physiology and Neurobiology 185, 553-561.

Song, N., Zhang, G., Geng, W., Liu, Z., Li, L., Cao, Y., Zhu, D., Yu, J., 2012. Acid sensing ion channel 1 in lateral hypothalamus contributes to breathing control. PLoS One 7, e39982.

Stinner, J.N., 1994. Overwintering Behavior of Adult Bullfrogs, *Rana catesbeiana*, in North Eastern Ohio. Ohio Journal of Science 94, 8-13.

Su, J., Yang, L., Zhang, X., Rojas, A., Shi, Y., Jiang, C., 2007. High CO_2 chemosensitivity *versus* wide sensing spectrum: a paradoxical problem and its solutions in cultured brainstem neurons. Journal of Physiology 578.3, 831-841.

Tang, L.S., Goeritz, M.L., Caplan, J.S., Taylor, A.L., Fisek, M., Marder, E., 2010. Precise temperature compensation of phase in a rhythm motor pattern. PLoS Biology 8, e1000469.

Tattersall, G.J., Boutilier, R.G., 1999. Constant set points for pH- and Pco_2 in cold-submerged skin-breathing frogs. Respiration Physiology 118 49-59.

Taylor, B.E., Harris, M.B., Leiter, J.C., Gdovin, M.J., 2003. Ontogeny of central CO2 chemoreception: chemosensitivity in the ventral medulla of developing bull-frogs. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 285, R1461–R1472.

Taylor, B.E., Croll, A.E., Drucker, M.L., Wilson, A.L., 2008. Developmental exposure to ethanol or nicotine inhibits the hypercapnic ventilatory response in tadpoles. Respiratory Physiology & Neurobiology 160, 83–90.

Thannickal, V.J., Fanburg, B.L., 2000. Reactive oxygen species in cell signaling. American Journal of Physiology- Lung Cell and Molecular Physiology 279, 1005-1028.

Torgerson, C.S., Gdovin, M.J., Brandt, R., Remmers, J.E., 2001. Location of central respiratory chemoreceptors in the developing tadpole. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 280, R921–R928.

Wang, W., Pizzonia, J.H., Richerson, G.B., 1998. Chemosensitivity of rat medullary raphe neurones in primary tissue culture. Journal of Physiology 511, 433–450.

West, N.H., Topor, Z.L., Van Vliet, B.N., 1987. Hypoxemic threshold for lung ventilation in the toad. Respiration Physiology 70, 377-390.

Wilson, R.J.A., Vasilaksos, K., Remmers, J.E., 2006. Phylogeny of vertebrate respiratory rhythm generators: the oscillator homology hypothesis. Respiratory Physiology and Neurobiology 154, 47-60.

Winmill, R.E., Hedrick, M.S., 2003. Gap junction blockade with carbenoxolone differentially affects fictive breathing in larval and adult bullfrogs. Respiratory Physiology and Neurobiology 138, 239–251.

Wood, S.C., Glass, M.L., Johansen, K., 1977. Effects of temperature on respiration and acid-base balance in a monitor lizard. Journal of Comparative Physiology 116, 287-296.

Zimmer, B. M., Milsom, W.K., 2004. Effect of hypothermia on respiratory rhythm generation in
hamster brainstem–spinal cord preparations. Respiratory Physiology and Neurobiology 142, 237-249.